

**Role of cyclic adenosine monophosphate  
(cAMP), cyclic guanosine monophosphate  
(cGMP) and p38 mitogen activated protein  
kinase (p38 MAPK) in preconditioning of  
the ischaemic myocardium**



**Dissertation presented for the Degree of Doctor of Philosophy  
(Medical Biochemistry) at the University of Stellenbosch**

**Promotors: Prof. A. Lochner**

**December 2002**

**Prof. J.A. Moolman**

## **Declaration**

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or part submitted it at any university for a degree.

## Abstract

Ischaemic preconditioning (PC) is the phenomenon whereby a short episode of coronary occlusion followed by reperfusion protects the myocardium against a subsequent period of prolonged (also called index or sustained) ischaemia. Even though the exact mechanism of PC remains to be established, it implies that the heart has an endogenous protective mechanism against ischaemia which, if identified, may have important clinical implications. The importance of establishing the mechanism of PC lies in the potential to convert this biological phenomenon into a therapeutic modality to be used clinically. If mediated by certain components of a signal transduction pathway, such a goal will be achievable.

Several triggers and signal transduction pathways have been implicated in the mechanism of protection induced by PC: for example, receptor-dependent endogenous triggers (such as adenosine and opioids) and receptor-independent endogenous triggers (such as free radicals and calcium). However, the involvement of both the  $\beta$ -adrenergic signalling pathway as well as nitric oxide (NO) in PC has not been defined.

It has been suggested that all triggers are linked to a common final pathway, for example, activation of protein kinase C (PKC) and/or the mitogen-activated kinases (MAPKs), in particular p38 MAPK. However, the role of the latter is still controversial.

The aim of this study was to:

- (A) characterize changes in the cyclic nucleotides, cAMP and cGMP, and p38 MAPK occurring during the entire experimental procedure in an attempt to gain insights into the possible mechanisms involved in ischaemic PC (Chapter 3);
- (B) establish the significance of the changes observed in cAMP and cGMP by pharmacological manipulation of their respective pathways (Chapters 4 and 5);
- (C) establish the role of p38 MAPK in ischaemic PC: trigger or mediator involvement (Chapter 6).

Isolated perfused working rat hearts were preconditioned by 3 x 5 min global ischaemia, interspersed by 5 min reperfusion, followed by 25 min global ischaemia and 30 min reperfusion. Functional recovery during reperfusion was used as end-point. Hearts were freeze-clamped at different times during the PC protocol, sustained ischaemia, as

well as during reperfusion. Tissue cyclic nucleotides (cAMP and cGMP), cyclic nucleotide phosphodiesterase (cAMP- and cGMP-PDE) activities, adenylyl cyclase and protein kinase A activities and  $\beta$ -adrenergic receptor characteristics were determined. p38 MAPK activation was also assessed by Western blotting, using dual phospho-p38 MAPK (Thr180/Tyr182) antibody as well as activating transcription factor 2 (ATF2) activation. In addition, to evaluate the role of p38 MAPK in PC protection, the effect of inhibition of p38 MAPK activation, by SB203580, was determined in adult isolated rat cardiomyocytes as well as in isolated perfused rat hearts.

Based on the results obtained, it is proposed that during a multi-cycle ischaemic PC protocol triggers (presumably endogenous catecholamines and NO) are released which induce cyclic changes in cyclic nucleotides, cAMP and cGMP. Both these cyclic nucleotides transiently activate the downstream stress kinase, p38 MAPK, which may trigger further downstream adaptive processes.

Furthermore, the sustained ischaemic period of PC hearts was characterized by attenuated cAMP and elevated cGMP levels, as well as attenuated activation of p38 MAPK, which was associated with cardioprotection. In addition, pharmacological attenuation of p38 MAPK activation during sustained ischaemia led to functional recovery. It is concluded that the cardioprotection of PC is due to attenuation of ischaemia-induced p38 MAPK activation. Pharmacological manipulation of this kinase should be considered as a therapeutic modality in the future.

## Opsomming

Isgemiese prekondisionering (PK) verwys na die verskynsel waardeur 'n kort, verbygaande episode van isgemie gevolg deur herperfusie, die miokardium teen 'n daaropvolgende langdurige periode van isgemie beskerm. Die presiese meganisme van beskerming van PK moet nog opgeklar word, maar dit impliseer dat die hart oor 'n endogene beskermingsmeganisme beskik wat, indien geïdentifiseer, belangrike kliniese implikasies mag hê. Die belang van opklaring van die meganisme van PK lê daarin dat 'n biologiese verskynsel in 'n terapeutiese modaliteit vir kliniese gebruik, omgeskakel kan word. Sou dit deur bepaalde komponente van 'n seintransduksiepad gemedieër word, is so 'n doel bereikbaar.

Verskeie stimuli en seintransduksiepaaie is in PK betrokke: byvoorbeeld, reseptor-afhanklike endogene stimuli (soos adenosien en opioïde), asook reseptor-onafhanklike endogene stimuli (soos vrye radikale en kalsium). Die betrokkenheid van die  $\beta$ -adrenerge seintransduksiepad asook stikstofoksied (NO) in PK egter nog nie behoorlik evalueer nie.

Dit is voorgestel dat alle stimuli op 'n finale algemene pad uitloop, soos byvoorbeeld die aktivering van proteïen kinase C (PKC) en/of die mitogeen-geaktiveerde kinases (MAPKs), spesifiek die p38 MAPKs. Laasgenoemde se rol in PK is steeds kontroversieël.

### Die doel van die studie was dus:

- (A) karakterisering van die veranderinge in die sikliese nukleotiede, cAMP en cGMP, en p38 MAPK wat tydens die hele eksperimentele prosedure plaasvind, in 'n poging om meer insig te verkry aangaande moontlike meganismes betrokke in isgemiese PK (Hoofstuk 3);
- (B) bepaling van die belang van die waargenome veranderinge in cAMP en cGMP deur hul onderskeie paaie farmakologies te manipuleer (Hoofstukke 4 en 5);
- (C) bepaling van die rol van p38 MAPK in PK: betrokkenheid as stimulus of mediator (Hoofstuk 6).

Geïsoleerde, geperfuseerde werkende rotharte is geprekondisioneer deur blootstelling aan 3 x 5 min globale isgemie, afgewissel met 5 min herperfusie, gevolg deur 25 min

globale isemie en 30 min herperfusie. Funksionele herstel tydens herperfusie is as eindpunt gebruik. Harte is op verskillende tye tydens die PK protokol, volgehoue isemie, asook herperfusie gevriesklamp. Weefsel sikliese nukleotiede (cAMP en cGMP), die aktiwiteit van sikliese nukleotied fosfodiesterases (cAMP- en cGMP-PDE), adeniel siklase en proteïen kinase A (PKA) asook die eienskappe van die  $\beta$ -adrenerge reseptor is gemeet. p38 MAPK aktivering is met Westerse oordragtegnieke bepaal, deur van dubbel gefosforileerde p38 MAPK (Thr180/Tyr182) antiligggame asook geaktiveerde transkripsie faktor 2 (ATF2) gebruik te maak. Die rol van p38 MAPK in PK beskerming is evalueer deur die effek van inhibisie van p38 MAPK aktivering met SB 203580, in volwasse geïsoleerde rot kardiomyosiete asook in geïsoleerde geperfuseerde rotharte, te bepaal.

Na aanleiding van die resultate, is voorgestel dat, tydens 'n multi-siklus isemie PK protokol, stimuli (moontlik endogene katekolamiene en NO) vrygestel word wat die sikliese veranderinge in sikliese nukleotiede, cAMP en cGMP, veroorsaak. Beide hierdie sikliese nukleotiede aktiveer die distale stres kinase, p38 MAPK, op 'n betekenisvolle, maar verbygaande manier. Hierdie kinase mag verdere distale aanpassingsprosesse stimuleer.

Die volgehoue isemiese periode van PK harte is gekenmerk deur verminderde cAMP en verhoogde cGMP vlakke, asook verminderde aktivering van p38 MAPK. Hierdie veranderinge is met beskerming van die hart teen isemie geassosieer. Daarbenewens, farmakologiese vermindering van p38 MAPK aktivering tydens volgehoue isemie het tot verbeterde funksionele herstel gelei. Die gevolgtrekking is gemaak dat die beskermende effek van PK die gevolg is van verminderde aktivering van isemies-geïnduseerde p38 MAPK. Farmakologiese manipulasie van hierdie kinase moet in die toekoms as terapeutiese modaliteit oorweeg word.

## Acknowledgements

My deepest gratitude and thanks to my heavenly Father, for only through His grace and strength it was possible to do this work.

I would also like to express my sincerest thanks to the following persons:

**Prof. Amanda Lochner** for her constant support, positive encouragement, excellent supervision, constructive criticism and patience, as well as **Prof. Johan Moolman** for his guidance and support during the study and in the preparation of this manuscript.

**Mrs. Sonia Genade** for her excellent technical assistance, eagerness, constant support and patience while teaching me the perfusion technique.

**Dr. Thomas Podzuweit** (Max-Planck Institute, Bad Nauheim, Germany) for determination of the phosphodiesterases activities.

**Mss. Johanna van Wyk and Lolita Bailey** for valuable technical assistance.

**Dr. Barbara Huisamen** for her expert advice and support in the preparation of this manuscript.

The Department of Medical Physiology and Biochemistry (University of Stellenbosch), the Medical Research Council and the Harry Crossley Fund for financial support.

**My family, friends and colleagues** for their support and encouragement during this study.

**My father and mother** for their unwavering support, love and prayers.

**My husband, Adriaan** for his constant encouragement, patience and devoted love.

## Index

	Page No.
<b>Declaration</b>	ii
<b>Abstract</b>	iii
<b>Opsomming</b>	v
<b>Acknowledgements</b>	vii
<b>List of Tables</b>	xviii
<b>List of Figures</b>	xx
<b>Chapter 1. Literature Review</b>	<b>1</b>
<b>1.1 Ischaemic preconditioning</b>	<b>1</b>
1.1.1 Introduction	1
1.1.2 Factors affecting classic preconditioning	2
1.1.2.1 Duration of protection	2
1.1.2.2 Triggering	2
1.1.2.3 Endpoint	3
1.1.3 Possible signal transduction mediated mechanisms of preconditioning	4
1.1.3.1 Introduction	4
1.1.3.2 Triggers	5
1.1.3.3 Mediators	6
1.1.3.3.1 Protein kinase C (PKC)	6
1.1.3.3.2 Other kinases	7
1.1.3.4 Effectors	8
1.1.3.4.1 Na <sup>+</sup> /H <sup>+</sup> exchanger	8
1.1.3.4.2 Other potential end-effectors	8
1.1.4 Metabolic changes in classic preconditioning	9
1.1.4.1 Energy utilization	9
1.1.4.2 Attenuation of acidosis	9
<b>1.2 The role of cyclic nucleotides in classic preconditioning</b>	<b>11</b>
1.2.1 Introduction	11
1.2.2 $\beta$ -Adrenergic signalling pathway and cAMP	11
1.2.2.1 Catecholamines	11
1.2.2.2 $\beta$ -Adrenergic receptors	12

1.2.2.3	Adenylyl cyclases (ACs)	12
1.2.2.4	Cyclic Adenosine Monophosphate (cAMP)	13
1.2.2.4.1	Introduction	13
1.2.2.4.2	Background	13
1.2.2.4.3	Mechanism of activation of cAMP	14
1.2.2.4.4	Regulation of cAMP	14
1.2.2.4.4.1	Phosphodiesterases (PDEs)	14
1.2.2.4.4.2	Receptor desensitization	17
1.2.2.5	$\beta$ -Adrenergic signalling and myocardial ischaemia	18
1.2.2.5.1	Catecholamines and ischaemia	18
1.2.2.5.2	$\beta$ -Adrenergic receptor and adenylyl cyclase in ischaemia	19
1.2.2.5.3	cAMP and ischaemia	19
1.2.2.6	The role of adrenergic signalling in preconditioning	20
1.2.2.6.1	Catecholamines in preconditioning	20
1.2.2.6.2	$\alpha_1$ -Adrenergic signalling in preconditioning	21
1.2.2.6.3	$\beta$ -Adrenergic signalling in preconditioning	22
<b>1.2.3</b>	<b>Nitric oxide (NO)-Cyclic Guanosine Monophosphate (cGMP) pathway</b>	<b>24</b>
1.2.3.1	Introduction	24
1.2.3.2	Nitric oxide (NO)	24
1.2.3.3	Metabolism of cGMP	25
1.2.3.3.1	Guanylyl cyclase (GC)	25
1.2.3.3.2	Phosphodiesterase (PDE)	26
1.2.3.4	Effect of NO-cGMP pathway	26
1.2.3.4.1	In vascular smooth muscle	26
1.2.3.4.2	In myocardium	27
1.2.3.5	NO-cGMP pathway and ischaemic-reperfusion	28
1.2.3.5.1	NO-cGMP pathway: deleterious?	29
1.2.3.5.2	NO-cGMP pathway: protective?	29
1.2.3.6	NO-cGMP pathway in classic preconditioning	31
1.2.3.6.1	NO and transient ischaemia	31
1.2.3.6.2	Role of NO: trigger or mediator?	31
<b>1.3</b>	<b>Mitogen activated protein kinase (MAPK)</b>	<b>33</b>
1.3.1	Introduction	33

1.3.2	Overview of MAPKs	33
1.3.2.1	Major subfamilies of MAPKs	34
1.3.2.1.1	ERK	34
1.3.2.1.2	SAPK/JNK	35
1.3.2.1.3	p38 MAPK	35
1.3.2.1.4	Other SAPKs	36
1.3.3	Upstream kinases of MAPKs	36
1.3.3.1	Upstream of ERK	37
1.3.3.2	Upstream of SAPKs (JNK and p38 MAPK)	37
1.3.4	Activation of MAPKs via G protein-coupled receptors (GPCR)	38
1.3.4.1	Receptor tyrosine kinases and ERK pathway	38
1.3.4.2	G protein-coupled receptors (GPCRs) and ERK pathway	39
1.3.4.2.1	G $\beta\gamma$ -protein	39
1.3.4.2.2	G $\alpha$ -protein	40
1.3.4.3	G protein-coupled receptors (GPCRs) and JNK pathway	41
1.3.4.4	G protein-coupled receptors (GPCRs) and p38 MAPK pathway	42
1.3.5	Downstream substrates of MAPKs	43
1.3.5.1	Protein kinases	43
1.3.5.2	Transcription factors	44
1.3.6	Inactivation of MAPKs by phosphatases	47
1.3.7	Biological function of p38 MAPK activation	49
1.3.7.1	Introduction	49
1.3.7.2	Role of MAPKs in cell death or survival	49
1.3.7.2.1	p38 MAPK and cell death in ischaemia-reperfusion	50
1.3.7.2.1.1	Introduction	50
1.3.7.2.1.2	Inhibitors of p38 MAPK and ischaemia-reperfusion	51
1.3.7.2.1.3	Overexpression of p38 MAPK	52
1.3.7.2.2	p38 MAPK and cell survival in preconditioning	53
1.3.7.2.2.1	Introduction	53
1.3.7.2.2.2	p38 MAPK and preconditioning	53
1.3.7.2.2.3	Pharmacological activation of p38 MAPK	54
1.3.7.2.2.4	Pharmacological inhibition of p38 MAPK	55
1.3.8	Nitric oxide (NO) and p38 MAPK	56
1.3.8.1	Introduction	56
1.3.8.2	p38 MAPK: up- and downstream of NO	56

1.3.8.3	p38 MAPK and NO in preconditioning	57
1.3.9	Other MAPKs and preconditioning	57
1.3.10	Conclusion	58
<b>1.4</b>	<b>Motivation and aims</b>	<b>59</b>
1.4.1	General	59
1.4.2	Hypothesis	60
1.4.4	Specific aims	60
<b>Chapter 2.</b>	<b>Materials and Methods</b>	<b>62</b>
2.1	Animals	62
2.2	Perfusion Technique	62
2.3	Standard perfusion protocol	63
2.4	Biochemical analysis	63
2.4.1	Tissue high energy phosphate, cAMP and cGMP analysis	63
2.4.2	Tissue cAMP-dependent PKA activity	64
2.4.3	Tissue cAMP- and cGMP-phosphodiesterase activity	64
2.4.4	Characterization of $\beta$ -adrenergic response	65
2.4.4.1	Membrane preparation	65
2.4.4.2	$\beta$ -Adrenergic receptor assay	65
2.4.4.3	Adenylyl cyclase activity	66
2.4.5	Western blotting for p38 mitogen-activated protein kinase	66
2.4.5.1	Preparation of lysates	66
2.4.5.2	p38 MAPK dual phosphorylation	67
2.4.5.3	p38 MAPK activity	67
2.5	Drugs	67
2.6	Statistics	68
<b>Chapter 3.</b>	<b>Evaluation of events in Ischaemic Preconditioning</b>	<b>69</b>
<b>3.1</b>	<b>Introduction</b>	<b>69</b>
3.1.1	$\beta$ -Adrenergic signal transduction pathway	69
3.1.2	Mitogen activated kinases (MAPKs)	70
3.1.3	The aim	70

<b>3.2</b>	<b>Experimental Protocols and Results</b>	<b>72</b>
3.2.1	Evaluation of events during the ischaemic preconditioning protocol	72
3.2.1.1	Experimental protocol	72
3.2.1.2	Results	72
3.2.1.2.1	Evaluation of changes in tissue cyclic nucleotides	72
3.2.1.2.2	Evaluation of changes in tissue phosphodiesterases	73
3.2.1.2.3	Evaluation of changes in tissue high energy phosphates	73
3.2.1.2.4	Characterization of changes in $\beta$ -adrenergic signalling pathway during the preconditioning protocol	74
3.2.1.2.4.1	Role of release of endogenous catecholamines	74
3.2.1.2.4.2	Evaluation of changes in $\beta$ -adrenergic signalling pathway	74
3.2.1.2.4.3	Evaluation of $\beta$ -adrenergic receptor responsiveness	75
3.2.1.2.5	Evaluation of p38 MAPK activation during the PC protocol	76
3.2.2	Evaluation of events during Sustained Ischaemia	78
3.2.2.1	Experimental protocol	78
3.2.2.2	Results	78
3.2.2.2.1	Changes in tissue cyclic nucleotides	78
3.2.2.2.2	Changes in tissue phosphodiesterase activity	79
3.2.2.2.3	Changes in tissue high energy phosphates	79
3.2.2.2.4	Evaluation of p38 MAPK activation during sustained ischaemia	79
3.2.3	Evaluation of events during Reperfusion Phase	81
3.2.3.1	Experimental protocol	81
3.2.3.2	Results	81
3.2.3.2.1	Effect of ischaemic preconditioning on functional recovery	81
3.2.3.2.2	High energy phosphates	82
3.2.3.2.3	p38 MAPK activation during reperfusion	82
<b>3.3</b>	<b>Discussion of ischaemic preconditioning</b>	<b>83</b>
3.3.1	Validity of experimental model in this study	83
3.3.1.1	Working heart vs. retrograde perfusion	83
3.3.1.2	Endpoints	84
3.3.1.3	Reasons for choice	84
3.3.1.4	High energy phosphates (HEPs)	85
3.3.1.4.1	Changes in HEPs during the PC protocol	86

3.3.1.4.2	Changes in HEPs at end of sustained ischaemia	86
3.3.2	Changes during the ischaemic preconditioning protocol and subsequent sustained ischaemia	87
3.3.2.1	Cyclic increases in cAMP and cGMP during preconditioning?	87
3.3.2.1.1	Tissue cAMP	87
3.3.2.1.2	Tissue cGMP	90
3.3.2.1.3	cAMP and cGMP	90
3.3.2.2	p38 MAPK activation during the preconditioning protocol	91
3.3.3	Changes during sustained ischaemia	92
3.3.3.1	Changes in cAMP and cGMP	92
3.3.3.2	Attenuation of the $\beta$ -adrenergic response to sustained ischaemia	93
3.3.3.2.1	Phosphodiesterase activity	93
3.3.3.2.2	Desensitization	94
3.3.3.2.3	Endogenous catecholamines	96
3.3.3.3	p38 MAPK activation during sustained ischaemia and reperfusion	97
<b>3.4</b>	<b>SUMMARY</b>	<b>100</b>
3.4.1	Effect of ischaemic preconditioning on cyclic nucleotides	100
3.4.2	Effect of ischaemic preconditioning on p38 MAPK activation	100
<b>Chapter 4. Manipulation of cyclic adenosine monophosphate (cAMP)</b>		<b>102</b>
<b>4.1</b>	<b>Introduction</b>	<b>102</b>
4.1.1	The aim	103
<b>4.2</b>	<b>Experimental Protocols and Results</b>	<b>104</b>
4.2.1	Effect of $\beta$ -adrenergic receptor manipulation during the ischaemic preconditioning (PC) protocol	104
4.2.1.1	$\beta$ -adrenergic receptor blockade during the PC protocol	104
4.2.1.1.1	Choice of $\beta$ -adrenergic receptor blocker	104
4.2.1.2	Repeated $\beta$ -adrenergic stimulation: effect of cAMP	105
4.2.1.3	Repeated $\beta$ -adrenergic stimulation: effect on $\beta$ -adrenergic receptor responsiveness	106
4.2.1.4	Evaluation of p38 MAPK activation	107

4.2.1.4.1	Effects of $\beta$ -adrenergic receptor stimulation on p38 MAPK activation	107
4.2.1.4.1.1	Effects of time and dosage of different $\beta$ -adrenergic stimulants	107
4.2.1.4.1.2	Manipulation of p38 MAPK activation induced by 5 min ischaemia or isoproterenol	108
4.2.2	Effect of $\beta$ -adrenergic manipulation during sustained ischaemia	110
4.2.2.1	$\beta$ -adrenergic receptor blockade during PC protocol	110
4.2.2.2	Repeated $\beta$ -adrenergic receptor stimulation prior to sustained ischaemia	110
4.2.3	Evaluation of events after sustained ischaemia during reperfusion	112
4.2.3.1	Effect of $\beta$ -adrenergic receptor blockade during PC protocol on functional recovery	112
4.2.3.1.1	Experimental protocol	112
4.2.3.1.2	Results	113
4.2.3.2	Effect of repeated agonist-induced increases in cAMP prior to sustained ischaemia on functional recovery	114
4.2.3.2.1	Experimental protocol	114
4.2.3.2.2	Results	114
4.2.3.3	p38 MAPK activation during sustained ischaemia and reperfusion of $\beta$ -adrenergic receptor manipulated hearts	115
4.2.3.3.1	Experimental protocol	115
4.2.3.3.2	Results	116
<b>4.3</b>	<b>Discussion</b>	<b>117</b>
4.3.1	Prevention of cAMP generation during the PC protocol	117
4.3.2	Simulation of ischaemic-induced cAMP increases	118
4.3.3	Mechanism of protection: cAMP related?	120
4.3.4	Protein kinase A and Protein kinase C	122
4.3.5	Further downstream events of $\beta$ -adrenergic stimulation and p38 MAPK	123
4.3.5.1	Characteristics of $\beta$ -adrenergic-mediated stimulation of p38 MAPK	124
4.3.5.2	Manipulation of ischaemia- or isoproterenol-induced p38 MAPK activation	125

<b>4.4</b>	<b>SUMMARY</b>	<b>126</b>
4.4.1	Effect of $\beta$ -adrenergic stimulation	126
4.4.2	Downstream events of $\beta$ -adrenergic manipulation: role of p38 MAPK	127
<b>Chapter 5. Manipulation of cyclic guanosine monophosphate (cGMP)</b>		<b>129</b>
<b>5.1</b>	<b>Introduction</b>	<b>129</b>
5.1.1	The aim	130
<b>5.2</b>	<b>Experimental Protocols and Results</b>	<b>132</b>
5.2.1	Manipulation of the nitric oxide (NO)-cGMP pathway during PC protocol	132
5.2.1.1	Inhibition of cyclic increases in cGMP during ischaemic PC protocol	132
5.2.1.1.1	Experimental protocol	132
5.2.1.1.2	Results	132
5.2.1.2	Repeated increases in cGMP by NO donors before sustained ischaemia	133
5.2.1.2.1	Experimental protocol	133
5.2.1.2.2	Results	133
5.2.1.3	$\beta$ -adrenergic receptor responsiveness after repeated NO stimulation	134
5.2.1.3.1	Experimental protocol	135
5.2.1.3.2	Results	135
5.2.1.4	Manipulation of p38 MAPK activation by inhibition or stimulation of NO-cGMP pathway during the PC protocol	135
5.2.1.4.1	Experimental protocol	135
5.2.1.4.2	Results	135
5.2.2	Evaluation of events during Sustained Ischaemia	137
5.2.2.1	Effect of prior manipulation of the NO-cGMP pathway on cyclic nucleotides during sustained ischaemia	137
5.2.2.1.1	Experimental protocol	137
5.2.2.1.2	Results	137
5.2.2.2	Effect of NO-cGMP pathway manipulations on p38 MAPK activation at the end of sustained ischaemia	138
5.2.2.2.1	Experimental protocol	138

5.2.2.2.2	Results	139
5.2.3	Evaluation of events after sustained ischaemia during reperfusion	140
5.2.3.1	Effect of inhibition of guanylyl cyclase (GC) or nitric oxide synthase (NOS) during PC protocol on functional recovery	140
5.2.3.1.1	Experimental protocol	140
5.2.3.1.2	Results	141
5.2.3.2	Effect of NO donors on functional recovery	144
5.2.3.2.1	Experimental protocol	144
5.2.3.2.2	Results	144
<b>5.3</b>	<b>Discussion</b>	<b>146</b>
5.3.1	Abolishment of protection by prevention of cyclic increases in cGMP during preconditioning	146
5.3.1.1	Inhibitors and vehicles	146
5.3.1.2	Preconditioning and inhibitors	147
5.3.1.3	Non-preconditioning and inhibitors	149
5.3.2	Mimicking of preconditioning by using NO donors as triggers	150
5.3.2.1	PC protocol	150
5.3.2.2	Ischaemia-Reperfusion	150
5.3.2.3	Contractile Reserve	152
5.3.3	How does NO-cGMP pathway trigger protection?	152
5.3.3.1	“Cross-talk”	153
5.3.3.2	Reactive oxygen species (ROS)	153
5.3.3.3	p38 MAPK	154
5.3.4	How does NO-cGMP pathway mediate protection during sustained ischaemia?	155
5.3.4.1	Calcium	156
5.3.4.2	Phosphodiesterase	156
5.3.4.3	p38 MAPK	156
5.3.4.4	ATP-sensitive potassium (KATP)-channels	158
<b>5.4</b>	<b>SUMMARY</b>	<b>159</b>
	<b>Chapter 6. Evaluation of the role of p38 MAPK</b>	<b>160</b>
<b>6.1</b>	<b>Introduction</b>	<b>160</b>

6.1.1	p38 MAPK activation: trigger or mediator?	160
6.1.2	The aim	161
<b>6.2</b>	<b>Experimental Protocols and Results</b>	<b>162</b>
6.2.1	Inhibition of p38 MAPK during global ischaemia in the perfused rat heart	162
6.2.1.1	Experimental protocol	162
6.2.1.2	Results	163
<b>6.3</b>	<b>Discussion</b>	<b>165</b>
6.3.1	p38 MAPK activation during the PC protocol: a trigger for eliciting protection?	165
6.3.2	p38 MAPK during ischaemia and reperfusion: a mediator of protection?	167
6.3.3	p38 MAPK inhibition: good or bad?	169
6.3.4	Mechanism of p38 MAPK in preconditioning protection	171
6.3.4.1	Free radicals and NFkB	171
6.3.4.2	Heat-shock protein 27 (HSP27)	171
6.3.4.3	cAMP response element-binding protein (CREB)	172
6.3.4.4	Stress-responsive genes (ATF-2, MEF-2, c-Jun)	172
6.3.4.5	Phosphatases	172
6.3.4.6	ATP-sensitive potassium (KATP) channels	173
<b>6.4</b>	<b>SUMMARY</b>	<b>174</b>
	<b>Chapter 7. Conclusions</b>	<b>175</b>
	<b>Addendum: List of publications</b>	<b>177</b>
	<b>References</b>	<b>178</b>

## List of Tables

### Chapter 1

**Table 1.1** Summary of studies on pharmacological inhibition of p38 MAPK in PC

### Chapter 3

**Table 3.1** Effect of 3 x 5 min preconditioning on tissue cGMP and cAMP levels and the cAMP/cGMP ratio before onset of sustained ischaemia.

**Table 3.2** The cAMP/cGMP ratios in non-preconditioned (non-PC) and preconditioned (PC) hearts during sustained ischaemia.

### Chapter 4

**Table 4.1** Metabolic changes during administration of forskolin or isoproterenol.

**Table 4.2** Effect of Adrenaline ( $10^{-6}$  M) on mechanical performance during reperfusion of hearts pretreated with Alprenolol.

**Table 4.3** Effect of  $\beta$ -adrenergic stimulation with isoproterenol or forskolin on mechanical function during 30 min of reperfusion.

### Chapter 5

**Table 5.1** Effects of ODQ, an inhibitor of guanylyl cyclase on tissue cyclic nucleotide levels and ratio during a 3 x 5 min preconditioning protocol.

**Table 5.2** Effects of administration of NO donors (SNAP or SNP) on tissue cyclic nucleotides before onset of sustained ischaemia.

**Table 5.3** Tissue cyclic nucleotides and cAMP/cGMP ratio at the end of 25 min sustained ischaemia.

**Table 5.4** Effect of ischaemic preconditioning, ODQ, L-NAME and L-NA on functional recovery during reperfusion after 25 min sustained ischaemia.

**Table 5.5** Effect of Adrenaline ( $10^{-6}$  M) on mechanical performance during reperfusion of hearts after treatment with inhibitors.

**Table 5.6** Effect of ischaemic preconditioning and NO donors on functional recovery after 25 min sustained ischaemia

**Table 5.7** Effect of Adrenaline ( $10^{-6}$  M) on mechanical performance during reperfusion of hearts on NO donors.

## **Chapter 6**

**Table 6.1** Mechanical function after ischaemia during 30 min reperfusion: Effect of SB 203580, an inhibitor of p38 MAPK.

## List of Figures

### Chapter 1

- Fig 1.1** Schematic representation of the  $\beta$ -adrenergic signalling pathway.
- Fig 1.2** A model of how Gs protein couples receptor activation to adenylyl cyclase activation.
- Fig 1.3(a)** Molecular structure of cyclic nucleotides.
- Fig 1.3(b)** The mechanism of elevation or reduction of intracellular levels of cAMP and cGMP.
- Fig 1.4** A model of  $\beta$ -adrenergic receptor desensitization.
- Fig 1.5** Regulation of contractility of arterial smooth muscle by nitric oxide (NO) and cGMP.
- Fig 1.6** Second-messenger pathways for the activation of nitric oxide (NO) in cardiac myocytes.
- Fig 1.7** Basic assembly of mitogen-activated protein kinase (MAPK) pathways.
- Fig 1.8** The different MAPK pathways.
- Fig 1.9** Multiple pathways linking G protein-coupled receptors to ERK1/2.
- Fig 1.10** Molecules linking G protein-coupled receptors to JNK and p38 MAPKs.

### Chapter 2

- Fig 2.1** Standard perfusion protocol for non-preconditioned and preconditioned hearts.

### Chapter 3

- Fig 3.1** Evaluation of the changes occurring during the preconditioning (PC) protocol: Experimental protocol.
- Fig 3.2** Cyclical changes in tissue cAMP and cGMP during the PC protocol.
- Fig 3.3** Cyclical changes in cAMP- and cGMP- phosphodiesterase activities during the PC protocol.
- Fig 3.4** Cyclic changes in tissue high energy phosphates during the PC protocol.
- Fig 3.5** Tissue cAMP of non-reserpinized and reserpinized hearts during the PC protocol.
- Fig 3.6**  $\beta$ -Adrenergic receptor population during the PC protocol.
- Fig 3.7** Adenylyl cyclase and protein kinase A activities during the PC protocol.

- Fig 3.8**  $\beta$ -Adrenergic pathway responsiveness after the PC protocol: Experimental protocol.
- Fig 3.9**  $\beta$ -Adrenergic pathway responsiveness after the PC protocol: cAMP generation in response to different agonists.
- Fig 3.10**  $\beta$ -Adrenergic receptor responsiveness to isoproterenol after the PC protocol: Experimental protocol.
- Fig 3.11**  $\beta$ -Adrenergic receptor responsiveness to isoproterenol after the PC protocol: Aortic output and total work performance.
- Fig 3.12** The ischaemic PC protocol: Analysis of p38 MAPK dual phosphorylation and activity.
- Fig 3.13** Tissue cAMP and cGMP levels during sustained global ischaemia.
- Fig 3.14A** Evaluation of the tissue phosphodiesterase levels during sustained ischaemia: Experimental protocol.
- Fig 3.14B,C** cAMP- and cGMP- phosphodiesterase (PDE) activities during sustained ischaemia.
- Fig 3.15** Changes in tissue high energy phosphates during sustained ischaemia.
- Fig 3.16** Sustained ischaemia: Analysis of p38 MAPK dual phosphorylation and activity.
- Fig 3.17** Evaluation of events after sustained ischaemia during the reperfusion phase: Experimental protocol.
- Fig 3.18** Effect of ischaemic preconditioning on functional recovery after sustained ischaemia.
- Fig 3.19** Changes in high energy phosphates after 30 min reperfusion.
- Fig 3.20** Sampling for p38 MAPK in non-preconditioned and preconditioned hearts: Experimental protocol.
- Fig 3.21** Reperfusion: Analysis of p38 MAPK dual phosphorylation and activity.

#### Chapter 4

- Fig 4.1** Cardiac output of hearts treated with  $\beta$ -adrenergic blocker.
- Fig 4.2** Dose-response with  $\beta$ -adrenergic blocker, alprenolol.
- Fig 4.3**  $\beta$ -Adrenergic blockade during PC protocol: Experimental protocol
- Fig 4.4** Tissue cAMP levels after  $\beta$ -adrenergic blockade during PC protocol.
- Fig 4.5** Dose-response with isoproterenol and forskolin.
- Fig 4.6** Repeated  $\beta$ -adrenergic stimulation.

- Fig 4.7**  $\beta$ -Adrenergic receptor responsiveness after repeated  $\beta$ -adrenergic stimulation: Experimental protocol.
- Fig 4.8**  $\beta$ -Adrenergic receptor responsiveness after repeated  $\beta$ -adrenergic stimulation with isoproterenol: Tissue cAMP levels.
- Fig 4.9**  $\beta$ -Adrenergic receptor responsiveness after repeated  $\beta$ -adrenergic stimulation with forskolin: Tissue cAMP levels.
- Fig 4.10** Characterization of effects of  $\beta$ -adrenergic stimulation on p38 MAPK activation: Effect of different isoproterenol concentrations.
- Fig 4.11** Characterization of effects of  $\beta$ -adrenergic stimulation on p38 MAPK activation: Effect of different agonists.
- Fig 4.12** Isoproterenol dose-response comparison of dual phosphorylation and kinase activity of p38 MAPK.
- Fig 4.13** Manipulation of brief ischaemia-induced p38 MAPK activation.
- Fig 4.14** Manipulation of brief isoproterenol-induced p38 MAPK activation.
- Fig 4.15** Effect of brief ischaemia and  $\beta$ -adrenergic blockade on phosphorylation and activity of p38 MAPK.
- Fig 4.16**  $\beta$ -Adrenergic response to 25 min sustained global ischaemia: Experimental protocol.
- Fig 4.17**  $\beta$ -Adrenergic response to 25 min sustained global ischaemia after  $\beta$ -adrenergic blockade: Tissue cAMP levels.
- Fig 4.18**  $\beta$ -Adrenergic response to 25 min sustained global ischaemia after forskolin stimulation: Tissue cAMP levels.
- Fig 4.19**  $\beta$ -Adrenergic response to 25 min sustained global ischaemia after isoproterenol stimulation: Tissue cAMP levels.
- Fig 4.20** Functional recovery during reperfusion after  $\beta$ -adrenergic blockade: Experimental protocol.
- Fig 4.21** Functional recovery during reperfusion after  $\beta$ -adrenergic blockade: Aortic output and total work performance.
- Fig 4.22** Functional recovery during reperfusion after repeated pharmacological increases in cAMP: Experimental protocol.
- Fig 4.23** Functional recovery during reperfusion after repeated increases in cAMP with isoproterenol.
- Fig 4.24** Functional recovery during reperfusion after repeated increases in cAMP with forskolin.

- Fig 4.25** Effect of  $\beta$ -adrenergic manipulation on p38 MAPK activation during sustained ischaemia and reperfusion: Experimental protocol.
- Fig 4.26** Effect of  $\beta$ -adrenergic preconditioning with isoproterenol on p38 MAPK activation during sustained ischaemia and reperfusion.
- Fig 4.27** Effect of  $\beta$ -adrenergic blockade on p38 MAPK activation during sustained ischaemia and reperfusion.
- Fig 4.28** Comparison of dual phosphorylation and kinase activity of p38 MAPK in non-preconditioned, isoproterenol-preconditioned, ischaemic-preconditioned and alprenolol-treated hearts.

## Chapter 5

- Fig 5.1** Inhibition of cyclic increases in cGMP during the ischaemic preconditioning protocol: Experimental protocol.
- Fig 5.2** Manipulation of cGMP and cAMP levels during the PC protocol with ODQ and DMSO.
- Fig 5.3** Repetitive increases in cGMP by NO donors: Experimental protocol.
- Fig 5.4** Repetitive increases in cGMP by NO donors: Tissue cGMP levels.
- Fig 5.5** Effect of repetitive administration of NO donors on cAMP levels.
- Fig 5.6**  $\beta$ -Adrenergic receptor responsiveness after repeated NO stimulation: Experimental protocol.
- Fig 5.7**  $\beta$ -Adrenergic receptor responsiveness after repeated NO stimulation: Tissue cAMP levels.
- Fig 5.8** Manipulation of ischaemic-induced p38 MAPK activation by inhibition of NO-cGMP pathway.
- Fig 5.9** Mimicking ischaemic-induced p38 MAPK activation by stimulation of NO-cGMP pathway.
- Fig 5.10** Tissue cyclic nucleotide responses to 25 min sustained global ischaemia after prior manipulation of the NO-cGMP pathway: Experimental protocol.
- Fig 5.11** Tissue cAMP and cGMP levels after sustained ischaemia: effect of manipulation with guanylyl cyclase inhibitor, ODQ.
- Fig 5.12** Tissue cAMP and cGMP levels after sustained ischaemia: effect of manipulation with NO donors, SNAP and SNP.
- Fig 5.13** Effect of NO-cGMP pathway inhibition on p38 MAPK activation at the end of sustained ischaemia.

- Fig 5.14** Effect of NO-cGMP pathway stimulation on p38 MAPK activation at the end of sustained ischaemia.
- Fig 5.15** Effect of inhibition of the NO-cGMP pathway on functional recovery during reperfusion: Experimental protocol.
- Fig 5.16** Effect of ODQ pretreatment on functional recovery during reperfusion of non-PC and PC hearts.
- Fig 5.17** Effect of L-NAME pretreatment on functional recovery during reperfusion of non-PC and PC hearts.
- Fig 5.18** Effect of prior stimulation of the NO-cGMP pathway on functional recovery during reperfusion: Experimental protocol.
- Fig 5.19** Effect of prior stimulation of the NO-cGMP pathway on functional recovery during reperfusion.

## **Chapter 6**

- Fig 6.1** Effect of SB 203580, an inhibitor of p38 MAPK, pretreatment on non-preconditioned, ischaemic-preconditioned and isoproterenol perfused rat hearts: Experimental protocol.
- Fig 6.2** Effect of SB 203580 (1 $\mu$ M) on p38 MAPK activation induced by brief global ischaemia in the perfused rat heart.
- Fig 6.3** Effect of SB 203580 (1 $\mu$ M) on p38 MAPK activation induced by isoproterenol in the perfused rat heart.
- Fig 6.4** Functional recovery during reperfusion after 25 min global ischaemia of perfused rat hearts: Effect of inhibition of p38 MAPK.

## CHAPTER 1

### Literature Review

#### 1.1 Ischaemic preconditioning

##### 1.1.1 Introduction

Ischaemic preconditioning (PC) describes the powerful myocardial protection against ischaemic damage that follows a short episode of sub-lethal ischaemia. This was recently considered at a NHBLI (National Heart, Lung and Blood Institute) workshop to be the most potent form of myocardial protection against ischaemia and infarction yet described (Kloner *et al.*, 1998). Since the first observation in dogs (Murry *et al.*, 1986), the protective effect of PC has been demonstrated in rats, rabbits, pigs, as well as in humans (Kloner and Yellon 1994). Because of PC's potential clinical use its mechanism has been the subject of intense research during the last 15 years, with over 1500 papers published on the subject (Medline search of "ischemic preconditioning"). However, considerable controversy still exists regarding the mechanism whereby protection against ischaemia is elicited.

This phenomenon was first described by Murry *et al.* (1986): canine myocardium subjected to brief, transient episodes of coronary artery occlusion – too brief in themselves to result in myocyte death – exhibited an increased resistance to infarction caused by a subsequent sustained ischaemic insult. This original study described what is now referred to as "classic" or "early" ischaemic PC which, although powerful, only lasts 1 to 2 hours. It was subsequently discovered that the cardioprotection reappears 12 to 24 hours after the PC incident and although the benefits are less potent, they can last up to 3 days (Kuzuya *et al.*, 1993, Marber *et al.*, 1993). This second phase of protection is known as the "late" or "delayed" phase of ischaemic PC and is also referred to as the "second window of protection" (SWOP). However, in this study the focus will be on mechanisms involved in classic ischaemic PC.

Despite the obvious potential benefit of ischaemic PC, the risk of subjecting patients to ischaemia to elicit this protection remains. Furthermore, hearts of hypertensive (Moolman *et al.*, 1997) and diabetic rats (Kersten *et al.*, 2000) and pathological human myocardium (Ghosh *et al.*, 2001) did not precondition successfully with a PC protocol

that elicited protection in normal myocardial tissue. Since subjecting the heart to ischaemia, however short, it is clearly not ideal, the importance of establishing the mechanism of PC lies in the potential to convert this biological phenomenon into a therapeutic modality to be used clinically. If it is mediated by a ligand-receptor interaction or signal transduction intermediate, such a goal will be achievable. Furthermore, pharmacological manipulations could also be utilized to define the mechanism of PC protection.

## **1.1.2 Factors affecting classic preconditioning**

### **1.1.2.1 Duration of protection**

There are certain limits to the benefits of PC. As mentioned before, the protection afforded by brief episodes of ischaemia is transient and disappears after 1 to 2 hours of reperfusion and although it reappears after 12 to 24 hours, the latter protection is less potent. In addition, if the duration of prolonged (sustained) ischaemia exceeds 90 min (in some models), then the benefits of PC are lost (Nao *et al.*, 1990).

### **1.1.2.2 Triggering**

Obviously, the factor triggering protection is operative during the short periods of ischaemia or reperfusion of ischaemic PC. The majority of evidence suggests that it is the ischaemia and not the reperfusion that starts the PC cascade (Dekker 1998). For example: Ischaemic PC by partial occlusion (Koning *et al.*, 1994) or a brief episode of no-flow ischaemia (Schulz *et al.* 1995) without intermittent reperfusion, still provides protection against subsequent sustained ischaemia. On the other hand, there is a definite time frame for reperfusion between PC and sustained ischaemia: no protection occurs if reperfusion is less than 30 s in duration (Alkhulaifi *et al.*, 1993) or if the interval is prolonged beyond 1-2 h in most species (van Winkle *et al.*, 1991, Murry *et al.*, 1991, Sack *et al.*, 1993).

A PC regime of only 1 or 2 min of ischaemia with subsequent reperfusion prior to sustained ischaemia has a protective effect in rats (Takeshima *et al.*, 1997), but not in rabbits (van Winkle *et al.*, 1991), pigs (Schulz *et al.* 1998) and humans (Matsubara *et al.*, 2000). A single 5 min episode of global ischaemia provided optimal functional

protection in isolated perfused rat hearts (Moolman *et al.*, 1995, Cave and Hearse 1992, Asimakis *et al.*, 1992, Steenbergen *et al.*, 1993). Conversely, it has been reported that multiple episodes of PC are more protective than a single episode in the rat (Liu and Downey 1992, Moolman *et al.*, 1995) and rabbit heart (Sandhu *et al.*, 1997). In a regional ischaemic model the latter protocol protected against arrhythmias only and multiple episodes were necessary to limit infarct size (Liu and Downey 1992). However, increasing the number of cycles used to induce PC does not result in a cumulative increase in protection against necrosis (Walker and Yellon 1992, Jenkins *et al.*, 1995), whereas it increased protection against arrhythmias (Lawson and Hearse 1994, Seyfarth *et al.*, 1994). Furthermore, if the intensity of the PC stimulus exceeds a certain threshold by extending or too frequently repeating the period of ischaemia, the protective effect is lost (Iliodromitis *et al.*, 1997).

Classically, the stimulus for PC is a critical reduction of myocardial blood flow, and the end-point is a reduction in myocardial necrosis (infarct size) (Yellon *et al.*, 1998). However, PC is also induced by a reduction of coronary flow in buffer perfused (Cohen *et al.*, 1995), or hypoxic buffer perfused (Cohen *et al.*, 1995) and blood perfused hearts (Mei and Gross 1995). Furthermore, PC can also be induced in isolated cardiac muscle preparations when ischaemia is simulated by a combination of hypoxia, substrate free perfusion and pacing stress (Speechly *et al.*, 1995) and even in isolated cardiomyocytes subjected to transient hypoxia and lack of glucose substrate (Armstrong *et al.*, 1994).

### **1.1.2.3 Endpoint**

Many endpoints have been adopted to assess protection afforded by ischaemic PC, including limitation of necrosis or infarct size (Liu and Downey 1992, Speechly-Dick *et al.*, 1994, Bugge and Ytrehus 1995a,b, Suematsu *et al.*, 2001, Yue *et al.*, 2001), recovery of contractile function (stunning) (Cave 1995, Asimakis *et al.*, 1992, Moolman *et al.*, 1995), reduction in arrhythmias (Hagar *et al.*, 1991, Shiki and Hearse 1987) and reduction in cardiac enzyme release (Cave and Hearse 1992, Volovsek *et al.*, 1992). It is unknown whether the protection afforded by PC against stunning and arrhythmias is mediated by the same mechanism that mediates its protection against lethal cell injury (Connaughton *et al.*, 1996, Przyklenk and Kloner 1995). For example, the efficacy of ischaemic PC in reducing stunning (Ovize *et al.*, 1992) and arrhythmias (Ovize *et al.*, 1995) has not been as consistent as its ability to reduce necrosis. In addition,

ischaemic PC in rabbits protects from cell death and necrosis, but does not improve functional recovery (Asimakis *et al.*, 1996, Sandhu *et al.*, 1993). However, a lack of functional recovery during short-periods of reperfusion cannot be used to deny PC's morphological protection (anti-necrosis effect), because PC does not improve myocardial function within the first hours of reperfusion (thus it does not attenuate stunning) (Cohen *et al.*, 1999). In addition, indications are that improved recovery of function is more difficult to achieve than a reduction in infarct size (Lochner *et al.* unpublished data).

Thus, as long as the signal cascade of ischaemic PC is not clear, caution should be exercised when extrapolating findings from recovery of contractile function and protection against arrhythmias to reduction of infarct size. To a large extent, the use of different end-points may be responsible for the many controversial results that hampered the progress in the research on PC.

### **1.1.3 Possible signal transduction mediated mechanisms of preconditioning**

#### **1.1.3.1 Introduction**

The signalling cascades that initiate the two distinct phases of PC protection may have similar biochemical components. However, the protective effects of classic PC are protein synthesis-independent, mediated primarily by phosphorylation of key proteins, and therefore short-lived, whereas, the effects of late PC require new protein synthesis and could be sustained for days (Nandagopal *et al.*, 2001).

Studying the mechanisms of early and late ischaemic PC has led to a signalling paradigm that can be divided into three linear events: (i) triggers, i.e. factors released during the PC stimulus that initiate the protective process; (ii) mediators, i.e. protein kinases responsible for integrating and relaying the signal initiated by the triggers and modulating the (iii) effectors, which could confer myocardial protection (reviewed by Baines, *et al.*, 2001, Schulz *et al.*, 2001).

### 1.1.3.2 Triggers

It is generally accepted that PC is a receptor-mediated process, initiated by numerous possible triggers that could stimulate a variety of G protein-coupled receptors. For example, adenosine acting via A<sub>1</sub> and A<sub>3</sub> adenosine receptors, opioids acting mainly via  $\delta_1$  opioid receptors, bradykinin via B<sub>2</sub> receptors, acetylcholine via muscarinic receptors, angiotensin via AT<sub>1</sub> receptors, endothelin via ET<sub>1</sub>- receptors and noradrenaline acting via  $\alpha_1$ - and  $\beta$ -adrenergic receptors (for review see Dekker 1998, Yellon *et al.*, 1998, Cohen *et al.*, 2000, Schulz *et al.*, 2001). The importance of different triggers varies among different species; for example, in the rat opioid receptors have been suggested to be the principal ones involved in PC (Schultz *et al.*, 1998b), and it is difficult to show a role for adenosine (Li and Kloner 1993, Cave *et al.*, 1993) or bradykinin (Bugge and Ytrehus 1996), while in rabbits and pigs opioids, adenosine and bradykinin may all be importance.

A number of these ligands have been implicated as both triggers and mediators, including adenosine (Mullane *et al.*, 1995), acetylcholine (Yao *et al.*, 1993) and bradykinin (Goto *et al.*, 1995). These substances could also generate nitric oxide (NO) by activating the endothelially derived constitutive nitric oxide synthase enzyme (eNOS). This has implicated NO as a potential trigger and/or mediator (Rakhit *et al.*, 1999).

Receptor-independent endogenous triggers of PC also exist. For example, free radicals can also trigger PC since the administration of antioxidants during the brief triggering ischaemia has been found to prevent both early and late PC (Sun *et al.*, 1996). An alternative candidate for initiating PC is a pre-ischaemic mild increase of intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>): short episodes of ischaemia and reperfusion induce a loss of Ca<sup>2+</sup> from the sarcoplasmic reticulum and an accumulation of Ca<sup>2+</sup> in the cytosol (Kaplan *et al.*, 1992, Wu and Feher 1995). Furthermore, it was shown that a small increase of [Ca<sup>2+</sup>]<sub>i</sub> acts as mediator of PC (Miyawaki *et al.*, 1996) via activation of protein kinase C (PKC) (Miyawaki and Ashraf 1997a).

### 1.1.3.3 Mediators

#### 1.1.3.3.1 Protein kinase C (PKC)

It has been suggested that several triggers may be linked to a common final pathway by sharing a key protein, for example protein kinase C (PKC) (for review see Nakano *et al.*, 2000d, Cohen *et al.*, 2000). This has been demonstrated in rat (Cave and Apstein 1994, Speechly-Dick *et al.*, 1994, Mitchell *et al.*, 1995), rabbit (Ytrehus *et al.*, 1994, Armstrong *et al.*, 1994) and human studies (Ikonomidis *et al.*, 1997). However, a pivotal role for PKC could not be corroborated by all workers in the field (for review see Brooks and Hearse 1996, Simkhovich *et al.*, 1998), for example inhibition of PKC did not prevent cardioprotection in rat (Kolocassides and Galinanes 1994), rabbit (Simkhovich *et al.*, 1996), pig (Vahlhaus *et al.*, 1996) and dog hearts (Przyklenk *et al.*, 1995).

One of the key features of PKC activation is translocation of the kinase from the cytosol to membranes and cytoskeletal structures. Therefore, as long as PKC remains translocated, the heart will be in a preconditioned state. However, data supporting the translocation hypothesis are still controversial. For example, disruption of microtubules, which inhibits intracellular translocation, blocked PC in rabbits (Liu *et al.*, 1994), whereas a subsequent study in rabbit myocytes was unable to show translocation of PKC following PC (Armstrong *et al.*, 1996). In addition, it is proposed that PKC activity is not required during the period of PC, but may be essential only during the sustained ischaemia (Yang *et al.*, 1997).

Another confounding factor in examining the effects of PKC is the presence of 11 isozymes (Ping *et al.*, 1997). However, not all isozymes of PKC are translocated by PC. Ping *et al.* (1997) documented selective translocation of the PKC- $\epsilon$  and  $\eta$  isozymes following brief ischaemia and reperfusion in the rabbit, while Mitchell *et al.* (1995) reported movement of PKC- $\epsilon$  and  $\delta$  in preconditioned rat hearts. Recently Vondriska *et al.* (2001) used functional proteomics to confirm that PKC $\epsilon$  is the isoform involved in PC. Therefore measurement of total PKC translocation or activity simply does not reflect the subtle alterations in the subcellular distribution of specific isozymes.

Furthermore, evidence indicates that translocation is dependent on PKC binding to a family of proteins called receptors of activated C-kinase (RACKs) (Gray *et al.*, 1997).

The RACKs were considered to be highly isozyme-specific, i.e. for PKC $\epsilon$  it was thought to be RACK 2. This was confirmed by studying a peptide antagonist for RACK 2 (Johnson *et al.*, 1996), which blocked the ability of PC to protect both rat (Gray *et al.*, 1997) and rabbit (Liu *et al.*, 1999) cardiomyocytes.

Despite earlier controversies, it would seem that PKC is important in the context of PC, but new evidence indicate that it is certainly not the only kinase involved in the cascade of events during PC.

#### **1.1.3.3.2 Other kinases**

Although it is likely that PKC may be part of the pathway leading to protection of PC, it is still unclear what lies beyond PKC. Baines *et al.* (1998) showed that selective tyrosine kinase antagonists, genistein and lavendustin A, abolished protection induced by PMA, a direct activator of PKC, in rabbit hearts. Therefore tyrosine kinases appear to be downstream of PKC. However, in other species, tyrosine kinases may also be present in a second pathway that bypasses PKC. For example, in the pig (Vahlhaus *et al.*, 1996) and rat (Fryer *et al.*, 1999) heart antagonists of PKC or tyrosine kinase alone could not block protection from ischaemic PC. However if they were combined, protection was completely abolished. This suggests that these kinases may work through parallel pathways.

Potential downstream targets of PKC and tyrosine kinases are the mitogen-activated protein kinases (MAPKs). Each subfamily of the MAPKs, namely extracellular signal-regulated kinases (ERK 1/2), p46 and p54 c-Jun NH<sub>2</sub>-terminal kinases (JNK) and p38 MAPK, has been suggested to play a role in the cardioprotection achieved by ischaemic PC (refer to section 1.3, reviewed by Ping and Murphy 2000, Michel *et al.*, 2001). Therefore, these MAPKs may be possible contenders for the "final common pathway" of PC, specifically the stress-activated kinase p38 MAPK (Maulik *et al.*, 1996, Weinbrenner *et al.*, 1997).

### 1.1.3.4 Effectors

#### 1.1.3.4.1 Na<sup>+</sup>/H<sup>+</sup> exchanger

It is well-established that protons which are produced during ischaemia exit myocytes via the Na<sup>+</sup>/H<sup>+</sup> exchanger during ischaemia-reperfusion. The resulting Na<sup>+</sup> loading can increase ATP consumption by increasing Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during the initial phases of ischaemia. Subsequently, deleterious Ca<sup>2+</sup> loading occurs as Na<sup>+</sup>, in turn, leaves the cell via the Na<sup>+</sup>/Ca<sup>2+</sup> - exchanger (Piper *et al.*, 1996). The resulting rise in [Ca<sup>2+</sup>]<sub>i</sub> could trigger Ca<sup>2+</sup> -activated proteases and phospholipases that cause the cellular damage (Tani 1990, Pierce *et al.*, 1995).

On the basis of this mechanism, Xiao and Allen (1999) have suggested that the Na<sup>+</sup>/H<sup>+</sup> exchanger might be an end-effector of PC. They showed that PC of rat hearts slowed the rate of pH<sub>i</sub> recovery after ischaemia and blocked the Na<sup>+</sup>/H<sup>+</sup> exchanger during early reperfusion. This inhibition prevented Na<sup>+</sup> entry and Ca<sup>2+</sup> loading, which may be part of the protective pathways in PC. However, Bugge and Ytrehus (1995b) indicated that a reduced Na<sup>+</sup>/H<sup>+</sup> exchange might not be the only cause of protection, since the beneficial effects of classic PC and Na<sup>+</sup>/H<sup>+</sup> exchange inhibition seem to be partially additive. Furthermore, other data (for review see Avkiran 1999) do not support a role of the Na<sup>+</sup>/H<sup>+</sup> exchanger as the end-effector of PC, but rather suggest distinct pathways and mechanisms.

#### 1.1.3.4.2 Other potential end-effectors

The roles of the mitochondrial ATP sensitive potassium (KATP) channel (O'Rourke 2000) and downstream effectors of p38 MAPK, such as MAPKAPK2 and heat shock proteins, particularly HSP27 (Sugden and Clerk, 1998) are currently being investigated as end-effectors (Cohen *et al.*, 2000). Furthermore, in late PC, inducible nitric oxide (iNOS) and antioxidants, such as mitochondrial superoxide dismutase (MnSOD), may also act as effectors in cardioprotection (Baines *et al.*, 2001).

Therefore, with the increase in the knowledge of PC it became more apparent that there are different means within the cell to attain protection and it may even be possible that there is no "common pathway".

## **1.1.4 Metabolic changes in classic preconditioning**

### **1.1.4.1 Energy utilization**

Murry *et al.* (1990) pointed out that the preconditioned myocardium (in dogs) is characterized by a decrease in energy utilization (reduced ATP depletion and lactate accumulation) that may be related to delayed cell death. Studies in rat hearts, however, produced diverse results, with PC either reducing (Volovsek *et al.*, 1992, Vuorinen *et al.*, 1995), increasing (Schaefer *et al.*, 1995, Kolocassides *et al.*, 1996) or having no effect (Cave and Hearse 1992, Chen *et al.*, 1995) on the rate of ATP consumption during sustained ischaemia. The differences between these studies may relate to the PC procedure.

Conversely, the relatively small influence of classic PC on the rate of energy depletion during prolonged ischaemia is probably not sufficient to explain its cardioprotective effect (Yellon *et al.*, 1998). Therefore, the energy-sparing effect of PC might only be one of the possible mechanisms whereby cardioprotection is mediated.

### **1.1.4.2 Attenuation of acidosis**

Several observations support an important role of reduced catabolite accumulation in the protection of PC. In the isolated rat heart ischaemic PC attenuates acidosis (Asimakis *et al.*, 1992, Finegan *et al.*, 1995). During brief PC episodes of ischaemia, glycogen stores are depleted and lactate and protons accumulate. During the reperfusion periods following these episodes, accumulated catabolites are rapidly washed out (Kida *et al.*, 1991, Van Wylen 1994). The synthesis of glycogen proceeds at a much slower rate, and glycogen stores remain substantially depleted over a period of time, which coincides with the duration of classic ischaemic PC (Wolfe *et al.*, 1993). When glycogen-depleted myocardium is exposed to prolonged ischaemia, lactate accumulation is slowed (Kida *et al.*, 1991). The consequent attenuation of proton production is the main reason for the slower rate of progression of ischaemic acidosis in preconditioned myocardium. This effect is much more prominent than that on energy depletion, and the delay in pH reduction is more prolonged than the delay in ATP depletion (Steenbergen *et al.*, 1993). However, reduced lactate accumulation per se

does not explain the anti-infarct effect of classic PC. Glycogen-depleted myocardium can still be preconditioned, and washout of catabolites during ischaemia has no effect on infarct size (Sanz *et al.*, 1995).

Thus, reduced proton and catabolite accumulation during prolonged ischaemia are prominent effects of classic PC. However, it is not yet clear whether these metabolic changes are mere markers of PC or whether they are necessary for protection, because it is difficult to distinguish between cause and effect of certain observations.

## **1.2 The role of cyclic nucleotides in classic preconditioning**

### **1.2.1 Introduction**

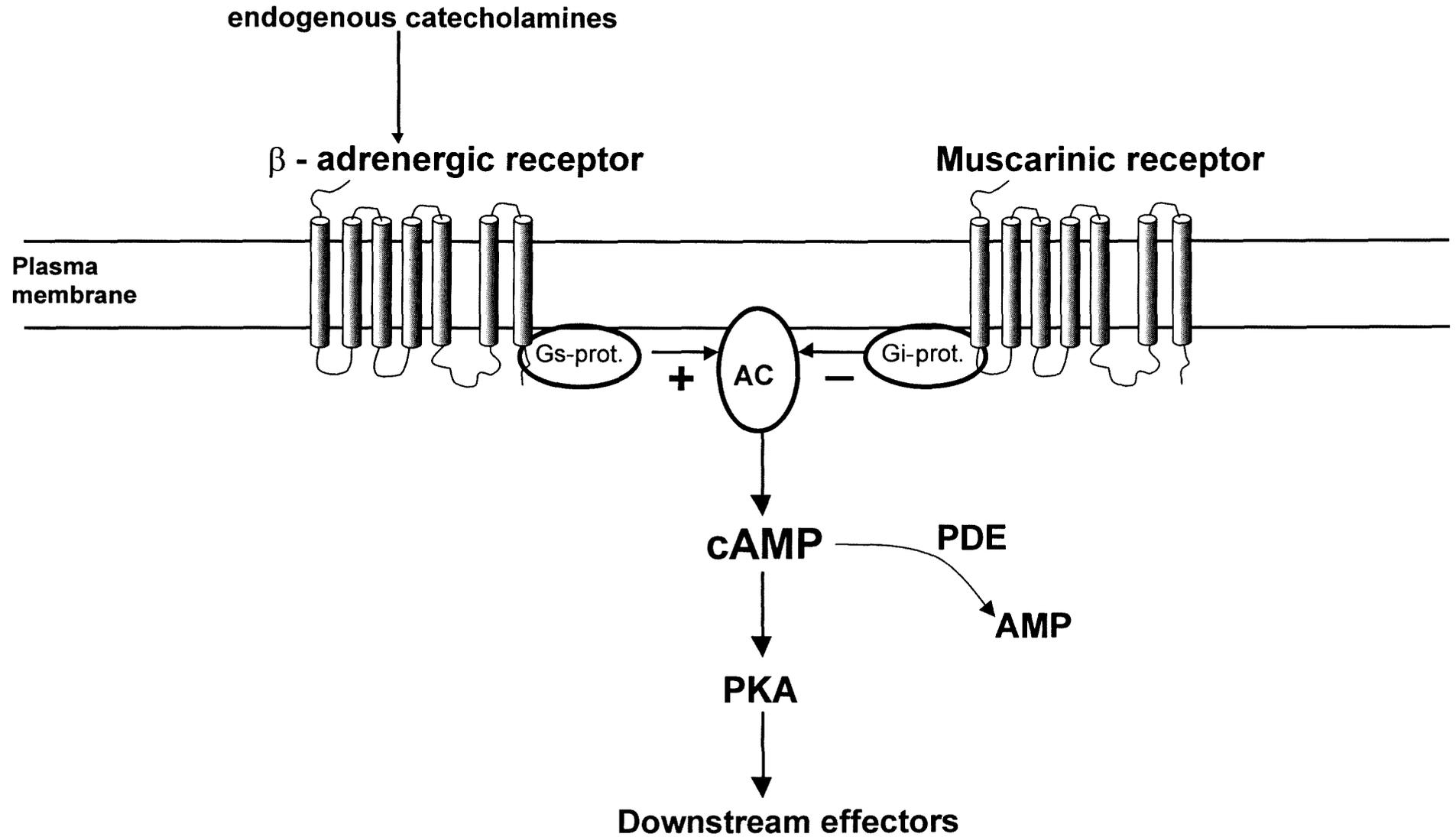
Despite the uncertainty regarding the exact mechanism whereby PC elicits protection, it is generally accepted that it is triggered by stimulation of a variety of G protein-coupled receptors (i.e. adenosine  $A_1$ ,  $\alpha_1$ -adrenergic, muscarinic, bradykinin receptors) with activation of protein kinase C as the common denominator in eliciting protection (reviewed by Brooks and Hearse, 1996, Baxter 1997, Simkhovich *et al.*, 1998). However, several other signal transduction pathways may also participate. For example, Banerjee *et al.* (1993) showed that catecholamine release occurs within minutes after the onset of myocardial ischaemia and this could trigger the  $\beta$ -adrenergic signal transduction pathway (Strasser *et al.*, 1990), whereas bradykinin release early in ischaemia has been accepted as a trigger for the release of nitric oxide (NO) (Parratt *et al.*, 1993). This suggests the possible involvement of these pathways in early PC. However, both these pathways have received little or no attention regarding their significance in the protection elicited by classic PC.

### **1.2.2 $\beta$ -Adrenergic signalling pathway and cyclic adenosine monophosphate (cAMP) (Fig. 1.1)**

#### **1.2.2.1 Catecholamines**

Catecholamines are released locally from the autonomic nerve endings in the myocardium, increasing the interstitial concentration of mainly noradrenaline, where it can bind and activate adrenergic receptors (Schömig *et al.*, 1984). Noradrenaline is measurable in the coronary effluent during reperfusion following brief myocardial ischaemia (Schömig *et al.*, 1984, Banerjee *et al.*, 1993) and could be a possible trigger for PC.

Ahlquist (1948) proposed that adrenergic stimulation interacted with two types of adrenergic receptors:  $\alpha_1$ -adrenergic and  $\beta$ -adrenergic. Convincing evidence has been presented for ischaemic PC-mediated noradrenaline release and subsequent  $\alpha_1$ -



**Fig. 1.1** The  $\beta$ -adrenergic signalling pathway. See text for details and abbreviations.

adrenergic receptor stimulation (Bankwala *et al.*, 1994, Banerjee *et al.*, 1993, Tosaki *et al.*, 1995, Thornton *et al.*, 1993, Banerjee *et al.*, 1993). However, previous findings from our laboratory (Moolman *et al.*, 1996) as well as others (Asimakis and Inners-McBride 1993, Bugge and Ytrehus 1995a) indicated that ischaemic PC was not mediated through  $\alpha_1$ -adrenergic receptors in the isolated rat heart. Therefore, the focus of this study will be on the involvement of the  $\beta$ -adrenergic signalling pathway in PC.

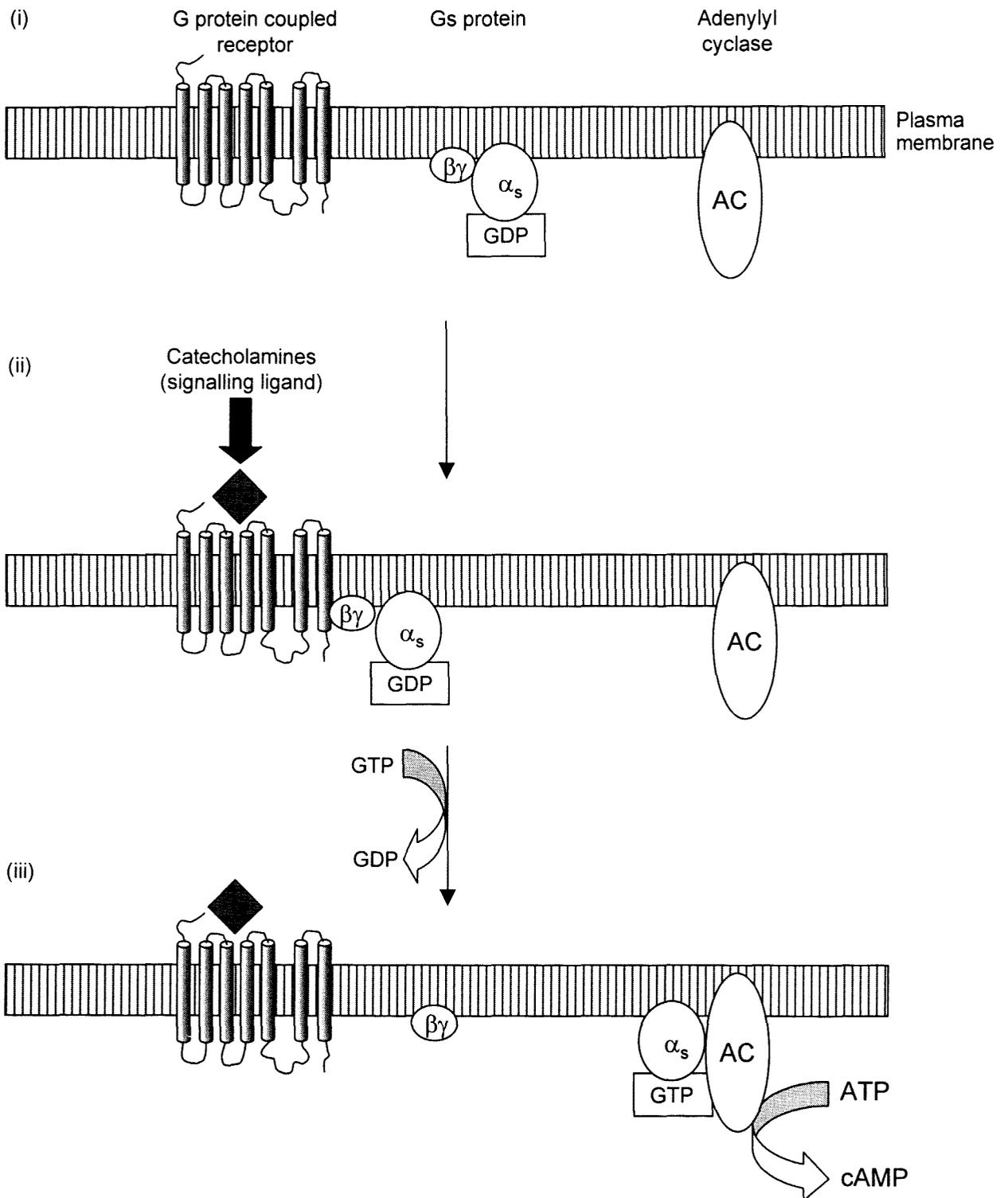
### 1.2.2.2 $\beta$ -Adrenergic receptors

The  $\beta$ -adrenergic receptor is a G-protein-coupled receptor, GPCR, with seven transmembrane-spanning segments. Studies have revealed three subtypes of  $\beta$ -adrenergic receptors in the heart, namely  $\beta$ -1,  $\beta$ -2 and  $\beta$ -3. The  $\beta$ -1 subtype usually predominates (~85%) in the mammalian and rat heart. The ratio of  $\beta$ -1 and  $\beta$ -2 differs in different regions of the human heart, where the percentage of  $\beta$ -1 is higher in the ventricle than the atrium (Buxton *et al.*, 1987). Furthermore, Shen *et al.* (1996) showed that  $\beta$ -3 adrenergic receptor stimulation was most profound in dogs, diminished but still significant in rats and absent in primates. Evidence was also provided for the expression of  $\beta$ -3 adrenergic receptors in human ventricles (Gauthier *et al.*, 1996).

### 1.2.2.3 Adenylyl cyclases (ACs) (Fig 1.2)

Binding of the ligands, such as noradrenaline (from the adrenergic nerve terminals in the heart) and adrenaline (from the adrenal gland), to the  $\beta$ -adrenergic receptor activates the GTP-binding regulatory proteins (or trimeric guanine nucleotide-binding proteins or G-proteins) containing the  $\alpha_s$  (or stimulatory) subunit. The  $\alpha$ -subunit of the Gs protein dissociates from the  $\beta\gamma$ -subunits and binds to the catalytic subunit of the enzyme adenylyl cyclase generating the second messenger cyclic adenosine monophosphate (cAMP) from ATP (Opie 1991, Alberts *et al.*, 1994).

Even though adenylyl cyclase isoforms are widely distributed, only types V and VI have been identified in ventricular heart tissue (Ishikawa and Homcy 1997). Expression of type VI is most abundant in the fetus, but declines with age. As all the other isoforms, these two types are also stimulated by G  $\alpha_s$ , but are not affected by calcium/calmodulin as opposed to the other types (Ishikawa and Homcy 1997).



**Fig. 1.2** A model of how Gs protein couples receptor activation to adenylyl cyclase activation. See text for details and definitions of terms and abbreviations. (modified from Alberts *et al.*, 1994)

A second G-protein,  $G_i$ , is responsible for inhibition of many isoforms of adenylyl cyclase, including type V and VI. For example, cholinergic stimulation of the muscarinic receptor, by coupling to  $G_i$  rather to  $G_s$ , exerts inhibitory influences on the heart at least in part by decreasing the rate of formation of cAMP (Opie 1998).

#### **1.2.2.4 Cyclic adenosine phosphate (cAMP)**

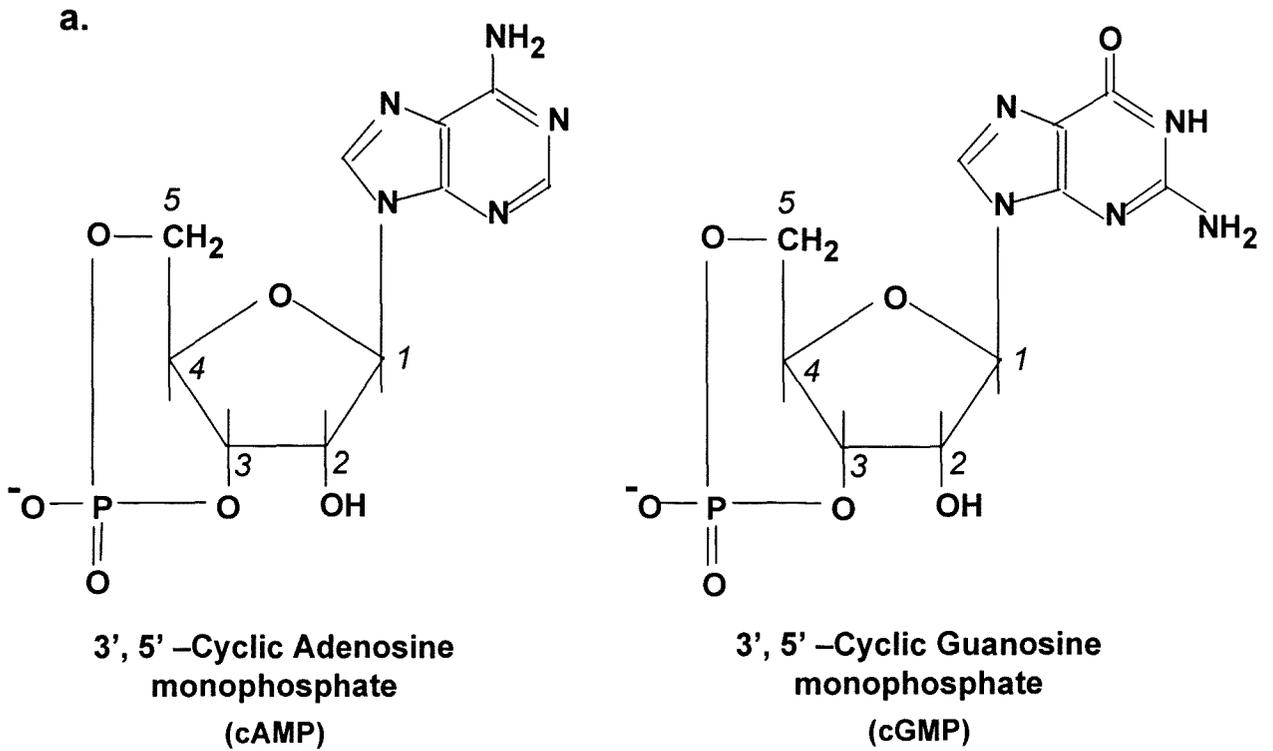
##### **1.2.2.4.1 Introduction**

Previously, it was shown that ventricular overdrive pacing (VOP) induced PC protection in anaesthetized rabbits (Szilvassy *et al.*, 1994). This was associated with alterations in cardiac cyclic nucleotide contents: a slight increase in cGMP and a profound elevation in cAMP within 5 min of VOP. When VOP was preceded by a brief preconditioning VOP, the cGMP increase was amplified, whereas the cAMP increase was significantly attenuated. Therefore, it may be of great importance to investigate the role of these cyclic nucleotides as triggers in the process of ischaemic PC.

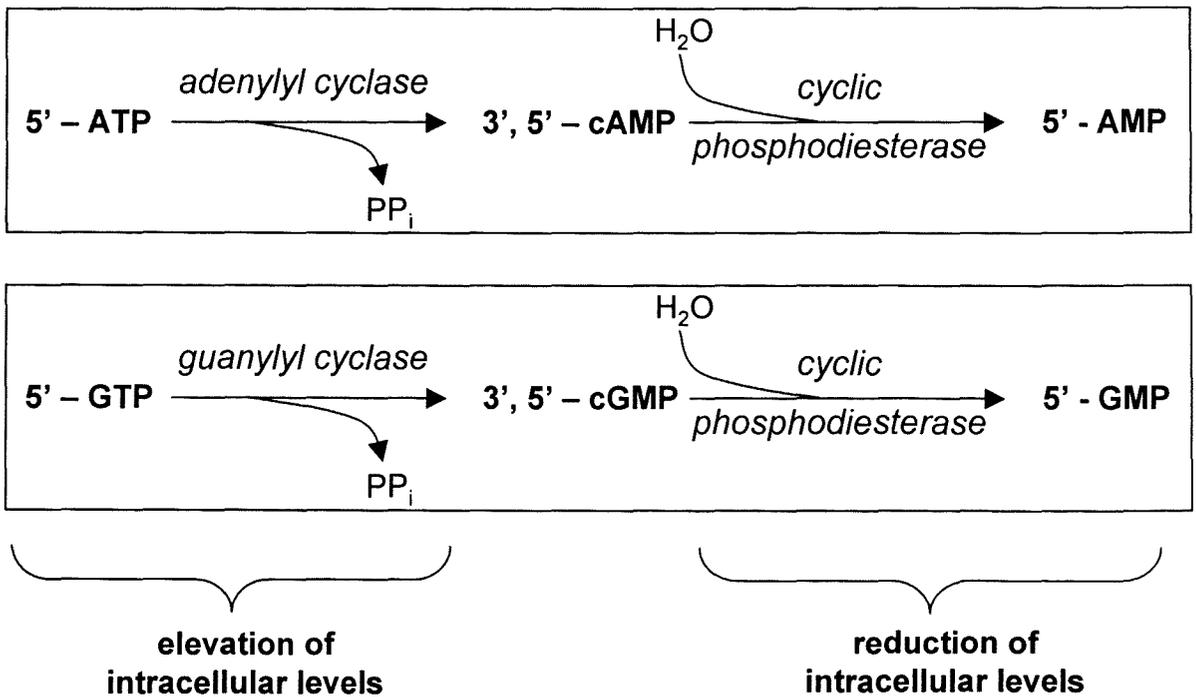
##### **1.2.2.4.2 Background**

The 3', 5'-cyclic adenosine monophosphate (cAMP) was first discovered as an intracellular mediator (second messenger) of hormone action, such as adrenaline, in 1959 by E. Sutherland (Nobel Prize, 1971). It has since been found to act as a second messenger in all procaryotic and animal cells studied (Pastan, 1972). Another cyclic nucleotide that has also been identified as a second messenger, was 3', 5'-cyclic guanosine monophosphate (cGMP). However, cGMP is proposed to act in the same manner as cAMP, but in reverse (see Fig. 1.3a, Bohinski 1983)

The cyclic nucleotides, cAMP and cGMP, are formed from the corresponding triphosphonucleotides by the enzymes adenylyl cyclase and guanylyl cyclase, respectively. The metabolic turnover of cyclic nucleotides is rapid and involves cyclic phosphodiesterases (PDEs), which catalyze the degradation of the cyclic diester on the 3' side, yielding the corresponding 5'-monophosphonucleotide (see Fig. 1.3b, Bohinski 1983).



**b.**



**Fig. 1.3 (a)** Molecular structure of cyclic nucleotides, cAMP and cGMP. **(b)** The mechanism of elevation or reduction of intracellular levels of cAMP and cGMP. See text for details. (modified from Bohinski, 1983).

#### 1.2.2.4.3 Mechanism of action of cAMP

The  $\beta$ -adrenergic effect on the heart is to increase the force and rate of contraction (increased chronotropy and inotropy, respectively) and to increase the rate of relaxation (lusitropic effect). These effects are mainly mediated by cAMP through the activation of a protein kinase, cAMP-dependent protein kinase (PKA) which phosphorylates, (i) the L-type  $\text{Ca}^{2+}$  channels with the consequent increased entry of  $\text{Ca}^{2+}$  through the plasma membrane and subsequent calcium-induced calcium release from the sarcoplasmic reticulum (SR). This leads to the increase of  $[\text{Ca}^{2+}]_i$  (intracellular free calcium ion concentration), causing an increase in force of contraction as  $\text{Ca}^{2+}$  interacts with troponin C (Gibbons 1986, Morad *et al.*, 1987, Opie 1998); (ii) the ryanodine receptor on the SR to release  $\text{Ca}^{2+}$  and increase  $[\text{Ca}^{2+}]_i$  (Valdivia *et al.*, 1995); (iii) phospholamban to enhance  $\text{Ca}^{2+}$  uptake by the SR ATPase pump, resulting in enhanced myocardial relaxation (Katz 1979); (iv) troponin-I to increase the rate of crossbridge detachment and relaxation (Stull 1980, Robertson *et al.*, 1982); and (v) the  $\text{Na}^+/\text{Ca}^{2+}$  exchange protein to stimulate the rate of forward  $\text{Na}^+/\text{Ca}^{2+}$  exchange which would increase  $\text{Ca}^{2+}$  extrusion resulting in a decline in  $[\text{Ca}^{2+}]_i$  and a faster relaxation (Perchenet *et al.*, 2000).

#### 1.2.2.4.4 Regulation of cAMP

An interesting aspect of the catecholamine-induced inotropic response is that it occurs transiently, even though the stimulus is maintained. Reasons for this phenomenon may be: (i) When cAMP increases in the myocardial cell, the enhanced cytosolic calcium concentration also activates calmodulin, which in turn enhances the activity of phosphodiesterases (PDEs), so that the rate of cAMP breakdown is increased. (ii) Also, the intense stimulation of the  $\beta$ -adrenergic receptor leads to activation of  $\beta$ -ARK and receptor downregulation that desensitizes the receptor to further stimulation (described in 1.2.3.5.2) (Opie 1998).

##### 1.2.2.4.4.1 Phosphodiesterases (PDEs)

Four different cAMP hydrolyzing PDEs have been shown to coexist in the heart muscle and are characterized by their substrate specificity and dependence towards calcium and calmodulin: (i) a PDE regulated by calcium/calmodulin (PDE1); (ii) a cGMP-

stimulated PDE (PDE2); (iii) a cGMP-inhibited PDE (PDE3); and (iv) a low  $K_m$  cAMP-specific PDE (PDE4) (Shahid 1990, Beavo 1995, Podzuweit *et al.*, 1995).

#### **1.2.2.4.4.1.1 PDE1 (calcium/calmodulin-stimulated cGMP PDE)**

This PDE is present in nonmyocyte cells of whole rat ventricles (Bode *et al.*, 1991). PDE1 is stimulated by calcium and calmodulin ( $Ca^{2+}/CaM$ ), and preferentially hydrolyses cGMP over cAMP (Bode *et al.*, 1991). However, the regulation of the overall cyclic nucleotide concentration by PDE1 isoform is expected to be complex because of the competition for the active  $Ca^{2+}/CaM$  cofactor between this PDE and other  $Ca^{2+}/CaM$ -binding proteins, such as protein phosphatases and constitutive nitric oxide synthases (NOSs) (Balligand 1999). Furthermore the activity of PDE1 is regulated by cross-talk between  $Ca^{2+}$  and cAMP signalling: PDE1 can be phosphorylated by PKA, resulting in a decrease in the enzyme's affinity for CaM, whereas the phosphorylation of PDE1 is blocked by  $Ca^{2+}$  and CaM (Sharma 1995).

#### **1.2.2.4.4.1.2 PDE2 (cGMP-stimulated PDE)**

PDE2 hydrolyses both cAMP and cGMP, but the addition of low concentrations of cGMP leads to enhanced cAMP breakdown (Bode *et al.*, 1991, Han *et al.*, 1998). It has been purified in bovine adrenal and heart tissue (Martins *et al.*, 1982). PDE2 contains two fundamentally distinct domains; (i) a non-catalytic binding site having high specificity for cGMP and when cGMP binds to this site, it increased the affinity of (ii) the catalytic site by allosteric interaction. This PDE is expressed in tissues in which the effects of cGMP are opposite to those of cAMP (Hartzell *et al.*, 1986): cGMP has negligible effects on basal L-type calcium current, but elevated intracellular cAMP via  $\beta$ -adrenergic agonists initiates the cGMP stimulation of PDE2 leading to cAMP hydrolysis and a decrease in L-type calcium current. PDE2 represented a minor component of the total hydrolytic activity (Lugnier *et al.*, 1999).

#### **1.2.2.4.4.1.3 PDE3 (cGMP-inhibited PDE)**

This PDE which selectively hydrolyses cAMP, was fractionated into two forms: one was inhibited by cGMP (PDE3) and the second was insensitive to cGMP (PDE4, see 1.2.2.4.4.1.4) (Komas *et al.*, 1989). Both these PDEs are insensitive to  $Ca^{2+}/CaM$

(Lugnier *et al.*, 1999). In the heart, such inhibition of PDE3 by cGMP would initially potentiate the increase in intracellular cAMP, thereby producing a positive inotropic effect maybe through an increase in L-type calcium current. However the net result of cGMP increases in the heart is difficult to predict, given the co-expression of both PDE2 and PDE3 isoforms in myocytes in rats (Bode *et al.*, 1991).

#### **1.2.2.4.4.1.4 PDE4 (cAMP-specific PDE)**

The PDE4, recognized in human cardiac muscle, was insensitive to cGMP and like PDE3, also insensitive to calmodulin (Reeves *et al.*, 1987) and has a high affinity for cAMP. PDE4 was localized on the plasma membranes of rat cardiac and endothelial cells, while PDE3 was localized on the plasma membrane of cardiac cells only (Okruhlicova *et al.*, 1996). However, Lugnier *et al.* (1999) suggested that most of the activity of these PDEs was associated with the nuclear membrane.

Verde *et al.* (1999) demonstrated the PDE3 and PDE4 are dominant PDE subtypes involved in the regulation of basal L-type calcium current in rat ventricular myocytes, while all four PDEs determine the response of L-type calcium current to a stimulus activating cAMP production, with the rank order of potency PDE4 > PDE3 > PDE2 > PDE1. Likewise, in the human myocardium PDE4 might contribute significantly to the regulation of intracellular cAMP when PDE3 is already inhibited or when the myocardium is under  $\beta$ -adrenergic receptor-mediated stimulation (Kajimoto *et al.*, 1997).

The factors involved to produce either a potentiation or an attenuation of the cAMP effects in cardiac cells, include the relative differences in affinity of this cyclic nucleotide between the isoforms of PDE and the localization of each isoform together with effector proteins for cAMP in the same subcellular compartment (Balligand 1999). For example; Dodge *et al.* (2001) demonstrated that muscle-selective A-kinase anchoring protein (mAKAP), maintains a cAMP signalling module, by including PKA and PDE4 in heart tissue. Furthermore, PKA activation stimulates mAKAP-associated PDE4 activity, whereas PDE4 activity reduced the activity of the anchored PKA. This suggests that the mAKAP signalling complex forms a negative feedback loop to restore basal cAMP levels.

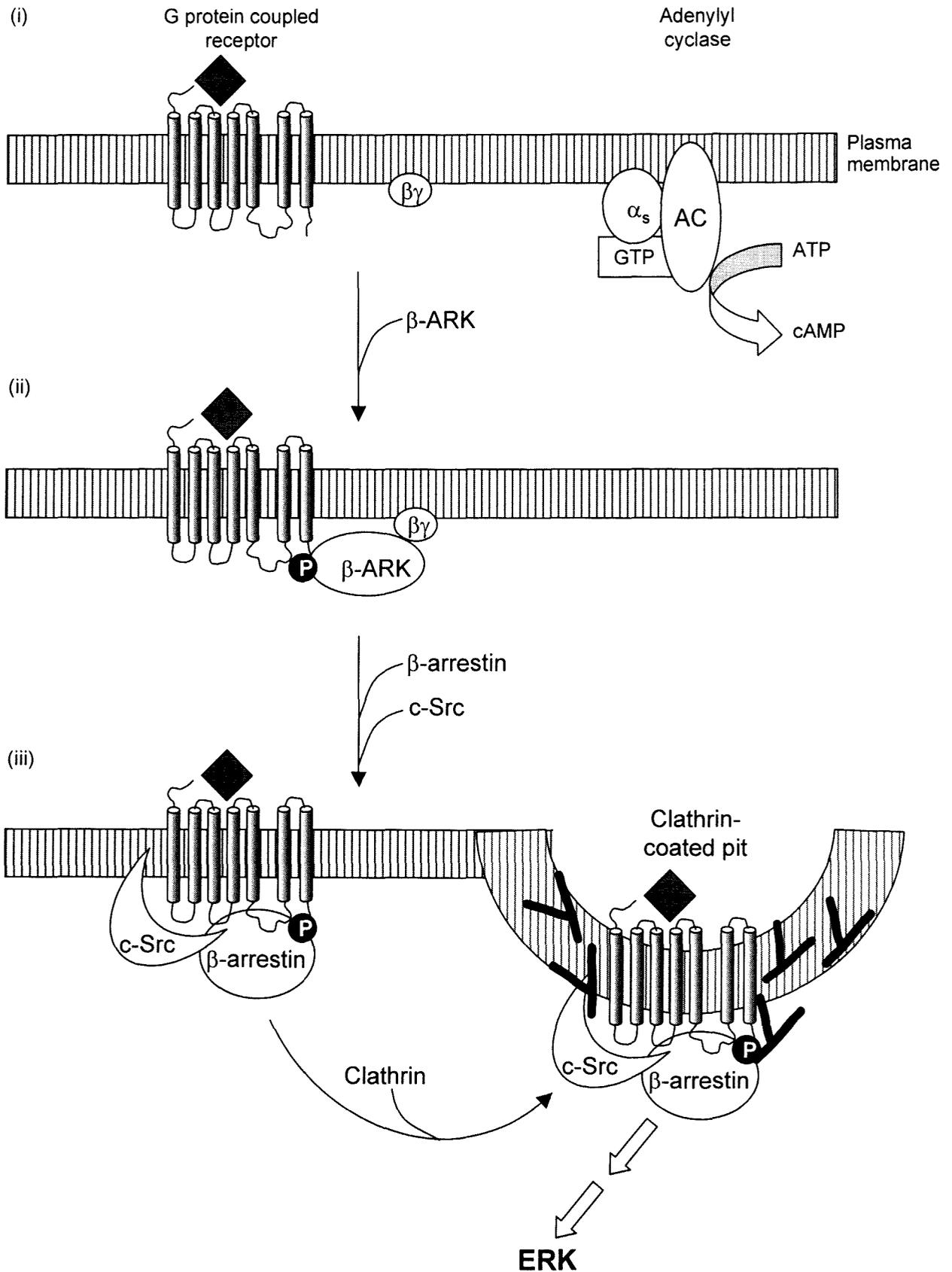
#### **1.2.2.4.4.2 Receptor desensitization (Fig. 1.4)**

##### **1.2.2.4.4.2.1 Loss of the receptor function**

Exposure of GPCRs to an agonist often results in rapid attenuation of receptor responsiveness, a process termed desensitization (Strasser 1989, Lefkowitz, 1990). There are three types of  $\beta$ -adrenergic receptor desensitization. (i) Uncoupling is initiated by prolonged  $\beta$ -agonist stimulation, which leads to activation of G-protein-coupled receptor kinase (GRK) or the  $\beta$ -adrenergic receptor kinase ( $\beta$ -ARK) (Lefkowitz, 1993). Phosphorylation of the cytoplasmic domain of the  $\beta$ -receptor by  $\beta$ -ARK appears to change the molecular configuration in such a way that the G-protein cannot interact with the receptor, leading to functional uncoupling. Many GPCRs are phosphorylated by second messenger-dependent kinases, such as PKA and PKC, on sites different from GRKs, which also leads to a significant loss of the receptor function (Lefkowitz *et al.*, 1990). (ii) The receptor may then be resensitized if it is dephosphorylated by a phosphatase and is then again be able to link to Gs. This process is aided by the fact that after the  $\beta$ -agonist stimulation is over,  $\beta$ -ARK ceases to be active (Opie, 1991). (iii) Alternatively, the receptor can be internalized by either sequestration or downregulation. In sequestration an internal vesicle forms that can be reincorporated into the cell membrane. On the other hand, during downregulation the receptor numbers decrease because there is degradation of the receptor, possibly by a lysosomal pathway. Downregulation results from exposure to high concentrations of agonist (Ungerer *et al.*, 1996, Opie, 1998).

##### **1.2.2.4.4.2.2 Extracellular mitogen-activated protein kinase (ERK) signalling**

Recent evidence suggests that GPCR desensitization and internalization are also signalling events. Desensitized  $\beta$ -adrenergic receptors could function as scaffolds for Src-dependent activation of ERK signalling pathways, while the  $\beta$ -arrestins function as adapter proteins that link G-protein coupled receptors to tyrosine kinase-dependent pathways (Luttrell *et al.*, 1999). In this case, binding of the  $\beta$ -adrenergic agonists to their G-protein linked receptors results in the rapid phosphorylation of the agonist-occupied receptor by the G protein-coupled receptor kinase (GRK) and the consequent recruitment of a protein known as  $\beta$ -arrestin to the GRK-phosphorylated receptor. In



**Fig. 1.4** A model of  $\beta$ -adrenergic receptor desensitization. Both  $\beta$ -arrestin-mediated recruitment and receptor targeting to clathrin-coated pits are required for  $\beta$ -adrenergic activation of the ERK pathway. See text for details and definitions of terms and abbreviations (modified from Luttrell *et al.*, 1999).

turn,  $\beta$ -arrestin functions as an adaptor protein, bringing Src to the agonist-occupied receptor, and targeting both to clathrin-coated pits. Both Src binding and clathrin-targeting are required for  $\beta_2$ -AR-mediated ERK activation.

### **1.2.2.5 $\beta$ -Adrenergic signalling and myocardial ischaemia**

#### **1.2.2.5.1 Catecholamines and ischaemia**

As mentioned before, endogenous catecholamines are released during ischaemia (1.2.2.1). Fleckenstein (1971) described how myocardial necrosis occurred in response to high doses of catecholamine, with a greatly increased uptake of calcium by the myocardium. Rona (1985) later confirmed that excess catecholamines could induce extensive injury, very similar to that caused by infarction, namely myofibrillar lysis, swollen mitochondria and disturbed cell morphology. This injury can be prevented by the  $\beta$ -adrenergic receptor blocker, propranolol or selected  $\text{Ca}^{2+}$  channel blockers administered simultaneously with the  $\beta$ -adrenergic receptor agonist, isoproterenol (Opie *et al.*, 1979). The catecholamine-induced injury appears to be the result of an interaction between intracellular  $\text{Ca}^{2+}$  overload, high energy phosphate depletion and "oxygen wastage" due to cAMP-mediated hydrolysis of triglyceride into free fatty acids and glycerol (Kjekhus 1975, Opie *et al.*, 1979). Furthermore, the harmful effects of excess cAMP are also well-established (Opie 1991). Even in the absence of ischaemia, elevations in cAMP have been shown to produce myocardial necrosis (Martorana 1971). High levels of cAMP are also known (i) to increase sarcolemmal  $\text{Ca}^{2+}$  entry (Tsien 1983) which in turn results in intracellular  $\text{Ca}^{2+}$  overload and (ii) to increase the activation of cardiac lipases (Opie 1982), effects known to aggravate ischaemic injury. Therefore an attenuation of increases in cAMP during sustained ischaemia would be expected to protect against necrosis. However, this is not always the case (1.2.2.5.3).

Adrenergic stimulation during ischaemia is also believed to be deleterious to the myocardium, both on a structural and electrophysiological level, leading to cellular damage and arrhythmias (Waldenstrom *et al.*, 1978). However, the elimination of the effect of catecholamines during ischaemia, such as the administration of  $\beta$ -blockers does not seem to limit infarct size, but only delay the development of infarction and arrhythmias (Opie and Lubbe 1979, Hearse *et al.*, 1986, Tsuchida *et al.*, 1993).

### 1.2.2.5.2 $\beta$ -Adrenergic receptor and adenylyl cyclase in ischaemia

Under normal physiological conditions, stimulation of the  $\beta$ -receptors is followed by their rapid inactivation and desensitization (Strasser 1989, Lefkowitz *et al.*, 1990). However, early myocardial ischaemia is characterized by dual sensitization of the  $\beta$ -adrenergic system (Strasser *et al.*, 1990), namely independent upregulation of the  $\beta$ -adrenergic receptors and increased adenylyl cyclase activity, which could occur via three distinct mechanisms: (i) An increase in number of functionally coupled  $\beta$ -adrenergic receptor at cell surface (Strasser *et al.*, 1988, Thandroyen *et al.*, 1990) due to the loss of high energy phosphates like ATP (Strasser *et al.*, 1988) or due to receptor synthesis by rapid upregulation of the mRNA levels of the receptor (Ihl-Vahl *et al.*, 1995). (ii) An impairment of catecholamine-promoted uncoupling and internalization (Strasser *et al.*, 1988), because the stimulatory Gs-protein remains coupled to the receptor, while the coupling to the inhibitory Gi-protein is rapidly impaired (Strasser *et al.*, 1990). (iii) The adenylyl cyclase is transiently sensitized during ischaemia: its activity increases rapidly after the onset of ischaemia (5 - 15 min) followed by a gradual loss of activity during sustained ischaemia (30 - 50 min) (Strasser *et al.*, 1990). The early sensitization of adenylyl cyclase may be due to a rapidly reversible covalent modification (Strasser *et al.*, 1990) or due to Gi-protein impairment and therefore removal of the inhibitory effect on adenylyl cyclase (Strasser *et al.*, 1990) or due to activation by PKC independently of  $\alpha_1$ -adrenergic receptors (Strasser *et al.*, 1992), while late inactivation of adenylyl cyclase occurred independently of the  $\beta$ -adrenergic receptor activation (Strasser *et al.*, 1990). Therefore the persistent sensitization at the receptor level then meets an unresponsive adenylyl cyclase.

### 1.2.2.5.3 cAMP and ischaemia

It is well known that ischaemia causes an increase in tissue levels of cAMP, the second messenger of the  $\beta$ -adrenergic system (Wollenberger *et al.*, 1969). Strasser *et al.* (1990) reaffirmed that cAMP levels increased within 10 min after onset of ischaemia in the rat myocardium, reaching maximum values after 20 min of severe ischaemia. This may be linked to an increased tendency to ventricular arrhythmias at the onset of a heart attack (Lubbe *et al.*, 1992). Stimulation of the sensitized  $\beta$ -adrenergic receptor by locally released endogenous catecholamines (as mentioned before) may contribute to

this phenomenon, but results on this issue are inconclusive: both Rabinowitz *et al.* (1975) and Krause *et al.* (1983) found that the increase in tissue cAMP after coronary occlusion and aortic clamping, respectively, was prevented by blocking the  $\beta$ -adrenergic receptor with a  $\beta$ -blocker, such as propranolol. Therefore their results support the notion that the increase in cAMP was attributed to the sensitization or the increase in  $\beta$ -adrenergic receptor density. In contrast, Muller *et al.* (1986) and Lubbe *et al.* (1981) showed that  $\beta$ -adrenergic receptor blockade had no effect on the increase in tissue cAMP levels of the ischaemic area in pigs and rat hearts, respectively.

There may also be other reasons for the increase in tissue cAMP in the ischaemic myocardium. One possibility is the formation of lysophospholipids, such as lysophosphatidyl choline, during ischaemia that may activate adenylyl cyclase (Ahumada *et al.*, 1979). Another possibility is the inactivation of myocardial phosphodiesterases in the pig heart, which may be caused by acidosis during ischaemia (Podzuweit *et al.*, 1994, 1996). However, the role of these factors in the increase in cAMP in the ischaemic myocardium has not yet been elucidated.

### **1.2.2.6 The role of adrenergic signalling in preconditioning**

#### **1.2.2.6.1 Catecholamines in preconditioning**

As endogenous catecholamines are released during short episodes of ischaemia (Schömig *et al.*, 1984), it is possible that it may play a triggering role in ischaemic PC. However, indications are that the release thereof only occurs after 10 min of ischaemia (Schömig *et al.*, 1984). On the other hand, Banerjee *et al.* (1993) showed that catecholamines were released within 2 min of transient global ischaemia, although this amounted to less than 3% of the total estimated quantity of rat heart noradrenaline. Therefore, it is possible that either no or minute quantities of catecholamines are released during the brief episodes of ischaemia associated with PC.

To investigate the role of catecholamines in ischaemic PC, Vander Heide *et al.* (1993) depleted dog heart of endogenous catecholamines with reserpine. They found that the infarct size in reserpinated dogs was slightly smaller than untreated animals, though not significantly. Furthermore, ischaemic PC could limit infarct size in both treated and untreated animals. Therefore they concluded that endogenous catecholamines might

not be required to mediate the cardioprotective effect of PC. In addition, studies in rat (Weselcouch *et al.*, 1995a, Moolman *et al.*, 1996a) and rabbit (Ardell *et al.*, 1994) hearts could also not provide any evidence for the involvement of endogenous catecholamines. In contrast, Banerjee *et al.* (1993) and Toombs *et al.* (1993) showed that ischaemic PC in the rat and rabbit heart, respectively, was abolished by depletion of endogenous catecholamines by prior reserpination.

Although Seyfarth *et al.* (1996) did not observe catecholamine release during the cycles of transient ischaemia in rat hearts, these ischaemic episodes reduced catecholamine release during sustained ischaemia by protecting the neural tissue as it does myocardial tissue.

#### **1.2.2.6.2 $\alpha_1$ -Adrenergic signalling in preconditioning**

Convincing evidence has been presented for ischaemia-mediated noradrenaline release and subsequent  $\alpha_1$ -adrenergic receptor stimulation and PKC activation (1.1.3.3.1): administration of  $\alpha_1$ -agonists before sustained ischaemia elicit PC in rat (Banerjee *et al.*, 1993, Tosaki *et al.*, 1995) or rabbit (Bankwala *et al.*, 1994, Tsuchida *et al.*, 1994) while  $\alpha_1$ -adrenoceptor antagonists in rat (Banerjee *et al.*, 1993) or rabbit (Thornton *et al.*, 1993a) or PKC blockers in rat (Cave and Apstein 1994, Speechly-Dick *et al.*, 1994, Mitchell *et al.*, 1995) or rabbit (Ytrehus *et al.*, 1994, Armstrong *et al.*, 1994) have been shown to abolish PC. Although investigators like Bugge and Ytrehus (1995a) indicated that ischaemic PC in rat was not mediated through  $\alpha_1$ -adrenergic or adenosine receptors, they found that it was still dependent on the activation of PKC. Furthermore, Weinbrenner *et al.* (1993) showed that a brief episode of ischaemia (2 – 5 min) caused rapid translocation of PKC and although Strasser *et al.* (1992) found this to be  $\alpha_1$ -independent, it is possibly involved in sensitization of the adenylyl cyclase system. However, our laboratory could not find any evidence of either  $\alpha_1$ -adrenergic or PKC activation in the mechanism of ischaemic PC in rat hearts (Moolman *et al.*, 1996). Similar negative findings were reported by others: Asimakis and Inners-McBride (1993) failed to demonstrate involvement of  $\alpha_1$ -adrenergic receptor stimulation in the attenuated postischaemic contractile dysfunction associated with ischaemic PC. Likewise, inhibition of PKC did not prevent cardioprotection in rat (Kolocassides and Galinanes 1994), rabbit (Simkhovich *et al.*, 1996), pig (Vahlhaus *et al.*, 1996) and dog hearts (Przyklenk *et al.*, 1995).

Thus there is not yet clarity about the involvement of the  $\alpha_1$ -adrenergic receptor signalling pathway in ischaemic PC. However, the differences in end-points or PC protocols could be responsible for these discrepancies. For example, although both Banerjee *et al.* (1993) and Asimakis and Inners-McBride (1993) evaluated the effect of  $\alpha_1$ -agonists before sustained ischaemia in rats, their results were contradictory. This is probably due to use of different end-points *viz* infarct size reduction vs. functional recovery, respectively. Since these two end-points may be mediated by different mechanisms, results from one model should not be extrapolated to the other. Furthermore, Fryer *et al.* (1999) indicated that multiple cycles of PC could overcome the abolishment of protection by PKC inhibitors in rats. The discrepancy between the results obtained with PKC inhibitor in a one-cycle and a multi-cycle ischaemic-PC protocol may thus be due to the fact that the latter provides more effective protection than a one-cycle protocol and is less susceptible to blockade by inhibitors (Sandhu *et al.*, 1997). It is now well-established that several receptor systems contribute to triggering ischaemic-PC and one particular inhibitor often fails to inhibit protection (Cohen *et al.*, 2000).

Since catecholamines also have the capacity to stimulate the  $\beta$ -adrenergic receptors and its signal transduction pathway, it should also be considered as an additional trigger of the PC process.

#### **1.2.2.6.3 $\beta$ -Adrenergic signalling and preconditioning**

Apart from one study by Asimakis *et al.* (1994), showing that transient  $\beta$ -adrenergic receptor stimulation by noradrenaline could mimic PC, the involvement of the  $\beta$ -adrenergic receptor as a trigger in classic PC has received little or no attention thus far.

Although, Fu *et al.* (1993) have shown that adenylyl cyclase activity of the preconditioned segment was not different from those of the non-preconditioned segment in pig myocardium, Strasser *et al.* (1992) convincingly demonstrated that acute myocardial ischaemia induces a rapid increased activity of adenylyl cyclase after the onset of ischaemia (5-15 min) and this is rapidly reversible on reperfusion in isolated rat hearts. Preservation of adenylyl cyclase activity and improvement in the functional activity of the stimulatory G-protein (Gs) have been shown to occur in preconditioned

rabbit hearts (Iwase *et al.*, 1993). On the other hand, it has been shown that the activity of the inhibitory G-protein (Gi) was enhanced in PC (Niroomand *et al.*, 1995), an effect that would be expected to decrease cAMP. Previously, our laboratory (Moolman *et al.*, 1996) also indicated that prior PC significantly attenuated cAMP accumulation during sustained ischaemia in isolated rat hearts. It is not known whether this is the cause or effect of prior protection to ischaemic damage. Sandhu *et al.* (1996) reported that PC did not affect the receptor responsiveness in the *in vivo* rabbit model, but mediated attenuation in noradrenaline release resulting in a reduction in cAMP levels during sustained ischaemia. However, they found that attenuation of the rise in cAMP levels with PC during sustained ischaemia was not necessary for its protection against necrosis, since raising cAMP via an activator of adenylyl cyclase, NKH477, did not block the protection of a multi-cycle PC protocol. They subsequently showed that when PC was induced with only a single cycle of transient ischaemia and reperfusion, raising cAMP levels with NKH477 produces a block of PC (Sandhu *et al.*, 1997). Therefore, a multi-cycle ischaemic-PC protocol may provide a more effective protection than a one-cycle protocol and is less susceptible to pharmacological manipulation (Sandhu *et al.*, 1997).

Thus, although the  $\beta$ -adrenergic signal transduction system has been implicated in the phenomenon of PC, results are controversial. Furthermore, both the role of the  $\beta$ -adrenergic receptor-adenylyl cyclase system and the changes in tissue cAMP levels during the PC protocol were still undefined at the initiation of our studies. In addition, changes in the downstream events of the  $\beta$ -adrenergic-cAMP pathway, involved in PC, are still unknown.

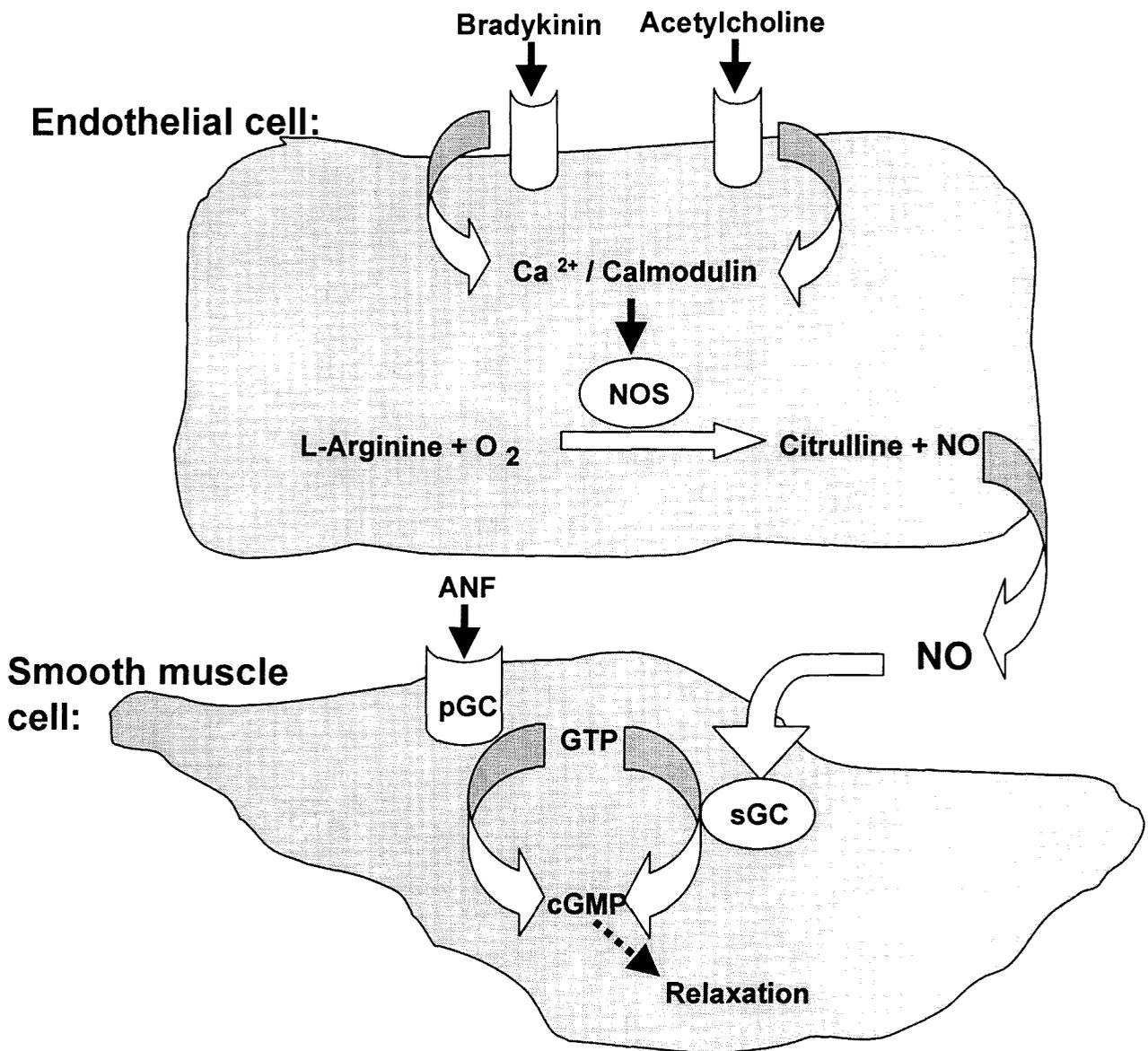
### **1.2.3 Nitric oxide (NO)- cyclic Guanosine Monophosphate (cGMP) pathway**

#### **1.2.3.1 Introduction**

It has been proposed that nitric oxide (NO), a gas known to give rise to the formation of cGMP, may play a role in ischaemic PC. Parratt and colleagues concluded that NO was involved in pacing-induced PC against arrhythmias in dog heart (Parratt, 1994, Kis *et al.*, 1999). The involvement of NO in the late phase of protection seen 24 hr after ischaemic PC has been the focus of intense investigation. Bolli *et al.* (1998) recently postulated a dual role for NO in this scenario: initially a trigger and subsequently as mediator of protection. They proposed that triggering the development of late PC on *day one*, involves generation of NO after a brief ischaemic stress (probably via endothelial nitric oxide synthase, eNOS) (Bolli *et al.*, 1997) and subsequent activation of PKC (Ping *et al.*, 1999c). The latter could in turn trigger a complex signalling cascade that involves protein tyrosine kinases (Imagawa *et al.*, 1997). Furthermore, the cardioprotective effects of late PC observed on *day two* might be due to upregulation of inducible NOS (iNOS) (Takano *et al.*, 1998a, Takano *et al.*, 1998b). On the other hand, the role of NO in early or classic PC is currently unclear, mainly because it was recently shown that NO is not a trigger or mediator of classic PC against infarction in either rabbits (Nakano *et al.*, 2000) or pigs (Post *et al.*, 2000).

