

Catecholamine Release as Mediator of Intracellular Enzyme Activation in Ischaemic Perfused Rat Hearts

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SUMMARY

Isolated rat hearts perfused at suboptimal pressures have been studied as a model for generalised myocardial ischaemia. Glycogen phosphorylase *a* and hormone-sensitive triglyceridase activities, measured as markers for endogenous catecholamine release, were significantly increased at low perfusion pressures.

Pharmacological blockage of noradrenaline re-uptake accentuated these effects, and depletion of catecholamine reserves eliminated them. This phenomenon may be important in the pathophysiology of cardiac ischaemia and its serious complications.

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Norepinephrine (NE) homeostasis is a fundamental regulatory component of the contractile machinery and the intermediary metabolism of the heart. Functioning as a neuro-endocrine organ, the heart is capable of synthesising and packaging dopamine into storage vesicles for enzymatic conversion into NE, of transporting these vesicles to the terminal *boutons* via the microtubular apparatus of the adrenergic axons, and of releasing stored vesicle contents by the process of exocytosis, upon appropriate stimulation. Released NE interacts with membrane receptors on post-synaptic effector cells, while termination of effects is mediated mainly by rapid cellular uptake, which leads to degradation by intracellular monoamine oxidase and other enzymes, or to storage for future release.¹

It has now become well established that the intracellular effects of catecholamines are mediated largely by variations in the concentration of cyclic AMP (cAMP), which reflect a fine balance between synthesis via the hormone-sensitive membrane enzyme, adenylyl cyclase, and degradation by a family of nucleotide phosphodiesterase isoenzymes.² cAMP activates a number of protein kinases, which phosphorylate specific key intracellular proteins, including certain regulatory contractile proteins (troponins), glycogen phosphorylase, triglyceridase and certain mem-

brane proteins involved in Ca^{2+} fluxes; all these are in turn dephosphorylated by specific phosphatases.³

Although catecholamines are essential for normal cardiac function, excessive release can cause functional and structural damage to the heart. Recently reported catecholamine-mediated cardiomyopathies in both experimental animal models (isoproterenol cardiomyopathy⁴) and in clinical medicine (phaeochromocytoma, tetanus, cardiac surgery⁵), prompted us to examine the effect of ischaemia on catecholamine release in the isolated rat heart.

In this study, use has been made of the known stimulatory effect of catecholamines on cardiac glycogenolysis and lipolysis,² activations of the respectively rate-limiting enzymes glycogen phosphorylase and hormone-sensitive triglyceride lipase (HSL) being used as indirect parameters of NE release. These enzyme activities were characterised, and particular care was taken to ensure that contaminating lipoprotein lipase was not measured. Rat hearts, perfused at low pressure by the retrograde Langendorff procedure, served as working models for myocardial ischaemia, and the effects on these enzymes were evaluated by comparisons with hearts perfused at normal pressures. Finally, the question as to whether observed changes were indeed catecholamine-mediated was examined by testing the effects in this system of: (a) inhibiting catecholamine uptake and subsequent degradation, and (b) depleting cardiac catecholamine stores by reserpine pretreatment, or by prolonged perfusion.

METHODS

Perfusion Technique and Tissue Preparation

After an overnight fast (12 - 20 h), inbred albino Wistar rats (150 - 250 g) of both sexes were anaesthetised by intraperitoneal injection of pentobarbital sodium (30 mg/kg) and the hearts were rapidly removed 30 minutes later, to be perfused at 37°C by the Langendorff technique, using Krebs-Ringer bicarbonate medium gassed with 95% O_2 : 5% CO_2 and containing 15 mM glucose.⁶ Routine perfusions were carried out for 30 minutes at a constant perfusion pressure of 70 mmHg. Low-pressure perfusions, unless otherwise specified, consisted of an initial 10-minute perfusion at 70 mmHg, followed by 20 minutes at 20 mmHg.