#### **1.2.3.2 Nitric oxide (Fig. 1.5)**

Furchgott and Zawadzki (1980) made the fundamental discovery that acetylcholine, the neurotransmitter of the cholinergic nervous system, dilated healthy blood vessels but caused vasoconstriction when the endothelium was damaged, which led to the discovery of an endothelial-derived relaxing factor (EDRF). Moncada and colleagues later found EDRF to be nitric oxide (NO), an extremely short-lived free-radical (Palmer *et al.*, 1987, Ignarro *et al.*, 1987). Since then it has become clear that NO also play an important role in cardiovascular disease. Although initially characterized in the vasculature, NO is also present in heart muscle (Schulz *et al.*, 1992), where it is involved in regulation of cardiac function (Balligand 1999).



**Fig. 1.5** Regulation of contractility of arterial smooth muscle by nitric oxide (NO) and cGMP. See text for details and definitions of terms and abbreviations (modified from Moncada and Higgs 1993, Lowenstein *et al.*, 1994).

Nitric oxide (NO) and citrulline production, via a live-electron oxidation of a guanidino nitrogen from L-arginine, are catalyzed by NO synthase (NOS). The latter consists of several isoforms, such as endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS or NOS2). Two of these (eNOS and nNOS) are constitutively expressed and activated by  $\text{Ca}^{2+}$  and release small amounts of NO for brief periods to signal adjacent cells. The other, iNOS, is transcriptionally regulated by a variety of inflammatory cytokines, and releases large amounts of NO (Schmidt *et al.*, 1993, Lowenstein *et al.*, 1994). In addition, in cardiac myocytes the activation of the extracellular mitogen-activated protein kinase 1 and 2 (ERK1/2) may be required for induction of iNOS by cytokines (Singh *et al.*, 1996 and see MAPKs ).

### **1.2.3.3 Metabolism of cGMP**

Although cGMP was discovered more than thirty years ago, its role as a second messenger has long been overshadowed by that of cAMP (Fig. 1.3). In the heart muscle, cGMP could oppose the inotropic action of catecholamines after  $\beta$ -adrenergic stimulation (Balligand and Cannon, 1997). Cyclic GMP has a well-established role as vasodilatory messenger in vascular smooth muscle, in contrast to its still controversial role in the myocardium.

#### **1.2.3.3.1 Guanylyl cyclase (GC) (Fig. 1.5)**

Synthesis of cGMP is catalyzed by two types of guanylyl cyclases: a soluble cytosolic (sGC) and a particulate transmembrane form (pGC). The latter has a ligand-binding domain on the outer surface of the plasma membrane for interaction with certain peptide hormones, such as atrial natriuretic factor (ANF) (Vaandrager and De Jonge 1996). Binding of the ligand to this receptor promotes the catalytic activity of the intracellular guanylyl cyclase cytosolic domain, leading to formation of cGMP from GTP.

Furthermore, soluble guanylyl cyclases (sGC) are activated several hundred-fold by nitric oxide (NO). These enzymes are heterodimers and contain a bound heme molecule that interacts with both subunits. Binding of NO to the heme leads to a conformational change in the enzyme and stimulates its catalytic activity, leading to conversion of GTP to cGMP (Schmidt *et al.*, 1993, Vaandrager and De Jonge 1996).

### 1.2.3.3.2 Phosphodiesterases (PDEs)

PDE1 ( $\text{Ca}^{2+}$ /calmodulin-sensitive PDE, described before) and PDE5 (cGMP-specific PDE) contribute largely to the hydrolysis of cGMP, both in the presence and absence of  $\text{Ca}^{2+}$ /calmodulin, respectively. The cGMP-binding cGMP-specific phosphodiesterase (PDE5), which is very specific for cGMP hydrolysis, was first purified from rat lung (Francis *et al.*, 1980). Selective inhibition of PDE5 had no effect on cardiac function, suggesting that this isoform is not important in the rat myocardium (Cohen *et al.*, 1996). In fact, there was no detectable level of PDE5 in the heart (Wallis *et al.*, 1999). However, PDE5A mRNA genes are produced in aortic smooth muscle cells, heart, placenta, skeletal muscle and pancreas and to a much lesser extent in brain, liver and lung of humans (Loughney *et al.*, 1998). The major PDE activity in the human cardiac ventricle was shown to be PDE1.

In addition to its cGMP-selective catalytic site, PDE5 contains two allosteric cGMP-binding sites and at least one phosphorylation site (Ser92) on each subunit (Thomas *et al.*, 1990). Both cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) catalyse the phosphorylation of PDE5, causing an increase in enzyme activity and also increased affinity of cGMP for the allosteric cGMP-binding site. Considerably higher concentrations of PKA than of PKG are required for activation. Furthermore, phosphorylation of PDE5 by PKG could be involved in a physiological negative-feedback regulation of cGMP in bovine lungs (Corbin *et al.*, 2000).

### 1.2.3.4 Effect of NO-cGMP pathway

#### 1.2.3.4.1 In vascular smooth muscle (Fig. 1.5)

The contractility of arterial smooth muscles is regulated by NO and cGMP. Receptor activation by bradykinin or acetylcholine results in an influx of  $\text{Ca}^{2+}$  into endothelial cells lining blood vessels. After  $\text{Ca}^{2+}$  binds to calmodulin, the resulting complex stimulates the activity of eNOS. The NO that is synthesized diffuses from endothelial cells and activates soluble guanylyl cyclase in nearby smooth muscle cells. The resulting rise in cGMP mediates inhibition of voltage dependent L-type  $\text{Ca}^{2+}$  channels. This results in a reduction of cytosolic calcium that leads to the relaxation of the muscle and vasodilation (Moncada and Higgs 1993, Ishikawa *et al.*, 1993, Lowenstein *et al.*, 1994).

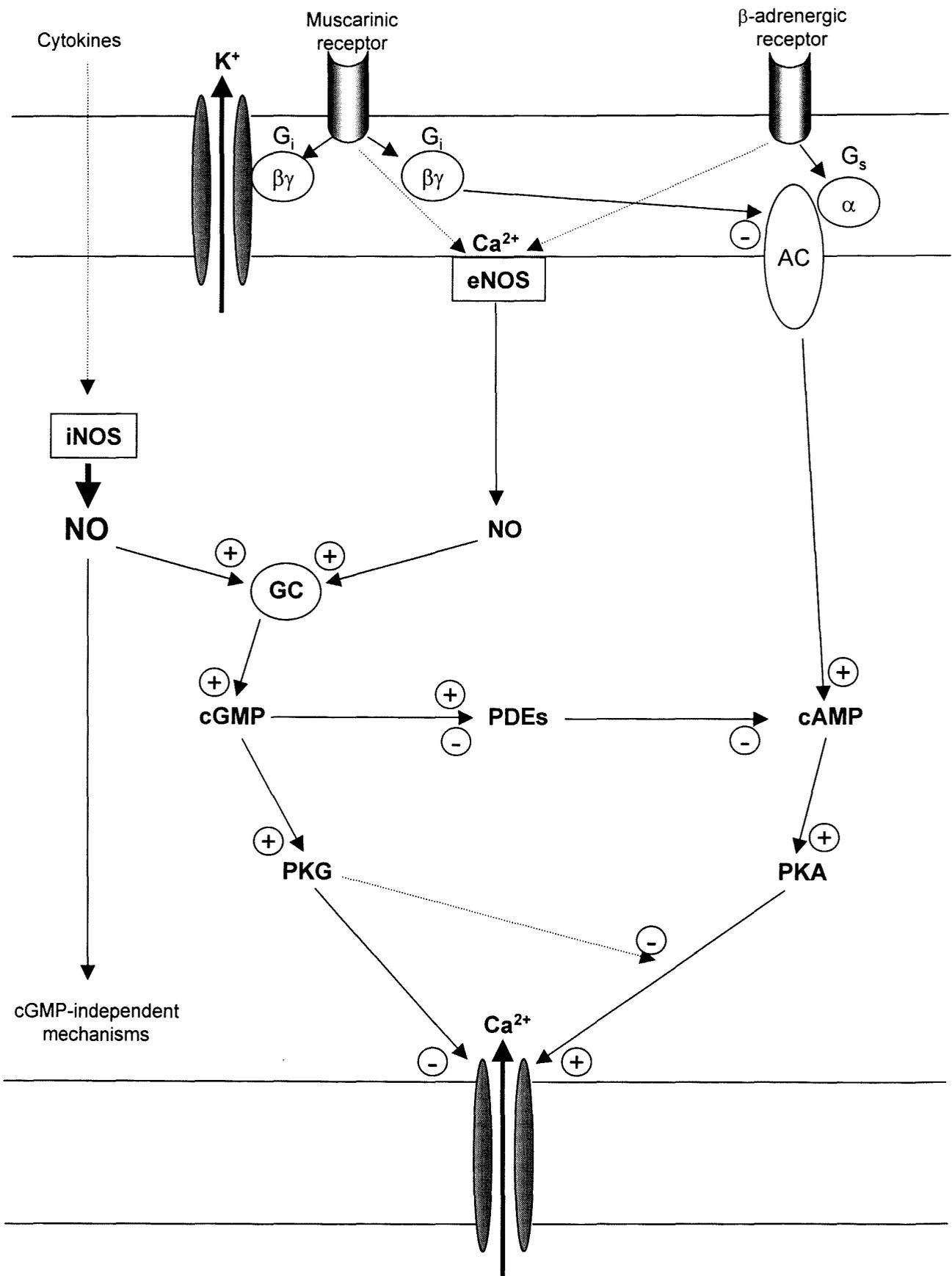
Furthermore, smooth muscle relaxation can also be mediated by  $K^+$  channels via both cAMP and cGMP after agonist stimulation, such as  $\beta$ -adrenergic agents and ANF, respectively. These activate the cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), respectively, which in turn phosphorylate  $K^+$  channels, thus hyperpolarizing the cell membrane. This hyperpolarization inactivates the L-type  $Ca^{2+}$  channels, resulting in decreased  $Ca^{2+}$  influx and subsequent relaxation (Walsh 1993, Somlyo and Somlyo 1994).

#### 1.2.3.4.2 In myocardium (Fig. 1.6)

cGMP has several intracellular targets, including cGMP-gated ion channels (L-type  $Ca^{2+}$  channels), a cGMP-activated phosphodiesterase (PDE2), a cGMP-inhibited phosphodiesterase (PDE3) and a cGMP-dependent protein kinase (PKG) (Butt *et al.*, 1993, Schmidt *et al.*, 1993, Vaandrager and De Jonge 1996).

Cyclic GMP may stimulate PKG to decrease heart rate and to have a negative inotropic effect (Lohmann *et al.*, 1991). Such formation of cGMP could occur in response to either cholinergic stimulation or activity of the NO pathway in the cardiac myocyte. These mechanisms are not mutually exclusive. Cholinergic stimulation induces the activity of the myocardial constitutive nitric oxide synthase (eNOS) to increase formation of NO (Kelly *et al.*, 1996). However, whether the myocardial NO system plays a physiological role in the myocardium is still controversial.

Balligand (1999) proposed a mechanism by which NO could regulate cardiac function (see Fig. 1.6): As described before,  $\beta$ -adrenergic stimulation produces a positive inotropic effect through  $G\alpha_s$ -coupled activation of adenylyl cyclase (AC), leading to increases in intracellular cAMP. Subsequent activation of PKA leads to downstream phosphorylation of target proteins, including those on the L-type  $Ca^{2+}$ -channel. The resultant increased influx of  $Ca^{2+}$  activates intracellular  $Ca^{2+}$ -induced  $Ca^{2+}$  release and enhances myofibrillar contraction. Inducible NOS (iNOS) gene transcription and protein expression is induced in cardiac myocytes on exposure to cytokines or other inflammatory mediators. Direct eNOS activation in cardiac myocytes occurs in response to either muscarinic cholinergic or adrenergic receptor stimulation through unidentified mechanisms. NO produced by either eNOS or iNOS activates the soluble



**Fig. 1.6** Second-messenger pathways for the action of nitric oxide (NO) in cardiac myocytes. See text for details and definitions of terms and abbreviations (modified from Balligand and Cannon, 1997).

guanylyl cyclase to increase intracellular cGMP, which opposes the positive inotropic effects of cAMP through (i) activation of the cGMP-stimulated PDE (PDE2) to enhance the breakdown of cAMP (on the contrary, cGMP may also potentiate the effects of cAMP through inhibition of PDE3); (ii) activation of PKG may in turn decrease the L-type  $\text{Ca}^{2+}$  current stimulated by PKA via phosphorylation of an intermediate protein opposing the effect of PKA. PKG also promotes relaxation by decreasing myofilament sensitivity to calcium. Besides eNOS activation, muscarinic cholinergic receptor stimulation results in  $\text{G}\beta\gamma$ -mediated inhibition of adenylyl cyclase (AC) and activation of distinct  $\text{K}^+$  channels.

In addition, NO may affect myocyte contraction through mechanisms independent of cGMP elevations such as: (i) oxidation of contractile regulatory proteins, including the cardiac  $\text{Ca}^{2+}$  release channel (ryanodine receptor) and L-type  $\text{Ca}^{2+}$  channel (Xu *et al.*, 1998); (ii) NO and its redox-related derivative (peroxynitrite) may also inactivate key enzymes, including creatine kinase and cytochrome C oxidase, that regulate oxygen consumption and ATP generation in heart muscle. (Torres *et al.*, 1995).

#### **1.2.3.5 NO-cGMP pathway and ischaemia-reperfusion**

In cardiac myocytes, vascular endocardial endothelium and specific cardiac neurons, NO is synthesized by a constitutively expressed and  $\text{Ca}^{2+}$ -dependent NOS, such as eNOS and nNOS. This NO plays an important role in the regulation of coronary circulation and cardiac contractile function (Balligand *et al.*, 1997).

Myocardial ischaemia led to increased activity of  $\text{Ca}^{2+}$ -dependent eNOS (Depré *et al.*, 1997) and cGMP content (Depré *et al.*, 1994) in perfused rabbit hearts. Direct measurement of myocardial NO indicated marked increases after 30 min of ischaemia in Langendorff-perfused (Zweier *et al.*, 1995) and working rat hearts (Csonka *et al.*, 1999). Others previously suggested ischaemia-induced increases in NOS on the basis of indirect pharmacological evidence (Kitakaze *et al.*, 1995, Node *et al.*, 1996). However, contradictory viewpoints exist about whether endogenous NO is detrimental or protective in ischaemia-reperfusion and will be discussed in the following sections (1.2.3.5.1 and 1.2.3.5.2).

#### 1.2.3.5.1 NO-cGMP pathway: deleterious?

NO may combine with superoxide ( $O_2^-$ ) to generate peroxynitrite ( $ONOO^-$ ) which decomposes to yield hydroxyl radicals ( $OH^-$ ) and other reactive oxygen species (ROS) (Beckman *et al.*, 1990). The latter are known to play a major role in myocardial ischaemia, and particularly reperfusion injury (McCord, 1985).

Results obtained by Schulz *et al.* (1995) and Woolfson *et al.* (1995) indicated that inhibition of NOS protects against ischaemia-reperfusion injury. In addition, Wang and Zweier (1996) reported that NOS inhibitors at concentrations that decreased NO during ischaemia/ reperfusion reduce peroxynitrite generation as well as ischaemia/ reperfusion injury. It was suggested that the harmful effects of NO accumulation in the ischaemic heart (Zweier *et al.*, 1995, Csonka *et al.*, 1999) was not due to NO itself but rather to peroxynitrite, a reaction product of NO and superoxide (Beckman *et al.*, 1996, reviewed by Rakhit *et al.*, 1999). Addressing this controversy, Yasmin *et al.* (1997) demonstrated that in rat hearts the NO-dependent ischaemia-reperfusion injury was mediated by peroxynitrite generation. They showed that a NOS inhibitor ( $N^G$ -monomethyl-L-arginine, L-NAME) could abolish the latter and was cardioprotective. However, a NO donor (S-nitroso-N-acetyl-penicillamine, SNAP) was also cardioprotective in their model. This may be because NO in itself is an antioxidant that interferes with peroxynitrite-mediated radical chain propagation reactions (Rubbo *et al.*, 1994). These ambiguous effects of NO within ischaemia-reperfusion were reinforced by a recent study (Du Toit *et al.*, 1998) in the isolated rat heart in which the NO donor sodium nitroprusside (SNP) and the NOS inhibitor  $N^G$ -nitro-L-arginine (LNA) both reduced ischaemia-reperfusion injury. They proposed that the cardioprotective effect of NO donors might be due to their ability to elevate cGMP levels. However, because low concentrations of NOS inhibitors were used, it did not affect the cGMP levels and their beneficial effects may be mediated via an additional mechanism independent of NOS activity.

#### 1.2.3.5.2 NO-cGMP pathway: protective?

Numerous studies have indicated that NO was directly protective during myocardial ischaemia-reperfusion by administration of exogenous NO (Johnson *et al.* 1991, Ferdinandy *et al.*, 1995a, Nossuli *et al.*, 1997, Yasmin *et al.* (1997), Du Toit *et al.*

(1998). They could be protective via numerous mechanisms which will be discussed in the following sections (reviewed by Rakhit *et al.*, 1999). However, the exact mechanism by which these compounds act is unknown.

#### **1.2.3.5.2.1 Calcium**

There is strong evidence for a role of cytosolic  $\text{Ca}^{2+}$  overload in myocardial ischaemic-reperfusion injury (Kusuoka *et al.*, 1987, Marban *et al.*, 1989). Factors that contribute to the elevation in cytosolic  $\text{Ca}^{2+}$  levels during ischaemia and reperfusion include adrenergic stimulation leading to elevated cAMP levels (Wollenberger *et al.*, 1969) and increased  $\text{Ca}^{2+}$  influx through sarcolemmal  $\text{Ca}^{2+}$  channels (Du Toit and Opie, 1992). NO causes relaxation of vascular smooth muscle by increasing cGMP levels which in turn activate cAMP phosphodiesterases (PDEs), reducing cAMP and intracellular  $\text{Ca}^{2+}$  levels (Lohmann *et al.*, 1991). Exogenous administration of NO (Du Toit *et al.*, 1998) or activation of myocardial NOS (Du Toit *et al.*, 1998, Depré *et al.*, 1994, Depré *et al.*, 1997) increases cGMP levels by increasing guanylyl cyclase activity. If, as in vascular smooth muscle cells, elevations in myocardial cGMP lead to a decrease in cytosolic  $\text{Ca}^{2+}$  levels, then NO-cGMP pathway stimulation may protect the ischaemic heart against ischaemia-reperfusion induced  $\text{Ca}^{2+}$  overload.

It has indeed been shown that elevated tissue cGMP levels counter the cAMP induced increase in the slow inward  $\text{Ca}^{2+}$  current and decreases cytosolic  $\text{Ca}^{2+}$  levels in frog (Hartzell and Fischmeister, 1986) and rat myocytes (Sumii and Sperelakis, 1995) and thereby inducing protection.

#### **1.2.3.5.2.2 Phosphodiesterase (PDE)**

There is also pharmacological evidence that the guanylyl cyclase-cGMP-PDE system contributes to cardioprotection. For example, Ljusegren and Axelsson (1993) described that an increase in myocardial cGMP by either SNP or ANP or zaprinast (an inhibitor of cGMP-specific PDE5), caused a reduction of lactate accumulation in isolated hypoxic rat ventricular myocardium.

### 1.2.3.5.2.3 Reactive oxygen species (ROS)

NO can act both as a pro-oxidant as well as an antioxidant depending on the relative concentrations of individual ROS. As mentioned before, the pro-oxidant reactions of NO will occur after reacting with superoxide ( $O_2^-$ ) to yield a potent secondary oxidant peroxynitrite ( $ONOO^-$ ), while the antioxidant effects of NO are a consequence of direct reaction with lipid radical species, such as alkoxyl ( $LO^\cdot$ ) and peroxy ( $LOO^\cdot$ ) radicals, resulting in termination of membrane lipid radical chain propagation reactions (Rubbo *et al.*, 1994). Furthermore, ROS could also result in activation of PKC in the PC scenario, such as protection elicited by late PC (Ping *et al.*, 1999c).

### 1.2.3.6 NO-cGMP pathway in classic preconditioning

#### 1.2.3.6.1 NO and transient ischaemia (as in PC protocol)

Free radicals can also trigger PC protection, since the administration of antioxidants during the brief triggering ischaemia has been found to prevent both early and late PC (Sun *et al.*, 1996). Therefore, a detrimental component of NO (NO-derived radicals) can paradoxically trigger subsequent protection.

Bradykinin is released early in ischaemia (Parratt *et al.*, 1993) and has been postulated as a trigger for the release of NO. Bradykinin is thought to act via  $B_2$  receptors to activate nitric oxide synthase (NOS) in endothelial cells, leading to stimulation of soluble guanylate cyclase in myocytes with a resultant increase in intracellular cGMP (Schini *et al.*, 1990). Elevated cGMP may reduce calcium influx, stimulate cGMP phosphodiesterase (PDE) and therefore reduce cAMP levels, and decrease myocardial oxygen demand via NO-mediated reduction in myocardial contractility (Brady *et al.*, 1993). Pharmacological manipulation of this pathway could be utilized to define the role for NO in PC protection.

#### 1.2.3.6.2 Role of NO: trigger or mediator?

The role of NO as a trigger or mediator in the phenomenon of classic PC is however much less defined and the few studies done thus far focused mainly on outcomes such as reperfusion dysrhythmias. The results obtained were controversial: some studies

reported that the anti-arrhythmic effects of PC were independent of NO (Lu *et al.*, 1995, Siegfried *et al.*, 1992), and that NOS-inhibition by L-NAME had no effect on PC-induced reduction in infarct size (Woolfson *et al.*, 1995) or improvement in functional recovery (Weselcouch *et al.*, 1995b); others showed that NO donors could mimic (Bilinska *et al.*, 1996) or NOS-inhibitors abolish (Vegh *et al.*, 1992) the protective effects of PC on arrhythmias. Further evidence for involvement of NO in PC was the recent observation that cardiac NO biosynthesis was essential to trigger, but not to mediate PC (Csonka *et al.*, 1999): when they added an inhibitor of NOS, N<sup>G</sup>-nitro-L-arginine (LNA), during the PC protocol to reduce the basal NO synthesis, PC failed to protect against ischaemia-reperfusion. Furthermore, when LNA was applied after the PC protocol, the PC-induced protection was not affected. Thus far, the effect of NO donors on outcomes such as infarct size or functional recovery during reperfusion has not been studied.

## 1.3 Final common pathway of preconditioning: Mitogen Activated Protein Kinase (MAPK)?

### 1.3.1 Introduction

Potential downstream targets of PKA, PKC and tyrosine kinases are the mitogen activated protein kinases (MAPKs). Each of the three major subfamilies, namely ERK (extracellular signal-regulated kinase) and the two stress-activated protein kinases (SAPKs), p38 MAPK and JNK (c-Jun NH<sub>2</sub>-terminal kinases), has been suggested to play a role in the cardioprotection achieved by PC (for review see Ping and Murphy 2000, Michel *et al.*, 2001, Schulz *et al.*, 2001). These three different signalling modules will be discussed in detail, as well as the transcription factors involved, especially the importance of the latter in the identification of a final effector of PC.

All three of the subfamilies of MAPKs are expressed in rodent, rabbit, as well as human hearts (Cook *et al.*, 1999). They are activated in response to a wide variety of stimuli, including growth factors, G protein-coupled receptor agonists and environmental stresses (Abe *et al.*, 2000). Their activation has been implicated in many cardiac functions, such as cardiac hypertrophy (reviewed in Schaub *et al.*, 1997), induction (MacKay *et al.*, 1999, Wang *et al.*, 1998, Ma *et al.*, 1999) or inhibition of cell death (apoptosis) (Zechner *et al.*, 1998) and ischaemic PC (Weinbrenner *et al.*, 1997). It is suggested that ERK1/2 is part of a "survival" pathway whereas p38 MAPK and JNK mediate a "death" pathway (Yue *et al.*, 2000, Abe *et al.*, 2000). In addition, the activities of JNK and p38 were increased in heart failure secondary to ischaemic heart disease (Cook *et al.*, 1999).

### 1.3.2 Overview of MAPKs

The identification of a superfamily of related serine/threonine protein kinases, known as mitogen-activated protein kinase (MAPK) (reviewed by Widmann *et al.*, 1999; Bogoyevitch 2000; Kyriakis and Avruch 2001) are among the most widespread mechanisms of eukaryotic cell regulation. All eukaryotic cells possess multiple MAPK pathways, each recruited by distinct sets of stimuli, thereby allowing the cell to respond to multiple divergent inputs. Mammalian MAPK pathways can be activated by a wide variety of different stimuli acting through diverse receptor families, including (i)

hormones and growth factors that act through cytokine receptors (i.e. growth hormone) or receptor tyrosine kinases (i.e. insulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors), (ii) vasoactive peptides acting through G protein-coupled seven-transmembrane spanning receptors (i.e. angiotensin, endothelin), (iii) transforming growth factor (TGF)- $\beta$ -related polypeptide, acting through Ser-Thr kinase receptors, (iv) inflammatory cytokines of the tumor necrosis factor (TNF) family and (v) environmental stresses such as osmotic shock, ionizing radiation and ischaemic injury. MAPK pathways, in turn, coordinate activation of gene transcription, protein synthesis, cell cycle machinery, cell death and differentiation.

### 1.3.2.1 Major subfamilies of MAPKs

#### 1.3.2.1.1 ERK

The initial molecule identified to mediate the mitogenic effects of growth factors which stimulate receptor protein tyrosine kinases (RPTK), was a tyrosine-phosphorylated protein of 42 kD (Cooper and Hunter 1983). Soon after, this molecule was identified as a p42 mitogen-activated protein kinase (MAPK), which requires a dual phosphorylation of both threonine and tyrosine for full activation (Ray and Sturgill 1988, Rossomando 1989). Molecular cloning of this molecule and a highly related protein, p44 MAPK, revealed that they belong to a family of serine-threonine kinases related to a yeast MAPK involved in the pheromone-induced mating response (Boulton *et al.*, 1990, Boulton *et al.*, 1991). In mammalian cells, p44 MAPK and p42 MAPK, also known as ERK1 and ERK2, respectively, are believed to be central components of proliferative pathways. A wide variety of growth promoting or hypertrophic agents activate these kinases in cardiac myocytes, fibroblasts, smooth muscle cells or endothelial cells (Bogoyevitch 2000). Activation of ERK may also follow exposure to a variety of extracellular stresses. Such activation of a potent growth factor-stimulated pathway by stress may seem contradictory, but if stress-activated ERK is chemically inhibited, cell death is accelerated (Wang *et al.*, 1998). Therefore ERKs may contribute to cardiovascular cell survival. Accordingly, recent findings suggest that ERK promote cell survival by both inhibiting components of the cell death machinery (i.e. the proapoptotic Bcl-2 family protein, BAD) and increasing transcription of pro-survival genes (i.e. cAMP-response element binding protein, CREB) (Bonni *et al.*, 1999, Tan *et al.*, 1999).

### 1.3.2.1.2 JNK

There has been a recent focus on other MAPK subfamilies that are activated by a diverse range of extracellular stresses such as irradiation, ultraviolet light, mechanical stress (shear stress evoked by blood flow or stretch), hyperosmotic shock, heat shock, hypoxia/reoxygenation, reactive oxygen species (ROS), proinflammatory cytokines (such as interleukin, IL-1 and tumor necrosis factor, TNF- $\alpha$ ) and to a lesser degree by growth factors (Sugden and Clerk 1998, Bogoyevitch 2000).

A subfamily of MAPK, structurally related to ERKs, termed c-Jun NH<sub>2</sub>-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs), was discovered as a cycloheximide (a protein synthesis inhibitor) activated 54 kDa protein kinase (Kyriakis and Avruch 1990). Molecular cloning revealed three genes that encode the 46- and 54-kDa isoforms of JNK: SAPK $\alpha$ /JNK2, SAPK $\beta$ /JNK3, SAPK $\gamma$ /JNK1 (Kyriakis *et al.*, 1994, Derijard *et al.*, 1994). These kinases selectively phosphorylate the NH<sub>2</sub>-terminal transactivation domain of the transcription factor c-Jun (Hibi *et al.*, 1993), which results in gene expression of *c-jun* (Karin 1994). JNKs are important for cytokine biosynthesis and are involved in cell transformation, stress responses and apoptosis (programmed cell death) (Kyriakis and Avruch 1996, Dong *et al.*, 1998, Kuan *et al.*, 1999).

### 1.3.2.1.3 p38 MAPK

The p38 MAPK subgroup (also referred to as CSBP / Dp38 / HOG1 / Mxi2 / p40 / Phh1 / RK / SAPK2 / Spc1 / Sty1 / XMpk2) was discovered as a lipopolysaccharide (LPS)-induced tyrosine phosphoprotein and as the target of a drug developed to inhibit LPS-induced tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) biosynthesis (Han *et al.*, 1994, Lee *et al.*, 1994). There are six isoforms of p38 MAPK: p38  $\alpha_1$  /  $\alpha_2$  (also CSBP1 / SAPK2a), p38  $\beta_1$  /  $\beta_2$  (also SAPK2b / p38-2), p38 $\gamma$  (also SAPK3 / ERK6) and p38 $\delta$  (also SAPK4) (Sugden and Clerk, 1998, Kyriakis and Avruch 2001). Like the JNKs, they are strongly activated by environmental stresses and inflammatory cytokines and therefore also referred to as stress-activated protein kinases. In almost all instances, the same stimuli that recruit the JNKs also recruit the p38 MAPKs (Kyriakis and Avruch 1996). One exception is ischaemia-reperfusion. JNKs are not activated during ischaemia, but rather during reperfusion, whereas p38 MAPKs are activated during ischaemia and remain

active during reperfusion (Pombo *et al.*, 1994, Bogoyevitch *et al.*, 1996, Kyriakis and Avruch 1996). The basis for this difference is unknown.

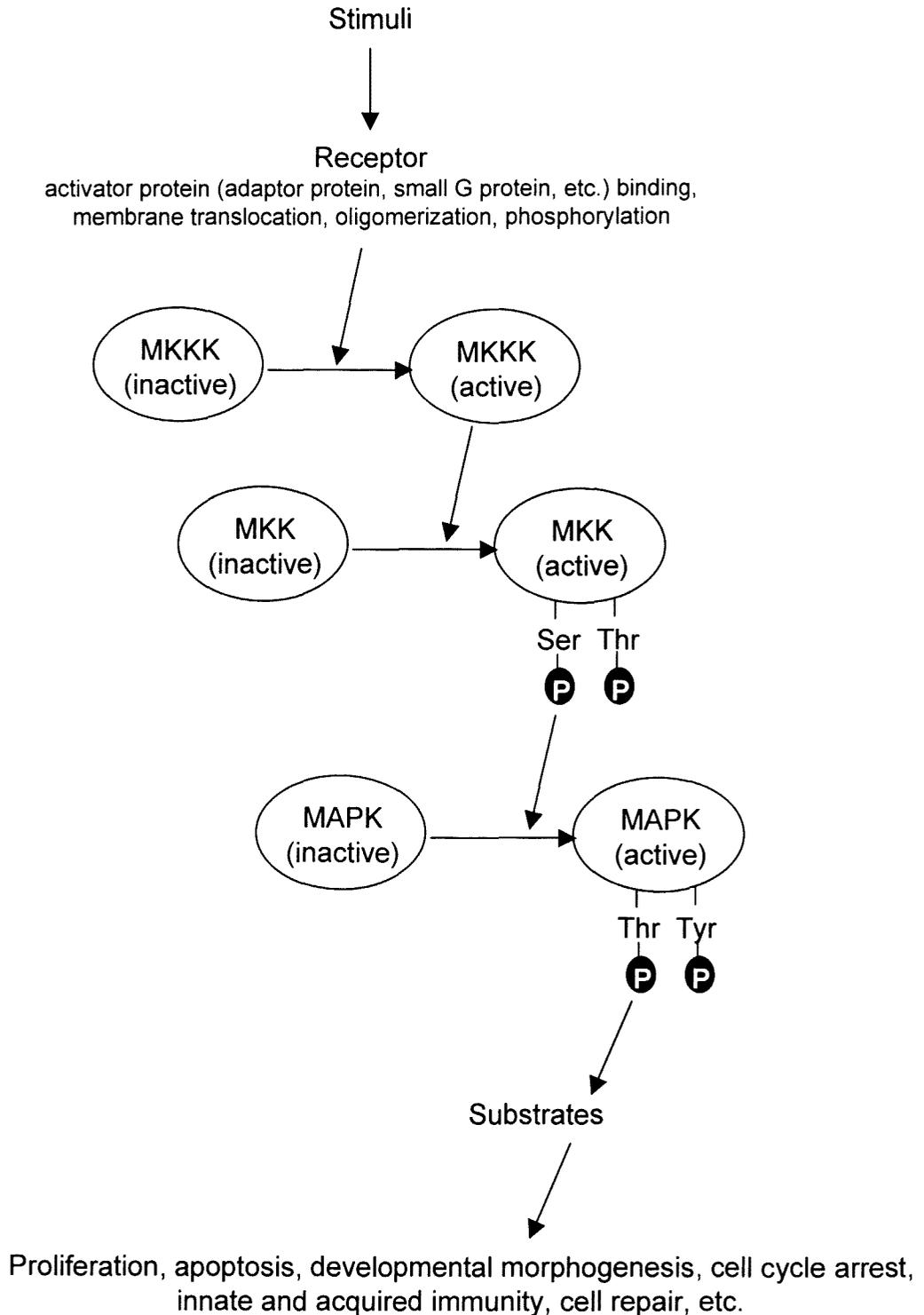
Activation of p38 MAPKs has been implicated in transcription, protein synthesis, cell surface receptor expression and cytoskeletal structure, ultimately affecting cell survival or leading to programmed cell death (apoptosis) (Obata *et al.*, 2000). In addition, p38 may also play a role in embryonic development (Mikkola *et al.*, 1999) and organogenesis (Ganiatsas *et al.*, 1998). However, the significance of activation of p38 MAPKs and its downstream events in myocytes has not been fully characterized.

#### **1.3.2.1.4 Other SAPKs**

A less understood MAPK, Big MAPK (BMK1) or ERK5, was identified independently as a 98 kDa protein kinase (Lee *et al.*, 1995, Zhou *et al.*, 1995). ERK5 was originally cloned as a homolog of ERK1/2, but differs in its activation loop sequence and is activated by a distinct upstream kinase, MEK5 (Zhou *et al.*, 1995). ERK5 is activated in vascular smooth muscle cells or aortic endothelial cells exposed to oxidative stress, osmotic shock or shear stress but not by angiotensin II or growth factors (Abe *et al.*, 1996, Yan *et al.*, 1999). Although the expression of ERK5 is high in the heart, the manner and role of its activation in cardiac myocytes remains uncharacterized (Zhou *et al.*, 1995).

### **1.3.3 Upstream components of MAPKs**

Although each MAPK subfamily member acts within a distinct signalling pathway, all MAPKs are the final components of a basic assembly of a three-component protein kinase cascade (Fig. 1.7). This basic assembly of MAPKs are themselves regulated by a wide variety of stimuli (1.3.2). The first kinase of the assembly is a MAPK kinase kinase (MKKK) (Fanger *et al.*, 1997) that could be activated either by (i) phosphorylation by a MAPK kinase kinase kinase (MKKKK), or (ii) by small G-proteins which are recruited when hormones or growth factors interacts with its receptor, or (iii) oligomerization and subcellular relocalization. The MKKK are serine/threonine kinases which, when activated, phosphorylate and activate the next kinase in the assembly, a MAPK kinase (MKK) (Siow *et al.*, 1997). The MKK recognize and phosphorylate a Thr-X-Tyr motif (X can be different amino acids among the MAPKs) in the activation loop of



**Fig. 1.7** Basic assembly of mitogen-activated protein kinase (MAPK) pathways is a three-component cascade conserved from yeast to humans. Divergent stimuli (activators) feed into core MAPK kinase kinase (MKKK), MAPK kinase (MKK) and MAPK pathways that are sequentially activated by phosphorylation, which then recruit appropriate substrates and responses (modified from Widmann *et al.*, 1999; Kyriakis and Avruch 2001).

MAPK (Gartner *et al.*, 1992), defining MKK as a dual-specificity kinase. The MAPKs are the final kinases in the three-kinase module and phosphorylate substrates on serine and threonine residues. The vast majority of substrates for MAPKs are transcription factors. However, MAPK have the ability to phosphorylate many other substrates including other protein kinases, phospholipases and cytoskeleton-associated proteins.

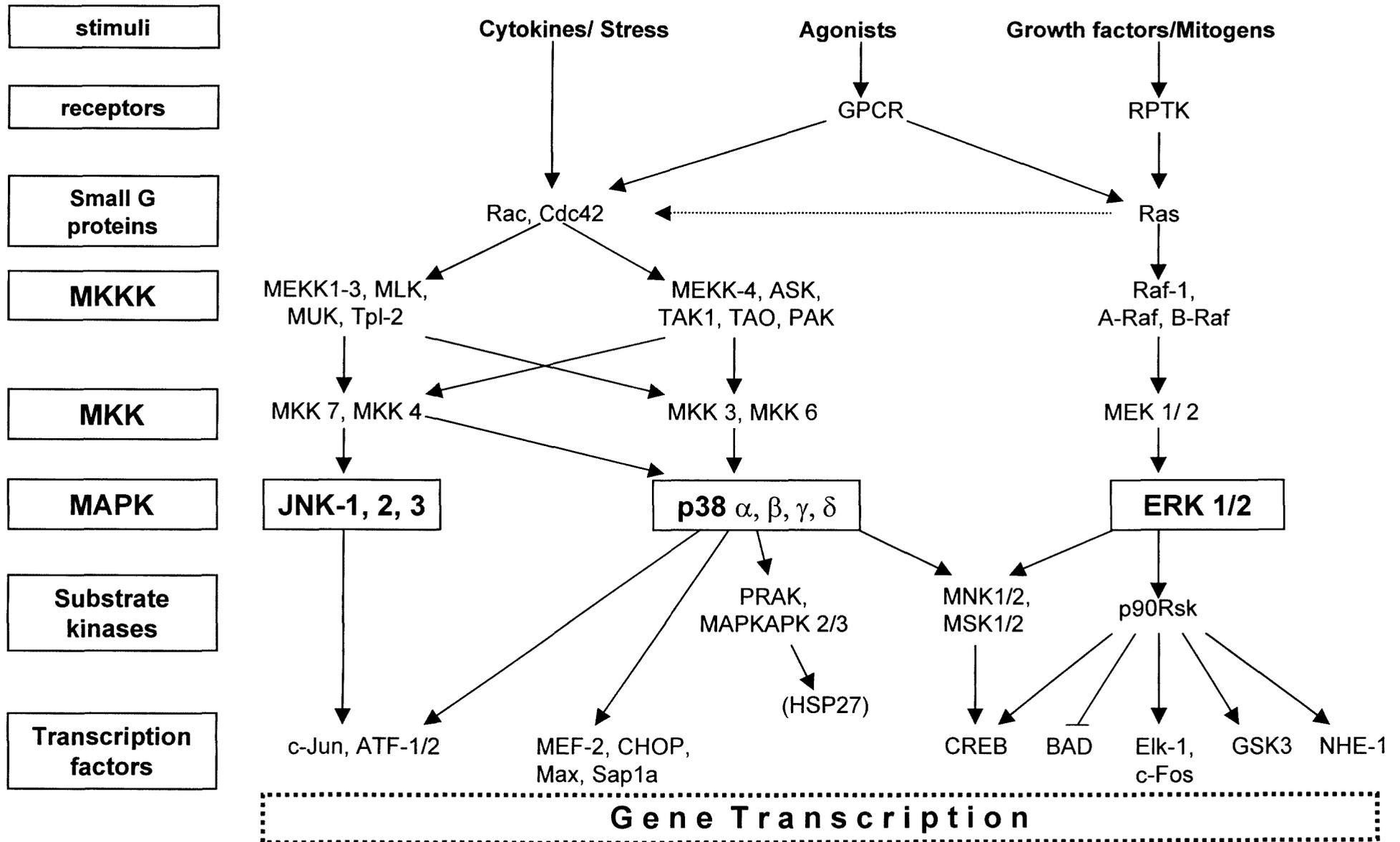
In mammalian systems the three major MAPKs, the ERKs, JNKs and p38 MAPK appear to have distinct upstream kinases.

### 1.3.3.1 Upstream components of ERK: (Fig.1.8)

In the ERK1/2 cascade, the first of the three-component kinase assembly or MKKK is a Raf isoform, Raf-1. Activation of Raf-1 usually requires Ras, although other mechanisms, like PKC, have been proposed (Kolch *et al.*, 1993, Morrison *et al.*, 1997, also see Fig 1.9). Once activated, Raf-1 then phosphorylates two residues, either serine or threonine, to activate the protein kinases known as ERK kinases (MEK1 and MEK2) or MKKs (Kyriakis *et al.*, 1992). These kinases are a family of dual-specificity protein kinases that phosphorylate two residues, tyrosine and threonine, to selectively activate their MAPK targets (Payne *et al.*, 1991). MEK1/2 phosphorylate and activate only ERK1/2 and no other MAPK family member (Crews *et al.*, 1992, Wu *et al.*, 1993). There are two additional MKKKs, A-Raf and B-Raf, whose pattern of expression is more restricted than that of Raf-1 (Storm *et al.*, 1990). The B-Raf appears to be the major MEK (MKK) activator in brain (Catling *et al.*, 1994), while A-Raf activates MEK in cardiac myocytes after stimulation with hypertrophic agonists (Bogoyevitch *et al.*, 1995b).

### 1.3.3.2 Upstream components of JNK and p38 MAPK: (Fig 1.8)

Members of the MKKK (MAPKKK, MEKK) superfamily responsible for activating JNK and p38 MAPK pathways that have been identified, include MEKK-1, MEKK-2, MEKK-3, MEKK-4 (MTK), MUK (MAPK upstream kinase), MLK (mixed lineage kinase), Tpl-2 (tumor progression locus-2), TAK-1 (TGF- $\beta$  activated kinase 1), ASK-1 (apoptosis signal-regulation kinase) and TAO (thousand and one amino acid protein kinase) (reviewed in Obata *et al.*, 2000, Gutkind 2000 and Widmann *et al.*, 1999). These



**Fig. 1.8** The different mitogen-activated protein kinase (MAPK) pathways: The upstream activators of the different mitogen-activated protein kinase (MAPK) cascades and their downstream substrates. Cross-talk between the different pathways has been observed. See text for details and abbreviations (modified from Bogoyevitch *et al.*, 2000, Obata *et al.*, 2000 and Widmann *et al.*, 1999).

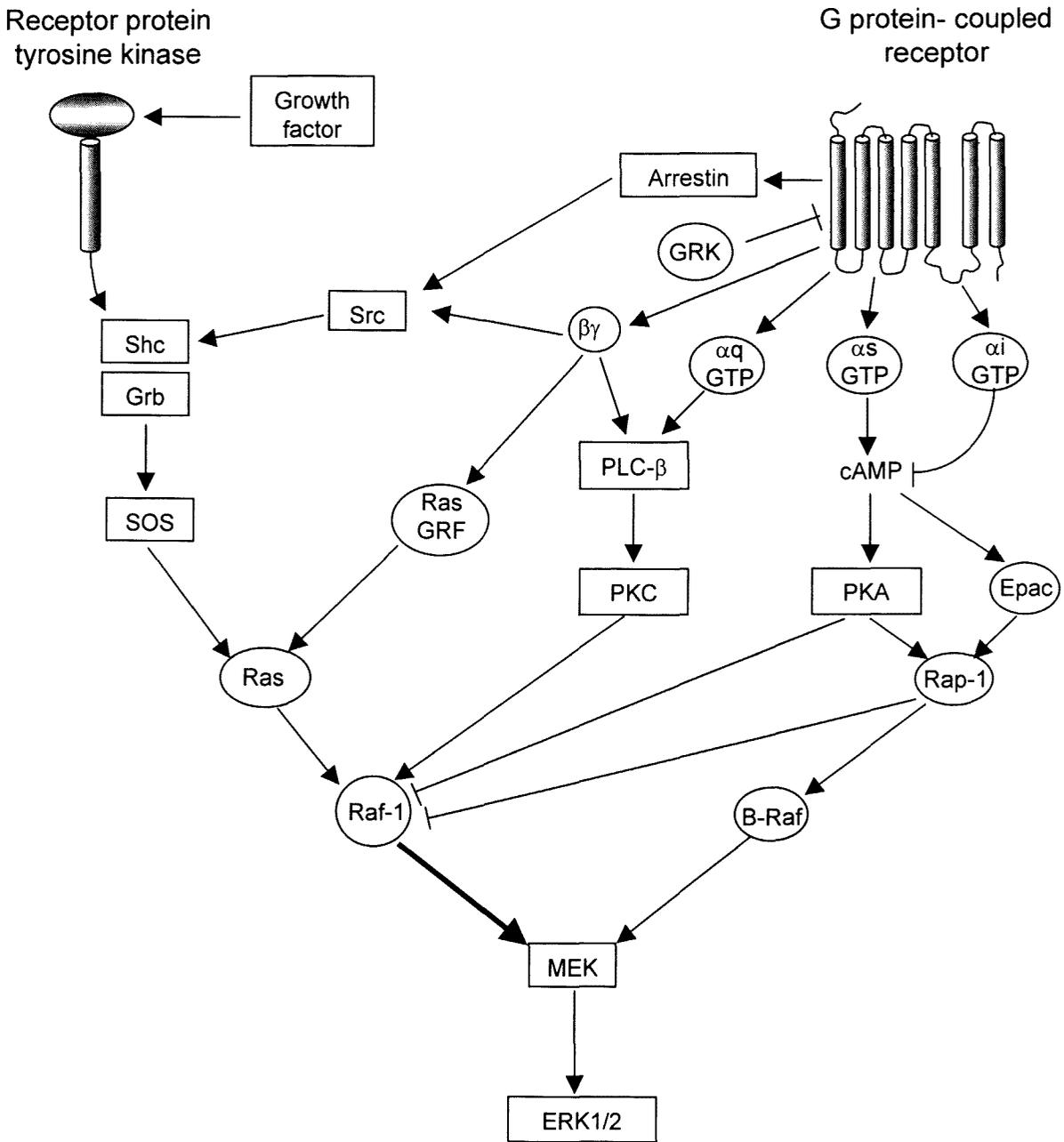
MKKKs are regulated by Rac and Cdc42, two small guanosine 5'-phosphate (GTP)-binding proteins of the Rho family, in a manner similar to that of Ras acting on Raf (Minden *et al.*, 1995). At least a portion of this JNK/p38 MAPK activation by small GTP-binding proteins involves activation of a serine/threonine kinase called PAK (p21-activated kinases) (Bagrodia *et al.*, 1995). The relative contribution of each MKKK to either JNK or p38 MAPK pathways has not yet been clearly defined. MEKK-1, MEKK-2, MEKK-3, MUK, MLK and Tpl-2 seemed to preferentially activate JNK, rather than p38 (Fanger *et al.*, 1997, Obata *et al.*, 2000, Gutkind 2000), through two MKKs, MKK4 (Sek1, JNKK1) (Sanchez *et al.*, 1994) and MKK7 (Sek2) (Tournier *et al.*, 1997). On the other hand, MEKK-4, TAK-1, ASK-1 and TAO appear to be more effective activators of p38 MAPK (Obata *et al.*, 2000), through MKK6 and MKK3 (Moriguchi *et al.*, 1996). MKK6 appears to phosphorylate all p38 MAPK isoforms, whereas MKK3 phosphorylates only p38 $\alpha$ ,  $\gamma$  and  $\delta$  (Enslin *et al.*, 1998).

Cross-talk between the JNK and p38 MAPK pathways has also been observed, since MEKK-1 can activate p38 MAPK through MKK-4 in NIH 3T3 cells (Guan *et al.*, 1998), and similarly, MEKK-2 or MEKK-3 can couple to p38 MAPK pathway via MKK6 (Deacon and Blank, 1999).

### **1.3.4 Activation of MAPKs via G protein-coupled receptors (GPCR)**

#### **1.3.4.1 Receptor protein tyrosine kinases (RPTK) and ERK pathway (Fig. 1.9)**

Different receptor types are able to activate the ERK 1/2 pathway (reviewed in Widmann *et al.* 1999 and Gutkind 2000). Originally ERKs were activated in response to activation of RPTK by growth factors (1.3.2.1.1, Cooper and Hunter 1983). Upon stimulation of RPTKs with growth factors their intrinsic domains are activated, leading to phosphorylation of specific substrates including the receptor itself. Tyrosine phosphorylation of the receptor allows the binding of adapter proteins, like Shc, to the receptor. The association of Shc permits phosphorylation thereof by the receptor itself or by intracellular tyrosine kinases such as Src. This phosphorylation allows the binding of another adaptor protein, Grb2, which also results in the recruitment of SOS. The latter stimulates the exchange of GDP bound to Ras for GTP (Downward 1996) and initiates the protein kinase cascade that results in phosphorylation and activation of ERK1/2 (as mentioned in 1.3.3.1).



**Fig. 1.9** Multiple pathways linking G protein-coupled receptors to the MAPKs (i.e. ERK1/2). See text for details and definitions of terms and abbreviations. Arrows, positive stimulation; blocked lines, inhibition. (modified from Gutkind, 2000)

### 1.3.4.2 G protein-coupled receptors (GPCRs) and ERK pathway (Fig. 1.9)

It has become increasingly apparent that like the RPTKs, GPCRs are involved in the regulation of cell growth and differentiation (reviewed in Post and Brown 1996). The activation of ERKs in response to agonists acting on seven transmembrane-spanning receptors linked to heterotrimeric G proteins, the so-called G protein-coupled receptors (GPCRs), is also well documented (reviewed in Sugden and Clerk 1997). In the case of Gi-coupled m2 muscarinic and  $\alpha_2$ -adrenergic receptors (Koch *et al.*, 1994, Crespo *et al.*, 1994) and G<sub>s</sub>-coupled  $\beta$ -adrenergic receptors (Crespo *et al.*, 1995, Faure *et al.*, 1994), the activation of ERKs is mediated primarily by G $\beta\gamma$  subunits. On the other hand, Gq-coupled m1 muscarinic acetylcholine and  $\alpha_1$ -adrenergic receptors induce ERK activation mainly through G $\alpha$  subunits (Koch *et al.*, 1994, Hawes *et al.*, 1995). However, the nature of the biochemical pathways linking GPCRs to ERKs remains the subject of intense investigation (Zheng 2000).

#### 1.3.4.2.1 G $\beta\gamma$ -protein (Fig. 1.9)

One major pathway of GPCR-mediated activation of MAPKs is dependent on “trans-activation” of a panel of receptor tyrosine kinases. Specifically, stimulation of GPCRs leads to the release of free G $\beta\gamma$  subunits, which in turn, could activate the Src kinases that have been found to mediate the phosphorylation of Shc on tyrosine residues, resulting in activation of ERK1/2 (Hordijk *et al.*, 1994, Luttrell *et al.*, 1996). In addition, alternate biochemical routes might also cooperate with those described above to connect GPCR to ERKs. For example, Ras-GRF (Ras-guanine-nucleotide releasing factor) is a distinct Ras- guanine-nucleotide exchange factor that is activated in response to GPCR stimulation or upon coexpression of G $\beta\gamma$  by a mechanism involving Ca<sup>2+</sup> and thereby linking GPCR and G $\beta\gamma$  to Ras and ERKs (Mattingly and Macara, 1996).

### 1.3.4.2.2 G $\alpha$ -protein (Fig. 1.9)

#### 1.3.4.2.2.1 Phospholipase C

The fact that G $\beta\gamma$  subunits or G $\alpha_q$  coupled receptors can stimulate phospholipase C (PLC) suggest that the subsequent stimulation of protein kinase C (PKC) may contribute to MAPK activation by GPCRs (Gutkind 2000). Although PKCs can activate Raf by direct phosphorylation (Kolch *et al.*, 1993) this alone does not result in an ability of Raf to phosphorylate MEK. Thus, PKC might act directly on Raf to facilitate full activation of Raf upon binding to Ras (Burgering and Bos, 1995).

#### 1.3.4.2.2.2 PKA

Daaka *et al.* (1997) proposed a model for  $\beta_2$ -AR mediated ERK activation in which PKA-mediated phosphorylation of the receptor enables it to couple to G $\alpha_i$ -proteins: Upon agonist binding the receptor activation of G $\alpha_s$  stimulates adenylyl cyclase activity and the resulting rise in cAMP activates PKA, which could phosphorylate the receptor. This phosphorylation of the  $\beta_2$ -AR by PKA switches receptor coupling from the adenylyl cyclase stimulatory G-protein G $_s$  to G $_i$ -proteins. GTP-bound G $\alpha_i$  dissociates from the G $\beta\gamma$  subunit and free G $\beta\gamma$  mediates activation of the ERKs.

Activation of G protein  $\alpha_s$  subunits may also inhibit ERKs via a PKA-dependent or – independent pathway. For example: (i) inhibition of ERK1/2 is mediated by the negative regulation of Raf-1 by PKA, due to direct phosphorylation of serine 621 (Mischak *et al.*, 1996) or activation of the Ras-like GTPase, Rap-1 (itself a PKA substrate) can block ERK activation by competing with Ras for binding to Raf (Kawasaki *et al.*, 1998); (ii) cAMP can directly activate Rap-1, in a PKA-independent manner, via a guanine-nucleotide exchange factor named Epac (de Rooij *et al.*, 1998). On the other hand, Vossler *et al.* (1997) proposed that PKA activation of Rap-1 could stimulate ERKs through the activation of another Raf isoform, B-Raf. However, B-Raf has a more restricted distribution than the ubiquitously expressed Raf-1.

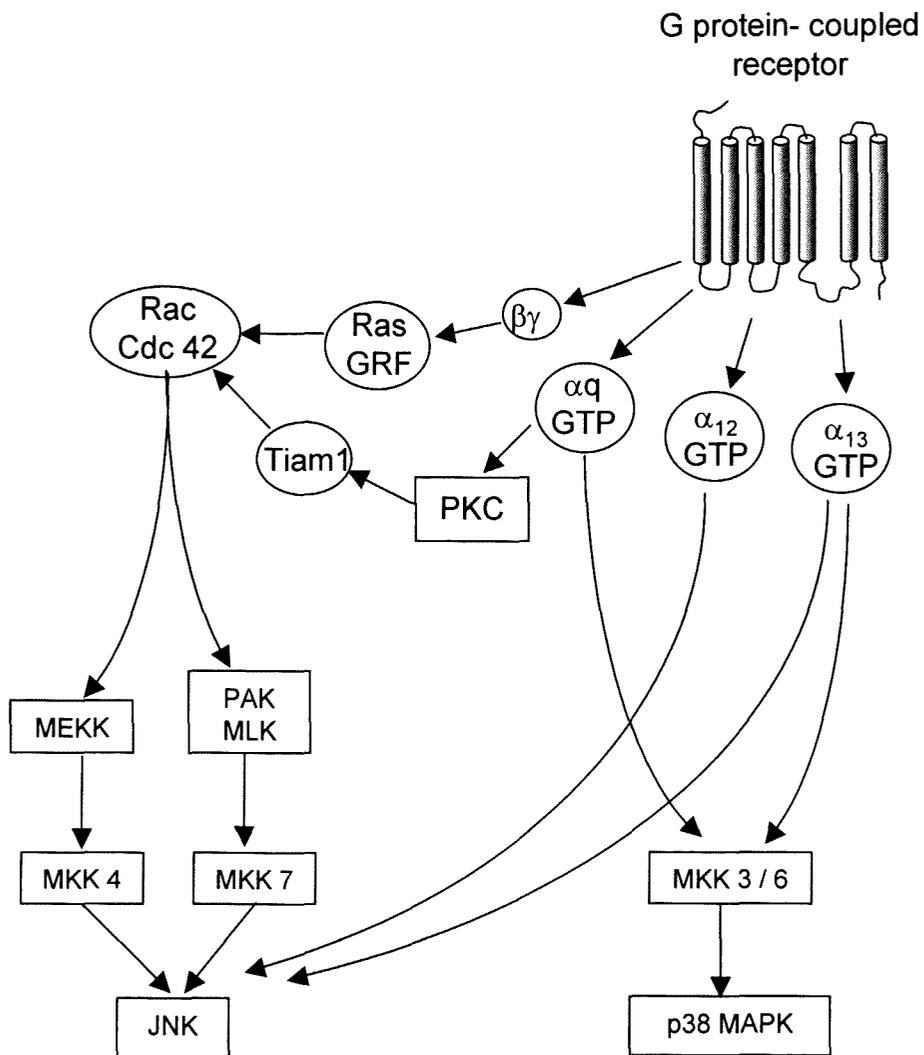
#### 1.3.4.2.2.3 Receptor desensitization (GRKs and Arrestins) (Fig. 1.4)

Recent evidence suggests that GPCR desensitization and internalization are also signalling events. Exposure of GPCRs to an agonist often results in rapid attenuation of receptor responsiveness, a process termed desensitization (as mentioned before, Lefkowitz, 1993). Desensitized  $\beta$ -adrenergic receptors could function as scaffolds for Src-dependent activation of ERK signalling pathways, while the  $\beta$ -arrestins function as adapter proteins that link G-protein coupled receptors to tyrosine kinase-dependent pathways (Luttrell *et al.*, 1999). In this case, binding of the  $\beta$ -adrenergic agonists to their G-protein linked receptors results in the rapid phosphorylation of the agonist-occupied receptor by the G protein-coupled receptor kinase (GRK) and the subsequent recruitment of a protein known as  $\beta$ -arrestin to the GRK-phosphorylated receptor. In turn,  $\beta$ -arrestin functions as an adaptor protein, bringing Src to the agonist-occupied receptor, and targeting both to clathrin-coated pits. Both Src binding and clathrin-targeting are required for  $\beta_2$ -AR-mediated ERK activation.

#### 1.3.4.3 G protein-coupled receptors (GPCRs) and JNK pathway (Fig. 1.10)

In contrast to the wealth of knowledge available regarding GPCR-mediated ERK1/2 activation, the role of GPCRs in regulating JNK and p38 MAPK activity is still under investigation. For example, the molecular mechanisms by which extracellular signals are transmitted from the membrane receptors to most of the upstream kinases in the JNK and p38 signalling modules are not fully understood.

It was observed that GPCRs, but not tyrosine kinase receptors, potently activate JNK (Coso *et al.*, 1995). Signalling from GPCRs to the JNK might be regulated by a distinct set of upstream signalling molecules. For example: Rac1 and Cdc42, two small GTPases of the Rho family, were found to initiate an independent kinase cascade regulating JNK activity (Minden *et al.*, 1995). Regulation of guanine-nucleotide exchange factors (GEFs) may link  $\beta\gamma$  to Rac1 and Cdc42. For example: Ras-GRF1, which upon  $\beta\gamma$  binding can act as a Ras GEF and can induce guanine-nucleotide exchange on Rac1 (Kiyono *et al.*, 1999), while Tiam1, a Rac-specific GEF is phosphorylated on threonine residues in a  $Gq\alpha$  - PKC – dependent manner (Fleming *et al.*, 1997).



**Fig. 1.10** Molecules linking G protein-coupled receptors to JNK and p38 MAPKs. See text for details and definitions of terms and abbreviations. Arrows, positive stimulation. (modified from Gutkind, 2000)

Constitutively active forms of G proteins,  $G\alpha_{12}$  and  $G\alpha_{13}$ , with less well defined signalling pathways also activate the SAPK pathways (Prasad *et al.*, 1995). Both these G-protein subunits can activate the JNK through small GTPases, Rac1 or Cdc42, while inhibiting ERK pathway in a Ras-and Raf-independent fashion (Collins *et al.*, 1996, Voyno-Yasenetskaya *et al.*, 1996). Only the  $G\alpha_{13}$  subunit has been shown to be involved in the activation of p38 MAPKs (Wilk-Blaszczak *et al.*, 1998).

#### 1.3.4.4 G protein-coupled receptors (GPCRs) and p38 MAPK pathway (Fig. 1.10)

In HEK 293 (human embryonal kidney 293) cells, p38 MAPK can be activated through GPCRs, such as  $G_{q/11}$ -coupled m1 and the  $G_i$ -coupled m2 muscarinic acetylcholine receptors and  $G_s$ -coupled  $\beta$ -adrenergic receptors. Overexpression of  $G\beta\gamma$  and  $G\alpha_q$ , but not  $G\alpha_s$  and  $G\alpha_i$ , can stimulate p38 MAPK, suggesting that the activation of p38 MAPK by the  $\beta$ -adrenergic and m2 receptors occurs mainly by  $G\beta\gamma$ , but not by  $G\alpha_s$  and  $G\alpha_i$ , whereas the activation via m1 muscarinic receptors is mediated by both  $G\beta\gamma$  and  $G\alpha_q$  (Yamauchi *et al.*, 1997). Furthermore, Lazou *et al.* (1998) indicated that in perfused rat hearts,  $\alpha_1$ -adrenergic receptor agonists, such as phenylephrine, also activate p38 MAPK via  $G\alpha_q$ . Thus, depending on the GPCR, p38 MAPK activation can be mediated by the  $G\beta\gamma$ -subunit or the  $G\alpha$ -subunit of heterotrimeric G proteins. However, Communal *et al.* (2000) reported that in adult rat ventricular myocytes both  $\beta_1$ - and  $\beta_2$ -adrenergic receptor subtypes stimulate p38 MAPK in a pertussis toxin sensitive manner, therefore via  $G_i$  proteins. Likewise, stimulation of  $G_i$  via the activation of m2 muscarinic receptors by treatment with carbachol, also activate p38 MAPK. On the other hand, Zheng *et al.* (2000) showed that in adult mouse cardiac myocytes,  $\beta_2$ -adrenergic receptor activation of p38 MAPK occurs via a cAMP dependent protein kinase (PKA) pathway, rather than by  $G_i$  or the  $G\beta\gamma$  subunit. Similarly, in rat epididymal fat cells, a cell permeable cAMP analogue increased the p38 MAPK activity to a similar extent to isoproterenol, suggesting that the effect of the  $\beta$ -adrenergic agonist is mediated via cAMP and PKA (Moule *et al.*, 1998).

Furthermore, it has recently been reported that PKA activation causes a increase in p38 MAPK activity through inactivation of protein tyrosine phosphatases in rat fibroblasts,

HEK 293 and COS cells (Saxena *et al.*, 1999, Blanco-Aparicio *et al.*, 1999). Both protein tyrosine phosphatases, HePTP and PTP-SL bind to the MAPKs, p38 $\alpha$  and ERK1/2, resulting in inactivation of the MAPKs by dephosphorylating the critical phosphorylated tyrosine residue in their activation loop. The PKA-mediated release of the MAPKs from the phosphatases is sufficient to activate the kinases.

Despite the above uncertainties regarding the regulation of p38 MAPK by different G protein subfamilies,  $\beta$ -adrenergic stimulation could activate p38 MAPK. Therefore  $\beta$ -adrenergic stimulation could be a useful tool to evaluate the contribution of this stress kinase to cardioprotection in ischaemic or  $\beta$ -adrenergic PC.

### 1.3.5 Downstream substrates of MAPK (Fig. 1.8)

#### 1.3.5.1 Protein kinases

MAPKs phosphorylate both transcription factors and other protein kinases (Kyriakis and Avruch, 2001). The latter substrates allow signal diversification and amplification downstream of the MAPKs.

One of the selective protein kinase targets of ERK is MAPKAPK-1/p90Rsk (MAPK-activated protein kinase-1/p90 ribosomal S6 kinase, Erkson 1991). Essential functions of p90Rsk (Frodin and Gammeltoft, 1999) include (i) regulation of gene expression via phosphorylation of transcription factors, including c-Fos (Seger and Krebs, 1995) and cyclic AMP response element-binding protein, CREB (Xing *et al.*, 1996); (ii) regulation of protein synthesis by phosphorylation of polyribosomal proteins and glycogen synthase kinase-3 (GSK3); the latter has also been shown to negatively regulate c-Jun (Widmann *et al.*, 1999); (iii) stimulation of Na<sup>+</sup>/H<sup>+</sup> exchanger by phosphorylating Na<sup>+</sup>/H<sup>+</sup> exchanger isoform-1 (NHE-1) kinase (Takahashi *et al.*, 1999); (iv) phosphorylation and inactivation of BAD (a proapoptotic Bcl-2 family protein) which suppressed apoptotic cell death (Bonni *et al.*, 1999, Tan *et al.*, 1999).

Stress-activated MAPKs, in particular p38 $\alpha$  and p38 $\beta$  MAPKs (but not by p38 $\gamma$  or p38 $\delta$ ) selectively recruit protein kinase substrates, namely MAPKAPK-2 and -3 (mitogen-activated protein kinase-activated protein kinase-2 and -3), as well as PRAK (p38-regulated and activated kinase) (Freshney *et al.*, 1994, Rouse *et al.*, 1994). These

protein kinases are unrelated to MAPKAPK-1/Rsks. The phosphorylated and activated MAPKAPK-2, a Ser/Thr kinase, can phosphorylate and activate small 27-kDa heat shock protein, HSP27 (Rouse *et al.*, 1994). This activation coincides with the dissociation of HSP27 into monomers and dimers, and with the redistribution of HSP27 to the actin cytoskeleton (Huot *et al.*, 1997).

Other protein kinase substrates, like MNKs (MAPK-interacting kinases) and MSKs (mitogen- and stress-activated protein kinases), are activated by both p38 MAPK and ERKs and consequently intergrate both stress and mitogen signalling pathways. The MNKs phosphorylates the mRNA 5' cap-binding protein, eIF-4E (eukaryotic initiation factor-4E), which is a key participant in the control of gene expression at the translation level that is important for cell growth and proliferation (Sonenberg and Gingras, 1998). The MSKs are potent stress- and mitogen activated CREB (see transcription factors) kinases (Deak 1998). These kinases may also phosphorylate nucleosomal proteins such as histone H3 and HMG-14 (high mobility group-14) (Thomson *et al.*, 1999). These proteins are involved in chromatin remodelling that enables the transcription machinery to gain access to genes.

### **1.3.5.2 Transcription factors**

#### **1.3.5.2.1 Introduction**

Transcription factors form a major group of MAPK substrates, although some should be considered as indirect targets of the MAPK pathways. These factors regulate gene expression and modulate long-term changes in the cell (Bogoyevitch 2000).

Activation of MAPKs, such as ERKs and JNKs, takes place in the cytoplasm and the activated enzyme moves to the nucleus. This translocation is essential to enable the kinase to phosphorylate transcription factors (Obata *et al.*, 2000). Although, p38 MAPK was reported to be present in both the nucleus and the cytoplasm, it also translocates from the cytoplasm to the nucleus in response to stress stimuli (Raingeaud *et al.*, 1996, Maulik *et al.*, 1998). In contrast, a complex of p38 MAPK bound to one of its effector molecules, MAPKAPK-2, appears to be exported from the nucleus to the cytoplasm following p38 MAPK activation (Ben-Levy *et al.*, 1998), which permits them to phosphorylate substrates in the cytoplasm.

### 1.3.5.2.2 Major transcription factors

The MAPK pathway phosphorylates and enhances the activity of some of the major transcription factors, including Elk-1 (Ets-related ternary complex factor), Sap-1 (serum response factor accessory protein), ATF-1/2 (activating transcription factor), c-Jun, CREB (cyclic AMP response element-binding protein), CHOP/GADD153 (CREB homologous protein/growth arrest and DNA damage), Max, MEF-2A/2C (myocyte enhancer factor) and HSF-1 (heat shock factor) (Obata *et al.*, 2000). Elk-1 can be activated as substrate for all the MAPKs, while others such as ATF-1/2 are suitable for only p38 MAPK and JNK. Other substrates are suitable for only one MAPK subfamily, such as c-Jun for JNK; and Sap1a, CHOP, Max and MEF2A/C for p38 MAPK (Bogoyevitch 2000, Kyriakis and Avruch 2001). CREB may be considered as an indirect target of the ERK and p38 MAPK pathways, as it is a substrate for kinases downstream of these MAPKs.

The transcription factors which have been well-characterized and which play significant roles within cells of the cardiovascular system, include Elk-1, c-Jun, ATF-2, MEF-2 and CREB (Bogoyevitch, 2000).

#### 1.3.5.2.2.1 Elk-1

Elk-1 and Sap1a, are ternary complex factors (TNFs), which together with serum-response factor (SRF) controls transcription from the serum-response element (SRE) and MEF-2 transcription factors (Marais *et al.*, 1993). These factors mediate the expression of many immediate-early genes, including c-fos and Egr-1 that regulate muscle-specific and immune cell gene expression (Han *et al.*, 1997). ERK, as well as JNK but not p38 MAPK, can phosphorylate Elk-1, while p38 MAPK can efficiently phosphorylate Sap1a (Janknecht and Hunter, 1997). By these processes, MAPKs activated by both stress and mitogens can come together to contribute to c-fos induction. However, skeletal  $\alpha$ -actin, cardiac  $\alpha$ -actin and ANF (atrial natriuretic factor) promoters possess SREs that do not have Elk-binding sequences and expression may be dependent on other transcription factors, such as ATFs (Hines *et al.*, 1999, Thuerauf *et al.*, 1998).

#### 1.3.5.2.2.2 c-Jun

One of the major substrates of JNKs is c-Jun (a product of one of the immediate-early genes, *c-jun*), which is also a member of the bZip (basic leucine zipper, that enables homo- and heterodimerization) family of transcription factors (Force *et al.*, 1996). c-Jun functions as a heterodimer with c-Fos or ATF-2 (a member of the CREB family). When complexed with c-Fos, the dimer is targeted to AP-1 (activator protein-1) containing promoters of a number of genes of importance in the heart, such as skeletal muscle  $\alpha$ -actin (Bishopric *et al.*, 1992). However, when complexed with the ATF-2, the dimer prefers CRE (cAMP response element) sequences and *c-jun* promoters containing AP-1 variants, which controls the induction of *c-jun* (Force *et al.*, 1996). In addition, c-Jun plays an important role in regulation of cytochrome c expression during electrical pacing of myocytes. In these hypertrophied myocytes, enhanced c-Jun transcriptional activity allows for increases in mitochondria number and cytochrome oxidase activity, which are essential to satisfy the increased energy demand (Xia *et al.*, 1998).

#### 1.3.5.2.2.3 ATF-2

ATF-2, unlike the related CREB, is not activated by increased cAMP, but by stimuli that activate SAPKs. Both JNK and p38 MAPKs regulate ATF-2 and its mechanism of regulation of transcriptional activating activity appears to be similar to that of c-Jun (Gupta *et al.*, 1995). Phosphorylated ATF-2 can form dimers with ATF-3, CREB, c-Jun or NF- $\kappa$ B. This suggests a potential for widespread roles in promoter activation (Bogoyevitch 2000). Furthermore, the ATF family is implicated in the hypertrophic gene programme. For example, in cardiac myocytes p38 MAPK phosphorylated ATF-6, which resulted in ANF gene binding to the serum response factor, independent of Elk-1, and participated in ANF induction (Thuerauf *et al.*, 1998).

#### 1.3.5.2.2.4 MEF-2

The MEF-2 transcription factors can bind to DNA sequences in promoters of muscle-specific genes, such as the  $\alpha$ -cardiac myosin heavy chain promoter (Lee *et al.*, 1997). Consequently, an essential role for MEF-2C in cardiac development has been demonstrated (Lin *et al.*, 1997). Furthermore p38 MAPK phosphorylation of MEF-2C

(Han *et al.*, 1997) leads to the increase in *c-jun* transcription, thus linking p38 MAPK and JNK pathways.

#### **1.3.5.2.2.5 CREB**

CREB is also a bZIP transcription factor that trans-activates pro-survival genes containing CRE (Kyriakis and Avruch 2001). As mentioned before, PKA (protein kinase A), which is activated by agonist elevation of cAMP, represents the major kinase to phosphorylate CREB (Habener 1990). Furthermore, both growth- and stress-regulated pathways may also be responsible for CREB phosphorylation. On the one hand, p90Rsk2, the downstream kinase of ERK, phosphorylate CREB in the nucleus, allowing *c-Fos* expression (Xing *et al.*, 1996). Furthermore, in cardiomyocytes MEK-1 mediated activation of CREB, leads to the induction of expression of the antiapoptotic factor Bcl-2 (Mehrhof *et al.*, 2001). On the other hand, two downstream kinases of p38 MAPK, namely MAPKAPK-2 and MSK-1, may also mediate activation of CREB (Tan *et al.*, 1996, Deak *et al.*, 1998). The phosphorylation of CREB is implicated in cardiac disease, since inactivation of CREB, by overexpression of a negative CREB protein, results in cardiac dilation, fibrosis and chronic venous congestion (Fentzke *et al.*, 1998).

#### **1.3.6 Inactivation of MAPKs by phosphatases**

Protein phosphatases have been suggested to play a central role in regulation of signalling pathways mediated by the MAPKs, resulting in inactivation and down-regulation of these pathways (reviewed in English *et al.*, 1999, Bogoyevitch 2000). However, compared to the wealth of knowledge on MAPKs, relatively little is known about these phosphatases.

Protein phosphatases include three families based upon their substrate specificity. These are the serine/threonine protein phosphatases (Cohen, 1989), the tyrosine protein phosphatases (Fischer *et al.*, 1991) and the dual-specificity tyrosine protein phosphatases (Franklin and Kraft 1997). MAPKs contain both phosphothreonine and phosphotyrosine and are therefore potential substrates for all classes of phosphatases.

One group of phosphatases, the MAPK phosphatases (MKPs), have dual-specificity for both threonine and tyrosine residues of the MAPK family, leading to dephosphorylation

and inactivation (Zheng and Guan 1993). Nine members of this group have been reported, including MKP-1 (CL-100, VH1), MKP-2 (hVH2, TYP-1), MKP-3 (Pyst1, rVH6), MKP-4 (Pyst3), MKP-5, PAC1, hVH2 (B-23), M3/6 (hVH5) and Pyst1 (Obata *et al.*, 2000). All the MKPs, with the exception of Pyst1, are immediate-early genes that are induced by various mitogens, growth factors and stresses. MKP-1, the first isolated MKP is a dual specificity phosphatase that dephosphorylates MAPKs (Sun *et al.*, 1993). This MKP-1 has been shown to inhibit JNK and p38 MAPKs more potently than ERKs (Franklin *et al.*, 1998). In addition, Chu *et al.* (1996) showed that MKP-1, MKP-2 and PAC1 have unique substrate specificity and reduced activity *in vivo* toward the ERK2. Similarly, M3/6 and MKP-5 were also found to inactivate both JNK and p38 MAPK, but not ERK, whereas MKP-3 and MKP-4 preferably dephosphorylate ERK over JNK and p38 MAPK (Muda *et al.*, 1996, Tanoue *et al.*, 1999). Other phosphatases, namely protein phosphatase 2A and 1 (PP2A and PP1) are also important in inactivating MAPKs (Alessi *et al.*, 1995, Sontag *et al.*, 1993). For example, the use of okadaic acid, a cell-permeable inhibitor of PP2A, caused activation of both JNKs, in ventricular myocytes (Fischer *et al.*, 1998), and ERKs in ventricular myocytes, fibroblasts and H9c2 myoblasts (Andersson *et al.*, 1998).

According to Bogoyevitch (2000) it remains possible that phosphatase inhibition during ischaemia will facilitate higher levels and/or prolonged phosphorylation of proteins that provide protection. Furthermore, it has been shown that protein phosphatase inhibitors could mimic the protective effects of PC in rabbit cardiomyocytes (Armstrong and Ganote 1992, Armstrong *et al.*, 1996). In addition, pre-incubation of the latter cells with calyculin A, a protein phosphatase PP1/2A inhibitor, induced high levels of phosphorylation in p38 MAPK during a prolonged period of ischaemia, which resulted in protection (Armstrong *et al.*, 1998). In contrast, Mackay and Mochly-Rosen (2000) used a tyrosine phosphatase inhibitor, vanadate, to prevent p38 MAPK inactivation, extending the strength and length of p38 MAPK activation during ischaemia, which resulted a higher susceptibility to cell death. Therefore they concluded that a tyrosine phosphatase is inactivated during ischaemia, resulting in prolonged activation of p38 MAPK which causes cell death.

### 1.3.7 Biological function of p38 MAPK activation

#### 1.3.7.1 Introduction

All three major subfamilies of MAPKs, namely ERK, JNK and p38 MAPK can be activated by short episodes of ischaemia-reperfusion (as occurs during the ischaemic PC protocol) (Ping *et al.*, 1999a, Ping *et al.*, 1999b), as well as by sustained ischaemia-reperfusion (Bogoyevitch *et al.*, 1996, Ma *et al.*, 1999). In particular, activation of p38 MAPK is under investigation as a candidate kinase in protection elicited by ischaemic PC. However, the importance of p38 MAPK activation for cardioprotection remains controversial. Furthermore, the relationship between p38 MAPK activation and the underlying cellular mechanisms of cardioprotection by ischaemic PC is not yet clear. Therefore, the remainder of the chapter will focus on the role of p38 MAPK activation.

Various GPCR-mediated stimuli are involved in induction of ischaemic PC (as described before). It has been reported that PC can be mimicked pharmacologically by transient  $\beta$ -adrenergic receptor stimulation in isolated rat hearts (Asimakis *et al.*, 1994, Miyawaki and Ashraf 1997b, Yabe *et al.*, 1998). Since  $\beta$ -adrenergic receptor stimulation also stimulates p38 MAPK activation in rat (Communal *et al.*, 2000) and mouse cardiac myocytes (Zheng *et al.*, 2000), it could be a useful tool to evaluate the contribution of this stress kinase to cardioprotection in ischaemic or  $\beta$ -adrenergic PC.

#### 1.3.7.2 Role of MAPKs in cell death or survival

Emerging evidence suggest that in models of myocardial ischaemia-reperfusion, the MAPKs, including ERK, p38 MAPK and JNK, may be important determinants of cell death or survival, but their respective roles in this regard and their interaction with one another are not well understood (Bogoyevitch *et al.*, 1996, Yue *et al.*, 2000, Gysembergh *et al.*, 2001).

Although it is predicted that the extent and duration of activation of the MAPKs will affect their final cellular responses, the ultimate role of the SAPKs (JNK and p38 MAPK) remains to be established. For example: (i) SAPKs may contribute to signal transduction pathways that culminate in cell death following stress, while (ii) on the

other hand, these SAPKs may also lead to cell survival by preventing wide-spread cell death, limit damage or mediate adaptation (Bogoyevitch 2000).

In response to ischaemia-reperfusion in the heart, the most severely affected myocytes will die, either by necrosis or controlled apoptosis. The pathways regulated by both p38 MAPK and JNK may contribute to a significant extent to apoptosis (Abe *et al.*, 2000). However, the role of p38 MAPK in myocyte apoptosis is complicated since this kinase can also mediate cell survival, particularly in ischaemic PC. Therefore the role of p38 MAPK has been investigated in ischaemia-reperfusion, as well as in PC and will be discussed separately.

### **1.3.7.2.1 p38 MAPK and Cell Death in Ischaemia-Reperfusion**

#### **1.3.7.2.1.1 Introduction**

Growing evidence indicates that apoptosis or programmed cell death plays a role in myocardial reperfusion injury (Gottlieb *et al.*, 1994). Originally Xia *et al.* (1995) examined the role for MAPKs in regulating apoptotic cell death after withdrawal of growth factors from neuronal cells. They demonstrated that activation of JNK and p38 MAPK and concurrent inhibition of ERK are critical in promoting apoptosis. The JNKs and p38 MAPKs have also been implicated in apoptosis in other cell types (Ichijo *et al.*, 1997, Chauhan *et al.*, 1997), but studies in the heart are in their early stages.

In cultured cardiac myocytes (Aikawa *et al.*, 1997, Krown *et al.*, 1996) activation of JNK and p38 MAPK, for example by proinflammatory cytokines and reactive oxygen species (ROS) (Clerk *et al.*, 1997, Aikawa *et al.*, 1997), induces apoptosis. However, the role of p38 MAPK activation in myocardial injury caused by ischaemia-reperfusion, an extreme stress to the heart, is unclear.

Bogoyevitch *et al.* (1996) were the first to demonstrate that myocardial ischaemia-reperfusion activates p38 MAPK and JNK. They found that only p38 MAPK was activated during ischaemia with no activation of JNK, but the latter was activated only during reperfusion following ischaemia. Furthermore, ERK was not activated by either ischaemia or ischaemia-reperfusion. Yin *et al.* (1997) correlated these events with tissue damage: a significant increase in DNA fragmentation (a marker of apoptosis)

was evident during ischaemia-reperfusion concomitant with the activation of JNK and p38 MAPK. However, the specific assessment of the role of SAPKs in this scenario requires the use of specific inhibitors or overexpression of MAPKs.

#### **1.3.7.2.1.2 Inhibitors of p38 MAPK and ischaemia-reperfusion**

Using chemical inhibitors has led to the conclusion that activation of the p38 MAPKs promote cardiac myocyte death during extended periods of ischaemia (Mackay and Mochly-Rosen 1999, Saurin *et al.*, 2000, Barancik *et al.*, 2000). In a cultured neonatal rat cardiac myocyte model, inhibition of p38 MAPK protects against ischaemic injury as indicated by decreased LDH release (marker for cell damage) (Mackay and Mochly-Rosen 1999, Saurin *et al.*, 2000). In addition, Barancik *et al.* (2000) reported that a specific inhibitor of p38 MAPK, SB203580, protected pig myocardium against ischaemic injury in an *in vivo* model by reducing infarct size.

Several studies indicated that p38 MAPK plays a pivotal role in promoting myocardial apoptosis (Ma *et al.*, 1999, Mackay and Mochly-Rosen 1999, Yue *et al.*, 2000). Ma *et al.* (1999) demonstrated that in isolated perfused rabbit hearts, ischaemia alone caused a moderate but transient (peaked at 15 min, declined at 30 min and maintained low levels thereafter) increase in p38 MAPK activity. Reperfusion further activated p38 MAPK after 10 min and remained elevated throughout reperfusion (20 min). Administering SB203580 before ischaemia and during reperfusion completely inhibited p38 activation and exerted significant cardioprotective effects, characterized by decreased myocardial apoptosis and improved post-ischaemic cardiac function, as well as attenuated myocardial necrotic injury. In contrast, administering SB203580 10 min after reperfusion (a time point when maximal MAPK activation had already been achieved) failed to convey significant cardioprotection. Mackay and Mochly-Rosen (1999) indicated that in neonatal rat cardiac myocytes, two distinct phases of p38 activation were observed during ischaemia: the first phase began within 10 min and lasted for less than 1 h, and the second began after 2 h and lasted throughout the ischaemic period. They demonstrated that SB203580 also protected cardiac myocytes against ischaemia by reducing activation of caspase-3 (a key event in apoptosis). However, the protective effect was seen even when the inhibitor was present during only the second, sustained phase of p38 activation. Subsequent studies by Yue *et al.* (2000), exposing rat neonatal cardiomyocytes to ischaemia showed a rapid and

transient activation of p38 and JNK. On reoxygenation, further activation of SAPKs was noted. With pretreatment of cells with SB203580 apoptotic cells were reduced, suggesting that p38 activation mediates apoptosis in rat cardiomyocytes subjected to ischaemia-reoxygenation. In addition, Yue *et al.* (2000) also showed that in isolated rat hearts SB203580 improved cardiac contractile function of ischaemic hearts.

Inhibition of p38 MAPK activation was therefore correlated with cardioprotection against ischaemia-reperfusion in cardiac myocytes as well as isolated hearts.

#### 1.3.7.2.1.3 Overexpression of p38 MAPK

MKK6 appears to phosphorylate all p38 MAPK isoforms, whereas MKK3 phosphorylates only p38 $\alpha$ ,  $\gamma$  and  $\delta$  (Enslin *et al.*, 1998). Zechner *et al.* (1998) reported that overexpression of MKK6, an upstream activator of p38 MAPK, resulted in protection of cardiac myocytes from apoptosis induced by either anisomycin or MEKK1, an upstream activator of JNK pathway. In addition, expression of MKK6 elicited a hypertrophic response, which was enhanced by co-infection of p38 $\beta$  (Wang *et al.*, 1998). Therefore a distinct isoform of p38 MAPK, p38 $\beta$ , may participate in mediating cell survival. In contrast, overexpression of MKK3 in mouse cardiomyocytes led to programmed cell death (apoptosis), which was increased by co-infection of p38 $\alpha$  (Wang *et al.*, 1998). Therefore, differential activation of p38 MAPKs isoforms may exert opposing effects: p38 $\alpha$  is implicated in cell death, while p38 $\beta$  may mediate myocardial survival.

To determine whether p38 MAPK activation was isoform selective, rat neonatal cardiomyocytes were infected with adenoviruses encoding wild-type p38 $\alpha$  or p38 $\beta$  (Saurin *et al.*, 2000). They showed that transfected p38 $\alpha$  and p38 $\beta$  were differentially activated during sustained ischaemia, with p38 $\alpha$  remaining activated but p38 $\beta$  deactivated. Furthermore, cells expressing a dominant negative p38 $\alpha$ , which prevented ischaemia-induced p38 MAPK activation, were resistant to sustained ischaemic injury. Therefore, activation of p38 $\alpha$  MAPK isoform during ischaemia is detrimental.

In addition, Hreniuk *et al.* (2001) demonstrated that treatment of rat cardiac myocytes with antisense oligonucleotides that specifically targeted either JNK1 or JNK2 and

significantly reduced both mRNA and protein expression of the target isoforms, resulted in almost complete attenuation of reperfusion-apoptosis. These observations suggest that also JNK activation contribute directly to apoptotic cell death.

### **1.3.7.2.2 p38 MAPK and Cell Survival in Preconditioning**

#### **1.3.7.2.2.1 Introduction**

In contrast to the previous mentioned evidence, that increased p38 MAPK activation during ischaemia-reperfusion mediate cell death, some studies could not demonstrate this association (Weinbrenner *et al.*, 1997, Maulik *et al.*, 1998d, Armstrong *et al.*, 1999, Omura *et al.*, 1999, Nakano *et al.*, 2000). Omura *et al.*, 1999 even indicated that p38 MAPK decreased during sustained coronary occlusion in an *in vivo* rat model. In other well-defined cell systems, i.e. fibroblasts (Roulston *et al.*, 1998) and HeLa cells (Assefa *et al.*, 1999), activation of JNK and p38 MAPKs enhances cell survival. It has therefore been suggested that an increase in p38 MAPK activation may also play a role in cell survival leading to cardioprotection (as in the phenomenon of PC, Weinbrenner *et al.*, 1997).

#### **1.3.7.2.2.2 p38 MAPK and Preconditioning**

It is well established that stress stimuli can elicit a survival signal, as occurs in PC, which is characterized by attenuated apoptosis during ischaemia-reperfusion (Maulik *et al.*, 1998c). The role of the p38 MAPK signalling pathway in the protection mediated by PC has been extensively investigated (Weinbrenner *et al.*, 1997, Maulik *et al.*, 1998d, Armstrong *et al.*, 1999, Nakano *et al.*, 2000, Nagarkatti *et al.*, 1998, Saurin *et al.*, 1999, Schneider *et al.*, 1999, Barancik *et al.*, 2000). These studies have sought to determine (i) whether prior PC induces the activation of p38 MAPKs during sustained ischaemia and (ii) whether inhibition of p38 MAPKs abolishes the cardioprotective effect. However, these observations are inconsistent, and the role of p38 MAPK in classic PC at this stage seems to be controversial.

On the one hand, it has been shown that PC is associated with an increased phosphorylation of p38 MAPK during sustained ischaemia (Weinbrenner *et al.*, 1997, Maulik *et al.*, 1998d, Armstrong *et al.*, 1999, Nakano *et al.*, 2000a). Weinbrenner *et al.*

(1997) indicated that isolated rabbit hearts preconditioned, with 5 min ischaemia and 10 min reperfusion, enhanced the phosphorylation of p38 MAPK after 10 and 20 min of ischaemia. Maulik *et al.* (1998d) subjected isolated rat hearts to a 4-cycle ischaemia-reperfusion PC protocol, which resulted in an increase in p38 MAPK phosphorylation that moved to the nucleus during the subsequent sustained ischaemic period and translocated to the cytoplasm after 10 min reperfusion. This PC-induced increase in p38 MAPK was also associated with enhanced MAPKAPK2 activity, the downstream substrate of p38 MAPK (Maulik *et al.*, 1998d), which in turn could result in the phosphorylation of HSP27 (Rouse *et al.*, 1994). The latter has been implicated in regulating cytoplasmic actin (Zu *et al.*, 1997). Similarly, Armstrong *et al.* (1999) showed that PC increased the dual phosphorylation of p38 MAPK as well as HSP27 phosphorylation in isolated adult hypoxic rabbit cardiomyocytes. In addition, Nakano *et al.* (2000a) also confirmed that the p38 MAPK/MAPKAPK2 pathway is activated during ischaemia only if the heart is in a preconditioned state.

On the other hand, Nagarkatti and Sha'afi (1998) found that exposure of the rat myoblast cell line H9C2 to PC led to a reduction in ischaemia-induced p38 MAPK activation and protection against a second lethal stress. In addition, several studies have reported that activation of p38 MAPK during a multi-cycle ischaemic PC protocol is transient and does not correlate with the PC effect (Ping *et al.*, 1999a, Behrends *et al.*, 2000), thereby questioning the significance of p38 MAPK activation in PC. Furthermore, Saurin *et al.* (2000) showed that prior PC prevented the activation of p38 $\alpha$  MAPK isoform during the subsequent sustained ischaemia in neonatal rat myocytes. Therefore, inhibition of the p38 $\alpha$  MAPK activation during ischaemia reduces injury and may contribute to PC-induced cardioprotection.

Therefore, there are two opposing theories for the role of p38 MAPK in PC. PC-induced cardioprotection is either associated with (i) an increased activation of p38 MAPK or (ii) an attenuation of p38 MAPK activation during sustained ischaemia.

#### **1.3.7.2.2.3 Pharmacological activation of p38 MAPK**

In the cardiovascular system, evidence also supports a pro-survival role for p38 MAPK activation: Pharmacological activation of p38 MAPK by administration of anisomycin before the onset of sustained ischaemia, elicited cardioprotection in isolated rabbit

cardiomyocytes (Weinbrenner *et al.*, 1997), protected rat neonatal cardiac myocytes (Mackay and Mochly-Rosen, 1999), perfused rat hearts (Nakano *et al.*, 2000a) and pig hearts (Barancik *et al.*, 1999). However, anisomycin, an inhibitor of protein synthesis, is also a powerful stimulant of JNK (Barancik *et al.*, 1999, Schneider *et al.*, 2001) and is therefore unsuitable for use in studies aimed at elucidating the role of p38 MAPK *per se*.

#### 1.3.7.2.2.4 Pharmacological inhibition of p38 MAPK in preconditioning

Besides determining the activation status of p38 MAPK during ischaemic PC, pharmacological inhibition of p38 MAPK was also used to elucidate whether p38 MAPK activation was the cause or effect of protection. However, data obtained using the specific p38 MAPK inhibitors (SB 203580 or SB 202190) also produced controversial results (see Table 1.1). Careful evaluation of data showed that the timing of SB 203580 administration to preconditioned hearts may account for the controversial findings. For example, Maulik *et al.* (1998d) and Nagarkatti *et al.* (1998) noted that pretreatment of rat hearts and a rat myoblast cell line, respectively, with SB 203580 before the ischaemic PC protocol completely abolished protection. Furthermore, a recent study in dogs showed that SB 203580 blocked cardioprotection only when given prior to the PC protocol, but not when given prior to sustained ischaemia (Sanada *et al.*, 2001). This was also confirmed by Sato *et al.* (2000) in working rat hearts. In contrast, according to a preliminary report by Schneider *et al.* (1999) SB 202190 pretreatment during a multi-cycle PC protocol could not abolish protection in isolated perfused rat hearts. In addition, Barancik *et al.* (2000) also confirmed that infusion of SB 203580 before and during the PC protocol did not influence the infarct size reduction mediated by PC in an *in vivo* pig model. On the other hand, Mocanu *et al.* (2000) recently showed that induction of SB 203580 during the PC protocol did not abolish protection, but administration after the PC protocol, prior to sustained ischaemia only, completely blocked protection. Furthermore, Nakano *et al.* (2000a, 2000c) also indicated that inhibition of p38 MAPK prior to sustained ischaemia only, completely abolished PC-induced protection. Therefore, exact timing of inhibitor administration may help clarify whether p38 MAPK acts as a trigger during the PC process or whether this kinase acts as a mediator of protection during the sustained ischaemia.

**Table 1.1****Summary of studies on pharmacological inhibition of p38 MAPK in preconditioning**

SB 203580 abolish PC induced protection:

Authors	Species	Model	Protocol	SB concentration	Endpoint
Maulik <i>et al</i> 1998d	rat	Isolated perfused heart	PC: 4x(5minI+10minR) I/R: 15minI+120minR SB: before PC	5 $\mu$ M	↓ LVDP ↑ Infarct size
Nagarkatti and Sha'afi 1998	rat	Myoblasts (H9C2)	PC: 5minI+60minR I: 45minI SB: before PC	15 $\mu$ M	↓ Cell survival
Sato <i>et al</i> 2000	rat	Isolated perfused heart	PC: 4x(5minI+5minR) I/R: 30minI+120minR SB: before PC+10min washout	5 $\mu$ M	↑ Infarct size
Nakano <i>et al</i> 2000a and 2000c	rabbit	Isolated perfused heart	PC: 5minI+10minR I/R: 30minRI+120minR SB: PC+20minSB starting 5min before RI	10 $\mu$ M and 2 $\mu$ M	↑ Infarct size
Mocanu <i>et al</i> 2000	rat	Isolated perfused heart	PC: 2x(5minI+10minR) I/R: 35minRI+120minR SB: PC+SB+I/R	10 $\mu$ M	↑ Infarct size
Sanada <i>et al</i> 2001	dogs	In vivo	PC: 4x(5minI+5minR) I/R: 90minCO+6hR SB: before PC	1 $\mu$ M	↑ Infarct size

SB 203580 could not abolish PC induced protection:

Authors	Species	Model	Protocol	SB concentration	Endpoint
Schneider <i>et al</i> 1999	rat	Isolated perfused heart	PC: 4x(5minI+5minR) I/R: 20minI+30minR SB: before and during PC	10 $\mu$ M	↓ Necrosis ↑ LVDP
Barancik <i>et al</i> 2000	pigs	In vivo	PC: 2x(10minCO+10minR) I/R: 60minCO+60minR SB: before and during PC	unknown	↓ Infarct size
Mocanu <i>et al</i> 2000	rat	Isolated perfused heart	PC: 2x(5minI+10minR) I/R: 35minRI+120minR SB: during PC	10 $\mu$ M	↓ Infarct size
Sanada <i>et al</i> 2001	dogs	In vivo	PC: 4x(5minI+5minR) I/R: 90minCO+6hR SB: PC+SB+I/R	1 $\mu$ M	↓ Infarct size

PC = preconditioning  
 I = global ischaemia  
 R = reperfusion  
 RI = regional ischaemia  
 CO = coronary occlusion  
 LVDP = left ventricular developed pressure

A further cause for concern is the drugs used as inhibitors to elucidate the role of p38-MAPK. The pyridinyl imidazole inhibitors have been widely used, particularly SB203580 and the related SB202190, however their efficacy and selectivity are still unclear. Although they have a potent effect on the activity of p38  $\alpha$  and p38  $\beta$  MAPKs, their efficacy for the p38  $\gamma$  and p38  $\delta$  isoforms is low (Lee *et al.*, 1999). Although inhibition of p38 MAPK has been demonstrated at  $< 1 \mu\text{M}$ , these inhibitors are normally used at the concentrations ( $\geq 10 \mu\text{M}$ ) that could also inhibit JNKs ( $\text{IC}_{50} = 5 \mu\text{M}$ ) (Clerk *et al.*, 1998b, Yue *et al.*, 2000) and phosphatidylinositol-3-kinase (PI3K) / protein kinase B (PKB) ( $\text{IC}_{50} = 3\text{-}5 \mu\text{M}$ ) (Lali *et al.*, 2000). Both the JNKs and PI3Ks have been shown to be recruited by ischaemic PC (Ping *et al.*, 1999a, Tong *et al.*, 1999). Therefore, these data suggest that extreme caution should be taken when interpreting data where SB203580 has been used at concentrations above  $1\text{-}2 \mu\text{M}$ . However, comparison of the concentrations used in the different protocols (Table 1.1) showed that although a broad range was used, it did not affect the outcome: For example, both  $10 \mu\text{M}$  and  $2 \mu\text{M}$  SB203580 abolished PC induced protection in the same protocol (Nakano *et al.* 2000a and 2000c).

### **1.3.8 Nitric oxide (NO) and p38 MAPK**

#### **1.3.8.1 Introduction**

Although several observations suggested that nitric oxide (NO) might act as a trigger for PC, conflicting results were published (as mentioned before). There are numerous mechanisms by which the NO/cGMP system and its downstream effectors could elicit protection (reviewed by Rakhit, *et al.*, 1999). However, its role in classic PC is less well defined.

#### **1.3.8.2 p38 MAPK: up- and downstream of NO**

p38 MAPK may be involved in the generation of NO at two points. First, the p38 pathway can enhance NO production by increasing the intracellular concentration of the substrate for NO synthetase via stimulation of arginine transporter activity (Caivano 1998). Second, p38 MAPK activity is required for increased gene expression of the inducible form of NO synthetase (iNOS, NOS2) (Guan *et al.*, 1997). p38 MAPK may also be involved in the transduction of NO signalling. Activation of p38 MAPK in human

neutrophils following lipopolysaccharide (LPS) stimulation is attenuated by inhibitors of NOS and by NO scavengers whereas treatment with NO releasing agents (donors) increased MKK3, 6 activity and p38 MAPK phosphorylation (Browning *et al.*, 1999). Similarly, the NO-induced apoptosis in a neutrophil-like cell line (HL-60) requires p38 MAPK activation (Jun *et al.*, 1999).

### **1.3.8.3 p38 MAPK and NO in preconditioning**

The role of the stress kinase, p38 MAPK and that of the mitochondrial KATP channel in the mechanism of ischaemic PC is currently being investigated. Activation of the mitochondrial KATP channel by NO has indeed been demonstrated (MacKay and Mochly-Rosen, 1999; Sasaki *et al.*, 2000), but to our knowledge the relationship between NO and p38 MAPK in PC has not been investigated previously. There is also no data concerning the downstream effects of cGMP in the context of PC.

A recent study (Kim *et al.*, 2000) investigated the role of sodium nitroprusside (SNP), a commonly used vasodilator that acts as a NO donor, in the regulation of the MAPKs in isolated adult rat cardiomyocytes. SNP maximally activated ERK1, ERK2, p38 MAPK and MAPKAPK2 within 5 – 10 min. They showed that activation of MAPKAPK2 by SNP was blocked by the soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one (ODQ) and the p38 MAPK inhibitor SB203580. The activation of ERK1 was insensitive to ODQ but completely blocked by the MEK1 inhibitor PD98059. The membrane-permeable homologue of cGMP, 8-Br-cGMP, also activated p38 MAPK, but not ERK1 or ERK2. Their results indicated that p38 MAPK and MAPKAPK2 are activated by NO in cGMP-dependent-pathways, while ERK1 activation by NO is independent of cGMP levels.

### **1.3.9 Other MAPKs and preconditioning**

One should also consider the role that ERK plays in the response to stress. The well-defined role of ERKs in modulating growth responses indicates that this subfamily could mediate cell survival. In support of this, cells overexpressing an active MEK (upstream of ERK) are more likely to survive than cells expressing dominant-negative MEKs (Guyton *et al.*, 1996). Although Bogoyevitch *et al.* (1996) showed that neither global ischaemia nor ischaemic reperfusion did activate the ERKs in perfused rat hearts, Seko

*et al.* (1996) demonstrated that, both hypoxia and hypoxia/reoxygenation caused rapid activation of Raf-1, followed by ERK activation in cardiac myocytes. In addition, coronary reperfusion increased ERKs in an *in vivo* rat model (Omura *et al.*, 1999). Aikawa *et al.* (1997) reported that oxidative stress activates ERK and suggested that it may be important for protecting cardiac myocytes from apoptotic cell death. Furthermore, in the presence of PD98059 (a MEK1/MEK2/ERK inhibitor), ischaemia-triggered myocyte apoptosis was significantly enhanced, confirming that the ERK pathway is important for survival of cells (Yue *et al.*, 2000).

Furthermore, ERK (Ping *et al.*, 1999b) and JNK (Ping *et al.*, 1999a) remained activated throughout a multi-cycle PC protocol, which was associated with activation of PKC $\epsilon$  in conscious rabbits. These data implicate PKC $\epsilon$  as the specific isoform responsible for PKC-induced MAPK activation and suggest that both ERK and JNK contribute to PKC $\epsilon$ -mediated protection against ischaemia (Ping *et al.*, 1999a).

#### **1.3.10 Conclusion**

The above indicated that no consensus has been reached regarding the significance of particularly p38 MAPK in PC. Thus, in view of the controversy regarding the time and role of p38 MAPK activation in ischaemia-reperfusion and ischaemic PC, careful evaluation and manipulation of the activation of this kinase is required, with meticulous attention to protocols and endpoints.

## 1.4 Motivation and Aims

### 1.4.1 General

Classic ischaemic PC implies that the heart has an endogenous protective mechanism against ischaemia. However the exact mechanism of this protection remains to be established, which if identified, may have important clinical implications. It is now generally accepted that several ligand-receptor interactions and signalling pathways are involved as triggers and mediators/effectors, respectively (as discussed in Chapter 1).

Endogenous triggers of PC may include substances released during the transient ischaemia and reperfusion (PC protocol), namely adenosine, opioids, bradykinin, acetylcholine, angiotensin and endothelin, as well as free radicals, nitric oxide (NO) and calcium. The release of endogenous catecholamines, i.e. noradrenaline, during the PC protocol with subsequent activation of the  $\alpha$ -adrenergic system has been implicated as one of the important triggers in the mechanism of PC (Banerjee *et al.*, 1993, Bankwala *et al.*, 1994). However, there is also evidence to the contrary from others (Asimakis and Inners-McBride 1993, Bugge and Ytrehus 1995), as well as from our laboratory (Moolman *et al.*, 1996). Another pathway which may also be involved in PC via the release of endogenous catecholamines, is the  $\beta$ -adrenergic signal transduction pathway. Although Strasser *et al.*, (1990) confirmed that activation of this pathway occurs within minutes after the onset of myocardial ischaemia, the contribution of  $\beta$ -adrenergic signalling to the phenomenon of PC has received relatively little attention thus far. Other receptor-independent endogenous triggers, namely free radicals, nitric oxide (NO) and calcium may also be involved in PC (reviewed by Schulz *et al.*, 2001). The involvement of NO as an initial trigger and subsequently as mediator, in the late phase of PC is well-established (Bolli *et al.*, 1998). However, although nitric oxide synthase (NOS) activity increased within 5 min of global ischaemia (Depré *et al.*, 1997), the role of NO in classic PC has not been defined.

It has been suggested that all triggers may be linked to a common final pathway. One such pathway may be via activation of protein kinase C (PKC) (Ytrehus *et al.*, 1994, Brooks and Hearse 1996, Baxter 1997). Lately, the mitogen-activated kinases (MAPKs) have received attention as possible role players. Particularly the p38 MAPK has been

investigated by a number of workers (Weinbrenner *et al.*, 1997), but its role is still controversial.

### **1.4.2 Hypothesis**

Knowledge of events occurring during the PC protocol is a prerequisite for elucidating the significance of the different riggers released during repetitive short episodes of ischaemia and reperfusion. At the time when this study was initiated, most previous studies focussed on events during sustained ischaemia only, while ignoring events during the PC protocol, as well as reperfusion after ischaemia.

(i) We propose that careful characterization of events during the entire experimental procedure of PC is required to elucidate the significance and interaction of these complex mechanisms.

(ii) Based on previous studies we hypothesize a role for catecholamines, in particular  $\beta$ -adrenergic signalling, and nitric oxide in PC. Accordingly, abolishment or attenuation of these events during either the PC protocol phase or during sustained ischaemia, causing inhibition of protection during reperfusion, may indicate a role as trigger or mediator, respectively. Conversely, pharmacological manipulation of these events during either the PC protocol or during sustained ischaemia, causing protection during reperfusion, may confirm a role as trigger or mediator, respectively.

(iii) We further hypothesize that, due to the sensitivity of p38 MAPK to stress, a multi-cycle ischaemic PC protocol *per se* should also elicit marked activation of this enzyme, which in turn, could be an important downstream event of both the  $\beta$ -adrenergic signalling and NO-cGMP pathways.

### **1.4.3 Specific aims:**

#### **1.4.3.1 Evaluation of events in ischaemic preconditioning: (Chapter 3)**

To elucidate the role of endogenous catecholamines and nitric oxide release as triggers, evaluating changes in tissue levels of cyclic nucleotides (cAMP and cGMP), cyclic nucleotide phosphodiesterases (cAMP-PDE and cGMP-PDE), as well as p38 MAPK

activation during the PC protocol, sustained ischaemia as well as during reperfusion in the isolated perfused working rat heart, using functional recovery during reperfusion as end-point.

#### **1.4.3.2 Manipulation of cyclic nucleotides: (Chapter 4 and 5)**

The next aim was to establish the significance of the changes observed. Therefore use will be made of pharmacological interventions, such as selective blockers or activators of either the  $\beta$ -adrenergic (Chapter 4) or the NO-cGMP (Chapter 5) pathway, which would abolish or mimic the ischaemia-induced changes in cyclic nucleotides and p38 MAPK activation during the PC protocol, respectively.

#### **1.4.3.3 Role of p38 MAPK activation: trigger or mediator? (Chapter 6)**

Timing of protein kinase activation appears to be critical for cardioprotection achieved by ischaemic PC. Therefore our final aim was to evaluate the role of p38 MAPK as trigger or mediator in ischaemic PC as well as in  $\beta$ -adrenergic-induced PC by using a p38 MAPK inhibitor, SB203580.

## CHAPTER 2

### 2. Materials and Methods

#### 2.1 Animals

Male Wistar rats weighing between 250 and 350g were used. Before experimentation, rats were allowed free access to food and water. This investigation was approved by the Ethics committee of the University of Stellenbosch (Faculty of Medical Sciences) and conforms with *the Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

#### 2.2 Perfusion Technique

The well-characterized isolated working rat heart was used as experimental model as described previously (Edoute, *et al.*, 1988). Rats were anaesthetized by injecting sodium pentobarbitol (30 mg/rat) intraperitoneally. The hearts were rapidly excised and arrested in ice-cold Krebs-Henseleit bicarbonate buffer, pH 7.4, containing (in mM): NaCl 119, NaHCO<sub>3</sub> 25, KCl 4.75, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 0.6, NaSO<sub>4</sub> 0.6, CaCl<sub>2</sub> 1.25, and glucose 10. After removal, the hearts were perfused uniformly via the aorta cannula, by the Langendorff technique (Langendorff, 1895). This was done in a retrograde, non-recirculating manner at 100 cm H<sub>2</sub>O (not electrically stimulated) with Krebs-Henseleit buffer oxygenated with 95% O<sub>2</sub> / 5% CO<sub>2</sub> at 37°C. The left atrium was cannulated to allow atrial perfusion (atrial pressure 15 cm H<sub>2</sub>O) according to the working heart model of Neely (Neely *et al.*, 1967) as modified by Opie and coworkers (1971).

Normothermic zero-flow global ischaemia was utilized for both ischaemic preconditioning as well as for induction of sustained ischaemia. Global ischaemia was induced by simultaneous clamping of both the aortic and left atrial supply tubes. Hearts were surrounded by a small tightly stoppered water-jacketed chamber to prevent cooling. Temperature was maintained at 36.5°C during global ischaemia and monitored continuously by a temperature probe inserted into the pulmonary artery.

Reperfusion was initiated by unclamping the tube leading to the aortic cannula, allowing retrograde perfusion. Functional recovery was assessed by measuring the mechanical activity before (during 15 min of working heart mode) and after global ischaemia (at the end of the reperfusion period), so that each heart served as its own control. Mechanical activity was determined by manually measuring the aorta output ( $Q_a$ ) and coronary flow ( $Q_e$ ), with their sum being the cardiac output (CO) in ml/min. Aorta pressure was obtained through a side arm of the aortic cannula, which was connected to a pressure transducer (Viggo Spectromed) and was recorded as a function of time by a computer. Peak systolic pressure (PSP) and heart rate (HR) were obtained from these recordings. The external power produced by the left ventricle as measured by total work performance ( $W_{tot}$ ) was calculated according to the following formula (Kannengieser *et al.*, 1979):  $W_{tot} = 0.002222 \times \text{PSP} \times \text{CO}$ .

### **2.3 Standard perfusion protocol: (Fig. 2.1)**

In all hearts (at least 6 per series), a stabilization period of 15 min retrograde perfusion was followed by 15 min in the working heart mode. Non-preconditioned hearts were subsequently perfused retrogradely for a further 30 min, whereas the hearts to be preconditioned (PC) were subjected to three episodes of 5 min global ischaemia (PC1-, 2-, 3-), interspersed by 5 min retrograde perfusion (PC1+, 2+, 3+) (total duration 30 min). All hearts were then subjected to 25 minutes of total global ischaemia followed by 30 min reperfusion (10 min retrograde and 20 min working heart) for evaluation of functional recovery.

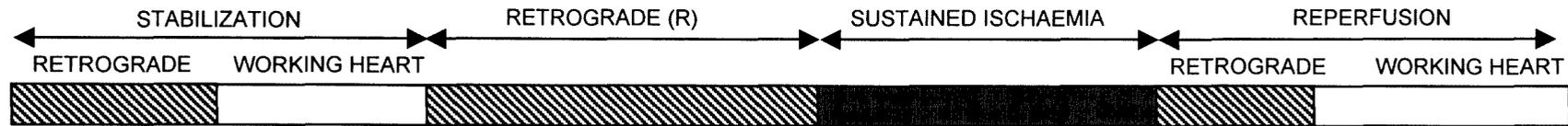
### **2.4 Biochemical analysis**

#### **2.4.1 Tissue high energy phosphate (HEP), cAMP and cGMP analysis**

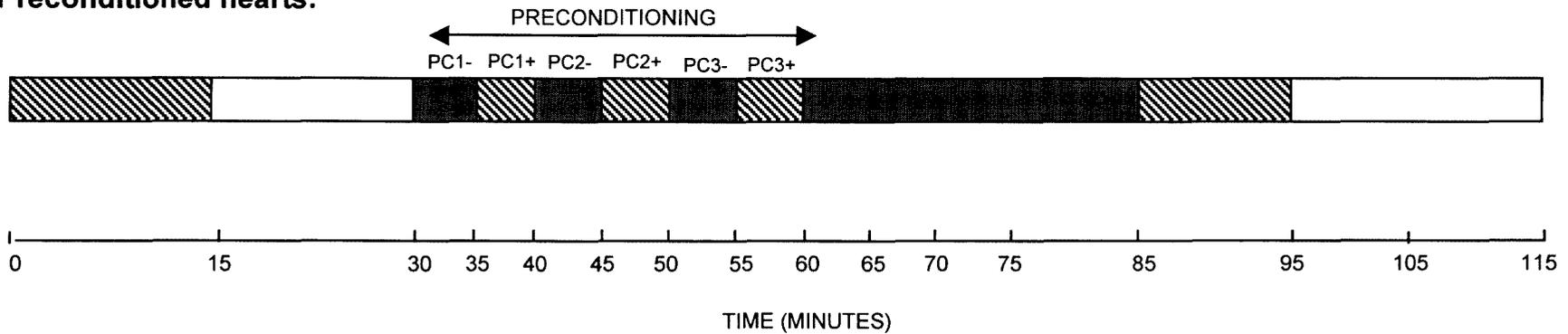
Hearts were freeze-clamped at various times during the perfusion protocol (see detail in following chapters) with precooled Wollenberger tongs and immediately plunged into and stored in liquid nitrogen.

For tissue HEP and cAMP analyses, 100-200 mg tissue was extracted with 1.2 ml 6% perchloric acid. Extracts were neutralized and filtrated through a 0.45 $\mu$ m (Millipore) filter. Analysis of HEP (adenosine triphosphate, ATP and creatine phosphate, CrP) was

**Non-preconditioned hearts:**



**Preconditioned hearts:**



**Fig 2.1** Standard perfusion protocol for non-preconditioned and preconditioned hearts. PC1-, PC2- and PC3- indicate hearts exposed to 5 min of global ischaemia; PC1+, PC2+ and PC3+ indicate hearts reperfused for 5 min after global ischaemia.

done by the reversed phase high pressure liquid chromatography (HPLC) technique, developed by Victor and coworkers (1987). Samples were separated by HPLC (LUNA 5 $\mu$  C18(2), 250 x 4.6 mm, Phenomenex) with on-line UV detection (210 nm) and quantitated with appropriate standards (Sigma). The mobile phase (flow rate 2.0 ml/min) consisted of a buffer, pH 4.0, containing (in mM): KH<sub>2</sub>PO<sub>4</sub> 257, tetrabutylammoniumphosphate 1.18 and HPLC graded methanol 12.5% (v/v).

Tissue cAMP content was determined using Amersham's cAMP [<sup>3</sup>H] assay system. This assay is based on the competition between unlabelled cAMP and a fixed quantity of [<sup>3</sup>H] cAMP for binding to a protein with high affinity and specificity for cAMP. A standard curve ranging from 0.125-16 picomol cAMP was included in each assay.

Tissue cGMP (~100 mg tissue) was extracted with 1.2 ml 5% trichloroacetic acid. The extracts were washed 4 times with 3.0 ml ether before analysis with Amersham's cGMP [<sup>125</sup>I] assay system. This assay is based on the competition between unlabelled cGMP and a fixed quantity of [<sup>125</sup>I] cGMP for a limited number of binding sites on a cGMP-specific antibody. The concentration of unlabelled cGMP in the samples was determined by interpolation from a standard curve (2-128 fmol cGMP).

#### **2.4.2 Tissue cAMP-dependent PKA activity**

Tissue cAMP-dependent PKA activity was determined with an assay from Gibco. Frozen tissue was homogenized in an extraction buffer (5 mmol/L EDTA, 50 mmol/L Tris, pH 7.5) and centrifuged for 10 min at 600g, and the supernatant was diluted to ~5  $\mu$ g protein/10  $\mu$ l extract. Four assay conditions were used for each sample (with and without inhibitor as well as with and without cAMP). Results were expressed as pmol activated PKA  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>. The protein content of samples was determined by either the Lowry et al., (1951), or the Kaplan and Pedersen (1985) technique.

#### **2.4.3 Tissue cAMP- and cGMP- phosphodiesterase (PDE) activity**

Phosphodiesterase (PDE) activity assays were performed on freeze-dried material, which was homogenized in water (50  $\mu$ l/mg dry weight). Since the homogenate was concentrated, it contained endogenous cyclic nucleotides as well as Mg<sup>2+</sup> in a concentration close to that found *in vivo*.

The PDE activity of the homogenate was determined using a radiotracer dilution method. The reaction was initiated by adding 100  $\mu$ l of homogenate to 20  $\mu$ l incubation mixture containing c[8-<sup>3</sup>H]AMP (SA 962 GBq/mmol) and c[8-<sup>3</sup>H]GMP (SA 363 GBq/mmol) (ca 200 000 cpm). After 10 min incubation (temperature 25°C) the reaction was terminated by addition of 20  $\mu$ l 60% perchloric acid. After centrifugation (2 min, 12 000g) the supernatant was separated by reversed phase HPLC (Purospher RP 18, 5 microspher, Merck) with on-line radiochemical detection of labelled nucleotides. The mobile phase consisted of (A) 100 mM ammoniumphosphate pH 5.5 and (B) Buffer A plus 6% acetonitrile. A linear gradient was used from 100% A to 100% B in 20 min (flow rate 0.5 ml/min). PDE activity was calculated as the percentage of cleavage products to total radioactivity (which was equivalent to the radioactive cyclic nucleotide initially present in the reaction mixture). This was done by Dr. Thomas Podzuweit (Centre for Experimental Cardiology, Max-Plank Institute, Bad Nauheim, Germany).

#### **2.4.4 Characterization of $\beta$ -adrenergic response**

##### **2.4.4.1 Membrane preparation**

To determine the characteristics of the  $\beta$ -adrenergic receptor population, a sarcolemmal enriched membrane fraction was prepared by a modification of the method described by Strasser et al., (1992). Tissue was homogenized in 20 ml buffer A (50mM Tris, 5mM EDTA, 2mM EGTA, 1mM dithiotreitol (DTT), pH 7.2 at 4° C) with a polytron PT 10 homogenizer for 3 x 10 sec at maximum speed. This homogenate was centrifuged for 10 min at 755 x g at 4° C. The resultant supernatant was centrifuged for 15 min at 45 000 x g at 4° C. The pellet obtained was washed twice with 5 ml buffer A and once with 5 ml buffer B (75mM Tris, 12.5mM MgCl<sub>2</sub>, 1.5mM EDTA, pH 7.4 with HCl at 4° C) and each time centrifuged for 15 min at 45 000 x g. The final pellet was suspended in buffer B to give a concentration of 10 mg protein/ml. Depending on the amount of protein, the protein content of each membrane preparation was determined by either the Lowry et al., (1951), or the Kaplan and Pedersen (1985) technique.

##### **2.4.4.2 $\beta$ -Adrenergic receptor assay**

The characteristics of the  $\beta$ -adrenergic receptor population were determined using the radiolabelled  $\beta$ -antagonist [<sup>125</sup>I]iodocyanopindolol as specific ligand as described by

Strasser et al., (1990). The number of  $\beta$ -receptors was determined in saturation experiments using increasing concentrations of ligand (0.01-0.5 nM) in an assay volume of 250  $\mu$ l, containing 10-20  $\mu$ g membrane protein/tube. Non-specific binding was determined by the nondisplaceable binding in the presence of the unlabeled  $\beta$ -antagonist, alprenolol ( $10^{-6}$ M). All determinations were done in triplicate. After 1 hour of incubation at 25° C, the reaction was terminated by addition of 0.6ml 25% polyethylene glycol (MW 8000) and 50 $\mu$ l (containing 125 $\mu$ g) gamma-globulin. After centrifugation for 30 min at 3 000 rpm, the pellets were washed once with buffer B and counted for radioactivity.  $K_d$  and  $B_{max}$  values of the receptor population were calculated, using the Enzfitter computer programme (Robin J Leatherbarrow, published by Elsevier-Biosoft).

#### **2.4.4.3 Adenylyl cyclase activity**

Adenylyl cyclase activity was determined as described by Salomon et al., (1974). The incubation medium contained 75mM Tris-HCl, pH 7.5, 12.5mM  $MgCl_2$ , 1mM EDTA, 0.1mM GTP, 0.001mM DTT, 0.1mM cAMP, 1mM isobutylmethylxanthine, 20mM phosphocreatine, 2 units creatine kinase and 0.5mM [ $\alpha$ - $^{32}P$ ]ATP (~ 200 000 cpm). For stimulation, forskolin ( $10^{-6}$  M) was added. The reaction was started by the addition of ~ 100  $\mu$ g membrane protein per tube, incubated for 10min at 37° C and stopped by addition of 500  $\mu$ l ice-cold  $NaHCO_3$  (120mM) and 500 $\mu$ l Zinc acetate (125mM). The radiolabelled cAMP was eluted from aluminium oxide columns prepared as described by Jakobs *et al.* (1976) (recovery 80-90%) and the eluates counted in 10ml Instagel for radio-activity.

#### **2.4.5 Western blotting for p38 Mitogen Activated Protein Kinases (MAPK)**

##### **2.4.5.1 Preparation of lysates**

Cytosolic p38 MAPK was determined by homogenizing freeze-clamped hearts with a lysis buffer containing (in mM): Tris 20; p-nitrophenylphosphate 20; EGTA 1; NaF 50; sodium orthovanadate 0.1; phenylmethyl sulphonyl fluoride (PMSF) 1; dithiotreitol (DTT) 1; aprotinin 10  $\mu$ g/ml; leupeptin 10  $\mu$ g/ml. These lysates were diluted in Laemmli sample buffer, boiled for 5 min and 10 $\mu$ g protein was separated by electrophoresis on a 12% polyacrylamide gel, using the standard Bio-RAD Mini-PROTEAN II system. The lysate protein content was determined using the Bradford

technique (Bradford 1976). The separated proteins were transferred to a PVDF membrane (Immobilon<sup>TM</sup>-P, from Millipore). These membranes were stained with Ponceau S red (reversible stain) to visualize the proteins. To assess the quality and quantity of the transfer, these membranes were laser-scanned and densitometrically analysed (UN-SCAN-IT, Silkscience). Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline, 0.1% Tween-20 (TBST).

#### **2.4.5.2 p38 MAPK dual phosphorylation**

The activated p38 MAPK was visualized with an appropriate primary phospho-antibody (phospho-p38 MAPK (Thr180/Tyr182) Antibody, New England Biolabs). Membranes were washed with large volumes of TBST (5 x 5min) and the immobilized primary antibody was conjugated with a diluted horseradish peroxidase-labelled secondary antibody (purchased from Amersham Life Science). After thorough washing with TBST, membranes were covered with ECL<sup>TM</sup> detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method (ECL<sup>TM</sup> Western blotting from Amersham Pharmacia Biotech). Films were densitometrically analysed and activated p38 MAPK values were corrected for minor differences in protein loading, if required.

#### **2.4.5.3 p38 MAPK activity**

For analysis of p38 MAPK activity using an assay kit, tissue lysates were prepared (using the same buffer as described above) containing 200 µg total protein/200 µl cell lysate. A monoclonal phospho-specific antibody to p38 MAPK (Thr180/Tyr182) was used to selectively immunoprecipitate the p38 MAPK from the lysate. The resulting immunoprecipitate was then incubated with ATF2 fusion protein in the presence of 200 µM ATP. Phosphorylation of ATF2 at Thr71 was measured by Western blotting using a phospho-ATF2 (Thr71) antibody. Films were densitometrically analysed as described above.

### **2.5 Drugs**

The drugs that were used in this study include: a direct activator of adenylyl cyclase, forskolin was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 10<sup>-3</sup> mol/l

and diluted to final concentrations of  $10^{-8}$ ,  $10^{-7}$  or  $10^{-6}$  mol/l (0.04% DMSO);  $\beta_1$ -adrenergic receptor agonist, isoproterenol ( $10^{-8}$ ,  $10^{-7}$  or  $10^{-6}$  mol/l);  $\beta_2$ -adrenergic receptor agonist, zinterol ( $10^{-7}$ ,  $10^{-6}$  or  $10^{-5}$  mol/l), nonselective  $\beta_1/\beta_2$ -adrenergic blocker alprenolol ( $10^{-5}$  or  $7.5 \times 10^{-5}$  mol/l);  $\alpha_1$ -adrenergic receptor antagonist, prazosin (0.3  $\mu$ M), selective adenosine  $A_1$  receptor antagonist, CPT or 8-cyclopentyltheophylline ( $10^{-5}$  and  $2 \times 10^{-5}$  mol/l) from Research Biochemicals Incorporated;  $Ca^{2+}$  channel blocker, verapamil (0.5, 1 and 2  $\mu$ M).

NO-donors, s-nitroso-penicillamine (SNAP) dissolved in DMSO and diluted in buffer to 10 or 50  $\mu$ M (0.04% DMSO) and sodiumnitroprusside or SNP (100  $\mu$ M); a selective inhibitor of guanylyl cyclase, ODQ or 1H-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one purchased from TOCRIS was either dissolved in DMSO or ethanol and diluted in buffer to final concentrations of 10 or 20  $\mu$ M (0.04% DMSO, 2% ethanol); L-arginine (10mM); NOS inhibitors, L-NAME or  $N^G$ -nitro-L-arginine methyl ester (50 $\mu$ M) and  $N\omega$ Nitro-L-Arginine or L-NNA (50 $\mu$ M); Intramed adrenaline ( $10^{-6}$ M) was used for  $\beta$ -adrenergic stimulation; highly selective p38 MAPK inhibitor, SB 203580 (1 or 10 $\mu$ M) from CALBIOCHEM. Routine chemicals were obtained from Merck, Cape Town.

## 2.6 Statistics

Results are given as mean  $\pm$  standard error of the mean (SEM). Multiple comparisons were analyzed by one-way analyses of variance (ANOVA) followed by the Bonferroni correction as *post hoc* test. In the case of p38 MAPK activity determinations, data scattered due to differences in overall blot densities, which might introduce type-II statistical errors, therefore unpaired t-test was used. A  $p < 0.05$  was considered significant.

## CHAPTER 3

### Evaluation of events in Ischaemic Preconditioning

#### 3.1 Introduction

Despite intensive efforts, elucidation of the exact mechanism(s) of ischaemic preconditioning (PC) has still not been accomplished. Involvement of several signal transduction pathways has been implicated. For example, stimulation of a variety of G protein-coupled receptors (i.e. adenosine A<sub>1</sub>,  $\alpha_1$  –adrenergic, muscarinic, bradykinin receptors) results in activation of protein kinase C, the latter being suggested as the common denominator in eliciting protection (Downey *et al.*, 1996, Lawson *et al.*, 1993). However, involvement of other signal transduction pathways has been proposed, including the  $\beta$ -adrenergic signal transduction pathway (Asimakis *et al.*, 1994, Sandhu *et al.*, 1996) and mitogen activated kinases (MAPKs) (Brooks *et al.*, 1996).

##### 3.1.1 $\beta$ -Adrenergic signal transduction pathway

Activation of the  $\beta$ -adrenergic signal transduction pathway occurs within minutes after the onset of myocardial ischaemia (Strasser *et al.*, 1990), therefore suggesting the possible involvement of this pathway in PC. Furthermore, our laboratory (Moolman *et al.*, 1996) and others (Sandhu *et al.*, 1996) indicated that prior PC significantly attenuated cAMP accumulation during sustained ischaemia in isolated rat and rabbit hearts. This observation may be due to, (a) upstream effects, i.e. reduced noradrenaline release or desensitization of the  $\beta$ -receptor (Sandhu *et al.*, 1996), changes in function of stimulatory- (Gs) or inhibitory- (Gi) G-proteins or adenylyl cyclase activity (Niroomand *et al.*, 1995, Iwase *et al.*, 1993) and/or (b) downstream mechanisms, such as less inhibited phosphodiesterase (PDE) activity. The activity of the PDEs is also influenced by cGMP and other reputed mediators of PC (such as nitric oxide or bradykinin) (Parrat *et al.*, 1995), that in turn increase tissue cGMP levels. The changes in downstream factors involved in cAMP metabolism/ breakdown in PC are presently unknown.

### 3.1.2 Mitogen activated kinases (MAPKs)

Emerging evidence suggests that, in models of myocardial ischaemia-reperfusion, mitogen activated protein kinases (MAPKs) – including p38 MAPK, extracellular signal-regulated kinases (ERK1 and ERK2), c-Jun N-terminal kinases (JNK1 and JNK2) and big MAPK-1 – may be important determinants of myocyte viability (Abe *et al.*, 2000). Although all members of the MAPK family have been investigated in the setting of PC-induced cardioprotection, the greatest interest was shown in p38 MAPK (which appears to be the more sensitive kinase to stress signals) (Bogoyevitch *et al.*, 1996, Clerk *et al.*, 1998, Gysembergh *et al.*, 2001). Therefore, it has been studied intensively as a possible mediator in the transduction of the stress signal (i.e. ischaemic PC) into the generation of a protective protein or activation of another downstream protective kinase. However, whether the ischaemia-induced activation of p38 MAPK is beneficial or deleterious is still controversial.

Most previous studies focussed on events during sustained ischaemia only, while ignoring events during the PC protocol, as well as during reperfusion after ischaemia. We therefore hypothesized that, in view of the sensitivity of p38 MAPK to stress, the multicycle ischaemic PC protocol *per se* should also elicit marked activation of this enzyme, which in turn, could affect subsequent events during sustained ischaemia and reperfusion.

### 3.1.3 The aim:

The aim of this study was therefore to evaluate myocardial levels of cAMP, cGMP and high energy phosphates (HEPs), as well as the activity of the PDEs and p38 MAPKs during (i) the PC (PC) protocol, (ii) sustained ischaemia and (iii) during subsequent reperfusion.

After having demonstrated cyclic increases in tissue cAMP during a multicycle PC protocol, we further hypothesized that these changes were caused by the release of endogenous catecholamines, resulting in downregulation of the  $\beta$ -adrenergic signal transduction pathway, contributing to attenuation of cAMP during sustained ischaemia and functional improvement during reperfusion. Therefore we also evaluated whether:

- pretreatment with reserpine would abolish the changes in cAMP during the PC protocol and
- downregulation of the  $\beta$ -adrenergic signal transduction pathway during sustained ischaemia occurred by evaluation of the (i)  $\beta$ -adrenergic receptor characteristics, (ii) PKA activity and (iii)  $\beta$ -adrenergic responsiveness of preconditioned myocardial tissue.

## **3.2 Experimental Protocols and Results**

### **3.2.1 Evaluation of events during the ischaemic PC protocol (preceding sustained ischaemia)**

#### **3.2.1.1 Experimental protocol**

##### **Non-preconditioned or control hearts:**

Hearts were stabilized by perfusing retrogradely for a period of 15 min, followed by 15 min in the working mode. Non-preconditioned hearts were subsequently perfused retrogradely for a further 30 min. Hearts were freeze-clamped at 30 and 60 min total perfusion time (Fig. 3.1).

##### **Preconditioned hearts:**

Hearts were stabilized for a period of 30 min (15 min retrograde, 15 min working mode) and then preconditioned by subjecting the hearts to three episodes of 5 min global ischaemia designated as PC1-, 2-, 3-, alternated by 5 min of retrograde perfusion designated as PC1+, 2+, 3+. Hearts were freeze-clamped at the beginning and end of each period of 5 min ischaemia, as well as at the end of the third reperfusion phase (Fig. 3.1). At least six hearts were freeze-clamped at each time point.

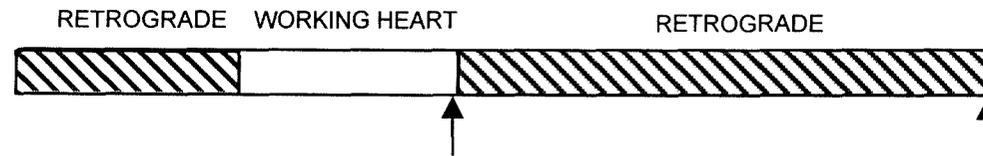
#### **3.2.1.2 Results**

##### **3.2.1.2.1 Evaluation of changes in tissue cyclic nucleotides (cAMP and cGMP)**

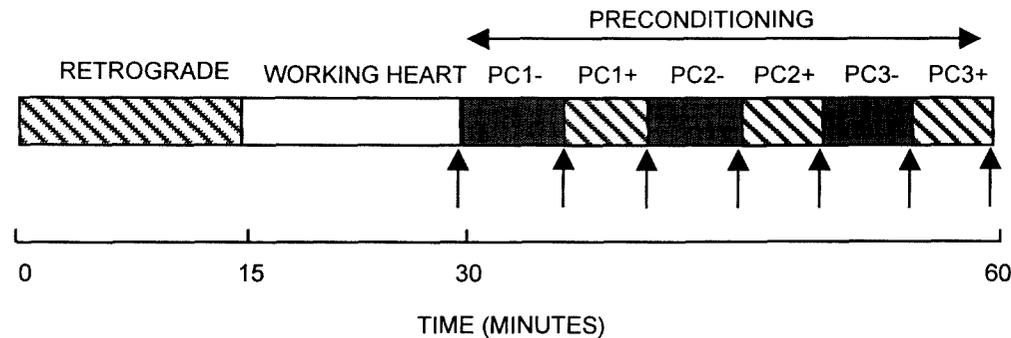
Control values for cyclic nucleotides were found to be similar after 30 and 60 min perfusion. Values were therefore pooled.

Hearts exposed to three episodes of 5 min global ischaemia (PC1-, 2-, 3-), showed significant alternating increases in myocardial content of cAMP when compared to the control. During each reperfusion episode (PC1+, PC2+, PC3+) the cAMP levels

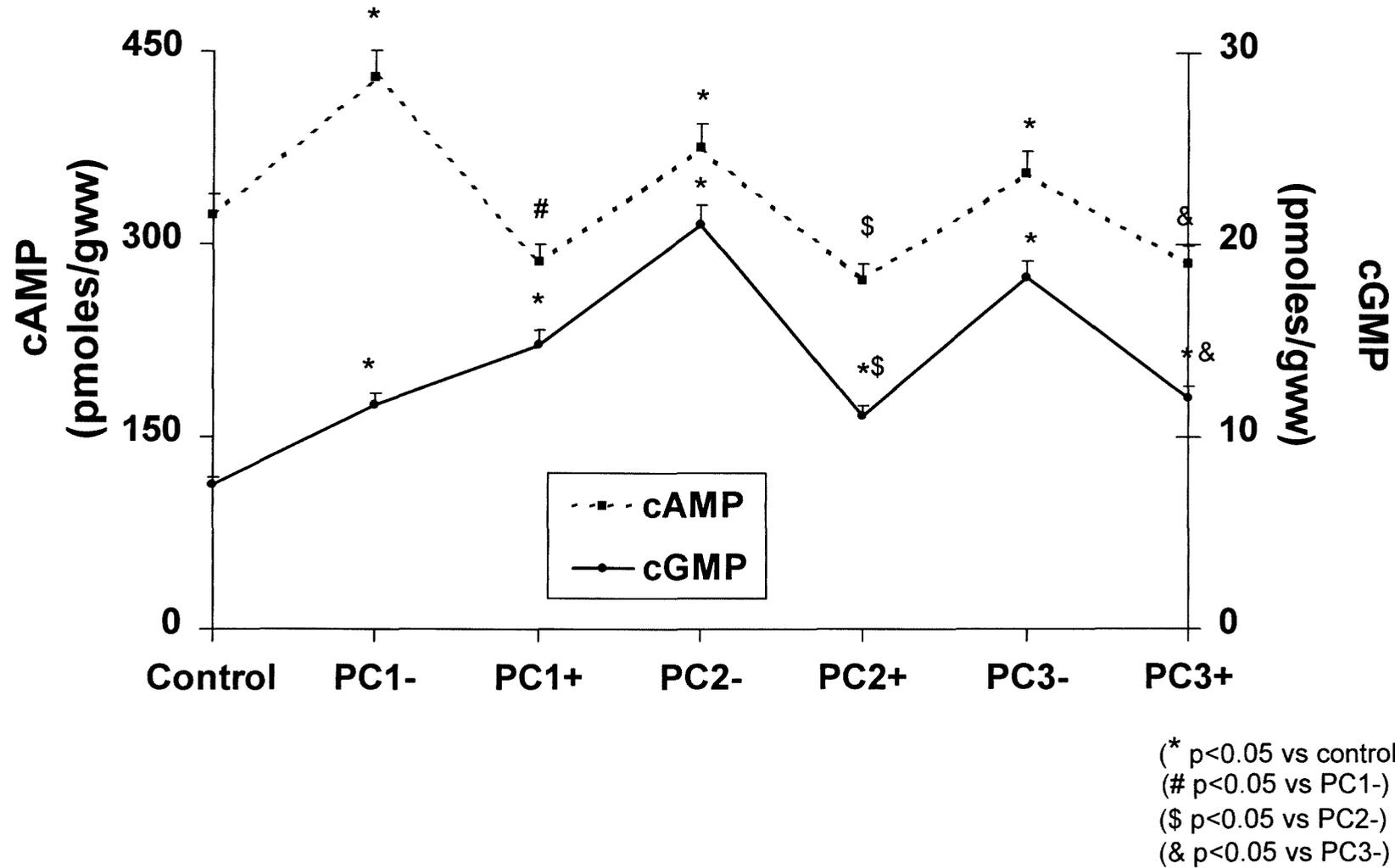
**Non-preconditioned hearts:**



**Preconditioned hearts:**



**Fig 3.1** Evaluation of the changes occurring during the preconditioning protocol. Experimental protocol. PC1-, PC2- and PC3- indicate hearts exposed to 5 min of global ischaemia; PC1+, PC2+ and PC3+ indicate hearts reperfused for 5 min after global ischaemia. Arrows indicate time of freeze-clamping. Controls were freeze-clamped after 30 min and 60 min perfusion. Six hearts were studied at each time interval for each experimental series.



**Fig 3.2** Cyclic changes in tissue cAMP and cGMP during the preconditioning protocol. Abbreviations as in Fig 3.1.

**Table 3.1**

**Effects of 3 x 5 min preconditioning on tissue cGMP and cAMP levels and the cAMP/cGMP ratio before onset of sustained ischaemia.**

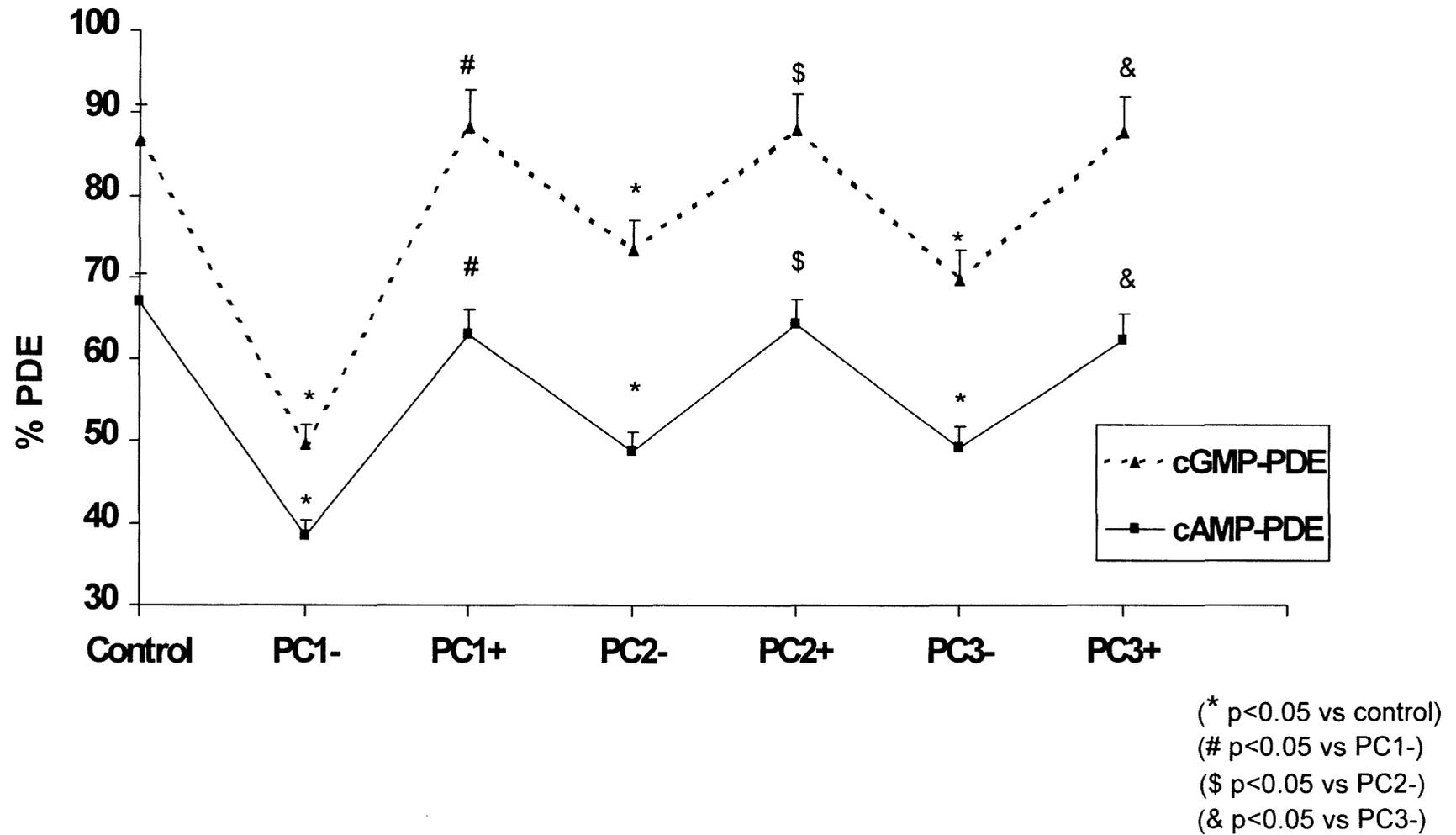
	<b>cGMP</b>	<b>cAMP</b>	<b>cAMP/cGMP</b>
Control (n = 11)	8.42 ± 0.35	317.0 ± 9.27	37.65
<u>Preconditioning</u> (n = ≥ 9/series)			
PC1-	12.18 ± 0.71 *	402.8 ± 13.23 *	33.07
PC1+	12.71 ± 1.37	307.0 ± 13.51 δ	24.15
PC2-	20.22 ± 0.91 *	368.7 ± 13.84 *	18.23
PC2+	13.12 ± 1.65 *δ	283.0 ± 10.34 δ	21.57
PC3-	16.79 ± 1.12 *	370.2 ± 9.74 *	22.05
PC3+	12.54 ± 0.69 *δ	277.1 ± 9.16 δ	22.10

Values are means ± SE in pmoles/g wet weight. Control hearts were perfused for 60 min (15 min retrograde, 15 min working heart, 30 min retrograde perfusion) (similar values were obtained at 30 min of control perfusion conditions).

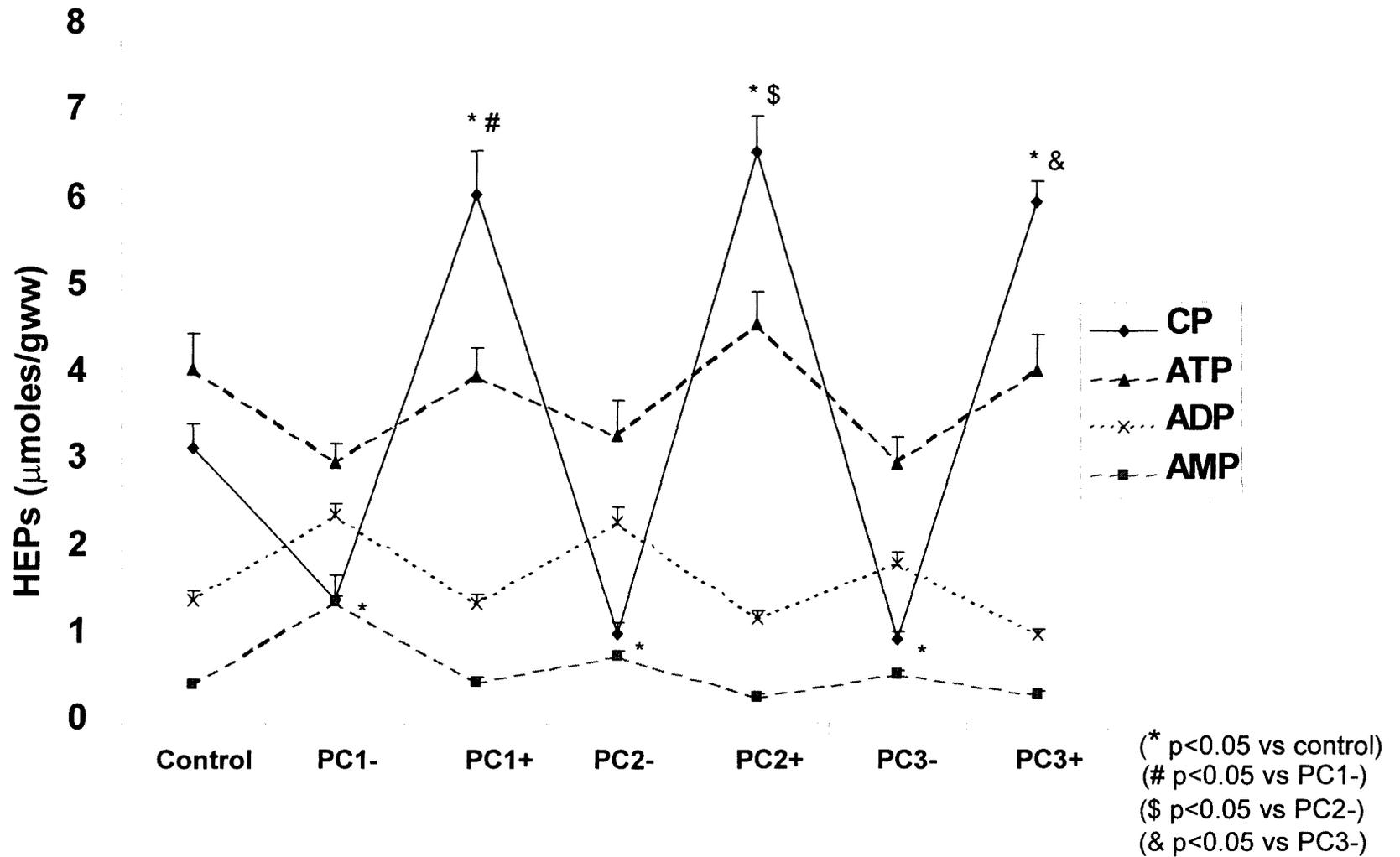
Individual cAMP/cGMP ratios not calculated in control and preconditioned series as values were obtained from different hearts.

δ p < 0.05 PC- vs PC+

\* p < 0.05 vs control (untreated)



**Fig 3.3** Cyclic changes in cAMP- and cGMP- phosphodiesterase activities during the preconditioning protocol. Abbreviations as in Fig 3.1.



**Fig 3.4** Cyclic changes in tissue high energy phosphates (HEPs) during the preconditioning protocol. Please note the significant changes in tissue creatine phosphate (CP). Abbreviations as in Fig 3.1.

recovered to those of control hearts. Furthermore, in each of the three episodes, tissue cAMP levels were significantly higher at the end of ischaemia (PC1-, 2-, 3-), than after reperfusion (PC1+, 2+, 3+) (Fig. 3.2 and Table 3.1).

Tissue cGMP levels at the end of each ischaemic episode (PC1-, PC2-, PC3-) showed the same tendency as the cAMP levels, viz. a significant increase. With the exception of the first reperfusion episode (PC1+), reperfusion caused a significant reduction in tissue cGMP, although not to baseline values (Fig. 3.2). Therefore tissue cGMP at the end of the PC protocol was significantly higher than control.

Since tissue cAMP and cGMP levels were determined in two separate series of experiments, cAMP/cGMP ratios could not be calculated for each individual heart and the values given in Table 3.1 were derived from the means of each series. Since the percentages increase in cGMP at PC1-, PC2- and PC3- (45, 140 and 99%, respectively) exceeded those of cAMP (27, 16 and 17%, respectively), the cAMP/cGMP ratios at these time points were reduced, when compared to controls.

#### **3.2.1.2.2 Evaluation of changes in tissue phosphodiesterase (PDE) activity**

The marked fluctuations in myocardial cAMP and cGMP levels during ischaemic-reperfusion cycles were reflected by opposite changes in both cAMP- and cGMP-hydrolysing PDE activities. At PC1-, both phosphodiesterases showed a significant reduction in their activities, followed by a return to baseline values during PC1+. Similar observations were made at the second and third PC episodes, however, inhibition of PDEs was less than during PC1- (Fig. 3.3).

#### **3.2.1.2.3 Evaluation of changes in tissue high energy phosphates (HEPs)**

During the ischaemic PC protocol, the tissue creatine phosphate (CP) values were significantly higher at each reperfusion episode (PC1+, PC2+, PC3+) than those of the controls, while each 5 min ischaemic episode (PC1-, PC2-, PC3-) lowered these values significantly. Although tissue ATP showed similar fluctuations, the changes were not significant and did not differ from controls. Tissue ADP and AMP showed opposite fluctuations which were also not significant from controls (Fig. 3.4).

### **3.2.1.2.4 Characterization of changes in $\beta$ -adrenergic signalling pathway during the PC protocol**

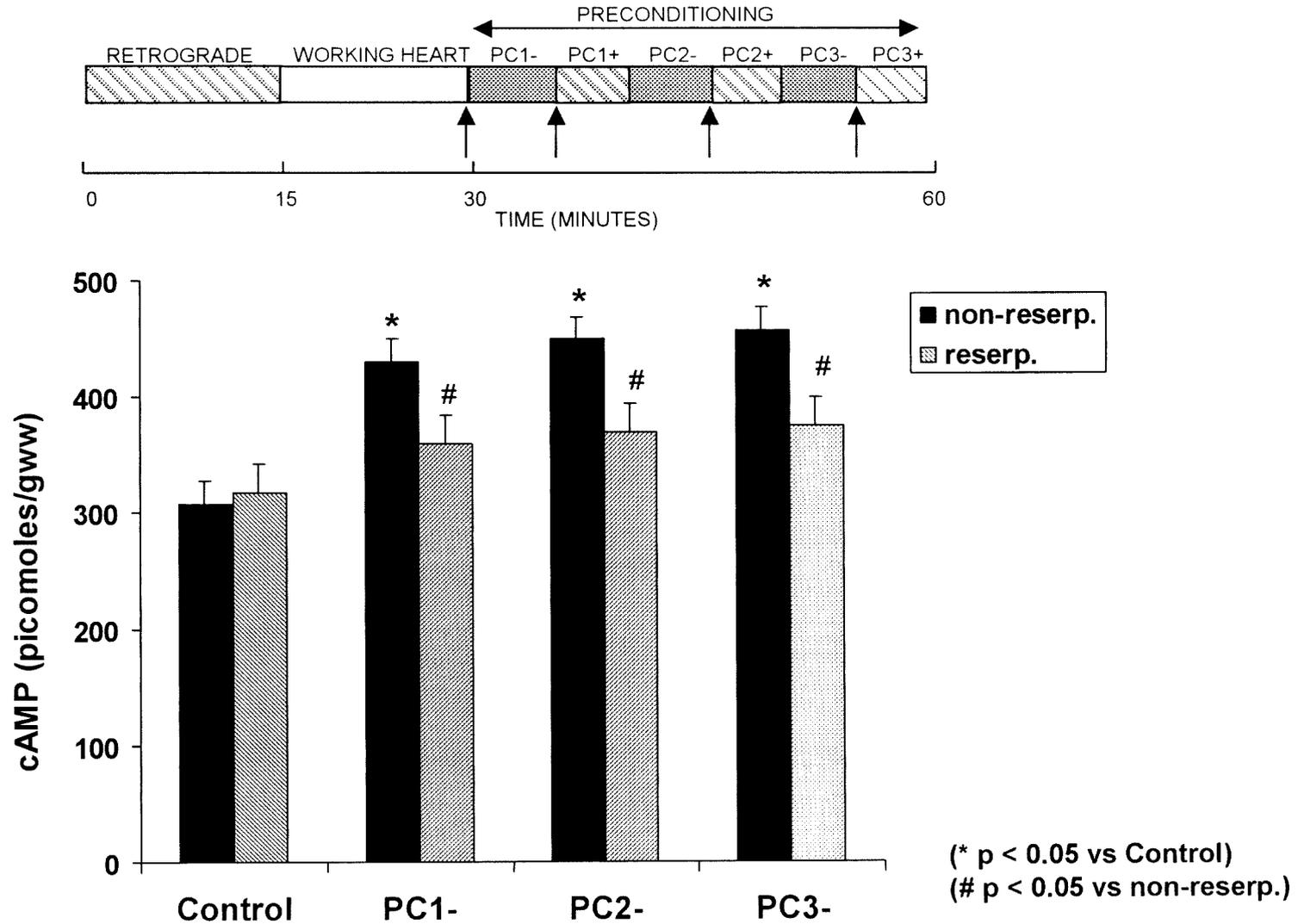
#### **3.2.1.2.4.1 Role of release of endogenous catecholamines in causing increases in cAMP**

Depletion of endogenous catecholamines was attained by intraperitoneal administration of reserpine (7 mg/kg) 24 h before experimentation (dose according to Toombs, *et al.*, 1993). Hearts from reserpinized and untreated animals were first perfused retrogradely (15 min), then for 15 min in the working mode (mimicking the exact technique used previously, Moolman, *et al.*, 1995). Non-preconditioned (Non-PC) hearts were subsequently perfused retrogradely for 30 min, whereas preconditioned (PC) hearts were subjected to three episodes of 5 min global ischaemia, alternated by 5 min retrograde reperfusion. Hearts were freeze-clamped after 30 min. total perfusion time (controls) as well as at the end of each 5 min period of global ischaemia (PC1-, PC2-, and PC3-) (Fig. 3.1).

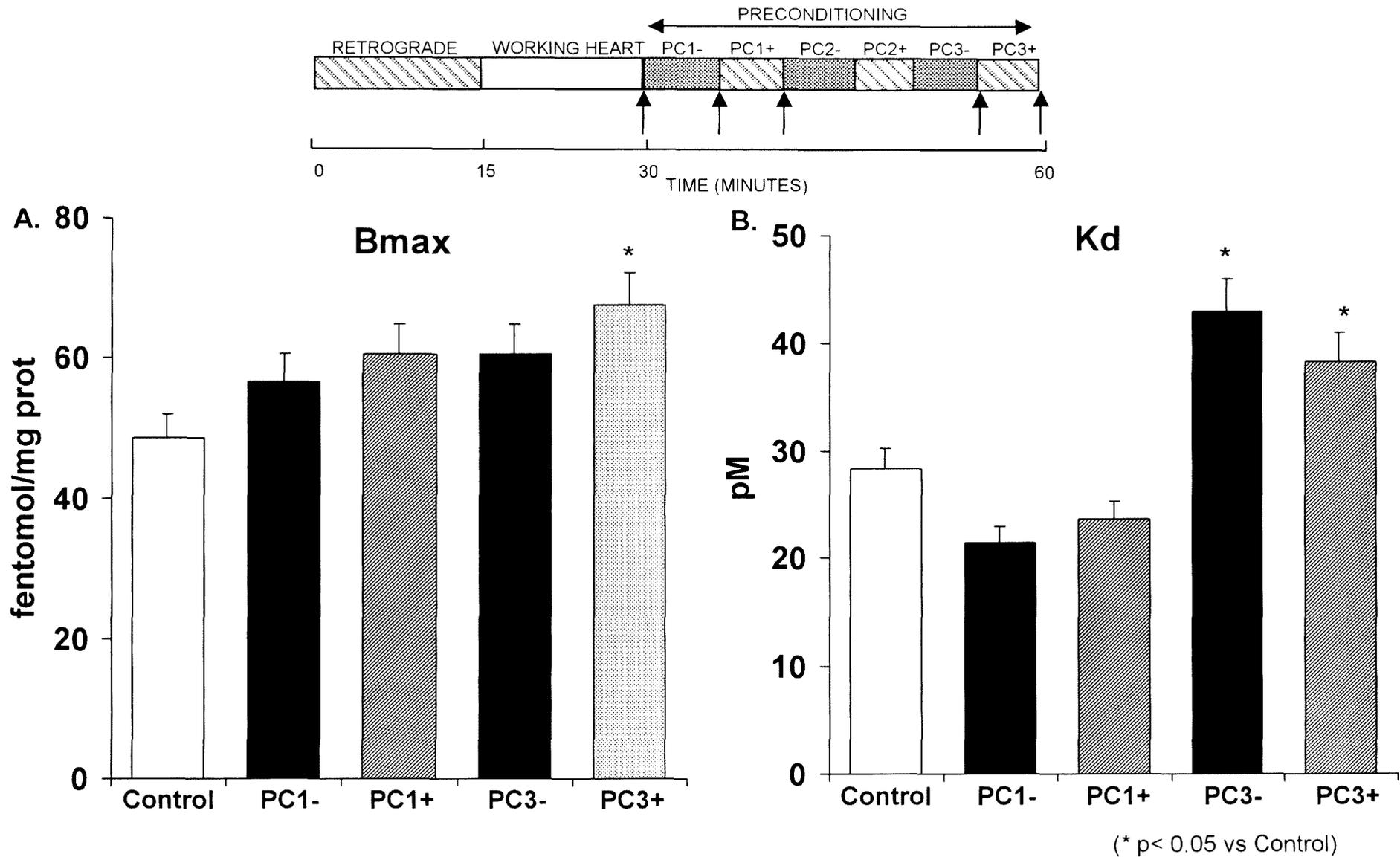
Results indicate that hearts of non-reserpinized rats subjected to PC exhibited similar increases in tissue cAMP as shown in Fig. 3.2, whereas depletion of catecholamines by prior reserpination abolished these increases at every time-point studied ( $p > 0.05$  vs. reserpinated controls at each time point) (Fig. 3.5).

#### **3.2.1.2.4.2 Evaluation of changes in $\beta$ -adrenergic signalling pathway**

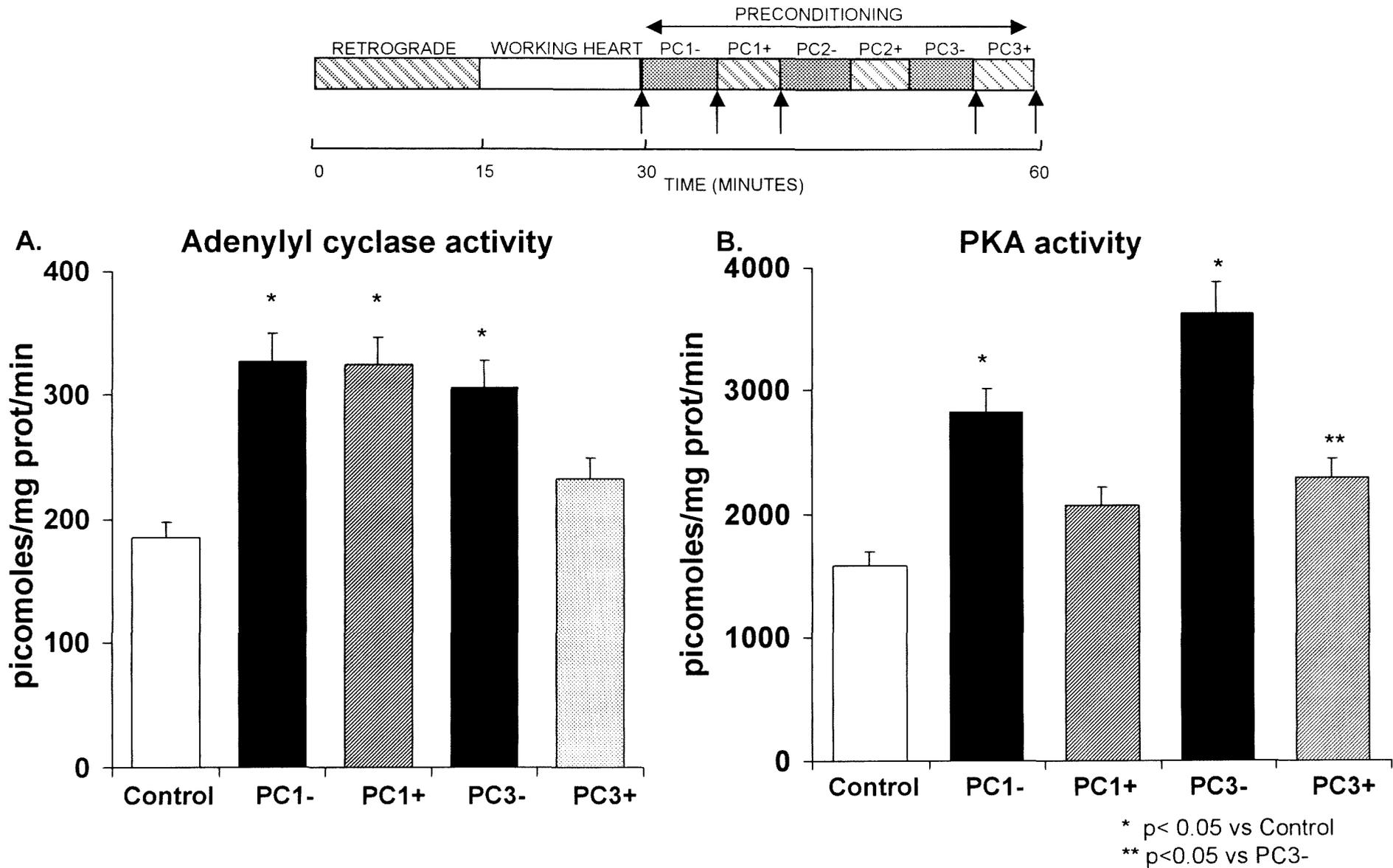
Non-PC hearts were perfused (as described in 3.2.1.1) and freeze-clamped after 30 and 60 min total perfusion time. The changes in the  $\beta$ -adrenergic signalling pathway were evaluated at the beginning and end of the PC protocol. Thus, PC hearts were freeze-clamped after 30 min total perfusion time (before onset of PC), at PC1- and PC3- and at the end of PC1+ and PC3+ for determination of  $\beta$ -adrenergic receptor characteristics, adenylyl cyclase and PKA activities (In view of the inordinate large number of rats required for protocols like these, it was decided not to study hearts at PC2- and PC2+).



**Fig. 3.5** Tissue cAMP of non-reserpinized and reserpinized hearts during the preconditioning protocol: Depletion of endogenous catecholamines by prior reserpination abolished the ischaemia-induced increases in cAMP. Arrows indicate time of freeze-clamping. Abbreviations as in Fig 3.1.



**Fig 3.6**  $\beta$ -Adrenergic receptor population during the preconditioning protocol: Preconditioning caused a gradual increase in receptor density (Bmax) and Kd (i.e. decrease in affinity). Arrows indicate time of freeze-clamping. Abbreviations as in Fig 3.1.



**Fig 3.7** Adenylyl cyclase activity (forskolin-stimulated) and total activated cAMP-dependent PKA activity during PC protocol: Both their activities were increased from PC1- and PC3- and reduced at PC3+. Arrows indicate time of freeze-clamping. Abbreviations as in Fig 3.1.

**$\beta$ -receptor population:**

Results from Fig. 3.6 A and B indicate that PC caused a gradual increase in receptor density ( $B_{max}$ ), and  $K_d$  (i.e. decrease in affinity), which became significant at PC3-. At the end of PC (PC3+),  $B_{max}$  was increased by 39% and affinity decreased by 35.5%.

**Adenylyl cyclase activity:**

Although the forskolin-stimulated adenylyl cyclase activity (Fig. 3.7A) was increased significantly at PC1- and PC3-, it was reduced at PC3+ (i.e. the activity at onset of sustained ischaemia did not differ from those of controls).

**PKA activity:**

cAMP-dependent PKA activity (Fig. 3.7B) changed in the same way as tissue cAMP levels, being significantly elevated at the beginning (PC1-) and the end (PC3-) of the PC ischaemic episodes, while returning to control levels upon reperfusion (PC1+, PC3+). PKA activity at the end of PC3+ was significantly lower than at PC3-.

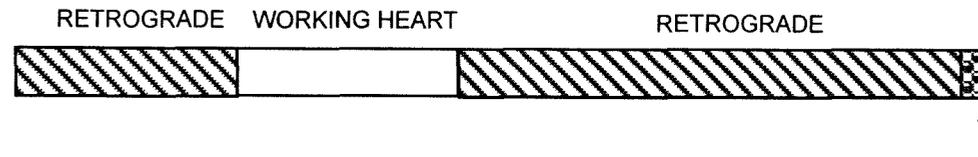
**3.2.1.2.4.3 Evaluation of  $\beta$ -adrenergic receptor responsiveness**

To determine whether the  $\beta$ -adrenergic signalling pathway was being desensitized during repeated transient ischaemic insults (for example, during a multi-cycle PC protocol),  $\beta$ -adrenergic responsiveness was evaluated by measuring changes in tissue cAMP content after administration of  $\beta$ -adrenergic agonists for a period of 2 min.

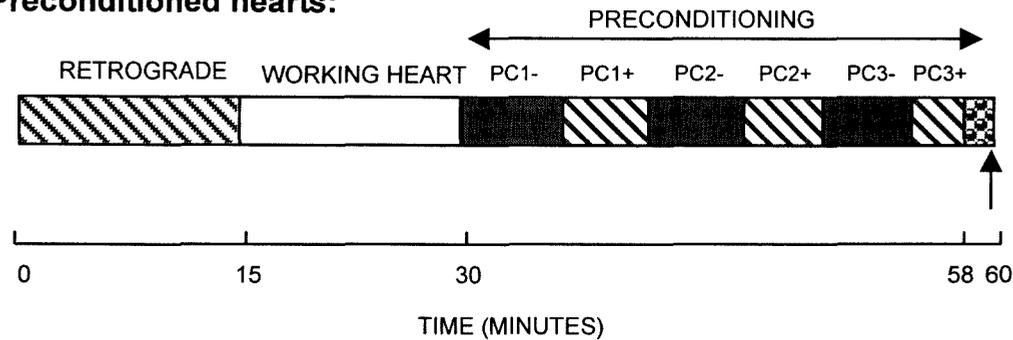
Non-PC and PC hearts were perfused as described in 3.2.1.1. At 58 minutes perfusion time, either forskolin ( $10^{-6}$  mol/l) or isoproterenol ( $10^{-8}$ ,  $10^{-7}$  or  $10^{-6}$  mol/l) was added to the perfusate and the hearts freeze-clamped after 2 minutes for cAMP determination (see Fig. 3.8 for protocol). Control non-PC and PC samples were taken from hearts freeze-clamped after 60 minutes perfusion without administration of drugs.

From the results (Fig. 3.9) it can be seen that isoproterenol ( $10^{-8}$ ,  $10^{-7}$  or  $10^{-6}$  mol/l) caused a significant dose-dependent increase in cAMP content of non-PC hearts compared to untreated control hearts, whereas cAMP of all PC groups remained unchanged, indicating desensitization of the  $\beta$ -adrenergic response. Forskolin ( $10^{-6}$

**Non-preconditioned hearts:**

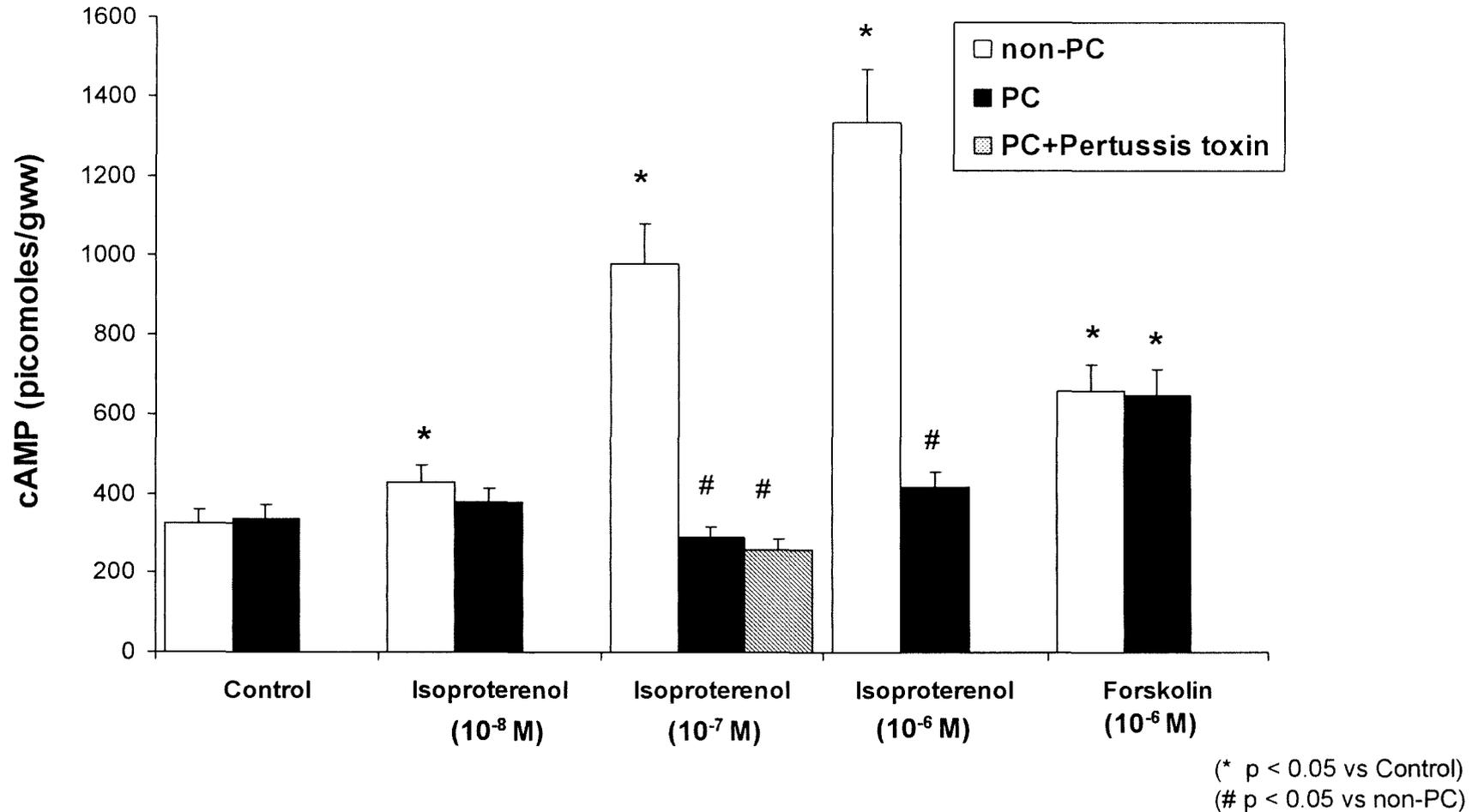


**Preconditioned hearts:**



 Isoproterenol ( $10^{-8}$  or  $10^{-7}$  or  $10^{-6}$  M) or Forskolin ( $10^{-6}$  M) for 2 min

**Fig 3.8**  $\beta$ -adrenergic pathway responsiveness after the ischaemic preconditioning protocol: Experimental protocol. Abbreviations as in Fig 3.1. At 58 min of perfusion time of non-PC and PC hearts, either isoproterenol  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M or forskolin  $10^{-6}$  M was added to the perfusate and hearts were freeze-clamped (as indicated by arrows) after 2 min for cAMP determination. Control hearts were freeze-clamped after 60 min of perfusion without administration of drugs.  $n = 6$  hearts per series.



**Fig 3.9**  $\beta$ -adrenergic pathway responsiveness after the ischaemic preconditioning protocol: cAMP generation in response to different agonists: Comparison of  $\beta$ -adrenergic response of non-PC and PC rat hearts to stimulation with isoproterenol  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M or forskolin  $10^{-6}$  M for 2 min. One series of hearts was pretreated with pertussis toxin 48h before experimentation. **Isoproterenol** caused a dose-dependent increase in cAMP of non-PC hearts, whereas cAMP of all PC groups remained unchanged, indicating desensitization of the  $\beta$ -adrenergic response. **Forskolin** elicited similar increases in cAMP in both non-PC and PC hearts, indicating that repeated ischaemic PC induces changes on  $\beta$ -adrenergic receptor level. No difference in cAMP response to isoproterenol ( $10^{-7}$  M) between untreated PC and **pertussis toxin**- treated PC hearts, indicates that the attenuation in  $\beta$ -adrenergic response was not due to increased Gi protein activity.

mol/l) elicited a similar two-fold increase in tissue cAMP in both non-PC and PC hearts, indicating that prior ischaemic PC induced a change at  $\beta$ -adrenergic receptor level.

To evaluate the role of the inhibitory G-protein ( $G_i$  protein), similar experiments were performed on rats pre-treated with pertussis toxin in saline (25  $\mu$ g/kg) (Lawson, *et al.*, 1993) or saline only, 24 hours before experimentation. After 58 min total perfusion time isoproterenol ( $10^{-7}$  mol/l) was added to the perfusate and the hearts freeze-clamped after 2 min. The cAMP generation in response to isoproterenol  $10^{-7}$  mol/l by hearts from pertussis toxin treated rats did not differ from hearts of untreated animals (Fig. 3.9), indicating that the diminished response to  $\beta$ -adrenergic stimulation by PC hearts was not due to increased  $G_i$  protein activity.

In addition to these experiments, the  $\beta$ -adrenergic receptor responsiveness of the ischaemic PC myocardium was also evaluated by measuring work performance after administration of the  $\beta$ -adrenergic agonist, isoproterenol, in a dose dependent manner. Non-PC and PC hearts were perfused as described in 3.2.1.1. At 65 min perfusion time, isoproterenol  $10^{-8}$  M was added to the perfusate for 5 min, increasing the dose to  $10^{-7}$  M and  $10^{-6}$  M at 5 min intervals. Work performance (aortic output and total work) were measured every 2 min after the first addition of isoproterenol. The initial 30 min perfusion time served as controls for non-PC and PC hearts (see Fig. 3.10 for protocol).

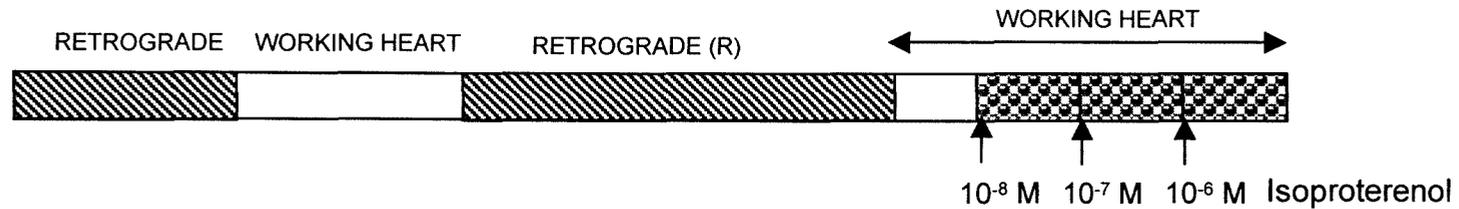
The results (Fig. 3.11 A, B) demonstrate that isoproterenol ( $10^{-8}$ ,  $10^{-7}$  or  $10^{-6}$  mol/l) caused a transient, but significant increase in aortic output and work performance 2 min after drug administration. The response to  $10^{-7}$  M isoproterenol elicited in the PC hearts was significantly lower compared to non-PC hearts, whereas the PC hearts were desensitised to  $10^{-6}$  M isoproterenol.

#### **3.2.1.2.5 Evaluation of p38 MAPK activation during the PC protocol**

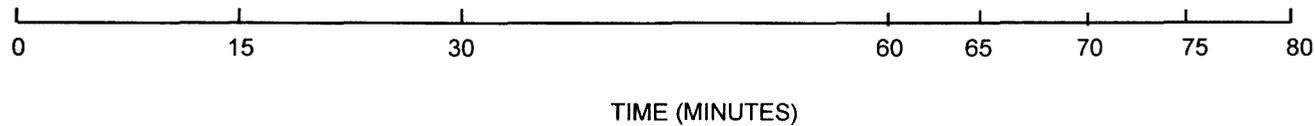
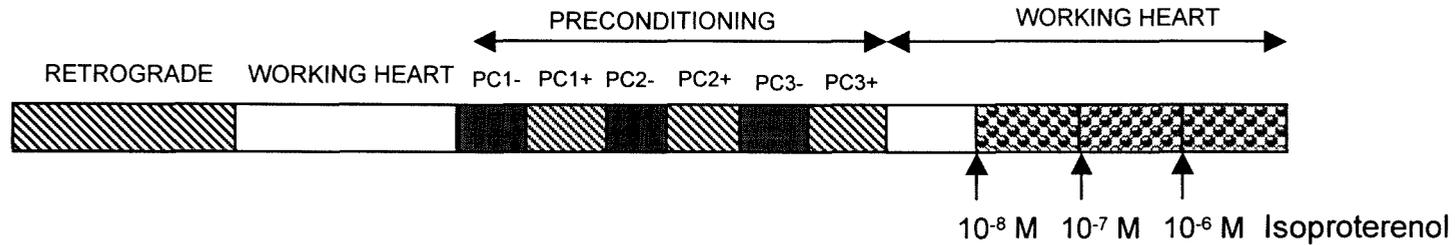
##### **Experimental protocol**

Dual phosphorylation of p38 MAPK (residues Thr 180 / Tyr 182 are phosphorylated) was assayed at PC1-, PC2-, PC3-, as well as at PC1+, PC2+ and PC3+ (4 - 6 hearts/series). In all series studied the activation of p38 MAPK, as indicated by its dual

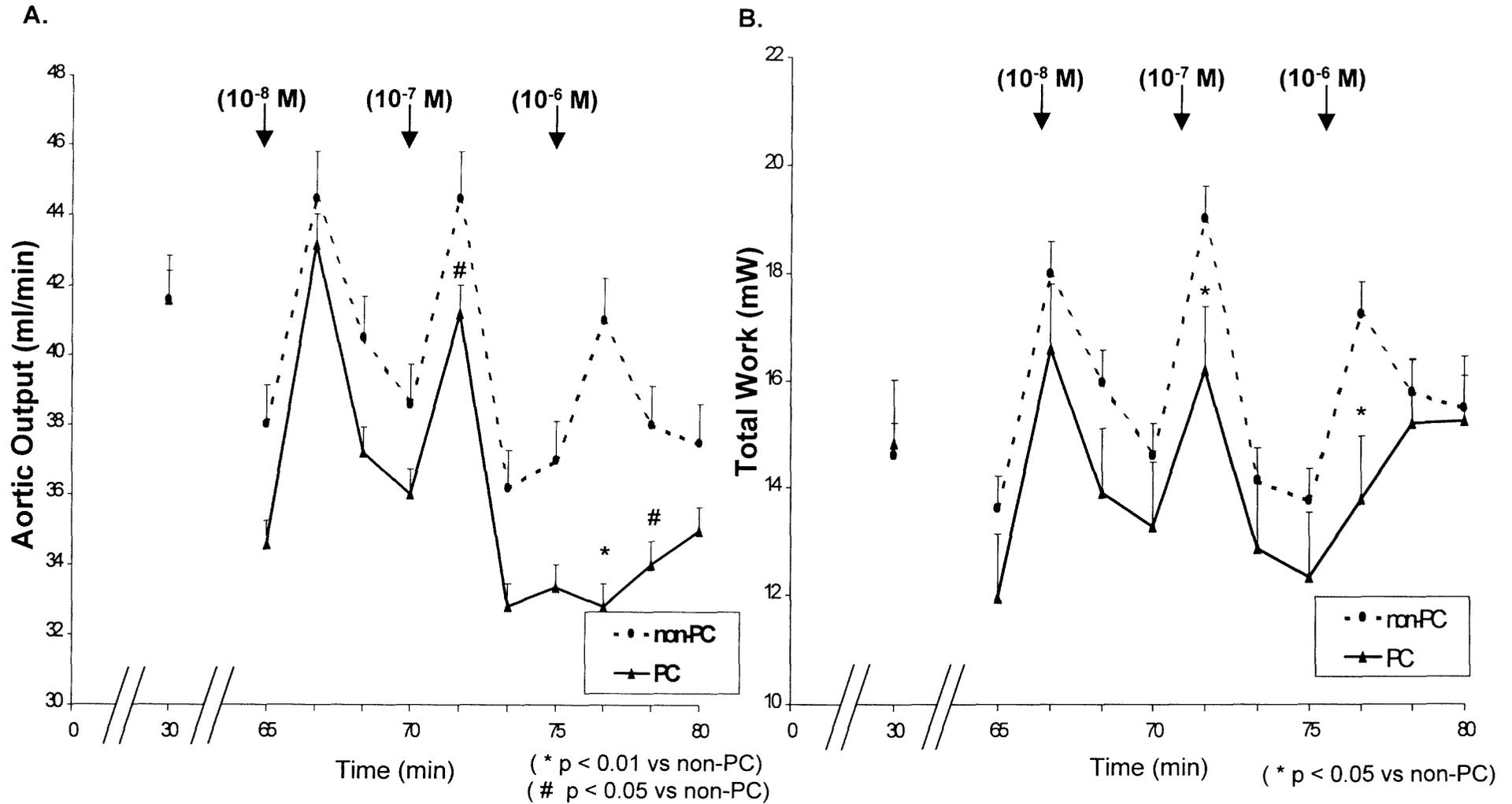
**Non-preconditioned hearts:**



**Preconditioned hearts:**



**Fig 3.10**  $\beta$ -adrenergic receptor responsiveness to isoproterenol after the ischaemic preconditioning protocol: Experimental protocol. Abbreviations as in Fig 3.1. Arrows indicate time of isoproterenol administration in a concentration range from  $10^{-8}$  to  $10^{-6}$  M. Mechanical function was measured every 2 min after the first addition of isoproterenol.  $n = 6$  hearts per series.

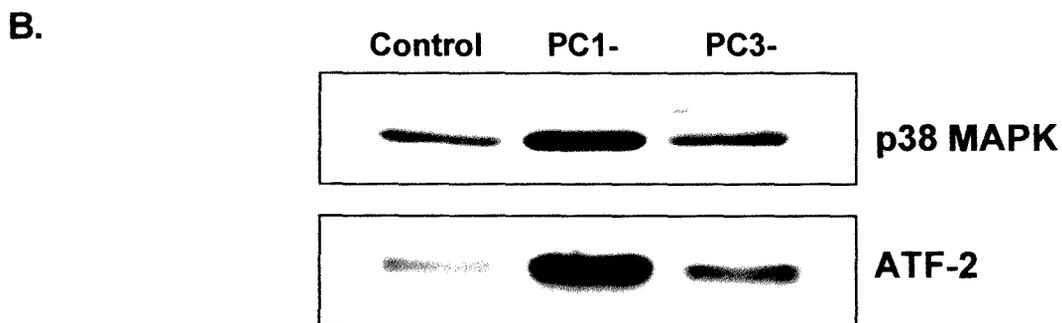
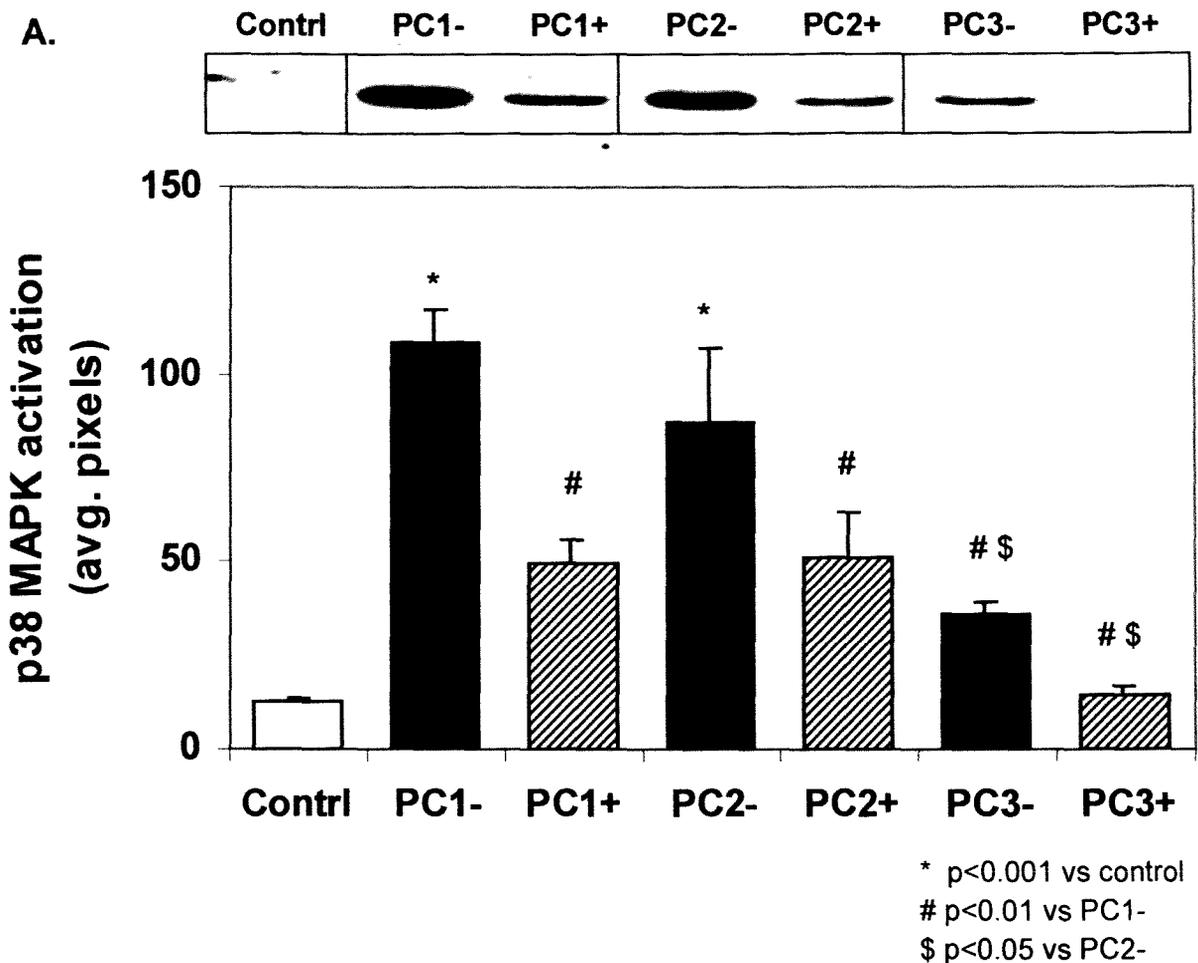


**Fig 3.11**  $\beta$ -adrenergic receptor responsiveness to isoproterenol after the ischaemic preconditioning protocol. Aortic output (A) and total work performance (B) were transiently increased by each administration of isoproterenol. The response to  $10^{-7}$  M isoproterenol elicited in PC hearts was lower compared to non-PC hearts, whereas PC hearts were desensitized to  $10^{-6}$  M isoproterenol.

phosphorylation, was reflected by its kinase activity as detected by phosphorylation of the substrate ATF-2. The latter was determined at selected intervals (3 hearts/series).

## Results

Exposure of the heart to 5 min global ischaemia (PC1-) caused a significant dual phosphorylation of p38 MAPK (Fig. 3.12A) as well as activation of ATF-2 (Fig. 3.12B), when compared to controls (obtained from hearts perfused for 30 min). This significant activation was also seen at the end of the second PC episode (PC2-), but activation at the end of the third episode (PC3-) was no longer significant. Reperfusion after the first PC episode (PC1+) caused a significant decline in p38 MAPK activation, compared to PC1-. Therefore, although dual phosphorylation and activation of p38 MAPK were observed during all three PC episodes, the activation was maximal during PC1-, becoming progressively less during PC2- and PC3-.



**Fig 3.12** The ischaemic preconditioning protocol: **A.** Analysis of p38 MAPK dual phosphorylation state (n = 4-6 hearts/series). **B.** Comparison with its kinase activity with *in vitro* phosphorylation of ATF-2 using anti-p38 immunoprecipitates (n = 3 hearts/series). Representative blots are given. p38 MAPK is transiently phosphorylated and activated during the ischaemic preconditioning protocol.

## **3.2.2 Evaluation of events during Sustained Ischaemia:**

### **3.2.2.1 Experimental protocol:**

#### **Non-preconditioned or control hearts:**

To evaluate the changes in tissue nucleotides during sustained global ischaemia, non-preconditioned hearts were stabilized for 60 min (15 min retrograde, 15 min working mode, 30 min retrograde), followed by 25 min sustained global ischaemia. Non-PC hearts were freeze-clamped before onset of sustained ischaemia (at 60 min total perfusion time), as well as at 10 min and 25 min during sustained ischaemia (Fig. 3.13A).

#### **Preconditioned hearts:**

Hearts were stabilized for a period of 30 min (15 min retrograde, 15 min working mode), followed by a PC protocol of 3 x 5 min global ischaemia and a subsequent period of 25 min global ischaemia. Similar to non-PC hearts, PC hearts were also freeze-clamped before onset of sustained ischaemia (at 60 min total perfusion time), as well as at 10 min and 25 min during sustained ischaemia. Four to six hearts were freeze-clamped at each time point (Fig. 3.13A).

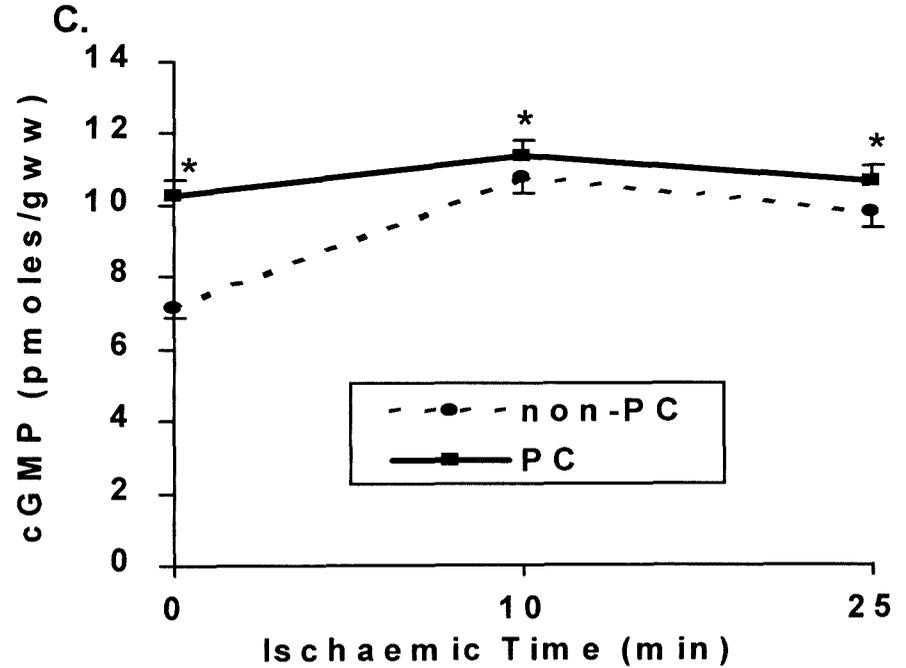
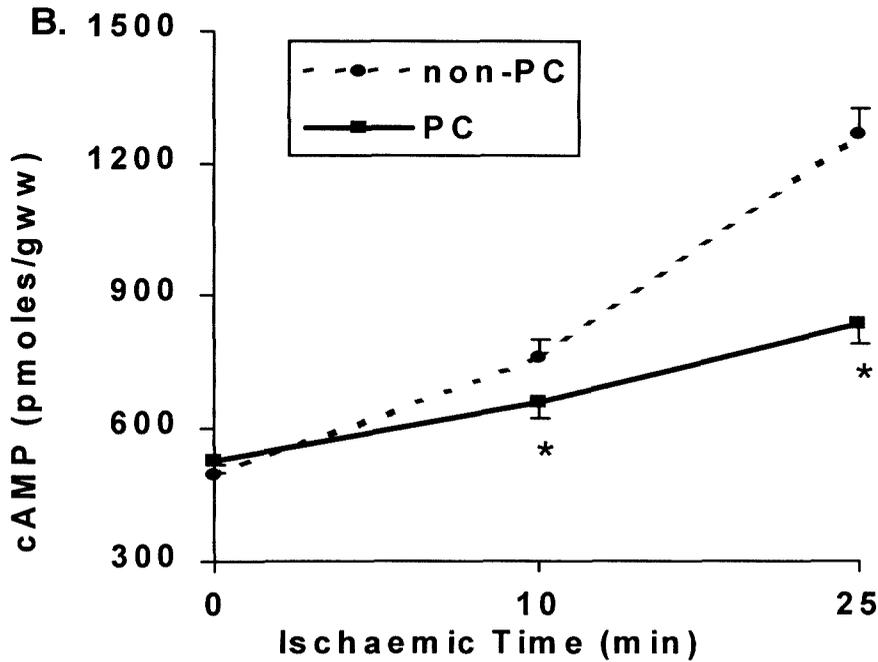
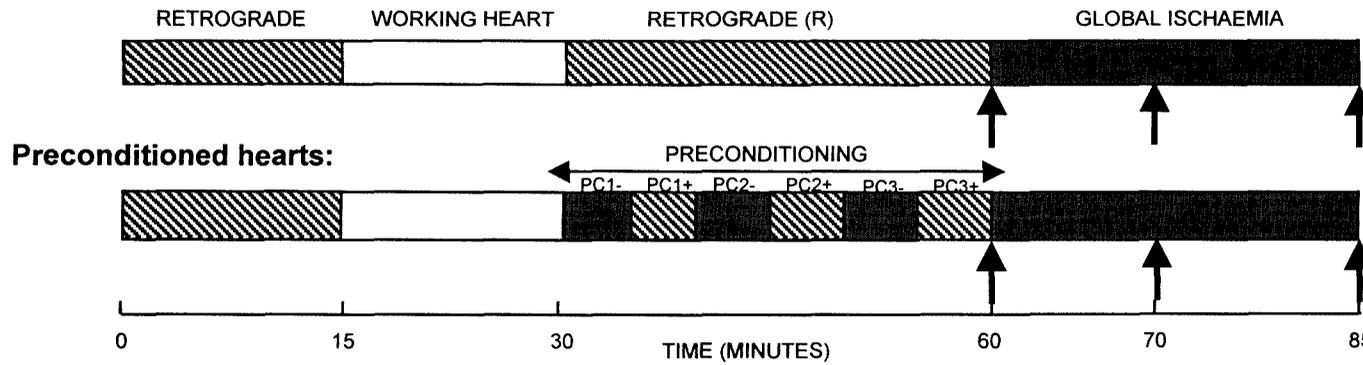
### **3.2.2.2 Results**

#### **3.2.2.2.1 Changes in tissue cyclic nucleotides**

Tissue cAMP at the onset of sustained ischaemia (zero-time) was similar in both non-PC and PC groups, and increased significantly at all durations of ischaemia (10 and 25 min of ischaemic time) in both groups compared to zero-time. However, cAMP levels were significantly lower in PC hearts compared to non-PC hearts at both 10 and 25 min ischaemia (Fig. 3.13B).

In contrast, cGMP levels of PC hearts were significantly higher than those of non-PC hearts at the onset of sustained ischaemia and remained higher in the PC hearts throughout sustained ischaemia compared to non-PC hearts (Fig. 3.13C).

**A. Non-preconditioned hearts:**



(\* p<0.05 vs non-PC)

**Fig 3.13** Tissue cAMP and cGMP levels during sustained global ischaemia. Two way analysis of variance of cAMP showed significant interaction between time and group. Tissue cAMP was significantly lower in PC hearts at both 10 and 25 min ischaemia, while tissue cGMP of PC hearts was significantly higher than non-PC hearts throughout sustained ischaemia.

**Table 3.2**

**The cAMP/cGMP ratios in non-preconditioned (non-PC) and preconditioned (PC) hearts during sustained ischaemia.**

<b>Sustained Ischaemia</b>	<b>cAMP/cGMP ratios</b>	
	<b>Non-PC</b>	<b>PC</b>
Zero-time	69.6 ± 6.4 $\delta$	51.7 ± 1.4* $\#$
10 min	72.6 ± 8.3 $\delta$	59.7 ± 5.4* $\#$
25 min	134.2 ± 14.6	78.9 ± 5.2*

Values are means ± SE of individual cAMP/cGMP ratios (tissue cAMP and cGMP levels in Fig. 3.13). n = 6 hearts per series.

\* p < 0.05 vs non-PC

$\delta$  p < 0.05 vs 25min non-PC

$\#$  p < 0.05 vs 25min PC

In both groups the cAMP/cGMP ratios increased markedly with prolongation of the ischaemic time (Table 3.2). At all time intervals studied (also zero-time values obtained immediately before onset of sustained ischaemia), this ratio was lower in PC hearts.

#### **3.2.2.2.2 Changes in tissue PDE activity**

In both PC and non-PC hearts, cAMP- and cGMP-PDE activities declined over the ischaemic time period of 10 - 25 min. This inhibition was maximal after 15 min. With increasing periods of global ischaemia, both PDE activities were significantly less inhibited in PC than in non-PC hearts (Figs. 3.14 A, B & C).

#### **3.2.2.2.3 Changes in tissue HEPs**

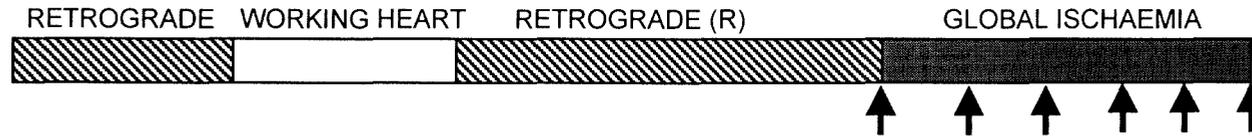
Before the onset of sustained ischaemia, the total adenine nucleotides (TAdN, which includes ATP, ADP and AMP) levels differed significantly between the different groups. This was attributable to differences in ATP and ADP which were both lower in the PC group than those of the non-PC control group. Tissue CP values also differed significantly, with the PC group having higher CP levels than that of the non-PC control group (Fig. 3.15). At the end of 25 min sustained global ischaemia the TAdN levels as well as the CP contents of non-PC and PC hearts were similar (Fig. 3.15). Consequently, more TAdN was utilized in non-PC than PC hearts, whereas more CP was utilized in PC hearts.

#### **3.2.2.2.4 Evaluation of p38 MAPK activation during sustained ischaemia**

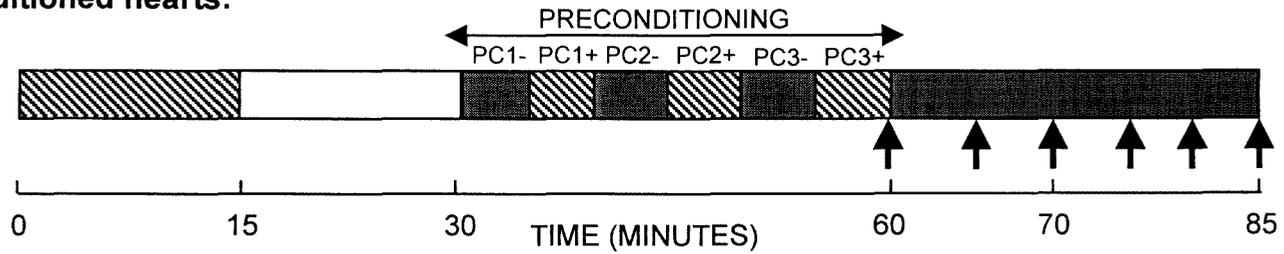
### **Experimental protocol**

During sustained ischaemia, non-PC and PC hearts were freeze-clamped at 5, 10, 15 and 25 min (Fig. 3.14A). Dual phosphorylation of p38 MAPK (residues Thr 180 / Tyr 182 are phosphorylated) was assayed at all the above mentioned time intervals (4 - 6 hearts/series). In all series studied the activation of p38 MAPK, as indicated by its dual phosphorylation, was reflected by its kinase activity as detected by phosphorylation of the substrate ATF-2. The latter was determined at selected intervals (3 hearts/series).

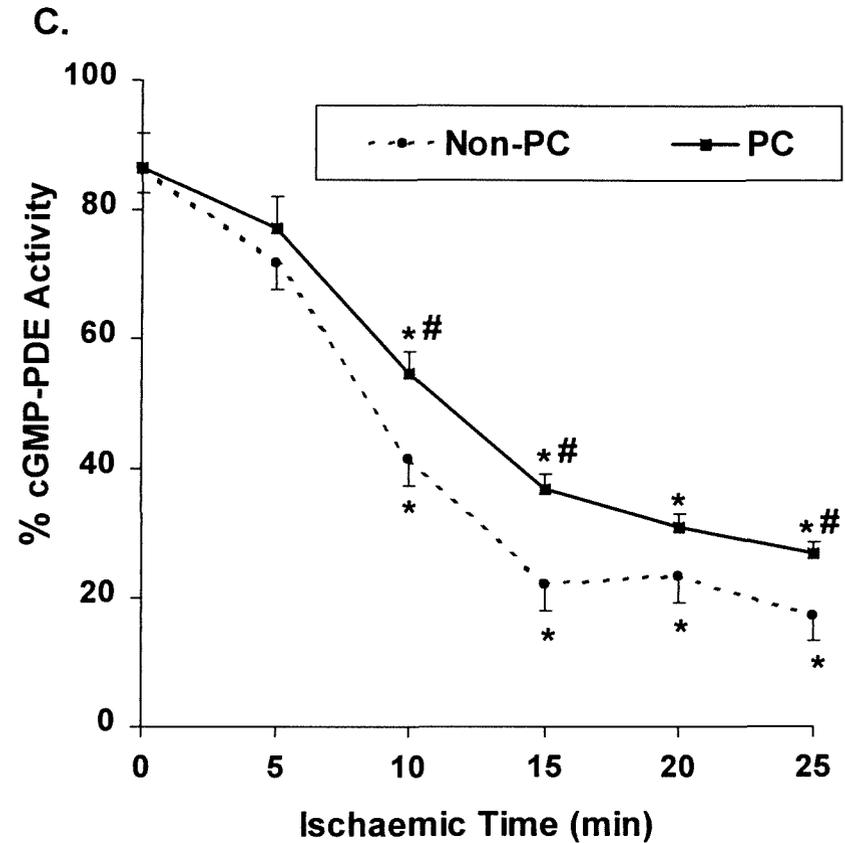
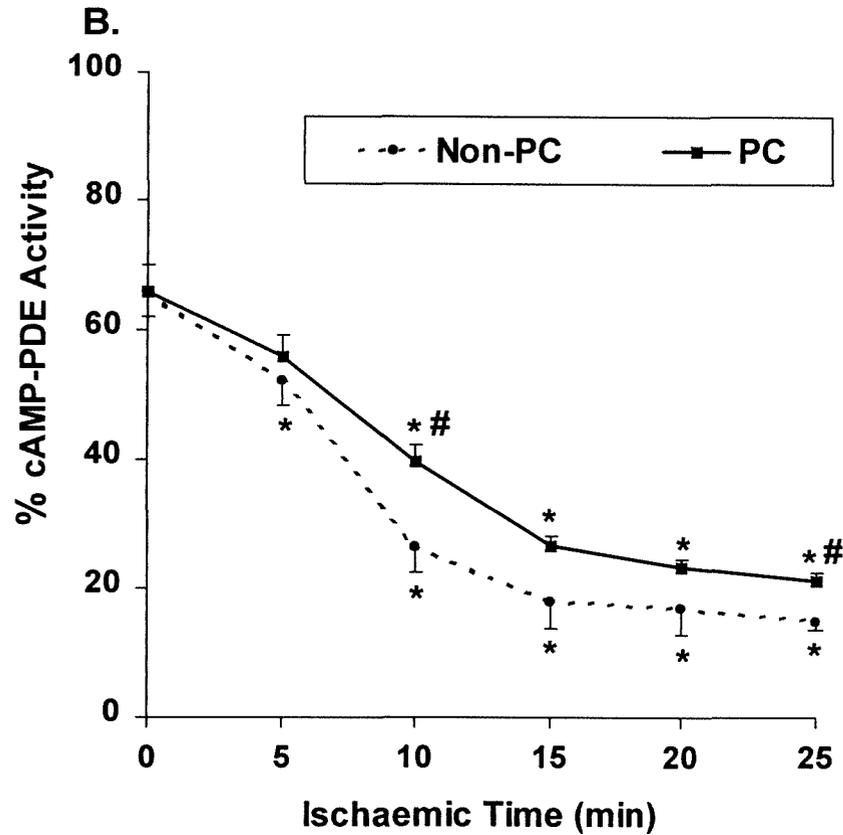
**Non-preconditioned hearts:**



**Preconditioned hearts:**



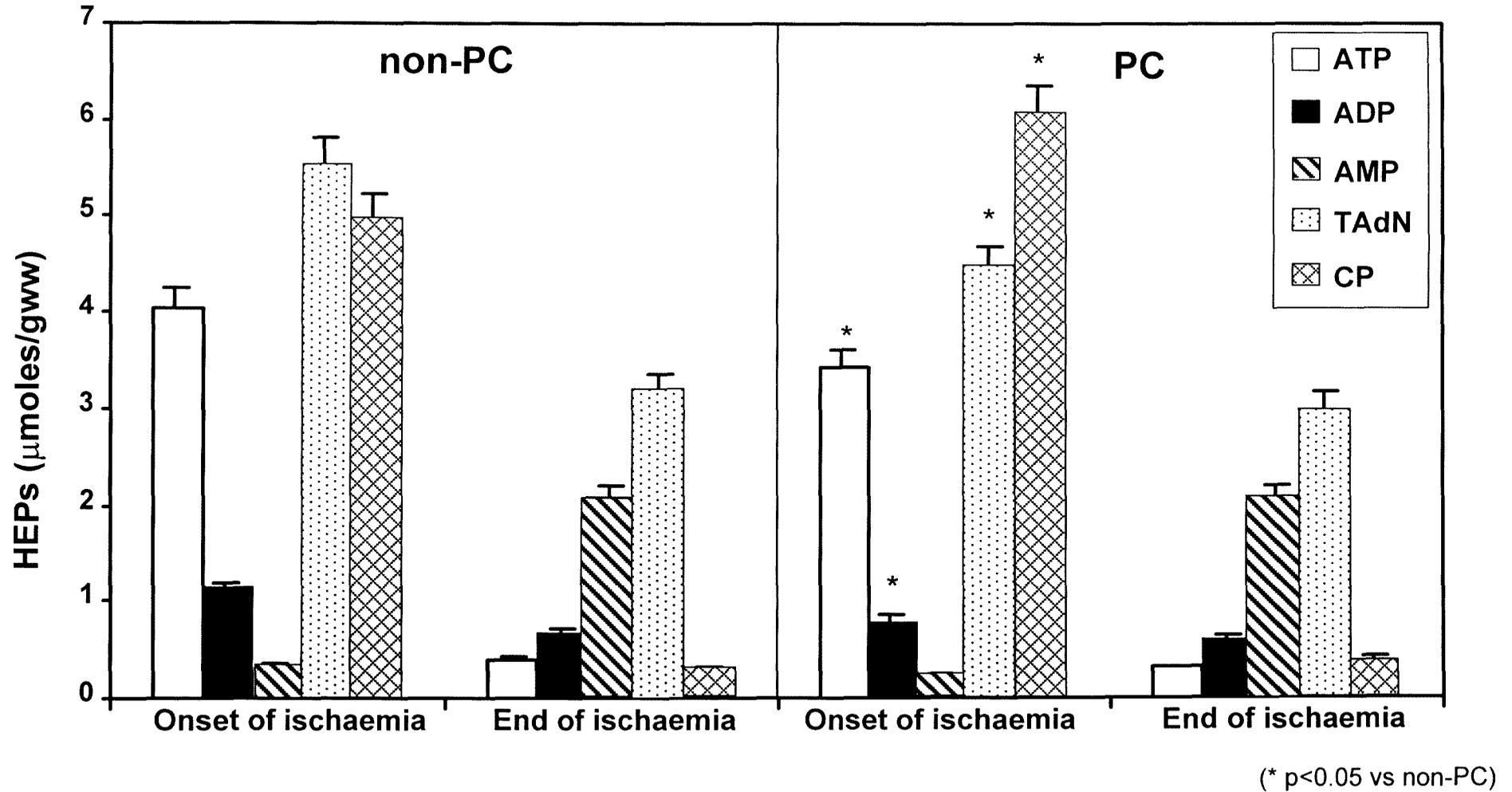
**Fig 3.14A** Evaluation of tissue PDE levels during sustained global ischaemia: Experimental protocol. Abbreviations as indicated in Fig 3.1. Arrows indicate time intervals of measurement of PDE activity. n = 6 hearts / time interval.



(\* p < 0.05 vs zero-time)

(# p < 0.05 vs non-PC)

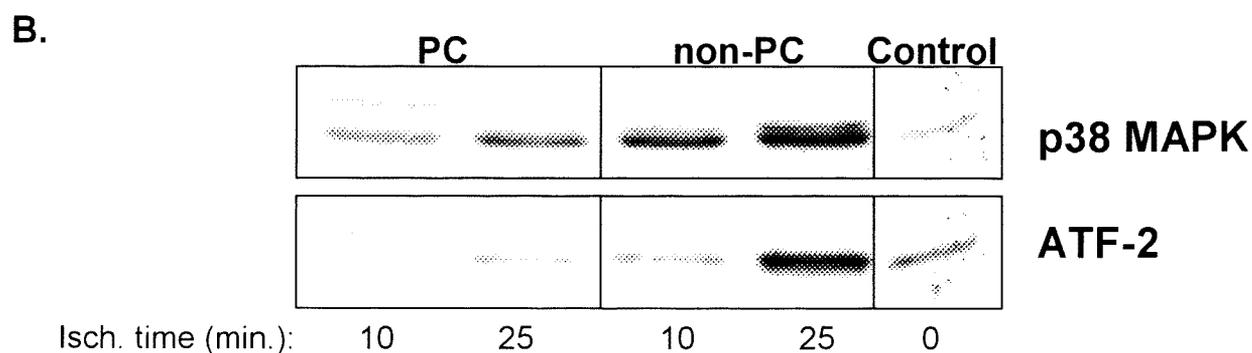
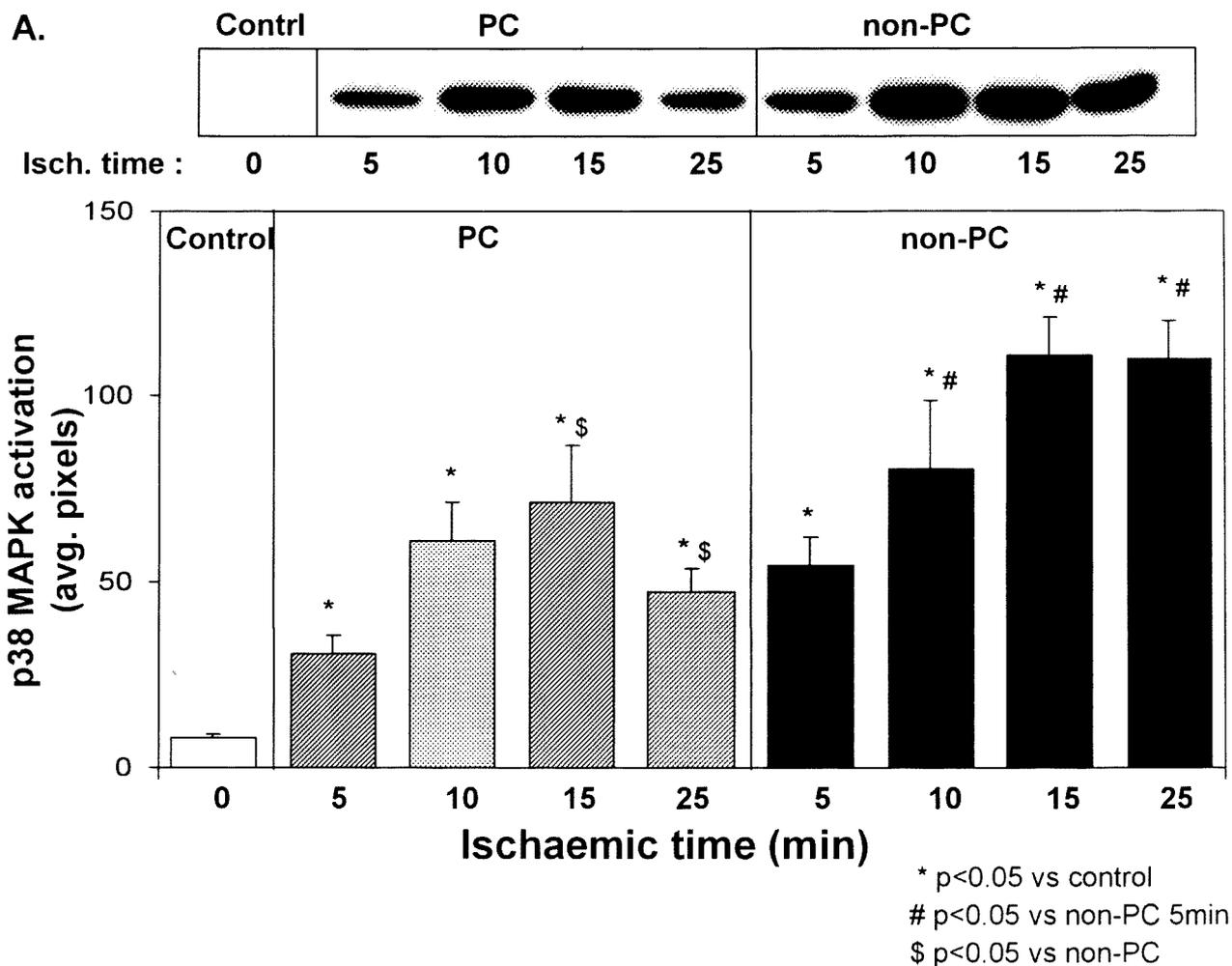
**Fig 3.14 B,C** cAMP- and cGMP-PDE activities during sustained global ischaemia. In both PC and non-PC hearts, cAMP- and cGMP-PDE activities declined with increasing periods of global ischaemia. This inhibition was maximal after 15 min. Two analysis of variance of PDE activities during sustained ischaemia, showed that they were significantly less inhibited in PC than in non-PC hearts.



**Fig 3.15** Changes in tissue high energy phosphates (HEPs) during sustained ischaemia. Before the onset of ischaemia, total adenine nucleotides (TAdN = ATP + ADP + AMP) levels in PC hearts were significantly lower than in non-PC hearts. However creatine phosphate (CP) levels were higher in PC than non-PC hearts. At the end of 25 min sustained ischaemia the TAdN levels as well as the CP levels in non-PC and PC hearts were similar.

## Results

Dual phosphorylation and activation of p38 MAPK was monitored at 5, 10, 15 and 25 min during sustained ischaemia of non-PC and PC hearts. Sustained global ischaemia caused significant activation of p38 MAPK within 5 min in non-PC hearts, reaching maximal values after 15 min compared to control perfused hearts (Fig. 3.16A). Although p38 MAPK phosphorylation in PC hearts was also significantly elevated compared to controls, it was significantly less at 15 and 25 min of ischaemia when compared to non-PC hearts (Fig. 3.16A). p38 MAPK activity (as reflected by ATF-2 phosphorylation, Fig. 3.16B) also reflected similar results as dual phosphorylation, indicating a significant activation of p38 MAPK at 25 min ischaemia in non-PC compared to PC hearts.



**Fig 3.16** Sustained ischaemia: **A.** Analysis of p38 MAPK dual phosphorylation state (n = 4-6 hearts/series). **B.** Comparison with its kinase activity with *in vitro* phosphorylation of ATF-2 (n = 3 hearts/series). Representative blots are given. p38 MAPK activation is attenuated during sustained ischaemia in preconditioned hearts.

### **3.2.3 Evaluation of events during Reperfusion Phase (after sustained ischaemia)**

#### **3.2.3.1 Experimental Protocol**

##### **Non-preconditioned hearts:**

Hearts were stabilized for 60 min (15 min retrograde, 15 min working mode, 30 min retrograde), followed by 25 min sustained global ischaemia. Hearts were subsequently reperfused for 30 min, which consisted of 10 min retrograde perfusion and 20 min working heart perfusion (Fig. 3.17). Functional recovery during reperfusion was used as endpoint to evaluate the extent of protection against ischaemic damage. Therefore the myocardial mechanical function, which included the coronary flow, aortic output, cardiac output, peak systolic pressure, heart rate and total work performance, was monitored during reperfusion (at 20 min and 30 min reperfusion time). These measurements were compared with the mechanical activity before sustained ischaemia (15 min of working heart mode during stabilization) - each heart served as its own control.

##### **Preconditioned hearts:**

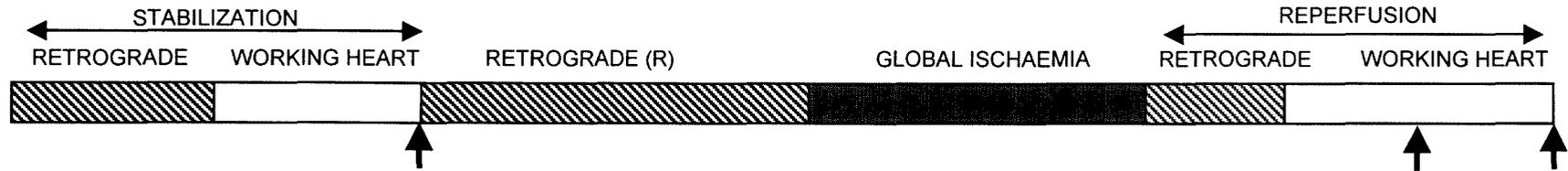
Hearts were stabilized for a period of 30 min (15 min retrograde, 15 min working mode). Preconditioned hearts were then subjected to 3 x 5 min global ischaemia, followed by a subsequent period of 25 min global ischaemia and 30 min reperfusion. Like non-PC hearts, the functional recovery of PC hearts was monitored at 20 min and 30 min reperfusion time. Four to six hearts were monitored at each time point (Fig. 3.17).

#### **3.2.3.2 Results**

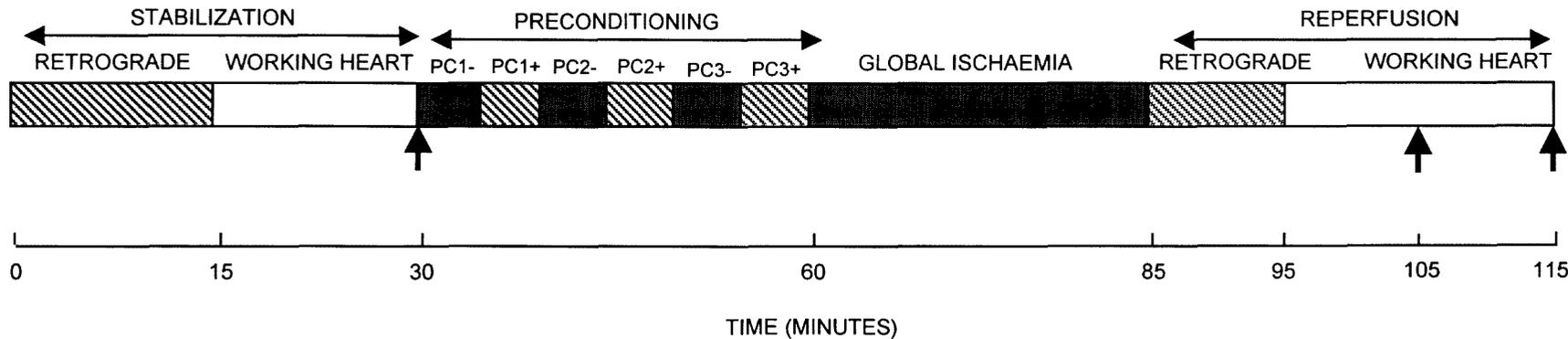
##### **3.2.3.2.1 Effect of ischaemic PC on functional recovery**

At the end of the initial stabilization phase (15 min retrograde and 15 min working heart perfusion), the myocardial function of both PC and non-PC groups was comparable (Fig. 3.18). In both experimental groups, exposure of the heart to 25 min sustained global ischaemia, caused a significant decline in all parameters (apart from coronary

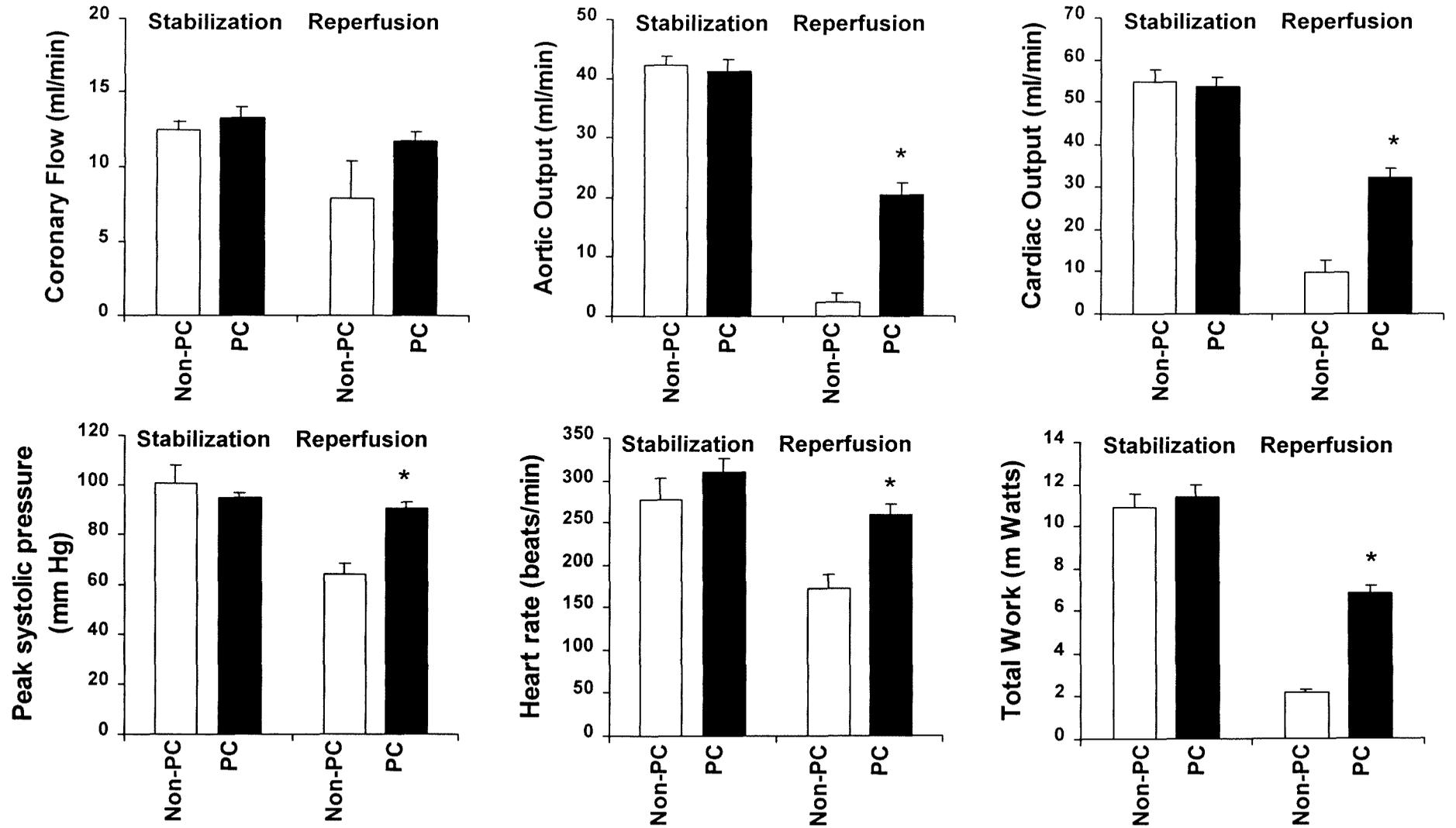
**Non-preconditioned hearts:**



**Preconditioned hearts:**

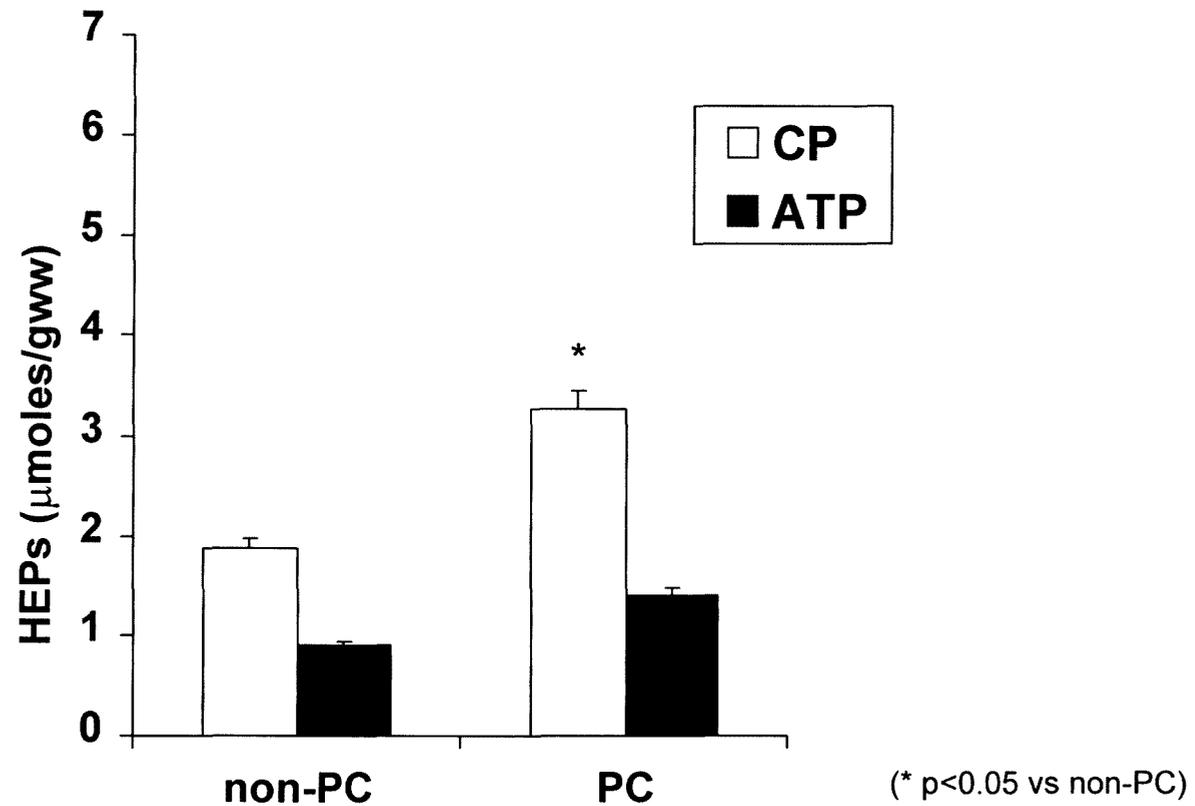


**Fig 3.17** Evaluation of events after sustained ischaemia during the reperfusion phase: Experimental protocol. Abbreviations as indicated in Fig 3.1. Arrows indicate time points of measurement of mechanical function, at the end of stabilization and during reperfusion (6 hearts / time point).



(\* p<0.05 vs non-PC during Reperfusion)

**Fig 3.18** Effect of ischaemic preconditioning on functional recovery after 25 min sustained ischaemia. At the end of stabilization, the function of both PC and non-PC hearts were comparable. Apart from the coronary flow rate, PC caused a significant improvement in all parameters of function measured during reperfusion compared to non-PC.



**Fig 3.19** High energy phosphate (HEP) levels after 30 min reperfusion. In PC hearts the tissue creatine phosphate (CP), but not tissue ATP levels, were significantly increased compared to that in non-PC hearts.

flow rate) of function measured during reperfusion as compared to pre-ischaemic function. However, comparison of the functional performance during reperfusion revealed significant differences between the groups. For example, PC with 3 episodes of 5 min ischaemia prior to sustained ischaemia, caused a significant improvement in the aortic output ( $20.5 \pm 2.0$  ml/min) of hearts during reperfusion after sustained ischaemia, that was significantly higher than the values obtained in non-PC hearts ( $2.4 \pm 1.2$  ml/min) (Fig. 3.18). This significant improvement in function by PC hearts compared to non-PC hearts was also confirmed by the cardiac output, peak systolic pressure, heart rate and total work performance.

### **3.2.3.2.2 High energy phosphates**

Measuring high energy phosphate levels after 30 min of reperfusion, indicated that tissue creatine phosphates (CP) of PC hearts were significantly higher than that of non-PC hearts (Fig. 3.19). On the other hand, tissue ATP levels did not differ significantly between these two groups.

### **3.2.3.2.3 p38 MAPK activation during reperfusion**

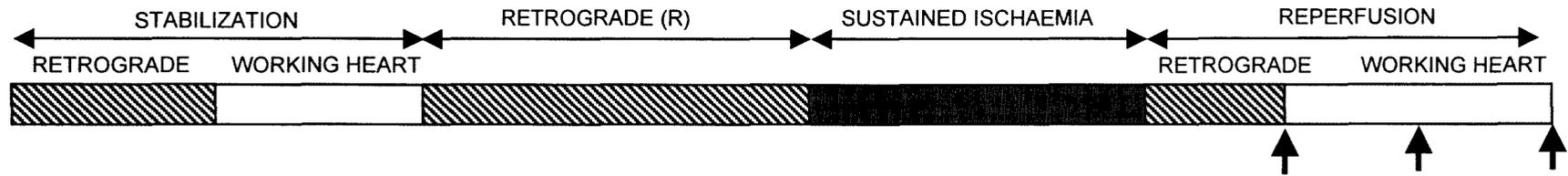
#### **Experimental protocol**

During reperfusion samples were taken at 10 min intervals (10, 20 and 30 min) (Fig. 3.20). Dual phosphorylation of p38 MAPK (residues Thr 180 / Tyr 182 are phosphorylated) was assayed at all the above mentioned time intervals (4 to 6 hearts/series). In all series studied the activation of p38 MAPK, as indicated by its dual phosphorylation, was reflected by its kinase activity as detected by phosphorylation of the substrate ATF-2. The latter was determined at selected intervals (3 hearts/series).

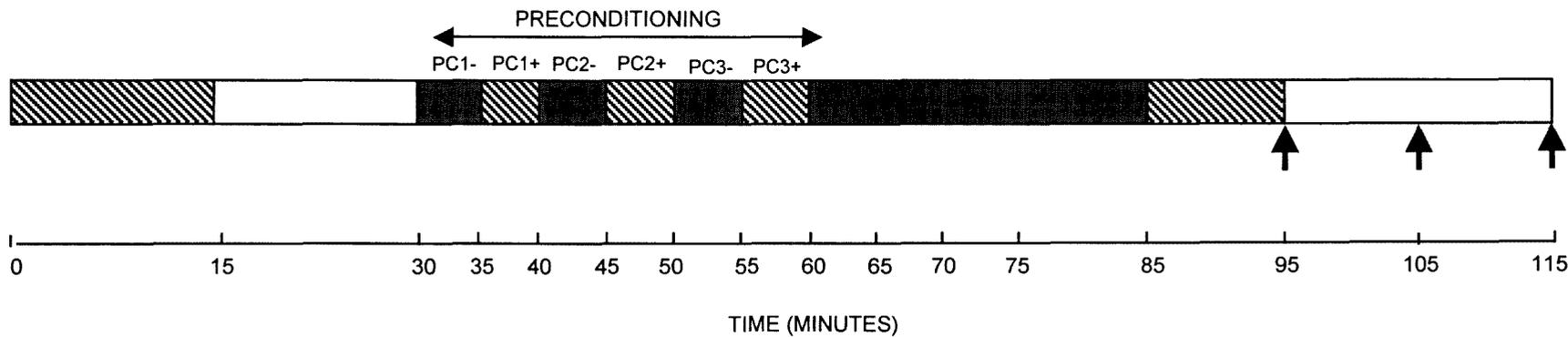
#### **Results**

Both non-PC and PC hearts showed a similar and significant activation of p38 MAPK within 10 min of reperfusion (Fig. 3.21A). However, in the non-PC hearts this activation was maintained for 30 min, whereas it significantly declined in the PC series. The p38 MAPK activity as reflected by ATF-2 phosphorylation, was significantly lower in PC hearts compared to non-PC hearts at both 20 and 30 min reperfusion time (Fig. 3.21B).

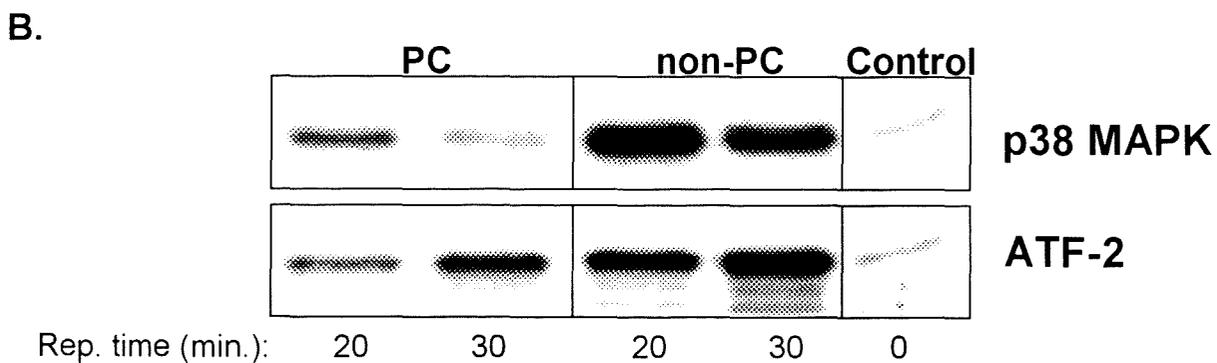
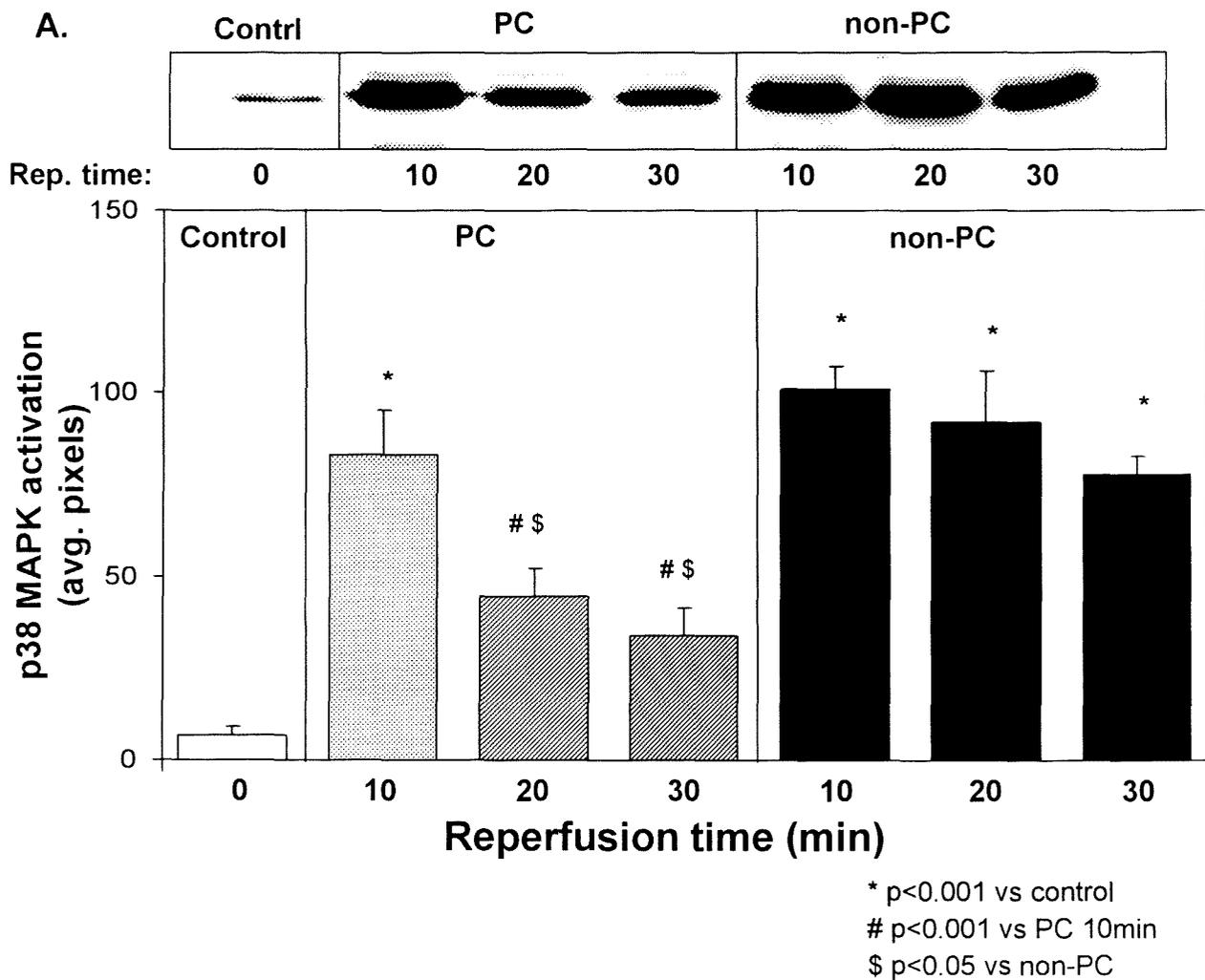
**Non-preconditioned hearts:**



**Preconditioned hearts:**



**Fig 3.20** Sampling for p38 MAPK in non-preconditioned and preconditioned hearts: Experimental protocol. Abbreviations as indicated in Fig 3.1. Arrows indicate time of freeze-clamping at 10, 20 and 30 min reperfusion. (n = 4 to 6 hearts in each series).



**Fig 3.21** Reperfusion: **A.** Analysis of p38 MAPK dual phosphorylation state (n = 4-6 hearts/series). **B.** Comparison with *in vitro* phosphorylation of ATF-2, to determine p38 MAPK activity (n = 3 hearts/series). Representative blots are given. p38 MAPK activation is attenuated during reperfusion in preconditioned hearts.

## **3.2 Discussion of ischaemic preconditioning (PC)**

### **3.3.1 Validity of experimental model in this study**

#### **3.3.1.1 Working heart vs. retrograde perfusion**

The phenomenon of ischaemic PC has been reported in the hearts of all species of animals studied thus far (reviewed by Kloner and Yellon 1994). In practical terms, the rat heart is by far, the best characterized and also the most frequently used for more complex perfusion preparations such as working and blood perfused hearts (reviewed by Sutherland and Hearse 2000). Two modes of perfusion have been used in studies on PC: (i) the retrogradely perfused heart (according to the technique of Langendorff 1895) fitted with an intraventricular balloon which allows monitoring of left ventricular developed pressure (LVDP), heart rate and the onset of contracture (in the case of sustained ischaemia). In most cases regional ischaemia was induced by left coronary artery ligation and infarct size has mostly been used as endpoint (Yellon *et al.*, 1992, Strasser *et al.*, 1989, Downey *et al.*, 1996); (ii) the working perfused rat heart (as originally described by Neely *et al.*, 1967), where the left atrium is cannulated to allow left atrium and ventricle perfusion. This model uses functional recovery as endpoint, as indicated by coronary flow rate, aortic output, developed pressure and heart rate. In this model, hearts were subjected to total global ischaemia, rather than regional ischaemia. This technique has been used by a number of workers (Moolman *et al.*, 1995, Cave and Hearse 1992, Volovsek *et al.*, 1992, Asimakis *et al.*, 1992, Steenbergen *et al.*, 1993, Tosaki *et al.*, 1995, Ferdinandy *et al.*, 1995b, Sandhu *et al.*, 1996).

This working perfused rat heart model is regarded to be more physiological than the retrograde perfused heart since it allows evaluation of the cardiac pump function at a fixed preload (15 – 20 cm H<sub>2</sub>O) and afterload (100 cm H<sub>2</sub>O), while the retrograde perfusion model is actually an empty beating heart which provides measurement of left ventricular systolic and diastolic pressures only (for review see Sutherland and Hearse 2000).

### 3.3.1.2 Endpoints

In the original study by Murry *et al.* (1986), which first described the phenomenon of PC, the endpoint was reduced infarct size. Since then the majority of workers in the field of PC used the infarct size as endpoint, which is now regarded as the “gold standard” by many (Liu and Downey 1992, Speechly-Dick *et al.*, 1994, Bugge and Ytrehus 1995a,b, Suematsu *et al.*, 2001, Yue *et al.*, 2001). However, other endpoints have been used for example: protection against post-ischaemic left ventricular dysfunction (stunning) (Cave 1995, Asimakis *et al.*, 1992) and arrhythmias (Shiki and Hearse 1987, Hagar *et al.*, 1991, Wang *et al.*, 2001) in the isolated rat heart. However, the efficacy of ischaemic PC in reducing stunning (Ovize *et al.*, 1992) and arrhythmias (Ovize *et al.*, 1995) has not been as consistent as its ability to reduce necrosis. In addition, in some models a reduction in infarct size is not always associated with improved function. For example, ischaemic PC in rabbits protects from cell death and necrosis, but does not improve functional recovery (Asimakis *et al.*, 1996, Sandhu *et al.*, 1993). Therefore, a lack of functional recovery during short-periods of reperfusion does not deny PC’s morphological protection (anti-necrosis effect) in animals such as pigs and rabbits (reviewed by Cohen *et al.*, 1999).

Furthermore, it is unknown whether the protection afforded by PC against stunning and arrhythmias is mediated by the same mechanism that mediates its protection against lethal cell injury (Connaughton *et al.*, 1996, Przyklenk and Kloner 1995). Thus, as long as the signalling cascade of ischaemic PC is not clear, great caution should be used when extrapolating findings from recovery of contractile function and protection against arrhythmias to reduction of infarct size. PC-induced protection against contractile dysfunction is likely to represent a combination of protection against cell death and stunning (Cave 2000).

### 3.3.1.3 Reasons for choice

Although infarct size reduction is regarded as the “gold standard” as endpoint in PC (Hatter Institute, 1998), many workers, including ourselves, prefer to use contractile recovery during reperfusion as endpoint, since assessment of infarct size can be complicated by the choice of staining and the degree of collateral flow (Farber *et al.*, 1993, Takahashi *et al.*, 2000, Cave, 2000). Therefore, myocardial function may

perhaps be the more physiological indicator of the beneficial effects of PC. In fact, indications are that improved recovery of function is more difficult to achieve than a reduction in infarct size (Lochner *et al.* submitted for publication).

In view of the above, it was decided to use the working rat heart as model throughout this study. Preference was given to global ischaemia, rather than regional ischaemia, to induce both the PC stimulus and sustained ischaemia. Careful characterization of this model (Moolman *et al.*, 1995) showed that improved contractile recovery of the working heart is always associated with morphological improvement (i.e. reduction in necrosis). Another advantage is that this model lends itself to measurement of both mechanical function and biochemical changes in the same heart and these measurements can be made in the absence of the confounding effects of other organs (reviewed by Sutherland and Hearse 2000). The globally ischaemic heart also has the advantage that it yields more tissue than regional ischaemia for biochemical analysis.

Since previous studies indicated that multiple (rather than a single) cycles induced the strongest protection against arrhythmias and infarct size (Liu *et al.*, 1992, Yellon *et al.*, 1992, Seyfarth *et al.*, 1996, Sandhu *et al.*, 1997), a protocol consisting of three episodes of transient ischaemia and reperfusion was utilized to elicit PC in this study and evaluated by using parameters such as changes in energy metabolism (changes in high energy phosphates, HEPs).

Additionally, subjecting the heart to ischaemia, however short, it is clearly not ideal, hence examining the possibility that the protection provided by ischaemic PC could be mimicked pharmacologically, is important. Therefore, the fact that the isolated working heart model is able to rapidly wash out drugs, as well as allowing controlled dose-response studies of drugs with speed, reproducibility and precise control over concentration, makes it well suited for pharmacological manipulations.

#### **3.3.1.4 High energy phosphates (HEPs)**

After 25 min of global ischaemia in the rat heart, varying degrees of stunning, apoptosis and necrosis may contribute to the reduced mechanical performance during reperfusion. We have previously shown that PC reduces the ischaemia induced structural damage in this model (Moolman *et al.*, 1995).

#### 3.3.1.4.1 Changes in HEPs during the PC protocol

Similar to our study, Bradamante *et al.* (1995) demonstrated that myocardial creatine phosphate (CP) levels decreased during each ischaemic period of a multi-cycle PC protocol, and recovered during reperfusion. However, in our study a significant overshoot in CP levels occurred during the reperfusion phases of the PC protocol (Fig 3.4) causing it to be high at the onset of sustained ischaemia (Fig. 3.15). Both Asimakis *et al.* (1992) and Volovsek *et al.* (1992) reported similar observations and proposed that this could have played a major role in ATP replenishment, thus conserving energy.

#### 3.3.1.4.2 Changes in HEPs at end of sustained ischaemia

At end of sustained ischaemia no difference in HEPs between PC and non-PC hearts was observed in this study (Figs. 3.15). However calculating the rate of HEP utilization of the ischaemic myocardium according to the equation of Murry *et al.* (1990), indicated that during 25 min ischaemia the adenine nucleotide utilization of non-PC (0.31  $\mu$ moles HEP/g wet weight/minute) was higher than that of PC hearts (0.26  $\mu$ moles HEP/g wet weight/minute), whereas CP made a substantial contribution to the energy utilization of PC (0.23  $\mu$ moles HEP /g wet weight/minute) compared to non-PC hearts (0.19  $\mu$ moles HEP /g wet weight/minute) (data from Moolman *et al.* 1996). Therefore the reduction in energy utilization observed during 25 min sustained ischaemia, was consistent with the known metabolic behaviour of PC hearts (Murry *et al.*, 1990), and functional data (Fig. 3.18) confirmed that this PC protocol protected preconditioned hearts effectively.

Furthermore, the finding that the mitochondrial oxidative phosphorylation of PC hearts upon 30 min reperfusion, as indicated by CP generation (Fig. 3.19), was superior to that of non-PC hearts may be indicative of more pronounced protection. Likewise, Volovsek *et al.* (1992) speculated that preservation of mitochondrial function and oxidative energy production is involved in PC protection.

Thus, we have demonstrated that utilizing a protocol consisting of three episodes of transient ischaemia and reperfusion to elicit PC conserved energy and resulted in a reduction in energy utilization during 25 min sustained global ischaemia and improvement of post-ischaemic functional recovery. These observations confirmed that

our perfusion protocol was effective in eliciting PC and could therefore be used to study the phenomenon.

### **3.3.2 Changes during the ischaemic PC protocol and subsequent sustained ischaemia**

In this study the results have shown that cyclic increases in tissue cAMP and cGMP levels (Fig. 3.2) and opposite changes in cAMP- and cGMP-PDE activities (Fig. 3.3), as well as high energy phosphates (i.e. creatine phosphate) (Fig. 3.4) occurred during a multi-cycle PC protocol. When exposing the preconditioned myocardium to a subsequent sustained global ischaemia, the rise in cGMP and cAMP was significantly higher and lower, respectively, than in the non-PC hearts (Fig. 3.13, Moolman *et al.*, 1996). This was associated with functional recovery significantly superior to that of non-PC hearts (Fig. 3.18). A major issue therefore was to establish whether these changes *per se* elicit protection against subsequent ischaemic damage or whether they are mere epiphenomena or mere reflection of protection initiated by another mechanism.

#### **3.3.2.1 Cyclic increases in cAMP and cGMP during PC?**

Although increases in tissue cAMP and cGMP during long periods of myocardial ischaemia have been described previously (Krause *et al.*, 1978, Podzuweit T *et al.*, 1995d), the present study is the first to describe the profiles of both these second messengers during repeated short episodes of ischaemia and reperfusion as occurs during the PC protocol (Fig. 3.2). Previously, it was shown that another model of PC, namely ventricular overdrive pacing (VOP), induces PC protection in anaesthetized rabbits (Szilvassy *et al.*, 1994). This was associated with alterations in cardiac cyclic nucleotide contents: a slight increase in cGMP and a profound elevation in cAMP within 5 min of VOP. When this VOP was preceded by a preconditioning VOP, the cGMP increase was amplified, whereas the cAMP increase was significantly attenuated.

##### **3.3.2.1.1 Tissue cAMP**

A rapid rise in tissue cAMP during 5 min global ischaemia may be due to several factors, for example, (i) release of endogenous catecholamines (Banerjee *et al.*, 1993), (ii) dual sensitization of the  $\beta$ -adrenergic system (Strasser *et al.*, 1990), or (iii) inhibition

of PDEs (Podzuweit T *et al.*, 1996), or (iv) increase in inhibitory G-protein (Gi) (Niroomand *et al.*, 1995). The observation that the cyclic increases in cAMP during the PC protocol could be abolished by prior reserpination, suggested that these increases were due to the release of endogenous catecholamines (Fig. 3.5). Furthermore, the cyclic increases in cAMP during the PC protocol were also accompanied by similar changes in PKA activity (Fig. 3.7B), increased adenylyl cyclase activity (Fig. 3.7B) and reduction in PDE activity (Fig. 3.3).

#### **3.3.2.1.1.1 Release of endogenous catecholamines**

According to our data the release of endogenous catecholamines is mainly responsible for the cyclic increases in tissue cAMP during PC, because prior reserpination abolished cAMP generation at PC1-, PC2- and PC3- (Fig. 3.5). The findings that release of noradrenaline occurred within 2 min of ischaemia in the rat heart (Banerjee *et al.*, 1993) and that a substantial reduction in synaptic noradrenaline store content occurred during brief ( $\geq 10$  min) ischaemia, while the release of noradrenaline stopped instantly upon reperfusion (Podzuweit *et al.*, 1995a), support our findings. A role for catecholamines and  $\alpha_1$ -adrenergic receptors has also been suggested in the reduction of infarct size in preconditioned rat (Banerjee *et al.*, 1993) and rabbit hearts (Toombs *et al.*, 1993, Thornton *et al.*, 1993, Bankwala *et al.*, 1994).

#### **3.3.2.1.1.2 Sensitization**

Another mechanism that may be involved in the cyclic increases in cAMP, which occur within minutes after the onset of ischaemia, is the dual sensitization of the  $\beta$ -adrenergic system (Strasser *et al.*, 1990): Firstly adenylyl cyclase activation was attributed to PKC activation and intracellular acidosis during acute myocardial ischaemia (Simonis *et al.*, 1998). Secondly upregulation of the  $\beta$ -adrenergic receptor has been shown to be due to loss of ATP in ischaemia: the internalization of  $\beta$ -adrenergic receptors is ATP dependent, and the increased receptor density during ischaemia has been shown to be the consequence of increased externalization (Strasser *et al.*, 1992). Thus, the increases in  $\beta$ -receptor  $B_{max}$  values at PC1-, although not significant, and the marked increase in adenylyl cyclase activity at this time-point in our study is in agreement with the concept of dual sensitization of the  $\beta$ -adrenergic system (Fig. 3.6). Strasser *et al.* (1990) observed upregulation of the  $\beta$ -receptor population to occur after exposure to 15 min ischaemia,

which may explain why the changes in  $B_{max}$  obtained in the present study only became significant towards the end of the PC protocol. However, its effects could be counteracted by the reduced affinity (a 52% increase in  $K_d$ ) at PC3- (Fig. 3.6). Therefore the rise in cAMP at PC3- was probably largely due to the continued increase in adenylyl cyclase activity and reduced PDE activity at this stage.

#### 3.3.2.1.1.3 Phosphodiesterase (PDE)

Another role player in the observed fluctuations in cAMP is the marked reduction in the activities of both cAMP- and cGMP- PDEs during PC1-, PC2- and PC3- (Figs 3.2, 3.3), which is supported by the finding that brief ( $\geq 10$  min) ischaemia caused a substantial inhibition in total PDE activity, that was readily reversed by reperfusion (Podzuweit *et al.*, 1995b). Podzuweit *et al.* (1996) suggested that acidosis is a major determinant of PDE inhibition in ischaemia, which would explain why PDE inhibition is readily reversed by reperfusion as hydrogen ions are washed out.

It should be pointed out that PDE as measured in this study indicates the total cAMP and cGMP hydrolyzing capacity of the isoenzymes present in heart muscle. The rat myocardium is reported to contain at least four PDE isoenzymes (Shahid *et al.*, 1990) of which PDE1 (calcium-calmodulin stimulated) and PDE2 (cGMP stimulated) hydrolyze cGMP, while PDE3 (cGMP inhibited) and PDE4 (cAMP specific) hydrolyze cAMP only. By offering cAMP or cGMP as substrate or isoenzyme-selective inhibitors, one can estimate which isoforms may be involved.

#### 3.3.2.1.1.4 Gi protein

Although  $\beta$ -adrenergic receptors do not transduce their signals via Gi proteins, Gi affects the balance between stimulatory and inhibitory effects on adenylyl cyclase and may thus contribute to the decreased response to  $\beta$ -adrenergic agonists (Hajjar *et al.*, 1998). Niroomand *et al.* (1995) have reported that in canine myocardium, Gi proteins were sensitized during reperfusion following a single 5 min period of transient ischaemia and that this sensitization was maintained during a subsequent period of ischaemia. In our study, pertussis toxin pretreatment failed to increase cAMP in preconditioned hearts, excluding a role for the Gi protein (Fig. 3.9). In addition, studies showed that PC against either infarction or arrhythmias in the rat, does not appear to involve a pertussis toxin

sensitive G protein (Liu *et al.*, 1993, Lawson *et al.*, 1993, Yabe *et al.*, 1995). However, pertussis toxin has been shown to abolish the cardioprotective effect of ischaemic PC in rat (Piacentini *et al.*, 1993, Schultz *et al.*, 1998), rabbit (Thornton *et al.*, 1993) and dog (Miura *et al.*, 1997) hearts.

### 3.3.2.1.2 Tissue cGMP

Tissue cGMP levels showed similar cyclic changes during PC2-/+ and PC3-/+ as the cAMP levels (Fig. 3.2). The significant inhibition of cGMP-PDE during PC2- and PC3- (Fig. 3.3) are possibly responsible for the rise in cGMP, whereas the rise in PDE activity during reperfusion may account for the reduction in cGMP during the reperfusion episodes. Depré and coworkers (1997) have shown that activation of NOS (suggested to be the soluble fraction of the eNOS isoenzyme) occurs within 5 min of ischaemia and returns to normal levels during reperfusion. This was recently confirmed by a study on conscious rabbits, in which brief myocardial ischaemia caused an immediate activation of cNOS (most likely eNOS) (Xaun *et al.*, 2000). Therefore the activation of NOS during the ischaemic episodes of PC and normalization during reperfusion, may also be responsible for the fluctuations in cGMP levels. The mechanism of the ischaemia-induced activation of NOS is not known, but could occur secondary to an increased  $[Ca^{2+}]_i$  (Kelly *et al.*, 1996).

The reason for the relatively minor change in tissue cGMP levels observed at PC1-, in the presence of marked inhibition of cGMP-PDE activity remains to be established. This rather modest change in cGMP, amongst others, may contribute to the observation that one episode of PC was less effective than three episodes in eliciting protection in the isolated perfused rat heart (Liu *et al.*, 1992, Downey and Cohen 1996, Sandhu *et al.*, 1997, reviewed by Cohen *et al.*, 2000).

### 3.3.2.1.3 cAMP and cGMP

Simultaneous generation of these two cyclic nucleotides could lead to considerable crosstalk. For example, it has been shown that NO (and thus cGMP) attenuates  $\beta$ -receptor mediated responses (Ebihara *et al.*, 1996), while in vitro (Balligand *et al.*, 1993) and in vivo studies (Keany *et al.*, 1996, Hare *et al.*, 1995) demonstrated that NOS inhibition and reduced cGMP levels could enhance the positive inotropic response to

$\beta$ -adrenergic stimulation (by i.e. isoproterenol). Therefore, the contribution of these cyclic changes in cAMP and cGMP, alone and in combination, to the protection conferred by PC remains to be established.

### 3.3.2.2 p38 MAPK activation during the PC protocol

Our observation that a robust increase in p38 MAPK activation occurred within 5 min of ischaemia (Fig. 3.12), is consistent with several studies obtained by immuno-blotting, in-gel kinase and biochemical assays in rat (Bogoyevitch *et al.*, 1996, Shimizu *et al.*, 1998, Maulik *et al.*, 1998d), rabbit (Ma *et al.*, 1999, Ping *et al.*, 1999a, Gysembergh *et al.*, 2001), pig hearts (Baranick *et al.*, 2000) and in isolated cardiomyocytes (Mackay and Mochly-Rosen 1999, Yue *et al.*, 2000). Apart from ourselves, only one group made an attempt to evaluate changes in p38 MAPK activation during the PC protocol (Ping *et al.*, 1999a).

We found that the activation of p38 MAPK (as evidenced by both dual phosphorylation and kinase activity) during the repetitive cycles of ischaemia-reperfusion (PC protocol) is transient, since the significant activation observed during the first two episodes (PC1- and PC2-), disappeared during PC3-. These data concur with the study by Ping *et al.* (1999a), who observed that a single 4-min period of ischaemia induced a robust activation of the p38 MAPK cascade, which was attenuated after 5 min reperfusion and disappeared after six cycles of 4-min ischaemia-reperfusion. These cyclic elevations in p38 MAPK activity are most likely due to the release of the many substances which could act as trigger in the PC process, for example,  $\alpha_1$ - and  $\beta$ -adrenergic receptor stimulation due to release of endogenous catecholamines (Schömig *et al.*, 1991), activation of nitric oxide synthase and generation of nitric oxide (NO) (Depré *et al.*, 1997), adenosine (Haq *et al.*, 1998) and generation of oxygen free radicals (Das *et al.*, 1999). The rapid decline in activity during the reperfusion periods probably occurs as a result of washout of these substances. Activation of phosphatases may also contribute, but the pattern of activation was not assessed in this study.

Furthermore, p44/p42 MAPK (ERK) (Ping *et al.*, 1999b) and c-Jun NH<sub>2</sub>-terminal kinase (JNK) (Ping *et al.*, 1999a), remained activated throughout a multi-cycle PC protocol, the latter via a PKC $\epsilon$  dependent signalling pathway (Ping *et al.*, 1999a). In view of the transient nature of p38 MAPK activation during a multi-cycle PC protocol while the

activation of PKC $\epsilon$  persisted, it was suggested that activation of p38 MAPK was not coupled to that of PKC $\epsilon$  (Ping *et al.*, 1999a). In addition, over-expression of PKC $\epsilon$  in cardiac myocytes failed to increase p38 MAPK activity (Ping *et al.*, 1999a). However, the transient activation of p38 MAPK during the PC protocol does not exclude its acting as a trigger: it may rapidly phosphorylate downstream substrates, setting in motion the cardioprotective response.

### 3.3.3 Changes during sustained ischaemia

#### 3.3.3.1 cAMP and cGMP

Even in the absence of ischaemia, elevations in cAMP have been shown to produce myocardial necrosis (Martorana 1971). High levels of cAMP are also known (i) to increase sarcolemmal calcium entry (Tsien 1983) which in turn results in intracellular calcium overload and (ii) to increase the activation of cardiac lipases (Opie 1982), effects known to aggravate ischaemic injury. Therefore an attenuation of increases in cAMP during sustained ischaemia may be necessary to protect against necrosis.

As observed before (Sandhu *et al.*, 1996 and Moolman *et al.*, 1996), PC leads to significant reduction in tissue cAMP during sustained ischaemia. Tissue cGMP, on the other hand, was significantly higher in PC hearts (Fig. 3.13). Similar findings in the *in vivo* rabbit heart also showed that cGMP is significantly higher during long ischaemia (30 min regional ischaemia) if previously exposed to 5 min ischaemia and 10 min reperfusion (Iliodromitis *et al.*, 1996), while no difference was found between baseline and values obtained at the end of sustained ischaemia in the ineffectively PC (with 1 min ischaemia) and control groups. It is not yet known whether these changes are merely the consequence or reflection of the PC-induced protection or whether they are protective by them.

It is possible that cAMP acts in synergism with cGMP during sustained ischaemia: these two cyclic nucleotides have opposing effects on the cardiac slow Ca<sup>2+</sup> channel (cAMP stimulating and cGMP inhibiting) (Sperelakis 1994). The lowering of tissue cAMP and elevation in cGMP may reduce Ca<sup>2+</sup> influx during ischaemia as well as during reperfusion. Elevation of endogenous cGMP has long been known to lower intracellular Ca<sup>2+</sup> (Lincoln 1989). As discussed before, NO (and thus cGMP) is an important endogenous inhibitory

regulator of the actions of adrenergic agonists and may thus play a role in the attenuated  $\beta$ -adrenergic response during sustained ischaemia.

Whether the decrease in cAMP is the cause or merely a reflection of the protection induced by PC remains to be established. Attempts to manipulate cAMP during the PC protocol by administration of forskolin immediately before sustained ischaemia (Moolman *et al.*, 1996) failed to demonstrate a clear effect between tissue cAMP and protection. In view of the observations and the known effects of ischaemia on the  $\beta$ -adrenergic signalling pathway (Strasser *et al.*, 1990), it was decided not to further examine the role of cAMP and thus also the effects of PC on adenylyl cyclase (AC) and protein kinase A (PKA) activation in sustained ischaemia. In addition, unpublished studies from our laboratory showed that PKA activation was significantly depressed throughout sustained ischaemia in PC hearts (Lochner *et al.* unpublished data).

### **3.3.3.2 Attenuation of the $\beta$ -adrenergic response to sustained ischaemia**

Although the decreased cAMP accumulation in PC hearts during sustained ischaemia is likely to be due to a reduced response, there is no proof as yet that the preconditioned myocardium is indeed less responsive to  $\beta$ -adrenergic stimulation. This attenuated response may be due to (i) less inhibited breakdown by cAMP- and cGMP-PDE (as shown in Fig. 3.14B, C) (ii) desensitization of the  $\beta$ -adrenergic receptor (unlikely according to Sandhu *et al.*, 1996) or (iii) diminished release of catecholamines (Seyfarth *et al.*, 1996, Takasaki *et al.*, 1998).

#### **3.3.3.2.1 PDE activity**

Despite the increased cGMP-PDE activity in PC hearts during sustained ischaemia, the cGMP levels remained somewhat elevated and therefore it appears that its generation overrides its breakdown. The latter was confirmed by Depré *et al.* (1994), who showed that activation of NOS is maintained throughout sustained ischaemia. The increased cGMP levels in PC hearts may also contribute to activation of the cGMP-stimulated PDE (Hardman *et al.*, 1971). However, the rise in cGMP also causes inhibition of PDE3 with little effect on total cAMP degrading capacity (Beavo *et al.*, 1995).

As discussed before, inhibition of PDE during ischaemia may be caused, in part, by acidosis (Podzuweit *et al.*, 1996). This corroborates the work by Wollenberger *et al.* (1968), who proposed that ischaemia-induced increase in cAMP was due to an inactivation of PDE activity caused by a lowering in pH. Since ischaemic PC significantly attenuates the decrease in pH induced by sustained ischaemia (Asimakis *et al.*, 1992; Steenbergen *et al.*, 1993, Miura *et al.*, 1997), the PDE activity of such hearts may be increased compared to that of non-PC hearts; as was the case in the present study (Fig. 3.14B, C). Podzuweit *et al.* (1995b) have reported that, in pig hearts, PC attenuates the inhibition of myocardial PDE activity during subsequent ischaemic episodes, thereby limiting the accumulation of cAMP. Reduced accumulation of cAMP may conceivably be protective because of the reduction in energy demand and calcium influx.

Although attenuated inhibition of PDE activity during sustained ischaemia may be of particular importance in conferring protection, this possibility has received relatively little attention and should be determined by use of appropriate blockers. Sanada *et al.* (2001b) recently showed that transient pre-treatment with PDE3 inhibitors could indeed limit infarct size in open chest dogs.

### 3.3.3.2.2 Desensitization

It is possible that the episodes of transient ischaemia in PC hearts results in the liberation of norepinephrine, as well as other mediators, which then act to desensitize the  $\beta$ -adrenergic pathway and prevent the rise in cAMP during subsequent sustained ischaemia. We focused on the  $\beta$ -adrenergic pathway, because the  $\beta$ -adrenergic receptor can desensitize fairly rapidly (Roth *et al.*, 1991), a requirement for a role in ischaemic PC. It is well-established that the process of desensitization can be mediated (i) by changes in the functional state of the receptor induced by phosphorylation (Hein *et al.*, 1997, January *et al.*, 1997) and (ii) by changes in the number of receptors present on the cell surface (Hein *et al.*, 1997).

In our study, at the end of the PC protocol (PC3+) before the onset of sustained ischaemia, the following situation prevails: a significant (39%) increase in  $\beta$ -adrenergic receptor density ( $B_{max}$ ) and a reduction in the affinity of the  $\beta$ -adrenergic receptor for its ligand (% increase in Kd: 45%), concomitant with normalization of adenylyl cyclase and

PDE activities (Fig. 3.6). Since the decrease in  $\beta$ -adrenergic receptor affinity was accompanied by an almost similar increase in density, it was not clear to us whether these two effects of similar magnitude would cancel each other. Our data show that, using cAMP generation in response to  $\beta$ -adrenergic receptor stimulation with isoproterenol as parameter, that the preconditioned myocardium is indeed less responsive, and that the  $\beta$ -adrenergic receptor itself must be implicated: isoproterenol elicited a marked increase in cAMP in non-PC hearts, but not in PC hearts, while forskolin caused similar increases in cAMP in both groups (Fig. 3.9). It is therefore possible that the reduced affinity of the receptor for its ligand overrides the effect of the increase in  $B_{max}$ , resulting in a desensitized but not internalized receptor.

On the other hand, Sandhu *et al.* (1996) could not demonstrate reduced  $\beta$ -adrenergic receptor sensitivity in a rabbit model after a PC protocol, because stimulation of the  $\beta$ -adrenergic receptor with isoproterenol produced similar increases in cAMP in non-ischaemic hearts and in hearts subjected to three cycles of transient ischaemia-reperfusion. Further evidence against  $\beta$ -adrenergic receptor desensitization in ischaemic PC comes from work by Iwase *et al.* (1993), who observed that coronary occlusion induced a rapid and progressive reduction in the  $B_{max}$ , stimulatory G protein (Gs) and adenylyl cyclase activities after 60 min of ischaemia in rabbit hearts. Their data also indicated that PC did not affect the reduction in  $B_{max}$ , although it prevented the early reductions in Gs and adenylyl cyclase activities after 10 and 20 min, but not 60 min of sustained ischaemia. Therefore, PC delays the ischaemia-induced reductions in  $\beta$ -adrenergic signal transduction. Thus, both Sandhu *et al.* (1996) and Iwase *et al.* (1993) suggest that the  $\beta$ -adrenergic signal transduction pathway are not desensitized by PC. This is contradictory to our findings and may be due to the differences in the species employed (both used rabbits).

### **Should cyclic increases in cAMP or PKA activity during PC protocol be important, how do they confer desensitization or protection?**

One possibility is that cAMP may act as a messenger or signal to elicit protection against subsequent ischaemic damage. The signal is, however, readily abolished during PC reperfusion, but we have convincingly demonstrated the downstream effector, PKA, is indeed activated under these circumstances. As mentioned before, desensitization can also be mediated by changes in the functional state of the receptor induced by

phosphorylation (Hein *et al.*, 1997). The significant elevation in PKA activity, especially during the third PC episode, could be particularly important in the desensitization process. There are two consensus sites for PKA phosphorylation in the  $\beta$ -adrenergic receptor, one in the third intracellular loop and one in the carboxy-terminus (Clark *et al.*, 1989). Evidence points to the former as being functionally the most important phosphorylation site. Likewise, the receptor could be phosphorylated by another class of protein kinases, the G protein coupled receptor kinases or  $\beta$ -adrenergic receptor kinases ( $\beta$ -ARK) (Hausdorff *et al.*, 1990, Premont *et al.*, 1993). These kinases, which require the inhibitory protein  $\beta$ -arrestin (Lohse *et al.*, 1990, Attramadal *et al.*, 1992), mediate a very rapid desensitizing process during ischaemia (Ungerer *et al.*, 1996), however their role in PC is unknown.

In addition, Ma and Huang (2002) demonstrated that  $G_{\alpha s}$  -and  $G_{\alpha i}$ -, but neither  $G_{\alpha q}$  -,  $G_{\alpha 12}$  – nor  $G_{\beta \gamma}$  -, coupled  $\beta$ -adrenergic receptors directly stimulate the kinase activity of a c-Src family tyrosine kinase, independent of cAMP and PKA. The c-Src tyrosine kinase also binds the  $\beta$ -adrenergic receptor via phospho-Tyr-350, phosphorylates  $\beta$ -ARK 2, and mediates agonist-induced receptor desensitization (Fan *et al.*, 2001). The desensitized  $\beta$ -adrenergic receptors could also function as scaffolds for Src-dependent activation of ERK signalling pathways, while the  $\beta$ -arrestins function as adapter proteins that link G-protein coupled receptors to tyrosine kinase-dependent pathways (Luttrell *et al.*, 1999).

#### **3.3.3.2.3 Endogenous catecholamines**

Endogenous catecholamine release may play a role in ischaemic PC either as a trigger or as a target within the process of PC. As mentioned before, some authors suggest a role of catecholamines and particularly the  $\alpha_1$ -adrenergic receptors in the reduction of infarct size by PC (Toombs *et al.*, 1993, Thornton *et al.*, 1993, Banerjee *et al.*, 1993, Bankwala *et al.*, 1994).

Schömig (1990) has shown that in non-PC rat hearts, periods of myocardial ischaemia of more than 10 min duration can increase norepinephrine release (called nonexocytotic release), which does not depend on local sympathetic activation, but rather on local metabolic factors to cause a reverse in the uptake-carrier to produce an efflux of

norepinephrine from the cytoplasm of the nerve terminals to the extracellular space. On the other hand, PC may lead to the preservation of myocardial autonomic nerve terminals (Miyazaki *et al.*, 1989), reduction in activity of the sympathetic nervous system (Takasaki *et al.*, 1998) and reduced release of endogenous catecholamines during sustained ischaemia (Seyfarth *et al.*, 1996, Sandhu *et al.*, 1996), – if so, it will add to the protective effect of the down-regulated  $\beta$ -adrenergic response and, consequently, reduced cAMP accumulation, as observed in our study. Conversely, Lawson *et al.* (1996) indicated that PC had no significant effect upon tissue catecholamine content in isolated rat hearts perfused with whole blood.

According to Seyfarth *et al.* (1996), a single 5 min ischaemic episode was sufficient to reduce norepinephrine release during sustained ischaemia. However the most pronounced effect of transient ischaemia was achieved with two to three ischaemia-reperfusion cycles. This suppression could not be further enhanced by an additional cycle of transient ischaemia. These findings further support the efficiency of our 3-cycle ischaemic PC model, as well as the previous studies in which multiple cycles induced the strongest protection against arrhythmias and infarct size (Liu *et al.*, 1992, Yellon *et al.*, 1992).

### **3.3.3.3 p38 MAPK activation during sustained ischaemia and reperfusion**

The time-dependent activation of the stress kinase during sustained ischaemia is probably due to the same factors as described above for the PC protocol as well as the osmotic imbalances characteristic of ischaemia (Jennings *et al.*, 1991). Some workers failed to demonstrate activation of p38 MAPK by 30 min global ischaemia in isolated rabbits (Weinbrenner *et al.*, 1997) and 30-120 min coronary ligation in *in vivo* rats (Omura *et al.*, 1999). On the other hand, most others reported a significant activation regardless of the experimental model, but their results are conflicting regarding the duration, with some reporting only transient activation (Yin *et al.*, 1997, Shimizu *et al.*, 1998, Ma *et al.*, 1999, Yue *et al.*, 2000), while others reported a sustained activation (Bogoyevitch *et al.*, 1996, Maulik *et al.*, 1996). Our results are in agreement with the latter reports in which ischaemia caused sustained activation of p38 MAPK (Fig. 3.16). Furthermore, the biphasic response to ischaemia observed in neonatal cardiomyocytes (the first phase began within 10 min and lasted less than 1 h, and the second began

after 2 h and lasted throughout the ischaemic period) (MacKay *et al.*, 1999), was not seen in our model where the duration of sustained ischaemia is shorter (25 min).

Although the p38 MAPK showed dual phosphorylation and increased activity, by phosphorylation of its substrate ATF-2, at all time intervals during sustained ischaemia of non-preconditioned and preconditioned hearts, the activation of the kinase was significantly less between 10 and 25 min in the preconditioned hearts. Using phosphorylation of ATF-2 and HSP27 as indicators of p38 MAPK activation, Nagarkatti and Sha'afi (1998) also showed significantly less activation during ischaemia of preconditioned rat heart myoblast cells (H9C2). These results as well as those obtained in the present study, are in direct contradiction to those observed by others (Weinbrenner *et al.*, 1997, Maulik *et al.*, 1998d, Nakano *et al.*, 2000): not only could these workers not demonstrate increased p38 MAPK activation during ischaemia in non-preconditioned hearts, but they also reported a significant increase in kinase activity in preconditioned hearts, suggesting that increased p38 MAPK activation may be a key step in cardioprotection. These completely controversial results may be due to differences in (i) species (rats vs rabbits) or (ii) experimental preparation (cardiomyocytes vs perfused heart) or (iii) ischaemia (regional vs global) or (iv) PC regimen (one vs multi-cycles) or (v) method of tissue collection (repeated biopsy vs sampling of whole heart at one time point) or (vi) isoenzyme distribution (p38 $\alpha$  vs p38 $\beta$  MAPK). Obviously it is of paramount importance to elucidate these discrepancies.

Another potentially important difference may be the use of antibodies against tyrosine-182 of p38 MAPK only (Weinbrenner *et al.*, 1997, Maulik *et al.*, 1998d) as opposed to the dual-phosphorylated p38 MAPK as well as p38 MAPK activity (the ability of the enzyme to phosphorylate and activate its downstream substrate, ATF-2) used in the present study. This discrepancy may be explained by the fact that activation of p38 MAPK requires dual phosphorylation of both tyrosine-182 and threonine-180 (Armstrong *et al.*, 1999, Kumar *et al.*, 1999), thus these data may not adequately reflect the activation status of the enzyme. This was verified by Gysembergh *et al.* (2001), who quantified p38 MAPK activity (the ability of the enzyme to phosphorylate and activate its downstream substrate, MAPKAPK2) and observed modest attenuation thereof at 5 min into coronary occlusion in PC rabbit hearts. They speculated that the difference in outcome – increased phosphorylation/activation of p38 MAPK, but unchanged or attenuated activity of the enzyme in their study – might imply a defect or uncoupling in

the p38 MAPK signalling pathway in the rabbit heart. However, Ping *et al.* (1999a) demonstrated that the p38 MAPK pathway is intact in the *in vivo* rabbit heart and our results in rat heart also confirmed this.

However, Omura *et al.* (1999) for example assessed the activity levels of p38 MAPK, by phosphorylation of ATF-2 and also could not find any increase during continuous coronary ligation (30-120 min) of an *in vivo* rat model, rather a decrease was observed. The reason for these discrepancies is not clear. In addition, a recent study by Pain *et al.* (2000) showed that, like PC, diazoxide-induced reduction in infarct size was also characterized by increased p38 MAPK activation during sustained ischaemia.

Although reperfusion of both ischaemic preconditioned and non-preconditioned hearts caused a significant activation of p38 MAPK within 10 min, this effect was transient in the former, with p38 MAPK activation returning to normal after 20 - 30 min of reperfusion (Fig. 3.21). The sustained activation of p38 MAPK during reperfusion in the non-preconditioned hearts was concurrent with findings in isolated perfused rabbit hearts (Ma *et al.*, 1999). The burst of activation seen during the early phase of reperfusion may have been due to generation of free radicals at this stage (Das *et al.*, 1999, Ferrari *et al.*, 1994).

## 3.4 SUMMARY

### 3.4.1 Effect of ischaemic PC on cyclic nucleotides:

The results obtained in the present study demonstrated significant changes in the cyclic nucleotides both during the trigger ischaemia of the *PC protocol* and during the subsequent *sustained ischaemia*.

In particular, the PC protocol was characterized by cyclic increases in tissue cAMP and cGMP that might be caused by the concomitant changes in cAMP- and cGMP- PDE activities. Furthermore, the changes in the  $\beta$ -adrenergic pathway was associated by marked fluctuations in adenylyl cyclase and protein kinase A (PKA), accompanied by a decrease in receptor affinity ( $K_d$ ) and responsiveness despite a gradual upregulation of the  $\beta$ -adrenergic receptor population ( $B_{max}$ ). In addition to the above, the rise in tissue cAMP during the PC protocol was shown to be dependent on the release of endogenous catecholamines. However, since catecholamines were not assessed during PC, it does not allow speculation whether PDE or catecholamines is the primary mechanism.

Sustained ischaemia of preconditioned hearts was characterized by an attenuated  $\beta$ -adrenergic response and elevated tissue cGMP, associated with less PDE inhibition and reduced energy utilization. Whether these events mediate the protection conferred by prior PC, or whether they are merely epiphenomena associated with myocardial protection against ischaemia, remains to be established. Nevertheless we suggest that both of these cyclic nucleotides may be involved in triggering the protection conferred by PC. Possible proof of involvement will be given in chapters 4 and 5.

### 3.4.2 Effect of ischaemic PC on p38 MAPK activation:

Although only one study attempted to evaluate changes in p38 MAPK during the repetitive ischaemia-reperfusion (PC) protocol (Ping *et al.*, 1999a), our study is the first attempt to evaluate changes in p38 MAPK activation throughout the entire experimental protocol of a preconditioned heart and to correlate these changes with functional recovery during reperfusion. Our results show activation of p38 MAPK during all three stages of the experimental protocol, while attenuation of p38 MAPK activity during both ischaemia and reperfusion is associated with cardioprotection. Indeed, a very recent

study suggested that while activation of p38 MAPK (via cAMP- PKA activation) during the PC protocol (stimulus) was required for early PC in dogs, the subsequent inhibition of p38 MAPK during the sustained ischaemia was also necessary (Sanada *et al.*, 2001b).

The pattern of changes observed in p38 MAPK activation follows the same pattern as observed in the cAMP levels – activation during the PC protocol and continued, but attenuated activation during sustained ischaemia. The cause and significance of these observations remain to be established.

## CHAPTER 4

### Manipulation of cyclic adenosine monophosphate (cAMP)

#### 4.1 Introduction

The previous chapter showed that myocardial protection, induced by ischaemic PC, is associated with attenuation of cAMP generation during the sustained ischaemic period. Therefore, manipulation of cAMP levels *per se* during sustained ischaemia by either  $\beta$ -adrenergic blockade or  $\beta$ -adrenergic stimulation should also elicit or abolish protection, respectively. Our laboratory has previously shown that attenuation of  $\beta$ -adrenergic effects during ischaemia by prior treatment with propranolol (Edoute *et al.*, 1981) or reserpination (Moolman *et al.*, 1996) elicited functional protection similar to that of PC. In contrast, increasing cAMP levels during sustained ischaemia by administration of either a  $\beta$ -adrenergic agonist, isoproterenol (Podzuweit *et al.*, 1996) or adenylyl cyclase activator, forskolin (Sandhu *et al.*, 1994, Moolman *et al.*, 1996) after PC but before the onset of sustained ischaemia, causing high cAMP levels throughout this period, failed to abolish PC. These experiments may illustrate the protective effect of PC against drug-induced cAMP increases, rather than elucidating the role of cAMP in the protective effect of PC.

Should cAMP be involved in PC, it is more likely to act as a trigger during the PC protocol, prior to sustained ischaemia. Consequently, the observation that transient  $\beta$ -adrenergic stimulation can precondition the isolated rat heart (Asimakis *et al.*, 1994; Miyawaki *et al.*, 1997b) suggests that these changes in cAMP content, at least, may be important. Asimakis *et al.* (1994) suggested that the increased workload produced by  $\beta$ -stimulation might cause a degree of demand ischaemia that, in turn, produced PC, probably via attenuation of  $\text{Ca}^{2+}$  overload during reperfusion.

According to the results from the previous chapter (Chapter 3), we have shown that a multiple-episode PC protocol is characterized by cyclic increases in cAMP, coinciding with opposite changes in phosphodiesterase (PDE) activity, indicating that this particular pathway is changed during both PC and sustained ischaemia.

In this chapter we address the question whether the cyclic increases in cAMP (and thus PKA activation) occurring during PC are involved in eliciting protection against ischaemia. Two possibilities should be considered – cAMP/PKA may play a role in  $\beta$ -adrenergic desensitization, and/or may phosphorylate an unknown downstream target. We investigated both possibilities.

#### 4.1.1 Aims

The aim therefore was to evaluate whether pharmacological interventions (i.e.  $\beta$ -adrenergic blockade or stimulation) would;

- (i) abolish or mimic the ischaemia-induced cyclic increases in cAMP during the PC protocol, respectively;
- (ii) lead to or cause  $\beta$ -adrenergic receptor desensitization and thus reduced cAMP generation during sustained ischaemia;
- (iii) prevent or elicit myocardial protection during ischaemia and reperfusion, respectively.

To elucidate the possibility of phosphorylation of a downstream target, we chose to investigate the role of p38 MAPK in  $\beta$ -adrenergic induced protection by;

- (i) characterization of the effects of  $\beta$ -adrenergic receptor stimulation (time, dose-dependency) on p38 MAPK activation and
- (ii) comparison of changes in p38 MAPK activation of  $\beta$ -adrenergic- and ischaemic-preconditioned hearts during sustained ischaemia as well as reperfusion.
- (iii) evaluation of the effects of  $\beta$ -adrenergic blockade during the PC protocol on p38 MAPK activation during sustained ischaemia and reperfusion
- (iv) determination of the effects of inhibition of  $\beta$ -adrenergic-induced p38 MAPK activation on myocyte viability during hypoxia.

## 4.2 Experimental Protocols and Results

### 4.2.1 Effect of $\beta$ -adrenergic receptor manipulation during the PC protocol

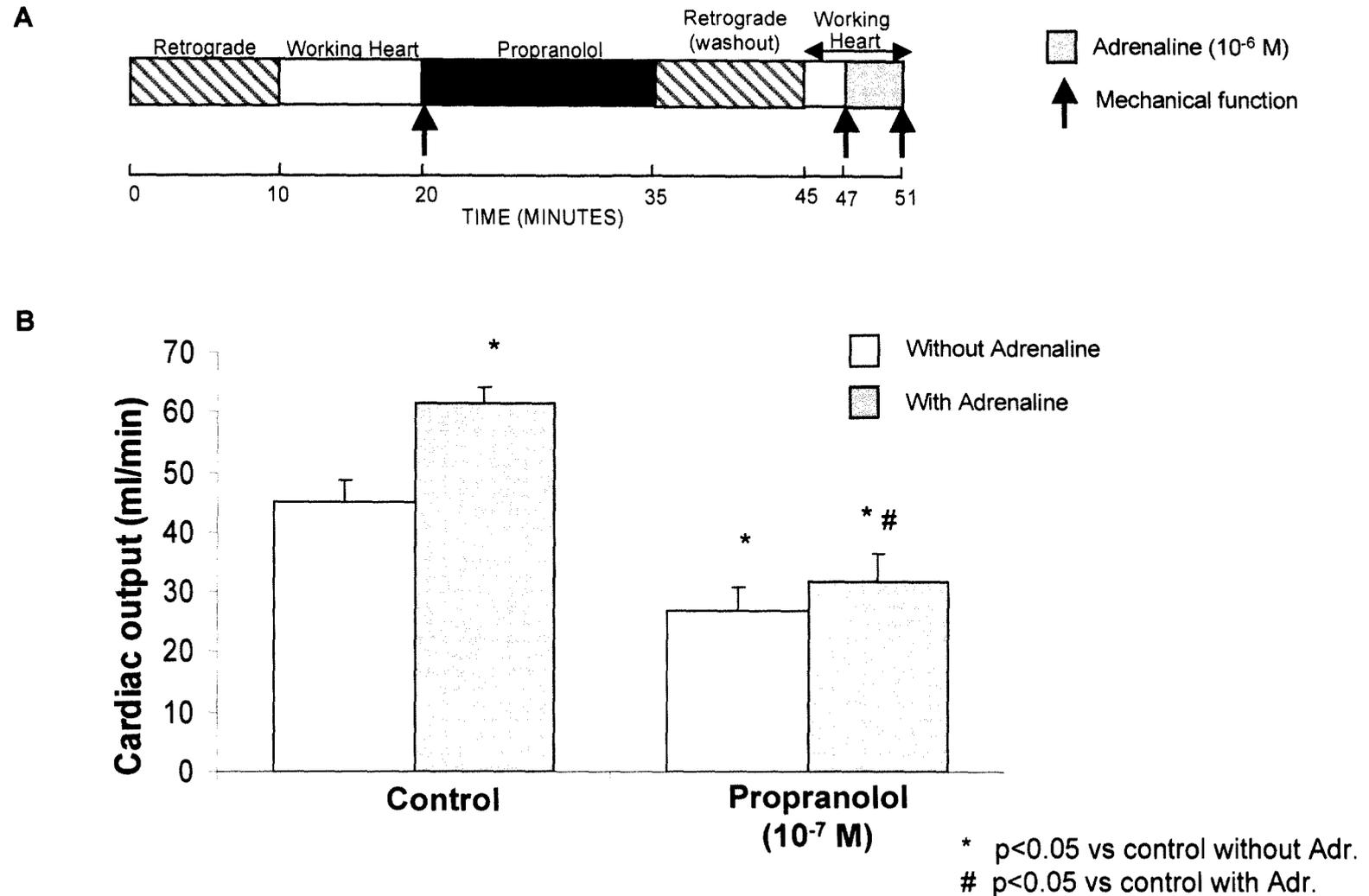
#### 4.2.1.1 $\beta$ -adrenergic receptor blockade during the PC protocol

##### 4.2.1.1.1 Choice of $\beta$ -adrenergic receptor blocker

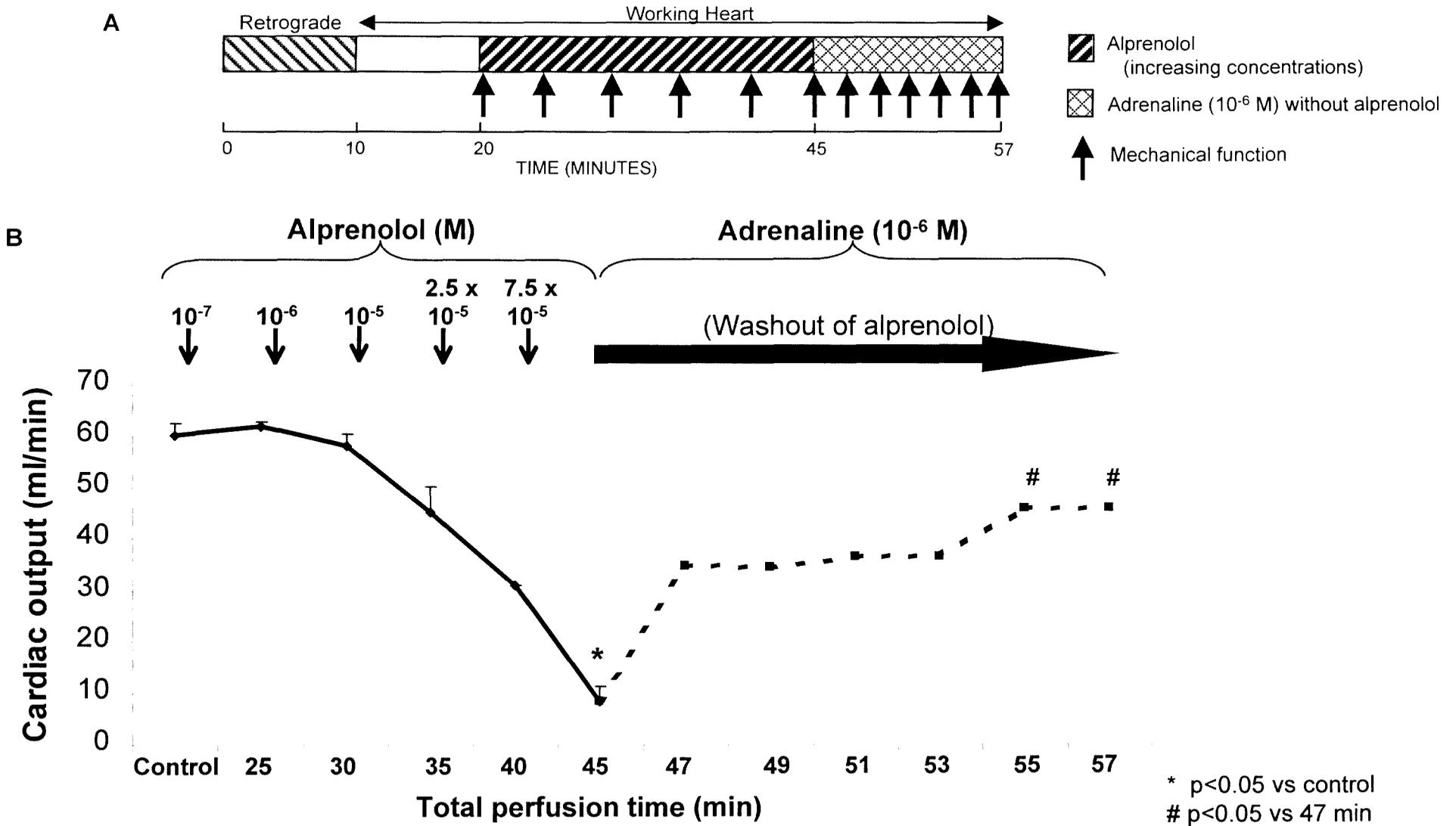
A prerequisite for assessing the effects of  $\beta$ -adrenergic receptor blockade during PC, was complete washout of the blocker before onset of sustained ischaemia as well as attenuation of cAMP generation during the PC protocol.

In the first series of experiments the  $\beta$ -adrenergic receptor blocker, propranolol, was evaluated for this purpose. Hearts were stabilized by perfusing retrogradely for a period of 10 min, followed by 10 min in the working mode. Control hearts were subsequently perfused retrogradely for a further 25 min, before administration of adrenaline ( $10^{-6}$  M). In another series, propranolol ( $10^{-7}$  M) was administered at 20 min total perfusion time for 15 min and washed out for 10 min, followed by administration of adrenaline (Fig. 4.1A). Control hearts respond significantly to 2 min of adrenaline (% increase in cardiac output from controls:  $31 \pm 3.1$ ) (Fig. 4.1B). Propranolol treatment significantly reduced the cardiac output compared to controls. However, these treated hearts could not respond to adrenaline ( $10^{-6}$  M) stimulation after the 10 min washout period (Fig. 4.1B), indicating that propranolol, a lipid-soluble antagonist, cannot be washed out under these conditions. It was thus decided to use the non-selective  $\beta_1/\beta_2$ -adrenergic blocker, alprenolol.

After the hearts were stabilized for 20 min (controls), alprenolol was administered every 2 min in a dose-dependent manner until the cardiac output was suppressed sufficiently. Drug administration was ceased and immediately followed by a 12 min period of adrenaline ( $10^{-6}$  M) administration (Fig. 4.2A). Results from fig. 4.2B indicate that  $7.5 \times 10^{-5}$  M alprenolol adequately suppressed the cardiac output ( $85.9 \pm 1.5$  % decrease in cardiac output from control). Furthermore, its effect could be reversed after 10 min



**Fig 4.1** Cardiac output of hearts treated with  $\beta$ -adrenergic blocker, propranolol ( $10^{-7}$ M). **A.** Experimental protocol. Arrows indicate time of measurement of mechanical function. **B.** Propranolol treated hearts could not respond to 2 min adrenaline (Adr.  $10^{-6}$ M) after 10 min washout. n = 4 hearts per series.



**Fig 4.2** Dose-response with alprenolol. **A.** Experimental protocol. Arrows indicate time of measurement of mechanical function. **B.** Dose effect of alprenolol on cardiac output, followed by washout thereof, as determined by the response to 12 min adrenaline ( $10^{-6}$  M). alprenolol could be washed out within 10 min.  $n = 3$  hearts per series.

cessation of alprenolol, as indicated by the response to adrenaline ( $10^{-6}$  M) [% decrease in cardiac output from control after alprenolol withdrawal: 2 min adrenaline (without alprenolol)  $41.9 \pm 2.0$  vs. 10 min adrenaline (without alprenolol)  $23.7 \pm 3.0$ ;  $n=4$ ]. Therefore indicating that alprenolol could be washed out within 10 min after being administered at its most effective concentration.

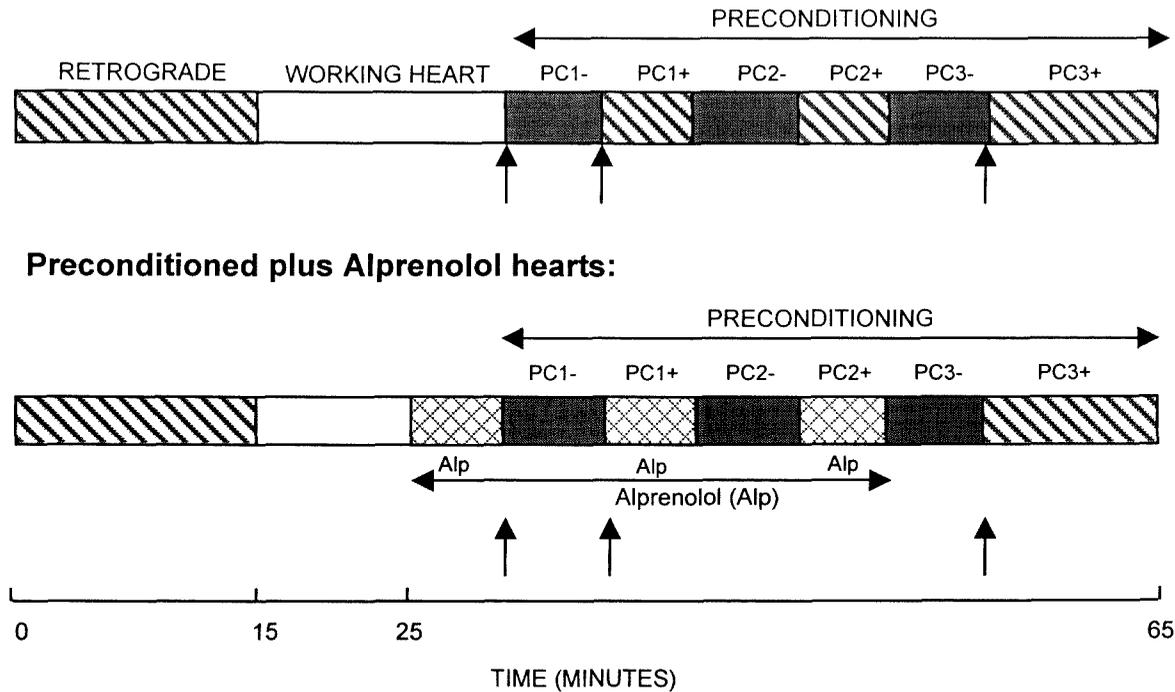
To establish whether  $7.5 \times 10^{-5}$  M alprenolol could attenuate cAMP generation during the PC protocol, hearts were preconditioned with 3 x 5 min ischaemia in the absence or presence of alprenolol. In the latter series, alprenolol was administered 5 min before the onset of PC1- and during PC1+ and PC2+ (Fig. 4.3). In a separate series, hearts were stabilized for 25 min followed by alprenolol administration for 3 episodes of 5 min alternated by 5 min periods of perfusion with buffer (protocol not shown), which indicated that alprenolol *per se* had no effect on control hearts (Fig. 4.4). However, transient administration of alprenolol ( $7.5 \times 10^{-5}$  M) during the PC protocol prevented the characteristic increases in cAMP (as shown in Fig. 3.2), while neither at PC1- nor at PC3- were the changes in cAMP content different from their corresponding controls (Fig. 4.4).

#### 4.2.1.2 Repeated $\beta$ -adrenergic stimulation: effect of cAMP

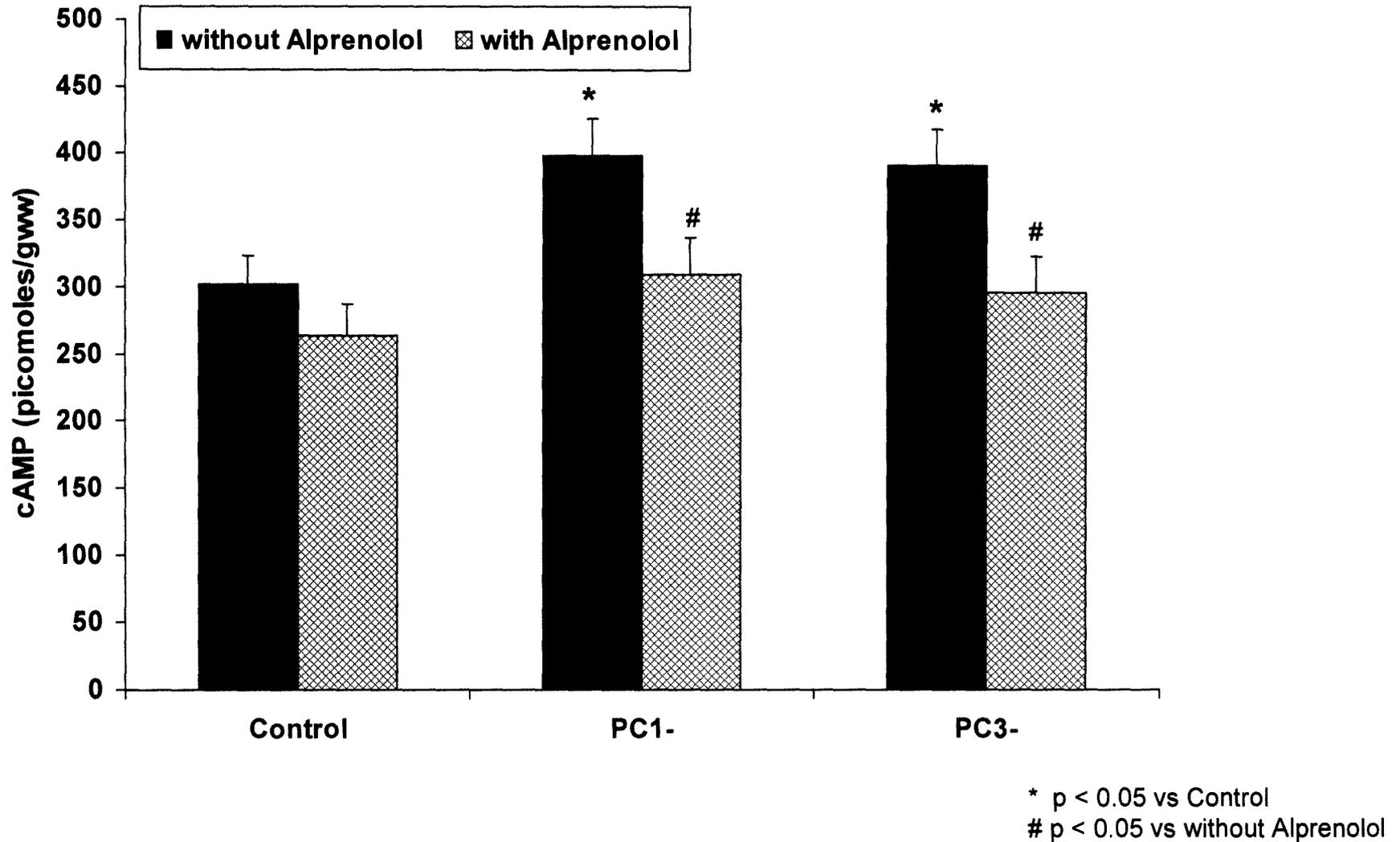
For meaningful simulation of PC-induced fluctuations in tissue cAMP, dose-response curves with forskolin and isoproterenol were required. A preliminary experiment was performed where hearts were perfused for 30 min (15 min retrograde, 15 min working mode) before administration of either forskolin ( $10^{-8}$ ,  $10^{-7}$  and  $10^{-6}$  M) or isoproterenol ( $10^{-8}$  or  $10^{-7}$  M) (Fig. 4.5A). After 5 min of drug administration hearts were freeze-clamped for subsequent cAMP analyses. Results showed that both drugs caused dose-dependent increases in tissue cAMP (Fig. 4.5B). We therefore decided to use forskolin at  $10^{-6}$  M and isoproterenol at  $10^{-7}$  M concentrations.

In a subsequent study, tissue cAMP and high energy phosphate levels were monitored during repeated administration (3 x 5min) of either forskolin ( $10^{-6}$  M) or isoproterenol ( $10^{-7}$  M). In this study hearts were freeze-clamped at the end of the first and third administration of the drug as well as after 5 min reperfusion with normal buffer (for protocol see Fig. 4.6A). At both administration times, forskolin caused a significant increase in tissue cAMP, while reperfusion caused a decline, although not to control

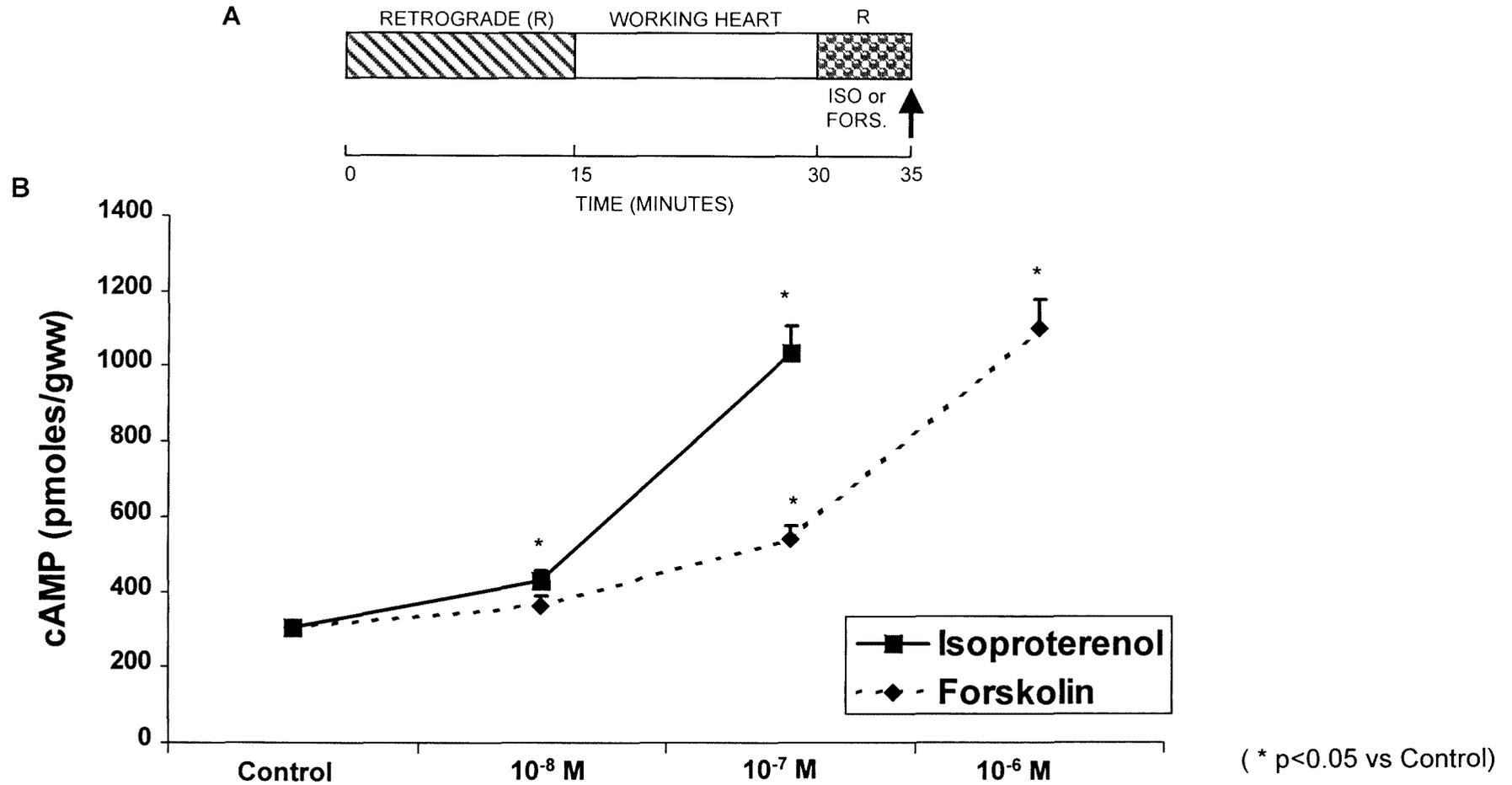
**Preconditioned hearts:**



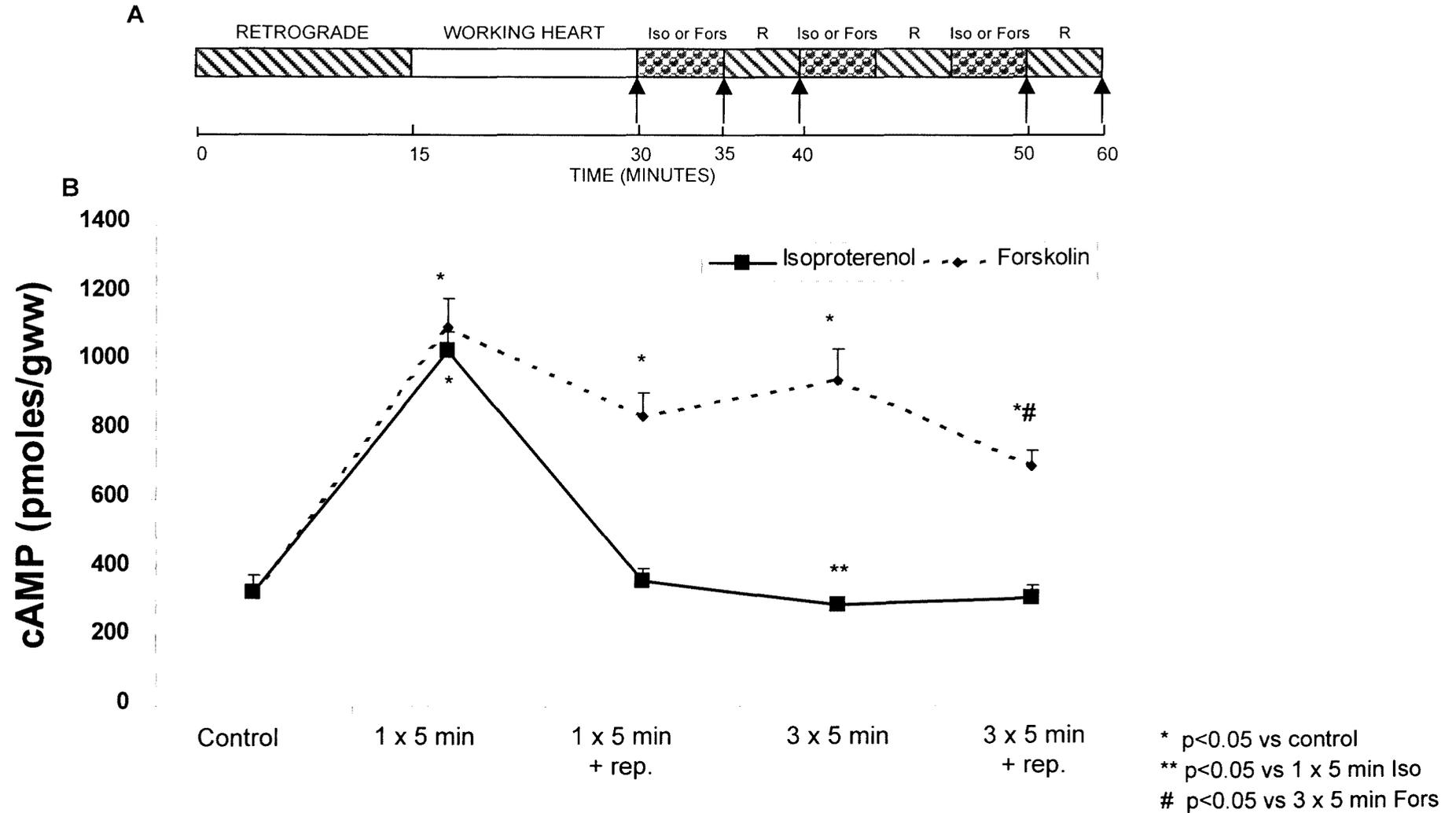
**Fig 4.3**  $\beta$ -adrenergic blockade with alprenolol ( $7.5 \times 10^{-5}$  M) during preconditioning protocol: Experimental protocol. PC1-, PC2-, PC3- indicate hearts exposed to 5 min of global ischaemia; PC1+, PC2+, PC3+, hearts reperfused for 5 min after global ischaemia. Arrows indicate time of freeze-clamping for cAMP determination. n = 6 hearts per series.



**Fig 4.4** Tissue cAMP levels after  $\beta$ -adrenergic blockade during preconditioning protocol. Alprenolol ( $7.5 \times 10^{-5}$  M) administration during the PC protocol prevented the characteristic increases in cAMP at PC1- and PC3-.



**Fig 4.5** Dose-response with isoproterenol and forskolin. A. Experimental protocol. Isoproterenol ( $10^{-8}$  or  $10^{-7}$  M) or forskolin ( $10^{-8}$ ,  $10^{-7}$  or  $10^{-6}$  M) administered for 5 min retrogradely. Arrows indicate time of freeze-clamping for cAMP determination.  $n = 6$  hearts per series. B. Tissue cAMP increased significantly at  $10^{-8}$  -  $10^{-7}$  M isoproterenol and at  $10^{-7}$  -  $10^{-6}$  M forskolin.



**Fig 4.6** Repeated  $\beta$ -adrenergic stimulation. A. Experimental protocol. Administration of isoproterenol ( $10^{-7}$  M) or forskolin ( $10^{-6}$  M) for 3 x 5 min interspersed by 5 min reperfusion(rep.) with buffer. Arrows indicate time of freeze-clamping for cAMP determination. n = 6 hearts per series. B. At both administration times, forskolin caused a increase in tissue cAMP. Repeated administration of isoproterenol for 3 x 5 min caused desensitization of the  $\beta$ -adrenergic receptor, as reflected by low cAMP levels after the third administration.

**Table 4.1****Metabolic changes during administration of forskolin or isoproterenol**

	<b>ATP</b>	<b>CP</b>
<b>Controls</b>	3.45 ± 0.18	4.25 ± 0.37
<b>Forskolin:</b>		
<b>10<sup>-6</sup> mol/l: (n=5)</b>		
1 x 5 min	3.64 ± 0.14	4.84 ± 0.28
1 x 5 min + 5 min reperfusion	3.26 ± 0.41	4.32 ± 0.37
3 x 5 min	3.02 ± 0.31	3.74 ± 0.67
3 x 5 min + 5 min reperfusion	3.40 ± 0.11	3.94 ± 0.19
<b>Isoproterenol:</b>		
<b>10<sup>-7</sup> mol/l: (n=5)</b>		
1 x 5 min	5.74 ± 0.30	4.36 ± 0.78
1 x 5 min + 5 min reperfusion	-	-
3 x 5 min	4.37 ± 0.59	4.07 ± 0.68
3 x 5 min + 5 min reperfusion	-	-

Hearts were freeze-clamped at the end of each intervention (n=5 for each series) (Fig. 4.6). High energy phosphates (ATP and creatine phosphate, CP) are expressed as  $\mu\text{moles/g}$  wet weight. Controls were perfused for 30 min before freeze-clamping.

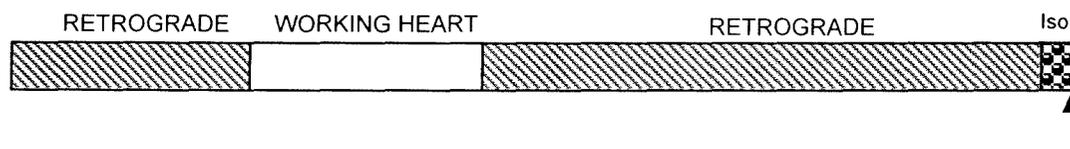
levels (Fig. 4.6B). Isoproterenol when administered for 5 min only caused a significant elevation in tissue cAMP, which returned to control levels when reperfused with normal buffer. However, repeated administration of isoproterenol for 3 x 5 min, caused complete desensitization of the  $\beta$ -adrenergic receptor, as reflected by the very low cAMP values after the third administration (Fig. 4.6B). To establish whether  $\beta$ -adrenergic stimulation induces a condition of demand ischaemia, high energy phosphate (HEP) levels were also measured. The HEP levels, such as tissue ATP and creatine phosphate (CP) levels, were not significantly affected by any of the above interventions (Table 4.1).

#### **4.2.1.3 Repeated $\beta$ -adrenergic stimulation: effect on $\beta$ -adrenergic receptor responsiveness**

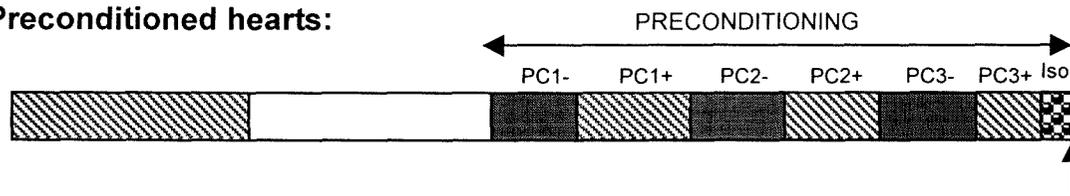
The following experiments were performed, to assess whether pharmacological stimulation of the  $\beta$ -adrenergic signalling pathway also reduced the  $\beta$ -adrenergic response comparable to PC hearts. Tissue cAMP levels were measured after additional  $\beta$ -adrenergic receptor stimulation, by administration of isoproterenol ( $10^{-7}$  M) for a period of 2 min after  $\beta$ -adrenergic stimulation with isoproterenol ( $10^{-8}$  or  $10^{-7}$  M for 1 x 5 min) or forskolin ( $10^{-7}$  or  $10^{-6}$  M for 1 x 5 min or 3 x 5 min) (Fig. 4.7). With each drug, administration of isoproterenol for 2 min was preceded by a 5 min washout period.

As observed previously (Fig. 3.9), the ischaemic PC protocol (3 x 5 min global ischaemia) significantly desensitizes the heart to further  $\beta$ -adrenergic receptor stimulation, when compared to that of non-PC hearts (Fig. 4.8). Hearts stimulated with isoproterenol ( $10^{-8}$  M) for 5 min only, was still sensitive to additional  $\beta$ -adrenergic stimulation, as evidenced by the significant increase in tissue cAMP (similar to that of non-PC hearts) (Fig. 4.8). However at  $10^{-7}$  M for 1 x 5 min or 3 x 5 min, isoproterenol caused a significant desensitization of the  $\beta$ -adrenergic response, with no increase in tissue cAMP. Forskolin, when administered for 1 x 5 min only, at  $10^{-6}$  M did not cause desensitization (Fig. 4.9). Similar results were obtained with forskolin at  $10^{-7}$  M, whether administered for 1 x 5 min or for 3 x 5 min. However, forskolin ( $10^{-6}$  M, 3 x 5 min) also caused a partial, but significant desensitization of the  $\beta$ -adrenergic response, as reflected by significantly reduced tissue cAMP compared to non-PC levels (Fig. 4.9).

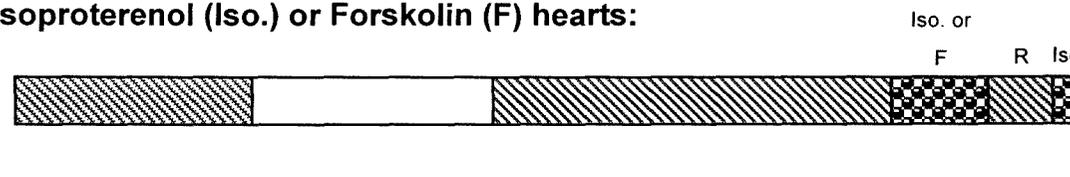
**Non-preconditioned hearts:**



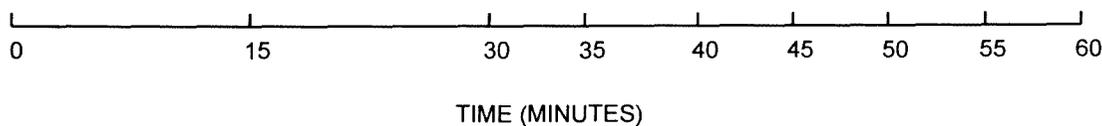
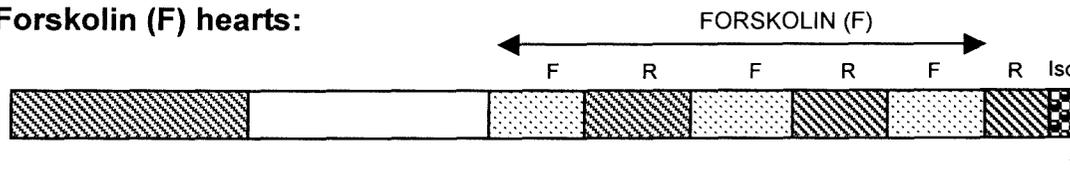
**Preconditioned hearts:**



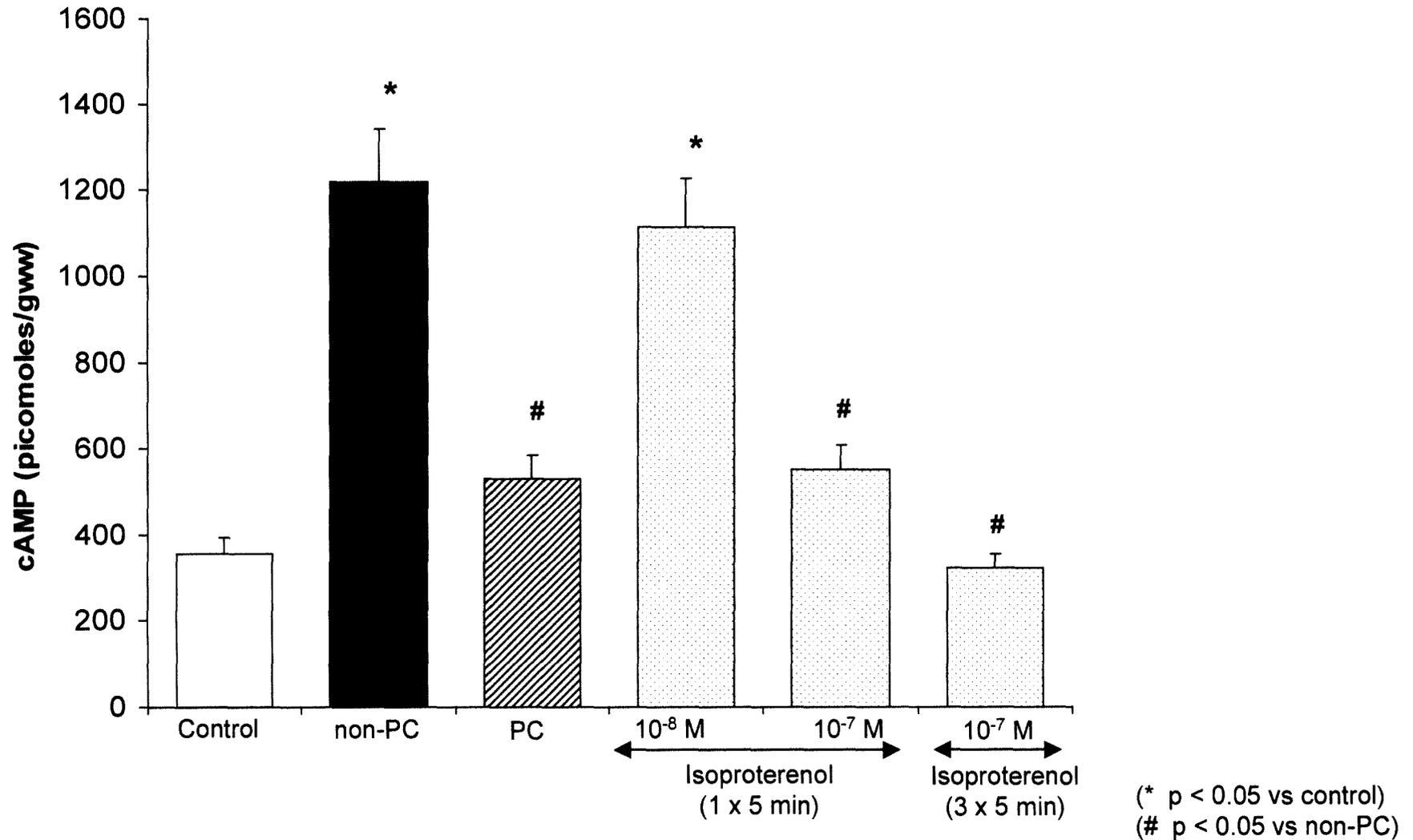
**Isoproterenol (Iso.) or Forskolin (F) hearts:**



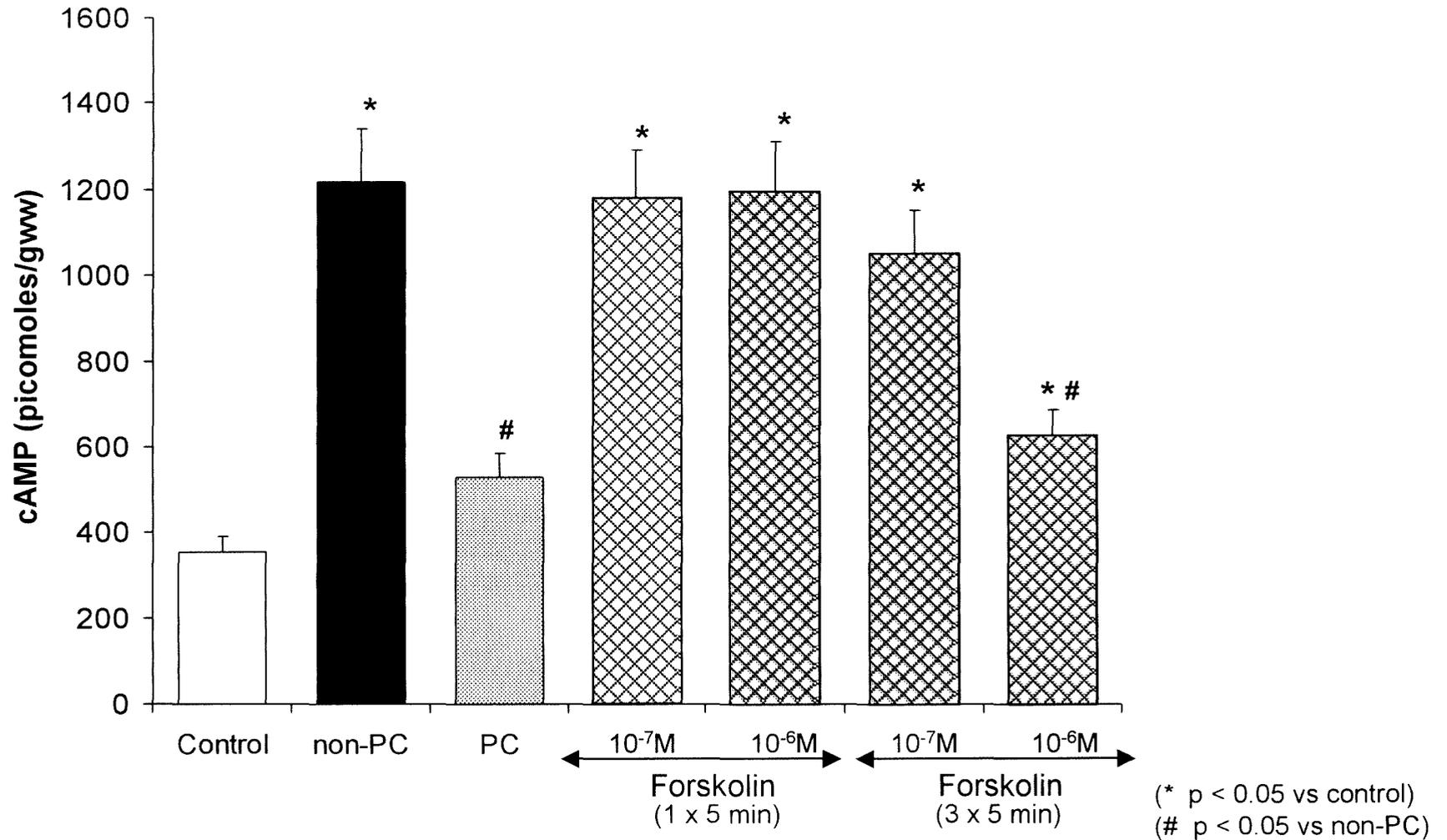
**Forskolin (F) hearts:**



**Fig 4.7**  $\beta$ -adrenergic receptor responsiveness after repeated  $\beta$ -adrenergic stimulation: Experimental protocol. Abbreviations as in fig 4.3.  $\beta$ -adrenergic stimulation with isoproterenol ( $10^{-8}$  or  $10^{-7}$  M, 1 x 5 min) or forskolin ( $10^{-7}$  or  $10^{-6}$  M, 1 x 5 min or 3 x 5 min) was followed by additional  $\beta$ -adrenergic receptor stimulation with isoproterenol ( $10^{-7}$  M) for 2 min. Arrows indicate time of freeze-clamping for cAMP determination. n = 6 hearts per series.



**Fig 4.8**  $\beta$ -adrenergic receptor responsiveness after repeated  $\beta$ -adrenergic stimulation with isoproterenol. Ischaemic PC hearts (3 x 5 min), as well as hearts treated with isoproterenol ( $10^{-7}$  M for 1 x 5 min or 3 x 5 min) were desensitized to further  $\beta$ -adrenergic receptor stimulation, with no increase in cAMP levels. However, hearts stimulated with isoproterenol ( $10^{-8}$  M) for 5 min only was still sensitive to additional  $\beta$ -adrenergic stimulation.



**Fig 4.9**  $\beta$ -adrenergic receptor responsiveness after repeated  $\beta$ -adrenergic stimulation with forskolin. Forskolin, when administered for 1 x 5 min only at 10<sup>-7</sup>M or 10<sup>-6</sup>M, or for 3 x 5 min at 10<sup>-7</sup>M, did not cause desensitization. However, repeated administration of forskolin (10<sup>-6</sup>M for 3 x 5 min) caused a partial, but significant desensitization of the  $\beta$ -adrenergic response, as reflected by reduced cAMP levels.

#### **4.2.1.4 Evaluation of p38 MAPK activation**

##### **4.2.1.4.1 Effects of $\beta$ -adrenergic receptor stimulation on p38 MAPK activation**

To establish the role of p38 MAPK in  $\beta$ -adrenergic induced protection, it was necessary to (i) characterize the effects of  $\beta$ -adrenergic receptor stimulation (time, dose-dependency) on p38 MAPK activation and (ii) study the effects of  $\alpha_1$ - or  $\beta$ -adrenergic blockade, as well as L-type  $\text{Ca}^{2+}$  channel or  $\text{A}_1$ -adenosine receptor blockade on ischaemia- or isoproterenol-induced activation of p38 MAPK.

##### **4.2.1.4.1.1 Effects of time and dosage of different $\beta$ -adrenergic stimulants**

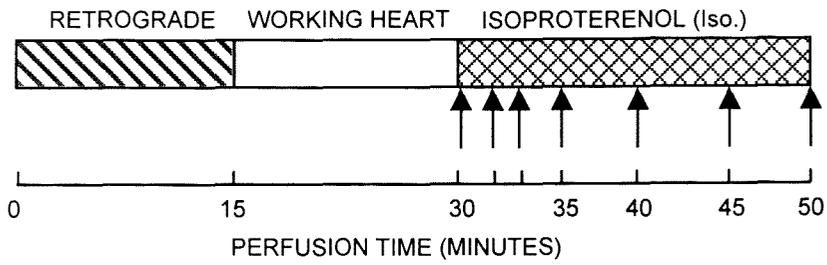
#### **Experimental protocol**

After 30 min of stabilization (15 min retrograde, 15 min working heart), hearts were perfused with isoproterenol ( $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  M) for 2, 3, 5, 10, 15 or 20 min before freeze-clamping (Fig. 4.10A). Zinterol ( $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$  M), a  $\beta_2$ -adrenergic receptor agonist, or forskolin ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M), an activator of adenylyl cyclase, was administered for 5 min only before freeze-clamping (Fig. 4.11A). At least 4 hearts were studied in each series. Dual phosphorylation of p38 MAPK was assayed at the above time intervals, while ATF-2 phosphorylation was studied at selected times.

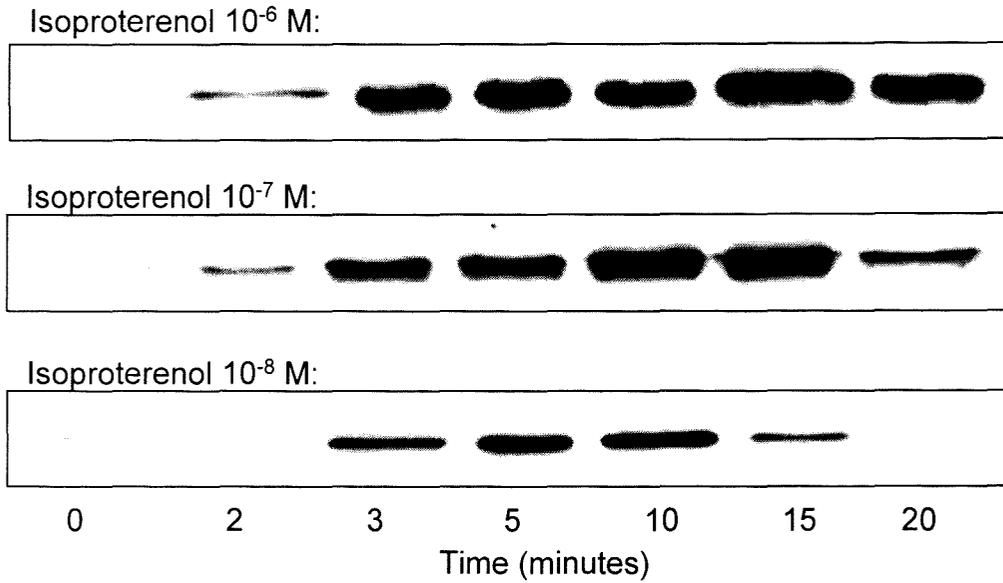
#### **Results**

As indicated by its dual phosphorylation, isoproterenol activated p38 MAPK in a dose- and time-dependent manner (Fig. 4.10B, C). During the first 2 min after administration of isoproterenol, its effects are dose-dependent, with  $10^{-8}$  M having a significantly smaller effect than  $10^{-7}$  and  $10^{-6}$  M. However, after 10 min p38 MAPK appears to be maximally activated, regardless of the concentration used. In the case of  $10^{-6}$  M isoproterenol the activation is maintained for at least 20 min, while in the case of  $10^{-8}$  and  $10^{-7}$  M the activation is transient and wanes after 15 min (Fig. 4.10B, C).

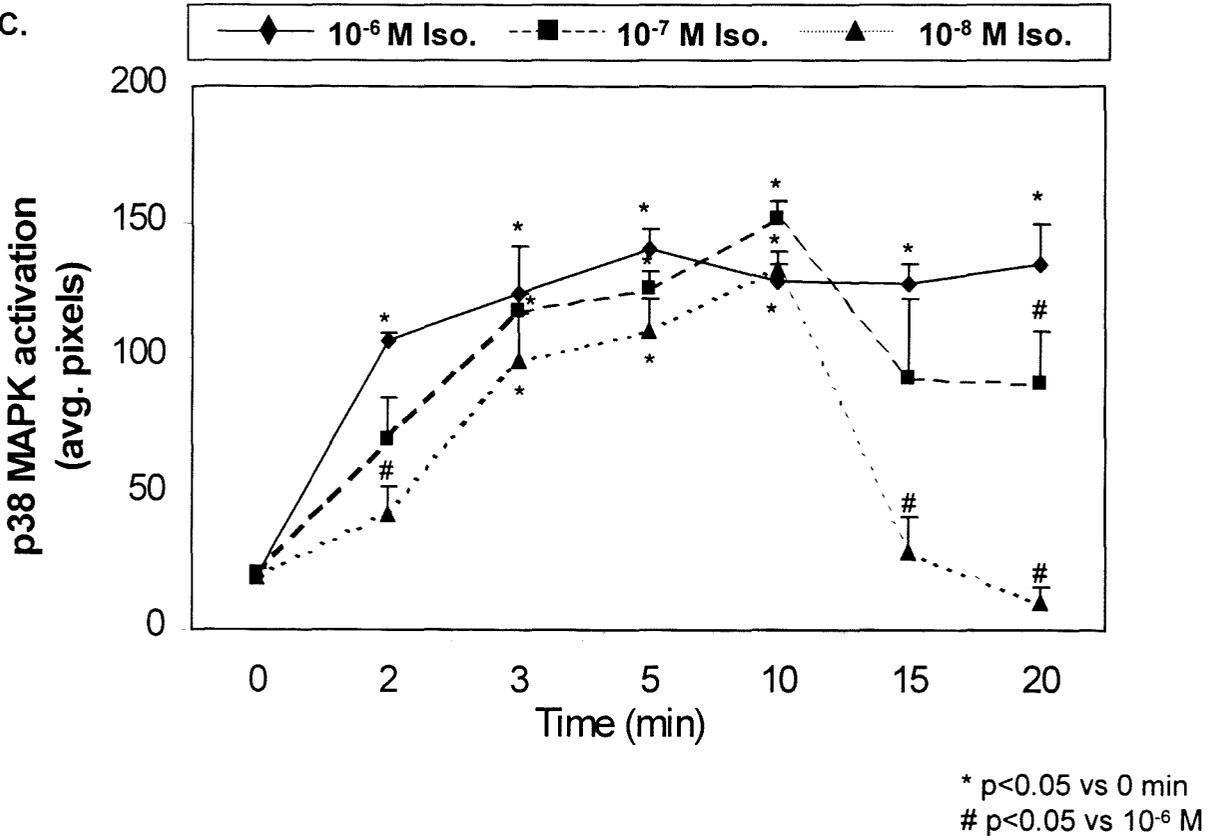
A.



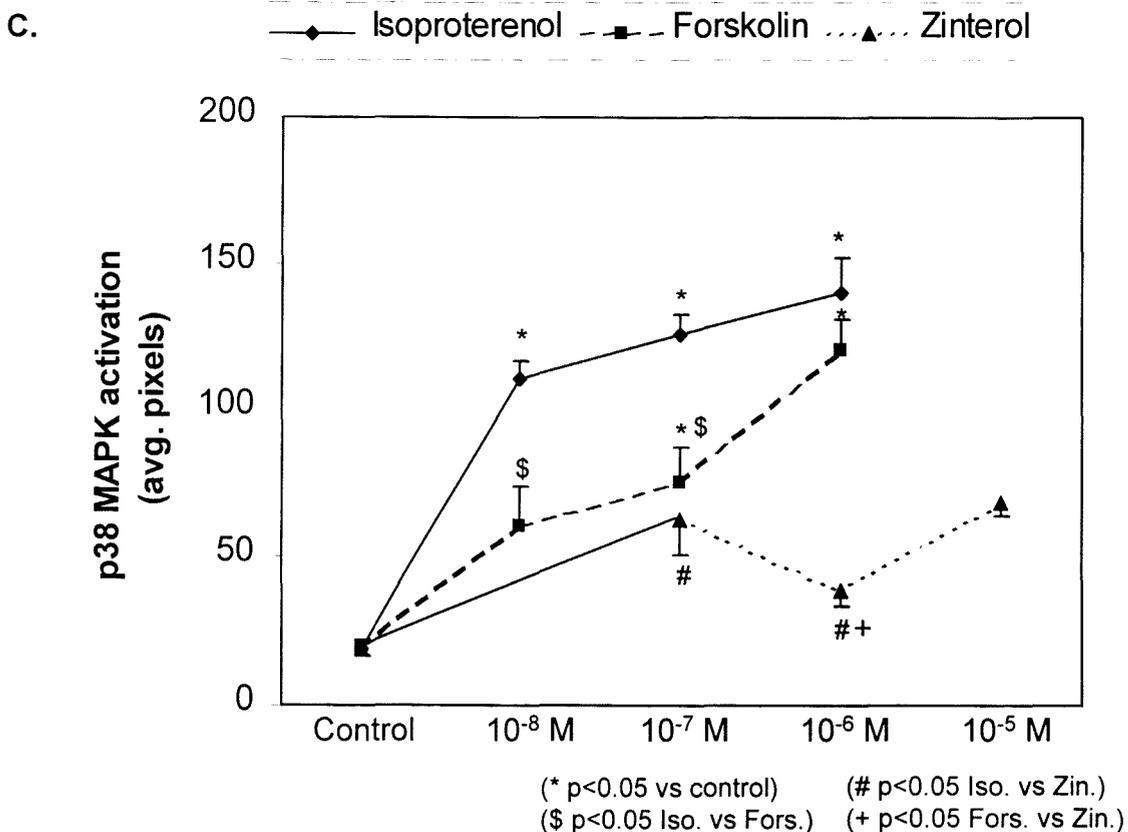
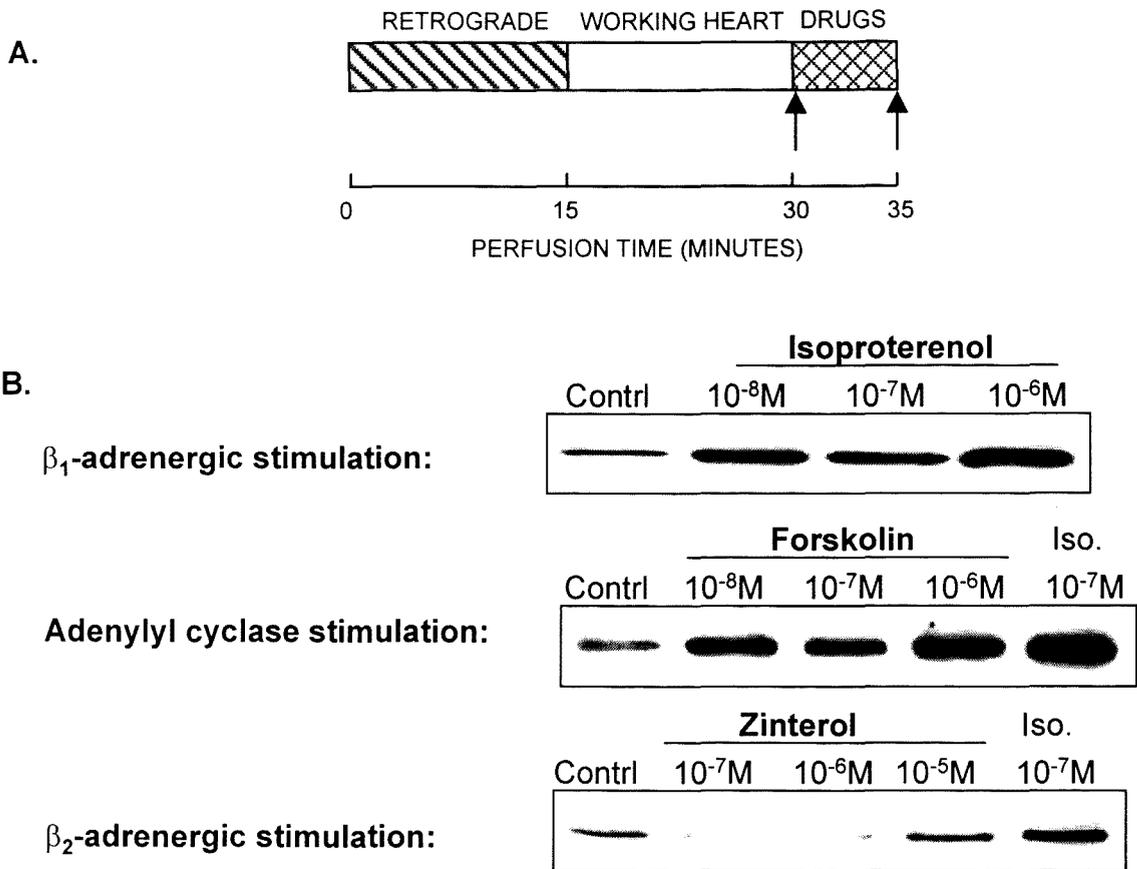
B.



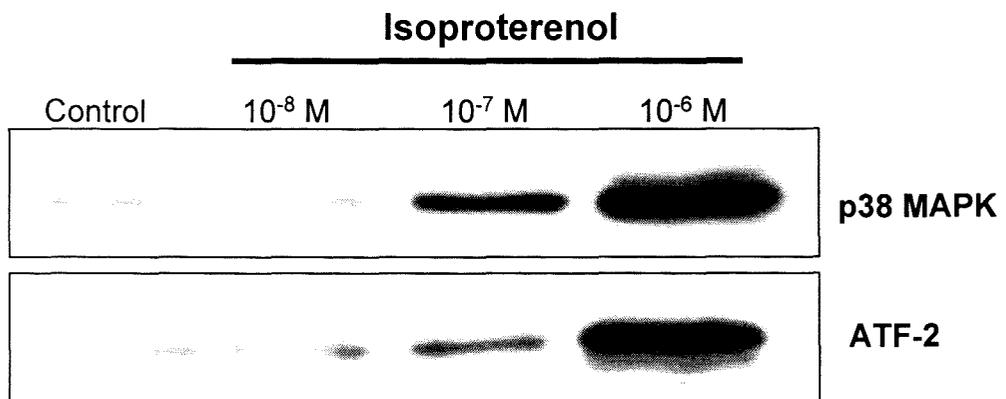
C.



**Fig 4.10** Characterization of effects of  $\beta$ -adrenergic stimulation on p38 MAPK activation. Effect of isoproterenol ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M) and time (0 - 20 min).  $\beta_1$ -adrenergic activation of p38 MAPK is dose- and time dependent. The blots given are representative of each series. Four hearts were studied at each time point.



**Fig 4.11** Characterization of effects of  $\beta$ -adrenergic stimulation on p38 MAPK activation. Comparison of effects of isoproterenol (10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> M), forskolin (10<sup>-8</sup>, 10<sup>-7</sup>, 10<sup>-6</sup> M) and zinterol (10<sup>-7</sup>, 10<sup>-6</sup>, 10<sup>-5</sup> M) administration for 5 min. The blots given are representative of each series. n = 4 hearts/series.



**Fig 4.12** Isoproterenol dose-response: comparison of dual phosphorylation and kinase activity (ATF-2 phosphorylation) of p38 MAPK. Effect of isoproterenol ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M) at time 20 min. The blots are representative of each experiment.  $n = 4$  hearts/series.

Forskolin, which directly activates adenylyl cyclase, also activated p38 MAPK in a dose-dependent manner within 5 minutes. Zinterol, a  $\beta_2$ -adrenergic receptor agonist, when administered for 5 min had a maximal effect on p38 MAPK at  $10^{-5}$  M, although significantly less than isoproterenol ( $10^{-7}$  M), (Fig. 4.11B, C).

Activation of p38 MAPK as indicated by its kinase activity (activation of ATF-2), was studied in the case of isoproterenol only: the pattern of activation was similar to that observed using dual phosphorylation as indicator (Fig. 4.12).

#### **4.2.1.4.1.2 Manipulation of p38 MAPK activation induced by 5 min ischaemia (PC1-) or isoproterenol**

##### **Experimental protocol**

After a stabilization period of 25 min, alprenolol ( $7.5 \times 10^{-5}$  M) or an  $\alpha_1$ -adrenergic receptor blocker, prazosin ( $3 \times 10^{-7}$  M) or a combination of both was administered 5 min before the onset of global ischaemia. Hearts were freeze-clamped after 5 min global ischaemia (PC1-). Appropriate controls were generated by perfusing hearts for 5 minutes with alprenolol or prazosin or a combination of both drugs (Fig. 4.13A).

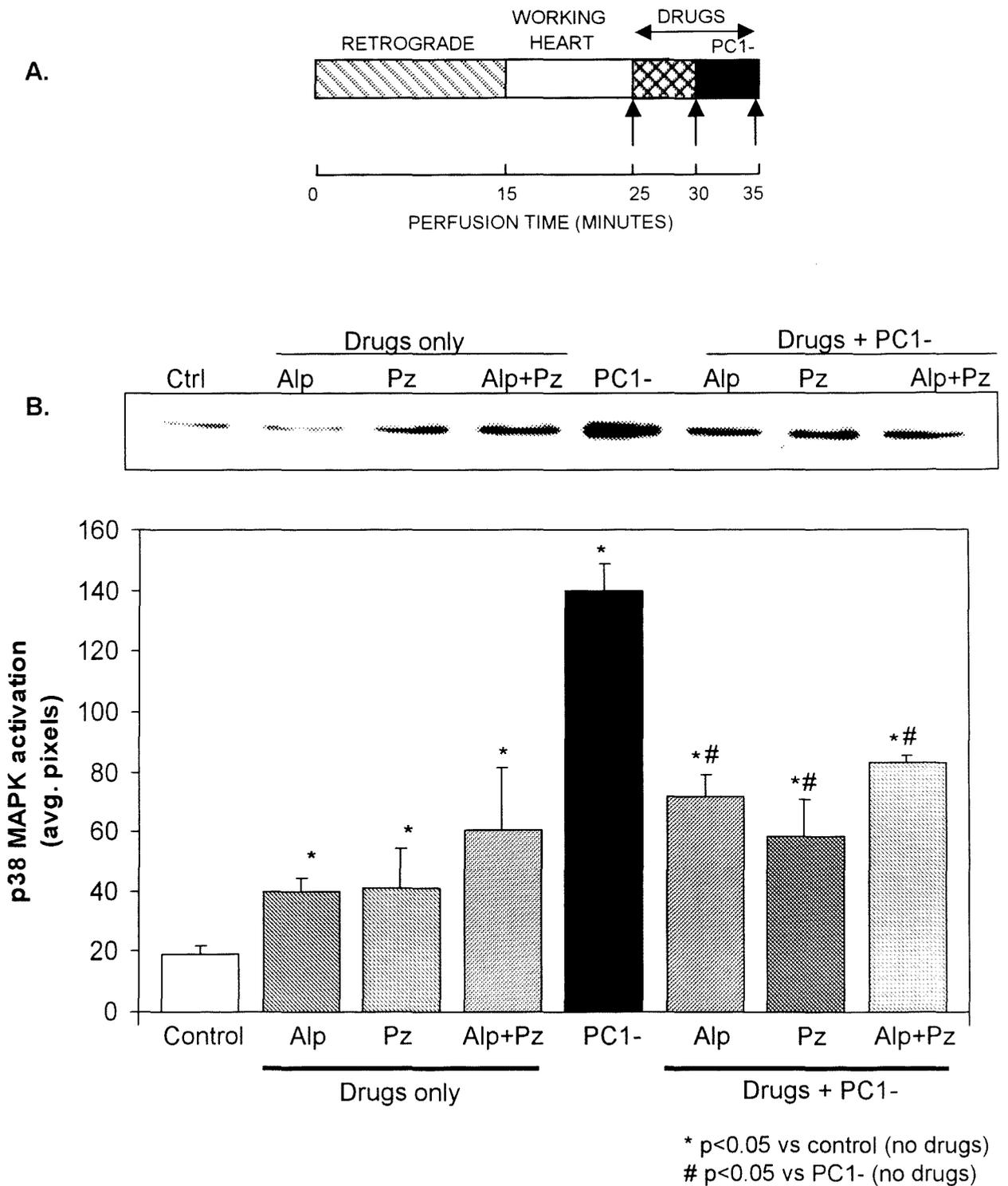
In the case of the isoproterenol-treated hearts, alprenolol ( $7.5 \times 10^{-5}$  M) or a selective  $A_1$ -adenosine receptor blocker, 8-cyclopentyltheophylline (CPT,  $10^{-5}$  M) or a L-type  $Ca^{2+}$  channel blocker, verapamil ( $5 \times 10^{-7}$  M) was administered for 10 min after the 25 min stabilization period. During the last 5 min of administration of either of these blockers, isoproterenol ( $10^{-7}$  M) was infused simultaneously. As before, appropriate controls were generated by 5 min infusion of each of these drugs (Fig. 4.14A).

##### **Results**

Ischaemia-induced (PC1-) activation of p38 MAPK as indicated by its dual phosphorylation (Fig. 4.13B) could be partially, but significantly inhibited by blockade of the  $\beta$ -adrenergic receptor (alprenolol,  $7.5 \times 10^{-5}$  M),  $\alpha_1$ -Adrenergic receptor blockade (prazosin,  $3 \times 10^{-7}$  M) as well as a combination of the drugs. This partial inhibition could be attributed to a drug effect, as the drugs themselves significantly increased p38 MAPK phosphorylation when compared to 30 min control perfused hearts (Fig. 4.13B).

Furthermore, the p38 MAPK activity (ATF-2 phosphorylation) was also significantly inhibited by alprenolol when administered to a 5 min global ischaemic heart (Fig. 4.15).

Similarly, isoproterenol-induced ( $10^{-7}$  M) phosphorylation of p38 MAPK could be blocked completely by alprenolol ( $7.5 \times 10^{-5}$  M) as well as by verapamil ( $5 \times 10^{-7}$  M), while  $A_1$ -adenosine receptor blockade by CPT ( $10^{-5}$  M) had no effect (Fig. 4.14B). These results prove that the  $\beta$ -adrenergic receptor as well as  $Ca^{2+}$ , but not adenosine was involved in  $\beta$ -adrenergic induced phosphorylation of p38 MAPK. The drugs, when administered in the absence of isoproterenol, had no significant effect on p38 MAPK activation when compared to control perfused hearts (Fig. 4.14B).



**Fig 4.13** Manipulation of brief ischaemic-induced p38 MAPK activation. Effects of alprenolol (Alp.,  $7.5 \times 10^{-5}$  M), prazosin (Pz.,  $3 \times 10^{-7}$  M) or a combination of the drugs when administered alone or 5 min before onset of 5 min global ischaemia (PC1-). A representative blot is given for each experiment. n = 4 hearts/series.

## **4.2.2 Effect of $\beta$ -adrenergic receptor manipulation during PC protocol on cyclic nucleotides during sustained ischaemia**

### **4.2.2.1 $\beta$ -adrenergic receptor blockade during the PC protocol**

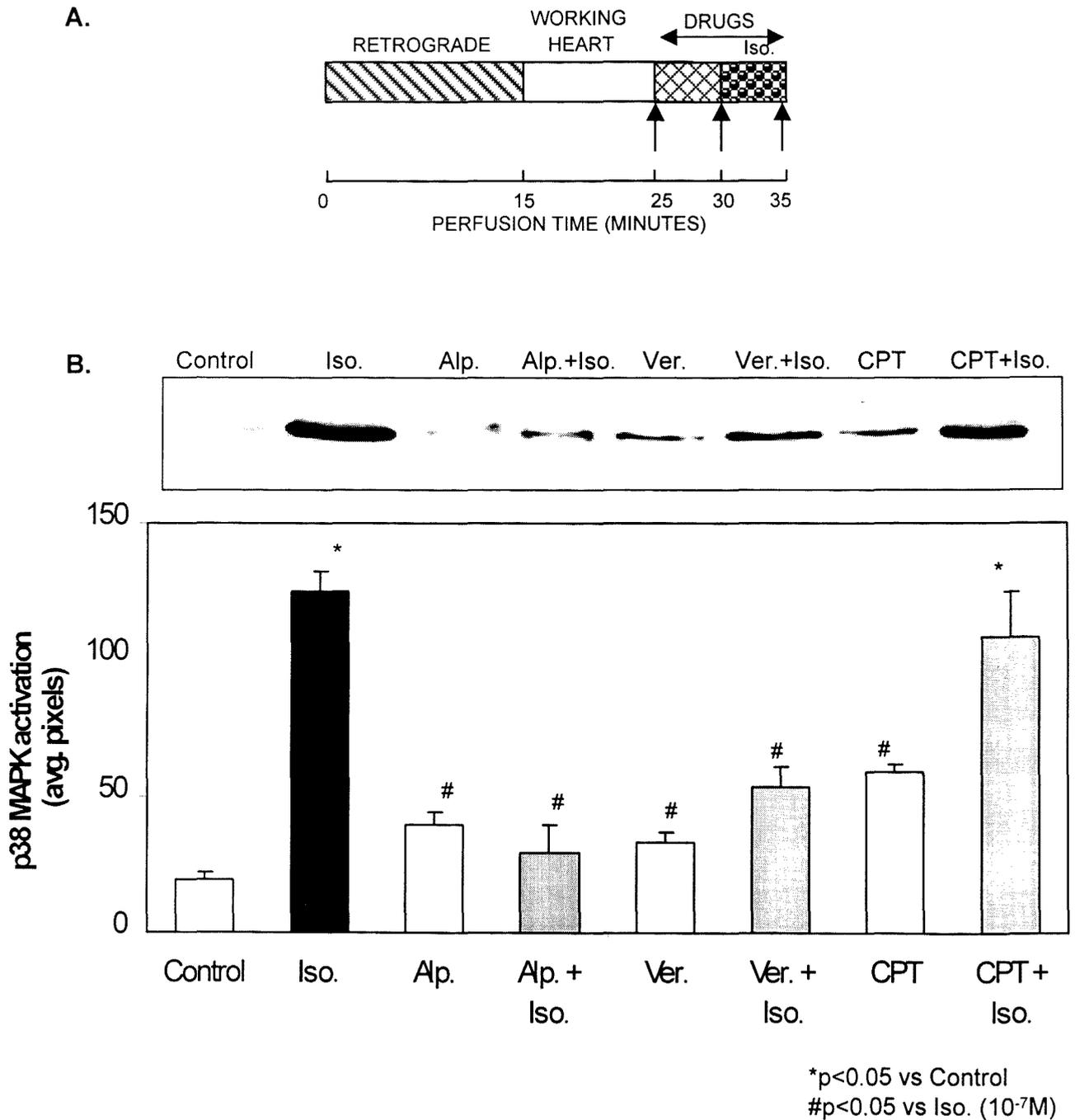
As described before, PC reduced the  $\beta$ -adrenergic response to subsequent sustained ischaemia, compared to non-PC. To establish whether  $\beta$ -adrenergic blockade could reverse this effect, PC hearts were pretreated with alprenolol ( $7.5 \times 10^{-5}$  M) during the PC protocol before sustained ischaemia. Alprenolol was administered 5 min before the onset of PC1-, during PC1+, PC2+ and washed out for 10 min following PC3- prior to 25 min sustained ischaemia (Fig. 4.16).

Alprenolol administered during the PC protocol resulted in a significant increase in tissue cAMP at the end of 25 min ischaemia, almost to the same extent as non-PC hearts, compared to untreated PC hearts. This increased cAMP level in PC hearts caused by  $\beta$ -adrenergic blockade was also significant compared to PC hearts, suggesting a partial involvement of the  $\beta$ -adrenergic receptor (Fig. 4.17).

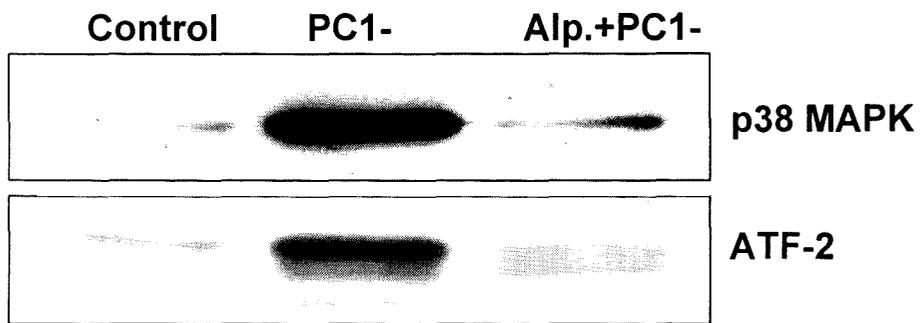
### **4.2.2.2 Repeated $\beta$ -adrenergic receptor stimulation prior to sustained ischaemia**

To assess whether repeated stimulation of the  $\beta$ -adrenergic receptor prior to ischaemia also reduce the  $\beta$ -adrenergic response to subsequent sustained ischaemia in a manner similar to PC hearts (see 3.2.2.2.1), tissue cAMP levels were compared after 25 min of global ischaemia in non-PC, PC, forskolin- and isoproterenol-pretreated hearts (Fig. 4.16).

One episode of isoproterenol ( $10^{-7}$  M, 1 x 5 min) stimulation, caused a similar significant reduction in tissue cAMP levels after 25 min of sustained ischaemia as observed in PC hearts, however  $10^{-8}$  M isoproterenol (1 x 5 min) did not affect tissue cAMP levels during this period (Fig. 4.18). Repeated forskolin ( $10^{-6}$  M, 3 x 5 min) pre-treatment caused a significant reduction in tissue cAMP levels at the end of sustained ischaemia and values similar to those of PC hearts were obtained (Fig. 4.19). Although one episode of forskolin ( $10^{-6}$  M, 1 x 5 min) reduced tissue cAMP levels, the values obtained were not



**Fig 4.14** Manipulation of brief isoproterenol-induced p38 MAPK activation. Effects of alprenolol (Alp.,  $7.5 \times 10^{-5}$  M), verapamil (Ver.,  $0.5 \times 10^{-6}$  M) and 8-cyclopentyladenosine (CPT,  $10^{-5}$  M) when administered for 10 min, while isoproterenol (Iso.,  $10^{-7}$  M) was administered simultaneously during the last 5 min. A representative blot is given for each experiment.  $n = 4$  hearts/series.



**Fig 4.15** Effect of brief ischaemia (5 min global ischaemia, PC1-) and  $\beta$ -blockade by alprenolol (Alp.,  $7.5 \times 10^{-5}$  M) on dual phosphorylation and kinase activity (ATF-2 phosphorylation) of p38 MAPK. The blots are representative of each experiment. n = 4 hearts/series.

significantly different from those of non-PC hearts. Forskolin at  $10^{-7}$  M, when administered for either 1 x 5 min or 3 x 5 min, had no effect on tissue cAMP levels at the end of sustained ischaemia (Fig. 4.19).

Therefore, ischaemic PC, isoproterenol ( $10^{-7}$  M, 1 x 5 min) and forskolin ( $10^{-6}$  M, 3 x 5 min) resulted in significant desensitization of the  $\beta$ -adrenergic response at the *onset* of sustained ischaemia (Fig. 4.8, 4.9), and a significant reduction in cAMP accumulation at the *end* of 25 min sustained ischaemia (Fig. 4.18, 4.19).

### **4.2.3 Evaluation of events after sustained ischaemia during reperfusion: Role of $\beta$ -adrenergic signalling pathway in eliciting protection against ischaemic damage**

To investigate the role of the  $\beta$ -adrenergic signalling pathway in eliciting protection against ischaemic damage, it was essential to determine whether the cyclic increases in tissue cAMP levels during the PC protocol acts as a trigger in eliciting protection. For this purpose isolated working rat hearts were subjected to (i)  $\beta$ -adrenergic blockade with alprenolol during the PC protocol or (ii) stimulation with either forskolin or isoproterenol before onset of sustained ischaemia. Functional recovery during reperfusion was used as endpoint to evaluate the extent of protection against ischaemic damage.

#### **4.2.3.1 Effect of $\beta$ -adrenergic receptor blockade during PC protocol on functional recovery**

##### **4.2.3.1.1 Experimental protocol**

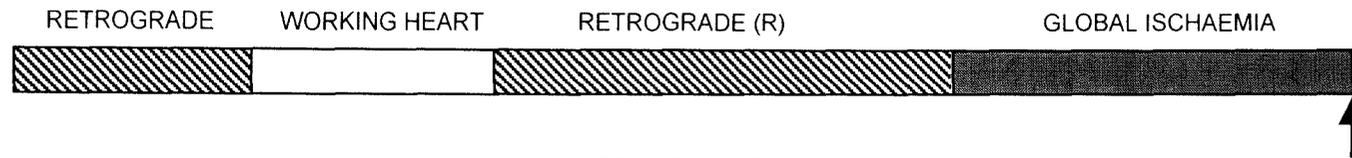
###### **Control hearts and alprenolol**

Control hearts were perfused for 115 min (Fig. 4.20A). In a separate series alprenolol ( $7.5 \times 10^{-5}$  M) was administered at 25 min total perfusion time for 3 episodes of 5 min interspersed by 5 min periods of perfusion with normal buffer, followed by perfusion in the retrograde (at 75 min for 10 min) and working (10 min) mode. Furthermore, evaluation of the recovery potential of the control hearts was done by adding adrenaline ( $10^{-6}$  M) after the 10 min working mode at 105 min total perfusion time. Subsequently, mechanical function was monitored for a further 10 min (Fig. 4.20A) at 115 min total perfusion time.

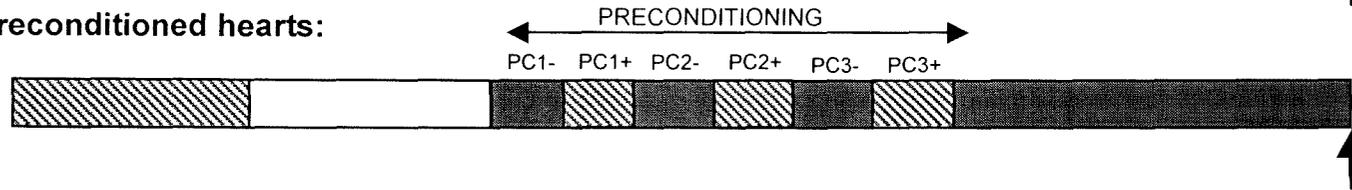
###### **Non-Preconditioned hearts and alprenolol:**

Non-Preconditioned hearts were perfused as above, in the absence or presence of alprenolol (Fig. 4.20B). After the last administration of the drug, the hearts were perfused with buffer for 10 min followed by 25 min sustained global ischaemia and 20 min reperfusion (10 min retrograde, 10 min working heart). Evaluation of the recovery potential of the non-preconditioned hearts was also done by adding adrenaline ( $10^{-6}$  M)

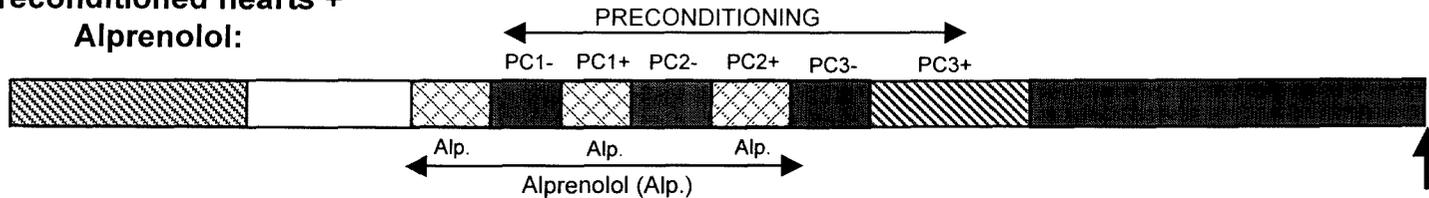
**Non-preconditioned hearts:**



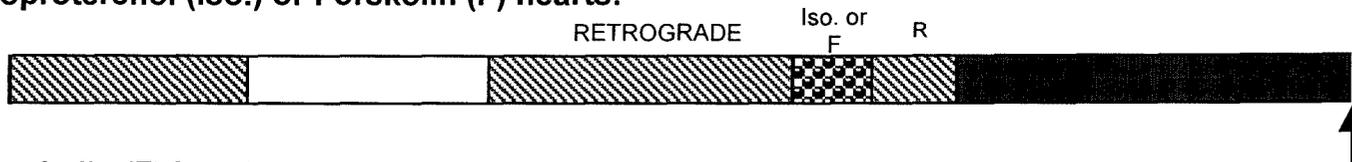
**Preconditioned hearts:**



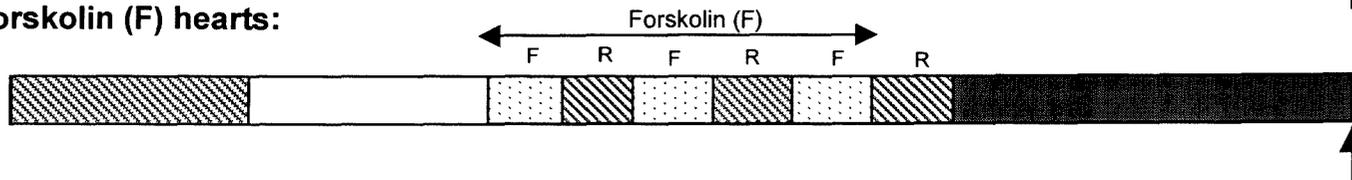
**Preconditioned hearts +  
Alprenolol:**



**Isoproterenol (Iso.) or Forskolin (F) hearts:**



**Forskolin (F) hearts:**



**Fig 4.16**  $\beta$ -adrenergic response to 25 min sustained global ischaemia: Experimental protocol. Abbreviations as in fig 4.3.  $\beta$ -adrenergic receptor blocker, alprenolol ( $7.5 \times 10^{-5}$  M), was administered 5 min before the the onset of PC1- and during PC1+ and PC2+. Following PC3-, the drug was washed out for 10 min before the onset of 25 min global ischaemia.  $\beta$ -adrenergic stimulation with isoproterenol ( $10^{-8}$  or  $10^{-7}$  M, 1 x 5 min) or forskolin ( $10^{-7}$  or  $10^{-6}$  M, 1 x 5 min or 3 x 5 min) replaced the ischaemic PC before 25 min global ischaemia. Arrows indicate time of freeze-clamping for cAMP determination. n = 6 hearts per series.

after the 20 min reperfusion phase at 105 min total perfusion time. Subsequently, mechanical function was monitored for 10 min (Fig. 4.20B) at 115 min total perfusion time.

### **Preconditioned hearts and alprenolol:**

Preconditioned hearts were preconditioned with 3 x 5 min ischaemia in the absence or presence of alprenolol (Fig. 4.20C). Alprenolol was administered 5 min before the onset of PC1- and during PC1+ and PC2+. Following PC3-, the drug was washed out for 10 min before the onset of 25 min sustained global ischaemia and 20 min reperfusion. Evaluation of the recovery potential of the preconditioned hearts was done as described above.

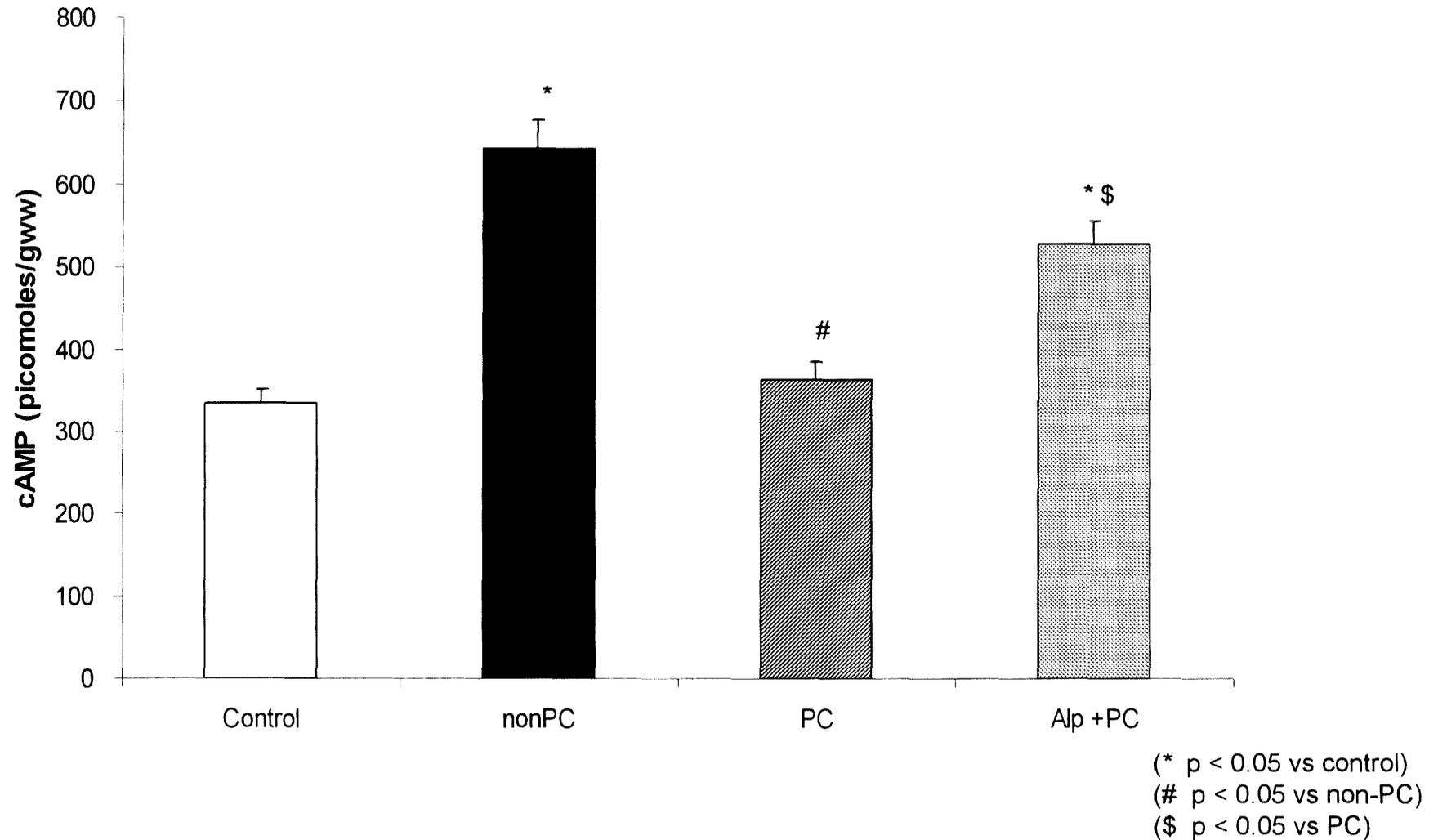
#### **4.2.3.1.2 Results**

##### **Effect of alprenolol administration on functional recovery:**

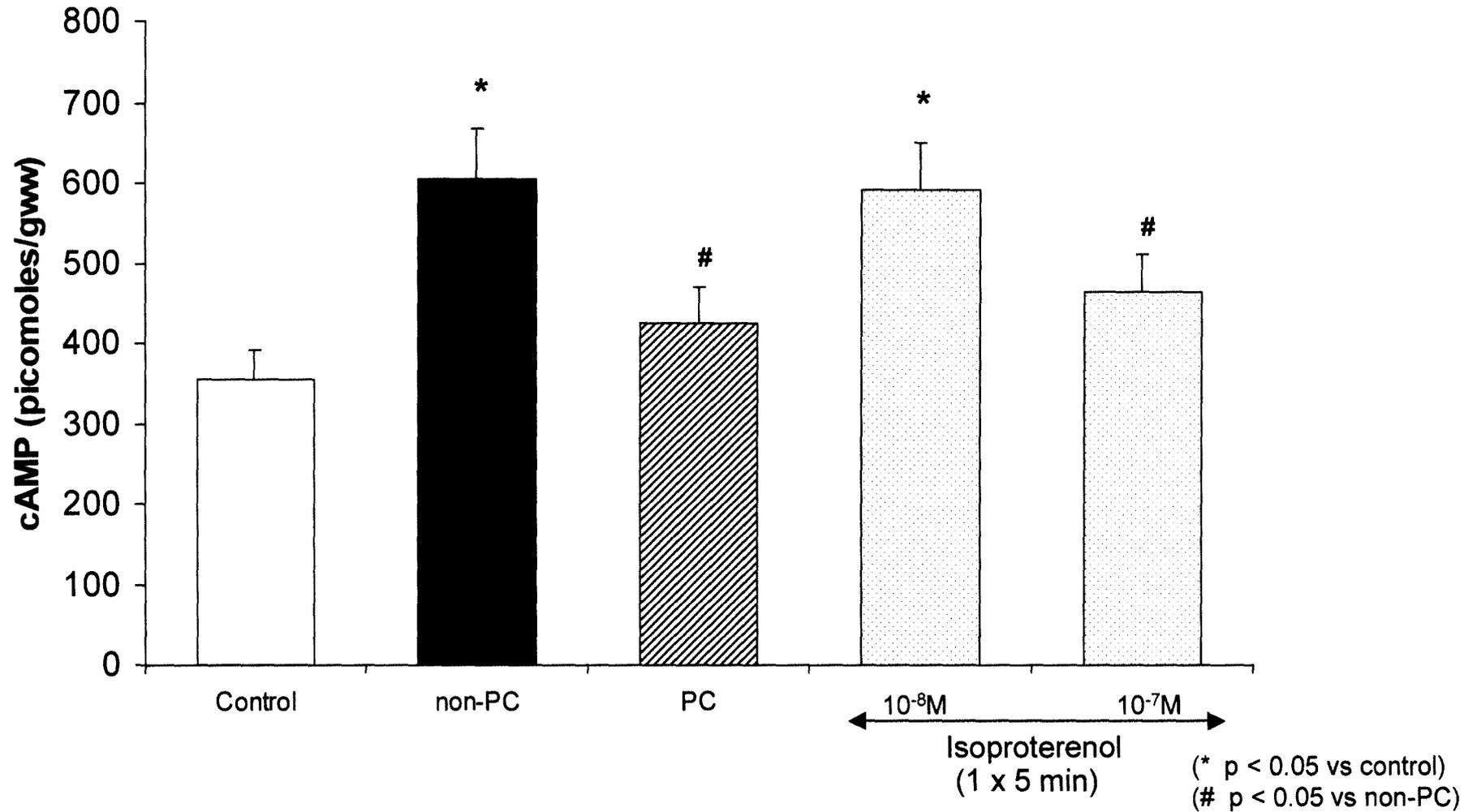
We observed that alprenolol had no effect on control hearts not subjected to ischaemia (Fig. 4.21). Neither did transient administration of alprenolol prior to the onset of sustained ischaemia in non-PC hearts have any effect on functional recovery, for both untreated and alprenolol treated hearts failed to produce a significant aortic output (Fig. 4.21A) or total work (Fig. 4.21B) during reperfusion. Alprenolol, when given prior to and during the PC protocol, significantly reduced functional recovery during reperfusion: in alprenolol treated PC hearts both the aortic output (Fig. 4.21A) and the total work performance (Fig. 4.21B), as well as the coronary flow, cardiac output and peak systolic pressure (Table 4.2) were significantly lower than those of untreated PC hearts, but significantly better than those of non-PC hearts. The latter indicates that alprenolol could only partially abolish the protective effect of PC.

##### **Effect of adrenaline on recovery potential of hearts treated with alprenolol:**

Although the aortic output and total work performance both alprenolol treated and untreated PC groups could be increased by adrenaline administration, the values remained lower in the alprenolol treated hearts (Fig. 4.21A, B). This was not due to a drug effect of alprenolol, as the response to adrenaline of control hearts (not subjected



**Fig 4.17**  $\beta$ -adrenergic response to 25 min sustained global ischaemia. Tissue cAMP accumulation was reduced in PC hearts compared to non-PC hearts.  $\beta$ -adrenergic receptor blocker, alprenolol ( $7.5 \times 10^{-5}$  M), administration to PC hearts before sustained ischaemia, partially abolished this reduction.



**Fig 4.18**  $\beta$ -adrenergic response to 25 min sustained global ischaemia. Isoproterenol pretreatment, for 1 x 5 min only at 10<sup>-8</sup>M did not cause a reduction in cAMP at the end of sustained ischaemia. On the other hand, one episode of 10<sup>-7</sup>M isoproterenol pretreatment reduced cAMP accumulation at the end of 25 min sustained ischaemia in a manner similar to ischaemic PC hearts.

to ischaemia) treated with alprenolol, was similar to that of untreated control hearts (Fig. 4.21A, B).

#### **4.2.3.2 Effect of repeated agonist-induced increases in cAMP prior to sustained ischaemia on functional recovery**

##### **4.2.3.2.1 Experimental protocol**

Hearts were stabilized by perfusing retrogradely for 15 min, followed by 15 min of working heart. In separate groups tissue cAMP was then elevated experimentally by:

- (i) 3 x 5 min global ischaemia, alternated by 5 min reperfusion (PC hearts) (Fig. 4.22B);
- (ii) 1 x 5 min isoproterenol ( $10^{-8}$  or  $10^{-7}$  M) followed by 5 min reperfusion (administration of isoproterenol was preceded by 20 min retrograde perfusion after initial stabilization) (Fig. 4.22C);
- (iii) 3 x 5 min isoproterenol ( $10^{-7}$  M), interspersed by 5 min reperfusion (Fig. 4.22D);
- (iv) 1 x 5 min forskolin ( $10^{-7}$ ,  $10^{-6}$  M) followed by 5 min reperfusion (administration of forskolin was preceded by 20 min retrograde perfusion after initial stabilization) (Fig. 4.22C);
- (v) 3 x 5 min forskolin ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M), interspersed by 5 min reperfusion (Fig. 4.22D).

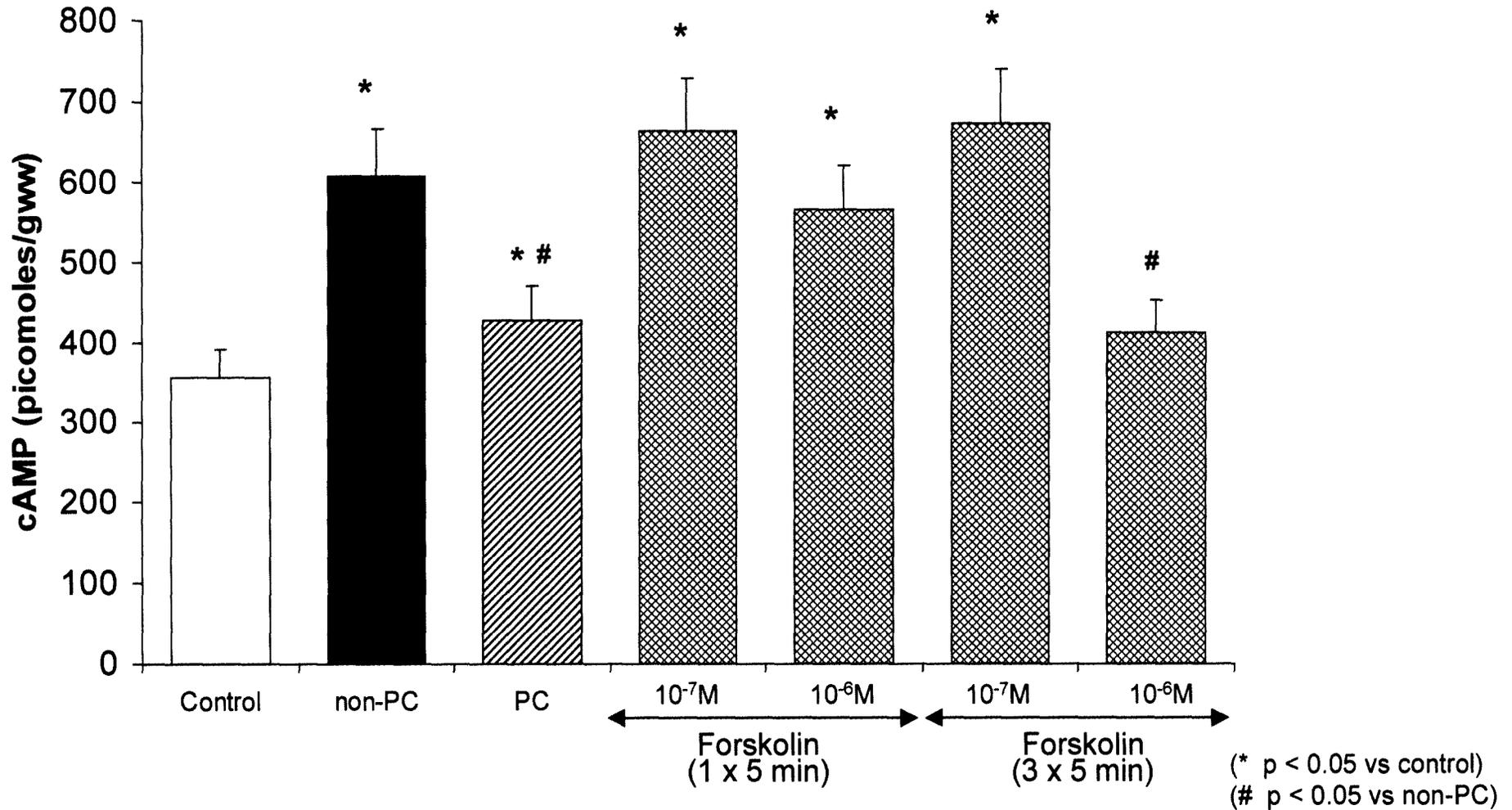
Non-PC hearts, which served as appropriate controls, were perfused retrogradely for 30 min after the initial stabilization period (Fig. 4.22A).

All hearts were then subjected to 25 min global ischaemia, followed by 30 min reperfusion (10 min retrograde, 20 min working heart) for evaluation of functional recovery (Fig. 4.22).

##### **4.2.3.2.2 Results**

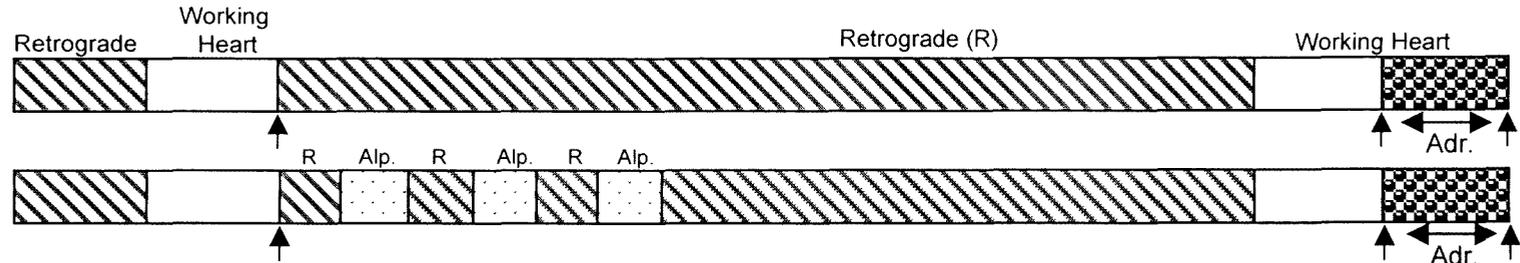
###### **Effect of isoproterenol on functional recovery**

At both concentrations ( $10^{-8}$  and  $10^{-7}$  M) studied, isoproterenol administered for 5 min only, caused a significant improvement in functional recovery during reperfusion. In these hearts aortic output (Fig. 4.23A), total work performance (Fig. 4.23B), as well as

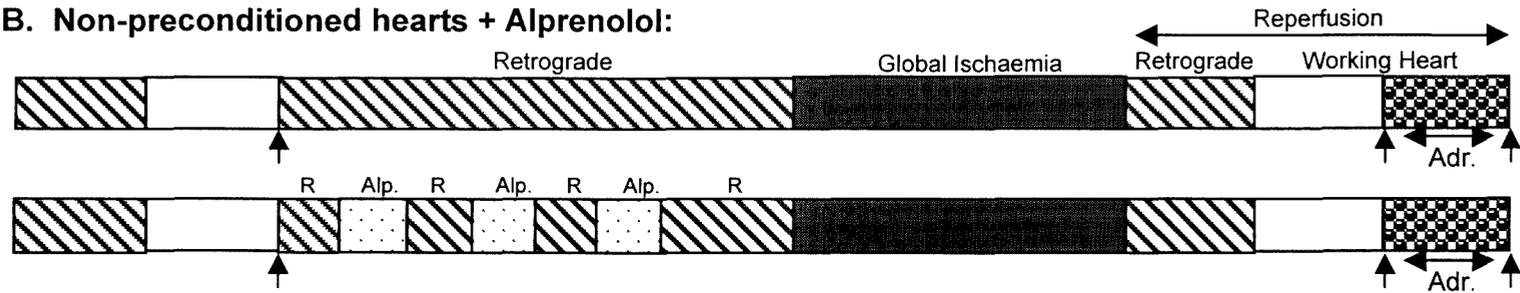


**Fig 4.19**  $\beta$ -adrenergic response to 25 min sustained global ischaemia. Forskolin pretreatment, for 1 x 5 min only at  $10^{-7}$ M or  $10^{-6}$ M, or for 3 x 5 min at  $10^{-7}$ M, did not cause a reduction in cAMP at the end of 25 min sustained ischaemia. However, repeated administration of forskolin ( $10^{-6}$ M for 3 x 5 min) reduced cAMP accumulation at the end of 25 min sustained ischaemia in a manner similar to ischaemic PC hearts.

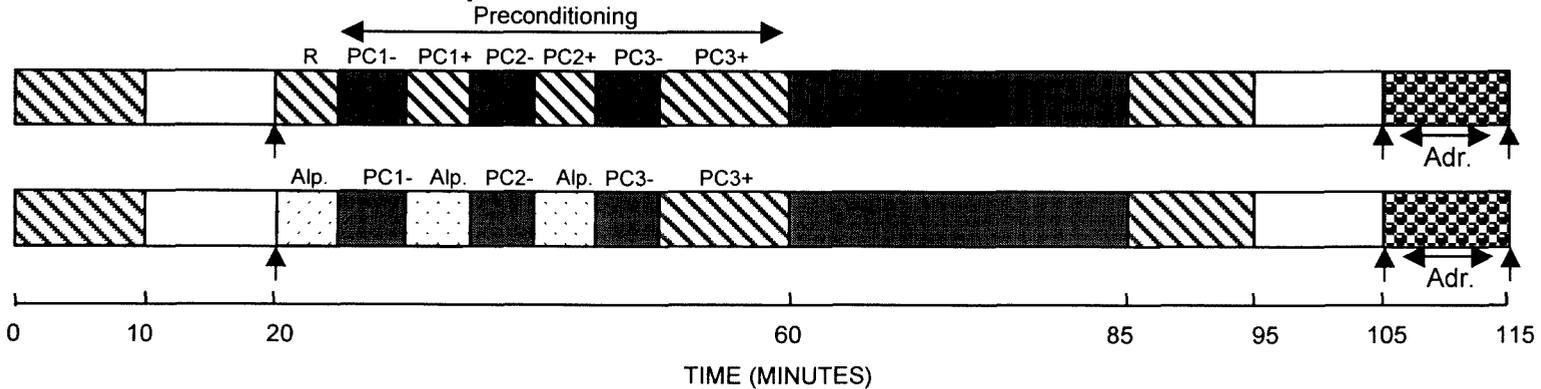
**A. Control hearts + Alprenolol:**



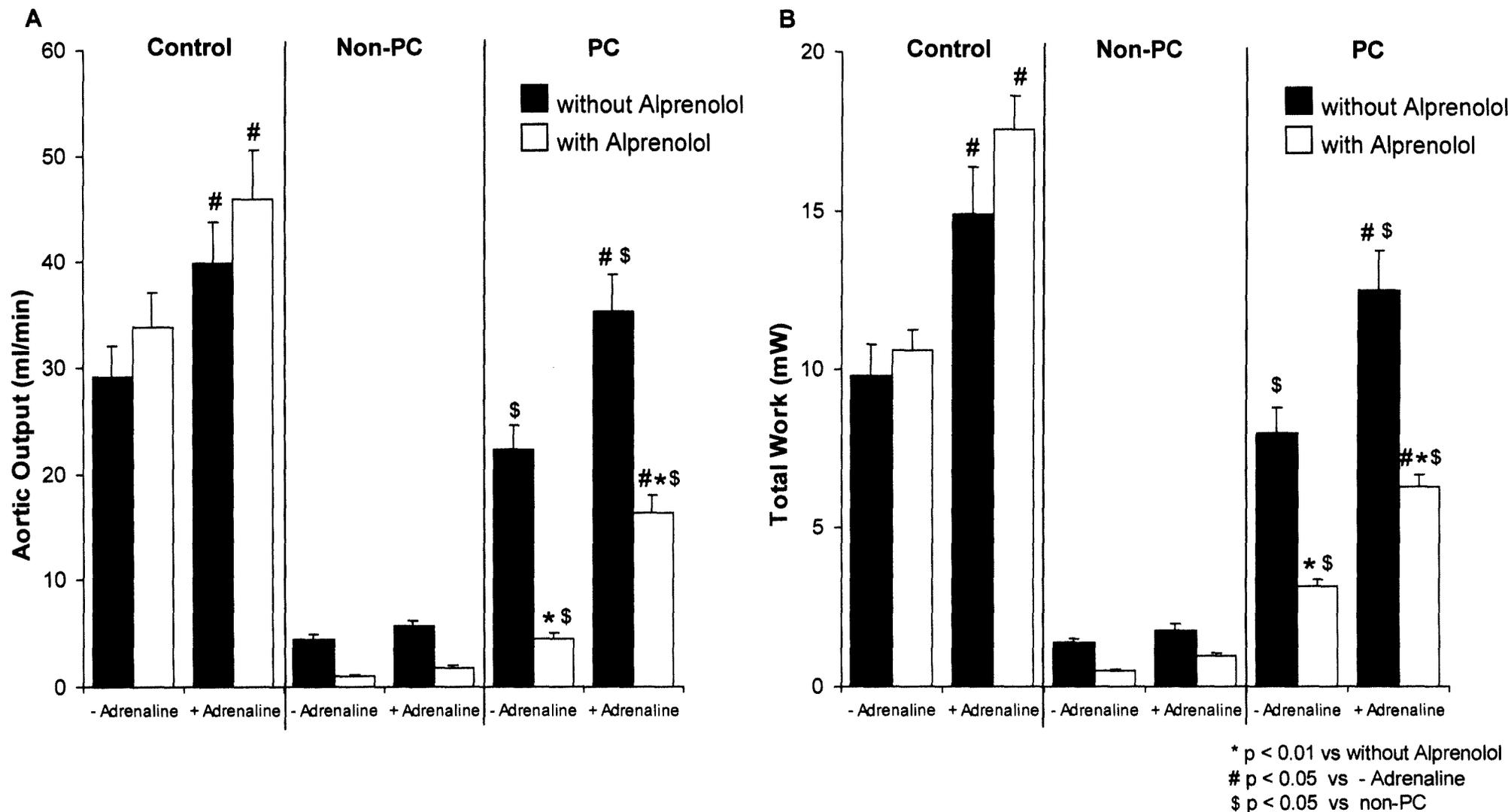
**B. Non-preconditioned hearts + Alprenolol:**



**C. Preconditioned hearts + Alprenolol:**



**Fig 4.20** Functional recovery during reperfusion after  $\beta$ -adrenergic receptor blockade with alprenolol ( $7.5 \times 10^{-5}$  M): Experimental protocol. Abbreviations as in fig 4.3. Control, non-PC and PC hearts were treated with alprenolol (Alp.) prior to 25 min of sustained global ischaemia. Adrenaline (Adr.)  $10^{-6}$  M was administered at the end of reperfusion (post-sustained ischaemia) of each experiment for a period of 10 min. Arrows indicate time of determination of mechanical function.  $n = 5$  hearts per series.



**Fig 4.21** Functional recovery after  $\beta$ -adrenergic receptor blockade with alprenolol ( $7.5 \times 10^{-5}$  M). In control hearts, alprenolol had no effect on aortic output nor on total work performance at 105 min perfusion time (- Adrenaline), these hearts respond to the same extent to Adrenaline  $10^{-6}$  M (+ Adrenaline, after 115 min). In non-PC hearts, both untreated and alprenolol treated hearts prior to sustained ischaemia, failed to recover significantly during reperfusion. Furthermore, adrenaline could not reverse these effects. In PC hearts, pretreatment with alprenolol during the PC protocol reduced both aortic output and total work performance during reperfusion (- Adrenaline). Although aortic output and total work performance in both alprenolol treated and untreated PC groups could be increased by adrenaline (+ Adrenaline), the values remained lower in the alprenolol treated hearts.

**Table 4.2****Effect of Adrenaline ( $10^{-6}$  M) on mechanical performance during reperfusion of hearts pretreated with Alprenolol.**

	Coronary flow (ml/min)	Cardiac output (ml/min)	Peak systolic Pressure (mm Hg)	Heart Rate (beats/min)
<u>Control:</u>				
- Before Arrest	15.1 ± 1.9	63.0 ± 0.9	110 ± 2.1	288 ± 15.7
<u>Control:</u>				
Without Alprenolol :				
- Adrenaline	13.8 ± 0.6	42.9 ± 3.2	101 ± 3.0	280 ± 21.0
+ Adrenaline	16.9 ± 1.0#	56.8 ± 5.3#	115 ± 6.4#	337 ± 6.6#
With Alprenolol :				
- Adrenaline	13.1 ± 1.1	47.0 ± 4.7	99.8 ± 3.0	283 ± 17.8
+ Adrenaline	19.5 ± 1.1#	65.5 ± 4.9#	117 ± 1.5#	317 ± 6.6#
<u>Non-Preconditioned:</u>				
Without Alprenolol :				
- Adrenaline	3.0 ± 2.9	7.5 ± 7.0	21.5 ± 20.0	72.0 ± 70
+ Adrenaline	4.3 ± 4.0	9.3 ± 9.0	21.9 ± 19.9	106 ± 99
With Alprenolol :				
- Adrenaline	0.0	0.0	0.0	0.0
+ Adrenaline	5.9 ± 2.1	6.6 ± 2.4	41.6 ± 6.6#	166 ± 11.8#
<u>Preconditioned:</u>				
Without Alprenolol :				
- Adrenaline	14.1 ± 0.9\$	36.5 ± 3.3\$	97.3 ± 2.2\$	247 ± 14.2\$
+ Adrenaline	17.7 ± 0.9#\$\$	53.1 ± 4.6#\$\$	104 ± 1.8#\$\$	361 ± 11.0#\$\$
With Alprenolol :				
- Adrenaline	11.0 ± 1.5*\$	15.8 ± 1.5*\$	79.8 ± 3.8*\$	254 ± 16\$
+ Adrenaline	12.6 ± 0.4*\$	29.0 ± 3.7*#\$\$	96.0 ± 2.0#\$\$	322 ± 9.0#\$\$

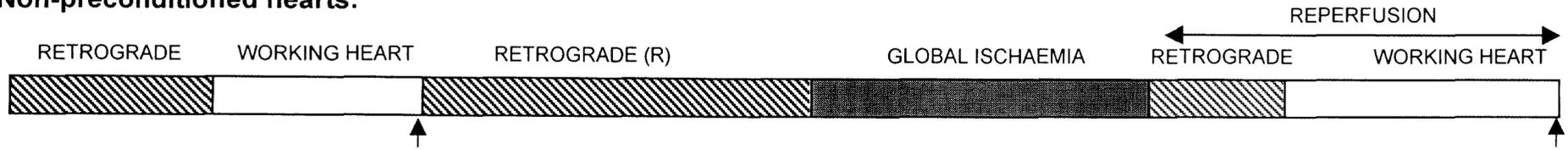
Results expressed as the mean values ± SE obtained after 20 min of reperfusion. Hearts were preconditioned or treated with the Alprenolol as shown in Fig. 4.20. Adrenaline was administered after 20 min of reperfusion and mechanical performance monitored after 10 min.

\* p < 0.05 vs without Alprenolol ( $7.5 \times 10^{-5}$  M)

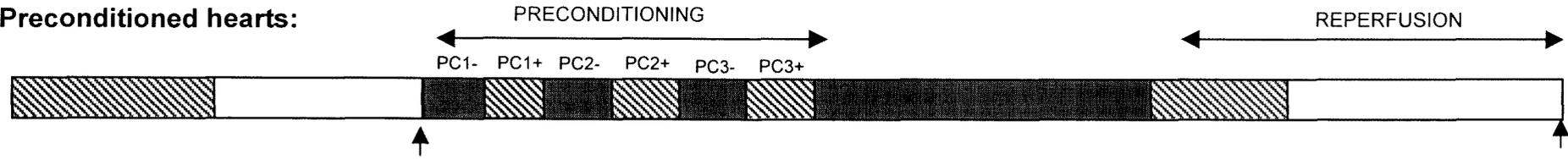
# p < 0.05 vs – Adrenaline ( $10^{-6}$  M)

\$ p < 0.05 vs non-PC

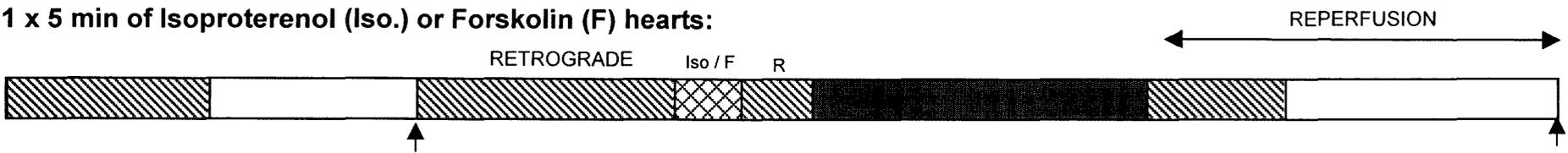
**A. Non-preconditioned hearts:**



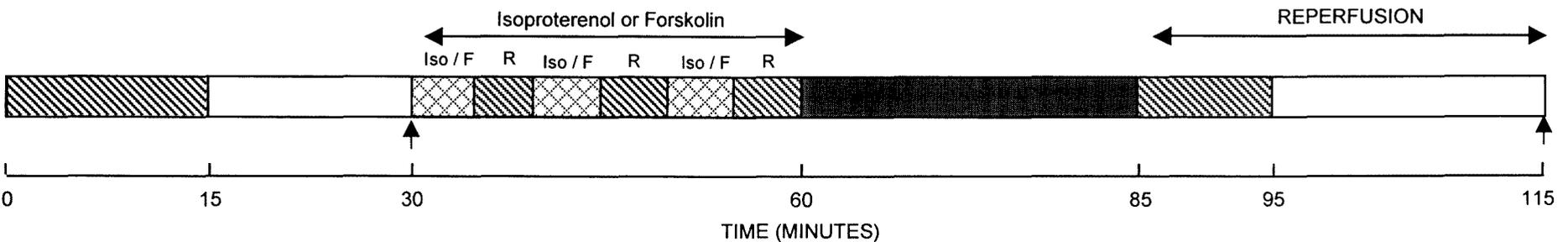
**B. Preconditioned hearts:**



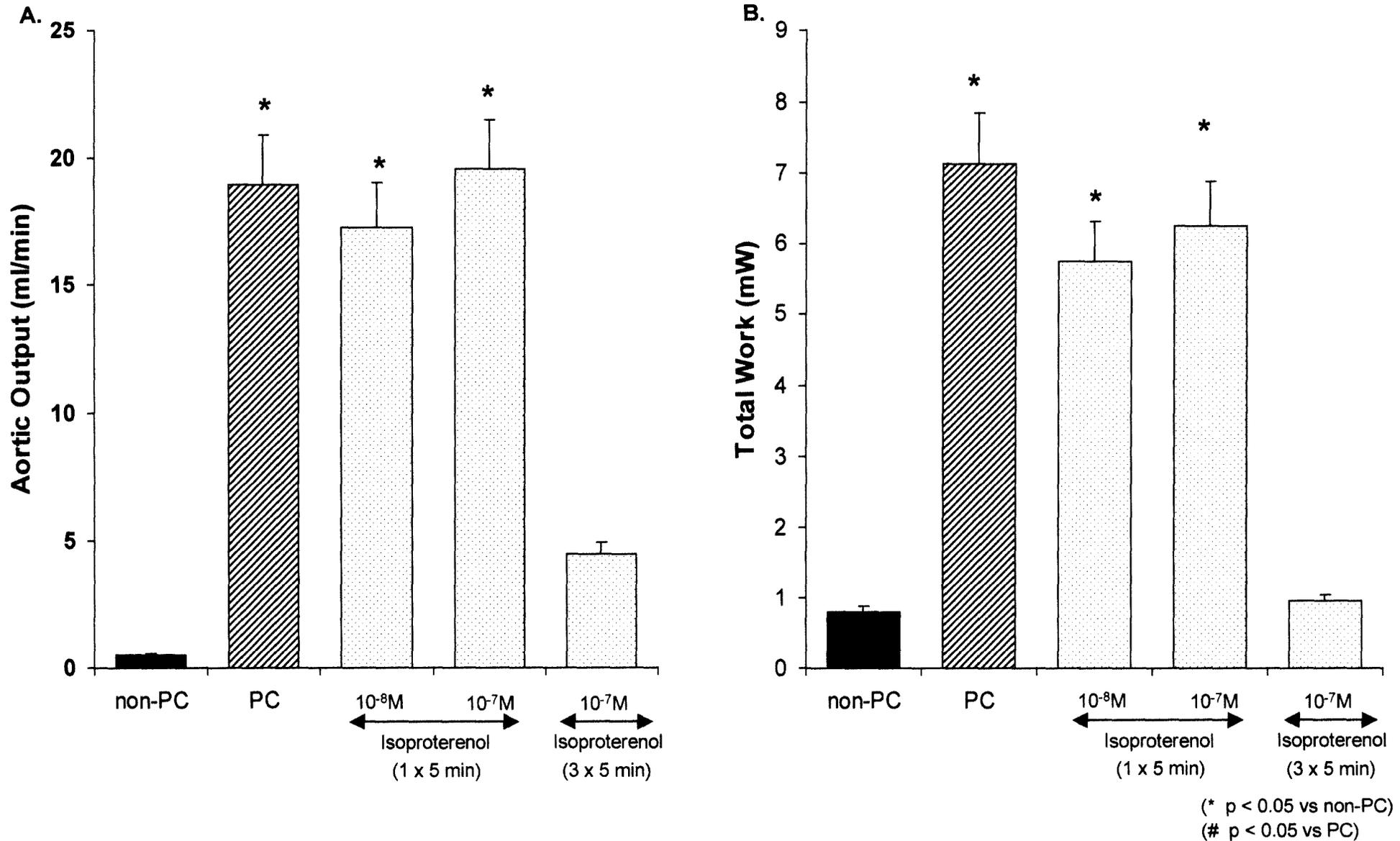
**C. 1 x 5 min of Isoproterenol (Iso.) or Forskolin (F) hearts:**



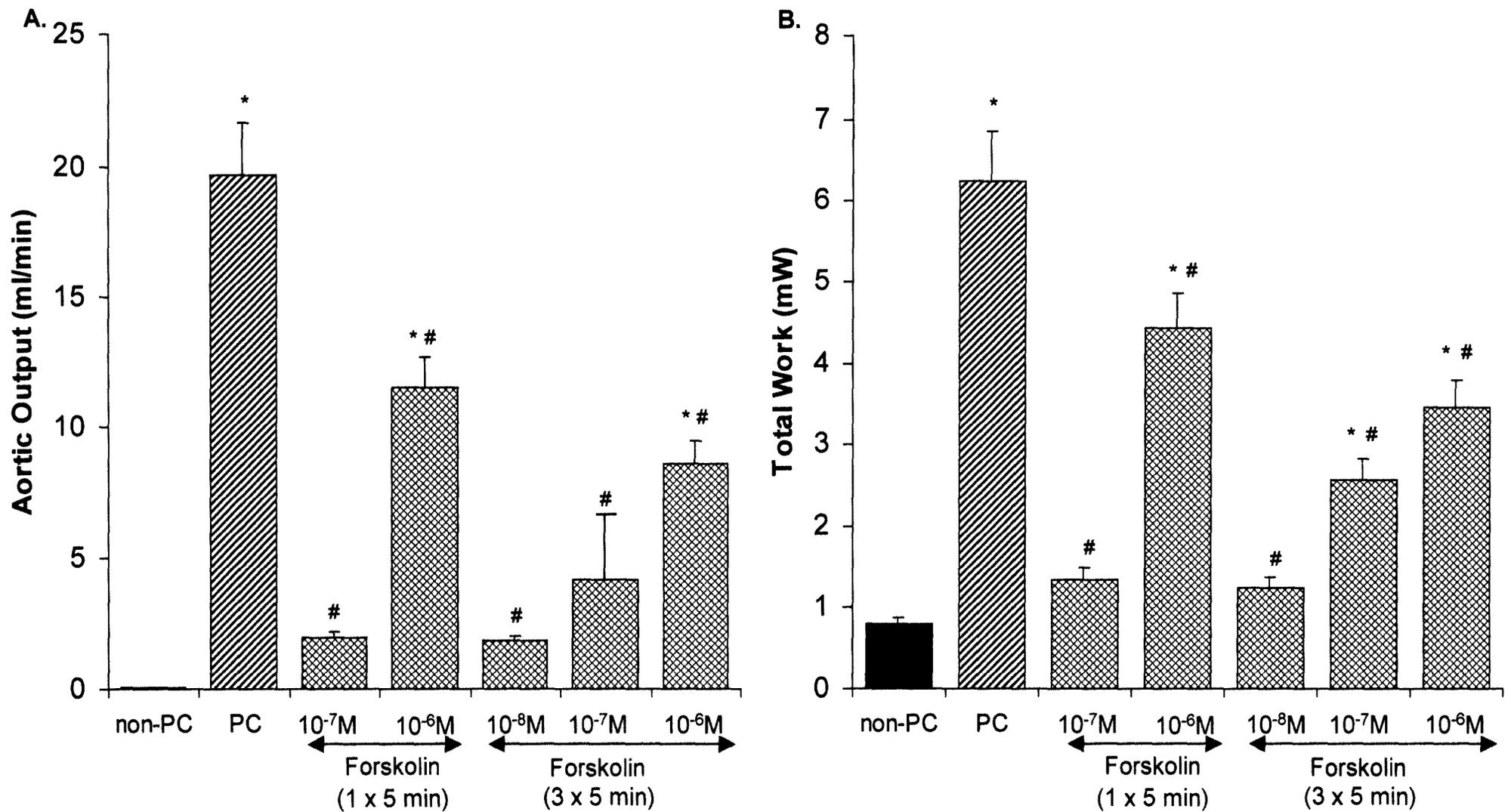
**D. 3 x 5 min of Isoproterenol (Iso.) or Forskolin (F) hearts:**



**Fig 4.22** Functional recovery after repeated pharmacological increases in cAMP as a trigger for eliciting preconditioning: Experimental protocol. Abbreviations as in fig 4.3.  $\beta$ -adrenergic stimulation for 1 x 5 min or 3 x 5 min with isoproterenol ( $10^{-8}$  or  $10^{-7}$  M) or forskolin ( $10^{-8}$ ,  $10^{-7}$  or  $10^{-6}$  M) replaced the ischaemic PC before 25 min global ischaemia followed by 30 min reperfusion. Arrows indicate time of determination of mechanical function. n = 6 to 8 hearts per series.



**Fig 4.23** Functional recovery after repeated increases in cAMP with isoproterenol. Both concentrations of isoproterenol (10<sup>-8</sup> and 10<sup>-7</sup> M) administered for 5 min only caused improved aortic output and total work performance during reperfusion, similar to PC hearts. However, repeated (3 x 5 min) isoproterenol (10<sup>-7</sup> M) administration caused complete mechanical failure.



(\* p < 0.05 vs non-PC)  
 (# p < 0.05 vs PC)

**Fig 4.24** Functional recovery after repeated increases in cAMP with forskolin. Only 10<sup>-6</sup> M forskolin, administered either once or three times, could improve aortic output and total work performance during reperfusion, although not to the same extent as PC hearts. On the other hand, both 10<sup>-8</sup> and 10<sup>-7</sup> M forskolin (1 x 5 min or 3 x 5 min) failed to improved functional recovery.

**Table 4.3**

**Effect of  $\beta$ -adrenergic stimulation with isoproterenol or forskolin on mechanical function during 30 min of reperfusion.**

	Coronary flow (ml/min)	Cardiac output (ml/min)	Peak systolic Pressure (mm Hg)	Heart Rate (beats/min)
Non-Preconditioned:	10.5 $\pm$ 1.0	10.5 $\pm$ 1.0	36.2 $\pm$ 13.4	122 $\pm$ 47.4
Preconditioned:	13.0 $\pm$ 0.6	32.7 $\pm$ 2.8*	88.6 $\pm$ 2.9*	235 $\pm$ 8.7*
1 x 5 min Isoproterenol:				
10 <sup>-7</sup> M	10.1 $\pm$ 0.9	29.7 $\pm$ 1.5*	94.2 $\pm$ 4.3*	202 $\pm$ 17.2*
10 <sup>-8</sup> M	10.6 $\pm$ 0.5	27.9 $\pm$ 2.5*	91.5 $\pm$ 1.4*	216 $\pm$ 9.8*
3 x 5 min Isoproterenol:				
10 <sup>-7</sup> M	4.3 $\pm$ 1.8*	8.8 $\pm$ 4.4	55.4 $\pm$ 14.3	160 $\pm$ 34.7
1 x 5 min Forskolin:				
10 <sup>-6</sup> M	10.6 $\pm$ 0.4	22.1 $\pm$ 3.0*	87.6 $\pm$ 1.4*	208 $\pm$ 12.7*
10 <sup>-7</sup> M	5.4 $\pm$ 2.0*	7.4 $\pm$ 3.4	47.2 $\pm$ 17.5	107 $\pm$ 39.5
3 x 5 min Forskolin:				
10 <sup>-6</sup> M	11.3 $\pm$ 0.9	19.9 $\pm$ 1.9*	77.0 $\pm$ 1.0*	225 $\pm$ 14.1*
10 <sup>-7</sup> M	9.8 $\pm$ 0.4	14.0 $\pm$ 4.3	70.8 $\pm$ 11.9	190 $\pm$ 12.6
10 <sup>-8</sup> M	5.3 $\pm$ 1.1*	7.1 $\pm$ 1.9	63.0 $\pm$ 11.2	208 $\pm$ 29.9

Results expressed as the mean values  $\pm$  SE obtained during 30 min reperfusion after 25 min sustained ischaemia. Hearts were stimulated with  $\beta$ -adrenergic stimulants before sustained ischaemia as shown in Fig. 4.22.

\* p < 0.05 vs non-PC

cardiac output, peak systolic pressure and heart rate (Table 4.3) improved significantly compared to non-PC hearts and a similar protection as in PC hearts was observed. On the other hand, isoproterenol ( $10^{-7}$  M) administration for 3 x 5 min, which resulted in no increases in cAMP after the first administration (Fig. 4.6), causing complete mechanical failure upon reperfusion (Fig. 4.23A, B and Table 4.3).

### **Effect of forskolin on functional recovery**

Forskolin ( $10^{-8}$  and  $10^{-7}$  M) administered either once or three times failed to improve functional recovery compared to non-PC hearts (Fig. 4.24A, B and Table 4.3). However, similar treatment with  $10^{-6}$  M forskolin, resulted in cyclic increases in cAMP (Fig. 4.6) before sustained ischaemia, followed by a significant improved functional recovery during reperfusion. Although forskolin ( $10^{-6}$  M) significantly increased aortic output (Fig. 4.24A), total work performance (Fig. 4.24B), cardiac output, peak systolic pressure and heart rate (Table 4.3) compared to non-PC hearts, the protection was less than that of PC hearts (Fig. 4.24A, B and Table 4.3).

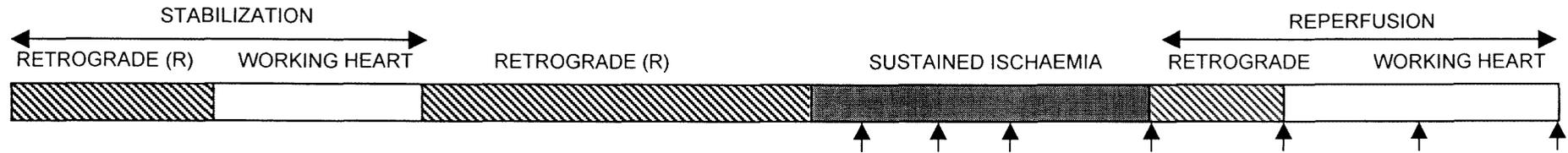
#### **4.2.3.3 p38 MAPK activation during sustained ischaemia and reperfusion of $\beta$ -adrenergic receptor manipulated hearts**

To establish the role of p38 MAPK in  $\beta$ -adrenergic induced protection, it was necessary to compare changes in p38 MAPK activation of  $\beta$ -adrenergic- and ischaemic-preconditioned hearts during sustained ischaemia and reperfusion.

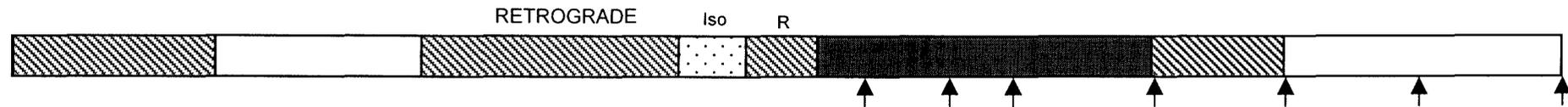
##### **4.2.3.3.1 Experimental protocol**

In this series, non-preconditioned and isoproterenol-treated hearts were freeze-clamped at 5, 10, 15 and 25 min global ischaemia and at 10, 20 and 30 min of reperfusion (Fig. 4.25). Ischaemic and alprenolol-treated preconditioned hearts were freeze-clamped at the end of 25 min global ischaemia, as well as 10, 20 and 30 min of reperfusion (Fig. 4.25). At least four hearts were studied at each time point. Dual phosphorylation of p38 MAPK was assayed at the above time intervals, while ATF-2 phosphorylation was studied at selected times.

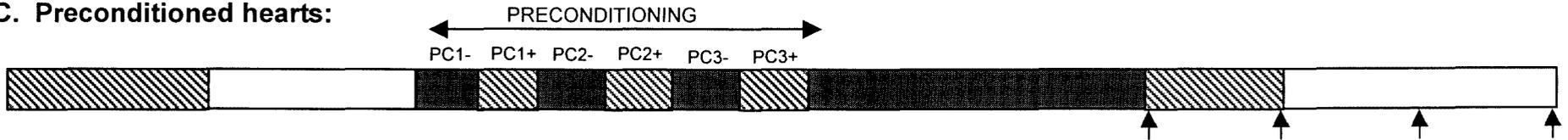
**A. Non-preconditioned hearts:**



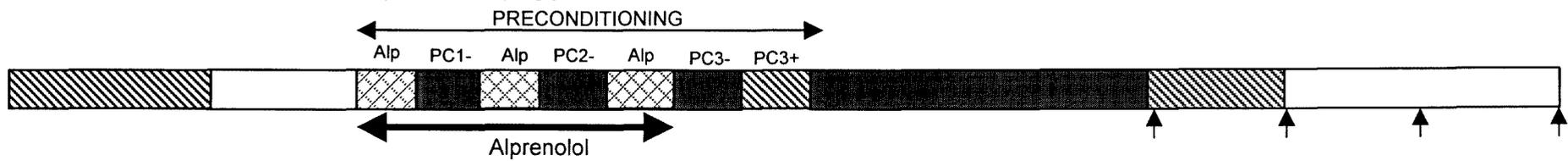
**B. Isoproterenol (Iso.) hearts:**



**C. Preconditioned hearts:**



**D. Preconditioned hearts + Alprenolol (Alp):**



0 15 30 60 85 95 115  
TIME (MINUTES)

**Fig 4.25** Effect of  $\beta$ -adrenergic manipulation (during the PC protocol) on p38 MAPK activation during sustained ischaemia and reperfusion: Experimental protocol. Manipulations before 25 min global ischaemia and 30 min reperfusion include either  $\beta$ -adrenergic stimulation with isoproterenol ( $10^{-7}$  M,  $1 \times 5$  min) replacing ischaemic PC or  $\beta$ -adrenergic blockade with alprenolol ( $7.5 \times 10^{-5}$  M) during the PC protocol. Abbreviations as in fig 4.3. Arrows indicate time of freeze-clamping for determination of dual phosphorylation of p38 MAPK, while ATF-2 phosphorylation was studied at selected times.  $n = 4$  to 6 hearts per series.

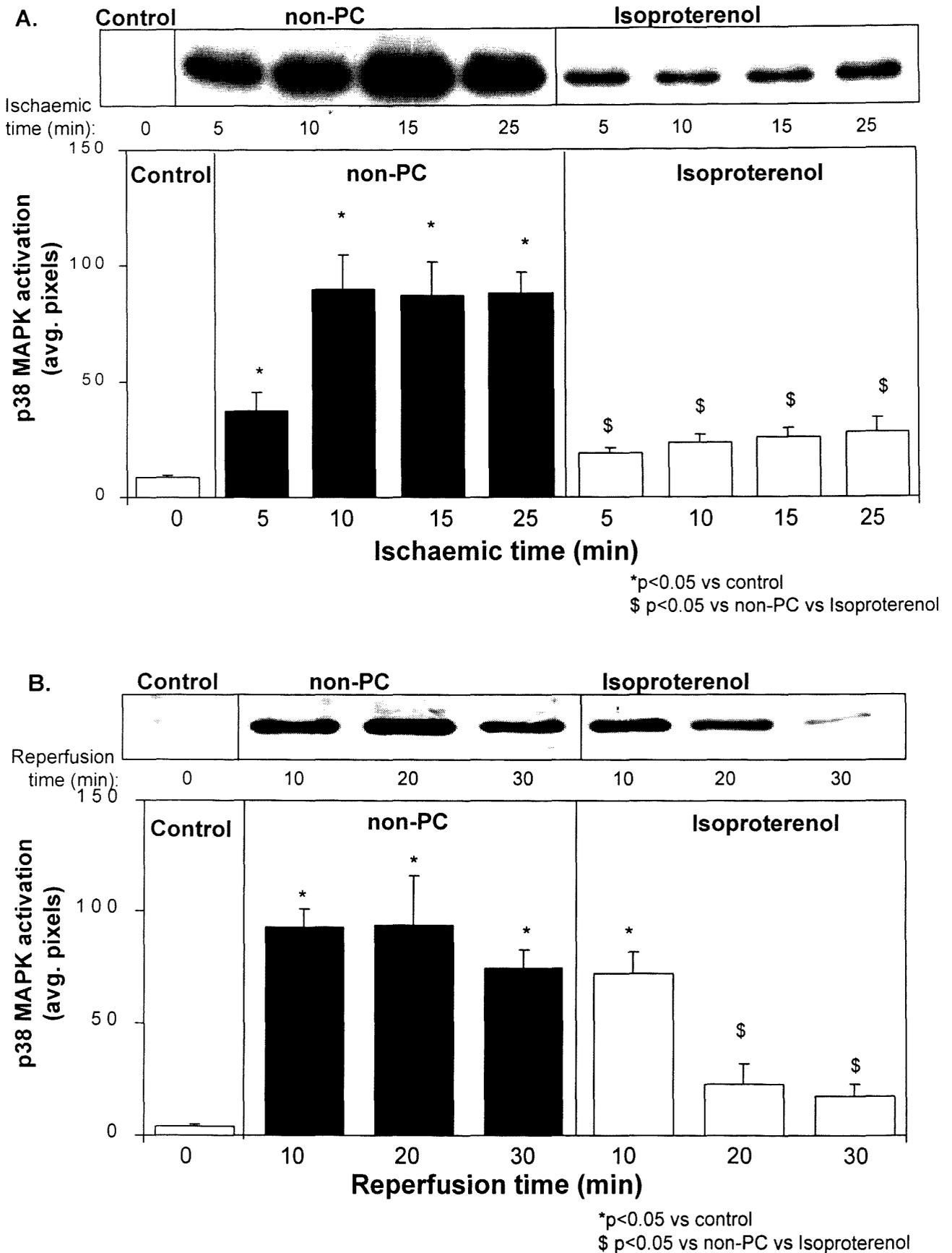
#### 4.2.3.3.2 Results

Dual phosphorylation of p38 MAPK by transient administration of isoproterenol (1 x 5 min,  $10^{-7}$  M) before the onset of sustained ischaemia, was associated with a significant reduction (although still higher than controls) in the activation of this stress kinase at all time intervals studied (5, 10, 15 and 25 min) during sustained ischaemia, when compared to the significant increases observed in untreated, non-PC hearts (Fig. 4.26A). These significant differences were due to the treatment and not to time (as determined by two way ANOVA).

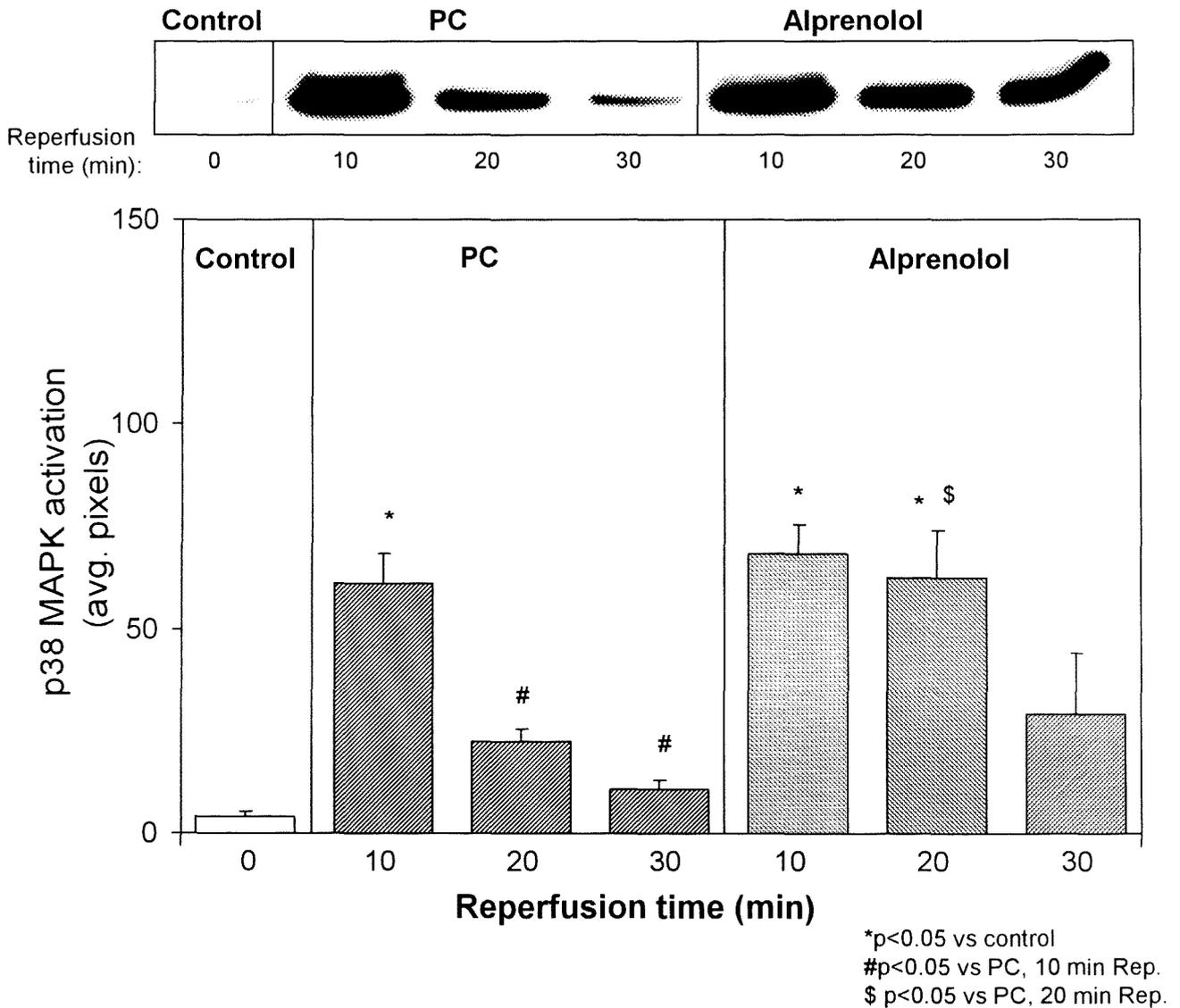
Furthermore, during reperfusion of non-PC hearts, p38 MAPK remained significantly elevated during the entire period, while that of the isoproterenol-treated hearts decreased over time, becoming significantly lower at 20 and 30 min of reperfusion when compared with 10 min. Therefore at 20 and 30 min reperfusion of isoproterenol-treated hearts p38 MAPK activation was also significantly attenuated compared to non-PC hearts (Fig. 4.26B).

Results obtained with isoproterenol-treated hearts were similar to those subjected to a 3 x 5 min ischaemic PC protocol: p38 MAPK activation was significantly attenuated at all times of global ischaemia studied (described in Fig. 3.16, only 25 min shown in Fig. 4.28), while its activation declined over time during reperfusion (Fig. 4.27). On the other hand, inhibition of p38 MAPK activation by alprenolol ( $7.5 \times 10^{-5}$  M) during the ischaemic PC protocol, led to a significant elevation in p38 MAPK activation at the end of 25 min global ischaemia, which remained elevated and unchanged throughout reperfusion, when compared to values obtained in preconditioned hearts (Fig. 4.27).

Activation of p38 MAPK as indicated by its kinase activity (ATF-2 phosphorylation) was evaluated at selected time intervals (25 min ischaemia and 20 min reperfusion) and compared with its dual phosphorylation. Similar results were obtained: attenuated activation (ATF-2) and dual phosphorylation (p38 MAPK) in isoproterenol-treated hearts, when compared to non-preconditioned hearts (Fig. 4.28A). On the other hand, alprenolol administration during the ischaemic PC protocol reversed its effects on attenuation of p38 MAPK activation (Fig. 4.28B).

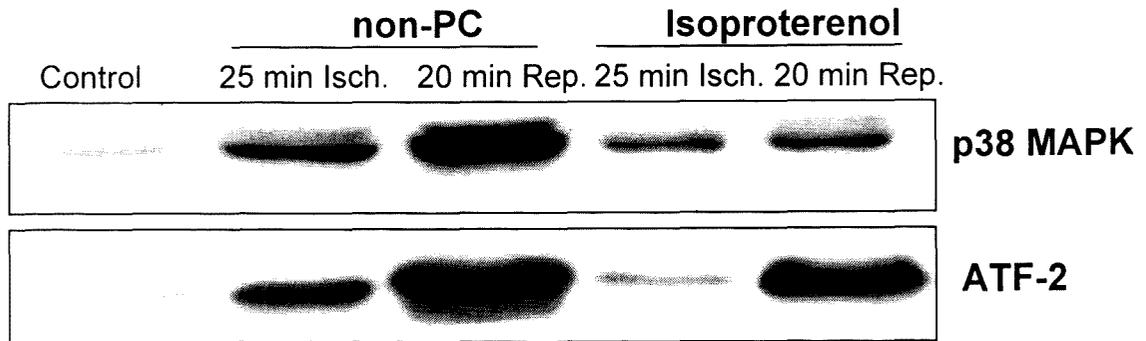


**Fig 4.26** Effect of  $\beta$ -adrenergic preconditioning with isoproterenol ( $10^{-7}$  M, 1 x 5 min) on p38 MAPK phosphorylation during sustained ischaemia (**A**) and reperfusion (**B**): comparison with non-PC. A representative blot is given for each series (n = 4 hearts / series). Two-way analysis of variance of p38 MAPK activation showed significant interaction between time and treatment; p38 MAPK activation is significantly attenuated during sustained ischaemia and reperfusion in isoproterenol-treated hearts.

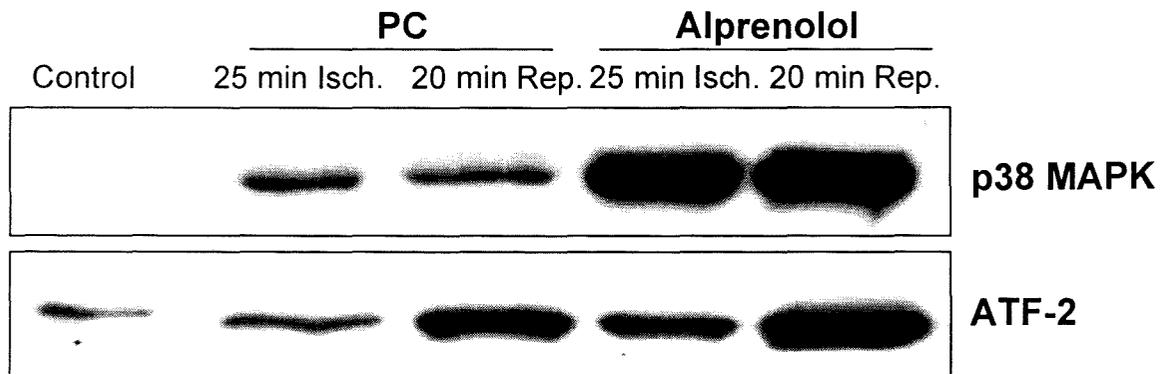


**Fig 4.27** Effect of  $\beta$ -adrenergic blockade with alprenolol ( $7.5 \times 10^{-5}$  M), administered during the ischaemic PC protocol) on p38 MAPK phosphorylation after 25 min sustained ischaemia and during reperfusion: comparison with ischaemic PC. A representative blot is given for each series. ( $n = 4$  hearts/series). Two-way analysis of variance of p38 MAPK activation showed significant interaction between time and treatment; Alprenolol administered during the ischaemic PC protocol, reversed its effects on attenuation of p38 MAPK activation.

**A**



**B**



**Fig 4.28** Comparison of dual phosphorylation and kinase activity of p38 MAPK in non-PC, isoproterenol-PC (**A**), ischaemic-PC and alprenolol-treated hearts (**B**) after 25 min sustained ischaemia and 20 min reperfusion. A representative blot is given for each series. (n = 4 hearts/series).

## 4.3 Discussion

Previously we have shown that cyclic increases in tissue cAMP occurred during a multi-cycle PC protocol (in chapter 3), which was accompanied by reduced cAMP generation by the preconditioned myocardium when subsequently exposed to sustained ischaemia (Moolman *et al.*, 1996). A role for  $\beta$ -adrenergic receptor stimulation and activation of the  $\beta$ -adrenergic signalling pathway as a trigger in PC has previously been suggested by Asimakis *et al.* (1994), who showed that 5 min of noradrenaline or isoprenaline administration followed by 5 min washout before 30 min global ischaemia mimicked the protection of PC and this could be prevented by  $\beta$ -adrenergic blockade. In this particular study no correlation was made between changes in tissue cAMP levels and functional recovery. It was suggested that the increased workload produced by these drugs might have resulted in a degree of demand ischaemia that was sufficient to produce ischaemic PC. A major issue therefore was to establish whether the changes, observed in the present study, *per se* elicit protection against subsequent ischaemic damage or whether they are mere epiphenomena or the result of PC protection initiated by another mechanism.

### 4.3.1 Prevention of cAMP generation during the PC protocol

One approach would be to establish whether abolishment of the cAMP fluctuations by  $\beta$ -adrenergic blockade during the PC protocol could prevent the beneficial effects of PC. To accomplish this, the drug to be used should be administered before the first PC episode (PC1-) and washed out before the onset of sustained ischaemia. The latter is particularly important since the presence of  $\beta$ -adrenergic blockers before and during the onset of sustained ischaemia could protect the rat heart against ischaemic damage (Edoute *et al.*, 1981) and elicit protection in a manner similar to PC (Moolman *et al.*, 1996). Propranolol was found to be unsuitable to test our hypothesis, due to its lipophilic properties: it could not be washed out within 10 minutes and such hearts showed a much-depressed response to adrenaline stimulation (Fig. 4.1).

Although some workers failed to demonstrate a lowering in tissue cAMP in ischaemia by  $\beta$ -receptor blockade (Muller *et al.*, 1986), we found that the non-specific  $\beta_1/\beta_2$  blocker, alprenolol, at a concentration of  $7.5 \times 10^{-5}$  M was suitable for our purpose: it

significantly attenuated the cyclic increases in cAMP during the PC protocol (Fig. 4.4) and could be washed out within 10 minutes, as was shown by the response of the control perfused hearts to adrenaline stimulation (Fig. 4.2).  $\beta$ -adrenergic receptor blockade during the PC protocol caused a significant reduction in functional recovery during reperfusion (Fig. 4.21) and although adrenaline could improve their performance, the values obtained were still significantly lower than those of the untreated PC hearts. These results emphasize the significance of activation of the  $\beta$ -adrenergic signal transduction pathway during the PC protocol in eliciting protection during sustained ischaemia.

#### 4.3.2 Simulation of ischaemia-induced cAMP increases

The reverse approach would be to establish whether repeated stimulation of cAMP generation before the onset of sustained ischaemia could elicit protection. Our data (Fig. 4.5), as well as work by Krause and Szekeres (1995), indicate a strong correlation between dose-dependent  $\beta$ -adrenergic increase in contractility and relaxation on one hand and the concomitant increase of myocardial cAMP levels on the other hand. These increases in cAMP were not due to demand ischaemia caused by administration of either forskolin or isoproterenol, because the tissue high energy phosphates levels (ATP and CP in Table 4.1) were not decreased by this intervention.

Furthermore, our study shows that, depending on the protocol and concentration, activation of the  $\beta$ -adrenergic signalling pathway by either isoproterenol (Fig. 4.23), or forskolin (Fig. 4.24) significantly improves functional recovery during reperfusion after 25 min global ischaemia, when compared to non-PC hearts.

A definite correlation between elevation in tissue cAMP levels prior to sustained ischaemia and subsequent protection, was not observed. Although isoproterenol ( $10^{-8}$  M), capable of generating cAMP levels comparable to those observed during a PC protocol ( $428 \pm 26$  vs.  $403 \pm 13$  pmoles/g wet weight respectively), elicited protection similar to that induced by PC (Fig. 4.23), further elevation of cAMP levels to  $1036 \pm 55$  pmoles/g wet weight by  $10^{-7}$  M isoproterenol (Fig. 4.5), prior to the onset of sustained ischaemia, did not further enhance recovery (Fig. 4.23).

On the other hand, forskolin, a direct activator of adenylyl cyclase, elicited protection in a dose-dependent manner: at  $10^{-6}$  M the drug was most effective, regardless of the frequency of administration, while at  $10^{-7}$  M the work performance did not increase significantly (Fig. 4.24). However, prior to sustained ischaemia forskolin ( $10^{-7}$  M) induced elevation of cAMP to levels comparable to those induced by PC1- ( $539 \pm 26$  vs.  $403 \pm 13$  pmoles/g wet weight, respectively) that had little effect on functional recovery and only at a concentration of  $10^{-6}$  M and cAMP levels of higher than 1000 pmoles/g wet weight did forskolin elicit protection, although significantly less than in PC hearts (Fig. 4.24).

Of interest is the observation that elevation in tissue cAMP to comparable levels by isoproterenol ( $10^{-7}$  M) and forskolin ( $10^{-6}$  M) (Fig. 4.5), did not lead to a similar degree of protection against ischaemic damage: aortic output of  $19.6 \pm 2.4$  ml/min vs.  $11.5 \pm 1.4$  ml/min, respectively (Figs. 4.23A and 4.24A). Additionally, a single isoproterenol administration proved to be significantly more protective than repeated administration of forskolin: aortic output of  $10^{-8}$  M:  $17.3 \pm 2.3$  vs.  $1.85 \pm 1.0$  ml/min and of  $10^{-7}$  M:  $19.6 \pm 2.4$  vs.  $4.17 \pm 4.0$  ml/min, respectively (Figs. 4.23A and 4.24A). A possible explanation for this phenomenon may be the fact that forskolin increases cAMP in a compartmentalized manner, so that AMP levels *per se* are not necessarily indicative of its effectiveness (Worthington *et al.*, 1992). Furthermore cAMP levels during repeated forskolin stimulation did not return to control levels, while only one episode of isoproterenol increased cAMP levels followed by desensitization of the response to further stimulation (Fig. 4.6). A further difference between the drugs is the fact that in contrast to forskolin, repeated stimulation with isoproterenol led to complete mechanical failure during reperfusion after sustained ischaemia (Fig. 4.23). This may be due to  $Ca^{2+}$  overloading, which is a well-established cause of damage (Opie 1991).

We therefore decided to use the 1 x 5 min ( $10^{-7}$  M) isoproterenol protocol to mimic the 3 x 5 min ischaemic PC protection (Fig. 4.23), because the 3 x 5 min isoproterenol could not mimic the cyclic changes in cAMP (Fig. 4.6) and resulted in complete mechanical failure (Fig. 4.23).

### 4.3.3 Mechanism of protection: cAMP related?

It remains to be established how transient stimulation of the  $\beta$ -adrenergic signalling pathway and cAMP generation lead to protection against ischaemic damage. Although cAMP is washed out during each reperfusion phase, activation of the downstream PKA occurs (as described in chapter 3), which in turn, may have further longer lasting downstream effects. The results obtained in this study suggest that PC-induced protection may result from down-regulation by the PC protocol of the  $\beta$ -adrenergic receptor responsiveness to subsequent ischaemia.

The mechanism of protection elicited by  $\beta$ -adrenergic agonists is complex. With ischaemic preconditioned or isoproterenol ( $10^{-7}$  M, 1 x 5 min) or forskolin ( $10^{-6}$  M, 3 x 5 min) treated hearts, the following pattern emerged: (i) a significant desensitization of the  $\beta$ -adrenergic receptor, as evidenced by the significant reduction in cAMP generation upon 2 min of isoproterenol administration (Figs. 4.8 and 4.9) and (ii) a significant reduction in cAMP content after 25 min sustained ischaemia (Figs. 4.18 and 4.19). Desensitization of hearts exposed to a short episode of ischaemia (Ungerer *et al.*, 1996) or isoproterenol (Mayor *et al.*, 1998) could probably be attributed to activation of both the  $\beta$ -adrenergic receptor kinase and PKA, whereas in the case of forskolin probably PKA only was involved. However, the mechanism of desensitization in PC hearts remains to be elucidated.

The above suggestion was also substantiated by the observation that in the case of forskolin ( $10^{-7}$  M; 1 x 5 min and 3 x 5 min) where no significant improvement in aortic output or work performance during reperfusion occurred (Fig. 4.24), no  $\beta$ -adrenergic receptor desensitization (Fig. 4.9) nor reduced cAMP during sustained ischaemia was observed (Fig. 4.19).

However, isoproterenol ( $10^{-8}$  M) or forskolin ( $10^{-6}$  M) when administered for 1 x 5 min did cause functional protection during reperfusion (Figs. 4.23 and 4.24), which was not associated with desensitization of the  $\beta$ -receptor (Figs. 4.8 and 4.9) or a reduction in cAMP levels at the end of sustained ischaemia (Figs. 4.18 and 4.19). It is possible that one 5 min episode was too short (in the case of forskolin) or the concentration of the agonist was too low (in the case of  $10^{-8}$  M isoproterenol) to elicit desensitization of the receptor and that the protection observed in these hearts may be due to the

characteristic increases in heart rate produced by both these agonists. It has recently been shown that increases in heart rate by rapid pacing can protect isolated working hearts against ischaemia, via production of nitric oxide (Ferdinandy *et al.*, 1996, Ferdinandy *et al.*, 1997). This possibility is supported by previous findings that transient stimulation with  $\beta$ -adrenergic agonists, which increased the heart rate, could mimic ischaemic PC (Asimakis *et al.*, 1994). These investigators suggested that the increased workload produced by these drugs might have resulted in a degree of demand ischaemia that was sufficient to produce ischaemic PC. Moreover, repeated  $\beta$ -adrenergic stress (induced by 5 intravenous administrations of isoproterenol, repeated at 10 min intervals in conscious rabbits) has also been shown to increase cardiac workload and induce a long-term cardiac adaptation manifested by a significant reduction of harmful ischaemic changes due to cardiac stress 24 and 48 h after PC (Kovanecz *et al.*, 1996).

It is thus possible that intermittent increases in heart rate, which was observed in all the protocols using isoproterenol or forskolin in our study, could contribute to the results obtained. It is unlikely though that the increased workload produced by either forskolin or isoproterenol caused a degree of demand ischaemia sufficient to produce ischaemic PC, since the tissue high energy phosphates were unchanged during repeated administration of high concentrations of these drugs (Table 4.1). In addition, even increases in heart rate by rapid pacing was associated with the release of catecholamines and activation of the adenylyl cyclase/cAMP pathway (Krause and Szekeres 1995, Parratt and Szekeres 1996).

Therefore, in view of these discrepancies, we suggest the following possible mechanisms: (i) the activation of PKA observed in all agonist treated hearts (results not shown) may act via desensitization of the  $\beta$ -adrenergic receptor and/or phosphorylation of a protective protein, (ii) agonist-induced increases in heart rate may protect against ischaemia via NO production. It is possible that both factors play a role in the protection observed.

#### 4.3.4 PKA and PKC

The results obtained provide proof that the protection elicited by ischaemic PC, may be partially dependent on activation of the  $\beta$ -adrenergic signalling pathway, implicating a role for PKA in downstream events.

Our observation that blockade by alprenolol could only partially, although effectively, abolish the protective effect of ischaemic PC, comes as no surprise in the view of the convincing evidence for involvement of other G-protein coupled receptors, acting via PKC activation (for review see Baxter 1997). In particular administration of  $\alpha_1$ -agonists before sustained ischaemia elicit PC (Bankwala *et al.*, 1994, Banerjee *et al.*, 1993, Tosaki *et al.*, 1995) while  $\alpha_1$ -adrenoceptor antagonists (Thornton *et al.*, 1993, Banerjee *et al.*, 1993) and PKC blockers (Ytrehus *et al.*, 1994, Armstrong *et al.*, 1994) have been shown to abolish PC. Although investigators like Bugge and Ytrehus (1995a) indicated that ischaemic PC was not mediated through  $\alpha$ -adrenergic or adenosine receptors, it was still dependent on the activation of PKC in isolated rat hearts. In addition, Yabe *et al.* (1998) observed that pharmacological PC induced by  $\beta$ -adrenergic stimulation (2 min of isoproterenol) is also mediated by activation of PKC in isolated rat hearts.

In spite of the fact that PKC activation has long been advocated as the main signal transduction pathway in PC, our laboratory could not find any evidence of either  $\alpha_1$ -adrenergic or PKC activation in the mechanism of ischaemic PC using the working perfused heart as model (Moolman *et al.*, 1996). Similar negative findings were reported by others (Kolocassides *et al.*, 1994, Asimakis *et al.*, 1993, Haessler *et al.*, 1996) and PKC as a major role player has been questioned (Brooks *et al.*, 1996, Simkhovich *et al.*, 1998).

Subsequent studies from our laboratory (Lochner *et al.*, submitted for publication) showed that the mode of perfusion, the PC protocol and the endpoint used (infarct size vs. functional recovery) may affect the outcome of PC and may be potential reasons for several of the current controversies. For example, the significance of the PC protocol has recently been emphasized by Sandhu *et al.* (1997), who demonstrated that a 3-cycle PC protocol provided more effective protection than one cycle and was less susceptible to specific inhibitors. However, the significance of  $\beta$ -adrenergic stimulation as a trigger of protection is underscored by the fact that  $\beta$ -adrenergic blockade could

abolish a 3-cycle induced protection (Fig. 4.21). Therefore failure of  $\alpha_1$ -adrenergic or PKC blockade to abolish protection in our particular model (isolated perfused rat heart) may be due to the use of a multi-cycle protocol and functional recovery as endpoint (Moolman *et al.*, 1996). In addition, most studies confirming the involvement of either  $\alpha_1$ -adrenergic system or PKC made use of infarct size, which may be a more tractable end-point (Lochner *et al.*, submitted for publication).

#### **4.3.5 Further downstream events of $\beta$ -adrenergic stimulation and p38 MAPK activation**

The results obtained in this study confirm that transient  $\beta$ -adrenergic stimulation (with  $10^{-7}$  M isoproterenol) of the isolated rat heart mimics the cardioprotective effect of our three-cycle ischaemic PC protocol (Fig. 4.23). During both  $\beta$ -adrenergic stimulation and ischaemic PC protocols generation of cAMP (Figs. 4.6 and Fig. 3.2, respectively) and transient activation of p38 MAPK occur (Figs. 3.12 and 4.11, respectively), which were associated with lowering of tissue cAMP (Figs. 4.19 and 3.13, respectively) and attenuation of p38 MAPK activity during sustained ischaemia and reperfusion (Figs. 3.16, 3.21 and 4.26A, B) as well as improvement in functional recovery (Fig. 4.23). These observations suggest that activation of p38 MAPK during the PC protocol may act as trigger, while attenuation of its activation during sustained ischaemia and reperfusion is associated with cardioprotection. The significance of activation of the  $\beta$ -adrenergic signalling pathway during ischaemic PC is emphasized by the observation that alprenolol-induced blockade of the receptor and thus attenuation of cAMP elevations (Fig. 4.4) and inhibition of p38 MAPK activation (Fig. 4.13) during the PC protocol only, results in increased tissue cAMP (Fig. 4.17) and activation of p38 MAPK (Fig. 4.27) during both sustained ischaemia and reperfusion with concurrent mechanical failure (Fig. 4.21), similar to the pattern observed in non-PC hearts.

As discussed in the previous chapter, it is still unsure whether p38 MAPK activation is indeed associated with cardioprotection. Increased activation of p38 MAPK signalling during sustained ischaemia in preconditioned hearts (Weinbrenner *et al.*, 1997, Armstrong *et al.*, 1999, Nakano *et al.*, 2000a) has been shown to be associated with cardioprotection, on the other hand activation of this kinase during sustained ischaemia/reperfusion has, in several studies, been associated with myocyte

necrosis/apoptosis (Bogoyevitch *et al.*, 1996, Ma *et al.*, 1999, Mackay *et al.*, 1999, Baranick *et al.*, 2000).

In view of the current controversy regarding the significance of p38 MAPK activation in ischaemic damage (Ping *et al.*, 2000), we therefore suggested that the PC-mimetic properties of isoproterenol, as well as the fact that it activates p38 MAPK, could be a useful experimental tool in evaluating the significance of this kinase in cardioprotection. Furthermore, in contrast to the wealth of knowledge as to G protein-coupled receptor (GPCR) -mediated ERK1/2 activation, the role of GPCRs in regulating p38 MAPK activity and its physiological relevance in heart muscle remains unclear. Therefore our study could further elucidate the relationship between the  $\beta$ -adrenergic receptor and p38 MAPK activation in myocardial protection.

#### **4.3.5.1 Characteristics of $\beta$ -adrenergic-mediated stimulation of p38 MAPK**

In order to use  $\beta$ -adrenergic stimulation of the isolated perfused rat heart as a tool in evaluating the role of p38 MAPK in cardioprotection, it was necessary to gain more information regarding its effects on the activity of the kinase.

We studied  $\beta_1$ -adrenergic stimulation of the isolated rat heart with isoproterenol and could also show a rapid onset (within 2 min), dose-dependent activation of p38 MAPK that is maintained for at least 15 min (Fig. 4.10). A similar maximal response after 15 min was also reported for stimulation of both the  $\beta_1$ - and  $\beta_2$ -adrenergic receptor in adult mouse and rat cardiomyocytes (Zheng *et al.*, 2000, Communal *et al.*, 2000). Using adult mouse cardiomyocytes expressing the  $\beta_2$ -adrenergic receptor, isoproterenol elicited a maximal response at  $\geq 1 \mu\text{M}$  (Zheng *et al.*, 2000), similar to the results obtained in this study (Fig. 4.10). A recent study regarding the activation of p38 MAPK by G protein-coupled receptors, reported that isoproterenol also activates p38 MAPK in a time and dose-dependent manner in HEK293 cells (Yamauchi *et al.*, 1997). Interestingly, Zinterol, a  $\beta_2$ -adrenergic receptor agonist, was much less effective in the isolated rat heart and even concentrations 100 x higher ( $10^{-5}$  M) than those used for isoproterenol could not elicit the same stimulation of p38 MAPK (Fig. 4.11). Despite these differences in susceptibility to  $\beta$ -stimulation (which may due to differences in species and experimental models), both  $\beta_1$ - and  $\beta_2$ -adrenergic receptor subtypes can

couple to the activation of p38 MAPK (Zheng *et al.*, 2000, Communal *et al.*, 2000). Furthermore, it is possible that the apparent ability of both  $\beta_1$ - and  $\beta_2$ -adrenergic receptor subtypes to activate p38 MAPK may reflect differential activation of p38 MAPK isoforms, which may exert opposing effects on apoptosis in cardiac myocytes (Wang *et al.*, 1998). However, our observations indicate that the marked activation of p38 MAPK, induced by either global ischaemia or isoproterenol, is mediated by the  $\beta_1$ -, rather than the  $\beta_2$ -, adrenergic receptor.

Direct stimulation of adenylyl cyclase by forskolin ( $10^{-6}$  M), is almost as effective as  $\beta_1$ -adrenergic stimulation by isoproterenol ( $10^{-7}$  M) in increasing tissue cAMP (Fig. 4.6) and activating p38 MAPK (Fig. 4.11). Furthermore, abolishment of cAMP increases by the  $\beta$ -adrenergic blocker alprenolol (Fig. 4.4) abolishes p38 MAPK activation (Figs. 4.13 and 4.14). Therefore activation of p38 MAPK by stimulation of the  $\beta$ -adrenergic signalling pathway is not receptor, but cAMP dependent. In addition, Zheng *et al.* (2000) showed that  $\beta_2$ -receptor activation of p38 MAPK occurs via a cAMP-PKA dependent pathway, rather than by  $G_i$  or the  $G\beta\gamma$  subunit. However, Communal *et al.* (2000) reported that both  $\beta_1$ - and  $\beta_2$ -adrenergic receptor subtypes stimulate p38 MAPK in a pertussis toxin sensitive manner, therefore via  $G_i$  protein. However, these aspects were not addressed in the present study.

#### **4.3.5.2 Manipulation of ischaemia- or isoproterenol-induced p38 MAPK activation**

The contribution of endogenous catecholamines to the phenomenon of ischaemic PC is well established and we therefore evaluated their contribution to the ischaemia-induced activation of p38 MAPK by administration of the appropriate blockers 5 min before the onset of 5 min of ischaemia (PC1- as indicated in Fig. 4.13). Ischaemia-induced activation of p38 MAPK, as occurs during an ischaemic-PC protocol, can be reduced significantly by both  $\alpha_1$  and  $\beta$ -blockade: when compensating for the effects of prazosin and alprenolol *per se* on p38 MAPK activation, it seems as if each blocker effectively abolishes ischaemia-induced activation of p38 MAPK. Although the contribution of other triggers cannot be ruled out, these results suggest that endogenous catecholamines play a significant role in p38 MAPK activation during an ischaemic PC protocol. Furthermore, although no role for the  $\alpha_1$ -adrenergic receptor stimulation (and

PKC) could be demonstrated in ischaemic-PC of the isolated perfused working rat heart model (our particular model of ischaemic PC) (Moolman *et al.*, 1996), it is possible that the marked activation of p38 MAPK which occurs within 5 min of ischaemia, may also be attributed to PKC activation.

As expected,  $\beta$ -adrenergic receptor induced (isoproterenol  $10^{-7}$  M) activation of p38 MAPK can be abolished completely by alprenolol (Fig. 4.14), whereas the  $\text{Ca}^{2+}$  antagonist verapamil was almost as effective, demonstrating a role for influx of extracellular  $\text{Ca}^{2+}$  in activating the kinase, as was also demonstrated in neonatal cardiomyocytes (Zhu *et al.*, 1999), PC 12 cells (Conrad *et al.*, 2000), mouse osteoblastic MC 3T3 - E1-cells (Yamaguchi *et al.*, 2000) and mesangial cells (Sodhi *et al.*, 2000). This observation suggests that the triggering of PC by elevation of intracellular  $[\text{Ca}^{2+}]_i$ , as demonstrated by Miyawaki and Ashraf (1997a, 1997b) may involve p38 MAPK activation.

Although the adenosine  $A_1$ -receptor is suggested to play an important role in triggering PC (Cohen *et al.*, 2000), it does not appear to be involved in isoproterenol-induced activation of p38 MAPK, ruling out adenosine as trigger in cardioprotection in this particular scenario (Fig. 4.14). This is in contrast to the results obtained by Weinbrenner and coworkers (1997), who showed that addition of 8-(p-sulfophenyl)-theophylline (8-SPT) blocked ischaemic PC-induced activation of p38 MAPK as well as the protective effects of PC in rabbits. However, our results are not surprising in view of the fact that adenosine plays little (Headrick 1996), if any (Liu and Downey 1992, Bugge and Ytrehus 1995a, Cave 2000), role in PC in rats.

## 4.4 SUMMARY

### 4.4.1 Effect of $\beta$ -adrenergic stimulation

Our results emphasize the significance of  $\beta$ -adrenergic stimulation as a trigger during the PC protocol: abolishment of the increases in cAMP during the PC protocol attenuated functional recovery during reperfusion. The reverse approach was to establish whether repeated generation of cAMP before the onset of sustained ischaemia could elicit protection. Although such a relationship could be established, it

depended on the mode of administration and concentration of the  $\beta$ -adrenergic receptor agonists.

In view of the results obtained in the present study, we propose that, in addition to stimulation of G-protein coupled receptors such as the  $\alpha_1$ -adrenergic, muscarinic, angiotensin II or opioid receptors (Banerjee *et al.*, 1993, Baxter *et al.*, 1997, Miki *et al.*, 1998), or PKC activation (Brooks *et al.*, 1996), ischaemia-induced activation of the  $\beta$ -adrenergic signalling pathway is an important trigger or contributory factor in eliciting PC.

It also appears as if the tissue cAMP levels at the end of sustained ischaemia may be merely a reflection of the protection conferred by the initial trigger mechanism. This is suggested by our previous findings (Moolman *et al.*, 1996), where preconditioned rat hearts were stimulated with forskolin before the onset of sustained ischaemia causing cAMP levels of PC hearts to increase to the same level after 25 min global ischaemia as observed in non-PC hearts. However, the protective effect of PC on functional recovery was not abolished by this intervention. This was also suggested by Sandhu *et al.*, (1994 and 1996), who observed that infusion of adenylyl cyclase activators, like forskolin and NKH477, throughout the three cycles of transient ischaemia in PC hearts also did not block the protection of PC against necrosis in rabbits, even though it significantly increased cAMP levels. The possibility also exists that forskolin increased cAMP in a compartmentalized manner (Worthington and Opie 1992), thus not allowing assessment of the effects of increased cAMP levels on the protection conferred by PC.

Finally, whether desensitization of the  $\beta$ -adrenergic receptor is the only way by which the ischaemic myocardium is protected by activation of the  $\beta$ -adrenergic signalling pathway or whether this is linked to the suggested subsequent phosphorylation of a membrane bound effector (Brooks *et al.*, 1996, Baxter *et al.*, 1997) still needs to be resolved.

#### **4.4.2 Downstream events of $\beta$ -adrenergic stimulation: role of p38 MAPK activation**

Furthermore, during  $\beta$ -adrenergic stimulation transient activation of p38 MAPK occurred, which was associated with attenuation of p38 MAPK activity during sustained

ischaemia and reperfusion as well as improvement in functional recovery. These observations suggest that activation of p38 MAPK during the PC protocol may act as trigger downstream of cAMP, while attenuation of its activation during sustained ischaemia and reperfusion is associated with cardioprotection. Therefore the importance of this will be investigated later in chapter 4.

The significance of activation of the  $\beta$ -adrenergic signalling pathway during ischaemic PC is emphasized by the observation that alprenolol-induced blockade of the receptor during the PC protocol only, resulted in abolishment of the increases in tissue cAMP and inhibition of p38 MAPK activation.  $\beta$ -Adrenergic receptor blockade with alprenolol resulted in increased activation of p38 MAPK during both sustained ischaemia and reperfusion with concurrent mechanical failure, similar to the pattern observed in non-PC hearts.

## CHAPTER 5

### Manipulation of cyclic guanosine monophosphate (cGMP)

#### 5.1 Introduction

We hypothesized previously that characterization of events during the PC protocol would yield more insight into the triggers and signalling pathways involved in the protective effect of classic PC. Subsequently, we showed that a 3 x 5 min PC protocol caused cyclic elevations in the cyclic nucleotides, cAMP and cGMP. The latter observation as well as the fact that nitric oxide (NO) synthase (NOS) activity is increased within 5 min of global ischaemia (Depré *et al.*, 1997) suggested the possibility that NO may also be a trigger in PC. In addition, it has been shown that rapid pacing can protect isolated working hearts against ischaemia, via production of nitric oxide, NO (Ferdinandy *et al.*, 1996, Ferdinandy *et al.*, 1997).

As mentioned previously, ischaemic preconditioning (PC) occurs in two phases: an *early* phase (classic PC), which develops immediately and lasts approximately 2 – 3 h after the ischaemic stimulus and a *delayed* phase (late PC or second window of protection), which appears 12 – 24 h later and lasts for 3 – 4 days (Kuzuya *et al.*, 1993, Marber *et al.*, 1993). Involvement of NO in the late phase of PC has been the focus of intense investigation. Bolli *et al.* (1998) recently postulated a dual role for NO in this scenario: initially a trigger and subsequently as mediator of protection. They proposed that triggering the development of late PC on *day one*, involves generation of NO after a brief ischaemic stress (probably via eNOS) (Bolli *et al.*, 1997) and subsequent activation of PKC (Ping *et al.*, 1999). The latter could in turn trigger a complex signalling cascade that involves protein tyrosine kinases (Imagawa *et al.*, 1997), the transcription factor nuclear factor- $\kappa$ B (Xuan *et al.*, 1999), and most likely other as yet unknown components. Furthermore, increasing evidence indicates that the cardioprotective effects of late PC observed on *day two* are due to upregulation of inducible NOS (Takano *et al.*, 1998a). Further convincing evidence of a role for NO in late PC is that NO donors mimic and NOS inhibitors abolish late protection (Takano *et al.*, 1998b).

The role of NO as a trigger or mediator in the phenomenon of classic PC is however not yet clear, because of the controversial results from the few studies done thus far. The

discrepancies are generally attributed to species differences, different PC stimuli and different study end points, i.e., myocardial function, arrhythmias or infarct size (Parrat 1994, Baxter *et al.*, 1996). Some studies reported that the anti-arrhythmic effects of PC were independent of NO (Lu *et al.*, 1995, Sun *et al.*, 1997), and that NOS-inhibition by L-NAME had no effect on PC-induced reduction in infarct size (Woolfson *et al.*, 1995) or improvement in functional recovery (Weselcouch *et al.*, 1995); however, others showed that NO donors could mimic (Bilinska *et al.*, 1996) or NOS-inhibitors could abolish (Vegh *et al.*, 1992) the protective effect of PC on arrhythmias. Further evidence for involvement of NO in classic PC was the recent observation that cardiac NO biosynthesis was essential to trigger, but not to mediate PC (Csonka *et al.*, 1999). Thus far, the effect of NO donors on outcomes such as infarct size or functional recovery during reperfusion, has not been studied.

Although there is ample evidence for generation of NO and peroxynitrite during ischaemia-reperfusion (Csonka *et al.*, 1999, Wang *et al.*, 1996, Weselcouch *et al.*, 1995, Yasmin *et al.*, 1997), no increase in tissue NO could be demonstrated during the PC protocol itself (Csonka *et al.*, 1999). However, in view of observations that NOS activation (Depré *et al.*, 1999) and increases in cGMP (from chapter 3) occur within 5 min of global ischaemia, it seems very likely that NO is generated during an ischaemic PC protocol and may act as trigger.

### **5.1.1 The aim:**

We therefore investigated whether the cyclic increases in tissue cGMP, elicited by multiple short episodes of ischaemia/reperfusion (as occurs during a multi-cycle PC protocol), indicated involvement of NO, and thus cGMP, as a trigger of classic PC in the isolated working rat heart model.

Our concept is that events occurring during the PC protocol serve to activate (i.e. trigger) the protective mechanism of PC. Biochemical events that take place during the subsequent phase of sustained ischaemia reflect the protection that was elicited by prior PC. Accordingly, abolishment or attenuation of biochemical events during either the PC phase or during the sustained ischaemia, causing inhibition of protection during reperfusion as reflected by mechanical recovery, may indicate a role as trigger or mediator, respectively.

The aim of this study was therefore to evaluate the role of NO and cGMP in classic PC by the following interventions:

- (i) evaluation of the role of NO and thus cGMP during the PC protocol (as *trigger*): by abolishing the cyclic changes in cGMP via inhibition of the NO-cGMP pathway before and during the PC protocol only.
- (ii) confirmation on the *trigger* effect of NO: by mimicking cyclic changes in cGMP via transient administration of exogenous NO donors.
- (iii) evaluation of the role of NO production during sustained ischaemia (as *mediator*): by inhibiting the NO-cGMP pathway after the PC protocol, before the onset of sustained ischaemia only.

Functional recovery during reperfusion of the globally ischaemic heart and tissue cyclic nucleotides at the end of sustained ischaemia will be used as indicators of protection.

In addition, the functional reserve of non-preconditioned, preconditioned and hearts pretreated with NO donors will be evaluated by administration of adrenaline during reperfusion.

Finally, in view of the putative role of p38 MAPK in ischaemic- and  $\beta$ -adrenergic PC, the downstream effects of NO and cGMP generation during the PC protocol and sustained ischaemia were evaluated by determination of p38 MAPK activation.

## **5.2 Experimental Protocols and Results**

### **5.2.1 Manipulation of the NO-cGMP pathway during PC protocol before sustained ischaemia**

#### **5.2.1.1 Inhibition of cyclic increases in cGMP during ischaemic PC protocol**

To investigate the role of the NO-cGMP pathway during ischaemic PC protocol, (i) cGMP generation was inhibited by a guanylyl cyclase (GC) inhibitor, ODQ and (ii) NO formation was suppressed by a nitric oxide synthase (NOS) inhibitor, L-NAME. The concentration of the inhibitors used, was such that it prevented the rise in cGMP during PC.

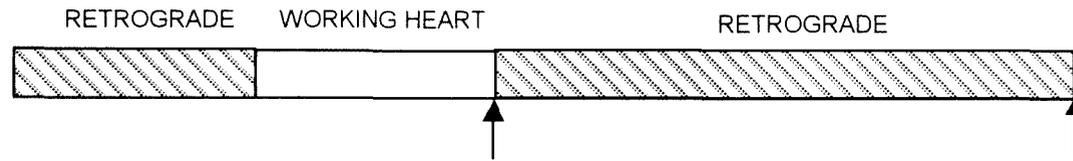
##### **5.2.1.1.1 Experimental protocol**

ODQ or 1H-[1,2,4]oxadiazolol[4,3-a]quinoxalin-1-one, a selective inhibitor of guanylyl cyclase, was administered 5 min preceding the first 5 min episode of ischaemic PC (PC1-) as well as during each 5 min reperfusion episode (PC1+, PC2+ and PC3+). Hearts were freeze-clamped at PC1-, PC1+, PC3- and PC3+ as indicated in Fig. 5.1. Appropriate controls and preconditioned hearts were perfused as described before (3.2.1.1). Hearts were freeze-clamped at 30 and 60 min total perfusion time for controls and at PC1-, PC1+, PC3- and PC3+ for PC hearts, as indicated in Fig. 5.1.

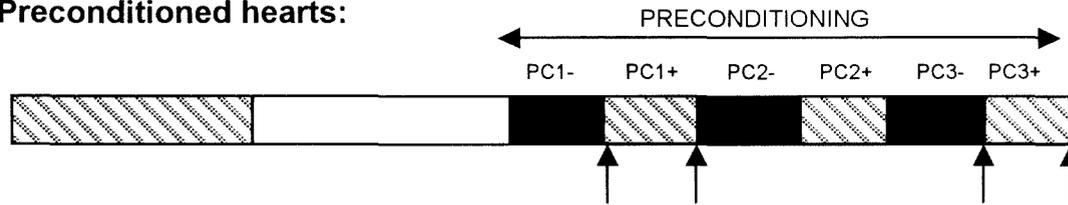
##### **5.2.1.1.2 Results**

The effect of inhibition of cGMP generation during the ischaemic PC protocol was studied with ODQ, which was administered 5 min before the onset of PC1- as well as during each reperfusion phase (Table 5.1). Since ODQ was dissolved in DMSO, a control series was included where DMSO (0.04%) plus ODQ (20  $\mu$ M) was administered retrogradely for 3 x 5 min after the usual stabilization period of 30 min. The combination of ODQ and DMSO significantly increased tissue cGMP levels (Untreated controls:  $8.42 \pm 0.35$  vs. ODQ+DMSO controls:  $11.22 \pm 0.43$  picomoles/g wet weight) while lowering cAMP levels (Untreated controls:  $317 \pm 9.3$  vs. ODQ+DMSO controls:  $241.5 \pm 10.1$  picomoles/g wet weight). The ODQ+DMSO controls were used for comparing the

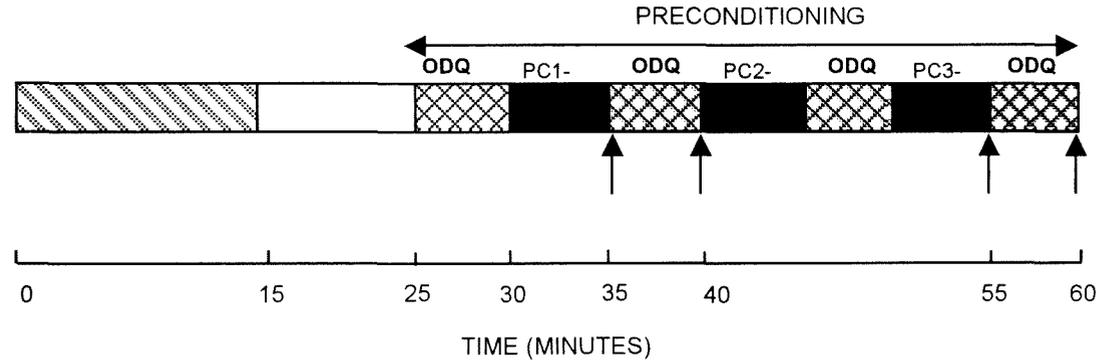
**Control hearts:**



**Preconditioned hearts:**

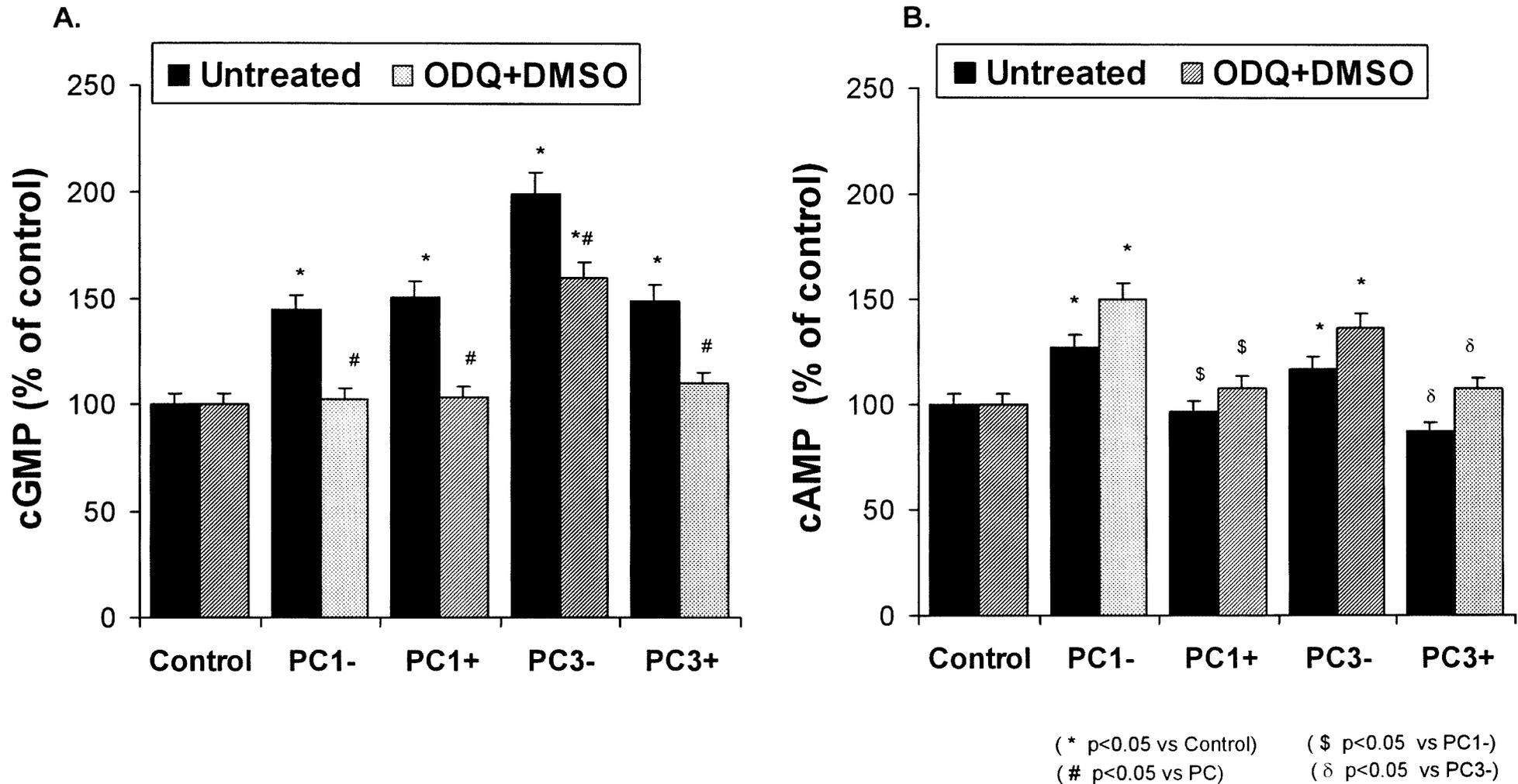


**Preconditioned hearts + GC inhibitor, ODQ (20  $\mu$ M) :**



0 15 25 30 35 40 55 60  
TIME (MINUTES)

**Fig 5.1** Inhibition of cyclic increases in cGMP during the ischaemic preconditioning protocol: Experimental protocol. Abbreviations as in Fig 3.1. The guanylyl cyclase (GC) inhibitor, ODQ (20 $\mu$ M), was administered 4 x 5 min during the preconditioning protocol. Arrows indicated time points of freeze-clamping for cGMP and cAMP determinations. (n = 6 hearts/series).



**Fig 5.2** Manipulations of cGMP and cAMP levels during the ischaemic preconditioning protocol with ODQ (20  $\mu$ M) + DMSO (0.04%). Untreated or ODQ + DMSO treated controls were taken as 100%, and were used to compare the effects of ischaemic PC on tissue cyclic nucleotides in the absence or presence of ODQ, respectively. ODQ + DMSO prevented the significant rise in cGMP, while not affecting the cyclic rises in cAMP, during the PC protocol.

**Table 5.1**

**Effects of ODQ, an inhibitor of guanylyl cyclase on tissue cyclic nucleotide levels and ratio during a 3 x 5 min preconditioning protocol.**

	<b>cGMP</b>	<b>cAMP</b>	<b>cAMP/cGMP</b>
<u>Untreated controls</u> (n = 11/series)	8.42 ± 0.35	317.0 ± 9.27	37.65
<u>Preconditioning:</u> (n = ≥ 9/series)			
PC1-	12.18 ± 0.71 *	402.8 ± 13.23 *	33.07
PC1+	12.71 ± 1.37	307.0 ± 13.51 δ	24.15
PC3-	16.79 ± 1.12 *	370.2 ± 9.74 *	22.05
PC3+	12.54 ± 0.69 *δ	277.1 ± 9.16 δ	22.10
<u>ODQ + DMSO:</u> (n = 6/series)			
Controls	11.22 ± 0.43 *	241.5 ± 10.1 *	24.60 ± 2.56
PC1-	11.51 ± 0.58 *	362.4 ± 39.7 **	30.90 ± 3.91
PC1+	11.60 ± 0.75 *	261.2 ± 16.2 δ	23.91 ± 2.18
PC3-	17.92 ± 1.00 *	330.0 ± 15.08 **	15.50 ± 1.71
PC3+	12.34 ± 0.51 *	259.7 ± 16.20 δ	20.59 ± 1.19

Values are means ± SE in pmoles/g wet weight. Untreated control hearts were perfused for 60 min (15 min retrograde, 15 min working heart, 30 min retrograde perfusion). ODQ + DMSO controls: ODQ (20 µM) + DMSO (0.04%) were administered for 3 x 5 min, as described above.

δ p < 0.05 PC- vs PC+

\* p < 0.05 vs control (untreated)

\*\* p < 0.05 vs controls, perfused with ODQ and DMSO

effects of preconditioning on cyclic nucleotides in the presence of ODQ. ODQ prevented the significant rise in cGMP occurring during the PC protocol at PC1- and PC3- (as observed in untreated hearts), whereas tissue cAMP increased in a similar manner as observed before (Fig. 5.2)

The nitric oxide synthase inhibitor, L-NAME (N(G)-nitro-L-arginine methyl ester), was also administered as described for ODQ, but these hearts were freeze-clamped at the end of PC3- only. L-NAME (50  $\mu$ M) also caused a reduction in cGMP, when measured at PC3- ( $11.32 \pm 0.85$  (n=6) vs.  $16.79 \pm 1.12$  (n=9) picomoles/g wet weight,  $p < 0.05$ ).

### **5.2.1.2 Repeated increases in cGMP by nitric oxide (NO) donors before sustained ischaemia**

The cyclic increases in tissue cGMP during a three-episode ischaemic PC protocol was mimicked by 3 x 5 min administration of the NO donors s-nitroso-penicillamine (SNAP), sodiumnitroprusside (SNP) or L-arginine in the *absence* of ischaemia.

#### **5.2.1.2.1 Experimental protocol**

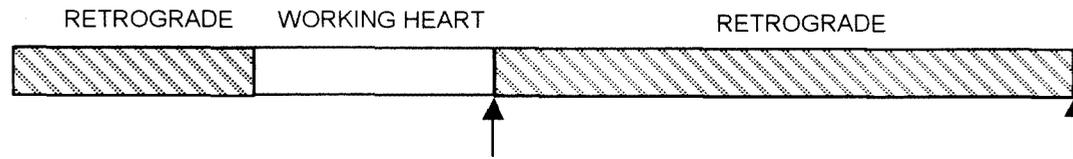
All hearts were stabilized for 30 min (15 min retrograde, 15 min working) as described before. The NO-donors SNAP, SNP or L-arginine or the solvent DMSO were then administered transiently for 3 x 5 min episodes, alternated by 5 min reperfusion with normal buffer. In these studies cGMP and cAMP were analyzed after freeze-clamping the hearts (at least six hearts at each time point) at the end of the first and third episodes of administration (1 x 5 min and 3 x 5 min) and also after 5 min of reperfusion (1 x 5 min + reperfusion and 3 x 5 min + reperfusion, as indicated in Fig. 5.3).

Appropriate controls and preconditioned hearts were perfused as described before (3.2.1.1). Hearts were freeze-clamped at 30 and 60 min total perfusion time for controls and at PC1-, PC1+, PC3- and PC3+ for PC hearts, as indicated in Fig. 5.3.

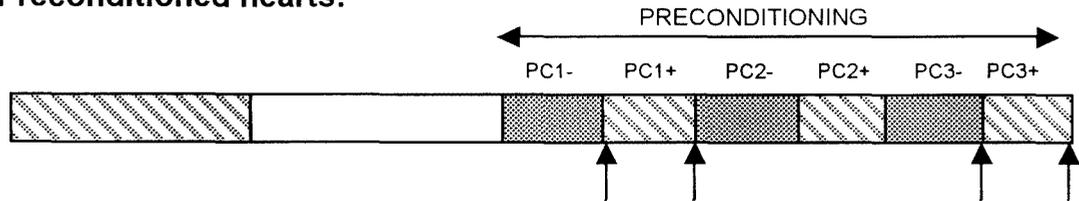
#### **5.2.1.2.2 Results**

Pilot studies were done to ensure that the concentration of donors used yielded cGMP levels equal to or exceeding those observed during the ischaemic PC protocol. The

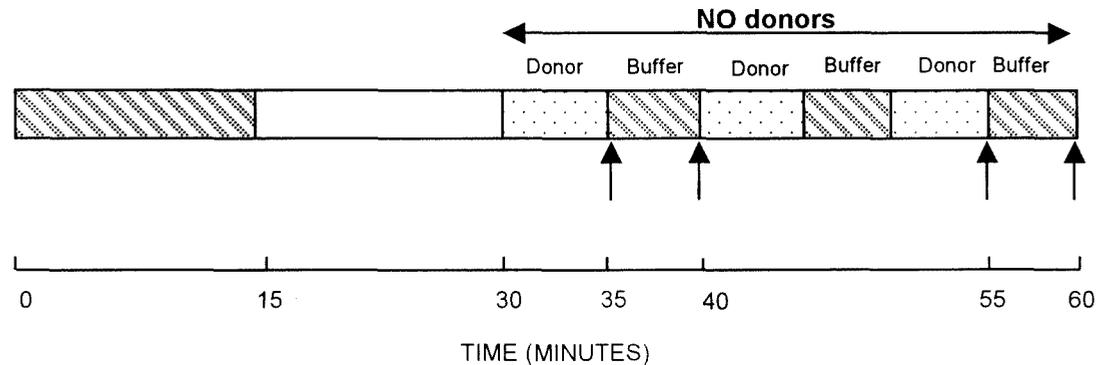
**Control hearts:**



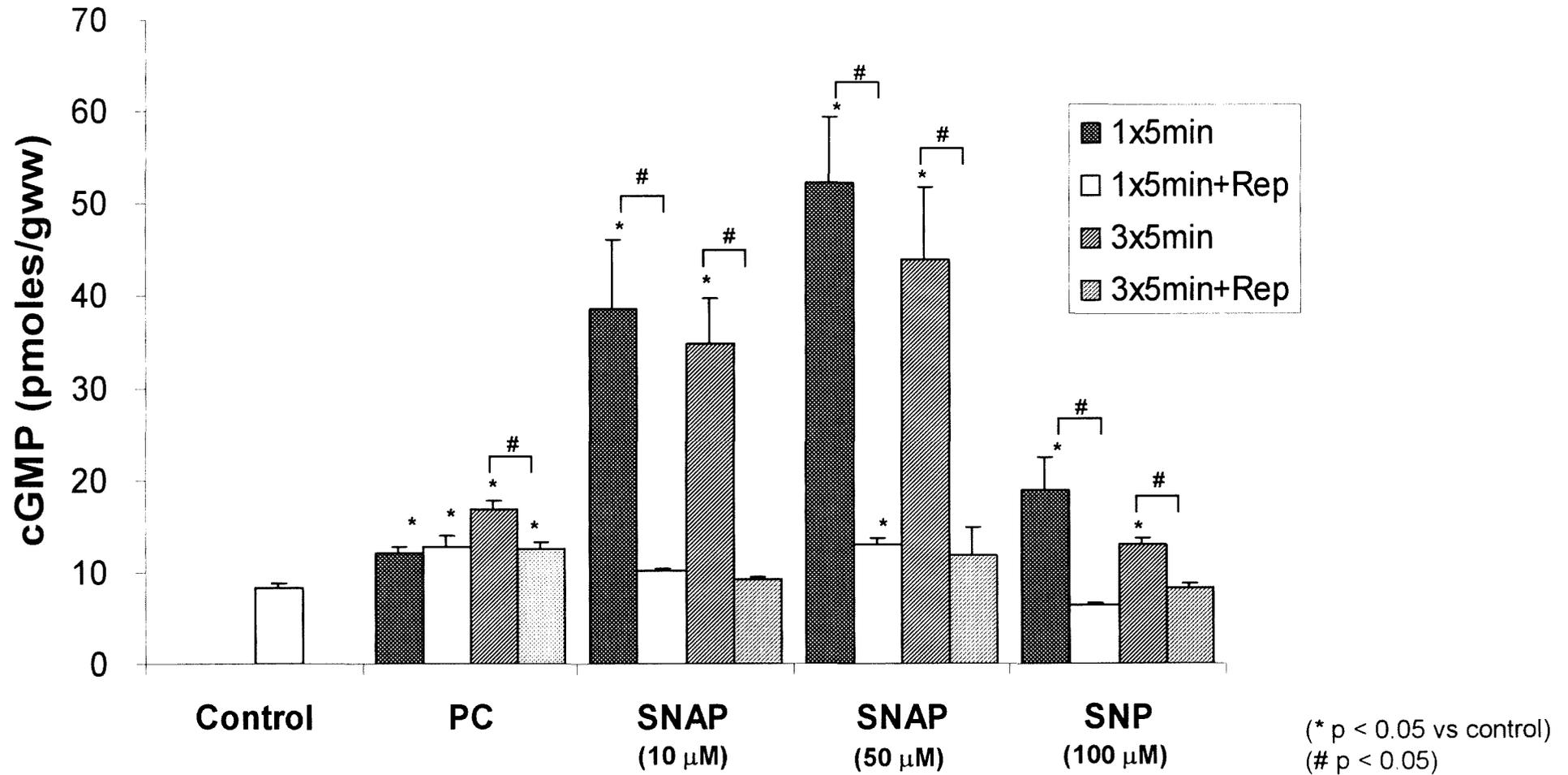
**Preconditioned hearts:**



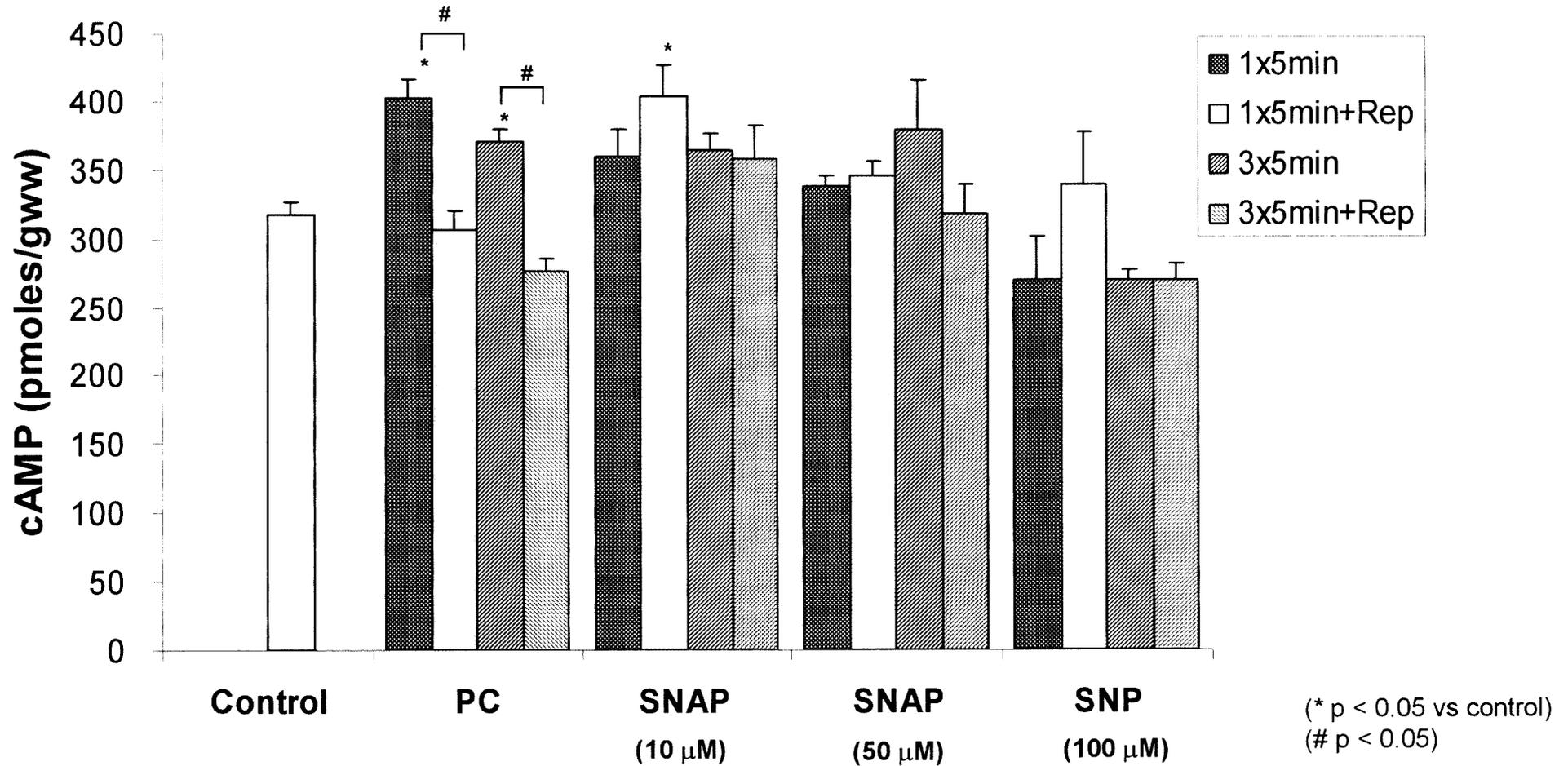
**3 x 5 min NO donors, SNAP (10  $\mu$ M or 50  $\mu$ M) or SNP (100  $\mu$ M):**



**Fig 5.3** Repetitive increases in cGMP by NO donors: Experimental protocol. Abbreviations as in Fig 3.1. The cyclic increases in cGMP during a three-episode ischaemic preconditioning protocol was mimicked by 3 x 5 min administration of the NO donors, SNAP (10  $\mu$ M or 50  $\mu$ M) or SNP (100  $\mu$ M) in the absence of ischaemia. Arrows indicated time points of freeze-clamping for cGMP and cAMP determinations. (n = 6 hearts/series).



**Fig 5.4** Repetitive increases in cGMP by NO donors. Cyclic increases in cGMP occurred after 1 x 5 min and 3 x 5 min episodes of both the NO donors, SNAP (10 μM or 50 μM) or SNP (100 μM) in the absence of ischaemia. Upon reperfusion (Rep.) with normal buffer, these values returned to control levels. The effect of SNAP on cGMP levels was dose-dependent and much more effective than SNP.



**Fig 5.5** Effect of repetitive administration of NO donors on cAMP levels. Cyclic increases in cAMP occurred during an ischaemic PC protocol, however after 1 x 5 min and 3 x 5 min episodes of both the NO donors, SNAP (10 μM or 50 μM) or SNP (100 μM) in the absence of ischaemia the cAMP levels remained unchanged (except for SNAP 10 μM, 1 x 5 min + reperfusion).

**Table 5.2****Effects of administration of NO donors (SNAP or SNP) on tissue cyclic nucleotides before onset of sustained ischaemia.**

	<b>cGMP</b>	<b>cAMP</b>	<b>cAMP/cGMP</b>
Control (n = 11)	8.42 ± 0.35	317.0 ± 9.27	37.65
<u>SNAP (10 µM)</u> (n = > 7/series)			
1 x 5 min	38.77 ± 7.56 *	360.5 ± 20.2	11.78 ± 2.02
1 x 5 min + Rep.	10.11 ± 0.26 δ	403.8 ± 23.7 *	43.63 ± 1.72
3 x 5 min	34.94 ± 4.87 *	364.7 ± 11.8	11.81 ± 2.23
3 x 5 min + Rep.	9.30 ± 0.28 δ	359.3 ± 24.3	41.47 ± 4.03
<u>SNAP (50 µM)</u> (n = 5/series)			
1 x 5 min	52.44 ± 7.13 *	339.7 ± 7.33	4.72 ± 0.99
1 x 5 min + Rep.	12.97 ± 0.80 δ	346.2 ± 10.43	28.30 ± 0.92
3 x 5 min	44.19 ± 7.74 *	379.4 ± 37.87	8.29 ± 1.15
3 x 5 min + Rep.	11.82 ± 3.05 δ	318.5 ± 21.89	20.23 ± 2.72
<u>SNP (100 µM)</u> (n = > 8/series)			
1 x 5 min	18.96 ± 3.67 *	270.7 ± 31.85	14.82 ± 2.10
1 x 5 min + Rep	6.38 ± 0.37 δ	340.9 ± 37.11	53.47 ± 8.2
3 x 5 min	13.05 ± 0.75 *	270.7 ± 7.32	18.46 ± 0.79
3 x 5 min + Rep.	8.22 ± 0.47 δ	270.9 ± 11.77	30.00 ± 2.87
<u>L-Arginine (10 mM)</u> (n = 5/series)			
1 x 5 min	8.14 ± 0.35	-	-
3 x 5 min	7.37 ± 0.58	-	-

Results expressed as pmoles/g wet weight. Control hearts were perfused for 60 min (15 min retrograde, 15 min working heart, 30 min retrograde perfusion) (similar values were obtained at 30 min of control perfusion conditions).

δ p < 0.05 1 x 5 min vs 1 x 5 min + Rep or 3 x 5 min vs 3 x 5 min + Rep

\* p < 0.05 vs control (untreated)

cyclic increases in tissue cGMP during a three-episode ischaemic PC protocol could be mimicked by 3 x 5 min administration of the NO donors, SNAP and SNP (in the absence of ischaemia) (Fig. 5.4). The effect of SNAP on tissue cGMP levels is dose-dependent: 1 x 5 min administration of SNAP at 10  $\mu$ M increased cGMP levels to  $38.77 \pm 7.56$ , compared to  $52.44 \pm 7.13$  pmoles/g wet weight with 50  $\mu$ M SNAP. Fig. 5.4 shows that cyclic increases in cGMP occur after 1 x 5 min and 3 x 5 min episodes of both 10 and 50  $\mu$ M SNAP administration. Upon reperfusion with normal buffer, these values returned to control levels. SNP appeared to be a less effective NO donor, since at 100  $\mu$ M cGMP levels were lower than those induced by 10  $\mu$ M SNAP (Fig. 5.4). However, it also caused significant increases in cGMP at both 1 x 5 min and 3 x 5 min of administration. L-arginine (L-Arg) had no effect on tissue cGMP values, because 1 x 5 min and 3 x 5 min administration of L-Arg (10 mM) resulted in  $8.14 \pm 0.35$  and  $7.37 \pm 0.58$  pmoles/g wet weight, respectively, which were not significantly different when compared to the control value of  $8.42 \pm 0.35$  pmoles/g wet weight.

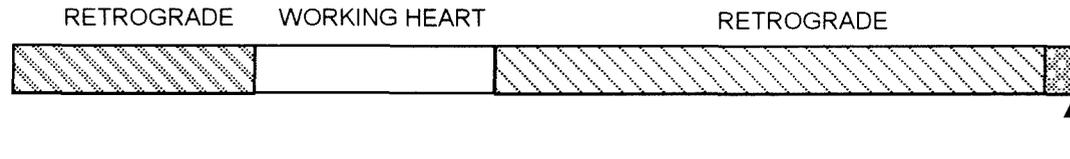
In order to study the effects of NO (or cGMP) in isolation, it was required that no concomitant changes in cAMP occur upon administration of SNAP or SNP. Although it appears that SNAP (10 and 50  $\mu$ M) increases tissue cAMP levels, the changes were not significant compared to the control levels (except for SNAP 10  $\mu$ M, 1 x 5 min + reperfusion) (Fig. 5.5).

Due to the marked increases in tissue cGMP upon administration of NO donors, SNAP (10 and 50  $\mu$ M) or SNP (100  $\mu$ M) (Fig. 5.4), the cAMP/cGMP ratios were severely depressed during 1 x 5 min and 3 x 5 min, compared to reperfusion with normal buffer during 1 x 5 min + Rep. and 3 x 5 min + Rep. (Table 5.2).

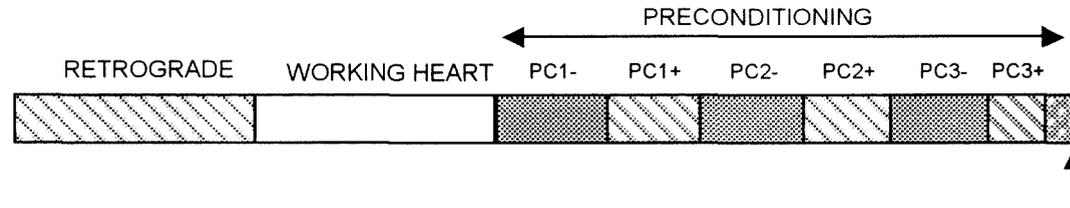
### **5.2.1.3 $\beta$ -adrenergic receptor responsiveness after repeated NO stimulation**

Should NO donors contribute to increases in cAMP, then repeated NO donor treatment would result in desensitization of the  $\beta$ -adrenergic receptor as observed after repeated increases in cAMP by ischaemic-PC or isoproterenol-PC (Fig. 4.8). Therefore the following experiments were performed to assess whether repeated stimulation of with the NO donor, SNP, might reduce the  $\beta$ -adrenergic response at the end of the simulated PC protocol before sustained ischaemia.

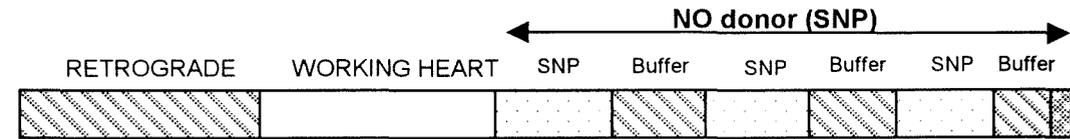
**Non-preconditioned hearts:**



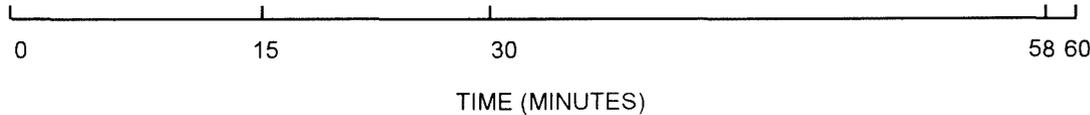
**Preconditioned hearts:**



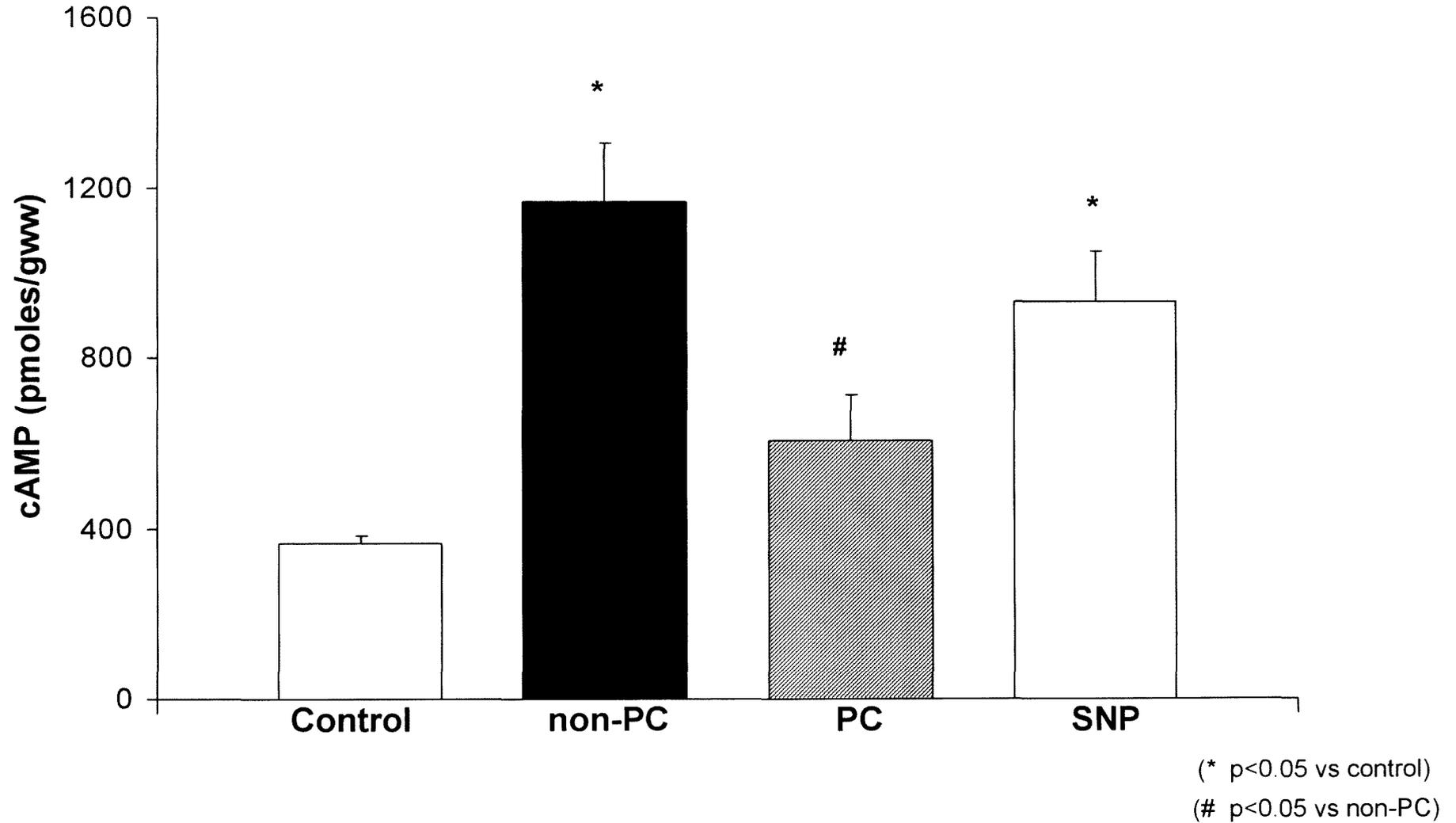
**NO donor, 100  $\mu$ M SNP (3 x 5 min) :**



 Isoproterenol ( $10^{-7}$  M)



**Fig 5.6**  $\beta$ -adrenergic receptor responsiveness after repeated NO stimulation. Experimental protocol. Abbreviations as in Fig 3.1. The cyclic increases in cGMP during a three-episode ischaemic preconditioning protocol was mimicked by 3 x 5 min administration of the NO donor, SNP (100  $\mu$ M) in the absence of ischaemia. At 58 min of perfusion time of non-PC, PC hearts and SNP treated hearts, isoproterenol ( $10^{-7}$  M) was added to the perfusate and hearts were freeze-clamped (as indicated by arrows) after 2 min for cAMP determination. (n = 6 hearts/series).



**Fig 5.7**  $\beta$ -adrenergic receptor responsiveness after repeated NO stimulation. Repeated administration of NO donor, SNP (3 x 5 min, 100  $\mu$ M), does not desensitize hearts to additional  $\beta$ -adrenergic stimulation, for the cAMP content was not significantly different from non-PC hearts. On the other hand, repeated ischaemic preconditioning (3 x 5 min global ischaemia) significantly desensitizes the heart to further  $\beta$ -adrenergic stimulation (as observed before).

#### **5.2.1.3.1 Experimental protocol**

Tissue cAMP levels were measured after additional  $\beta$ -adrenergic receptor stimulation, by administration of isoproterenol ( $10^{-7}$  mol/l) for a period of 2 min after repeated administration of 100  $\mu$ M SNP (Fig. 5.6).

#### **5.2.1.3.2 Results**

As observed previously (3.2.1.2.4.3 and Fig. 3.9), the ischaemic PC protocol (3 x 5 min global ischaemia) significantly desensitizes the heart to further  $\beta$ -adrenergic receptor stimulation, when compared to that of non-PC hearts. Hearts administered with SNP (100  $\mu$ M) for 3 x 5 min was still sensitive to additional  $\beta$ -adrenergic stimulation, for the cAMP content was not significantly different from that of non-PC hearts (Fig. 5.7).

#### **5.2.1.4 Manipulation of p38 MAPK activation by inhibition or stimulation of NO-cGMP pathway during the PC protocol**

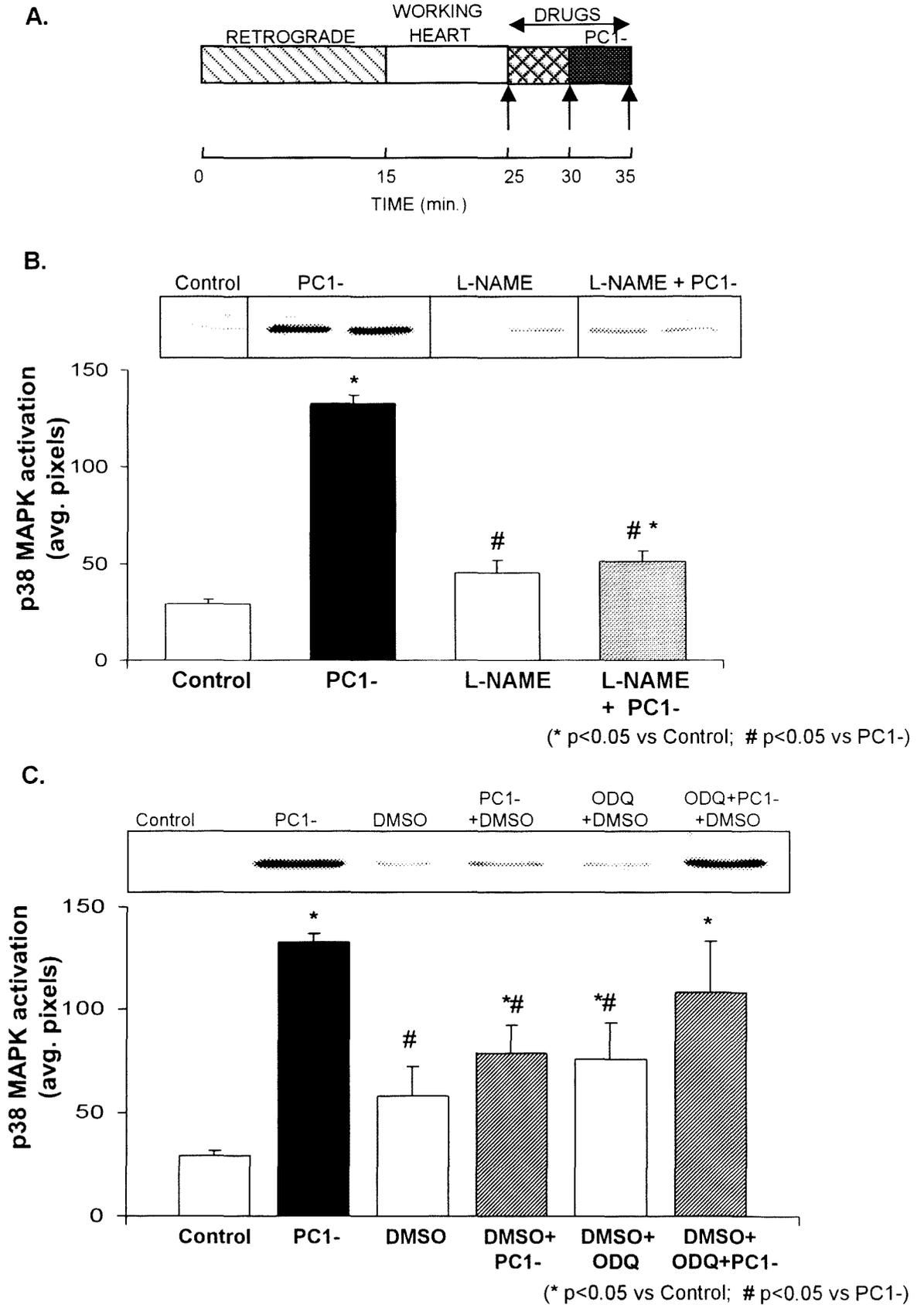
To assess whether repeated elevation of tissue cGMP during the PC protocol, had an effect on p38 MAPK activation, it was necessary to characterize the effects of manipulation of the NO-cGMP pathway on p38 MAPK activation during this protocol.

##### **5.2.1.4.1 Experimental protocol**

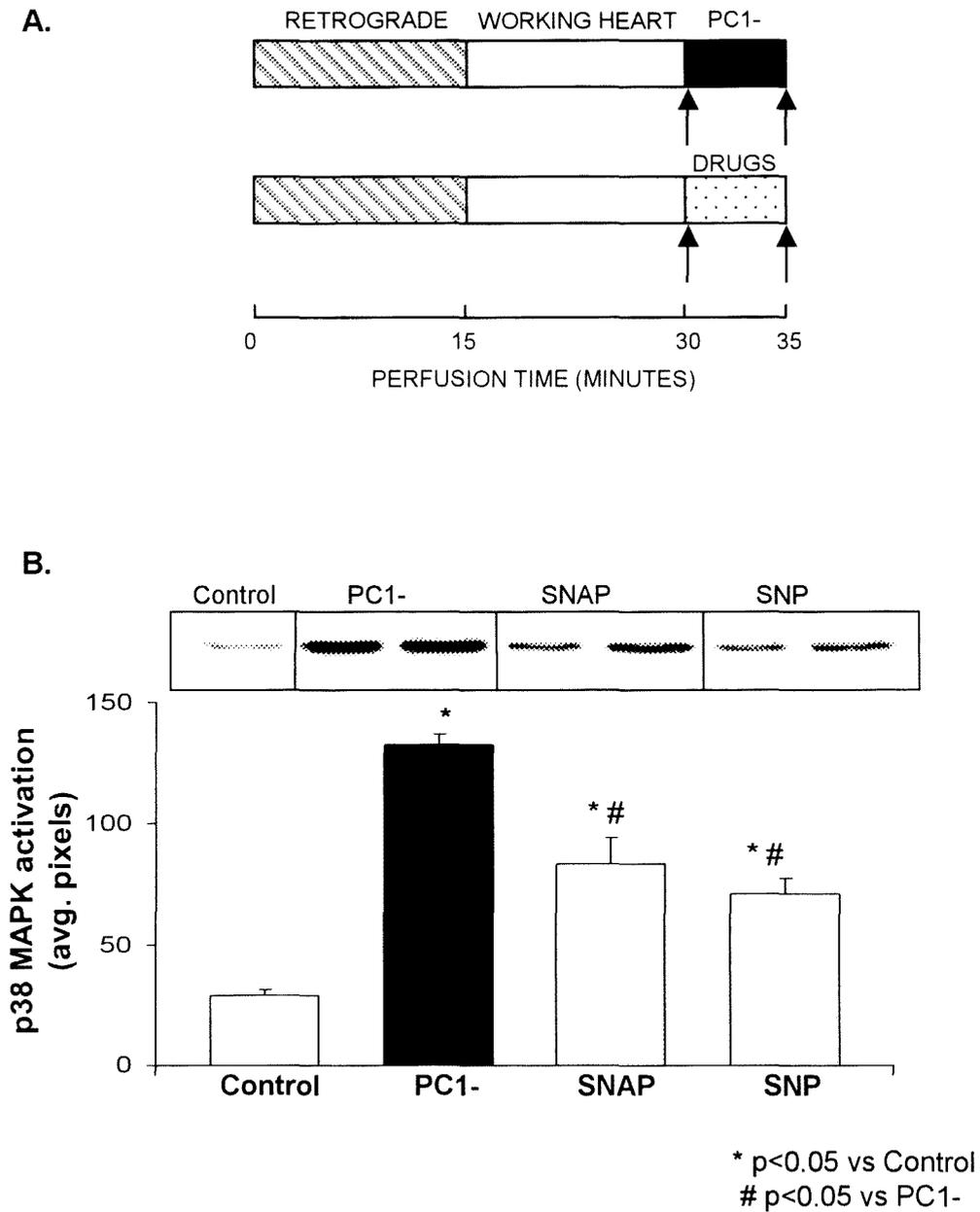
Following the stabilization period of 25 min, inhibitors of the NO-cGMP pathway ODQ (20  $\mu$ M), a guanylyl cyclase inhibitor or L-NAME (50  $\mu$ M) a nitric oxide synthase inhibitor, was introduced 5 min preceding 5 min global ischaemia (PC1-) (Fig. 5.8A). Furthermore, after 25 min of stabilization, hearts were also subjected to either 5 min global ischaemia or 5 min treatment with NO donors, SNP and SNAP (Fig. 5.9A).

##### **5.2.1.4.2 Results**

The ischaemia-induced (PC1-) activation of p38 MAPK, as indicated by its dual phosphorylation, was significantly inhibited by L-NAME (Fig. 5.8B). On the other hand, introducing ODQ prior to 5 min global ischaemia (PC1-), led to a slight, but not significant decrease of the ischaemia-induced activation of p38 MAPK (Fig. 5.8C, last



**Fig 5.8** Manipulation of ischaemia-induced p38 MAPK activation by inhibition of NO-cGMP pathway. **A.** L-NAME (50  $\mu$ M) or 20  $\mu$ M ODQ in DMSO (0.04%) was administered 5 min prior to 5 min global ischaemia (PC1-). **B.** L-NAME inhibited the ischaemia-induced p38 MAPK activation, **C.** however ODQ could not (last lane: DMSO+ODQ+PC1-). A representative blot is given for each experiment. n = 4-6 hearts/series.



**Fig 5.9** Mimicking ischaemia-induced p38 MAPK activation by stimulation of NO-cGMP pathway. **A.** NO donors, 50  $\mu$ M SNAP or 100  $\mu$ M SNP, were administered for 5 min in the absence of 5 min global ischaemia (PC1-). **B.** Both NO donors activated p38 MAPK. A representative blot is given for each experiment. n = 4-6 hearts/series.

lane, DMSO + ODQ + PC1-). Because ODQ was dissolved in DMSO, a control series with DMSO (0.04%) alone or DMSO + PC1- or DMSO + ODQ was also included. Although DMSO alone had no significant effect on p38 MAPK activation, it did however partially suppress the PC-induced p38 MAPK activation. Furthermore, DMSO + ODQ increased p38 MAPK activation to a much lesser extent than PC1- (Fig. 5.8C).

Both NO donors, SNP and SNAP, increased the phosphorylation p38 MAPK significantly compared to controls, however not to the same extent as that induced by 5 min ischaemia (PC1-) (Fig. 5.9B).

## **5.2.2 Evaluation of events during sustained ischaemia**

### **5.2.2.1 Effect of prior manipulation of the NO-cGMP pathway on cyclic nucleotides during sustained ischaemia**

To evaluate the effect of prior inhibition or stimulation of the NO-cGMP pathway on sustained ischaemia, it was necessary to determine the changes in cyclic nucleotides at the end of 25 min sustained ischaemia.

#### **5.2.2.1.1 Experimental protocol**

Six series of hearts (Non-PC, PC, or pre-treated with ODQ (20  $\mu$ M), SNAP (50  $\mu$ M) or SNP (100  $\mu$ M), as described above (5.2.1.1.1 and 5.2.1.2.1), were subjected to 25 min global sustained ischaemia and then freeze-clamped (6 hearts/series) (Fig. 5.10). The cAMP and cGMP responses to 25 min sustained ischaemia were determined as described in the methods (chapter 2). Cyclic nucleotides at this stage of the protocol were not assessed in L-arginine and L-NAME treated hearts.

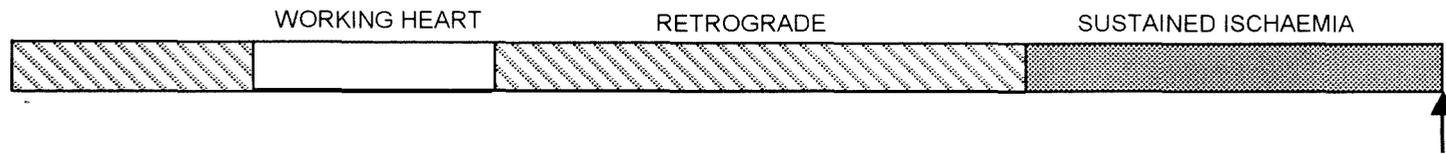
#### **5.2.2.1.2 Results**

##### **5.2.2.1.2.1 GC inhibition**

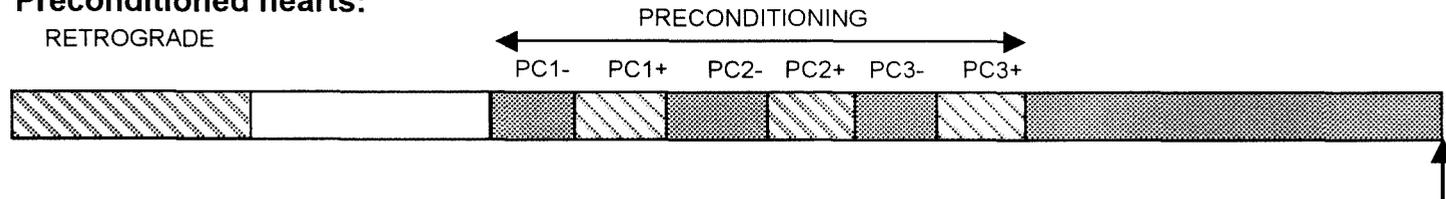
At the end of 25 min sustained ischaemia, the ischaemic PC protocol causes a significant increase in tissue cGMP when compared to non-PC hearts (Fig. 5.11A and Table 5.3). ODQ (20 $\mu$ M) significantly increased tissue cGMP compared to untreated controls (Fig. 5.11A and Table 5.3). Consequently, when ODQ + DMSO was introduced to PC hearts, no significant change in tissue cGMP occurred compared to control hearts treated with ODQ + DMSO. Therefore, introducing ODQ + DMSO to PC hearts diminished the significant percentage rise in cGMP occurring in untreated PC hearts after 25 min ischaemia from 42% (untreated control vs. untreated PC) to 15% (control + ODQ + DMSO vs. PC + ODQ + DMSO).

As indicated before (3.2.2.2.1), cAMP values at the end of 25 min sustained ischaemia were significantly increased in both non-PC and PC hearts compared to controls. However, PC significantly reduced cAMP when compared to non-PC hearts (Fig. 5.11B

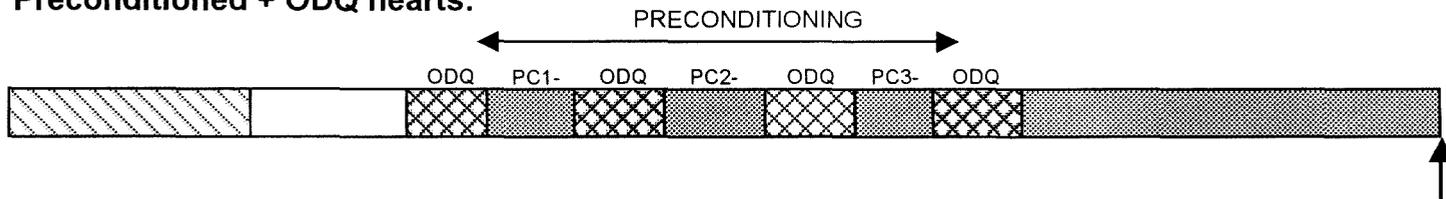
**Non-preconditioned hearts:**



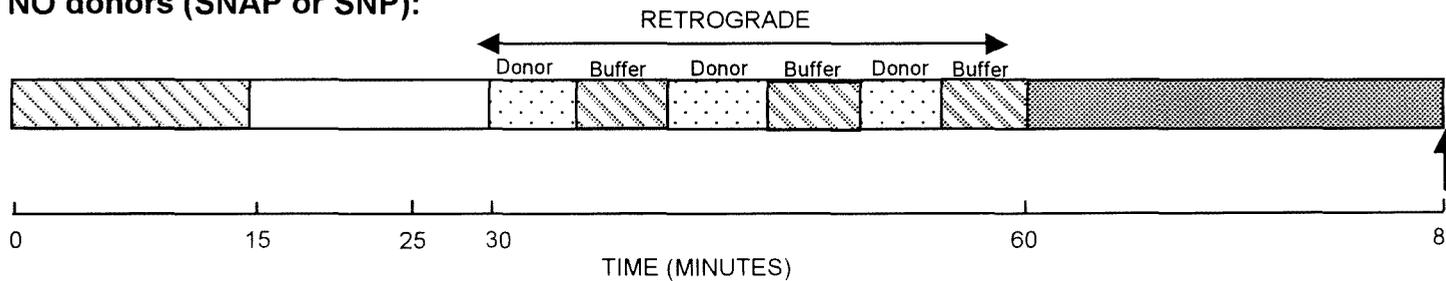
**Preconditioned hearts:**



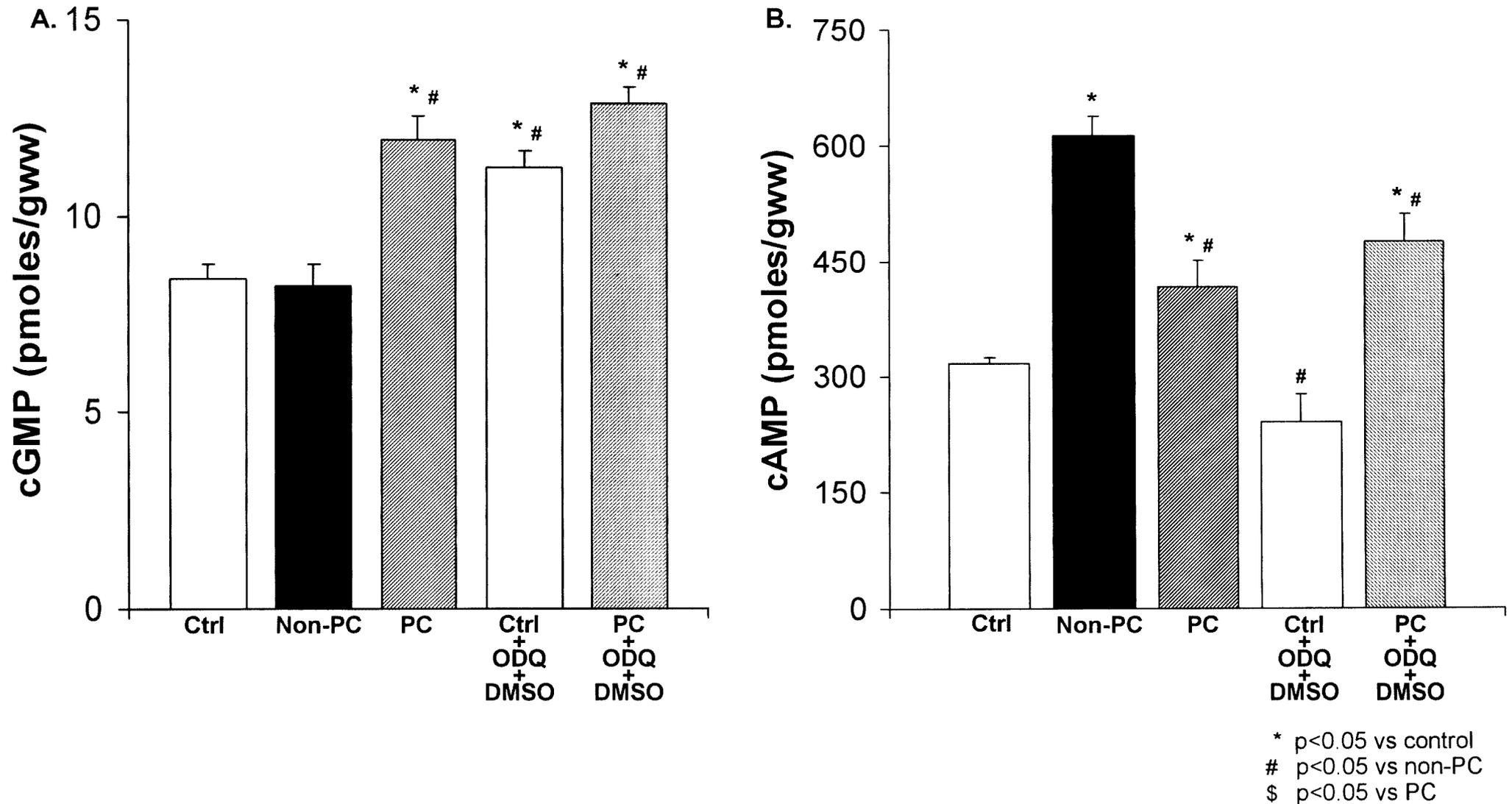
**Preconditioned + ODQ hearts:**



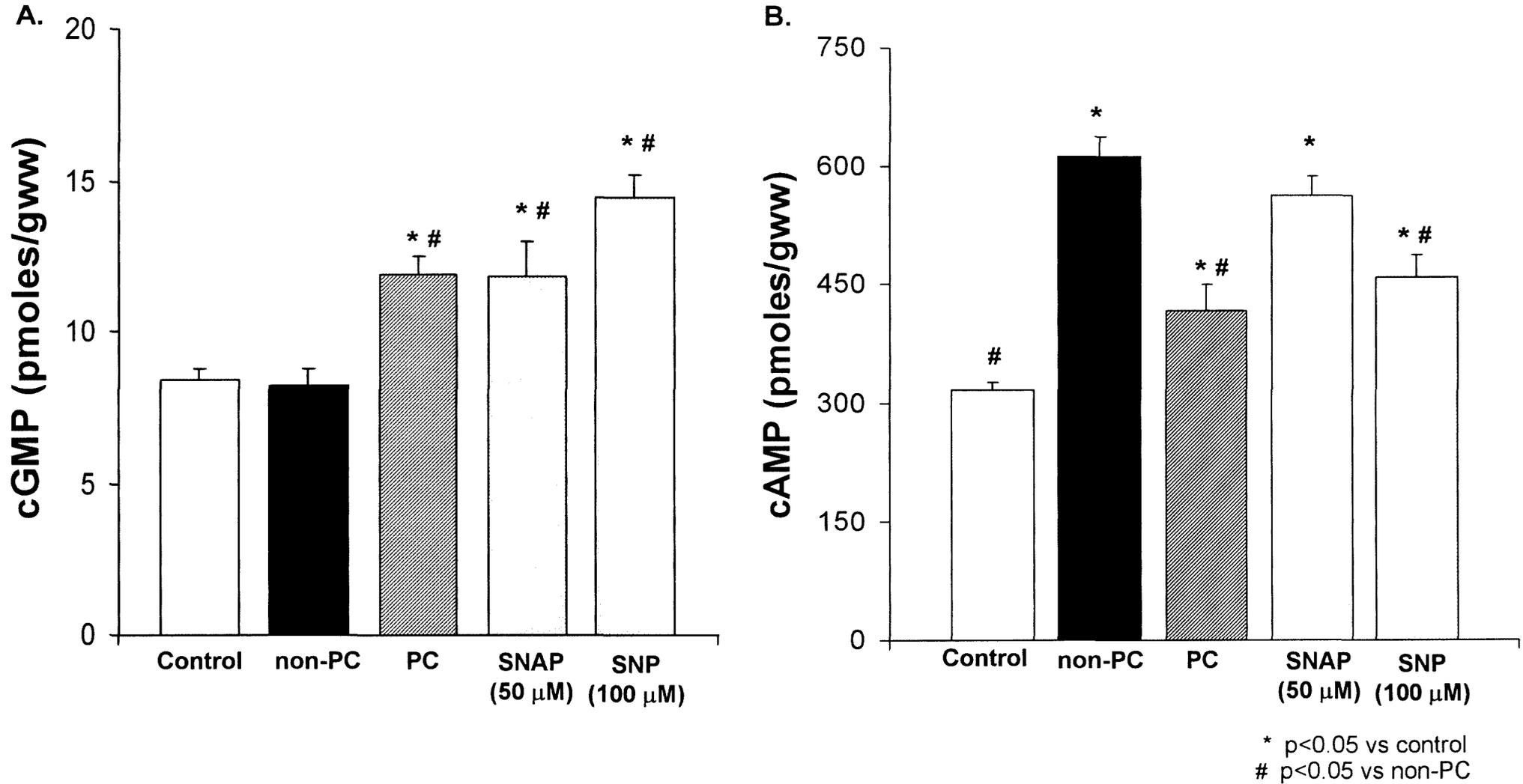
**NO donors (SNAP or SNP):**



**Fig 5.10** Tissue cyclic nucleotide responses to 25 min sustained global ischaemia after prior manipulation of the NO-cGMP pathway. Experimental protocol. Abbreviations as in Fig 3.1. Prior to 25 min sustained global ischaemia, the inhibitor of guanylyl cyclase, ODQ (4 x 5 min, 20  $\mu$ M), was administered before and during the ischaemic PC protocol, while the three-episode ischaemic preconditioning protocol was mimicked by 3 x 5 min administrations of the NO donors, SNAP (50  $\mu$ M) or SNP (100  $\mu$ M) in the absence of ischaemia. Arrows indicate time of freeze-clamping for cGMP and cAMP determination. (n = 6 hearts per series).



**Fig 5.11** Tissue cGMP and cAMP levels after 25 min sustained ischaemia: effect of manipulation with ODQ (20  $\mu$ M). Treating control hearts with ODQ + DMSO (administered for 3 x 5 min interspersed with drug-free buffer, after equilibration for 30 min) increased cGMP, while lowering cAMP levels, compared to untreated controls. Therefore, treating PC hearts with ODQ + DMSO, no change in cGMP but a significant increase in cAMP occurred compared to control hearts treated with ODQ+DMSO.



**Fig 5.12** Tissue cGMP and cAMP levels after 25 min sustained ischaemia: effect of manipulation with NO donors. Both SNAP (50 μM) and SNP (100 μM) increased the cGMP levels to the same extent as ischaemic PC, while reducing the cAMP levels at the end of sustained ischaemia. However, the SNAP induced reduction in cAMP levels was not significant.

**Table 5.3****Tissue cyclic nucleotides and cAMP/cGMP ratio at the end of 25 min sustained ischaemia**

	<b>cGMP</b>	<b>cAMP</b>	<b>cAMP/cGMP</b>
Controls	8.42 ± 0.38	317.0 ± 9.27 *	37.65
Controls (DMSO + ODQ)	11.22 ± 0.43 *	241.5 ± 10.10 *	24.60 ± 2.56
<u>After 25 min sustained ischaemia:</u>			
Non-PC	8.20 ± 0.58	612.7 ± 25.80	58.62 ± 6.37
PC	11.92 ± 0.62 *	417.5 ± 34.00 *	36.41 ± 3.19*
PC+ODQ (20 µM, in DMSO)	12.87 ± 0.42 *	476.4 ± 35.59 δ	37.02 ± 3.21*δ
SNAP (10 µM)	13.27 ± 0.76 *	554.3 ± 31.05	41.8 ± 1.58*
SNAP (50 µM)	11.86 ± 1.12 *	563.6 ± 25.44	22.24 ± 3.66*
SNP (100 µM)	14.45 ± 0.76 *	460.6 ± 27.42 *	31.93 ± 2.66*

Values are means ± SE in pmoles/g wet weight; n = >6 hearts /series. Controls were perfused for 60 min (15 min retrograde, 15 min working heart, 30 min retrograde). Controls + DMSO + ODQ: DMSO (0.04%) + ODQ (20 µM) were administered for 3 x 5 min interspersed with a drug-free buffer after equilibration for 30 min. PC+ODQ, SNAP and SNP were administered as shown in Fig. 5.12.

\* p < 0.05 vs non-PC

δ p < 0.05 vs controls + DMSO + ODQ

# p < 0.05 vs PC

and Table 5.3). ODQ dissolved in DMSO had no significant effect on the cAMP levels in either control or PC hearts compared to their untreated levels, respectively (Fig. 5.11B and Table 5.3). However, introducing ODQ + DMSO to PC hearts increased the significant percentage rise in cAMP occurring in untreated PC hearts after 25 min ischaemia from 32% (untreated control vs. untreated PC) to 97% (control + ODQ + DMSO vs. PC + ODQ + DMSO). Of interest is the similarity between the percentage increase in cAMP content of non-PC (93%) and ODQ + DMSO treated PC (97%) hearts, when compared with their respective controls (Fig. 5.11B).

Comparison of cGMP and cAMP values at the end of 25 min sustained ischaemia, showed that an ischaemic PC protocol caused a significant reduction in cAMP/cGMP ratio, when compared to non-PC hearts (Table 5.3). Although ODQ dissolved in DMSO significantly reduced cAMP/cGMP ratio in PC hearts compared to non-PC (Table 5.3), it was significantly increased compared to control hearts treated with ODQ + DMSO.

#### **5.2.2.1.2.2 NO donors**

At the end of sustained ischaemia the NO donors SNAP (50  $\mu$ M) and SNP (100  $\mu$ M) also caused significant increases in cGMP when compared to non-PC hearts (Fig. 5.12A). However, although both the donors significantly reduced the cAMP/cGMP ratio, SNAP had no effect on tissue cAMP levels, while SNP significantly lowered tissue cAMP compared to non-PC hearts (Fig. 5.12B and Table 5.3).

### **5.2.2.2 Effect of NO-cGMP pathway manipulations on p38 MAPK activation at the end of sustained ischaemia**

To assess whether p38 MAPK activation was involved in the protection induced by the cyclic elevation of cGMP during the PC protocol, it was necessary to compare changes in p38 MAPK activation during sustained ischaemia of NO or cGMP manipulated- and ischaemic- preconditioned hearts.

#### **5.2.2.2.1 Experimental protocol**

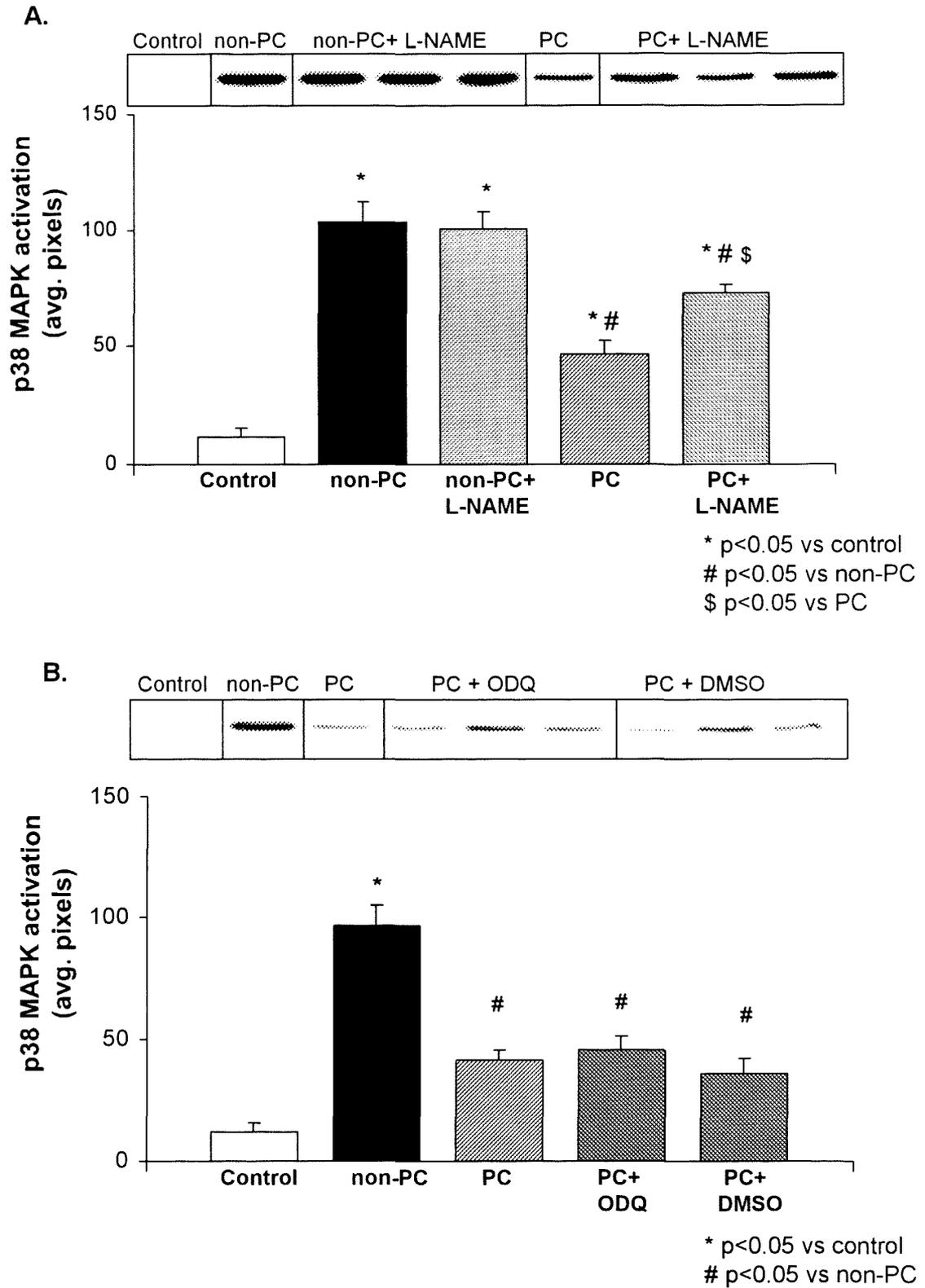
As described before, non-PC, ischaemic PC (with and without ODQ or L-NAME) and NO donor (SNAP or SNP)-treated hearts were freeze-clamped at the end of 25 min

global ischaemia (as Fig. 5.10). At least 4 hearts in each series were assayed for dual phosphorylation of p38 MAPK.

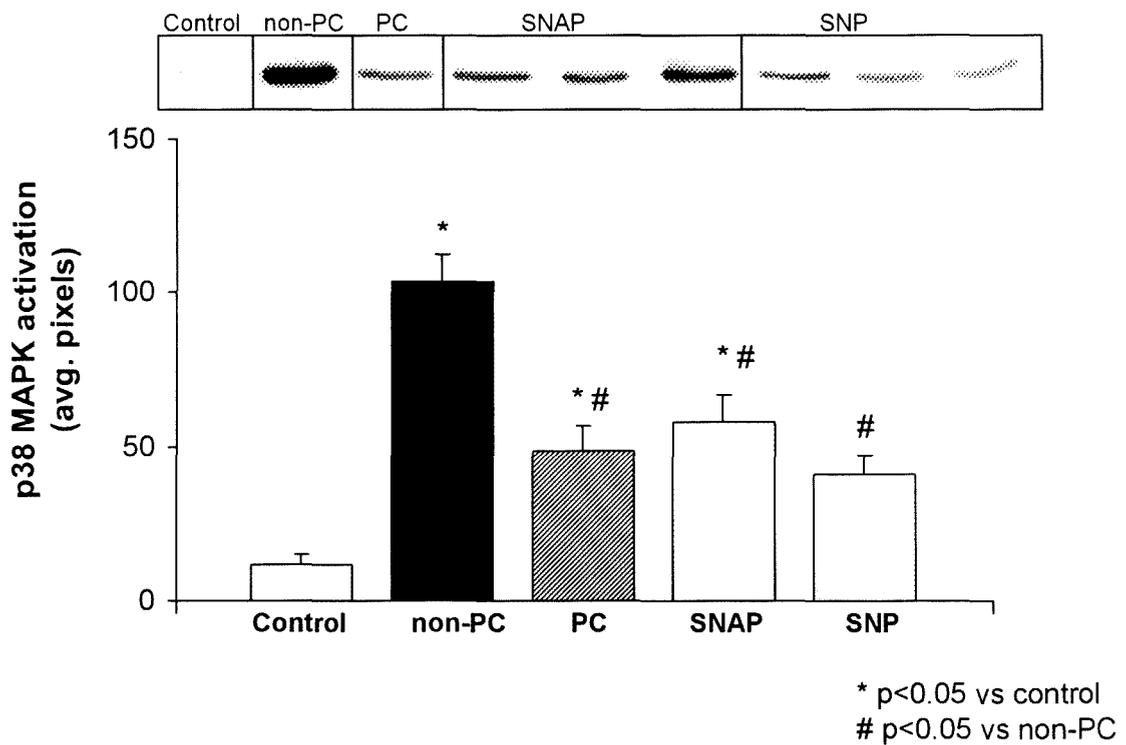
#### 5.2.2.2.2 Results

At the end of sustained ischaemia the significant increase in p38 MAPK activation in non-PC hearts, was unaffected by prior L-NAME-treatment (Fig. 5.13A). In contrast, inhibition of p38 MAPK activation during the PC protocol by L-NAME before sustained ischaemia (Fig. 5.8B), was associated with a significant increase in p38 MAPK activation when compared to untreated PC hearts at the end of sustained ischaemia (Fig. 5.13A). Conversely, as ODQ + DMSO or DMSO alone caused decreases in p38 MAPK activation during the PC protocol (Fig. 5.8C), p38 MAPK of ODQ-treated PC hearts remained attenuated at the end of sustained ischaemia similar to untreated PC hearts (Fig. 5.13B).

Furthermore, dual phosphorylation of p38 MAPK by NO donors (SNAP and SNP) before the onset of sustained ischaemia (Fig. 5.9) was associated with a significant attenuation in p38 MAPK activation at the end of sustained ischaemia (Fig. 5.14) when compared to untreated non-PC hearts. These results were comparable to activation levels of p38 MAPK in ischaemic PC hearts.



**Fig 5.13** Effect of NO-GMP pathway inhibition on p38 MAPK activation at the end of 25 min sustained ischaemia. **A.** Prior administration of L-NAME (50  $\mu$ M) to PC hearts increased p38 MAPK activation compared to untreated PC hearts, **B.** however ODQ (20  $\mu$ M) could not reverse PC-induced attenuation of p38 MAPK activation. A representative blot is given for each experiment. n = 4 – 6 hearts/series.



**Fig 5.14** Effect of NO-GMP pathway stimulation on p38 MAPK activation at the end of 25 min sustained ischaemia. Prior administration of NO donors, SNAP (50  $\mu$ M) or SNP (100  $\mu$ M), to non-PC hearts, significantly attenuated p38 MAPK activation at the end of sustained ischaemia. A representative blot is given for each experiment. n = 4 – 6 hearts/series.

## **5.2.3 Evaluation of events after sustained ischaemia during reperfusion: Role of NO-cGMP pathway in eliciting protection**

To establish whether NO release and thus the increases in tissue cGMP levels during the PC protocol acts as a trigger and/or a mediator in eliciting protection, isolated working rat hearts were subjected to (i) inhibition of guanylyl cyclase (GC) or nitric oxide synthase (NOS) with ODQ or L-NAME, respectively, during the PC protocol or (ii) pharmacological elevation of tissue cGMP levels by NO donors, SNAP or SNP, before onset of sustained ischaemia. Functional recovery during reperfusion was used as endpoint to evaluate the extent of protection against ischaemic damage.

### **5.2.3.1 Effect of inhibition of guanylyl cyclase (GC) or nitric oxide synthase (NOS) during PC protocol on functional recovery**

#### **5.2.3.1.1 Experimental protocol**

To establish whether NO acts as a trigger and/or mediator in ischaemic PC, the following protocols were used:

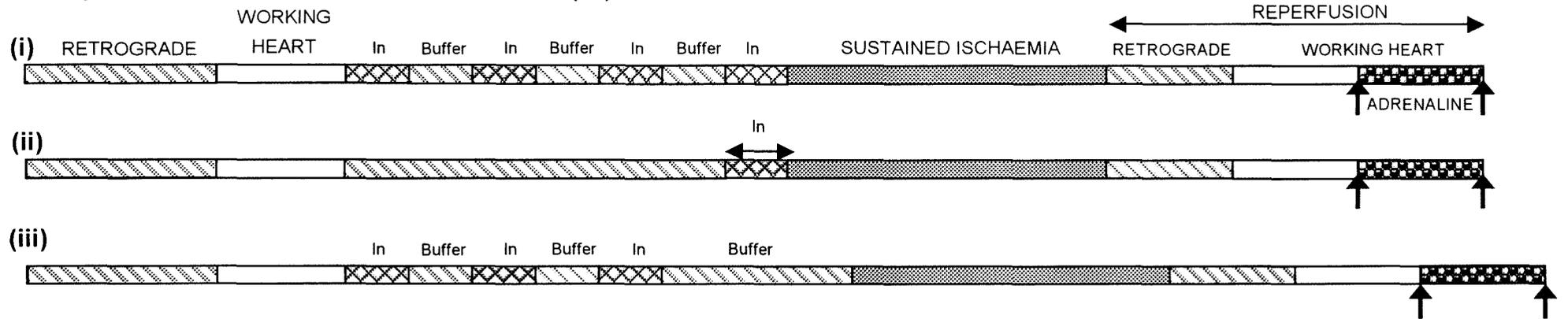
##### **(i) Trigger and Mediator:**

In the PC hearts, either ODQ (20  $\mu$ M) or L-NAME (50  $\mu$ M) or L-NNA (50  $\mu$ M) was administered during the PC protocol, 5 min before and after PC1- and during reperfusion after PC2- and PC3-. In non-PC hearts, each of these drugs was administered for 4 x 5 min, interspersed by 5 min perfusion with normal buffer before onset of sustained ischaemia. All hearts were subsequently subjected to 25 min global ischaemia followed by 30 min reperfusion (10 min retrograde, 20 min working heart) (Fig. 5.15i).

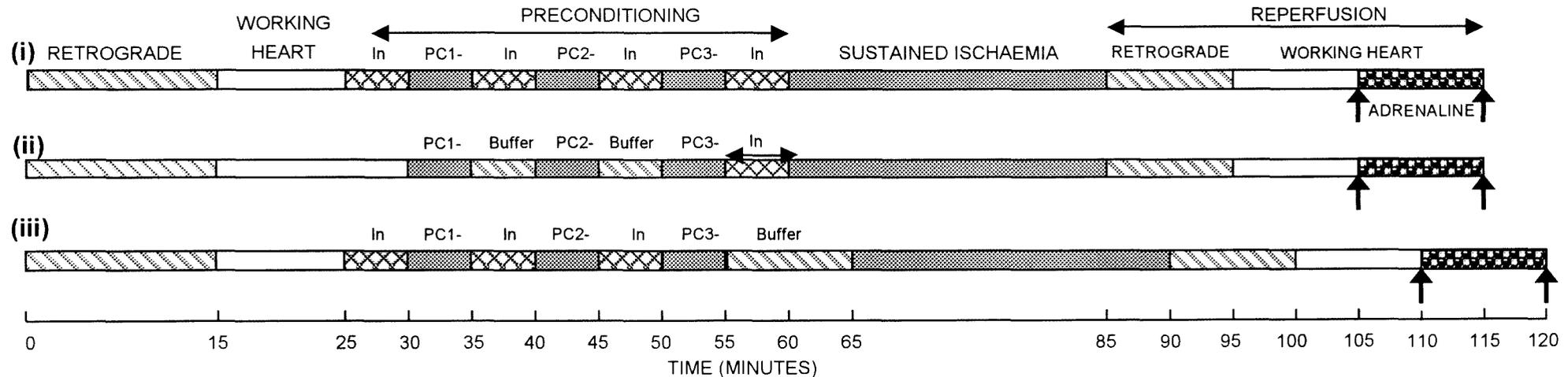
##### **(ii) Mediator:**

Hearts were preconditioned as described above and ODQ (20  $\mu$ M) or L-NAME (50  $\mu$ M) administered for 5 or 15 min during reperfusion after the PC protocol (PC3+), before the onset of sustained ischaemia. In non-PC hearts, drugs were given for 5 or 15 min before the onset of sustained ischaemia (Fig. 5.15ii).

**Non-preconditioned hearts + Inhibitors (In):**



**Preconditioned hearts + Inhibitors (In):**



**Fig 5.15** Effect of inhibition of the L-arginine-NO-cGMP pathway on functional recovery during reperfusion: Experimental protocol. (i) Trigger and mediator: Inhibitors (ODQ, 20  $\mu$ M or L-NAME, 50  $\mu$ M) were administered for 4x5 min until onset of sustained ischaemia, (ii) Mediator: Inhibitors were administered for 5 or 15 min until onset of sustained ischaemia, (iii) Trigger: Inhibitors were administered for 3x5 min and washed out for 10 min before onset of sustained ischaemia. Hearts were reperfused for 20 min after sustained ischaemia, then adrenaline ( $10^{-6}$  M) was added to the working heart. Arrows indicate time points of measurement of mechanical function during reperfusion (6 hearts / time point).

(iii) **Trigger:**

In PC hearts, either ODQ (20  $\mu$ M) or L-NAME (50  $\mu$ M) was administered during the PC protocol, 5 min before the onset of PC1- and during reperfusion after PC1- and PC2-. After PC3- hearts were perfused for 10 min with drug-free buffer only (to wash out the inhibitors), before onset of sustained ischaemia. In non-PC hearts the inhibitors were administered for 3 x 5 min alternated with perfusion with buffer and followed by a washout period before sustained ischaemia (Fig. 5.15iii).

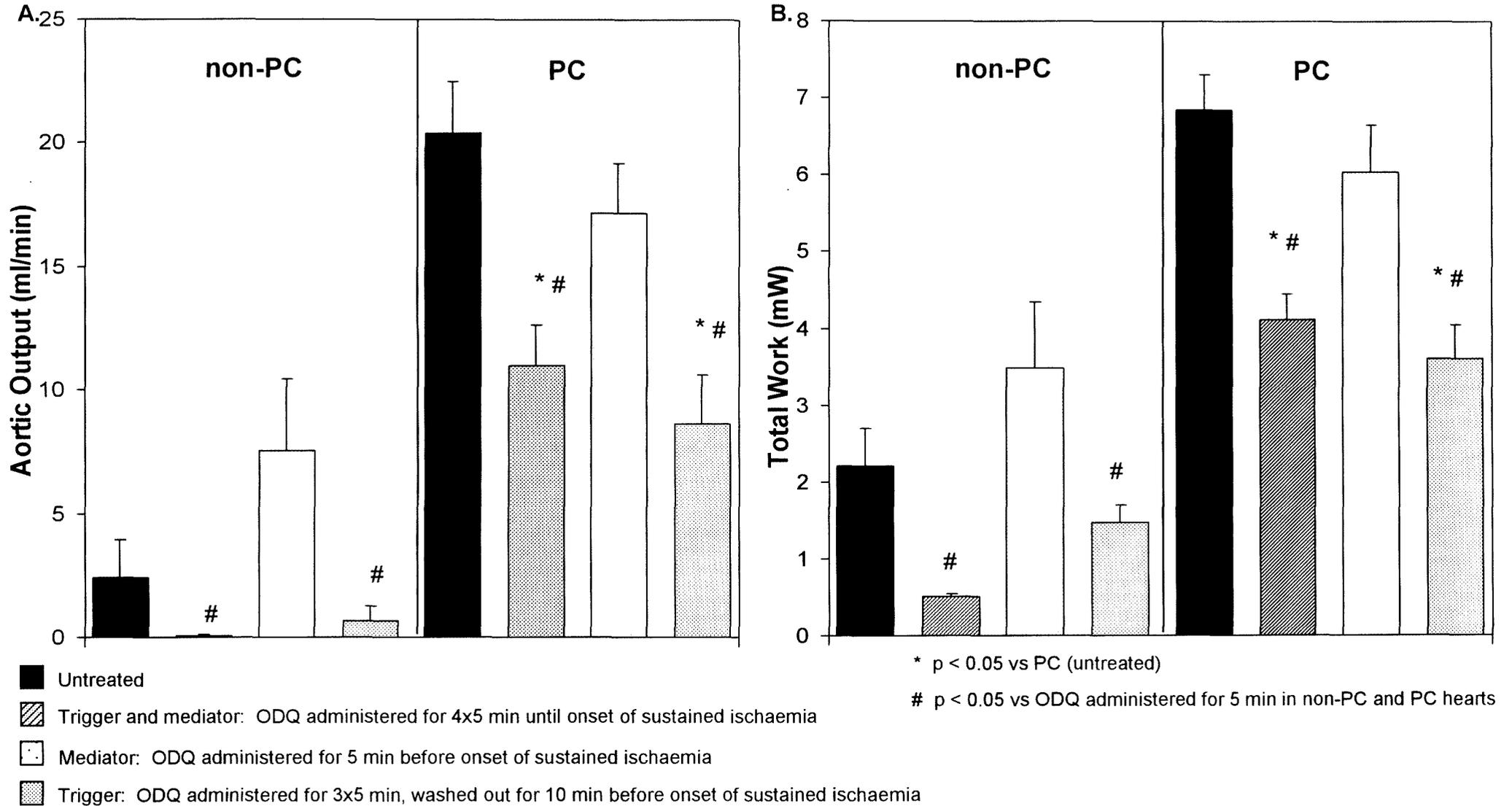
Functional recovery was measured after 20 min reperfusion (105 min or 110 min total perfusion time) of each experimental group, as indicated in Fig. 5.15. Table 5.4, Figs. 5.16 and 5.17 summarize the effects of the inhibitors ODQ, L-NAME and L-NA on the mechanical recovery during reperfusion.

The contractile reserve or recovery potential of hearts subjected to treatment with GC or NOS inhibitors was assessed by adding adrenaline ( $10^{-6}$  M) during the reperfusion phase for 10 min and monitoring changes in function occurred at 115 or 120 min total perfusion time (Fig. 5.15). The results were expressed as the percentage increase induced by adrenaline when compared to the values obtained during reperfusion before stimulation (Table 5.5).

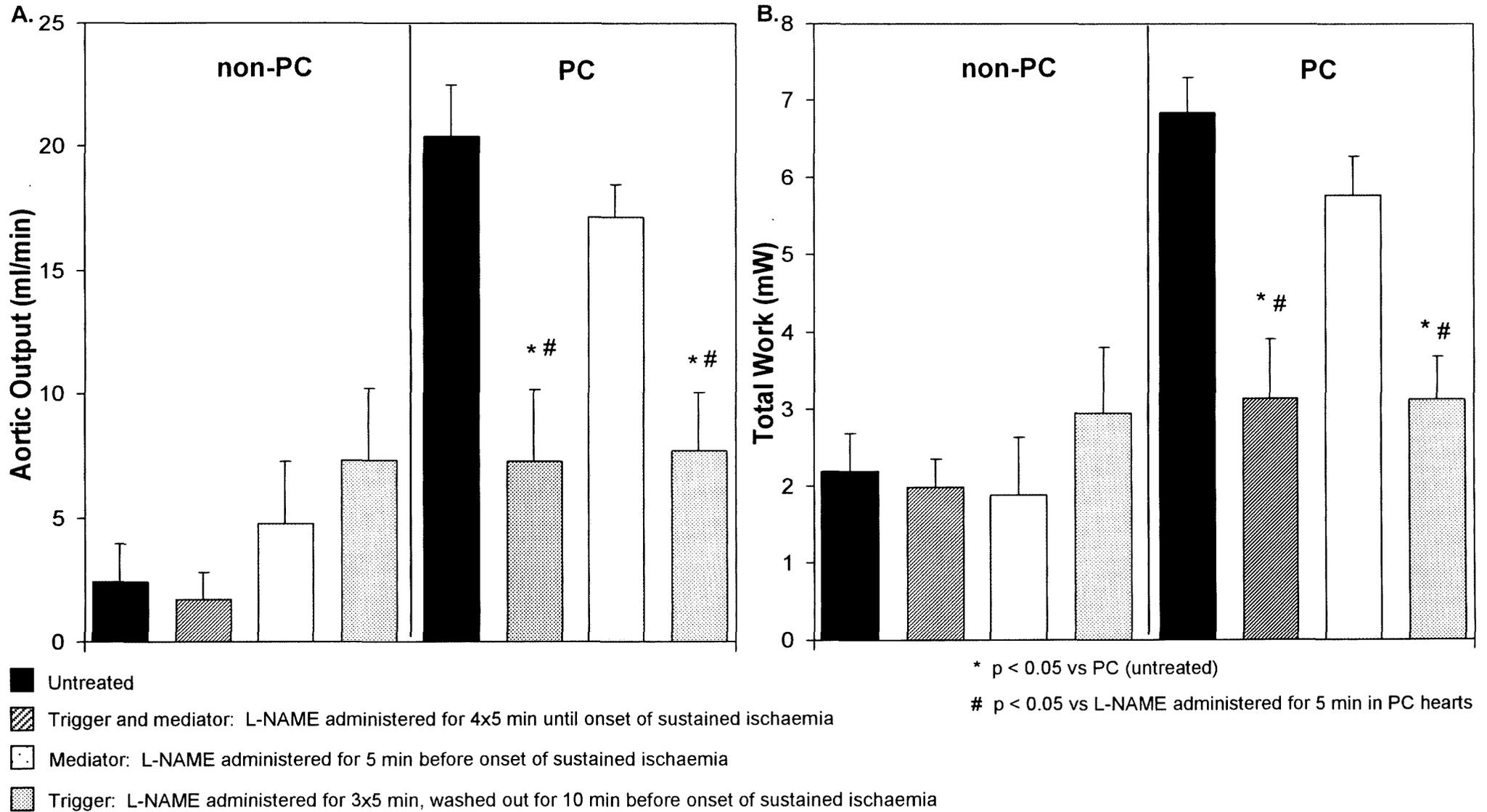
### 5.2.3.1.2 Results

#### 5.2.3.1.2.1 Vehicle

Since DMSO was used as a solvent for ODQ (L-NAME and L-NA being water soluble), a series of non-PC and PC hearts was studied in which DMSO alone was administered either 4 x 5 min or 15 min before onset of sustained ischaemia (Fig. 5.15). DMSO when administered for 4 x 5 min caused a significant reduction in coronary flow rate and cardiac output of non-PC hearts, while 15 min of administration had no significant effect (Table 5.4). However, DMSO (regardless of the protocol of administration) had no effect on any parameter of functional recovery in PC hearts.



**Fig 5.16** Effect of ODQ (20  $\mu$ M, dissolved in DMSO) pretreatment on functional recovery during reperfusion of non-PC and PC hearts. ODQ reduced the aortic output as well as the work performance of PC hearts when given during the PC protocol for either 4 x 5 min or 3 x 5 min, but washed out for 10 min before the onset of sustained ischaemia, however ODQ had no effect on functional recovery when given 5 min before the onset of sustained ischaemia. These results suggest a role for NO as a trigger.



**Fig 5.17** Effect of L-NAME (50  $\mu$ M) pretreatment on functional recovery during reperfusion of non-PC and PC hearts. L-NAME reduced the aortic output as well as the work performance of PC hearts when given during the PC protocol for either 4 x 5 min or 3 x 5 min, but washed out for 10 min before the onset of sustained ischaemia, however L-NAME had no effect on functional recovery when given 5 min before the onset of sustained ischaemia. These results also suggest a role for NO as a trigger.

**Table 5.4**

**Effect of ischaemic preconditioning, ODQ, L-NAME and L-NA on functional recovery during reperfusion after 25 min sustained ischaemia.**

	Coronary flow (ml/min)	Cardiac output (ml/min)	Peak systolic pressure (mmHg)	Heart Rate (beats/min)
<b>Before arrest</b> (7)	14.1 ± 0.4	56.1 ± 1.5	104 ± 3	271 ± 15
<b>During Reperfusion:</b>				
<b>Non-Preconditioned:</b>				
Untreated (6)	11.2 ± 1.5	13.6 ± 2.0 $\delta$	68 ± 3.7 $\delta$	213 ± 20 $\delta$
Vehicle:				
4x5 min DMSO (6)	4.0 ± 1.9 $\dagger$	4.5 ± 1.9 $\dagger$	34 ± 14	115 ± 39
15 min DMSO (3)	9.7 ± 0.4	13.1 ± 1.4	79 ± 1.4	192 ± 10
Trigger + mediator (4x5 min):				
20 $\mu$ M ODQ + DMSO (7)	5.4 ± 1.7	5.4 ± 1.7	32 ± 9 $\dagger$	120 ± 31
50 $\mu$ M L-NAME (6)	8.8 ± 2.4	10.5 ± 2.6	69 ± 13	183 ± 37
50 $\mu$ M L-NA (4)	8.6 ± 1.2	9.7 ± 1.8	68 ± 9	271 ± 13 $\dagger$
Mediator:				
5 min ODQ + DMSO (9)	10.5 ± 1.6	18.1 ± 3.7	80 ± 7	207 ± 10
15 min ODQ + DMSO (4)	3.1 ± 1.6 $\dagger$	3.1 ± 1.6 $\dagger$	26.3 ± 13	94 ± 49
5 min L-NAME (7)	5.8 ± 0.9 $\dagger$	10.6 ± 3.2	59 ± 12	175 ± 8
15 min L-NAME (4)	7.2 ± 0.4	16.0 ± 1.6	90 ± 1.0	211 ± 2.4
Trigger:				
3 x 5 min ODQ + DMSO (7)	8.6 ± 0.5	9.2 ± 1.0	70 ± 5	197 ± 17
3 x 5 min L-NAME (6)	9.6 ± 0.8	16.6 ± 2.5	82 ± 3	240 ± 16

Table 5.4 /...

.../ Table 5.4

	Coronary flow (ml/min)	Cardiac output (ml/min)	Peak systolic pressure (mmHg)	Heart Rate (beats/min)
<b>Before arrest (7)</b>	14.1 ± 0.4	56.1 ± 1.5	104 ± 3	271 ± 15
<b>During Reperfusion:</b>				
<b>Preconditioned:</b>				
Untreated (7)	13.7 ± 0.3	34.2 ± 2.2†	90 ± 2.5†	218 ± 7
Vehicle:				
4x5 min DMSO (7)	12.4 ± 1.1	27.7 ± 2.5†	83 ± 2.0	242 ± 6†
15 min DMSO (4)	11.6 ± 0.6	29.1 ± 3.5†	85 ± 1.5	208 ± 1.4
Trigger + mediator (4x5min):				
20 µM ODQ +DMSO (4)	10.3 ± 0.9	19.2 ± 0.6 †δ	86 ± 4	211 ± 14
50 µM L-NAME (8)	9.3 ± 1.0	16.6 ± 3.6 †δ	77 ± 6	229 ± 21
50 µM L-NA (6)	7.1 ± 0.5	14.85 ± 2.1 †δ	89 ± 3	202 ± 25
Mediator:				
5 min ODQ +DMSO (6)	11.8 ± 0.7	29.0 ± 3.0†	92 ± 3†	209 ± 17
15 min ODQ +DMSO (4)	11.0 ± 1.1	21.2 ± 0.1†	83 ± 1.1	188 ± 22
5 min L-NAME (6)	10.8 ± 0.8	28.1 ± 1.6†	90 ± 4†	207 ± 19
15min L-NAME (4)	8.2 ± 1.4	24.2 ± 5.9†	83 ± 9	184 ± 22
Trigger:				
3x5 min ODQ +DMSO (9)	10.0 ± 1.0	18.6 ± 2.5 †δ	86 ± 2	219 ± 15
3x5 min L-NAME (7)	9.0 ± 0.7	16.8 ± 2.8 †δ	83 ± 3	240 ± 19

Results expressed as the mean values ± SE obtained after 20 min of reperfusion. Numbers in parentheses indicate number of hearts.

In all series, the parameters of function measured during reperfusion differed significantly ( $p < 0.05$ ) vs before arrest (control values)

†  $p < 0.05$  vs non-PC

δ  $p < 0.05$  vs PC

### 5.2.3.1.2.2 Inhibitors to non-PC hearts

ODQ, regardless of its mode of administration, significantly affected functional recovery of non-PC hearts. For example, administration of ODQ for 5 min before onset of sustained ischaemia yielded values significantly higher than when administered for 3 x 5 or 4 x 5 min (Fig. 5.16). On the other hand, L-NAME administration had no effect on functional recovery during reperfusion of non-PC hearts, regardless of the protocol used (Fig. 5.17 and Table 5.4).

### 5.2.3.1.2.3 Inhibitors to PC hearts

#### (i) Trigger and Mediator:

Inhibitors administered before and during the PC protocol up to the onset of sustained ischaemia (4 x 5 min) will allow evaluation of the roles of NO and cGMP as trigger as well as mediator of protection.

Inhibition by ODQ + DMSO (4 x 5 min) caused partial inhibition of the protection induced by PC: in these hearts aortic output (Fig. 5.16A) and total work performance (Fig. 5.16B) during reperfusion averaged  $10.98 \pm 1.6$  ml/min and  $4.12 \pm 0.3$  mW respectively compared to  $20.5 \pm 2.1$  ml/min and  $6.9 \pm 0.5$  mW respectively in PC hearts. Inhibition of NOS activity by L-NAME or L-NA in a similar protocol also caused partial inhibition of the protection conferred by PC (Fig. 5.17A, B and Table 5.4).

#### (ii) Mediator:

Administration of ODQ (in DMSO) or L-NAME after the PC protocol, either for 5 min or 15 min before the onset of sustained ischaemia, had no effect and post-ischaemic function (aortic output: Figs 5.16A and 5.17A and total work: Figs 5.16B and 5.17B) was similar to that of PC hearts, suggesting that NO does not act as a *mediator*.

#### (iii) Trigger:

Both ODQ (in DMSO) and L-NAME, when given during the PC protocol, but washed out for 10 min before the onset of sustained ischaemia (Fig. 5.15iii), caused a significant reduction in aortic output (Figs 5.16A and 5.17A) and total work performance (Figs

5.16B and 5.17B), as well as cardiac output (Table 5.4), when compared with untreated PC hearts. These results suggest a role for NO as a *trigger*.

#### **5.2.3.1.2.4 Effect of Adrenaline on recovery potential of hearts treated with GC or NOS inhibitors**

The percentage increases in all parameters induced by adrenaline were similar in untreated PC and non-PC hearts. Similarly, neither of the protocols with ODQ (3 x 5 min or 5 min; in DMSO) or L-NAME caused a significant difference in recovery potential when compared with untreated PC and non-PC hearts. However, significantly higher increases in coronary flow rates, cardiac output and total work performance were observed in both PC and non-PC hearts treated with 3 x 5 min L-NAME. On the other hand, administration of ODQ for 15 min before the onset of sustained ischaemia, significantly impaired recovery potential in non-PC hearts (Table 5.5). Furthermore, 3 x 5 min ODQ caused a significant reduction in recovery potential of PC hearts.

**Table 5.5**

**Effect of Adrenaline ( $10^{-6}$  M) on mechanical performance during reperfusion of hearts after treatment with inhibitors.**

% increase	Coronary flow	Cardiac output	Peak systolic pressure	Heart rate	Total Work performance
<b>Non-Preconditioned:</b>					
Untreated (6)	31.0 ± 8.1	62.1 ± 28.5	13.7 ± 4.0	52.8 ± 21.2	97 ± 35
Vehicle: 15 min DMSO (3)	39.3 ± 7.6	104.7 ± 18.4	11.4 ± 0.4	57.9 ± 19.1	128.6 ± 18.4
Mediator: 5 min ODQ* (9)	41.8 ± 5.2	65.6 ± 6.3	16.7 ± 5.4	45.4 ± 6.5	94.3 ± 8.8
15 min ODQ* (4)	6.34 ± 14.1†	6.34 ± 14.1†	-21.1 ± 5.7†	1.91 ± 19.9†	-14.9 ± 17.5†
5 min L-NAME (7)	60.2 ± 11.9	72.9 ± 18.4	34.5 ± 10.9	46.1 ± 5.8	137.2 ± 33.0
15 min L-NAME (6)	53.3 ± 10.4	42.3 ± 13.5	6.3 ± 1.6	40.0 ± 3.2	52.6 ± 16.1
Trigger: 3 x 5 min ODQ* (7)	24.0 ± 3.0	26.8 ± 5.2	5.3 ± 2.7	56.0 ± 16.3	33.7 ± 7.5
3 x 5 min L-NAME (6)	66.3 ± 7.8†	149 ± 22.3†	20.3 ± 3.0	35.7 ± 9.1	205 ± 37.6†
<b>Preconditioned:</b>					
Untreated (9)	41.7 ± 5.5	42.3 ± 5.2	7.1 ± 2.5	46.5 ± 3.0	54.4 ± 7.6
Vehicle: 15 min DMSO (4)	42.3 ± 5.9	56.2 ± 7.7	9.6 ± 2.1	51.4 ± 6.7	72.8 ± 11.1
Mediator: 5 min ODQ* (6)	56.1 ± 4.7	38.0 ± 6.2	5.8 ± 0.9	55.1 ± 8.1	46.8 ± 6.9
15 min ODQ* (4)	43.3 ± 4.4	63.5 ± 12.8	9.4 ± 1.8	41.2 ± 7.2	81.0 ± 16.9
5 min L-NAME (6)	58.2 ± 7.9	78.6 ± 10.4	9.6 ± 2.1	51.3 ± 13.2	98.2 ± 13.3
15min L-NAME (6)	37.5 ± 9.1	34.0 ± 12.1	3.9 ± 2.0	32.1 ± 9.1	40.3 ± 15.1
Trigger: 3 x 5 min ODQ* (9)	24.5 ± 5.9	14.7 ± 8.2δ	1.3 ± 1.9	35.1 ± 9.1	16.9 ± 9.5δ
3 x 5 min L-NAME (5)	65.2 ± 8.5δ	116.0 ± 13.8δ	17.1 ± 1.5δ	33.6 ± 5.4	156.1 ± 17.6δ

Values expressed as the percentage increase from results obtained after 20 min of reperfusion. Adrenaline was administered after 20 min of reperfusion and mechanical performance monitored after 10 min. Hearts were preconditioned or treated with the NO inhibitors as shown in Fig 5.17. Numbers in parentheses indicate number of hearts. (\*ODQ dissolved in DMSO).

† p < 0.05 vs non-PC

δ p < 0.05 vs PC

### **5.2.3.2 Effect of NO donors on functional recovery**

#### **5.2.3.2.1 Experimental protocol**

After a stabilization period of 30 min, hearts were subjected to either 30 min retrograde perfusion (non-PC) or 3 x 5 min global ischaemia (PC) or 3 x 5 min SNAP (10 or 50  $\mu\text{M}$ ) or 3 x 5 min SNP (100  $\mu\text{M}$ ) or 3 x 5 min L-arginine (10 mM) prior to 25 min sustained global ischaemia, followed by 30 min reperfusion (10 min retrograde, 20 min working heart) (Fig. 5.18). Administration of donors was interspersed by normal buffer perfusion. Functional performance was evaluated in all series before the onset of sustained ischaemia (30 min total perfusion time) and during reperfusion (105 min total perfusion time (Fig. 5.18).

In order to assess and compare the contractile reserve of hearts subjected to treatment with NO donors, adrenaline ( $10^{-6}$  M) was administered during the reperfusion phase and the changes in function monitored. Non-PC, PC, SNAP or SNP treated hearts were perfused as described above until 20 min of reperfusion (105 min total perfusion time). After registration of mechanical performance at this time-point, adrenaline ( $10^{-6}$  M) was added to the working heart and the mechanical activity monitored after 10 min (Fig. 5.18).

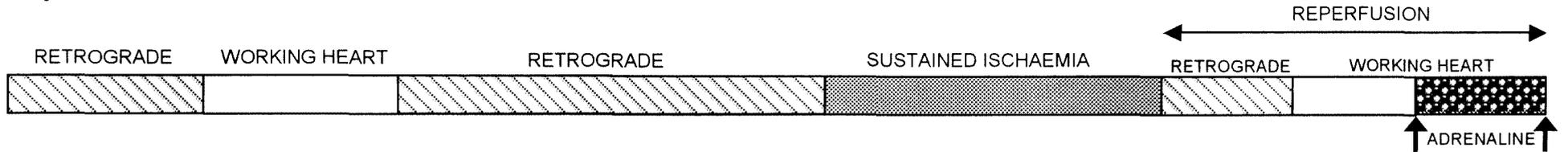
#### **5.2.3.2.2 Results**

##### **5.2.3.2.2.1 NO donors**

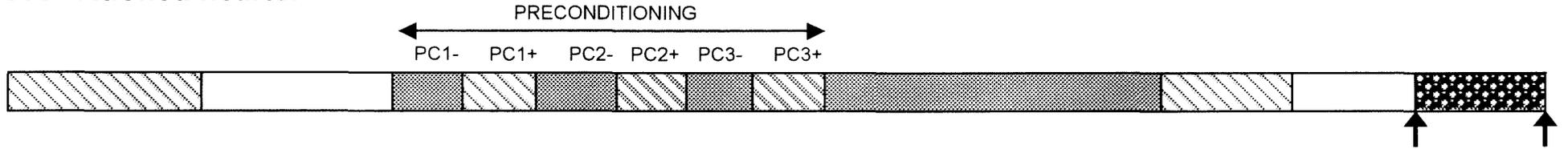
In all series studied, exposure of the heart to 25 min sustained global ischaemia caused a significant decline in all parameters of function measured during reperfusion compared to values obtained before arrest. However, comparison of the functional performance during reperfusion revealed significant differences between the groups (Table 5.6).

Intermittent administration (3 x 5 min) of NO donors SNAP (50  $\mu\text{M}$ ) or SNP (100  $\mu\text{M}$ ), prior to 25 min sustained ischaemia, significantly improved functional recovery during reperfusion, when compared to non-PC hearts. The protection afforded by these

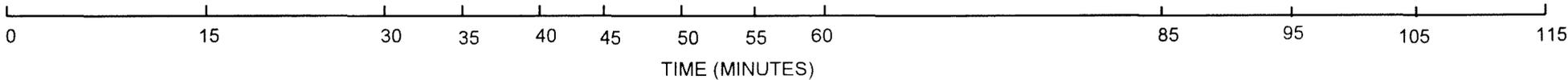
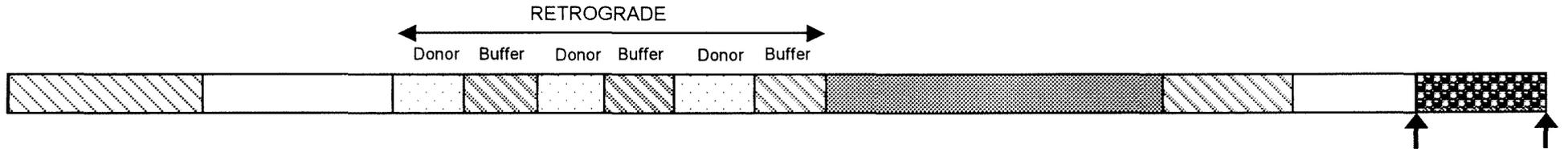
**Non-preconditioned hearts:**



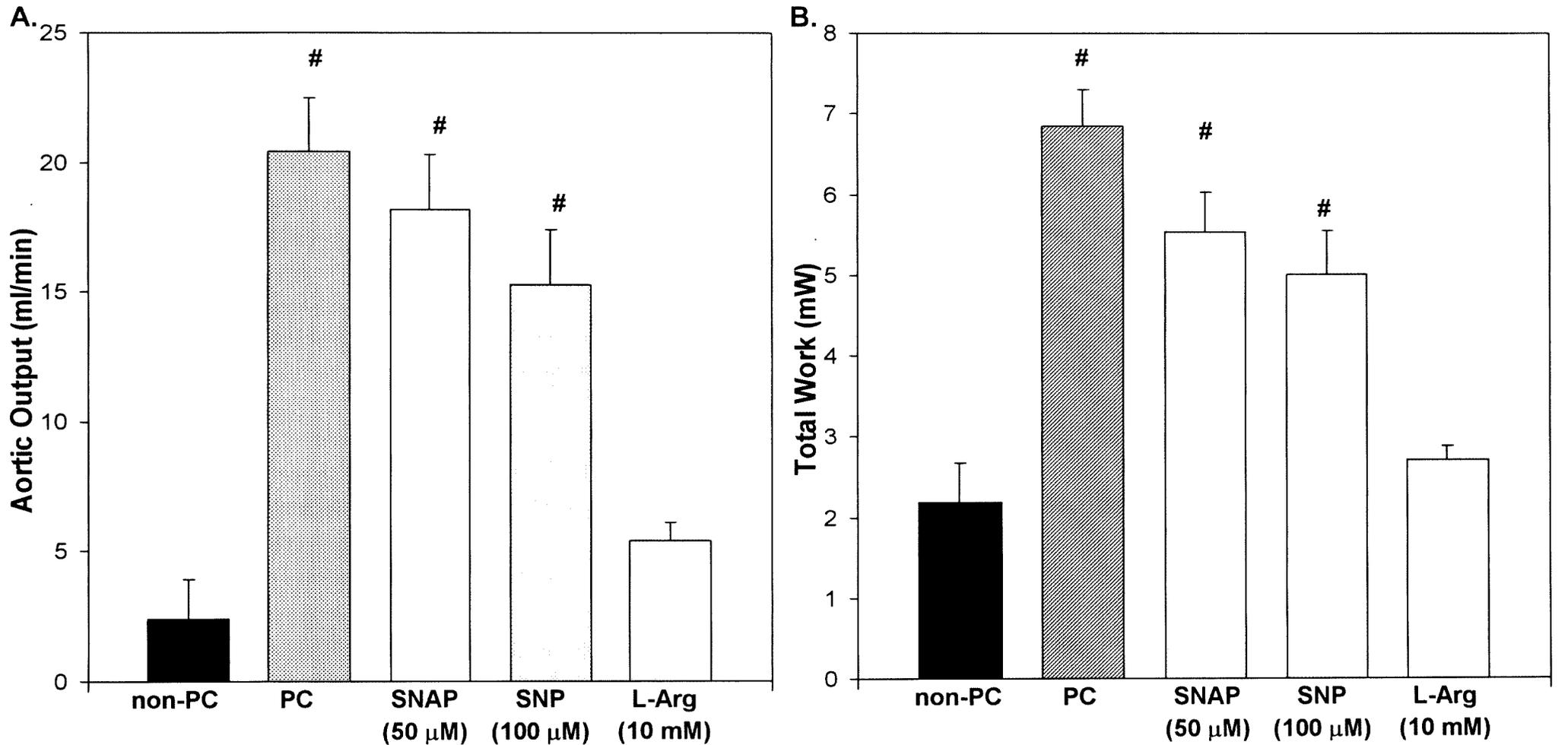
**Preconditioned hearts:**



**NO donors (SNAP or SNP or L-Arg):**



**Fig 5.18** Effect of prior stimulation of the L-arginine-NO-cGMP pathway on functional recovery during reperfusion. Experimental protocol. Abbreviations as in Fig 3.1. Prior to 25 min sustained global ischaemia, the three-episode ischaemic preconditioning protocol was mimicked by 3 x 5 min administrations of the NO donors, SNAP (50  $\mu$ M) or SNP (100  $\mu$ M), or L-arginine (10 mM) in the absence of ischaemia. Hearts were reperfused for 20 min after sustained ischaemia, then adrenaline ( $10^{-6}$  M) was added to the working heart. Arrows indicate time points of measurement of mechanical function during reperfusion (6 hearts / time point).



(#  $p < 0.05$  vs non-PC)

**Fig 5.19** Effect of prior stimulation of the L-arginine-NO-cGMP pathway on functional recovery during reperfusion. Intermittent administration (3 x 5 min) of NO donors (SNAP, 50 μM or SNP, 100 μM), in the absence of ischaemia, improved aortic output as well as work performance during reperfusion when compared to non-PC hearts. However, L-arginine (3 x 5 min, 10 mM) did not improve functional recovery.

**Table 5.6****Effect of ischaemic preconditioning and NO donors on functional recovery after 25 min sustained ischaemia**

	Coronary flow (ml/min)	Cardiac output (ml/min)	Peak systolic pressure (mmHg)	Heart rate (beats/min)
<u>Before arrest</u> (9)	12.4 ± 0.6	54.6 ± 2.0	101 ± 2	278 ± 9
<u>During reperfusion</u>				
Non-PC (6)	11.2 ± 1.5	13.6 ± 2.0*	68 ± 3.7*	213 ± 20*
PC (9)	13.7 ± 0.3	34.2 ± 2.2*†	90 ± 2.5†	218 ± 7
SNAP (10 µM) (2)	12.8 ± 0.53	30.8 ± 3.7*†	93 ± 0.5†	273 ± 11
SNAP (50 µM) (9)	8.5 ± 0.4	29.0 ± 2.6*†	89 ± 1†	248 ± 15
SNAP (100 µM) (2)	8.3 ± 0.5	25.3 ± 0.2*†	92 ± 0.7†	229 ± 8
SNP (100 µM) (10)	9.4 ± 0.8	30.5 ± 1.3*†	94 ± 2†	227 ± 5
L-Arginine (10 mM) (5)	9.4 ± 0.5	14.8 ± 0.8*δ	82 ± 1*	255 ± 13

Results expressed as the mean values ± SE obtained after 20 min of reperfusion. Numbers in parentheses indicate number of hearts.

In all series, the parameters of function measured during reperfusion differed significantly ( $p < 0.05$ ) vs before arrest (control values)

\*  $p < 0.05$  vs before arrest (control values)

†  $p < 0.05$  vs non-PC

δ  $p < 0.05$  vs PC

interventions was similar to that elicited by prior PC: aortic output (Fig. 5.19A), total work performance (Fig. 5.19B), cardiac output, peak systolic pressure and heart rate (Table 5.6) were similar in these groups. Different concentrations of SNAP induced similar protection: in hearts treated with 10, 50 and 100  $\mu\text{M}$  SNAP the aortic output averaged  $18.0 \pm 4.2$ ,  $20.5 \pm 2.5$  and  $17.0 \pm 0.7$  ml/min, respectively. L-arginine (10 mM; 3 x 5 min), on the other hand, did not improve functional recovery during reperfusion and values similar to those of non-PC hearts were obtained (Fig. 5.19A, B and Table 5.6).

#### **5.2.3.2.2.2 Effect of Adrenaline on recovery potential of hearts treated with NO donors**

The results in Table 5.7 were expressed as the percentage increase induced by adrenaline when compared to the values obtained during reperfusion of non-PC, PC, SNAP and SNP hearts before stimulation (Fig. 5.19A, B and Table 5.6). The percentage increases in all parameters induced by adrenaline were similar in PC and non-PC hearts. However, SNAP (50  $\mu\text{M}$ ) treated hearts showed significantly higher increases in coronary flow rates, cardiac output, peak systolic pressure and total work performance, when compared with non-PC, as well as PC hearts. Similarly, SNP (100  $\mu\text{M}$ ) treatment also caused significantly higher increases in cardiac output and total work performance (Table 5.7). These results indicate that hearts, in which protection was elicited by treatment with NO donors, have a superior recovery potential to those in whom ischaemia was used to induce protection.

**Table 5.7**

**Effect of Adrenaline ( $10^{-6}$  M) on mechanical performance during reperfusion of hearts on NO donors.**

<b>% increase</b>	<b>Coronary flow</b>	<b>Cardiac output</b>	<b>Peak systolic pressure</b>	<b>Heart rate</b>	<b>Total Work performance</b>
Non-Preconditioned	31.0 ± 8.1	62.1 ± 28.5	13.7 ± 4.0	52.8 ± 21.2	97 ± 35
Preconditioned	41.7 ± 5.5	42.3 ± 5.2	7.1 ± 2.5	46.5 ± 3.0	54.4 ± 9.3
SNAP (50 µM)	62.9 ± 7.5†	122.1 ± 18.0†δ	17.0 ± 0.5δ	50.2 ± 2.9	167.6 ± 22.7δ
SNP (100 µM)	44.0 ± 4.6	114.8 ± 13.3†δ	16.6 ± 1.3	53.1 ± 6.5	154.8 ± 16.8δ

Results expressed as the percentage increase from values obtained after 20 min of reperfusion. Adrenaline was administered after 20 min of reperfusion and mechanical performance monitored after 5 and 10 min. Hearts were preconditioned or treated with the NO donors as shown in Fig 5.20.

† p < 0.05 vs non-preconditioned

δ p < 0.05 vs Preconditioned

## 5.3 Discussion

The observation that a multi-cycle PC protocol elicits cyclic increases in both cGMP and cAMP (Fig. 3.2) suggests the possibility of a role for both as triggers in the phenomenon of ischaemic PC. To establish the relevance of the significant increases in cGMP *per se*, appropriate pharmacological agents were employed in the present study that allowed evaluation of such changes in the *absence* of concomitant changes in cAMP (Figs. 5.2 and 5.5). The results obtained suggest that NO and subsequent cGMP generation also act as a *trigger* in the phenomenon of classic PC – prevention of ischaemic PC-induced cyclic elevations in cGMP (by inhibition of either guanylyl cyclase or NOS activation) attenuated protection (Table 5.4, 5.5, Figs 5.16 and 5.17), while the non-endothelium dependent NO donors SNAP and SNP, both elicited protection against ischaemic damage (Table 5.6, 5.7, Fig. 5.19).

### 5.3.1 Abolishment of protection by prevention of cyclic increases in cGMP during PC

#### 5.3.1.1 Inhibitors and vehicles

The significance of the elevation of tissue cGMP during the PC protocol was investigated using L-NAME or L-NA, inhibitors of NOS, as well as ODQ, an inhibitor of soluble guanylyl cyclase. The latter has no effect on either NOS activity or NO generation (Hobbs *et al.*, 1997). Vehicle related studies were not required in the case of L-NAME or L-NA, since both are water soluble. According to the recommendations of the manufacturer (Tocris Cookson, Ltd), ODQ was dissolved in 0.04% DMSO. While DMSO alone had no effect on tissue cGMP levels, the combination of DMSO and ODQ caused an unexpected increase in basal cGMP levels. An explanation for this finding is not readily available.

In addition to the above, DMSO *per se*, when administered for 4 x 5 min before onset of sustained ischaemia caused a significant decline in functional recovery of the non-PC heart. Also in combination with ODQ, when administered for 4 x 5 min or for 15 min before the onset of sustained ischaemia, functional recovery (as evidenced by coronary flow, cardiac output and work performance) was significantly less than that of non-PC

hearts. These detrimental effects may be time-dependent, since they were not seen when ODQ and DMSO were administered for 5 min only (Table 5.4) and may indicate a direct toxic effect of ODQ+DMSO on the heart. This was also substantiated by the observation that the positive inotropic and chronotropic effects of adrenaline were absent in non-PC hearts pretreated with 15 min ODQ+DMSO (Table 5.5). However, these harmful effects were not seen in the PC hearts and regardless of the protocol used, similar values were obtained with ODQ+DMSO, L-NAME and L-NA. Whether the protection induced by ischaemic PC, also protects against the possible harmful effects of ODQ+DMSO, remains to be established.

The question thus arose whether any significance could be attached to observations made with ODQ+DMSO. After careful evaluation of the data, it was decided that, while recognizing its putative toxic effects on the non-PC heart, ODQ could be used as inhibitor in our studies, since (i) it had no detrimental effects on the PC heart and (ii) its effects were similar to those of L-NAME and L-NA.

### 5.3.1.2 PC and inhibitors

By using appropriate protocols (Fig. 5.15), it was shown convincingly that endogenous NO acts as a *trigger only* in the phenomenon of PC: both L-NAME and ODQ, if present before and throughout the PC procedure, but washed out before sustained ischaemia, significantly attenuated functional protection during reperfusion of preconditioned hearts, while having no inhibitory effects when added only after the PC protocol, before onset of sustained ischaemia (Figs 5.16 and 5.17). Similar conclusions were made by Csonka *et al.* (1999) in isolated working rat hearts; when N<sup>G</sup>-nitro-L-arginine (LNA, 4.6  $\mu$ M) was applied before the PC protocol, it abolished protection, while it failed to block the protective effects of PC when administered after the PC protocol, therefore confirming that NO synthesis by the heart is necessary to trigger classic PC. Although the results of Vegh *et al.* (1992) support the involvement of NO in PC, they did not distinguish whether NO act as mediator or trigger when they demonstrated that N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 10mg/kg) abolished the antiarrhythmic effect of PC in a coronary occlusion model in anesthetized dogs. Furthermore, recent results confirmed that endogenous NO is not a mediator of ischaemic PC, since L-NAME (3mM), when administered for 5 min after ischaemic PC (1 x 2 min) before 20 min

global ischaemia in Langendorff perfused rat hearts (Suematsu *et al.*, 2001), did not abolish the protective effect of PC.

Involvement of NO and cGMP as a trigger in cardioprotection has also been demonstrated in ventricular overdrive pacing (VOP) PC of a conscious rabbit model (Szilvassy *et al.*, 1994) and isolated working rat hearts (Ferdinandy *et al.*, 1996). In addition, Csonka and coworkers (1999) showed that intact basal NO synthesis was required to trigger PC, but that NO itself does not mediate protection, in both VOP-induced and no-flow ischaemia-induced PC of working rat hearts. Therefore, with reference to the role of NO, it appears that there is no difference between the mechanism of PC induced by VOP and no-flow ischaemia.

In contrast to our results, Lu *et al.* (1995), Sun *et al.* (1997), Weselcouch *et al.* (1995) and Nakano *et al.* (2000b), failed to demonstrate involvement of endogenous, endothelial-derived NO as trigger of PC in rat hearts. This discrepancy may be due to several minor, but possibly important differences in (i) protocol: 3 x 2 min coronary occlusion (Lu *et al.* 1995), 1 x 3 min coronary occlusion (Sun *et al.* 1997), 4 x 5 min global ischaemia (Weselcouch *et al.* 1995) and 1 x 5 min global ischaemia (Nakano *et al.* 2000b) vs. 3 x 5 min global ischaemia in the present study (ii) perfusion model: anesthetized rats (Lu *et al.* 1995, Sun *et al.* 1997) and retrogradely perfused rat or rabbit hearts fitted with an intraventricular balloon (Weselcouch *et al.* 1995, Nakano *et al.* 2000b) vs. our working rat hearts (iii) concentration of NOS inhibitors administered: 10mg/kg N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) or L-NAME (Lu *et al.* 1995), 10-100mg/kg L-NAME or 1-50mg/kg methylene blue (Sun *et al.* 1997), 30  $\mu$ M L-NAME (Weselcouch *et al.* 1995) 100  $\mu$ M L-NAME (Nakano *et al.* 2000b) vs. 50  $\mu$ M L-NAME in our study (iv) endpoints: arrhythmias (Lu *et al.* 1995, Sun *et al.* 1997), contractile function or enzyme release (Weselcouch *et al.* 1995) and infarct size (Nakano *et al.* 2000b) vs. functional recovery of the working rat heart in the present study.

Although we do not have an explanation as yet for the above discrepancies, it is of significance that, in our hands, inhibitors such as L-NAME or L-NA were able to abolish ischaemic PC induced by a multi-cycle protocol (3 x 5min), which was shown to elicit more effective protection than one or two cycles (Sandhu *et al.*, 1997).

### 5.3.1.3 Non-PC and inhibitors:

Inhibition of NOS activation by L-NAME had no effect on the functional recovery of non-preconditioned (non-PC) hearts, regardless of the protocol used (Fig. 5.17), suggesting that inhibition of NO generation during sustained ischaemia did not protect against cell damage in our model. Likewise, Weselcouch *et al.* (1995) and Nakano *et al.* (2000b) showed that NOS inhibition (L-NAME) alone had no effect on the severity of ischaemia in non-PC rat and rabbit hearts, respectively. In addition, we also demonstrated that guanylyl cyclase inhibition by ODQ did not improve functional recovery in non-preconditioned hearts, when administered for 3 x 5 or 4 x 5 min. In fact, when administered in this manner, ODQ significantly impaired functional recovery in non-preconditioned hearts, when compared to functional recovery observed in the protocol with a shorter (5 min) total exposure to ODQ. As stated previously (5.3.1.1) it would seem that repeated administration of the drug could have a detrimental effect (Fig. 5.16).

These results are in contrast to other studies where NOS inhibition protected against ischaemia-reperfusion (Woolfson *et al.*, 1995, Depré *et al.*, 1995, Naseem *et al.*, 1995, Csonka *et al.* 1999). This discrepancy could be due to differences in species, experimental protocol and end-points: coronary artery ligation of isolated rabbit hearts with infarct size as end-point (Woolfson *et al.*, 1995), low-flow ischaemia of isolated rabbit hearts with functional recovery (left ventricular developed pressure) (Depré *et al.*, 1995) or global ischaemia of retrogradely perfused rat hearts with functional recovery (arrhythmias) (Naseem *et al.*, 1995) compared to global ischaemia of isolated working rat heart with functional recovery (aortic flow) in our study. Although Csonka *et al.* (1999) also utilized global ischaemia in isolated working rat hearts with functional recovery (aortic flow), they induced a 30 min ischaemic period vs. 25 min in our model. The time of administration of NOS inhibitor may also be important, since L-NAME was administered continuously during 45 min of coronary artery ligation (Woolfson *et al.*, 1995), or L-NMMA was added 15 min before low-flow ischaemia (Depré *et al.*, 1995), or LNA was infused for either 30 min (Naseem *et al.*, 1995) or 20 min before no-flow ischaemia (Csonka *et al.* 1999). However, in our study L-NAME was administered for 5 min, or for 3 – 4 periods of 5 min alternating with drug-free perfusion and also for 15 min before onset of global ischaemia.

A recent study from our laboratory (unpublished results) showed that the perfusion mode (retrograde vs. working), grade of ischaemia (global vs. regional) and endpoint (functional recovery vs. infarct size) could all affect the outcome of the results in PC. Taking all these variables into account may help solve the discrepancies that so often hampered the experimental work on PC.

### **5.3.2 Mimicking of PC by using NO donors as triggers**

#### **5.3.2.1 PC protocol**

Elevation of cAMP *per se* acts as trigger during ischaemic PC (as observed in chapter 4). Therefore, to distinguish between the contribution of cAMP and cGMP as triggers, it was a prerequisite to use a NO donor that would not increase tissue cAMP levels before the onset of sustained ischaemia. Both SNAP and SNP caused dose-dependent increases in cGMP, while it had no significant effect on tissue cAMP at 50  $\mu$ M (Figs 5.4. and 5.5). However, at 10  $\mu$ M, SNAP caused slight but not significant increases in cAMP, except when administered for 1 x 5 min, followed by reperfusion. Low concentrations of SNAP (1  $\mu$ M) have also been shown to elevate cAMP content of isolated adult myocytes and to increase contractility; while at 100  $\mu$ M the drug had no effect on cAMP (Vila-Petroff *et al.*, 1999). These researchers attributed the increase in cAMP, induced by low levels of NO, to a novel cGMP-independent activation of adenylyl cyclase. In view of the above, it is possible but unlikely that generation of NO (as suggested by the cyclic increases in cGMP) during a multi-episode PC protocol in isolated rat hearts, contribute to the characteristic ischaemia-induced increases in cAMP during the PC protocol in the present study. Furthermore, if NO donors contribute to increases in cAMP, then repeated NO donor treatment would result in desensitization of the  $\beta$ -adrenergic receptor as observed after repeated increases in cAMP by ischaemic-PC or isoproterenol-PC (Fig. 4.8). However repetitive SNP administration does not desensitize the heart to additional  $\beta$ -adrenergic receptor stimulation (Fig. 5.7).

#### **5.3.2.2 Ischaemia-Reperfusion**

The improvement in functional recovery induced by NO donors was not dose-dependent in the range of tissue cGMP levels observed: elevation of cGMP to  $52.44 \pm 7.13$  (1 x 5

min 50  $\mu$ M SNAP) or  $18.96 \pm 3.67$  (1 x 5 min 100  $\mu$ M SNP) pmoles/g wet weight (Fig. 5.4) elicited the same degree of protection during reperfusion (Fig. 5.19, Table 5.3). Since  $20.22 \pm 0.91$  pmoles/g wet weight was the highest level to which cGMP increased during the ischaemic PC protocol (PC2-, Table 3.1) it seems as if maximal protection is achieved with cGMP levels in that range.

Failure of L-arginine (10 mM) to elicit protection (Fig. 5.19) was surprising in view of previous findings that this substrate protects against ischaemia-reperfusion injury (Engelman *et al.*, 1995a, Mizuno *et al.*, 1998). A possible explanation may be the failure of intermittent administration of L-arginine to increase cGMP in the present study (Table 5.2). On the other hand, it has also been shown that intracoronary administration of L-arginine aggravates myocardial stunning through production of peroxynitrite in dogs (Mori *et al.*, 1998). However, although the possibility exists that NO donors may contribute to improved function by reduction of stunning, as has been shown by others in large animals (Engelman *et al.*, 1995b, Ferdinandy *et al.*, 1996), this remains to be elucidated in our model.

As far as we know, this is the first direct demonstration that exogenous NO improves functional recovery during reperfusion in working rat hearts, by repeated transient administration and washout of NO donors before the onset of sustained ischaemia. (Fig. 5.19, Table 5.6). These results are consistent with the findings that exogenous NO donors mimic the effect of early PC on (i) reperfusion arrhythmias in rat hearts (Bilinska *et al.*, 1996), (ii) infarct size in rabbit hearts (Nakano *et al.*, 2000b), as well as on (iii) cell viability and enzyme release in cultured neonatal rat ventricular myocytes (Rakhit *et al.*, 2000).

Few studies have implicated a role for NO in early PC, and these largely involve animal models of protection against pacing (Vegh *et al.*, 1992) and reperfusion-induced arrhythmias (Bilinska *et al.*, 1996). The latter study showed that NO donors (glyceryl trinitrate, GTN and 3-morpholino-sydnonimine-hydrochloride, SIN-1), administered for 5 min and washed out for another 5min prior to ischaemia (10 min regional ischaemia), abolished reperfusion induced ventricular tachycardia in Langendorff perfused rat hearts. Recently, Nakano *et al.* (2000b) demonstrated that rabbit hearts treated with a NO donor, S-nitroso-N-acetylpenicillamine (SNAP, 2  $\mu$ M), for 5 min followed by a 10 min drug-free interval prior to 30 min regional ischaemia, reduced infarct size during

reperfusion. Furthermore, the study of Rakhit *et al.* (2000) in a simulated ischaemia model of early PC in rat neonatal cardiocytes suggested that the NO donor, SNAP, given for 90 min prior to lethal hypoxia (6 h) significantly protects against ischaemic injury. They also indicated that the NOS inhibitor, LNMMA, blocked the protective effect of PC. Hence, NO is implicated as a trigger in this model of early PC via activation of a constitutive NOS (eNOS) isoform.

Furthermore, Bell *et al.* (2001) recently used eNOS knockout mice to determine whether it is pivotal to early PC. They concluded that eNOS is not essential for robust (four cycles of 5 min ischaemia/reperfusion) PC, but may contribute to early PC by lowering the ischaemic threshold for protection. In addition they also demonstrated that triggering with NO (SNAP, 2  $\mu$ M) *per se* could be cardioprotective.

### 5.3.2.3 Contractile Reserve

In our studies there was no difference in the protection induced by either ischaemia or NO donors (Fig. 5.19, Table 5.6). Interestingly, the contractile reserve of hearts preconditioned with SNP or SNAP was significantly higher than that of ischaemic preconditioned hearts: for example, infusion of adrenaline ( $10^{-6}$  M) at the end of reperfusion, caused a ~3 fold higher percentage stimulation of cardiac output and Wt compared to preconditioned hearts (Table 5.7). This indicates that the superior protection induced by NO was unmasked by adrenaline, and that in fact, it offered a major advantage above PC with ischaemia. Therefore, these data suggest that NO-induced PC may be a useful clinical manipulation, and this possibility should be further investigated. It should, however, be kept in mind that the superior contractile reserve of NO donor treated hearts might be related to some of the other many actions of NO. Thus, whether the improved contractile reserve is due to prior preconditioning, or another mechanism, remains to be established.

### 5.3.3 How does NO-cGMP pathway trigger protection?

Establishing the relative importance of a trigger is difficult, since pharmacological mimicking of a trigger often elicits maximal protection, similar to that observed with ischaemic PC. Also in the present study where cyclic elevations in cGMP and cAMP occur simultaneously during the PC protocol (Fig. 3.2, Table 3.1), elevation in both

nucleotides is not a prerequisite for subsequent protection against ischaemic damage: elevation in cAMP *per se* by  $\beta$ -adrenergic stimulation (Fig. 4.6) or in cGMP *per se* by NO donors (Fig. 5.4) could induce protection similar to that induced by ischaemic PC (Figs. 4.23, 4.24 and 5.19). On the other hand, prevention of these cyclic increases in cAMP (Fig. 4.4) or cGMP (Table 5.1, Fig. 5.2), only partially suppressed PC induced protection (Figs. 4.21, 5.16 and 5.17), confirming involvement of more than one trigger.

### 5.3.3.1 “Cross-talk”

Considerable “cross-talk” may occur between the signal transduction pathways of the released triggers during an ischaemic PC protocol. For example, NO (and thus cGMP) attenuates  $\beta$ -receptor mediated responses (Ebihara *et al.*, 1996), whereas inhibition of NOS activity could enhance the  $\beta$ -response to isoproterenol in myocytes (Balligand *et al.*, 1993). Furthermore, NO-induced elevated cGMP can act by stimulating a cGMP sensitive phosphodiesterase with a resultant reduction in levels of cAMP (Parratt 1995). Therefore, in the present study, the very significant increases in cGMP during the PC protocol (PC2- and PC3-: 140 and 99%, respectively, refer to 3.2.1.2.1), suggests an important role for NO and could possibly account for the significantly smaller increases in cAMP (PC2- and PC3-: 16 and 17%, respectively) occurring simultaneously.

### 5.3.3.2 Reactive oxygen species (ROS)

Other possible mechanisms of action of NO in the PC process have recently been reviewed by Rakhit and coworkers (1999). NO can for example act as a free radical donor. NO combines with superoxide to generate peroxynitrite which, in turn, decomposes to yield hydroxyl radicals and other reactive oxygen species (ROS) (Beckman *et al.*, 1990). These NO-derived radicals are thought to aggravate injury under certain circumstances. However, free radicals can also trigger PC since administration of antioxidants during the brief triggering ischaemia has been found to prevent both early and late PC (Sun *et al.*, 1996). Furthermore, a recent study by Csonka *et al.* (2001) demonstrated that during a PC protocol (three intermittent periods of global ischaemia) peroxynitrite formation by the first period of ischaemia/reperfusion was gradually attenuated by the subsequent ischaemia/reperfusion periods, confirming that a detrimental component of NO can paradoxically trigger subsequent protection.

Recent studies, using appropriate blockers or scavengers, suggested that NO may use both cGMP and ROS as triggers during NO-induced PC: Rakhit *et al.* (2000) indicated that SNAP-triggered protection was completely abolished by ODQ in cultured rat ventricular myocytes, thus the NO-induced trigger is cGMP dependent. On the other hand, Nakano *et al.* (2000) has reported that the beneficial effect of exogenous NO production during SNAP pretreatment was blocked by a free radical scavenger, N-(2-mercaptopropionyl)-glycine or MPG, in isolated rabbit hearts.

In turn, several studies have demonstrated activation of PKC by ROS (Baines *et al.*, 1997, Konishi *et al.*, 1997). It has recently been shown (Ping *et al.*, 1999c) that activation of PKC during a 6 x 4 min PC protocol is NO dependent and that exogenous NO donors can translocate PKC  $\epsilon$  and  $\eta$  in the absence of ischaemia. In addition, Nakano *et al.* (2000) also reported that SNAP's protection could be blocked by chelerythrine, indicating that NO-triggered protection may be mediated by a PKC-dependent pathway.

### 5.3.3.3 p38 MAPK:

Protein tyrosine kinases are reported to be downstream of PKC for early and late ischaemic PC (Baines *et al.*, 1998, Ping *et al.*, 1999a,b). During the late phase of PC it was shown that protein tyrosine kinase signalling is essential for the increase of inducible NOS activity, indicating that iNOS is involved as a downstream element of protein tyrosine kinase (Dawn *et al.*, 1999). In addition, several studies have examined the potential role of the stress kinase, p38 MAPK as a component of the pathway of PC distal of PKC (Maulik *et al.*, 1996, Weinbrenner *et al.*, 1997). However, to our knowledge, the relationship between NO and p38 MAPK in early PC has not been investigated previously and there are also no data concerning the downstream effects of cGMP in the context of early PC.

In the present study, L-NAME (NOS inhibitor) could significantly attenuate p38 MAPK activation induced by 5 min ischaemia (PC1-), however this p38 MAPK activation was insensitive to ODQ (GC inhibitor) (Fig. 5.8). These results indicate that p38 MAPK are activated by 5 min ischaemia in a L-arginine-NO dependent way, but not by the rise in cGMP which occurs simultaneously.

Furthermore, our results indicate that 5 min administration of both NO donors, SNAP and SNP, significantly activated p38 MAPK, however not to the same extent as that induced by 5 min ischaemia (Fig. 5.9). Recently Kim *et al.* (2000) investigated the role of SNP in the regulation of the MAPKs in isolated adult rat cardiomyocytes and they also found that SNP maximally activated p38 MAPK, as well as its substrate MAPKAPK2, in 5 – 10 min. In addition, they blocked the activation of MAPKAPK2 by SNP with ODQ (GC inhibitor), indicating that p38 MAPK and MAPKAPK2 are activated by SNP in a cGMP-dependent way (which differs from ischaemia-induced p38 MAPK activation, Fig. 5.8). Furthermore, a study by Browning *et al.* (2000) demonstrated that exogenously supplied NO leads to activation of p38 MAPK that requires activation of cGMP-dependent protein kinase (PKG) in 293T fibroblasts.

The above mentioned studies indicate that triggering with NO donors induces p38 MAPK activation. However whether p38 MAPK activation acts as a trigger in NO-induced PC has not yet been studied - what is required is to study a NO donor in combination with a p38 MAPK inhibitor, like SB203580.

The physiological importance of p38 MAPK induced by brief surges of NO also remains to be investigated, but its downstream substrate, MAPKAPK2, can phosphorylate various transcription factors, i.e. HSP27 (Tan *et al.*, 1996). The phosphorylated HSP27 appears to stimulate the polymerization of actin and this might facilitate recovery of the actin microfilament networks, which are disrupted during cellular stresses like ischaemia.

#### **5.3.4 How does the NO-cGMP pathway mediate protection during sustained ischaemia?**

As observed in chapter 3, PC significantly attenuates the increase in tissue cAMP and stimulates an increase in cGMP in response to sustained ischaemia (Fig. 3.13). It is not yet known whether these changes are merely the consequence of or whether they contribute to the protection induced by ischaemic PC.

#### 5.3.4.1 Calcium ( $\text{Ca}^{2+}$ )

It is possible that the NO-cGMP pathway could be protective: elevated cGMP during sustained ischaemia may act by reducing the influx of  $\text{Ca}^{2+}$  through the L-type  $\text{Ca}^{2+}$  channels (Sperelakis, 1994) via protein kinase G (Sumii and Sperelakis 1995, Klein *et al.*, 2000), therefore limiting myocyte contractility (Brady *et al.*, 1992) that could serve to reduce oxygen consumption and energy demand. Similarly, Node *et al.* (1996) indicated that increased release of NO during ischaemia reduces myocardial contractility and improves metabolic dysfunction. In addition, Rakhit *et al.* (2001) recently indicated that pre-treatment with a NO donor (SNAP, 1mM for 90 min followed by 10 to 30 min washout) protects cardiomyocytes from ischaemia-reperfusion injury. They demonstrated a modest, sustained mitochondrial depolarization and a reduction in mitochondrial  $\text{Ca}^{2+}$  uptake that possibly reduces  $\text{Ca}^{2+}$  overload, thus providing a likely mechanism for NO-induced protection.

#### 5.3.4.2 Phosphodiesterase (PDE):

Elevated cGMP during sustained ischaemia may be due to stimulation of cGMP sensitive phosphodiesterase in PC hearts (Fig. 3.14) with resultant lowering of cAMP. It is possible that these two nucleotides act in synergism during sustained ischaemia: in view of their opposing effects on the  $\text{Ca}^{2+}$  slow channel, the simultaneous lowering of tissue cAMP and elevation in cGMP may reduce  $\text{Ca}^{2+}$  influx (Sperelakis, 1994) during ischaemia as well as during reperfusion.

In addition to the above, there is substantial pharmacological evidence that the guanylyl cyclase-cGMP-PDE system contributes to cardioprotection. For example, Ljusegren and Axelsson (1993) described that an increase in myocardial cGMP by SNP, atrial natriuretic peptide or zaprinast (an inhibitor of cGMP-specific PDE $_{\gamma}$ ) caused a reduction of lactate accumulation in isolated hypoxic rat ventricular myocardium.

#### 5.3.4.3 p38 MAPK:

In the previous chapters our results indicate that p38 MAPK plays a role as mediator of protection in ischaemia (chapter 3) and  $\beta$ -adrenergic (chapter 4) preconditioned hearts. The improved functional recovery of these hearts during reperfusion was associated

with a decrease in activation of p38 MAPK during the sustained period of ischaemia. The mechanism by which attenuated p38 MAPK activity could protect was suggested by Saurin *et al.* (2000), who have shown that PC protects by suppressing the activation of p38-alpha isoform, which is an activator of apoptosis. Consistent with these results, is our observation that the cardioprotective effect of both NO donors (SNAP and SNP) (Fig. 5.19) may be due to their ability to elicit the same attenuation in p38 MAPK as PC at the end of sustained ischaemia (Fig. 5.14). This indicates that prior triggering with NO donors may affect the p38 MAPK activity during sustained ischaemia. Our findings concur with a recent study by Rakhit *et al.* (2001), which indicated that SNAP pretreatment delayed peak activation of p38 $\alpha$  MAPK during ischaemia-reoxygenation in neonatal rat cardiomyocytes. Thus the delay in peak p38 MAPK activation may contribute to, rather than be the effect of, NO-induced (SNAP) cardioprotection.

Furthermore, administration of GC- or NOS- inhibitors, ODQ or L-NAME, respectively, during the ischaemic PC protocol, significantly attenuated functional recovery conferred by PC to the same extent as non-PC during reperfusion (Figs. 5.16 and 5.17). However ODQ did not affect the PC-induced attenuation of p38 MAPK activity, while L-NAME partially reversed this effect (Fig. 5.13A, B). This confirms our data on the triggering effect of NO during the PC protocol: L-NAME (NOS inhibitor) could significantly attenuate p38 MAPK activation induced by 5 min ischaemia (PC1-), however this p38 MAPK activation was insensitive to ODQ (GC inhibitor) (Fig. 5.8).

The results obtained suggest that early ischaemic PC and NO donors may trigger p38 MAPK via a pathway independent of cGMP, which could result in the attenuation of this stress kinase during sustained ischaemia and subsequent cardioprotection. As mentioned before, NO derived oxygen free radicals (i.e. peroxynitrite) may trigger the pathway leading to PC protection (Rakhit *et al.*, 1999). This free radical signalling involves p38 MAPK (Das *et al.*, 1999b). The latter might be upstream of the nuclear factor NF $\kappa$ B, which could be the link between PC and the adaptive protection mediated by expression of antioxidant genes during PC (Schieven *et al.*, 1993, Maulik *et al.*, 1998).

#### 5.3.4.4 ATP-sensitive potassium (KATP)-channels

Although further downstream events remain to be elucidated, it is suggested that PKC and the putative end-effector, the mitochondrial KATP channel are linked (Sato *et al.*, 1998). These channels may be involved in the end effect of both early and late ischaemic PC (reviewed by Sato and Marban, 2000). For example, Gross and Auchampach (1992) first demonstrated the involvement of the KATP channels in ischaemic PC in the canine model and demonstrated that glibenclamide, administered either before or after PC could abolish cardioprotection. These data imply that the KATP channels may set the heart into a preconditioned state but clearly demonstrate that the KATP channel is an end effector of PC. Additionally, in intact rabbit hearts, Ockaili *et al.* (1999) found that pretreatment with diazoxide 30 min or 24 hours before 30 min of ischaemia produced a reduction in infarct size. This was blocked by 5-hydroxydecanoate (5-HD) if administered after diazoxide in both the early and late phases of cardioprotection.

However, recent data from the laboratory of Downey indicated that mitochondrial KATP channels might act as a trigger rather than an end-effector of the protection of PC (Pain *et al.*, 1999). One possible hypothesis, which might explain the trigger action of mitochondrial KATP channels, involves mitochondrial generation of free radicals (Carroll *et al.*, 2001), which then stimulate downstream kinases. This theory is attractive since two of the kinases, which are thought to be involved in PC protection, PKC and p38 MAPK are known to be activated by free radicals (Downey and Cohen, 2000). Furthermore, Wang and Ashraf (1999) have reported that diazoxide's protection in rat heart can be blocked by a PKC antagonist, a result which would put KATP channels far upstream. Thus, it is possible that a different ion channel or an unidentified protein may still be the elusive end-effector.

Recent studies revealed that NO directly activates the mitochondrial KATP channels but not sarcolemmal KATP channels (MacKay and Mochly-Rosen, 1999, Sasaki *et al.*, 2000), while NO also potentiates the ability of KATP channel openers (i.e. diazoxide) to activate the potassium ( $K^+$ ) current in rabbit cardiomyocytes (Sasaki *et al.*, 2000). In addition, recently Han *et al.* (2001) provided the first direct evidence that KATP channels can be opened through PKG-dependent phosphorylation in rabbit ventricular

myocytes. Therefore, these data suggest that NO-induced protection may be mediated via direct opening of the KATP channels.

It appears that NO is involved as a *trigger* for protection in both VOP-induced or no-flow ischaemia-induced classic PC (Csonka *et al.*, 1999). However, the way they *mediate* their cardioprotective mechanism may be different in terms of the activation of the KATP-channel: glibenclamide (blocker of the KATP-channel) inhibited VOP-induced protection, but did not affect no-flow ischaemia-induced PC (Ferdinandy *et al.*, 1995b). In addition, in a simulated ischaemia model of early PC (rat neonatal cardiocytes), SNAP-triggered protection (accompanied by increased cGMP levels) was completely abolished by ODQ, but not by chelerythrine or glibenclamide plus 5-HD (a KATP-channel blocker) (Rakhit *et al.*, 2000). Therefore it was proposed that the mechanism of cardioprotection in early PC is cGMP dependent but independent of protein kinase C or KATP-channels. This differs from the proposed mechanism of NO-induced cardioprotection in late PC (Wang Y *et al.*, 2001).

## 5.4 SUMMARY

The results obtained suggest that NO and thus the generation of cGMP act as a trigger in classic PC. Cyclic elevation of cGMP by NO donors effectively protects against ischaemic damage, whereas inhibition of NOS or guanylyl cyclase partially abolishes the beneficial effects of PC.

Furthermore, our results suggest that NO mediated attenuation of the activation of p38 MAPK during sustained ischaemia plays a role in the mechanism of protection. However, conclusive interpretation of the relevance of p38 MAPK activation in cardioprotection cannot be made at this point in time. Amongst others, use of appropriate agonists or inhibitors as well as identification of the isoforms of p38 MAPK involved, are required.

## CHAPTER 6

### Evaluation of the role of p38 MAPK

#### 6.1 Introduction

As mentioned before in chapter 3, the signal transduction cascade of ischaemic PC can be divided into triggers and mediators. Blockade of a trigger's action during an ischaemic PC protocol will abolish its cardioprotective effects. In addition, an intervening washout period between the PC protocol and the onset of sustained ischaemia may be essential in order to determine an initiator of PC. Mediators are important during the sustained ischaemia, and blockade of a mediator's action during this phase will abolish protection. Careful manipulation of the protocol is therefore necessary to distinguish between a trigger and mediator effect of a particular signalling pathway.

##### 6.1.1 p38 MAPK activation: trigger or mediator?

Two potential roles for p38 MAPK have been proposed. (i) First, there is increasing evidence that even brief, non-lethal episodes of myocardial ischaemia (i.e. ischaemic PC) elicit a significant increase in the activity of p38 MAPK (Maulik *et al.*, 1998d, Yue *et al.*, 2000, Sato *et al.*, 2000) and further, that this activation of p38 MAPK during the PC stimulus may participate in triggering the benefits achieved with PC (Maulik *et al.*, 1998d, Sato *et al.*, 2000). However, several studies have reported that activation of p38 MAPK during ischaemia is transient and does not correlate with the PC effect (Ping *et al.*, 1999a, Behrends *et al.*, 2000), thereby questioning the significance of p38 MAPK as a trigger in PC. (ii) A second hypothesis is that p38 MAPK may mediate cardioprotection, with evidence of increased activation of p38 MAPK signalling during sustained ischaemia in preconditioned hearts, as well as in myocytes (Weinbrenner *et al.*, 1997, Armstrong *et al.*, 1999, Nakano *et al.*, 2000). However, activation of p38 MAPK during sustained ischaemia-reperfusion has, in several studies, been associated with myocyte necrosis/apoptosis which makes its contribution as mediator unlikely (Bogoyevitch *et al.*, 1996, Ma *et al.*, 1999, Mackay *et al.*, 1999, Barancik *et al.*, 2000).

Investigation into the role of p38 MAPK in PC was approached in most cases by evaluation of (i) the effects of ischaemia on the activation of the kinase and (ii) pharmacological inhibition of p38 MAPK and assessing the consequences thereof on cardioprotection. Unfortunately, in most studies the inhibitor was added immediately before the onset of sustained ischaemia, a protocol that allows for evaluation of its role as mediator during sustained ischaemia only.

Furthermore, the selectivity of the inhibitor of p38 MAPK is questionable (Ping *et al.*, 2000) (SB203580, the most commonly used, also inhibits JNK) and no suitable activator of p38 MAPK exists. Therefore, since  $\beta$ -adrenergic stimulation also stimulates p38 MAPK activation (Zheng *et al.*, 2000, Communal *et al.*, 2000), it could be a useful tool to evaluate whether this particular stress kinase acts as trigger or mediator in ischaemic or  $\beta$ -adrenergic PC.

### **6.1.2 The aim**

In view of the above, our subsequent aim was to determine the effects of inhibition of ischaemia or  $\beta$ -adrenergic-induced p38 MAPK activation on functional recovery during reperfusion after global ischaemia of isolated perfused rat hearts. The results obtained in this study may help clarify whether p38 MAPK acts as a trigger and/or mediator in the PC process, as well as whether its activation during sustained ischaemia is beneficial or not.

## 6.2 Experimental Protocols and Results

### 6.2.1 Inhibition of p38 MAPK during global ischaemia in the perfused rat heart

#### 6.2.1.1 Experimental protocol

##### Evaluation of functional recovery

##### Non-preconditioned hearts

Hearts were stabilized for 60 min (15 min retrograde, 15 min working mode, 30 min retrograde), followed by 25 min sustained global ischaemia and 30 min reperfusion (10 min retrograde, 20 min working mode) (Fig. 6.1A).

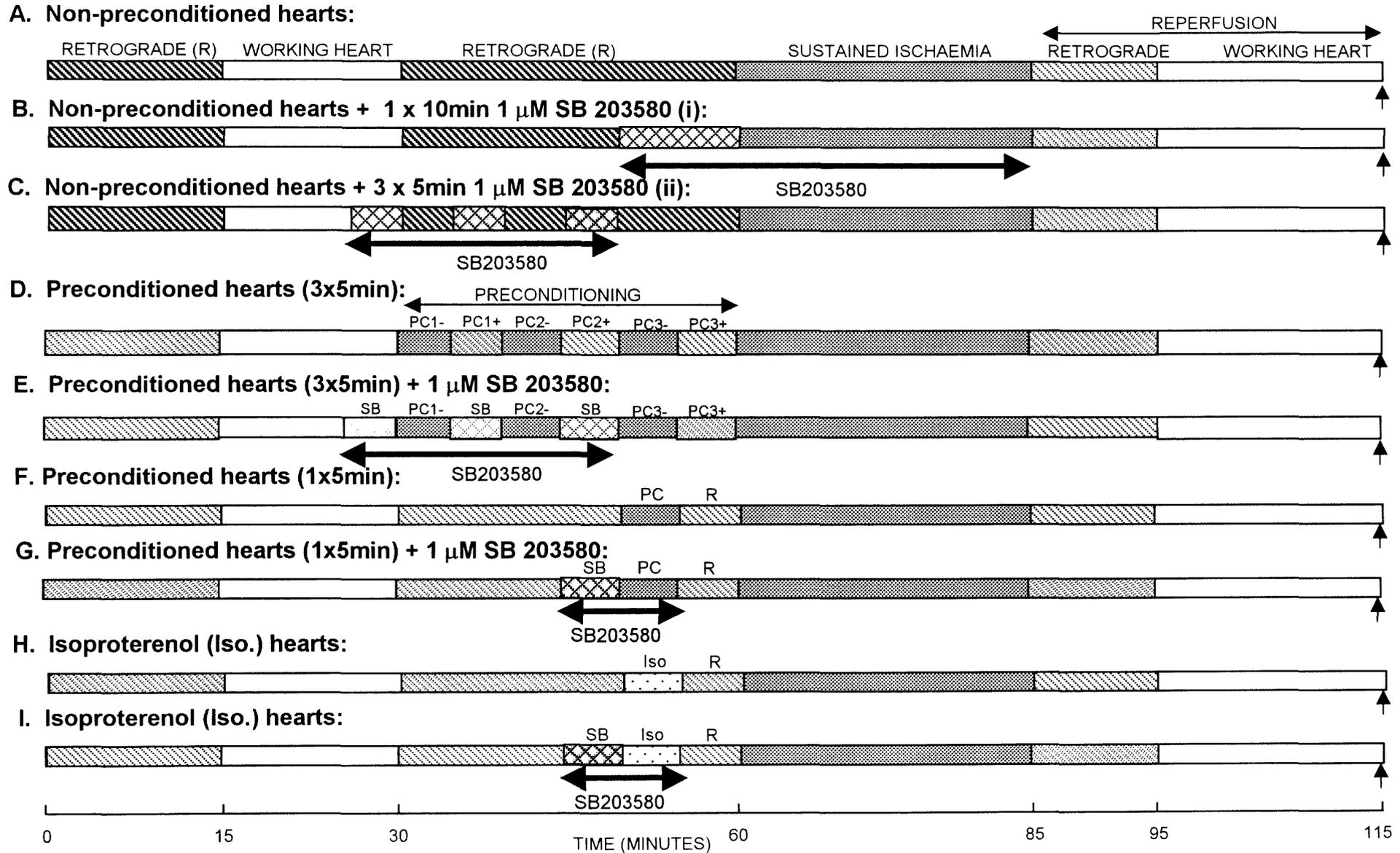
##### Non-preconditioned hearts plus SB 203580: p38 MAPK as mediator or trigger?

(i) Mediator: SB 203580 administered immediately before sustained ischaemia (Fig. 6.1B): hearts were stabilized for 50 min (15 min retrograde, 15 min working, 20 min retrograde) followed by administration of SB 203580 (1  $\mu$ M) for 10 min. Hearts were then subjected to 25 min global ischaemia and 30 min reperfusion (10 min retrograde, 20 min working heart).

(ii) Trigger: SB 203580 administered 3 x 5 min (Fig. 6.1C): hearts were stabilized for 30 min (15 min retrograde, 15 min working), followed by SB 203580 (1  $\mu$ M, 3 x 5 min), alternated with 5 min drug-free reperfusion. Hearts were then subjected to sustained ischaemia and reperfusion as described above.

##### Ischaemic preconditioned hearts

Hearts were stabilized for 30 min (15 min retrograde, 15 min working) followed by either a multi-cycle ischaemic PC protocol (3 x 5 min global ischaemia, alternated with 5 min of reperfusion) (Fig. 6.1D) or a single episode of PC (1 x 5 min global ischaemia and 5 min of reperfusion) (Fig. 6.1F). Hearts were then subjected to 25 min sustained global ischaemia and 30 min reperfusion as described above.



**Fig 6.1** Effect of SB 203580, an inhibitor of p38 MAPK activation, pretreatment on non-preconditioned, ischaemic-preconditioned and isoproterenol perfused rat hearts: Experimental protocol. See text for details on different protocols. Arrows indicate time(after 30 min reperfusion) of determination of mechanical function. n = 4 to 6 hearts per series.

**Ischaemic preconditioned hearts plus SB 203580: p38 MAPK as trigger?**

Multi-cycle ischaemic-PC hearts plus SB 203580 (Fig. 6.1E): hearts were perfused retrogradely for 15 min, followed by 10 min perfusion in the working mode. SB 203580 (1  $\mu$ M) was administered for 5 min before and after the first PC episode (PC1-) and also during reperfusion after the second PC episode (PC2-). After the third PC episode (PC3-) hearts were perfused for 10 min to ensure washout of the drug.

Single episode ischaemic-PC hearts plus SB 203580 (Fig. 6.1G): hearts were perfused retrogradely for 15 min, followed by 15 min in the working mode and 10 min retrogradely. SB 203580 was then administered for 5 min, followed by 5 min global ischaemia and 10 min washout. All hearts were then subjected to ischaemia-reperfusion as described above.

**Isoproterenol hearts**

Hearts were stabilized for 50 min (15 min retrograde, 15 min working, 20 min retrograde) followed by 5 min administration of  $10^{-7}$  M isoproterenol and 5 min drug-free perfusion (Fig. 6.1H). All hearts were then subjected to 25 min sustained global ischaemia and 30 min reperfusion as described above.

**Isoproterenol hearts plus SB 203580: p38 MAPK as trigger?**

Hearts were stabilized for 45 min (15 min retrograde, 15 min working, 15 min retrograde), followed by administration of SB 203580 (1  $\mu$ M) for 10 min (Fig. 6.1I). During the last 5 min of SB 203580 administration, isoproterenol ( $10^{-7}$  M) was given simultaneously, followed by 5 min drug-free perfusion. Hearts were then subjected to sustained ischaemia and reperfusion as described above.

**6.2.1.2 Results**

To determine the efficacy of the p38 MAPK inhibitor, SB 203580, at a concentration of 1  $\mu$ M in global ischaemia or during  $\beta$ -adrenergic treatment of perfused rat hearts, it was administered either (i) for 5 min before a brief 5 min global ischaemic episode or (ii) 5 min before and during isoproterenol-treatment (5 min). The results showed that while

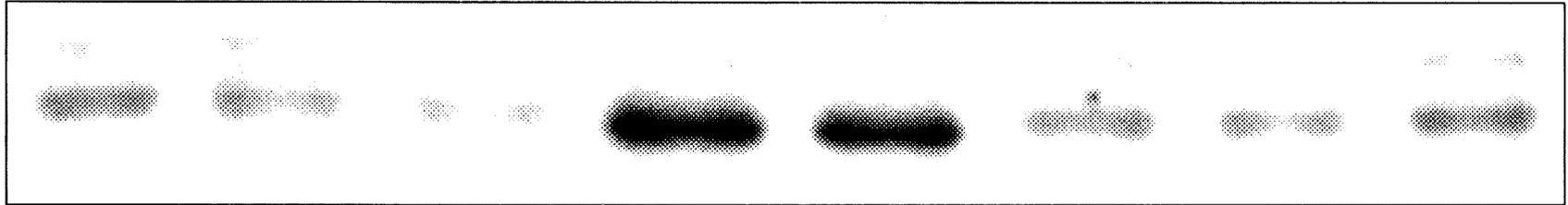
SB 203580 had no effect on the p38 MAPK activity of control perfused hearts, it completely abolished the activation induced by either global ischaemia (Fig. 6.2) or isoproterenol (Fig. 6.3). Therefore SB 203580 at a concentration of 1  $\mu$ M was used in all experiments.

### **Non-preconditioned hearts plus SB 203580: p38 MAPK as mediator or trigger?**

The effect of SB 203580 on the functional recovery of non-preconditioned hearts was dependent on the protocol used (Fig. 6.1B and C). When administered for 10 min immediately before the onset of sustained ischaemia (without washout), the effect on functional recovery during reperfusion after sustained ischaemia were similar to those of ischaemic-PC or isoproterenol hearts and significantly higher than those of untreated non-preconditioned hearts (Fig. 6.4, protocol i and Table 6.1). These findings indicate that p38 MAPK may act as a mediator. However, when administered for 3 x 5 min and washed out before the onset of sustained ischaemia (Fig. 6.4, protocol ii and Table 6.1), no improvement occurred and values similar to those of untreated non-preconditioned hearts were obtained, indicating that no triggering effect occurred and excluding the possibility that the drug was not washed out prior to sustained ischaemia.

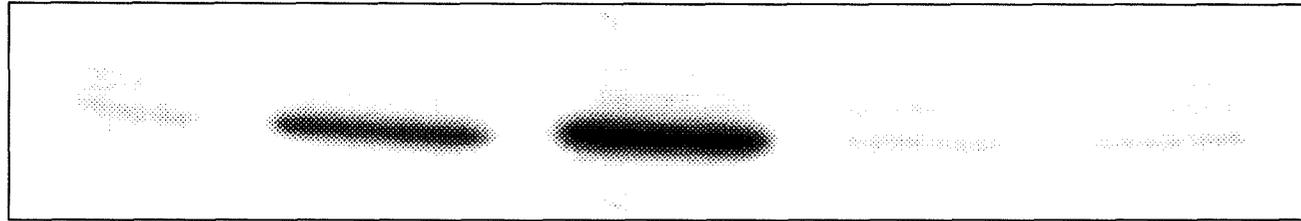
### **Preconditioned and isoproterenol hearts plus SB 203580: p38 MAPK as trigger?**

Bracketing the multi-cycle (3 x 5 min) ischaemic-PC protocol with SB 203580, did not abolish PC, but in fact the aortic output of these hearts was significantly higher than those of untreated ischaemic-preconditioned hearts (Fig. 6.4). However, the increases in cardiac output and total work performance were not significant (Table 6.1). Therefore p38 MAPK does not act as a trigger in a multi-cycle (3 x 5 min) ischaemic-PC protocol. On the other hand, bracketing of either the single episode (1 x 5 min) ischaemic-PC or the 5 min isoproterenol protocol with SB 203580, followed by 10 min washout, caused a significant decrease in functional recovery during reperfusion, when compared with either the single episode ischaemic-PC or the isoproterenol protocol alone, respectively (Fig. 6.4 and Table 6.1). Therefore p38 MAPK may act as a trigger during these protocols.



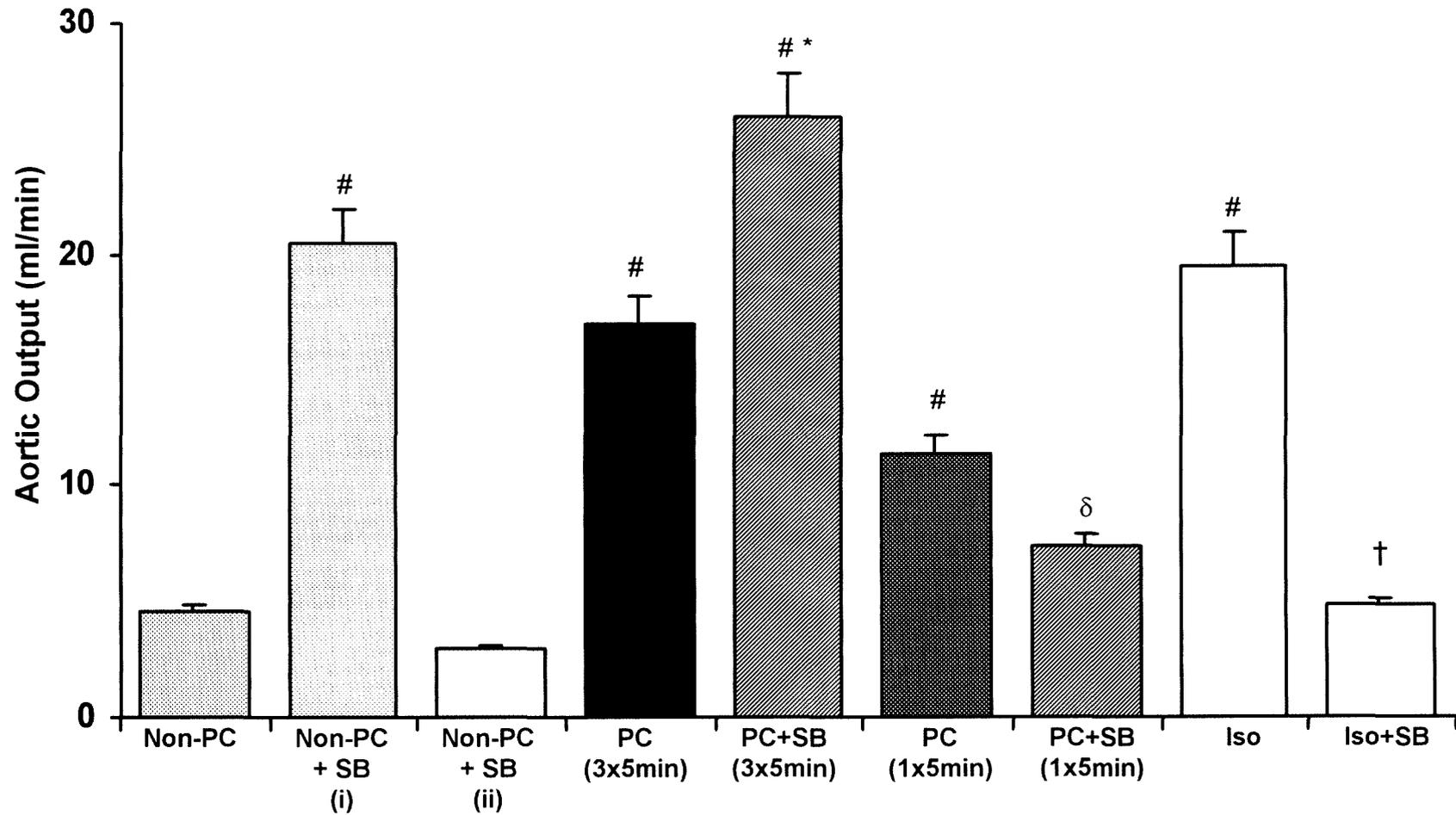
<b>Control</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>-</b>	<b>-</b>	<b>-</b>	<b>-</b>	<b>-</b>
<b>SB 203580</b>	<b>-</b>	<b>+</b>	<b>+</b>	<b>-</b>	<b>-</b>	<b>+</b>	<b>+</b>	<b>+</b>
<b>5 min Isch.</b>	<b>-</b>	<b>-</b>	<b>-</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>

**Fig 6.2** Effect of SB 203580 (1  $\mu$ M) on p38 MAPK activation: The p38 MAPK inhibitor SB 203580 prevents the dual activation of p38 MAPK induced by brief global ischaemia (5 min) in the perfused rat heart.



<b>Control</b>	<b>+</b>	<b>-</b>	<b>-</b>	<b>-</b>	<b>-</b>
<b>SB 203580</b>	<b>-</b>	<b>-</b>	<b>-</b>	<b>+</b>	<b>+</b>
<b>5 min Iso.</b>	<b>-</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>

**Fig 6.3** Effect of SB 203580 (1  $\mu$ M) on p38 MAPK activation: The p38 MAPK inhibitor SB 203580 prevents the dual activation of p38 MAPK induced by isoproterenol (5 min) in the perfused rat heart.



- (i) Mediator: SB 10 min before ischaemia  
 (ii) Trigger: SB 3 x 5 min, 10 min washout before ischaemia

#  $p < 0.05$  vs Non-PC without SB  
 \*  $p < 0.05$  vs PC (3x5min) without SB  
 δ  $p < 0.05$  vs PC (1x5min) without SB  
 †  $p < 0.05$  vs Iso without SB

**Fig 6.4** Functional recovery during reperfusion after 25 min global ischaemia of perfused rat hearts : Effect of inhibition of p38 MAPK with SB203580 on non-preconditioned, ischaemic-preconditioned and isoproterenol hearts.

**Table 6.1**

**Mechanical function after arrest during 30 min of reperfusion: Effect of SB203580, an inhibitor of p38 MAPK, on non-preconditioned (Non-PC), ischaemic-preconditioned (PC) and isoproterenol (Iso) hearts.**

	Coronary flow (ml/min)	Cardiac output (ml/min)	Peak systolic Pressure (mm Hg)	Heart Rate (beats/min)	Total work (mV)
<b>Non-Preconditioned:</b>					
Without SB (7)	8.0±1.4	11.9±2.5 *	80.0±2.0	211±15	2.6±0.4 *
With SB <sup>(i)</sup> (8)	12.0±0.7	31.3±2.8 #	88.6±1.3	262±20 #	6.3±0.7 #
With SB <sup>(ii)</sup> (8)	11.4±1.1	14.7±1.6 *	84.9±3.2	200±20	2.8±0.4 *
<b>Preconditioned (3 x 5 min):</b>					
Without SB (9)	11.1±0.6	27.7±2.1 #	86.0±1.5	250±13 #	5.5±0.5 #
With SB (8)	11.4±0.7	37.4±0.7 #	93.0±1.4 #	257±16 #	7.9±0.2 #
<b>Preconditioned (1 x 5 min):</b>					
Without SB (5)	12.5±0.5	23.9±1.3 #	91.0±1.1	228±6	5.1±0.4 #
With SB (8)	11.5±0.3	18.9±0.3 <sup>δ</sup>	86.0±0.8	206±15 <sup>δ</sup>	3.7±0.3 <sup>δ</sup>
<b>Isoproterenol (10<sup>-7</sup> M):</b>					
Without SB (6)	10.1±0.9	29.7±1.5 #	94.2±4.3 #	202±17	5.5±0.4 #
With SB (6)	10.8±1.0	15.7±1.6 <sup>†</sup>	85.8±0.8 <sup>†</sup>	202±11	3.0±0.2 <sup>†</sup>

Results expressed as the mean values ± SE obtained during 30 min reperfusion after 25 min sustained ischaemic arrest. Numbers in parentheses indicate number of hearts. Hearts were treated with SB 203580 before sustained ischaemia as shown in Fig. 6.1. For example; (i) Non-PC with 10 min SB before sustained ischaemia and (ii) Non-PC with 3 x 5 min SB followed by 10 min washout before sustained ischaemia.

# p < 0.05 vs Non-PC without SB

\* p < 0.05 vs PC (3x5min) without SB

δ p < 0.05 vs PC (1x5min) without SB

† p < 0.05 vs Iso without SB

## 6.3 Discussion on role of p38 MAPK

### 6.3.1 p38 MAPK activation during the PC protocol: a trigger for eliciting protection?

Since repeated but transient p38 MAPK activation could be observed during a multi-cycle (3 x 5 min) ischaemic PC protocol (Fig. 3.12), the question arises whether this phenomenon acts as a trigger in the PC process or whether this is merely an epiphenomenon of the multiple changes occurring in the myocardium during a multi-cycle ischaemic PC protocol. Some of the results obtained in the present study suggest that p38 MAPK activation may act as a trigger: experimental activation of the kinase with isoproterenol (Fig. 4.14) or transient ischaemia (as in ischaemic PC, PC1-, Fig. 4.13), elicits protection against subsequent sustained ischaemia (Fig. 4.23). Although these findings do not prove cause and effect, the fact that  $\beta$ -adrenergic blockade with alprenolol inhibited p38 MAPK activation during the ischaemic PC protocol (Fig. 4.13), completely abolishes functional recovery (Fig. 4.21), suggest a triggering action. Further evidence for a trigger role of this kinase, is the observation that inhibition of either a single episode (1 x 5min) ischaemic PC- or isoproterenol-induced p38 MAPK activation by the inhibitor SB 203580, completely abolished cardioprotection (Fig. 6.4 and Table 6.1). Sato *et al.* (2000), also using a working rat heart model, reported that activation of both JNK1 and p38 MAPK was obligatory as triggers for PC: SB 203580, when administered before a 4 x 5min ischaemic-PC protocol and washed out (10 min) before sustained ischaemia, completely abolished cardioprotection. In addition, Nagarkatti *et al.* (1998), who also washed away the inhibitor after PC (*viz.* brief duration of ischaemia) could also abolish the protective effect of PC in a rat myoblast cell line (see chapter 1, Table 1.1).

Despite the above, several observations argue against a role for p38 MAPK activation as trigger. For example, activation of p38 MAPK during a multi-cycle ischaemic-PC protocol is transient (Fig. 3.12, Ping *et al.*, 1999a, Schneider *et al.*, 2001), compared to the persistent activation of p46/p54 JNK (Ping *et al.*, 1999a) and p44/p42 MAPK (Ping *et al.*, 1999b). Although transient activation of p38 MAPK does not exclude a role as trigger, other observations also argue against activation of this kinase as the only trigger in a multi-cycle PC protocol: bracketing of the multi-cycle ischaemic-PC protocol with SB 203580 did not abolish cardioprotection in isolated working rat hearts (Fig. 6.1E and

Fig. 6.4). Contrary to expectations, this intervention caused a significant increase in aortic output during reperfusion (Fig. 6.4), suggesting that p38 MAPK activation during the ischaemic PC protocol may indeed be harmful. Schneider *et al.* (2001) also showed that p38 MAPK inhibition by SB 202190 (a related compound to SB 203580) during a multi-cycle PC protocol did not block the PC-induced improvement in post-ischaemic contractile function and reduction in necrosis in an isolated perfused rat heart model. A similar failure of SB 203580 to abolish protection was observed with a 2 x 5 min PC protocol in rat hearts (Mocanu *et al.*, 2000). In addition, SB 203580 also could not block PC protection in different models, for example in neonatal rat cardiomyocytes (Mockridge *et al.*, 2000) or in an *in vivo* pig model (Barancik *et al.*, 2000).

Contrary to the above mentioned observations that SB 203580 does not block the cardioprotection elicited by a multi-cycle PC protocol, the inhibitor causes a significant attenuation of protection when a single cycle ischaemic PC protocol was used (Fig 6.4 and Table 6.1). This observation is in agreement with the findings of Sandhu *et al.* (1997) that PC induced by a one-cycle was more susceptible to blockade by inhibitors than that induced by a multi-cycle protocol. It has been suggested that several receptor systems contribute to triggering multi-cycle ischaemic-PC and one particular inhibitor often fails to inhibit protection (Sandhu *et al.*, 1997, Cohen *et al.*, 2000). This concept was also demonstrated by Sakamoto *et al.* (2000) and Fryer *et al.* (2001): they could block a single cycle protocol with SB 203580, while Schneider *et al.* (2001), using SB 202190 failed to block a 4 x 5 min protocol in working hearts.

Apart from a few studies with anisomycin (Weinbrenner *et al.*, 1997, Sato *et al.*, 2000), the role of p38 MAPK activation acting as a trigger during a PC protocol has not been assessed with other activators of this kinase. Furthermore, anisomycin, an inhibitor of protein synthesis, is also a powerful stimulant of JNK (Barancik *et al.*, 1999, Schneider *et al.*, 2001) and is therefore unsuitable for use in studies aimed at elucidating the role of p38 MAPK *per se*. In view of the above, isoproterenol mediated protection is probably dependent on p38 MAPK activation before the onset of ischaemia (i.e. as trigger) and may be a useful tool to clarify the triggering role of this kinase. Further evidence for p38 MAPK activation as a contributing trigger for cardioprotection is the observation that SB 203580 inhibition of isoproterenol-induced p38 MAPK activation completely blocked functional recovery during reperfusion (Fig 6.4 and Table 6.1).

In addition, recent findings in our laboratory (Hartley, MSc Thesis) indicated that p38 MAPK activation act as a trigger: anisomycin (5  $\mu$ M) administration for 10 min, then washed out for 10 min prior to sustained ischaemia, resulted in significant protection against necrosis (decreased infarct size) and reduced apoptosis. These findings were also accompanied by attenuation of p38 MAPK activation during sustained ischaemia. Furthermore, the elucidation of the role of p38 MAPK would necessitate the use of anisomycin in the presence of an agent such as SP 600125, an inhibitor of JNK (work in progress).

### **6.3.2 p38 MAPK activation during ischaemia and reperfusion: a mediator of protection?**

Numerous studies have suggested a role for p38 MAPK activation as a mediator of protection against ischaemia: (i) pharmacological activation of p38 MAPK by administration of anisomycin before the onset of sustained ischaemia (without washout), elicited cardioprotection in isolated rabbit cardiomyocytes (Weinbrenner *et al.*, 1997), perfused rat hearts (Nakano *et al.*, 2000a) and pig hearts (Barancik *et al.*, 1999), while (ii) inhibition of p38 MAPK prior to sustained ischaemia only, completely abolished PC-induced protection (Nakano *et al.*, 2000a, Barancik *et al.*, 1999, Mocanu *et al.*, 2000, Nakano *et al.*, 2000c) again indicating a mediator role. However, though there is reasonable consensus that p38 MAPK acts as mediator, considerable controversy exists whether this occurs via increased or attenuated activation of this kinase.

In chapter 3 we showed that apart from the transient activation of p38 MAPK during the ischaemic PC protocol, p38 MAPK activation was significantly less in preconditioned than in non-preconditioned hearts during both sustained ischaemia (Fig. 3.16) and reperfusion (Fig. 3.21). This observation correlates with the reduction in cAMP accumulation occurring during sustained ischaemia in preconditioned hearts (Fig. 3.13) and could indicate that cAMP generation during sustained ischaemia (as in transient ischaemia) is one of the factors activating p38 MAPK. Since the marked attenuation in p38 MAPK activation in preconditioned hearts was associated with improved functional recovery, it was suggested that inhibition of the kinase might be an essential element in PC-induced cardioprotection.

This suggestion is supported by the findings obtained in chapter 4: the pattern of p38 MAPK activation (as indicated by its dual phosphorylation and kinase activity) during sustained ischaemia and reperfusion in hearts failing to recover functionally upon reperfusion after ischaemia, as in non-preconditioned hearts and alprenolol-treated preconditioned hearts, was similar (Fig. 4.27). Conversely, activation of p38 MAPK during both sustained ischaemia and reperfusion was significantly lower in ischaemic- (Figs. 3.16 and 3.21) and isoproterenol- preconditioned hearts (Fig. 4.26), associated with functional recovery during reperfusion. Interestingly, despite the possible differences in signalling during sustained ischaemia occurring in ischaemic and  $\beta$ -adrenergic preconditioned hearts, the pattern of p38 MAPK activation during both sustained ischaemia and reperfusion is similar in the two groups.

In addition to the above, considerable evidence exists suggesting that inhibition of p38 MAPK during sustained ischaemia may indeed be cardioprotective. For example, we have shown that administration of SB 203580 immediately before the onset of sustained ischaemia to non-preconditioned hearts (Fig. 6.4, protocol i) elicited cardioprotection similar to that seen in ischaemic or  $\beta$ -adrenergic preconditioned hearts (Fig. 6.4). In addition, not only does inhibition of p38 MAPK by SB 203580, protect myocytes against ischaemia (MacKay *et al.*, 1999), reduce apoptosis (Nagarkatti and Sha'afi, 1998) and limit infarct size (Sanada *et al.* 2001), but significantly less activation of p38 MAPK during ischaemia has been reported in preconditioned rat myoblast cells (Nagarkatti and Sha'afi 1998) and perfused hearts (Sato *et al.*, 2000). Schneider and coworkers (2001) also showed that another inhibitor of p38 MAPK, SB 202190, administered immediately before ischaemia, significantly improved functional recovery of the isolated perfused rat heart and reduced necrosis. Their data also indicated that although inhibition of p38 MAPK and PC are protective, their protection is not additive, which may suggest that PC and SB 202190 enhance protection via similar mechanisms. The latter finding is in contrast to our results, which showed additive protection (Fig. 6.4). Minor differences in the perfusion protocol and inhibitor used (SB 203580 vs. SB 202190) may account for this.

Perhaps the most convincing evidence thus far for the suggestion that attenuation of p38 MAPK activation during ischaemia is indeed protective comes from a recent study on cultured neonatal myocytes: not only did SB 203580 reduce ischaemic injury, but prior PC of these cells prevented p38 $\alpha$  MAPK activation during ischaemia. Moreover,

cells expressing a dominant negative p38 $\alpha$ , which prevented p38 activation, were resistant to lethal ischaemia (Saurin *et al.*, 2000). Selective activation of p38 $\alpha$  over p38 $\beta$  during ischaemia was shown to occur in these cells, therefore inhibition of the  $\alpha$ -isoform by SB 202190 was suggested to be involved in cardioprotection. Previous studies in neonatal cardiac myocytes supported a role for the  $\alpha$ -isoform in mediating apoptotic cell death (Wang *et al.*, 1998). However, in the present study no attempt was made to distinguish between the different isoforms of p38 MAPK, as the inhibitors currently available commercially do not allow distinction between isoforms.

Despite the above convincing evidence that attenuation of p38 MAPK activation during sustained ischaemia is beneficial, several other workers reported the contrary. Cardioprotection, associated with increased activation of p38 MAPK during sustained ischaemia, has been reported in rabbit cardiomyocytes (Armstrong *et al.*, 1999), isolated rabbit (Weinbrenner *et al.*, 1997, Nakano *et al.*, 2000a) as well as rat hearts (Liu *et al.*, 1998, Maulik *et al.*, 2000, Mocanu *et al.*, 2000) and linked to activation of its downstream signalling substrate MAPKAPK2 (Nakano *et al.*, 2000a, Maulik *et al.*, 2000). In addition, a recent study from Pain *et al.* (2000) showed that, like PC, diazoxide-induced (opening of the KATP channels) reduction in infarct size was characterized by increased p38 MAPK activation during sustained ischaemia. However, an explanation for the variability in results is not yet available and remains a matter of serious concern. Possible reasons for these discrepancies have recently been reviewed by Michel *et al.* (2001): differences in species (rat, rabbit, dog, pig, human) and model systems may be important. Most of the studies showing increased activation of p38 MAPK in cardioprotection, have been done in rabbits (Weinbrenner *et al.*, 1997, Armstrong *et al.*, 1999, Nakano *et al.*, 2000a). Furthermore, the rat model has been shown to be a complex model in PC, since contradictory results with regard to p38 MAPK activation in preconditioned rat hearts have also been reported: our study as well as work by Saurin *et al.* (2000) vs. the studies by Liu *et al.* (1998), Maulik *et al.* (2000) and Mocanu *et al.* (2000).

### 6.3.3 p38 MAPK inhibition: good or bad?

Activation of p38 MAPK during sustained ischaemia in non-preconditioned hearts has been demonstrated in several studies (Bogoyevitch *et al.*, 1996, Yin *et al.*, 1997, MacKay *et al.*, 1999, Ma *et al.*, 1999) and according to these studies this could be

harmful to the ischaemic myocardium. Studies using SB 203580, support this viewpoint: protection against ischaemic damage by SB 203580 has been shown in rat neonatal cells (MacKay *et al.*, 1999) and rat myoblast cell lines (Nagarkatti and Sha'afi, 1998), while it inhibits apoptosis in perfused rabbit hearts (Ma *et al.*, 1999) and limits necrosis in the *in vivo* pig model (Barancik *et al.*, 2000). Recently, Gysembergh *et al.* (2001) also indicated that SB 203580, which caused attenuation of p38 MAPK activity early (5 min) during sustained coronary occlusion of rabbit hearts, is cardioprotective. Although the dose of SB 203580 they employed (8  $\mu\text{M}$ ) was similar to the 5-10  $\mu\text{M}$  concentration used in most previous studies, recent evidence suggests that concentrations of SB in the 10  $\mu\text{M}$  range may also inhibit other kinases, including JNKs (Sugden *et al.*, 1998, Clerk *et al.*, 1998b). Therefore using 1  $\mu\text{M}$  SB 203580 in the present study, may selectively inhibit p38 MAPK and thereby exclude the possibility that inhibition of other kinases may have participated in the cardioprotection we observed. Yue *et al.*, 2000 used a selective inhibitor of p38 MAPK, SB242719, which had no effect on JNK or ERK in ischaemia-triggered myocytes and indicated that the protective effect of this compound was exclusively due to inhibition of p38 MAPK.

However, elucidating the exact role of p38 MAPK in cardioprotection is complicated by the identification of at least six isoforms of the enzyme (Sugden *et al.*, 1998). These isoforms may have distinct roles, with p38 $\alpha$  activation mediating apoptotic death in several cell types (Zia *et al.*, 1995, Kawasaki *et al.*, 1997, Kummer *et al.*, 1997), as well as in cardiomyocytes (MacKay and Mochly-Rosen 1999, Ma *et al.*, 1999, Saurin *et al.*, 2000, Sato *et al.*, 2000,) and p38 $\beta$  potentially contributing to cell survival (Wang *et al.*, 1998, Saurin *et al.*, 2000), while the presence and importance of p38 $\gamma$  and  $\delta$  in the myocardium remain to be determined. An additional confounding factor is that SB 203580 is a potent inhibitor of both p38 $\alpha$  and p38 $\beta$  MAPKs (Ping *et al.*, 2000). Therefore development of isoform-specific pharmacological inhibitors, selective monoclonal antibodies for all members of the p38 MAPK family or transgenic animals in which individual isoforms are manipulated, is essential.

A recent study by Martin *et al.* (2001), confirmed that SB 203580-mediated protection in adult rat cardiac myocytes depends on the inhibition of p38 $\alpha$  MAPK. In myocytes expressing wild-type p38 $\alpha$  MAPK, SB 203580 present during simulated ischaemia reduced injury, while SB 203580 did not protect cells expressing the SB 203580-

resistant form of p38 $\alpha$  MAPK. Therefore based on the evidence provided by Saurin *et al.* (2000) and Martin *et al.* (2001), it is possible that in ischaemic and  $\beta$ -adrenergic PC activation of p38 $\alpha$  MAPK is prevented during sustained ischaemia while only the protective  $\beta$ -isoform is expressed.

#### **6.3.4 Mechanism of p38 MAPK in PC protection:**

The relationship between p38 MAPK activation and its consequent underlying cellular mechanisms of cardioprotection by ischaemic PC is not yet clear. Furthermore, the end-effector of PC also needs to be elucidated. There are a number of candidates for the downstream effectors of p38 MAPK, which may also be the final effectors of cardioprotection. Although these effectors were not investigated in this study, we would like to speculate that they may indeed be involved in the cardioprotection of PC.

##### **6.3.4.1 Free radicals and NF $\kappa$ B:**

Our results indicated that during  $\beta$ -adrenergic preconditioning, cAMP induced transient p38 MAPK activation. This could have been mediated by cAMP-induced reduction of mitochondrial complex 1 activity (Scacco *et al.*, 2000) and generation of free radicals (Givertz *et al.*, 2001). Furthermore, Maulik *et al.* (1998a) proposed that during ischaemic adaptation (PC protocol) oxidative stress is developed by free radicals that function as second messengers resulting in the activation of p38 MAPK and MAPKAPK2 that could lead to the activation of NF $\kappa$ B (Maulik *et al.*, 1998a). The latter in turn may be involved in the induction of expression of antioxidant genes (Schieven *et al.*, 1993).

##### **6.3.4.2 Heat-shock protein (HSP) 27:**

Changes in localization of HSP27 may play a role in the mechanism of PC: redistribution of HSP27 from the cytosolic to the cytoskeletal fraction occurs after PC with 4 cycles of 5 min hypoxia-reoxygenation of rat myoblast cell line H9C2 (Sakamoto *et al.*, 1998). In addition, Sanada *et al.* (2001) concluded that phosphorylation and translocation of HSP27 occurs after ischaemic PC through the transient activation of p38 MAPK and protects myocardium from subsequent sustained ischaemic injuries. Phosphorylation of HSP27 is instrumental in polymerization and prevention of fragmentation of actin (Huot *et al.*, 1996, Bluhm *et al.*, 1998, Yoshida *et al.*, 1999),

thereby counteracting the disruptive effects of ischaemia on actin microfilaments (Das *et al.*, 1999).

#### **6.3.4.3 cAMP response element-binding protein (CREB):**

CREB could be activated by phosphorylation in response to a number of signalling pathways, including cAMP, calcium, stress and mitogenic stimuli (reviewed by Mayr and Montminy, 2001), as well as by transient global ischaemia (Williams and Ford 2001). Specifically, two downstream kinases of p38 MAPK, namely MAPKAPK-2 and MSK-1, may mediate activation of CREB (Tan *et al.*, 1996, Deak *et al.*, 1998). Activation of CREB induces the expression of the anti-apoptotic factor bcl-2, mediating cell survival of cardiomyocytes (Mehrhof *et al.*, 2001). Therefore the activation of CREB by cAMP, as well as p38 MAPK, during the ischaemic PC protocol could elicit a pro-survival response.

#### **6.3.4.4 Stress-responsive genes (ATF-2, MEF-2, c-Jun):**

ATF-2, unlike the related CREB, is not activated by increased cAMP, but by stimuli that activate SAPKs. Both JNK and p38 MAPK regulate ATF-2 and its function appears to be similar to that of c-Jun (Gupta *et al.*, 1995). Furthermore, p38 MAPK also phosphorylates the transcription factor MEF-2, which leads to the increase in *c-jun* transcription, thus linking p38 MAPK and JNK pathways (Han *et al.*, 1997). In addition, a significant increase in apoptotic cell death, was evident in the rat heart following ischaemia-reperfusion and correlated with the pattern of JNK and p38 MAPK activation, as well as expression of two stress-responsive genes, c-Jun and ATF3 (Yin *et al.*, 1997). Therefore, attenuation of p38 MAPK during sustained ischaemia by PC could result in a decreased expression of the stress-responsive genes and diminished cell death.

#### **6.3.4.5 Phosphatases:**

Protein phosphatase regulation also plays a role in regulating intracellular signalling pathways. According to Bogoyevitch (2000) it remains possible that phosphatase inhibition during ischaemia will facilitate higher levels and/or prolonged phosphorylation of proteins that provide protection. However, the deleterious effects of sustained p38

MAPK activation in ischaemia are emphasized by evidence that inactivation of a tyrosine phosphatase during ischaemia results in prolonged p38 MAPK activation that causes cell death (MacKay *et al.*, 2000). The identification of this phosphatase might assist in elucidating what cellular mechanism is responsible for the attenuation of the ischaemia-induced increase in p38 MAPK seen in preconditioned hearts (Gysembergh *et al.*, 2001).

#### **6.3.4.6 ATP-sensitive potassium (KATP) channels:**

For the past decade attention has focused on the KATP channel following the initial discovery by Gross and Auchampach (1992) that KATP channels appeared to be the elusive end-effector of ischaemic PC's protection. Since then, the involvement of the mitochondrial rather than the sarcolemmal KATP channel has been demonstrated by several workers (Garlid *et al.*, 1997, Liu *et al.*, 1998). Traditionally, KATP channels were thought to be located distal to MAPKs; e.g. Baines *et al.* (1999) reported that the opening of KATP channels on mitochondria are activated by p38 MAPK and could mediate cardioprotection. However, recent data indicated that mitochondrial KATP channels might act as a trigger rather than an end-effector of the protection of PC (Pain *et al.*, 1999, Wang and Ashraf (1999).

Besides the fact that there is controversy regarding these channels' location in the sequence of events leading to cell protection, it is also unknown in which manner the signalling events occurring in the preconditioned cell lead to opening of these channels and the exact mechanism of the latter. Garlid (2000) recently concluded that the only primary effect of K<sup>+</sup> flux through mitochondrial KATP channels is to regulate mitochondrial volume and that reported changes in membrane potential and Ca<sup>2+</sup> uptake are epiphenomena of supra-pharmacological drug doses.

Although, activation of a number of surface receptors probably precedes opening of the KATP channels prior to sustained ischaemia (Baines *et al.*, 1999), the link (if any) between these events remains to be established. Furthermore, it is possible that a different ion channel or an unidentified protein may still be the elusive end-effector.

## 6.4 SUMMARY

The results obtained in this study suggest that p38 MAPK plays a putative role in myocardial protection against ischaemia and that it is not a mere epiphenomenon of prior PC. Although transient activation of the kinase appears to be the trigger in  $\beta$ -adrenergic PC, and may contribute to triggering ischaemic PC, other triggers may also be involved in a multi-cycle protocol. This study showed that cardioprotection elicited by either ischaemic or pharmacological PC, is associated with attenuation of p38 MAPK activation during sustained ischaemia and reperfusion. In view of the fact that a blocker of p38 MAPK activation, SB 203580, also protects against ischaemic damage, we propose that activation of p38 MAPK during ischaemia-reperfusion is harmful and attenuation of activation is required for cardioprotection. Since the changes in p38 MAPK activation both during the PC protocol and during ischaemia follow the pattern observed in the  $\beta$ -adrenergic signal transduction pathway, the changes in p38 MAPK may be downstream to cAMP.

It remains intriguing that activation of the  $\beta$ -adrenergic signalling pathway and p38 MAPK acts as triggers, whereas attenuation of the same pathway during sustained ischaemia is required for cardioprotection. The exact cellular mechanism by which attenuation of the kinase occurs remains to be established.

## CHAPTER 7

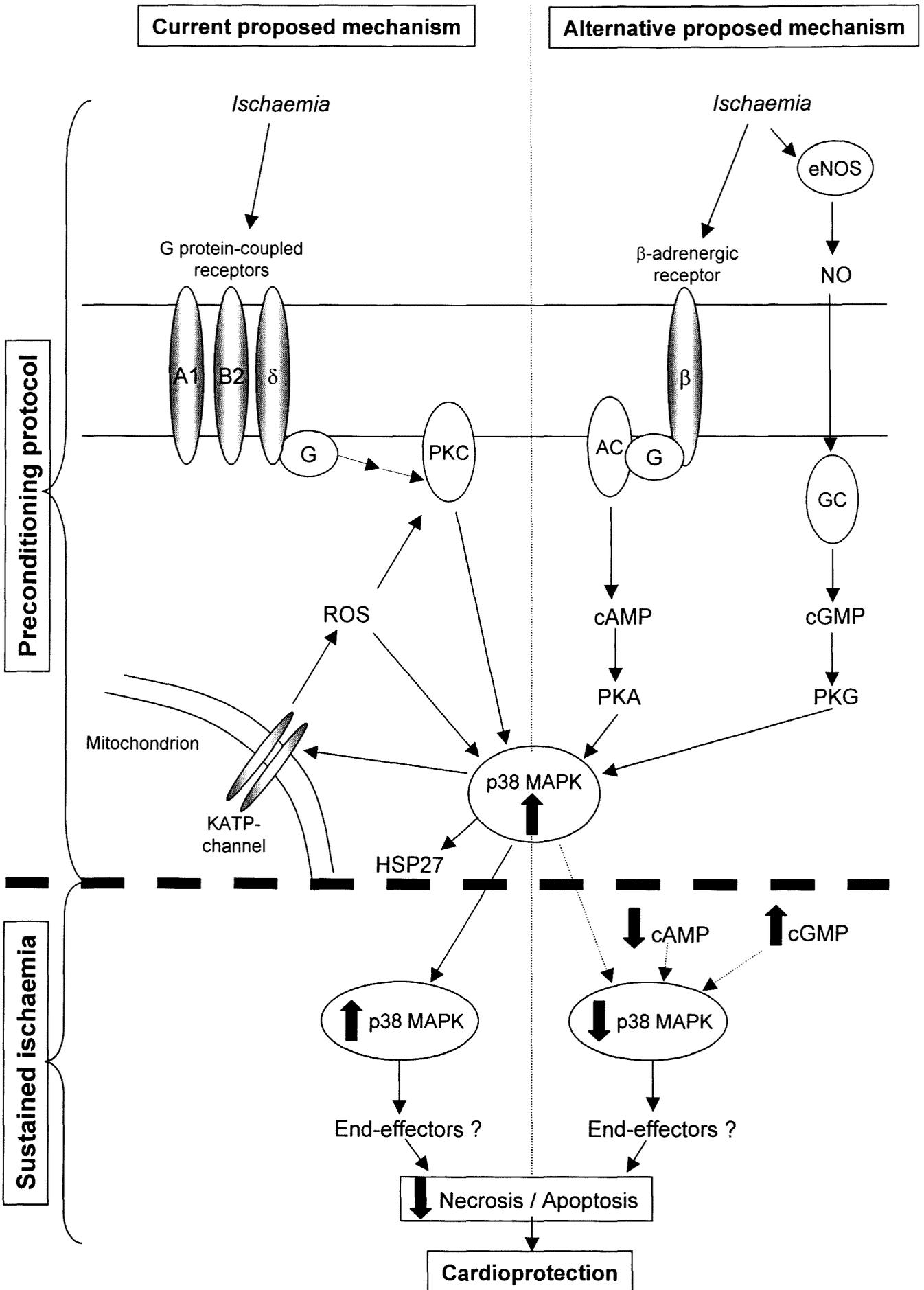
### Conclusions

In this study, we showed that during a multi-cycle ischaemic preconditioning protocol triggering agonists, for example catecholamines and nitric oxide, are released which induced cyclic changes in the cyclic nucleotides, cAMP and cGMP, with concomitant changes in cAMP- and cGMP- phosphodiesterase activities. The changes in the  $\beta$ -adrenergic pathway was associated by marked fluctuations in adenylyl cyclase and protein kinase A activity, accompanied by a decrease in receptor affinity ( $K_d$ ) and responsiveness despite a gradual upregulation of the  $\beta$ -adrenergic receptor population ( $B_{max}$ ). In addition, transient activation of the stress kinase, p38 MAPK was observed during the PC protocol. This kinase could set in motion further downstream adaptive processes, such as activation of HSP27 and mitochondrial ATP-sensitive potassium channel (mito. KATP-channel), which upon induction of the second period of sustained ischaemia, could protect the myocardium. The significance of these changes during the PC protocol and their role as triggers was investigated by using appropriate activators and inhibitors of cAMP, cGMP and p38 MAPK.

Furthermore, the sustained ischaemic period of PC hearts was characterized by reduced tissue cAMP levels, an attenuated  $\beta$ -adrenergic response and elevated cGMP levels, associated with less PDE inhibition and reduced energy utilization, as well as attenuated activation of p38 MAPK. This was associated with cardioprotection and confirmed by appropriate manipulations with drugs. In addition pharmacological inhibition of p38 MAPK during sustained ischaemia also protects against ischaemic damage. Therefore activation of p38 MAPK during sustained ischaemia is harmful and attenuation thereof, which may reduce necrosis/apoptosis, is required for cardioprotection.

Therefore, from the results obtained in this study, an alternative mechanism (Fig 7) is proposed to the current proposed mechanism of classic ischaemic PC as reviewed by Downey and colleagues (Pain *et al.*, 2000, Schulz *et al.*, 2001, Fig 7): They stated that ischaemic PC confers protection to the myocardium through a signal-transduction pathway that could be divided into 2 phases. The first is the trigger phase, which occurs before the sustained ischaemia, followed by the mediator/effector phase during the sustained ischaemia. The triggers of PC involve binding to membrane receptors such

as adenosine (A1), bradykinin (B2) and opioid ( $\delta$ ) receptors. Ligand-receptor binding is thought to initiate the intracellular mediator phase by activation of protein kinase C (PKC). Free radicals also contribute to PKC's activation. The kinase cascade that follows PKC activation has not been clearly defined, but appears to involve at least one tyrosine kinase and perhaps p38 MAPK. Finally, it has been assumed that the mito. KATP-channel may be the final effector of this protection. However, recent evidence suggests that mito. KATP-channel opening triggers protection through free radical generation. Once in the preconditioned state, kinases (such as p38 MAPK) become activated if the heart again becomes ischaemic (during sustained ischaemia), and these kinases mediate protection by modulating an as-yet-unknown end-effector.



**Fig. 7** Comparison between the current proposed (modified from Schulz, 2001) and alternative proposed (the present study) mechanisms of classic ischaemic preconditioning.

## **Addendum**

### **List of publications resulting from this study**

Lochner A, Genade S, Tromp E, Moolman JA, Opie LH, Thomas S, Podzuweit T. Role of cyclic nucleotide phosphodiesterases in ischaemic preconditioning. *Mol Cell Biochem* 1998; 186: 169 – 175.

Lochner A, Genade S, Marais E, Podzuweit T, Moolman JA. Ischaemic preconditioning and the  $\beta$ -adrenergic signal transduction pathway. *Circulation* 1999; 100: 958 - 966.

Lochner A, Marais E, Genade S, Moolman JA. Nitric oxide: a trigger for classic preconditioning. *Am J Physiol* 2000; 279: H2752 – H2765.

Marais E, Genade S, Huisamen B, Strijdom JG, Moolman JA, Lochner A. Activation of p38 MAPK induced by a multi-cycle ischaemic preconditioning protocol is associated with attenuated p38 MAPK activity during sustained ischaemia and reperfusion. *J Mol Cell Cardiol* 2001; 33: 769-778.

Marais, E., Genade, S., Strijdom, J.G., Moolman, J.A., Lochner, A. p38 MAPK activation triggers pharmacologically-induced beta-adrenergic preconditioning, but not ischaemic preconditioning. *J Mol Cell Cardiol* 2001; 33: 2157-77.

Lochner, A., Marais, E., du Toit, E.F., Moolman, J.A. Nitric oxide triggers classic ischaemic preconditioning. *Ann NY Acad Sci* 2002; 962 (34): 402-414.

## References

- Abe J-i, Baines CP, Berk BC. Role of mitogen-activated protein kinases in ischemia and reperfusion injury: the good and the bad. *Circ Res* 2000; 86: 607-609.
- Abe J-i, Kushuhara M, Ulevitch RJ, Berk BC, Lee J-D. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. *J Biol Chem* 1996; 271: 16586-16590.
- Ahlquist RP. A study of the adrenotropic receptors. *Am J Physiol* 1948; 153: 586-600.
- Ahumada GG, Bergmann SR, Carlson E, Corr PB, Sobel BE. Augmentation of cyclic AMP content induced by lysophosphatidyl choline in rabbit hearts. *Cardiovasc Res* 1979; 13: 377-382.
- Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, Shiojima I, Hiroi Y, Yazaki Y. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. *J Clin Invest* 1997; 100: 1813-1821.
- Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. *Molecular Biology of the Cell*. 3<sup>rd</sup> ed. New York and London. Garland Publishing 1994 Chapter 15.
- Alessi DR, Gomez N, Moorhead G, Lewis T, Keyse SM, Cohen P. Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr Biol* 1995; 5: 283-295.
- Alkhulaifi AM, Pugsley WB, Yellon DM. The influence of the time period between preconditioning ischemia and prolonged ischemia on myocardial protection. *Cardioscience* 1993; 4: 163-169.
- Andersson MB, Ketterman AJ, Bogoyevitch MA. Differential regulation of parallel mitogen-activated protein kinases in cardiac myocytes revealed by phosphatase inhibition. *Biochem Biophys Res Commun* 1998; 251: 328-333.
- Ardell JL, Yang XM, Thornton JD, Swafford A, Cohen MV, Downey JM. Depletion of norepinephrine by chronic surgical sympathectomy does not block preconditioning (Abstract). *Circulation* 1994; 90: I-108.
- Armstrong SC, Delacey MH, Ganote CE. Phosphorylation state of hsp27 and p38 MAPK during preconditioning and protein phosphatase inhibitor protection of rabbit cardiomyocytes. *J Mol Cell Cardiol* 1999; 31: 555 - 567.
- Armstrong SC, Downey JM, Ganote CE. Preconditioning of the isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. *Cardiovasc Res* 1994; 28: 72-77.
- Armstrong SC, Ganote CE. Effects of the protein phosphatase inhibitors okadaic acid and calyculin A on metabolically inhibited and ischemic isolated myocytes. *J Mol Cell Cardiol* 1992; 24: 869-884.
- Armstrong SC, Gao W, Lane JR, Ganote CE. Protein phosphatase inhibitors calyculin A and fostriecin protect rabbit cardiomyocytes in late ischemia. *J Mol Cell Cardiol* 1998; 30: 61-73.
- Armstrong SC, Hoover DB, Delacey MH, Ganote CE. Translocation of PKC, protein phosphatase inhibition and preconditioning of rabbit cardiomyocytes. *J Mol Cell Cardiol* 1996; 28: 1479-1492.
- Armstrong SC, Liu GS, Downey JM, Ganote CE. Potassium channels and preconditioning of isolated rabbit cardiomyocytes: effects of glyburide and pinacidil. *J Mol Cell Cardiol* 1995; 27: 1765-1774.
- Asimakis GK, Inners-McBride K, Conti VR, Yang C. Transient  $\beta$ -adrenergic stimulation can precondition the rat heart against postischaemic contractile dysfunction. *Cardiovasc Res* 1994; 28: 1726-1734.

- Asimakis GK, Inners-McBride K, Medellin G, Conti VR. Ischaemic preconditioning attenuates acidosis and postischaemic dysfunction in isolated rat heart. *Am J Physiol* 1992; 163: H887-H894.
- Asimakis GK, Inners-McBride K. Attenuated postischaemic contractile dysfunction associated with ischaemic preconditioning is not mediated by  $\alpha_1$ -adrenoceptor stimulation. *Circulation* 1993; 88(1): 1-43.
- Asimakis GK, Lick SD, Conti VR. Transient ischemia cannot precondition the rabbit against postischemic contractile dysfunction. *Ann Thorac Surg* 1996; 62: 543-549.
- Assefa Z, Vantieghem A, Declercq W. The activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. *J Biol Chem* 1999; 274: 8766-8796.
- Attramandal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ. Beta-arrestin 2, a novel member of the arrestin / beta-arrestin gene family. *J Biol Chem* 1992; 267:17882-17890.
- Avkiran M. Protection of the myocardium during ischemia and reperfusion.  $\text{Na}^+/\text{H}^+$  exchange inhibition versus ischemic preconditioning. *Circulation* 1999; 100: 2469-2472.
- Bagrodia S, Derijard B, Davis RJ, Cerione RA. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J Biol Chem* 1995; 270: 27995-27998.
- Baines CP, Cohen MV, Downey JM. Signal transduction in ischaemic preconditioning: the role of kinases and mitochondrial KATP channels. *J Cardiovasc Electro-physiol* 1999; 10: 741-754.
- Baines CP, Goto M, Downey JM. Oxygen radicals released during ischaemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J Mol Cell Cardiol* 1997; 29: 207-216.
- Baines CP, Pass JM, Ping P. Protein kinases and kinase-modulated effectors in the late phase of ischemic preconditioning. *Basic Res Cardiol* 2001; 96: 207-218.
- Baines CP, Wang L, Cohen MV, Downey JM. Protein tyrosine kinase is downstream of protein kinase C for ischaemic preconditioning's anti-infarct effect in the rabbit heart. *J Mol Cell Cardiol* 1998; 30: 383-392.
- Balligand JL, Cannon PJ. Nitric oxide synthases and cardiac muscle: autocrine and paracrine influences. *Arterioscler Thromb Vasc Biol* 1997; 17: 1846-1858.
- Balligand JL. Regulation of cardiac  $\beta$ -adrenergic response by nitric oxide. *Cardiovasc Res* 1999; 43: 607-620.
- Balligand JL., Kelly R.A., Marsden P.A., Smith T.W., Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signalling system. *Proc. Natl. Acad. Sci.* 1993; 90: 347 – 351.
- Banerjee A, Locke-Winter C, Rogers K, Mitchell MB, Brew EC, Cairns C, Bensard D, Harken AH. Preconditioning against myocardial dysfunction after ischaemia and reperfusion by an  $\alpha_1$ -adrenergic mechanism. *Circ Res* 1993; 73: 656 – 670.
- Bankwala Z, Hale SL, Kloner RA.  $\alpha$ -Adrenoceptor stimulation with exogenous norepinephrine or release of endogenous catecholamines mimics ischaemic preconditioning. *Circulation* 1994; 90: 1023-1028.
- Barancik M, Htun P, Schaper W. Okadaic acid and anisomycin are protective and stimulate the SAPK/JNK pathway. *J Cardiovasc Pharmacol* 1999; 34: 182 - 190.
- Barancik M, Htun P, Strohm C, Kilian S, Schaper W. Inhibition of cardiac p38 MAPK pathway by SB 203580 delays ischaemic cell death. *J Cardiovasc Pharmacol* 2000; 35: 474-483.
- Baxter G F. Ischaemic preconditioning of the myocardium. *Ann Med* 1997; 29: 345–352.

Baxter GF, Marber MS, Yellon DM. Myocardial stress response, cytoprotection proteins and the second window of protection against infarction. In: Wainwright CL, Parratt JR, eds. Myocardial Preconditioning. Georgetown, Tex: RG Landes Co; 1996: 233-250.

Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* 1995; 75: 725-748.

Beckman JS, Beckman TW, Chen J, Marshall PA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87: 1620-1624.

Beckman JS, Koppenol WH. Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. *Am J Physiol* 1996; 271: C1424-1437.

Behrends M, Schulz R, Post H, Alexandrov A, Belosjorow S, Michel MC, Heusch G. Inconsistent relation of MAPK activation to infarct size reduction by ischemic preconditioning in pigs. *Am J Physiol Heart Circ Physiol* 2000; 279: H1111-1119.

Bell RM, Yellon DM. The contribution of endothelial nitric oxide synthase to early ischaemic preconditioning: the lowering of the preconditioning threshold. An investigation in eNOS knockout mice. *Cardiovasc Res* 2001; 52(2): 274-280.

Ben-Levy R, Hooper S, Wilson R, Paterson HF, Marshall CJ. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr Biol* 1998; 8: 1049-1057.

Bilinska M., Maczewski M., Beresewicz A. Donors of nitric oxide mimic effects of ischaemic preconditioning on reperfusion induced arrhythmias in isolated rat hearts. *Mol. Cell. Biochem* 1996; 160 – 161: 265 – 271.

Bishopric NH, Jayasena V, Webster KA. Positive regulation of the skeletal  $\alpha$ -actin gene by Fos and Jun in cardiac myocytes. *J Biol Chem* 1992; 267: 25535-25540.

Blanco-Aparicio C, Torres J, Pulido R. A novel regulatory mechanism of MAP kinases activation and nuclear translocation mediated by PKA and the PTP-SL tyrosine phosphatase. *J Cell Biol* 1999; 147: 1129-1135.

Bluhm WF, Martin JL, Mestriell R, et al. Specific heat shock proteins protect microtubules during simulated ischemia in cardiac myocytes. *Am J Physiol* 1998; 275: H2243-H2249.

Bode DC, Kanter JR, Brunton LL. Cellular distribution of phosphodiesterase isoforms in rat cardiac tissue. *Circ Res* 1991; 68: 1070-1079.

Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH. Stimulation of the stress-activated mitogen-activated protein kinase subfamily in the perfused rat heart. p38/ERK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ Res* 1996; 79: 162 - 173.

Bogoyevitch MA, Ketterman AJ, Sugden PH. Cellular stresses differentially activate c-Jun N-terminal protein kinases and the extracellular signal-regulated protein kinases in cultured ventricular myocytes. *J Biol Chem* 1995a; 270: 29710 - 29717.

Bogoyevitch MA, Marshall CJ, Sugden PH. Hypertrophic agonists stimulate the activities of the protein kinases c-Raf and A-Raf in cultured ventricular myocytes. *J Biol Chem* 1995b; 270: 26303-26310.

Bogoyevitch MA. Signalling via stress-activated mitogen-activated protein kinases in the cardiovascular system. *Cardiovasc Res* 2000; 45: 826 - 842.

Bohinski RC. Modern concepts in biochemistry, 4<sup>th</sup> ed. Allyn and Bacon, Inc. 1983: 169.

Bolli R. The late phase of preconditioning. *Circ Res* 2000; 87: 972-983.

Bolli R., Bhatti Z.A., Tang X.L., Qiu Y., Zhang Q., Guo Y., Jadoon A.K. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ. Res.* 1997; 81: 42 – 52.

Bolli R., Dawn B., Tang X.L., Qiu Y., Ping P., Xuan Y.T., Jones W.K., Takano H., Guo Y., Zhang J. The nitric oxide hypothesis of late preconditioning. *Bas. Res. Cardiol* 1998; 93: 325 – 338.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and –independent mechanisms. *Science* 1999; 286: 1358-1362.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD. ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 1991; 65: 663-675.

Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* 1990; 249: 64-67.

Bradamante S, Piccinini F, Delu C, Janssen M, de Jong JW. NMR evaluation of changes in myocardial high energy metabolism produced by repeated short periods of ischemia. *Biochim Biohys Acta* 1995; 1243:1-8.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 71: 258 - 254.

Brady AJB, Poole-Wilson PA, Harding SE, Warren JB. Nitric oxide production within cardiac myocytes reduces their contractility in endotoxaemia. *Am J Physiol* 1992; 263: H1963-H1966.

Brady AJB, Warren JB, Poole-Wilson PA, Williams TJ. Nitric oxide attenuates cardiac myocyte contraction. *Am J Physiol* 1993; 265: H176-182.

Brooks G, Hearse DJ. Role of protein kinase C in ischaemic preconditioning: player or spectator? (Editorial). *Circ Res* 1996; 79: 627 - 630.

Browning DD, McShane MP, Marty C, Ye RD. Nitric oxide activation of p38 mitogen-activated protein kinase in 293T fibroblasts requires cGMP-dependent protein kinase. *J Biol Chem* 2000; 275(4): 2811-2816.

Browning DD, Windes ND, Ye RD. Activation of p38 mitogen-activated protein kinase by lipopolysaccharide in human neutrophils requires nitric oxide-dependent cGMP accumulation. *J Biol Chem* 1999; 274: 537-542.

Bugge E, Ytrehus K. Bradykinin protects against infarction but does not mediate ischemic preconditioning in the isolated rat heart. *J Mol Cell Cardiol* 1996; 28: 2333-2341.

Bugge E, Ytrehus K. Inhibition of sodium-hydrogen exchange reduces infarct size in the isolated rat heart – a protection additive to ischaemic preconditioning. *Cardiovasc Res* 1995b; 29: 269-274.

Bugge E, Ytrehus K. Ischaemic preconditioning is protein kinase C dependent but not through stimulation of alpha adrenergic or adenosine receptors in the isolated rat heart. *Cardiovasc Res* 1995a; 29(3): 401-406.

Burgering BM, Bos JL. Regulation of Ras-mediated signalling: More than one way to skin a cat. *Trends Biochem Sci* 1995; 20(1): 18-22.

Butt E, Geiger J, Jarchau T, Lohmann SM, Walter U. The cGMP-dependent protein kinase - gene, protein and function. *Neurochem Res* 1993; 18:27-42.

Buxton BF, Jones CR, Molenaar P, Summers RJ. Characterization and autoradiographic localization of  $\beta$ -adrenoreceptor subtypes in human cardiac tissues. *Br J Pharm* 1987; 92: 299-

310.

Caivano M. Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages. *FEBS Lett* 1998; 429: 249-253.

Carroll R, Grant VA, Yellon DM. Mitochondrial KATP channel opening protects a human atrial-derived cell line by a mechanism involving free radical generation. *Cardiovasc Res* 2001; 51: 691-700.

Catling AD, Reuter CW, Cox ME, Parsons SJ, Weber MJ. Partial purification of a mitogen-activated protein kinase kinase activator from bovine brain. Identification as B-Raf or a B-Raf-associated activity. *J Biol Chem* 1994; 269: 30014-30021.

Cave AC, Apstein CS. Inhibition of protein kinase C abolishes preconditioning against contractile dysfunction in the isolated perfused rat heart. *Circulation* 1994; 90: I-208.

Cave AC, Collis CS, Downey JM, Hearse DJ. Improved functional recovery by ischaemic preconditioning is not mediated by adenosine in the globally ischaemic isolated rat heart. *Cardiovasc Res* 1993; 27: 663-668.

Cave AC, Hearse DJ. Ischaemic preconditioning and contractile function: Studies with normothermic and hypothermic global ischaemia. *J Mol Cell Cardiol* 1992; 24: 1113-1123.

Cave AC. Improved functional recovery by ischaemic preconditioning is not mediated by adenosine in the globally ischaemic isolated rat heart. *Cardiovasc Res* 2000; 45: 131-133.

Cave AC. Preconditioning induced protection against post-ischaemic contractile dysfunction: characteristics and mechanisms. *J Mol Cell Cardiol* 1995; 27: 969-979.

Chauhan D, Kharbanda S, Ogata A, Urashima M, Teoh G, Robertson M, Anderson KC. Interleukin-6 inhibits Fas-induced apoptosis and stress-activated protein kinase activation in multiple myeloma cells. *Blood* 1997; 89: 227-234.

Chen W, Gabel S, Steenbergen C, Murohy E. A redox mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. *Circ Res* 1995; 77: 424-429.

Chu YF, Solski PA, Khosravifar R, Der CJ, Kelly K. The mitogen-activated protein kinase phosphatases PAC1, MKP-1 and MKP-2 have unique substrate specificity and reduced activity in vivo toward the ERK2 Sevenmaker mutation. *J Biol Chem* 1996; 271: 6497-6501.

Clark R B, Friedman J, Dixon R A F, Strader C D. Identification of a specific site required for rapid heterologous desensitization of the  $\beta$ -adrenergic receptor by cAMP-dependent protein kinase. *Mol Pharmacol* 1989; 36: 343 - 348.

Clerk A, Michael A, Sugden PH. Stimulation of multiple mitogen-activated protein kinase subfamilies by oxidative stress and phosphorylation of the small heat shock protein Hsp 27 in neonatal ventricular myocytes. *Biochem J* 1998a; 333: 581 - 589.

Clerk A, Sugden PH. Mitogen-activated protein kinases are activated by oxidative stress and cytokines in neonatal rat ventricular myocytes. *Biochem Soc Trans* 1997; 25: 566S.

Clerk A, Sugden PH. The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/cJun N-terminal kinases (SAPKs/JNKs). *FEBS Lett* 1998b; 426(1): 93-6.

Cohen AH, Hanson K, Morris K, Fouty B, McMurty IF, Clarke W, Rodman DM. Inhibition of cyclic 3'-5'- guanosine monophosphate-specific phosphodiesterase selectively vasodilates the pulmonary circulation in chronically hypoxic rats. *J Clin Invest* 1996; 97: 172-179.

Cohen MV, Baines CP, Downey JM. Ischemic preconditioning: From Adenosine Receptor to K<sub>ATP</sub> Channel. *Annu Rev Physiol* 2000, 62: 79 - 109.

Cohen MV, Walsh RS, Goto M, Downey JM. Hypoxia preconditions rabbit myocardium via

adenosine and catecholamine release. *J Mol Cell Cardiol* 1995; 27: 1527-1534.

Cohen P. The structure and regulation of protein phosphatases. *Ann Rev Biochem* 1989;58:453-508.

Cohn JN, Levine TB, Olivari MT, *et al.* Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N Engl J Med* 1984; 311: 819-823.

Collins LR, Minden A, Karin M, Brown JH.  $\alpha$ 12 stimulates c-Jun NH<sub>2</sub>-terminal kinase through the small G proteins Ras and Rac. *J Biol Chem*. 1996 Jul 19; 271(29): 17349-53.

Communal C, Colucci WS, Singh K. p38 Mitogen-activated protein kinase pathway protects adult rat ventricular myocytes against  $\beta$ -adrenergic receptor-stimulated apoptosis. Evidence for Gi-dependent activation. *J Biol Chem* 2000; 275: 19395 - 19400.

Connaughton M, Hearse DJ. Three questions about preconditioning. *Basic Res Cardiol* 1996; 91: 12-15.

Conrad PW, Millhorn DE, Beitner-Johnson D. Hypoxia differentially regulates the mitogen- and stress-activated protein kinases. Role of Ca<sup>2+</sup>/CaM in the activation of MAPK and p38 gamma. *Adv Exp Med Biol* 2000; 475: 293 - 302.

Cook SA, Sugden PH, Clerk A. Activation of c-Jun N-terminal kinases and p38-mitogen-activated protein kinases in human heart failure secondary to ischaemic heart disease. *J Mol Cell Cardiol* 1999; 31(8): 1429-34.

Cooper JA, Hunter T. Identification and characterization of cellular targets for tyrosine protein kinases. *J Biol Chem* 1983; 258: 1108-1115.

Corbin JD, Turko IV, Beasley A, Francis SH. Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur J Biochem* 2000; 267: 2760-2767.

Coso OA, Chiariello M, Kalinec G, Kyriakis JM, Woodgett J, Gutkind JS. Transforming G protein-coupled receptors potently activate JNK (SAPK). Evidence for a divergence from the tyrosine kinase signaling pathway. *J Biol Chem* 1995; 270: 5620-5624.

Crespo P, Cachero TG, Xu N, Gutkind JS. Dual effect of beta-adrenergic receptors on mitogen-activated protein kinase. Evidence for a beta gamma-dependent activation and a G alpha s-cAMP-mediated inhibition. *J Biol Chem* 1995; 270: 25259-25265.

Crespo P, Xu N, Simonds WF, Gutkind JS. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* 1994; 396: 418-420.

Crews C, Alessandrini A, Erikson R. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* 1992; 258: 478-480.

Csonka C, Csont T, Onody A, Ferdinandy P. Preconditioning decreases ischaemia/reperfusion-induced peroxynitrite formation. *Biochem Biophys Res Commun* 2001; 285(5): 1217-1219.

Csonka C., Szilvássy Z., Fülöp F., Pali T., Blasig I.E., Tosaki A., Schulz R., Ferdinandy P. Classic preconditioning decreases the harmful accumulation of nitric oxide during ischaemia and reperfusion in rat hearts. *Circulation* 1999; 100: 2260 – 2266.

Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the  $\beta$ 2-adrenergic receptor to different G proteins by protein kinase A. *Nature* 1997; 390: 88-91.

Das DK, Engelman RM, Maulik N. Oxygen free radical signaling in ischaemic preconditioning. *Ann NY Acad Sci* 1999a; 874: 49 - 65.

Das DK, Maulik N, Sato M, Ray PS. Reactive oxygen species function as second messenger during ischemic preconditioning of heart. *Mol Cell Biochem* 1999b; 196: 59-67.

- Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem* 1993; 268: 14553-14556.
- Dawn B, Xuan YT, Qiu Y, Takano H, Tang XL, Ping P, Banerjee S, Hill M, Bolli . Bifunctional role of protein kinases in late preconditioning against myocardial stunning in conscious rabbits. *Circ Res* 1999; 85: 1154-1163.
- De Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*; 1998; 396: 474-477.
- Deacon K, Blank JL. MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH<sub>2</sub>-terminal kinases. *J Biol Chem* 1999; 274: 16604-16610.
- Deak M, Clifton AD, Lucocq JM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J* 1998; 17: 4426-4441.
- Dekker LRC. Toward the heart of ischemic preconditioning. *Cardiovasc Res* 1998; 37: 14-20.
- Depré C, Fierain L, Hue L. Activation of nitric oxide synthase by ischaemia in the perfused heart. *Cardiovasc Res* 1997; 33: 82-87.
- Depré C, Vanoverschelde J-L, Goudemant J-F, Mottet I, Hue L. Protection against ischaemic injury by nonvasoactive concentrations of nitric oxide synthase inhibitors in the perfused rabbit heart. *Circulation* 1995; 92: 1911 – 1918.
- Depré C., Hue L. Cyclic GMP in the perfused rat heart. Effect of ischaemia, anoxia and nitric oxide synthase inhibitor. *FEBS Lett* 1994; 345: 241-245.
- Derijard B, Hibi M, Wu I-H, Barrett T, Su B, Deng T, Karin M, Davis RJ. JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 1994; 76: 1025-1037.
- Dodge KL, Khouangsathiene S, Kapiloff MS, Mouton R, Hill EV, Houslay MD, Langeberg LK, Scott JD. mAkap assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. *EMBO J* 2001; 20: 1921-1930.
- Dong C, Yang DD, Wysk M, Whitmarsh AJ, Davis RJ, Flavell RA. Defective T cell differentiation in the absence of Jnk1. *Science* 1998; 282: 2092-2095.
- Downey JM, Cohen MW. Do mitochondrial KATP channels serve as triggers rather than end-effectors of ischemic preconditioning's protection? *Basic Res Cardiol* 2000; 95: 272-274.
- Downey JM, Cohen MW. Mechanism of preconditioning: Correlates and epiphenomena. In M.M. Marber and D.M. Yellon (eds). *Ischaemia: Preconditioning and adaptation*. UCL Molecular Pathology Series, BIOS Scientific Publishers Limited, Oxford, UK, 1996, pp21-34.
- Downward J. Control of Ras activation. *Cancer Surv.* 1996; 27: 87-100.
- Du Toit EF, McCArthy J, Miyashiro J, Opie LH, Brunner F. Effect of nitrovasodilators and inhibitors of nitric oxide synthase on ischaemic and reperfusion function of rat isolated hearts. *Br J Pharmacol* 1998; 123: 1159-1167.
- Du Toit EF, Opie LH. Modulation of severity of reperfusion stunning in the isolated rat heart by altering calcium flux at the onset of reperfusion. *Circ Res* 1992; 70: 960-967.
- Ebihara Y., Karmazyn M. Inhibition of  $\beta$ , but not  $\alpha$ -mediated adrenergic responses in isolated hearts and cardiomyocytes by nitric oxide and 8-bromo-cyclic cGMP. *Cardiovasc. Res.* 32: 622 – 629, 1996.
- Edoute Y, Graney D, Sanan D, Kotzé JCN, Lochner A. Effects of propranolol on ultrastructure,

- mitochondrial function and high energy phosphates of working rat hearts with coronary artery ligation. *J Mol Cell Cardiol* 1981; 13: 619-639.
- Edoute Y, Van der Merwe E, Sanan D, Kotzé JCN, Steinmann C, Lochner A. Normothermic ischaemic arrest of the isolated working rat heart. Effect of time and reperfusion on myocardial ultrastructural, mitochondrial oxidative function and mechanical recovery. *Circ Res* 1988; 58: 663-678
- Engelman DT, Watanabe M, Engelman RM, Rousou JA, Flack JE 3rd, Deaton DW, Das DK. Constitutive nitric oxide release is impaired after ischaemia and reperfusion. *J Thorac Cardiovasc Surg* 1995a; 110(4Pt1): 1047-1053.
- Engelman DT, Watanabe M, Maulik N, Cordis GA, Engelman RM, Rousou JA, Flack JE 3rd, Deaton DW, Das DK. L-Arginine reduces endothelial inflammation and myocardial stunning during ischaemia/reperfusion. *Ann. Thorac. Surg.* 1995b; 60: 1275 – 1281.
- English J, Pearson G, Wilbacher J, Swantek J, Karandikar M, Xu S, Cobb MH. New insights into the control of MAP kinase pathways. *Exp Cell Res* 1999; 253: 255-270.
- Enslin H, Raingeaud J, Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J Biol Chem* 1998; 273: 1741-1748.
- Erikson RL. Structure, expression and regulation of protein kinases involved in phosphorylation of ribosomal protein S6. *J Biol Chem* 1991; 266: 6007-6010.
- Fan G, Shumay E, Malbon CC, Wang H. c-Src tyrosine kinase binds the  $\beta$ 2-adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. *J Biol Chem* 2001; 276: 13,240-13,247.
- Fanger GR, Gerwins P, Widmann C, Jarpe MB, Johnson GL. MEKKs, GCKs, MLKs, PAKs, TAKs and Tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr Opin Genet Dev* 1997; 7: 67-74.
- Farber A, Kolodgie, FD, Jones, RM, Jenkins M, Virmani R. Early detection and measurement of experimental infarcts with horseradish peroxidase. *J. Mol. Cell. Cardiol.* 1993; 25: 343 – 353.
- Faure M, Voyno-Yasenetskaya TA, Bourne HR. cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J Biol Chem* 1994; 269: 7851-7854.
- Fentzke RC, Korcarz CE, Lang RM, Lin H, Leiden JM. Dilated cardiomyopathy in transgenic mice expressing a dominant-negative CREB transcription factor in the heart. *J Clin Invest* 1998; 101: 2415-2426.
- Ferdinandy P, Csont T, Csonka C, Török M, Dux M, Nemeth J, Horvath L I, Dux L. Capsaicin-sensitive local sensory innervation is involved in pacing-induced preconditioning rat hearts: role of NO and cGRP? *Naunyn-Schmiedeberg's Arch Pharmacol* 1997; 356: 363.
- Ferdinandy P, Szilvassy Z, Balogh N, Csonka C, Csont T, Koltai M, Dux L. Nitric oxide is involved in active preconditioning in isolated working rat hearts. *Ann NY Acad Sci* 1996; 793; 489 – 493.
- Ferdinandy P, Szilvassy Z, Csont T, Csonka C, Nagy E, Koltai M, Dux L. Nitroglycerin-induced direct protection of the ischaemic myocardium in isolated working hearts of rats with vascular tolerance to nitroglycerin. *Br J Pharmacol* 1995a; 115: 1129-1131.
- Ferdinandy P, Szilvassy Z, Koltai M, Dux L. Ventricular overdrive pacing-induced preconditioning and no-flow ischaemia-induced preconditioning in isolated working rat hearts. *J Cardiovasc Pharmacol* 1995b; 25(1): 97-104.
- Ferrari R, Ceconi C, Curello C, Alfieri O, Visioli O. Myocardial damage during ischaemia and reperfusion. *Eur Heart J* 1994; 14 (Suppl G): 25 - 30.

- Finegan BA, Lopaschuk GD, Gandhi M, Clanachan AS. Ischemic preconditioning inhibits glycolysis and proton production in isolated working rat hearts. *Am J Physiol Heart Circ Physiol* 1995; 269: H1767-H1775.
- Fischer EH, Charbonneau H, Tonks NK. Protein tyrosine phosphatase: a diverse family of intracellular and transmembrane enzymes. *Science* 1991; 253: 401-406.
- Fischer TA, Singh K, O'Hara DS, Kaye DM, Kelly RA. Role of AT1 and AT2 receptors in regulation of MAPKs and MKP-1 by ANG II in adult cardiac myocytes. *Am J Physiol* 1998; 275: H906-H916.
- Fischer Y, Rose H, Kammermeier H. Highly insulin responsive isolated rat heart muscle cells yielded by a modified isolated method. *Life Science*, 1991; 49: 1679-1688.
- Fleckenstein A. Specific inhibitors and promoters of calcium action in the excitation-contraction coupling of heart muscle and their role in the prevention of myocardial lesions. In: Harris P, Opie LH (eds). *Calcium and the Heart*. New York: Academic, 1971: 135-188.
- Fleming IN, Elliott CM, Collard JG, Exton JH. Lysophosphatidic acid induces threonine phosphorylation of Tiam 1 in Swiss 3T3 fibroblasts via protein kinase C. *J Biol Chem* 1997; 272(52): 33105-33110.
- Force T, Pombo CM, Avruch JA, Bonventre JV, Kyriakis JM. Stress-activated protein kinases in cardiovascular disease. *Circ Res* 1996; 78: 947-953.
- Francis SH, Lincoln TM, Corbin JD. Characterization of a novel cGMP binding protein from rat lung. *J Biol Chem* 1980; 255: 620-626.
- Franklin CC, Kraft AS. Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. *J Biol Chem* 1997; 272: 16917-16923.
- Franklin CC, Srikanth S, Kraft AS. Conditional expression of mitogen-activated protein kinase phosphatase-1, MKP-1, is cardioprotective against UV-induced apoptosis. *Proc Natl Acad Sci USA* 1998; 95: 3014-3019.
- Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, Saklatvala J. Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* 1994; 78: 1039-1049.
- Frodin M, Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol*. 1999; 151:65-77.
- Fryer RM, Patel HH, Hsu AK, Gross GJ. Stress-activated protein kinase phosphorylation during cardioprotection in the ischemic myocardium. *Am J Physiol* 2001; 281: H1184-H1192.
- Fryer RM, Schultz JE, Hsu AK, Gross GJ. Importance of PKC and tyrosine kinase in single or multiple cycles of preconditioning in rat hearts. *Am J Physiol* 1999; 276: H1229-1235.
- Fu LX, Kirkeboen KA, Liang QM, Sjoegren KG, Hjalmarson A, Ilebek A. Free radical scavenging enzymes and G protein mediated receptor signalling systems in ischaemically preconditioned porcine myocardium. *Cardiovasc Res* 1993; 27: 612-616.
- Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; 288: 373-376.
- Furchgott RF. Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from retractor penis in organic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Vanhoutte PM, editor, *Vasodilatation: vascular smooth muscle, peptides, autonomic nerves and endothelium*, New York: Raven Press, 1988, 401-414.
- Ganiatsas S, Kwee L, Fujiwara Y, Perkins A, Ikeda T, Labow MA, Zon LI. SEK1 deficiency reveals mitogen-activated protein kinase cascade cross-regulation and leads to abnormal hepatogenesis.

Proc Natl Acad Sci USA 1998; 95: 6881-6886.

Ganote CE, Vander Heide RS. Irreversible injury of isolated adult rat myocytes: osmotic fragility during metabolic inhibition. *Am J Pathol* 1988; 132: 212-222.

Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, et al. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K<sup>+</sup> channels: possible mechanism of cardioprotection. *Circ Res* 1997; 81: 1072-82.

Garlid KD. Opening mitochondrial KATP in the heart – what happens, and what does not happen. *Basic Res Cardiol* 2000; 95: 275-279.

Gartner A, Nasmyth K, Ammerer G. Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of FUS3 and KSS1. *Genes Dev* 1992; 6: 1280-1292.

Gauthier C, Tavernier G, Charpentier F, Langin D, Le Marec H. Functional  $\beta$ 3-adrenoceptor in the human heart. *J Clin Invest* 1996; 98: 556-562.

Gibbons WR. Cellular control of cardiac contraction. In: Fozzard HA, editor. *The heart and cardiovascular system* (Scientific Foundation), New York: Raven Press 1986, (Vol1): pp747-778.

Givertz MM, Sawyer DB, Colucci WS. Antioxidants and myocardial contractility: Illuminating the "Dark Side" of beta-adrenergic receptor activation? *Circ* 2001; 103: 782-783.

Goto M, Liu Y, Yang XM. Role of bradykinin in protection of ischaemic preconditioning in rabbit hearts. *Circ Res* 1995; 77:611-621.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 1994; 94: 1621-1628.

Gray MO, Karliner JS, Mochly-Rosen D. A selective  $\epsilon$ -protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem* 1997; 272: 30945-30951.

Guan Z, Baier LD, Morrison AR. p38 mitogen-activated protein kinase down-regulates nitric oxide and upregulates prostaglandin E2 biosynthesis stimulated by interleukin-1beta. *J Biol Chem* 1997; 272: 8083-8089.

Guan Z, Buckman SY, Miller BW, Springer LD, Morrison AR. Interleukin-1beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH<sub>2</sub>-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. *J Biol Chem* 1998; 273: 28670-28676.

Gupta S, Campbell D, Derijard B, Davis R. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 1995; 267: 389-393.

Gutkind JS. Regulation of mitogen-activated protein kinase signalling networks by G protein-coupled receptors. *Science's STKE* 11 July 2000 (40): review1.

Guyton KZ, Liu YS, Gorospe M, Xu QB, Holbrook NJ. Activation of mitogen-activated protein kinase by H<sub>2</sub>O<sub>2</sub> – role in cell survival following oxidant injury. *J Biol Chem* 1996; 271: 4138-4142.

Gysembergh A, Simkhovich BZ, Kloner RA, Przyklenk K. p38 MAPK activity is not increased early during sustained coronary artery occlusion in preconditioned versus control rabbit heart. *J Mol Cell Cardiol* 2001; 33: 681-690.

Habener JF. Cyclic AMP-response element binding proteins: a cornucopia of transcription factors. *Mol Endocrinol* 1990; 4: 1087-1094.

Haessler R, Kuzume K, Wolff RA, Kuzume K, Chien GL, Davis RF, Van Winkle DM. Adrenergic activation confers cardioprotection mediated by adenosine, but not required for ischemic preconditioning. *Coron Artery Dis* 1996; 7(4): 305-314.

- Hagar JM, Hale SL, Kloner RA. Effect of preconditioning ischemia on reperfusion arrhythmias after coronary artery occlusion and reperfusion in the rat. *Circ Res* 1991; 68: 61-68.
- Hajjar RJ, Muller FU, Schmitz W, Schnabel P, Bohm M. Molecular aspects of adrenergic transduction in cardiac failure. *J Mol Med* 1998; 76: 747-755.
- Han J, Jiang Y, Li Z, Kravchenko VV, Ulevitch RJ. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* 1997; 386: 296-299.
- Han J, Kim N, Kim E, Ho W-K, Earm YE. Modulation of ATP-sensitive potassium channels by cGMP-dependent protein kinase in rabbit ventricular myocytes. *J Biol Chem* 2001; 276(25): 22140-22147.
- Han J, Lee J-D, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 1994; 265: 808-811.
- Han X, Kobzik L, Severson D, Shimoni Y. Characteristics of nitric oxide-mediated cholinergic modulation of calcium current in rabbit sino-atrial node. *J Physiol (Lond)* 1998; 15: 741-754.
- Haq SEA, Clerk A, Sugden PH. Activation of mitogen-activated protein kinases (p38 MAPKs, SAPK/JNKs and ERKs) by adenosine in the perfused rat heart. *FEBS Lett* 1998 Sep 4;434(3): 305-8.
- Hardman JG, Robison GA, Sutherland EW. Cyclic nucleotides. *Annu Rev Physiol* 1971; 33: 311-36.
- Hare JM, Loh E, Creager MA, Colucci WS. Nitric oxide inhibits the positive inotropic response to beta-adrenergic stimulation in humans with left ventricular dysfunction. *Circulation* 1995; 92: 2198-2203.
- Hartley S. The role of p38 MAPK activation in preconditioning mediated protection against ischaemia/reperfusion injury. MSc Thesis 2002.
- Hartzell HC, Fischmeister R. Opposite effects of cyclic GMP and AMP on Ca<sup>2+</sup> current in single heart cells. *Nature* 1986; 323: 272-275.
- Hasebe N, Shen Y-T, Vatner SF. Inhibition of endothelium-derived relaxing factor enhances myocardial stunning in conscious dogs. *Circulation* 88: 2862 – 2871, 1993.
- Hatter Institute. Meeting report. Hatter Institute Workshop on myocardial preconditioning. *Cardiovasc Drugs Ther* 1998; 12: 529-531.
- Hausdorff WP, Caron MG, Lefkowitz RJ. Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB J* 1990; 4: 2881-2889.
- Hawes BE, van Biesen T, Koch WJ, Luttrell LM, Lefkowitz RJ. Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. *J Biol Chem*. 1995; 270(29): 17148-53.
- Headrick JP. Ischemic preconditioning: bioenergetic and metabolic changes and the role of endogenous adenosine. *J Mol Cell Cardiol* 1996; 28: 1227-1240.
- Hearse DJ, Yellon DM, Downey JM. Can beta-blockers limit myocardial infarct size? *Eur Heart J* 1986, 7: 925-930.
- Hein L, Kobilka BK. Adrenergic receptors. From molecular structure to *in vivo* function. *Trends Cardiovasc Med* 1997; 7: 137-145.
- Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev* 1993; 7: 2135-2148.
- Hines WA, Thorburn J, Thorburn A. A low-affinity serum response element allows other transcription factors to activate inducible gene expression in cardiac myocytes. *Mol Cell Biol* 1999;

19: 1841-1852.

Hobbs AJ. Soluble guanylate cyclase: the forgotten sibling. *TIPS* 18: 484–491, 1997.

Hordijk PL, Verlaan I, Van Corven EJ, Moolenaar WH. Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of MAP kinase is mediated by the Gi-protein. *J Biol Chem* 1994; 269: 645-651.

Hreniuk D, Garay M, Gaarde W, Monia BP, McKay RA, Cioffi CL. Inhibition of c-Jun N-terminal kinase 1, but not c-Jun N-terminal kinase 2, suppresses apoptosis induced by ischemia/reperfusion in rat cardiac myocytes. *Mol Pharmacol* 2001; 59: 867-874.

Huot J, Houle F, Marceau F, Landry J. Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ Res* 1997; 80: 383-392.

Huot J, Houle F, Spitz DR, et al. HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. *Cancer Res* 1996; 56: 273-279.

Ichijo H, Nishida E, Irie K, Ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 1997; 275: 90-94.

Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987; 84: 9265-9269.

Ihl-Vahl R, Marquetant R, Bremerich MJ, Strasser RH. Regulation of  $\beta$ -adrenergic receptors in acute myocardial ischemia: subtype-selective increase of mRNA specific for  $\beta$ 1-adrenergic receptors. *J Mol Cell Cardiol* 1995; 27: 437-452.

Ikonomidis JS, Shirai T, Weisel RD, Derylo B, Rao V, Whiteside CI, Mickle DAG, Li RK. Preconditioning cultured human pediatric myocytes requires adenosine and protein kinase C. *Am J Physiol* 1997; 272: H1220-H1230.

Iliodromitis EK, Kremastinos DT, Katritsis DG, Papadopoulos CC, Hearse DJ. Multiple cycles of preconditioning cause loss of protection in open-chest rabbits. *J Mol Cell Cardio* 1997; 29: 915-920.

Iliodromitis EK, Papadopoulos CC, Markianos M, Paraskevaidis IA, Kyriakides ZS, Kremastinos DT. Alterations in circulating cyclic guanosine monophosphate (cGMP) during short and long ischaemia in preconditioning. *Basic Res Cardiol* 1996; 91: 234-239

Imagawa J, Baxter GF, Yellon DM. Genistein, a tyrosine kinase inhibitor, blocks the "second window of protection" 48 h after ischaemic preconditioning in the rabbit. *J. Mol. Cell. Cardiol.* 1997; 29: 1885 – 1893.

Ishikawa T, Hume JR, Keef KD. Regulation of  $Ca^{2+}$  channels by cAMP and cGMP in vascular smooth muscle cells. *Circ Res* 1993; 73: 1128-1137.

Ishikawa Y, Homcy CJ. The adenylyl cyclases as integrators of transmembrane signal transduction. *Circ Res* 1997; 80: 297-304.

Iwase T, Murakami T, Tomita T, Miki S, Nagai K, Sasayamas. Ischaemic preconditioning is associated with a delay in ischaemia-induced reduction of  $\beta$ -adrenergic signal transduction in rabbit hearts. *Circulation* 1993; 88: 2827-2837.

Jakobs KH, Saur W, Schultz G. Reduction of adenylyl cyclase activity in lysates of human platelets by the  $\alpha$ -adrenergic component of epinephrine. *J Cycl Nucl Res* 1976; 2: 381-392.

Janknecht R, Hunter T. Convergence of MAP kinase pathways on the ternary complex factor Sap1a. *EMBO J* 1997; 16: 1620-1627.

- January B, Seibold A, Whaley B, Hipkin R W, Lin D, Schonbrunn A, Barber R, Clark RB.  $\beta_2$ -adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. *J Biol Chem* 1997; 272: 23871-23879.
- Jenkins D, Baxter G, Yellon D. The pathophysiology of ischemic preconditioning. *Pharmacol Res* 1995; 31: 219-224.
- Jennings RB, Reimer KA. The cell biology of acute myocardial ischaemia. *Ann Rev Med* 1991; 42: 225 - 246.
- Johnson G, Tsao PS, Lefer AM. Cardioprotective effects of authentic nitric oxide in myocardial ischaemia with reperfusion. *Crit Care Med* 1991; 19: 244-252.
- Johnson JA, Gray MO, Chen C-H, Mochly-Rosen D. A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. *J Biol Chem* 1996; 271: 24962-24966.
- Jun CD, Oh CD, Kwak HJ, et al. Overexpression of protein kinase C isoforms protects RAW264 macrophages from nitric oxide-induced apoptosis: involvement of c-Jun N-terminal kinase/stress-activated protein kinase, p38 kinase, and CPP-32 protease pathways. *J Immunol* 1999; 162: 3395-3401.
- Kajimoto K, Hagiwara N, Kasanuki H, Hosoda S. Contribution of phosphodiesterase isozymes to the regulation of the L-type calcium current in human cardiac myocytes. *Br J Pharmacol* 1997; 121: 1549-1556.
- Kannengieser GJ, Opie LH, Van der Werff TJ. Impaired cardiac work and oxygen uptake after reperfusion of regionally ischaemic myocardium. *J Mol Cell Cardiol* 1979; 11: 197-207.
- Kaplan P, Hendikx M, Mattheussen M, Mubagwa K, Flameng W. Effect of ischemia and reperfusion on sarcoplasmic reticulum calcium uptake. *Circ Res* 1992; 71: 1123-1130.
- Kaplan RS, Pedersen PL. Determination of microgram quantities of protein in the presence of milligram levels of lipid with amido black 10B. *Anal Biochem* 1985; 150: 97-104.
- Karin M. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. *Curr Opin Cell Biol* 1994; 6: 415-424.
- Katz AM. Role of the contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: an historical review. *Adv Cyclic Nucleotide Res* 1979; 5: 453-472.
- Kawasaki H, Morooka T, Shimohama S, Kimura J, Hirano T, Gotoh Y, Nishida E. Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J Biol Chem* 1997; 272: 18518 - 18521.
- Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. A family of cAMP-binding proteins that directly activate Rap1. *Science* 1998; 282: 2275-2279.
- Keaney JF, Hare JM, Balligand JL, Loscalzo J, Smith TW, Colucci WS. Inhibition of nitric oxide synthase augments myocardial contractile responses to  $\beta$ -adrenergic stimulation. *Am J Physiol* 1996; 271: H2646-H2652.
- Kelly RA, Balligand J-L, Smith TW. Nitric oxide and cardiac function. *Circ Res* 1996; 79: 363-380.
- Kida M, Fujiwara H, Ishida M, Kawai C, Ohura M, Miura I, Yabuuchi Y. Ischemic preconditioning preserves creatine phosphate and intracellular pH. *Circulation* 1991; 84: 2495-2503.
- Kim SO, Xu Y, Katz S, Pelech S. Cyclic GMP-dependent and-independent regulation of MAP kinases by sodium nitroprusside in isolated cardiomyocytes. *Biochim Biophys Acta* 2000; 1496: 277-284.
- Kis A, Végh A, Papp J, Parratt JR. Pacing-induced delayed protection against arrhythmias is attenuated by aminoguanidine, an inhibitor of nitric oxide synthase. *Br J Pharmacol* 1999; 127:

1545-1550.

Kitakaze M, Node K, Komamura K, Minamino T, Inoue M, Hori M, Kamada T. Evidence for nitric oxide generation in the cardiomyocytes: its augmentation by hypoxia. *J Mol Cell Cardiol* 1995; 27(10): 2149-2154.

Kiyono M, Satoh T, Kaziro Y. G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mn). *Proc Natl Acad Sci USA* 1999; 96: 4826-4831.

Kjekhus JK. The role of free fatty acids in catecholamine-induced cardiac necrosis. In: *Recent advances in Studies on Cardiac Structure and Metabolism, Volume 6, Pathophysiology and Morphology of Myocardial Cell Alterations*. Eda. Fleckenstein A, Rona G. Baltimore; University Park Press: 1975: 183-191.

Klein G, Drexler H, Schroder F. Protein kinase G reverses all isoproterenol induced changes of cardiac single L-type calcium channel gating. *Cardiovasc Res* 2000; 48(3): 367-374.

Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E and Participants. Medical and cellular implications of stunning hibernation and preconditioning: An NHLBI Workshop. *Circulation* 1998; 97: 1848 – 1867.

Kloner RA, Yellon D. Does ischemic preconditioning occur in patients? *J Am Coll Cardiol* 1994; 24: 1133-1142.

Knight RJ, Buxton DB. Stimulation of c-Jun kinase and mitogen-activated protein kinase by ischaemia and reperfusion in the perfused rat heart. *Biochem Biophys Res Commun* 1996; 218: 83 - 88.

Koch WJ, Hawes BE, Allen LF, Lefkowitz RJ. Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. *Proc Natl Acad Sci U S A* 1994; 91: 12706-12710.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. Protein kinase C  $\alpha$  activates Raf-1 by direct phosphorylation. *Nature* 1993; 364(6434): 249-255.

Kolocassides KG, Galinanes M. The specific protein kinase C inhibitor chelerythrine fails to inhibit ischaemic preconditioning in the rat heart. *Circulation* 1994; 90:I-208.

Kolocassides KG, Seymour AM, Galinanes M, Hearse DJ. Paradoxical effect of ischemic preconditioning on ischemic contracture ? NMR studies of energy metabolism and intracellular pH in the rat heart. *J Mol Cell Cardiol* 1996; 28: 1045-1057.

Komas N, Lugnier C, Le Bec A, Serradeil-Le Gal C, Barthelemy G, Stoclet JC. Differential sensitivity to cardiotoxic drugs of cyclic AMP phosphodiesterase isolated from canine ventricular and sinoatrial-enriched tissues. *J Cardiovasc Pharmacol* 1989; 14: 213-220.

Koning MMG, Simonis LAJ, deZeeuw S, et al. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994; 28: 1146-51.

Konishi H, Tanaka M, Takemura Y *et al.* Activation of protein kinase C by tyrosine phosphorylation in response to H<sub>2</sub>O<sub>2</sub>. *Proc Natl Acad Sci USA* 1997; 94: 11233-11237

Kovanecz I, Papp J G, Szekeres L. Long-term ischaemic preconditioning of the heart induced by repeated beta-adrenergic stress. *Acta Physiol Hung* 1996; 84: 297 – 298.

Krause EG, Karczewski S, Lindenau KF. Cyclic nucleotides and changes in protein kinase activity ratio in the ischemic and non-ischemic myocardium. *Adv Myocardial* 1983; 4: 521-530.

Krause EG, Ziegelhöffner A, Fedelsova M, Styk J, Kostolankski S, Gabauer I, Blasig I, Wollenberger A. Myocardial cyclic nucleotide levels following coronary artery ligation. *Adv Cardiol* 1978; 25: 199-129.

- Krown KA, Page MT, Nguyen C, Zechner D, Gutierrez V, Comstock KL, Glembotski CC, Quintana PJE, Sabbadini RA. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes: involvement of the sphingolipid signaling cascade in cardiac cell death. *J Clin Invest* 1996; 98: 2854-2865.
- Kuan CY, Yang DD, Samanta RD, Davis RJ, Rakic P, Falvell RA. The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* 1999; 22: 667-676.
- Kumar S, Jiang MS, Adams JL, Lee JC. Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. *Biochim Biophys Res Commun* 1999; 263: 825-831.
- Kummer JL, Rao PK, Heidenreich KA. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J Biol Chem* 1997; 272: 20490 - 20494.
- Kusuoka H, Porterfield JK, Weisman HF et al. Pathophysiology and pathogenesis of the stunned myocardium: depressed calcium activation of contraction as a consequence of reperfusion induced cellular calcium overload in ferret hearts. *J Clin Invest* 1987; 79: 950-961.
- Kuzuya T, Hoshida S, Yamashita N, Fuji H, Horie M, Kodama T, Tada M. Delayed effects of sublethal ischaemia on the acquisition of tolerance to ischaemia. *Circ Res* 1993; 72: 1293-1299.
- Kyriakis JM, App H, Zhang X-F, Banerjee P, Brautigan DL, Rapp UR, Avruch J. Raf-1 activates MAP kinase-kinase. *Nature* 1992; 358: 417-421.
- Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Phys Rev* 2001; 81(2): 807-869.
- Kyriakis JM, Avruch J. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J Biol Chem* 1990; 265: 17355-17363.
- Kyriakis JM, Avruch J. Sounding the alarm: Protein kinase cascades activated by stress and inflammation. *J Biol Chem* 1996; 271: 24313-24316.
- Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 1994; 369: 156-160.
- Lali FV, Hunt AE, Turner SJ, Foxwell BM. The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. *J Biol Chem* 2000; 275: 7395-7402.
- Langendorff O. *Pflügers Archiv Für die gesamte Physiologie*. 1895; 61: 291-332.
- Lawson CS, Coltart DJ, Hearse DJ. The anti-arrhythmic action of ischaemic preconditioning in rat hearts does not involve functional Gi proteins. *Cardiovasc Res* 1993; 27: 681 - 687.
- Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res* 1993; 27: 542-550.
- Lawson CS, Hearse DJ. Anti-arrhythmic protection by ischaemic preconditioning in isolated rat hearts is not due to depletion of endogenous catecholamines. *Cardiovasc Res* 1996; 31(4): 655-662.
- Lawson CS, Hearse DJ. Preconditioning and ischemia- and reperfusion-induced arrhythmias. In: Przyklenk K, Kloner R, Yellon D, eds. *Ischemic Preconditioning: The Concept of Endogenous Cardioprotection*. Norwell, Mass: Kluwer Academic Publishers; 1994: 19-40.
- Lazou A, Sugden PH, Clerk A. Activation of mitogen-activated protein kinases (p38-MAPKs, SAPKs/JNKs and ERKs) by the G-protein-coupled receptor phenylephrine (PE) in the perfused rat

heart. *Biochem J* 1998; 332(pt2): 459-65.

Lee JC, Kassis S, Kumar S, Badger A, Adams JL. p38 Mitogen-activated protein kinase inhibitors: mechanisms and therapeutic potentials. *Pharmacol Ther* 1999; 82: 389-397.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 1994; 372: 739-746.

Lee J-D, Ulevitch RJ, Han J. Primary structure of BMK1: A new mammalian MAP kinase. *Biochem Biophys Res Commun* 1995; 213: 715-724.

Lee Y, Nadal-Ginard B, Mahdavi V, Izumo S. Myocyte-specific enhancer factor 2 and thyroid hormone receptor associate and synergistically activate the  $\alpha$ -cardiac myosin heavy-chain gene. *Mol Cell Biol* 1997; 17: 2745-2755.

Lefkowitz RJ, Hausdorff WP, Caron MG. Role of phosphorylation in desensitization of the beta-adrenoceptor. *Trends Pharmacol* 1990; 11: 190-194.

Lefkowitz RJ. G protein-coupled receptor kinases. *Cell* 1993; 74: 409-412.

Li Y, Kloner RA. The cardioprotective effects of ischemic preconditioning are not mediated by adenosine receptors in rat hearts. *Circulation* 1993; 87: 1642-1648.

Lin Q, Schwarz J, Bucana C, Olson EN. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* 1997; 276: 1404-1407.

Lincoln TM. Cyclic GMP and mechanism of vasodilation. *Pharmacol Ther* 1989; 41: 479-502.

Liu GS, Cohen MV, Mochly-Rosen D, Downey JM. Protein kinase C- $\epsilon$  is responsible for the protection of preconditioning in rabbit cardiomyocytes. *J Mol Cell Cardiol* 1999; 31: 1937-1948.

Liu YL, Downey JM. Ischaemic preconditioning protects against infarction in rat heart. *Am J Physiol* 1992; 263: H1107-H1112.

Liu YL, Downey JM. Preconditioning against infarction in the rat heart does not involve a pertussis toxin sensitive G protein. *Cardiovasc Res* 1993; 27(4):608-611.

Liu YL, Sato T, O'Rourke B, Marban E. Mitochondrial ATP-dependent potassium channels. Novel effectors of cardioprotection. *Circulation* 1998; 97:2463-2469.

Liu YL, Ytrehus K, Downey JM. Evidence that translocation of protein kinase C is a key event during ischaemic preconditioning of rabbit myocardium. *J. Mol. Cell. Cardiol.* 1994; 26: 661 – 668.

Ljusegren ME, Axelsson K. Lactate accumulation in isolated hypoxic rat ventricular myocardium: effect of different modulators of the cyclic GMP system. *Pharmacol. Toxicol.* 1993; 72: 56 – 60.

Lohmann SM, Fischmeister R, Walter U. Signal transduction by cGMP in the heart. *Basic Res in Cardiol* 1991; 86: 503-514.

Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. Beta-arrestin: a protein that regulates beta-adrenergic receptor function. *Science* 1990; 248: 1547-1550.

Loughney K, Hill TR, Florio VA, Uher L, *et al.*, Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3',5'-cyclic nucleotide phosphodiesterase. *Gene* 1998; 216: 139-147.

Lowenstein CJ, Dinerman JL, Snyder SH. Nitric oxide: a physiologic messenger. *Ann Intern Med* 1994; 120: 227-237.

Lowry AO, Rosenbrough NJ, Farr AL, Randall RJ. Protein with the folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.

- Lu HR, Remeysen P, de Clerck F. Does the antiarrhythmic effect of ischaemic preconditioning in rats involve the L-arginine nitric oxide pathway? *J. Cardiovasc. Pharmacol.* 1995; 25: 524 – 530.
- Lubbe WF, Muller CA, Worthington MG, McFayden L, Opie LH. Influence of propranolol isomers and atenolol on myocardial cyclic AMP, high energy phosphates and vulnerability to fibrillation after coronary ligation in the isolated rat heart. *Cardiovasc Res* 1981; 15: 690-699.
- Lubbe WF, Podzuweit T, Opie LH. Potential arrhythmogenic role of cyclic adenosine monophosphate (AMP) and cytosolic calcium overload: implications for prophylactic effects of beta-blockers in myocardial infarction and proarrhythmic effects of phosphodiesterase inhibitors. *J Am Coll Cardiol* 1992; 19: 1622-1633.
- Lugnier C, Keravis T, Le Bec A, Pauvert O, Rousseau E. Characterization of cyclic nucleotide phosphodiesterase isoforms associated to isolated cardiac nuclei. *Biochim Biophys Acta* 1999; 1472: 431-446.
- Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudskey S, Della Rocca GJ, Lin F, Kawakatsu H, Luttrell DK, Caron MG, Lefkowitz RJ.  $\beta$ -arrestin-dependent formation of  $\beta_2$ -adrenergic receptor kinase complexes. *Science* 1999; 283: 655-661.
- Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ. Role of c-Src tyrosine kinase in G protein-coupled receptor- and G $\beta\gamma$  subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* 1996; 271(32): 19443-19450.
- Ma XL, Kumar S, Gao F, Louden CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ, Yue T-L. Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischaemia and reperfusion. *Circulation* 1999; 99: 1685 - 1691.
- Ma Y-C, Huang X-Y. Novel signalling pathway through the  $\beta$ -adrenergic receptor. *Trends Cardiovasc Med* 2002; 12: 46-49.
- MacKay K, Mochly-Rosen D. An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischaemia. *J Biol Chem* 1999; 274: 6272 - 6279.
- Mackay K, Mochly-Rosen D. Involvement of a p38 mitogen-activated protein kinase phosphatase in protecting neonatal rat cardiac myocytes from ischaemia. *J Mol Cell Cardiol* 2000, 32: 1585 - 1588.
- Marais R, Wynne J, Treisman R. The SRF accessory protein Elk-1 contains a growth factor-regulated transcription activation domain. *Cell* 1993; 73: 381-393.
- Marban E, Koretsune Y, Corretti M, Chacko VP, Kusouka H. Calcium and its role in myocardial cell injury ischemia and reperfusion. *Circulation* 1989; 80 (suppl iv): IV17-22.
- Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischaemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993; 88: 1264-1272.
- Martin JL, Avkiran M, Quinlan RA, Cohen P, Marber MS. Antiischemic effects of SB203580 are mediated through the inhibition of p38 alpha mitogen-activated protein kinase: evidence from ectopic expression of an inhibition-resistant kinase. *Circ Res* 2001; 89: 882-890.
- Martins TJ, Mumby MC, Beavo JA. Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J Biol Chem* 1982; 257: 1973-1979.
- Martorana PA. The role of cyclic AMP in isoprenaline induced cardiac necroses in the rat. *J Pharm Pharmacol* 1971; 23: 200-203.
- Masuoka H, Ito M, Nakano T, Naka M, Tanaka T. Effects of amrinone and enoximone on the subclasses of cyclic AMP phosphodiesterase from human heart and kidney. *J Cardiovasc Pharmacol* 1990; 15: 302-307.

- Matsubara T, Minatoguchi S, Matsuo H, et al. Three minute, but not one minute, ischemia and nicorandil have a preconditioning effect in patients with coronary artery disease. *J Am Coll Cardiol* 2000; 35: 345-351.
- Mattingly RR and Macara IG. Phosphorylation-dependent activation of the Ras-GRF/CDC25Mn exchange factor by muscarinic receptors and G-protein  $\beta\gamma$  subunits. *Nature* 1996; 382: 268-272.
- Maulik N, Sato M, Price BD, Das DK. An essential role of NFkB in tyrosine signaling of p38 MAP kinase regulation of myocardial adaptation to ischaemia. *FEBS Lett* 1998a; 429: 365-369.
- Maulik N, Watanabe M, Zu YL, Huang CK, Cordis GA, Schley JA, Das DK. Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. *FEBS Lett* 1996; 396: 233-237.
- Maulik N, Yoshida T, Das DK. Oxidative stress developed during reperfusion of ischemic myocardium induces apoptosis. *Free Radic Biol Med* 1998b; 24: 869-875.
- Maulik N, Yoshida T, Engelman RM, Deaton D, Flack JE 3<sup>rd</sup>, Rousou JA, Das DK. Ischemic preconditioning attenuates apoptotic cell death associated with ischaemia/reperfusion. *Mol Cell Biochem* 1998c; 186: 139-145.
- Maulik N, Yoshida T, Zu YL, Sato M, Banerjee A, Das DK. Ischemic preconditioning triggers tyrosine kinase signalling: a potential role for MAPKAP kinase 2. *Am J Physiol* 1998d; 275: H1857 - H1864.
- Mayor F, Penela P, Ruiz-Gomez A. Role of G-protein coupled receptor kinase 2 and arrestins in  $\beta$ -adrenergic receptor internalisation. *Trends in Cardiovascular Medicine* 1998; 8: 234-240.
- Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature Reviews Mol Cell Biol* 2001; 2: 599-609.
- McCord JM. Oxygen derived free-radicals in post-ischemic tissue injury. *N Eng J Med* 1985; 312: 159-163.
- Mehrhof FB, Muller F-U, Bergmann MW, Li P, Wang Y, Schmitz W, Dietz R, von Harsdorf R. In cardiomyocyte hypoxia, insulin-like growth factor-I- induced antiapoptotic signaling requires phosphatidylinositol-3-OH-kinase-dependent and mitogen-activated protein kinase-dependent activation of the transcription factor cAMP response element-binding protein. *Circulation* 2001; 104: 2088.
- Mei DA, Gross GJ. Evidence for the involvement of the ATP-sensitive potassium channel in a novel model of hypoxia preconditioning in dogs. *Cardiovasc Res* 1995; 30: 222-230.
- Michel MC, Li Y, Heusch G. Mitogen-activated protein kinase in the heart. *Naunyn-Schmiedeberg's Arch Pharmacol* 2001; 363: 245-266.
- Mikkola I, Bruun JA, Bjorkoy G, Holm T, Johansen T. Phosphorylation of the transactivation domain of Pax6 by extracellular signal-regulated kinase and p38 mitogen-activated protein kinase. *J Biol Chem* 1999; 274: 15115-15126.
- Minden A, Lin A, Claret FX, Abo A, Karin M. Selective activation of the JNK signalling cascade and c-Jun transcriptional activity by the small GTPase Rac and Cdc42Hs. *Cell* 1995; 81: 1147-1157.
- Mischak H, Seitz T, Janosch P, Eulitz M, Steen H, Schellerer M, Philipp A, Kolch W. Negative regulation of Raf-1 by phosphorylation of serine 621. *Mol Cell Biol* 1996; 16: 5406-5418.
- Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Banerjee A. Preconditioning of isolated rat heart is mediated by protecin kinase C. *Circ Res* 1995; 75: 73-81.
- Miura K, Kano S, Nakai T, Satoh K, Hoshi K, Ichihara K. Inhibitory effects of glibenclamide and pertussis toxin on the attenuation of ischaemia-induced myocardial acidosis following ischaemic preconditioning in dogs. *Jpn Circ* 1997; 61(8): 709-714.

- Miyawaki H, Ashraf M.  $Ca^{2+}$  as a mediator of ischaemic preconditioning. *Circ Res* 1997a; 80: 790 - 799.
- Miyawaki H, Ashraf M. Isoproterenol mimics calcium preconditioning-induced protection against ischaemia. *Am J Physiol* 1997b; 272: H927 - H936.
- Miyawaki H, Zhou X, Ashraf M. Calcium preconditioning elicits strong protection against ischemic injury via protein kinase C signalling pathway. *Circ Res* 1996; 79: 137-146.
- Miyazaki T, Zipes DP. Protection against autonomic denervation following acute myocardial infarction by preconditioning ischaemia. *Circ Res* 1989; 64: 437 – 448.
- Mizuno T, Watanabe M, Sakamoto T, Sunamori M. L-Arginine, a nitric oxide precursor, attenuates ischaemia-reperfusion injury by inhibiting inositol-1,4,5-triphosphate. *J Thorac Cardiovasc Surg* 1998; 115: 931-936.
- Mocanu MM, Baxter GF, Yue Y, Critz SD, Yellon DM. The p38 MAPK inhibitor, SB 203580, abrogates ischaemic preconditioning in rat heart but timing of administration is critical. *Basic Res Cardiol* 2000; 95: 472-478.
- Mockridge JW, Punn A, Latchman DS, Marber MS, Heads RJ. PKC-dependent delayed metabolic preconditioning is independent of transient MAPK activation. *Am J Physiol* 2000; 279: H492 - H501.
- Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Eng J Med* 1993; 2002-2012.
- Moolman JA, Genade S, Tromp E, Lochner A. A comparison between ischaemic preconditioning and anti-adrenergic interventions: cAMP, energy metabolism and functional recovery. *Basic Res Cardiol* 1996a; 91: 219 - 233.
- Moolman JA, Genade S, Tromp E, Lochner A. No evidence for mediation of ischaemic preconditioning by  $\alpha_1$ -adrenergic signal transduction pathway or protein kinase C in the isolated rat heart. *Cardiovasc Drugs & Therapy* 1996b; 10: 125 - 136.
- Moolman JA, Genade S, Winterbach R, Harper I S, Williams K, Lochner A. Preconditioning with a single episode of ischaemia in the isolated working rat heart: effect on structure, mechanical function and energy metabolism for various durations of sustained ischaemia. *Cardiovasc Drugs Ther* 1995; 9: 103 - 115.
- Morad M, Cleeman L. Role of  $Ca^{2+}$  channel in development of tension in heart muscle. *J Mol Cell Cardiol* 1987; 19: 527-553.
- Mori E, Haramaki N, Ikeda H, Imaizumi T. Intracoronary administration of L-arginine aggravates myocardial stunning through production of peroxynitrite in dogs. *Cardiovasc. Res.* 1998; 40: 113 – 123.
- Moriguchi T, Kuroyanagi N, Yamaguchi K, Gotoh Y, Irie K, Kano T, Shirakabe K, Muro Y, Shibuya H, Matsumoto K, Nishida E, Hagiwara M. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J Biol Chem* 1996; 271: 13675-13679.
- Morrison DK, Cutler RE. The complexity of Raf-1 kinase. *Curr Opin Cell Biol* 1997; 9: 174-179.
- Moule SK, Denton RM. The activation of p28 MAPK by the beta-adrenergic agonist isoproterenol in rat epididymal fat cells. *FEBS Lett* 1998; 439: 287-290.
- Mouton R, Genade S, Boschmans SA, Perkins MF, Lochner A. The role of  $\alpha_1$ -adrenergic stimulation in inositol phosphate metabolism during post-ischaemic reperfusion. *Life Sciences* 1992; 51: 2033-2040.
- Muda M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A, Arkinstall S. The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J Biol Chem* 1996; 271: 27205-27208.

- Mullane K, Bullough D. Harnessing an endogenous cardioprotective mechanism: cellular sources and sites of action of adenosine. *J Mol Cell Cardiol* 1995; 27: 1041-1054.
- Muller CA, Opie LH, Hamm CW, Peisach M, Chiwala D. Prevention of ventricular fibrillation by metoprolol in pig model of acute myocardial ischaemia: absence of a major arrhythmogenic role for cAMP. *J Mol Cell Cardiol* 1986; 18: 375-387.
- Murry CE, Jennings RB, Reimer KA. Preconditioning with ischaemia: a delay of lethal injury in ischaemic myocardium. *Circulation* 1986; 74: 1124-1136.
- Murry CE, Richard VJ, Jennings RB, Reimer KA. Myocardial protection is lost before contractile function recovers from ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 1991; 260: H796-H804.
- Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischaemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischaemic episode. *Circ Res* 1990; 66: 913-931.
- Nagarkatti D, Sha'afi RI. Role of p38 MAP kinase in myocardial stress. *J Mol Cell Cardiol* 1998; 30: 1651 - 1664.
- Nakano A, Baines CP, Kim SO, Pelech SL, Downey JM, Cohen MV, Critz SD. Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. *Circ Res* 2000a; 86: 144 - 151.
- Nakano A, Cohen MV, Critz S, Downey JM. SB 203580, an inhibitor of p38 MAPK abolished infarct-limiting effect of ischaemic preconditioning in isolated rabbit hearts. *Basic Res Cardiol* 2000c; 95: 466-471.
- Nakano A, Cohen MV, Downey JM. Ischemic preconditioning: From basic mechanisms to clinical applications. *Pharm Therapeutics* 2000d; 86: 263-275.
- Nakano A, Liu GS, Heusch G, Downey JM, Cohen MV. Exogenous nitric oxide can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischaemic preconditioning. *J Mol Cell Cardiol* 2000b; 32: 1159-1167.
- Nandagopal K, Dawson TM, Dawson VL. Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. *Pharmacol Exp Ther* 2001; 297: 474-478.
- Nao BS, McClanahan TB, Groh MA, Schott RJ, Gallagher KP. The time limit of effective ischemic preconditioning in dogs. *Circulation* 1990; 82 (suppl III): III-271.
- Naseem SA, Kontos MC, Rao PS, *et al.* Sustained inhibition of nitric oxide by N<sup>G</sup>-nitro-L-arginine improves myocardial function following ischaemia/reperfusion in isolated perfused rat heart. *J. Mol. Cell Cardiol.* 1995; 27: 419 – 426.
- Neely JR, Liebermeister H, Battersby EJ, Morgan HE. Effect of pressure development on oxygen consumption by isolated rat heart. *Am J Physiol* 1967; 212(4): 804-814.
- Niroomand R, Weinbrenner C, Weis A, Bangert M, Schwenke C, Marquetant R, Beyer T, Strasser RH, Kubler W, Rauch B. Impaired function of inhibitory G-proteins during acute myocardial ischaemia of canine hearts and its reversal during reperfusion and a second period of ischaemia. *Circ Res* 1995; 76: 861-870.
- Node K, Kitakaze M, Kosaka H, Komamura K, Minamino T, Inoue M, Tada M, Hori M, Kamada T. Increased release of NO during ischemia reduces myocardial contractility and improves metabolic dysfunction. *Circulation* 1996; 39(2): 356-364.
- Nossuli TO, Hayward R, Scalia R, Lefer AM. Peroxynitrite reduces myocardial infarct size and preserves coronary endothelium after ischaemia and reperfusion in cats. *Circulation* 1997; 96: 2317-2324.

- O'Rourke B. Myocardial K<sup>+</sup>(ATP) channels in preconditioning. *Circ Res* 2000; 87: 845 – 855.
- Obata T, Brown GE, Yaffe MB. MAP kinase pathways activated by stress: The p38 MAPK pathway. *Crit Care Med* 2000; 28: N67-N77.
- Okruhlicova L, Tribulova N, Eckly A, Lugnier C, Slezak J. Cytochemical distribution of cyclic AMP-dependent 3',5' -nucleotide phosphodiesterase in the rat myocardium. *Histochem J* 1996; 28: 165-172.
- Omura T, Yoshiyama M, Shimada T, Shimizu N, Kim S, Iwao H, Takeuchi K, Yoshikawa J. Activation of mitogen-activated protein kinases in *in vivo* ischaemia/reperfused myocardium in rats. *J Mol Cell Cardiol* 1999; 31: 1269 - 1279.
- Opie LH, Lubbe WF. Catecholamine-mediated arrhythmias in acute myocardial infarction. Experimental evidence and role of beta-adrenergic blockade. *S Afr Med J* 1979; 56: 871-880.
- Opie LH, Mansford KRL, Owen R. Effects of increased heart work on glycolysis and adenine nucleotides in the perfused heart of normal and diabetic rats. *Biochem J* 1971; 124: 475-490.
- Opie LH, Thandroyen FT, Muller CA, Bricknell OL. Adrenaline induced "oxygen wastage" and enzyme release from the working rat heart. Effects of calcium antagonism, beta-blockade, nitric acid, and coronary ligation. *J Mol Cell Cardiol* 1979; 11: 1073-1094.
- Opie LH. Role of cyclic nucleotides in heart metabolism. *Cardiovasc Res* 1982; 17: 483-507.
- Opie LH. *The Heart: Physiology and Metabolism*. Raven Press, New York, 1991; 2<sup>nd</sup> Ed, p 454.
- Opie LH. *The Heart: Physiology, from Cell to Circulation*. Philadelphia: Lippincott-Raven Publishers, 1998, 3<sup>rd</sup> Ed, p173-207.
- Ovize M, Aupetit JF, Rioufol G, Loufoua J, Andre-Fouet X, Minaire Y, Faucon G. Preconditioning reduces infarct size but accelerates time to ventricular fibrillation in ischemic pig heart. *Am J Physiol* 1995; 269: H72-79.
- Ovize M, Przyklenk K, Kloner RA. Preconditioning does not attenuate myocardial stunning. *Circulation* 1992; 85: 2247-2254.
- Pain TS, Cohen MV, Downey JM. The mitochondrial K<sub>ATP</sub> channel may be a trigger rather than the end-effector of preconditioning's anti-infarct effect. *Circulation* 1999; 100(suppl I):I-342.
- Pain TS, Yang X-M, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. Opening of mitochondrial K<sub>ATP</sub> channels triggers the preconditioned state by generating free radicals. *Circ Res* 2000; 87: 460 - 466.
- Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; 327: 524-526.
- Parratt JR. Endogenous myocardial protective (antiarrhythmic) substances. *Cardiovasc Res* 1993; 27: 693-702.
- Parratt JR. Possibilities for the pharmacological exploitation of ischaemic preconditioning. *J Mol Cell Cardiol* 1995; 27: 991-1000.
- Parratt JR. Protection of the heart by ischaemic preconditioning: mechanisms and possibilities for pharmacological exploitation. *Trends Pharmacol Sci* 1994; 15: 19-25.
- Parratt JR. Protection of the heart by ischaemic preconditioning: mechanisms and possibilities for pharmacological exploitation. *Trends Pharmacol Sci*. 1994; 15: 19-25.
- Pastan I. Cyclic AMP. *Sci Am* 1972; 227(2): 97-105.
- Payne DM, Rossomando AJ, Martino P, Erickson AK, Her J-H, Shananowitz J, Hunt DF, Weber MJ, Sturgill TW. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated

protein kinase (MAP kinase). *EMBO J* 1991; 10: 885-892.

Perchenet L, Hinde AK, Patel KC, Hancox JC, Levi AJ. Stimulation of Na/Ca exchange by the beta-adrenergic/protein kinase A pathway in guinea-pig ventricular myocytes at 37 degrees C. *Plugers Arch – Eur J Phys* 2000; 439: 822-828.

Piacentini L, Wainwright CL, Parratt JR. The antiarrhythmic effect of ischaemic preconditioning in isolated rat heart involves a pertussis toxin sensitive mechanism. *Cardiovasc Res* 1993; 27(4):674-680.

Pierce GN, Czubryt MP. The contribution of ionic imbalance to ischemia/reperfusion-induced injury. *J Mol Cell Cardiol* 1995; 27: 53-63.

Ping P, Murphy E. Role of p38 mitogen-activated protein kinases in preconditioning. A detrimental factor or a protective kinase? *Circ Res* 2000; 86: 921 - 922.

Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RCX, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischaemia-induced preconditioning. *Circ. Res.* 1999c; 84: 587 – 604.

Ping P, Zhang J, Cao X, Li RC, Kong D, Tang XL, Qiu Y, Manchikalapudi S, Auchampach JA, Black RG, Bolli R. PKC-dependent activation of p44/p42 MAPKs during myocardial ischaemia-reperfusion in conscious rabbits. *Am J Physiol* 1999b; 276: H1468-H1481.

Ping P, Zhang J, Huang S, Cao X, Tang X-L, Li RC, Zheng Y-T, Qiu Y, Clerk A, Sugden P, Han J, Bolli R. PKC dependent activation of p46/p54 JNKS during ischaemic preconditioning in conscious rabbits. *Am J Physiol* 1999a; 277: H1771 - H1785.

Ping P, Zhang J, Qiu Y, Tang X-L, Manchikalapudi S, Cao X, Bolli R. Ischemic preconditioning induces selective translocation of protein kinase C isoforms  $\epsilon$  and  $\eta$  in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res* 1997; 81: 404-414.

Piper HM, Balsler C, Ladilov Y, Schafer M, Siegmund B, Ruiz-Meana M, Garcia-Dorado. The role of  $\text{Na}^+/\text{H}^+$  exchange in ischemia-reperfusion. *Cardiovasc Res* 1996; 91: 191-202.

Podzuweit T, Müller A, Thomas S, Bader R. Ischaemia induced inhibition on cyclic nucleotide phosphodiesterases in the pig heart. Cause of cAMP accumulation and arrhythmias? *Circulation* 1995c; 92: O641, (Abstract).

Podzuweit T, Nennstiel P, Bader R, Müller A. Ischaemia causes inhibition of cyclic nucleotide phosphodiesterases. *J Mol Cell Cardiol* 1994; 26: CXVI (Abstract).

Podzuweit T, Nennstiel P, Müller A. Isozyme selective inhibition of cGMP-stimulated cyclic nucleotide phosphodiesterase by erythro-9-(2-hydroxy-3-nonyl) adenine. *Cell Signal* 1995c; 7: 733-738.

Podzuweit T, Thomas S, Binz K, Müller A. Ischaemia and acidosis cause inhibition of cyclic nucleotide phosphodiesterases in pig heart. *J Mol Cell Cardiol* 1996; 28: 115, (Abstract).

Podzuweit T, Thomas S, Binz K, Müller A. Protective mechanisms 2. IP attenuates ischaemia induced PDE inhibition. *J Mol Cell Cardiol* 1995b; 27: A161, (Abstract).

Podzuweit T, Winkelmann A, Müller A, Vogt A. Protective mechanisms 1. IP modulates catecholamine release. *J Mol Cell Cardiol* 1995a; 27: A161, (Abstract).

Pombo CM, Bonventre JV, Avruch J, Woodgett JR, Kyriakis JM, Force T. The stress-activated protein kinases are major c-Jun amino-terminal kinases activated by ischemia and reperfusion. *J Biol Chem* 1994; 269: 26546-26551.

Post GR, Brown JH. G protein-coupled receptors and signaling pathways regulating growth

responses. *FASEB J* 1996; 10: 741-749.

Post H, Schulz R, Behrends M, et al. No involvement of endogenous nitric oxide in classical ischemic preconditioning in swine. *J Mol Cell Cardiol* 2000; 32: 725-733.

Prasad MVSV, Dermott JM, Heasley LE, Johnson GL, Dhanasekaran N. Activation of Jun kinase/stress-activated protein kinase by GTPase-deficient mutants of Ga12 and Ga13. *J Biol Chem* 1995; 270: 18655-18659.

Premont RT, Inglese J, Lefkowitz RJ. Protein kinases that phosphorylate activated G protein-coupled receptors. *J Biol Chem* 1993; 268: 23735-23738.

Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischaemic preconditioning protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993; 87: 893-899.

Przyklenk K, Kloner RA. Preconditioning: a balanced perspective. *Br Heart J*. 1995; 74: 575-577.

Przyklenk K, Sussman MA, Simkhovich BZ, Kloner RA. Does ischemic preconditioning trigger translocation of protein kinase C in the canine model? *Circulation* 1995; 92: 1546-1557.

Quantz M, Carsley L, Shum-Tim D, Tchervenkov C, Chiu RC. The enigma of myocardial preconditioning models. *J Card Surg* 1994; 9: 532-536.

Rabinowitz B, Kligerman B, Parmley WW. Alterations in myocardial and plasma cyclic adenosine monophosphate in experimental myocardial ischemia. *Recent Adv Stud Cardiac Struct Metab* 1975; 8: 251-261.

Raingaud J, Whitmarsh AJ, Barrett T, Derijard B, Davis RJ. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* 1996; 16: 1247-1255.

Rakhit RD, Edwards RJ, Marber MS. Nitric oxide, nitrates and ischaemic preconditioning. *Cadriovasc Res* 1999; 43: 621-627.

Rakhit RD, Edwards RJ, Mockridge JW, Baydoun AR, Wyatt AW, Mann GE, Marber MS. Nitric oxide-induced cardioprotection in cultured rat ventricular myocytes. *Am J Physiol Heart Circ Physiol* 2000; 278(4): H1211-1217.

Rakhit RD, Kabir AN, Mockridge JW, Saurin A, Marber MS. Role of G proteins and modulation of p38 MAPK activation in the protection by nitric oxide against ischemia-reoxygenation injury. *Biochem Biophys Res Commun* 2001; 286: 995-1002.

Ray LB, Sturgill TW. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. *Proc Natl Acad Sci USA* 1988; 85: 3753-3757.

Reeves ML, Leigh BK, England PJ. The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. Implications for the mechanism of action of selective phosphodiesterase inhibitors. *Biochem J* 1987; 241: 535-541.

Robertson SP, Johnson JD, Holroyde MJ, et al. The effect of troponin I phosphorylation on the Ca<sup>2+</sup> binding properties of the Ca<sup>2+</sup> - regulatory site of bovine cardiac troponin. *J Biol Chem* 1982; 257-263.

Rona G. Catecholamine cardiotoxicity. *J Mol Cell Cardiol* 1985; 17: 291-306.

Rossomando AJ, Payne DM, Weber MJ, Sturgill TW. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. *Proc Natl Acad Sci USA* 1989; 86: 6940-6943.

Roth NS, Campbell PT, Caron MG, Lefkowitz RJ, Lohse MJ. Comparative rates of desensitization of beta-adrenergic receptors by the beta-adrenergic receptor kinase and the cyclic AMP-dependent

protein kinase. *Proc Natl Acad Sci USA* 1991; 88: 6201-6204.

Roulston A, Reinhard C, Amiri P, Williams LT. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor- $\alpha$ . *J Biol Chem* 1998; 273: 10232-10239.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* 1994; 78: 1027-1037.

Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation: Formation of novel nitrogen-containing oxidized lipid derivatives. *J Biol Chem* 1994; 269: 26066-26075.

Sack S, Mohri M, Arras M, Schwarz ER, Schaper W. Ischaemic preconditioning – time course of renewal in the pig. *Cardiovasc Res* 1993; 27: 551-555.

Sakamoto K, Urushidani T, Nagao T. Translocation of HSP27 to cytoskeleton by repetitive hypoxia-reoxygenation in the rat myoblast cell line, H9c2. *Biochem Biophys Res Commun* 1998; 251: 576-579.

Sakamoto K, Urushidani T, Nagao T. Translocation of HSP27 to sarcomere induced by ischemic preconditioning in isolated rat hearts. *Biochem Biophys Res Commun* 2000; 269: 137-142.

Salomon Y, Londos C, Rodbell MA. A highly sensitive adenylyl cyclase assay. *Anal Biochem* 1974; 58: 541 - 548.

Sanada S, Kitakaze M, Papst P, Hatanaka K, Asanuma H, Aki T, Shinozaki Y, Ogita H, *et al.* Role of phasic dynamism of p38 mitogen activated protein kinase activation in ischaemic preconditioning of the canine heart. *Circ Res* 2001; 88: 175 - 180.

Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM, Zon LI. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. 1994; 372: 794-798.

Sandhu R, Diaz RJ, Mao GD, Wilson GJ. Ischaemic preconditioning. Differences in protection and susceptibility to blockade with single-cycle versus multicycle transient ischaemia. *Circulation* 1997; 96: 984 – 995.

Sandhu R, Diaz RJ, Wilson GJ. Comparison of ischaemic preconditioning in blood perfused and buffer perfused isolated heart models. *Cardiovasc Res* 1993; 27: 602-607.

Sandhu R, Diaz RJ, Wilson GJ. Raising cAMP levels using forskolin pretreatment does not block ischemic preconditioning. *J Mol Cell Cardiol* 1994; 26: 136. Abstract.

Sandhu R, Thomas U, Diaz RJ, Wilson GJ. Effect of ischaemic preconditioning of the myocardium on cAMP. *Circ Res* 1996; 78: 137 – 147.

Sanz E, Garcia-Dorrado D, Oliveras J, Barrabes JA, *et al.* Dissociation between anti-infarct effect and anti-edema effect of ischemic preconditioning. *Am J Physiol* 1995; 268: H233-241.

Sasaki, N., Sato, T., Ohler, A., O'Rourke, B., Marban, E. Activation of mitochondrial ATP-dependent potassium channels by nitric oxide. *Circulation* 101: 439 – 445, 2000.

Sato M, Cordis GA, Maulik N, Das DK. SAPKs regulation of ischaemic preconditioning. *Am J Physiol* 2000; 279: H901 - H907.

Sato T, Marban E. The role of mitochondrial KATP channels in cardioprotection. *Basic Res Cardiol* 2000; 95: 285-289.

Sato T, O'Rourke B, Marban E. Modulation of mitochondrial ATP-dependent K<sup>+</sup> channels by protein kinase C. *Circ Res* 1998; 83: 110-114.

- Saurin AT, Heads RJ, Foley C, Wang Y, Marber MS. Inhibition of p38 $\alpha$  activation may underlay protection in a surrogate model of ischemic preconditioning. *Circulation* 1999; 100(suppl I): I-492. Abstract.
- Saurin AT, Martin JC, Heads RJ, Foley C, Mockridge JW, Wright MJ, Wang Y, Marber S. The role of differential activation of p38 mitogen activated protein kinase in preconditioned ventricular myocytes. *Faseb J* 2000; 14: 2237 - 2246.
- Saxena M, Williams S, Tasken K, Mustelin T. Crosstalk between cAMP-dependent kinase and MAP kinase through a protein tyrosine phosphatase. *Nat Cell Biol* 1999; 1: 305-311.
- Scacco S, Vergari R, Scarpulla RC, Technikova-Dobrova Z, Sardanelli A, et al. cAMP-dependent phosphorylation of the nuclear encoded 18-kDa (IP) subunit of respiratory complex I and activation of the complex in serum-starved mouse fibroblast cultures. *J Biol Chem* 2000; 275: 17578-82.
- Schaefer S, Carr LJ, Prussel E, Ramasamy R. Effects of glycogen depletion on ischemic injury in isolated rat hearts: insights into preconditioning. *Am J Physiol Heart Circ Physiol* 1995; 268: H935-H944.
- Schaub MC, Hefti MA, Harder BA, Eppenberger HM. Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. *J Mol Med* 1997; 75: 901-920.
- Schieven GL, Kirihara JM, Myers DE, Ledbetter JA, Uckun FM. Reactive oxygen intermediates activate NF $\kappa$ B in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the p56 lck and p59 fyn tyrosine kinases in human lymphocytes. *Blood* 1993; 82: 1212-1220.
- Schini VB, Boulanger C, Regoli D, Vanhoutte PM. Bradykinin stimulates the production of cyclic GMP via activation of B<sub>2</sub>-kinin receptors in cultured porcine aortic endothelial cells. *J Pharmacol Exp Ther* 1990; 252: 581-585.
- Schmidt HHHW, Lohmann SM, Walter U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochem Biophys Acta* 1993; 1178:153-175
- Schneider S, Chen W, Hou J, Steenbergen C, Murphy E. Inhibition of p38 MAPK.  $\alpha/\beta$  reduces ischaemic injury and does not block protective effects of preconditioning. *Am J Physiol* 2001; 280: H499 - H508.
- Schneider S, Hou J, London RE, Steenbergen C, Murphy E. Inhibition of p38 MAPK reduces ischaemic injury and does not block the protective effects of preconditioning. *J Mol Cell Cardiol* 1999; 31: A43. Abstract.
- Schömig A, Dart AM, Dietz R, Mayer E, Kubler W. Release of endogenous catecholamines in the ischaemic myocardium of the rat. *Circ Res* 1984; 55: 689-701
- Schömig A, Haas M, Richard G. Catecholamine release and arrhythmias in acute myocardial ischaemia. *Europ H J* 1991; 12 (Suppl): 38 - 47.
- Schömig A. Catecholamines in myocardial ischaemia: systemic and cardiac release. *Circulation* 1990; 82 (2): II-13-II-22.
- Schultz JE, Hsu AK, Barbieri JT, Li PL, Gross GJ. Pertussis toxin abolishes the cardioprotective effect of ischaemic preconditioning in intact rat heart. *Am J Physiol* 1998; 275(2): H495-500.
- Schultz JE, Hsu AK, Gross GJ. Ischemic preconditioning in the intact rat heart is mediated by  $\delta$ 1- but not  $\mu$ - or  $\kappa$ -opioid receptors. *Circulation* 1998b; 97: 1282-1289.
- Schulz R, Cohen MV, Behrends M, Downey JM, Heusch G. Signal transduction of ischemic preconditioning. *Cardiovasc Res* 2001; 52: 181-198.
- Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a calcium-independent nitric oxide synthase in the myocardium. *Br J Pharmacol* 1992; 105: 575-580.

- Schulz R, Post H, Sakka S, Wallbridge DR, Heusch G. Intraischemic preconditioning: Increased tolerance to sustained low-flow ischemia by a brief episode of no-flow ischemia without intermittent reperfusion. *Circ Res* 1995; 76: 942-950.
- Schulz R, Post H, Vahlhaus C, Heusch G. Ischemic preconditioning in pigs: a graded phenomenon. Its relation to adenosine and bradykinin. *Circulation* 1998; 98: 1022-1029.
- Schulz R, Wambolt R. Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury. *Cardiovasc Res* 1995; 30: 432-439.
- Seeger R, Krebs EG. The MAPK signaling cascade. *FASEB* 1995; 9: 726-735.
- Seko Y, Tobe K, Ueki K, Kadowaki T, Yazaki Y. Hypoxia and hypoxia/reoxygenation activate Raf-1, mitogen-activated protein kinase kinase, mitogen-activated protein kinases, and S6 kinase in cultured rat cardiac myocytes. *Circ Res* 1996; 78: 82-90.
- Seyfarth M, Münch G, Schrieck J, Kurz T, Richardt G, Schömig A. Release of norepinephrine is suppressed by preconditioning in rat ischaemic hearts. *Circulation* 1994; 90: 1 – 108.
- Seyfarth M, Richardt G, Mizsnyak A, Kurz T, Schömig A. Transient ischaemia reduces norepinephrine release during sustained ischaemia. Neural preconditioning in the isolated rat heart. *Circ Res* 1996; 78: 573 - 580.
- Shahid M, Nicholson CD. Comparison of cyclic nucleotide phosphodiesterase isoenzymes in rat and rabbit ventricular myocardium: positive inotropic and phosphodiesterase inhibitory effects of Org 30029, milrinone and rolipram. *N Schmied Arch Pharmacol* 1990; 342: 698-705.
- Sharma RK. Signal transduction: regulation of cAMP concentration in cardiac muscle by calmodulin-dependent cyclic nucleotide phosphodiesterase. *Mol Cell Biochem* 1995; 149-150: 241-247.
- Shen YT, Cervoni P, Claus T, Vatner SF. Differences in beta 3-adrenergic cardiovascular regulation in conscious primates, rats and dogs. *J Pharmacol Exp Ther* 1996; 278: 1435-1443.
- Shih K, Hearse DJ. Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. *Am J Physiol* 1987; 253: H1470-H1476.
- Shimizu N, Yoshiyama M, Omura T, Hanatani A, Kim S, Takeuchi K, Iwao H, Yoshikawa J. Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats. *Cardiovasc Res* 1998; 38: 116-124.
- Shinbo A, Iijima T. Protection by nitric oxide of the ATP-sensitive K<sup>+</sup> current induced by K<sup>+</sup> channel openers in guinea-pig ventricular cells. *Br. J. Pharmacol.* 1997; 120: 1568 - 1574.
- Siegfried MR, Erhardt J, Rider T, Ma XL, Lefer AM. Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischaemia-reperfusion. *J. Pharm. Expt. Thera.* 1992; 260: 668 – 675.
- Simhovich BZ, Przyklenk K, Hale SL, Patterson M, Kloner RA. Direct evidence that ischemic preconditioning does not cause protein kinase C translocation in rabbit heart. *Cardiovasc Res* 1996; 32: 1064-1070.
- Simkhovich BZ, Przyklenk K, Kloner RA. Role of protein kinase C as a cellular mediator of ischemic preconditioning: a critical review. *Cardiovasc Res* 1998; 40: 9 – 22.
- Simonis G, Marquetant R, Rothele J, Strasser RH. The cardiac adrenergic system in ischaemia: differential role of acidosis and energy depletion. *Cardiovasc Res* 1998; 38: 646-654.
- Singh K, Balligand JL, Fischer TA, Smith TW, Kelly RA. Regulation of cytokine-inducible nitric oxide synthase (NOS2) in cardiac myocytes and microvascular endothelial cells: role of ERK1/2 (p44/p42) mitogen-activated protein kinases and STAT1 alpha. *J Biol Chem* 1996; 271: 1111-1117.

- Siow YL, Kalmar GB, Sanghera JS, Tai T, Oh SS, Pelech SL. Identification of two essential phosphorylated threonine residues in the catalytic domain of Mekk1. Indirect activation by Pak3 and protein kinase C. *J Biol Chem* 1997; 272: 7586-7594.
- Sodhi CP, Battle D, Sahai A. Osteopontin mediates hypoxia-induced proliferation of cultured mesangial cells: role of PKC and p38 MAPK. *Kidney Int* 2000; 58: 691 - 700.
- Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994; 372: 231-236.
- Sonenberg N, Gingras AC. The mRNA 5' cap-binding protein eIF4E and control of cell growth. *Curr Opin Cell Biol* 1998; 10: 268-275.
- Sontag E, Federov S, Robbins D, Cobb M, Mumby M. The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation. *Cell* 1993; 75: 887-897.
- Speechly-Dick ME, Grover GJ, Yellon DM. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K<sup>+</sup> channel? Studies of contractile function after simulated ischemia in an atrial in vitro model. *Circ Res* 1995; 77: 1030-1035.
- Speechly-Dick ME, Mocanu MM, Yellon DM. Protein kinase C. Its role in ischemic preconditioning in the rat. *Circ Res* 1994; 75: 586-590.
- Sperelakis, N. Regulation of calcium slow channels of heart by cyclic nucleotides and effects of ischaemia. *Adv. Pharmacol.* 1994; 31: 1 - 24.
- Steenbergen C, Perlman ME, London RE, Murphy E. Mechanism of Preconditioning. Ionic Alterations. *Circ Res* 1993, 72: 112-125.
- Storm SM, Cleveland JL, Rapp UR. Expression of raf family proto-oncogenes in normal mouse tissues. *Oncogene* 1990; 5: 345-351.
- Strasser RH, Braun-Dullaes R, Walendzik H, Marquetant R.  $\alpha_1$ -Receptor-independent activation of protein kinase C in acute myocardial ischaemia. Mechanisms for sensitization of the adenylyl cyclase system. *Circ Res* 1992; 70: 1304 - 1312.
- Strasser RH, Krimmer J, Braun-Dullaes R, Marquetant R, Kübler W. Dual sensitization of the adrenergic system in early myocardial ischaemia: independent regulation of the  $\beta$ -adrenergic receptors and the adenylyl cyclase. *J Mol Cell Cardiol* 1990; 22: 1405-1423.
- Strasser RH, Krimmer J, Marquetant R. Regulation of  $\beta$ -adrenergic receptors: impaired desensitization in myocardial ischaemia. *J Cardiovasc Pharmacol* 1988; 12: S15-S24.
- Strasser RH. Phosphorylation of the beta-adrenergic receptor: Mechanisms of desensitization. In: *Receptor Phosphorylation*. Ed. Moudgil VK. Boca Raton, Florida; CRC Press 1989; 199-226.
- Stull JT. Phosphorylation of contractile proteins in relation to muscle function. *Adv Cyclic Nucleotide Res* 1980; 13: 39-93.
- Suematsu Y, Ohtsuka T, Hirata Y, Maeda K, Imanka K, Takamoto S. L-Arginine given after ischaemic preconditioning can enhance cardioprotection in isolated rat hearts. *Euro J Cardio-Thor Surg* 2001; 19: 873-879.
- Sugden PH, Clerk A. "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circ Res* 1998; 83: 345 - 352.
- Sugden PH, Clerk A. Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal* 1997; 9: 337-351.
- Sumii K and Sperelakis N. cGMP-dependent protein kinase regulation of the L-type Ca<sup>2+</sup> current in

rat ventricular myocytes. *Circ Res* 1995; 7: 803-812.

Sun H, Charles CH, Lau LF, Tonks NK. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo*. *Cell* 1993; 75: 487-493.

Sun J-Z, Tang X-L, Park SW. Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J Clin Invest* 1996; 97: 562-776.

Sun W, Wainright CL. The role of nitric oxide in modulating ischaemia-induced arrhythmias in rats. *J. Cardiovasc. Pharmacol.* 29: 554 – 562, 1997.

Sutherland FJ, Hearse DJ. The isolated blood and perfusion fluid perfused heart. *Pham Res* 2000; 41: 613-627.

Szilvassy Z, Ferdinandy P, Bor P, Jakab I, Lonovics J, Koltai M. Ventricular overdrive pacing-induced anti-ischaemic effect: a conscious rabbit model of preconditioning. *Am. J. Physiol* 1994; 266: H2033 – H2041.

Takahashi E, Abe J, Gallis B, Aebersold R, Spring DJ, Krebs EG, Berk BC. p90RSK is a serum-stimulated NHE-1 kinase: regulatory phosphorylation of serine 703 of Na<sup>+</sup>/H<sup>+</sup> exchanger isoform-1. *J Biol Chem* 1999; 274: 20206-20214

Takahashi E, Ashraf M. Pathologic assessment of myocardial cell necrosis and apoptosis after ischaemia and apoptosis after ischaemia and reperfusion with molecular and morphological markers. *J. Mol. Cell. Cardiol.* 2000; 32: 209 – 224.

Takano H, Manchikalapudi S, Tang XL, Qiu Y, Rizvi A, Jadoon AK, Zhang Q, Bolli R. Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* 1998a ; 98: 441 – 449.

Takano H, Tang XL, Qiu Y, Guo Y, French BA, Bolli R. Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant-sensitive mechanism. *Circ. Res.* 1998b; 83: 73 – 84.

Takasaki Y, Adachi N, Dote K, Tsubote S, Yorozuya T, Arai T. Ischaemic preconditioning suppresses the noradrenaline turnover in the rat heart. *Cardiovasc Res* 1998; 39(2): 373-380.

Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ. FGF and stress regulate CREB and ATF-1 via a pathway involving RK/p38 MAP kinase and MAPKAP kinase-2. *EMBO J* 1996; 15: 4629-4642.

Tan Y, Ruan H, Demeter MR, Comb MJ. p90(RSK) blocks bad-mediated cell death via a protein kinase C-dependent pathway. *J Biol Chem* 1999; 274: 34859-34867.

Tani M. Mechanisms of Ca<sup>2+</sup> overload in reperfused ischemic myocardium. *Annu Rev Physiol* 1990; 52: 543-559.

Tanoue T, Moriguchi T, Nishida E. Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. *J Biol Chem* 1999; 274: 19949-19956.

Thandroyen FT, Muntz KH, Buja LM, Willerson JT. Alterations in  $\beta$ -adrenergic receptors, adenylate cyclase and cAMP concentrations during acute myocardial ischaemia and reperfusion. *Circulation* 1990; 82 (suppl II): II 30- II 37.

Thomas MK, Francis SH, Corbin JD. Substrate- and kinase-directed regulation of phosphorylation of a cGMP-binding phosphodiesterase by cGMP. *J Biol Chem* 1990; 265: 14971-14978.

Thomson A, Clayton A, Hazzalin CA, Rose S, Barratt MJ, Mahadevan LC. The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 is a potential H3/HMG-14 kinase. *EMBO J* 1999; 18: 4779-4793.

- Thornton JD, Daly JF, Cohen MV, Yang X-M, Downey JM. Catecholamines can induce adenosine receptor-mediated protection of the myocardium but not participate in ischaemic preconditioning in the rabbit. *Circ Res*. 1993a; 73: 649-655
- Thornton JD, Liu GS, Downey JM. Pretreatment with pertussis toxin blocks the protective effects of preconditioning: evidence for a G-protein mechanism. *J Mol Cell Cardiol* 1993b; 25(3): 311-320.
- Thuerauf DJ, Arnold ND, Zechner D, Hanford DS, De Martin KM, McDonough PM, Prywes R, Glembotski CC. p38 mitogen-activated protein kinase mediates the transcription induction of atrial natriuretic factor gene through a serum response element. A potential role for the transcription factor ATF6. *J Biol Chem* 1998; 273: 20636-20643.
- Todd JL, Tanner KG, Denu JM. Extracellular regulated kinases (ERK) 1 and ERK2 are authentic substrates for the dual-specificity protein-tyrosine phosphatase VHR. A novel role in down-regulating the ERK pathway. *J Biol Chem* 1999; 274: 13271-13280.
- Tong H, Chen W, London RE, Murphy E, Steenbergen C. Phosphoinositide-3-kinase (PI3K) is involved in preconditioning. *J Mol Cell Cardiol* 1999; 31: A19 Abstract.
- Toombs CF, Wiltse AL, Shebuski RJ. Ischemic preconditioning fails to limit infarct size in reserpinized rabbit myocardium: implication of norepinephrine release in the preconditioning effect. *Circulation* 1993; 88: 2351 – 2358.
- Torres J, Darley-Usmar V, Wilson MT. Inhibition of cytochrome c oxidase turnover by nitric oxide: mechanism and implications for control of respiration. *Biochem J* 1995; 212: 169-173.
- Tosaki A, Behjet NS, Engelman DT, Das DK. Alpha-1 adrenergic receptor agonist-induced preconditioning in isolated working rat hearts. *J Pharmacol Exp Ther* 1995; 273(2): 689-694.
- Tournier C, Whitmarsh AJ, Cavanagh J, Barrett T, Davis RJ. Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH<sub>2</sub>-terminal kinase. *Proc Natl Acad Sci USA* 1997; 94: 7337-7342.
- Tsien R. Calcium channels in excitable cell membranes. *Annu Rev Physiol*. 1983; 45: 341-358.
- Tsuchida A, Ardell J, Cohen MV, Downey J, Heymann A, Barron BA, Haamick M, Jones C. Elimination of myocardial catecholamines with ventricular sympathectomy fails to limit infarct size in canine hearts. *Circulation* 1993; 88 (suppl): I-42.
- Tsuchida A, Liu Y, Cohen MV, Downey JM. Alpha-1 adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ Res* 1994; 75: 576-585.
- Ungerer M, Kessebohm K, Kronsbein K, Lohse MJ, Richardt G. Activation of  $\beta$ -adrenergic receptor kinase during myocardial ischaemia. *Circ Res* 1996; 79: 455 – 460.
- Vaandrager AB, De Jonge HR. Signaling by cGMP-dependent protein kinases. *Mol Cell Biochem* 1996; 157:23-30.
- Vahlhaus C, Schulz R, Post H, Onallah R, Heusch G. No prevention of ischemic preconditioning by the protein kinase C inhibitor staurosporine in swine. *Circ Res* 1996; 79: 407-414.
- Valdivia HH, Kaplan JH, Ellis-Davies GC, Lederer WJ. Rapid adaptation of cardiac ryanodine receptors: Modulation by Mg<sup>2+</sup> and phosphorylation. *Science* 1995; 267: 1997-2000
- Valen G, Yan ZQ, Hansson GK. Nuclear factor kappa-B and the heart. *J Am Coll Cardiol* 2001; 38: 307-314.
- Van Winkle DM, Thornton JD, Downey DM, Downey JM. The natural history of preconditioning: cardioprotection depends on duration of transient ischemia and time to subsequent ischemia. *Coron Art Dis* 1991; 2: 613-619.

- Van Wylen DG. Effect of ischemic preconditioning on interstitial purine metabolite and lactate accumulation during myocardial ischemia. *Circulation* 1994; 89: 2283-2289.
- Vander Heide RS, Reimer KA, Jennings RB. Adenosine slows ischaemic metabolism in canine myocardium in vitro: relationship to ischaemic preconditioning. *Cardiovasc Res.* 1993 Apr;27(4):669-73.1993.
- Vander Heide RS, Rim D, Hohl CM, Ganote CE. An in vitro model of myocardial ischaemia utilizing isolated adult rat myocytes. *J Mol Cell Cardiol* 1990 Feb;22(2):165-181.
- Vegh A, Szekeres L, Parratt JR. Does nitric oxide play a role in ischaemic preconditioning? (abstract) *J Mol Cell Cardiol* 1991; 23 (suppl) 5):S72
- Vegh A, Szekeres L, Parratt JR. Preconditioning of the ischaemic myocardium; involvement of the L-arginine nitric oxide pathway. *Br. J. Pharmacol.* 1992; 107: 648–652.
- Verde I, Vandecasteele G, Lezoualc'h F, Fischmeister R. Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type Ca<sup>2+</sup> current in rat ventricular myocytes. *Br J Pharmacol* 1999; 127: 65-74.
- Victor T, Bester AJ, Lochner A. A sensitive and rapid method for separating adenine nucleotides and creatine phosphate by ion-pair-reversed-phase high-performance liquid chromatography. *J Chromatogr* 1987; 389: 339-44
- Vila-Petroff MG, Younes A, Egan J, Lakatta EG, Sollott SJ. Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ. Res.* 1999; 84: 1020 – 1031.
- Volovsek A, Subramanian R, Reboussin D. Effects of duration of ischaemia during preconditioning on mechanical function, enzyme release and energy production in the isolated working rat heart. *J Mol Cell Cardiol* 1992; 24: 1011-1019.
- Vondriska TM, Klein JB, Ping P. Use of functional proteomics to investigate PKC $\epsilon$ -mediated cardioprotection: the signaling module hypothesis. *Am J Physiol Heart Circ Physiol* 2001; 280: H1434-H1441.
- Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. cAMP activates MAP kinase and Elk-1 through a B-Raf and Rap1-dependent pathway. *Cell* 1997; 89: 73-82.
- Voyno-Yasenetskaya TA, Faure MP, Ahn NG, Bourne HR. Galpha12 and Galpha13 regulate extracellular signal-regulated kinase and c-Jun kinase pathways by different mechanisms in COS-7 cells. *J Biol Chem.* 1996;271(35):21081-7.
- Vuorinen K, Ylitalo K, Peuhkurinen K et al. Mechanisms of ischemic preconditioning in rat myocardium. Roles of adenosine, cellular energy state, and mitochondrial F1F0-ATPase. *Circulation* 1995; 91: 2810-2818.
- Waldenstrom AP, Hjalmarson AC, Thornell LT. A possible role of noradrenaline in the development of myocardial infarction. An experimental study in isolated rat heart. *Am Heart J* 1978; 95: 43-51.
- Walker D, Yellon D. Ischemic preconditioning: from mechanisms to exploitation. *Cardiovasc Res* 1992; 26: 734-739.
- Wallis RM, Corbin JD, Francis SH, Ellis P. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. *Am J Cardiol* 1999; 83: 3C-12C.
- Walsh MP. Regulation of vascular smooth muscle. *Can J Physiol Pharmacol* 1993; 72: 919-936.
- Wang L, Cherednichenko G, Hernandez L, Halow J, Camacho SA, Figueredo V, Schaefer S.

- Preconditioning limits mitochondrial Ca(2+) during ischemia in rat hearts: role of K(ATP)channels. *Am J Physiol Heart Circ Physiol* 2001; 280: H2321-H2328.
- Wang P, Zweier JL. Measurement of nitric oxide and peroxynitrite generation in postischaemic heart. *J Biol Chem*. 1996; 271: 29223 – 29230.
- Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochem J* 1998; 333:291-300.
- Wang Y and Ashraf M. Role of protein kinase C against Ca<sup>2+</sup> overload injury in rat myocardium. *Circ Res* 1999; 84: 1156-1165.
- Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J, Chien KR. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem* 1998; 273: 2161 - 2168.
- Wang Y, Kudo M, Xu M, Ayub A, Ashraf M. Mitochondrial K(ATP)Channel as an end effector of cardioprotection during late preconditioning: triggering role of nitric oxide. *J Mol Cell Cardiol* 2001; 33: 2037-2046.
- Weinbrenner C, Liu GS, Cohen MV, Downey JM. Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart. *J Mol Cell Cardiol* 1997; 29: 2383 - 2391.
- Weinbrenner CE, Simonis G, Marquetant R, Strasser RH. Selective regulation of calcium-dependent and calcium independent subtypes of protein-kinase C in acute and prolonged ischemia. *Circulation* 1993; 88: I-101.
- Weselcouch EO, Baird AJ, Sleph PG, Dzwonczyk S, Murray HN, Grover GJ. Endogenous catecholamines are not necessary for ischaemic preconditioning in the isolated perfused rat heart. *Cardiovasc Res* 1995a; 29: 126-132.
- Weselcouch EO, Baird AJ, Sleph PG, Grover GJ. Inhibition of nitric oxide synthesis does not affect ischaemic preconditioning in isolated perfused rat hearts. *Am. J. Physiol.* 1995b; 268: H242 – H249.
- Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. *Physiol Rev* 1999 (79): 143-180.
- Wilk-Blaszczak MA, Stein B, Xu S, Barbosa MS, Cobb MH, Belardetti F. The mitogen-activated protein kinase p38-2 is necessary for the inhibition of N-type calcium current by bradykinin. *J Neurosci* 1998; 18: 112-118.
- Williams SD, Ford DA. Calcium-independent phospholipase A<sub>2</sub> mediates CREB phosphorylation and *c-fos* expression during ischemia. *Am J Physiol Heart Circ Physiol* 2001; 281: H168-H176.
- Wolfe CL, Sievers RE, Visseren FL, Donnelly TJ. Loss of myocardial protection after preconditioning correlates with the time course of glycogen recovery within the preconditioned segment. *Circulation* 1993; 87: 881-892.
- Wollenberger A, Krause EG, Heier G. Stimulation of 3', 5' cyclic AMP formation in dog myocardium following arrest of blood flow. *Biochem Biophys Res Commun* 1969; 36: 664-670.
- Wollenberger A, Krause EG. Metabolic control characteristics of the acutely ischaemic myocardium. *Am J Cardiol* 1968; 22: 349-359.
- Woolfson RG, Patel VC, Neild GH, Yellon DM. Inhibition of nitric oxide synthesis reduces infarct size by an adenosine-dependent mechanism. *Circulation* 1995; 91: 1545 – 1551.
- Worthington MG, Opie LH. Contrasting effects of cAMP increase caused by  $\beta$ -adrenergic

stimulation or by adenylate cyclase activation on ventricular fibrillation threshold of isolated rat heart. *J Cardiovasc Pharmacol* 1992; 20: 595 – 600.

Wu J, Harrison JK, Vincent LA, Haystead C, Haystead TAJ, Michel H, Hunt DF, Lynch KR, Sturgill TW. Molecular structure of a protein-tyrosine/threonine kinase activating p42 mitogen-activated protein (MAP) kinase: MAP kinase kinase. *Proc Natl Acad Sci USA* 1993; 90: 173-177.

Wu QY, Feher JJ. Effect of ischemia and ischemia-reperfusion on ryanodine binding and  $Ca^{2+}$  uptake of cardiac sarcoplasmic reticulum. *J Mol Cell Cardiol* 1995; 27: 1965-1975.

Xia Y, Buja LM, McMillin JB. Activation of the cytochrome c gene by electrical stimulation in neonatal rat cardiac myocytes. Role of NRF-1 and c-Jun. *J Biol Chem* 1998; 273: 12593-12598.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995; 270: 1326 - 1331.

Xiao XH, Allen DG. Role of  $Na^{+}/H^{+}$  exchanger during ischemia and preconditioning in the isolated rat heart. *Circ Res* 1999; 85: 723-730.

Xing J, Ginty DD, Greenberg ME. Coupling of the Ras-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* 1996; 273: 959-963.

Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 1998; 279: 234-237.

Xuan Y-T, Tang XL, Banerjee S, Takano H, Li RCX, Han H, Qiu Y, Li JJ, Bolli R. Nuclear factor-kB plays an essential role in the late phase of ischaemic preconditioning in conscious rabbits. *Circ Res*. 1999; 84: 1095 – 1109.

Xuan Y-T, Tang XL, Qiu Y, Banerjee S, Takano H, Han H, Bolli R. Biphasic response of cardiac NO synthase isoforms to ischaemic preconditioning in conscious rabbits. *Am J Physiol Heart Circ Physiol* 2000; 279(5): H2360-H2371.

Yabe K, Ishishita H, Tanonaka K, Takeo S. Pharmacological preconditioning induced by  $\beta$ -adrenergic stimulation is mediated by activation of protein kinase C. *J Cardiovascular Pharmacology* 1998; 32(6): 962-968.

Yabe K, Nasa Y, Takeo S. Hypoxic preconditioning in isolated rat hearts: non-involvement of activation of adenosine A1 receptor, Gi protein, and ATP-sensitive  $K^{+}$  channel. *Heart Vessels*. 1995;10(6):294-303.

Yamaguchi T, Chattopadhyay N, Kifor O, Sanders JL, Brown EM. Activation of p42/44 and p38 mitogen-activated protein kinases by extracellular calcium-sensing receptor agonists induces mitogenic responses in the mouse osteoblastic MC3T3-E1 cell line. *Biochem Biophys Res Commun* 2000; 279: 363 - 368.

Yamauchi J, Nagao M, Kaziro Y, Itoh H. Activation of p38 mitogen-activated protein kinase by signalling through G protein-coupled receptors: involvement of  $G\beta\gamma$  and  $G\alpha_{q/11}$  subunits. *J Biol Chem* 1997; 272: 27771-27777.

Yan C, Takahashi M, Okuda M, Lee J-D, Berk BC. Fluid shear stress stimulates big mitogen-activated protein kinase (BMK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. *J Biol Chem* 1999; 274: 143-150.

Yang X-M, Sato H, Downey JM, Cohen MV. Protection of ischemic preconditioning is dependent upon a critical timing sequence of protein kinase C activation. *J Mol Cell Cardiol* 1997; 29: 991-999

Yao Z, Gross GJ. Role of nitric oxide, muscarinic receptors and the ATP-sensitive K channel in mediating the effects of acetylcholine to mimic preconditioning in dogs. *Circ Res* 1993; 73: 1193-1201.

Yasmin W, Strynadka KD, Schulz R. Generation of peroxynitrite contributes to ischaemia-

reperfusion injury in isolated rat hearts. *Cardiovasc Res* 1997; 33: 422 – 432.

Yellon DM, Alkhulaifi AM, Browne EE, Pugsley WB. Ischaemic preconditioning limits size in the rat heart. *Cardiovasc Res* 1992; 26: 983-987.

Yellon DM, Baxter GF, Garcia-Dorado D, Heusch G, Sumeray MS. Ischaemic preconditioning: present position and future directions. *Cardiovasc Res* 1998; 37: 21-33.

Yin T, Sandhu G, Wolfgang CD, Burrier A, Webb RL, Rigel DF, Hai T, Whelan J. Tissue-specific pattern of stress kinase activation in ischaemic/reperfused heart and kidney. *J Biol Chem* 1997; 272: 19943 - 19950.

Yoshida K, Aki T, Harada K, et al. Translocation of HSP27 and MKBP in ischemic heart. *Cell Struct Funct* 1999; 24: 181-185.

Ytrehus K, Liu Y, Downey JM. Preconditioning protects ischaemic rabbit heart by protein kinase C activation. *Am J Physiol* 1994; 266: H1145 – H1152.

Yue T-L, Wang C, Gu J-L, Ma X-L, Kumer S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B, Ohlstein EH. Inhibition of extracellular signal-regulated kinase enhances ischaemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ Res* 2000; 86: 692-699.

Zechner D, Craig R, Hanford DS, McDonough PM, Sabbadini RA, Glembotski CC. MKK6 activates myocardial cell NF- $\kappa$ B and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. *J Biol Chem* 1998; 273: 8232-8239.

Zheng C-F, Guan K. Dephosphorylation and inactivation of the mitogen-activated protein kinase by a mitogen-induced Thr/Tyr protein phosphatase. *J Biol Chem* 1993; 268: 16116-16119.

Zheng M, Zhang S-J, Zhu W-Z, Ziman B, Kobilka B, Ziao R-P.  $\beta_2$ -adrenergic receptor induced p38 activation is mediated by PKA rather than by  $G_i$  or  $G_{\beta\gamma}$  in adult mouse cardiomyocytes. *J Biol Chem* 2000; 275: 40635 - 40640.

Zhou G, Bao ZQ, Dixon JE. Components of a new human protein kinase signal transduction pathway. *J Biol Chem* 1995; 270: 12665-12669.

Zhu W, Zou Y, Aikawa R, Harada K, Kudoh S, Uozumi H, Hayashi D, Gu Y, Yamazaki T, Nagai R, Yazaki Y, Komuro I. MAPK superfamily plays an important role in daunomycin-induced apoptosis of myocytes. *Circulation* 1999; 100: 2100 - 2107.

Zia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995; 270: 1326 - 1331.

Zu YL, Ai Y, Gilchrist A, Maulik N, Watras J, Sha'afi RI, Das DK, Huang CK. High expression and activation of MAP kinase activated protein kinase 2 in myocardium. *J Mol Cell Cardiol* 1997; 29: 2159-2168.

Zweier JL, Wang P, Kuppusamy P. Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J Biol Chem* 1995; 270: 304-307.