Perfused hearts were blotted dry and vertically halved and the two parts were homogenised respectively in 10 vol. of

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buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate (Hepes), 20 mM KF, 1 mM EDTA; pH 6,1) and 7,5 vol. of buffer B (0,25M sucrose, 30 mM EDTA, 0,2% bovine serum albumin (B.S.A.), 15 mM sodium phosphate; pH 6,8), for 3×10 sec in the cold, at half-maximum speed with a Polytron PT10 homogeniser. The homogenates were centrifuged at 2°C, first at 1 000 *g* for 10 minutes and then at 12 500 *g* for a further 10 minutes.

Glycogen Phosphorylase and Triglyceride Lipase Determinations

Glycogen phosphorylase: The postmitochondrial supernatant in buffer A was used for phosphorylase determinations in the absence (phosphorylase *a* activity) and presence (total phosphorylase activity) of 5'-AMP, by a micro-modification of the method of Hers and van Hoof,⁷ using 50- μ l volumes of assay mixture and tissue extract, respectively. Inorganic phosphate formed was determined colorimetrically.⁸

Triglyceride lipases: The postmitochondrial supernatant and the crude mitochondrial fractions prepared in buffer B were incubated in buffer C (20 mM Hepes, 2% B.S.A., 15 mM mercapto-ethanol, 30 mM EDTA; pH 6,8) which also contained 10 mM substrate (pure glyceride in 0,5% B.S.A., after high-speed sonication with a Branson Sonifier, Mark IV), in a shaking waterbath for 60 or 90 minutes at 30°C. The mitochondrial sediment was resuspended in buffer B and sonicated (5×10 sec, low setting 5), prior to incubation. The reaction was terminated by the addition of 0,1 vol. of 30% cold perchloric acid, followed by high-speed centrifugation; perchlorate ions were removed, following neutralisation with KOH, by means of pressure filtration (Sartorius syringe filter; 0,45 μ l). The clear supernatant was used for the determination of glycerol by the double enzymatic method of Wieland,⁹ with the principal modification of the addition of 20 mM MgCl₂ to overcome EDTA inhibition. The justifiable assumption was made that triglyceridase, and not diglyceridase or monoglyceridase activity, was rate-limiting for the production of glycerol.

Manipulation of Cardiac Catecholamine Stores

Inhibition of catecholamine uptake and degradation was accomplished by the addition to the perfusion medium of 20 μ M phenoxybenzamine (PBA solution for injection), a known inhibitor of both neuronal and extraneuronal uptake processes,¹ immediately prior to mounting the heart.

Catecholamine stores were depleted *in vivo* by the intraperitoneal injection of reserpine (Serpasil 5 mg/kg) 2 hours prior to perfusion, as well as *in situ* by prolonged perfusion (see Fig. 4).

RESULTS

Table I illustrates some basic characteristics of the cAMP-dependent glycogen phosphorylase system. By means of

a dual action, fluoride ions are known to cause the activation of adenylyl cyclase as well as the inhibition of Mg²⁺-dependent phosphatase.³ It should be noted that inclusion of 20 mM KF, in combination with the chelating agent EDTA, brought about satisfactory stabilisation of phosphorylase *a* activity without the undue stimulation of adenylyl cyclase obtained in the presence of 100 mM KF. Decapitation, with its accompanying burst of adrenergic activity,¹⁰ gave inconsistently high phosphorylase *a* values, and barbiturate anaesthesia was accordingly preferred. Perfusion of isolated hearts gave both the most reproducible baseline, and the highest catecholamine-stimulated phosphorylase *a* activities.

TABLE I. GLYCOGEN PHOSPHORYLASE ACTIVITY IN DIFFERENT RAT HEART PREPARATIONS

	Phosphorylase <i>a</i>	
	Immediate assay	Assayed after 24 h
(i) Hearts from decapitated rats, with phosphorylase assay carried out in 20 mM Hepes alone ...	17 - 28%	10%
(ii) As (i) but with 100 mM KF ...	70%	48%
(iii) As (i) but with 20 mM KF + 1 mM EDTA ...	34%	34%
(iv) Anaesthesia with pentobarbital sodium (no decapitation), enzyme assayed in 100 mM KF + 20 mM Hepes ...	58%	
(v) Isolated heart perfused for 30 min; enzyme in 15 - 30 mM KF	4 - 20%	
(vi) Epinephrine, administered <i>in vivo</i> (5 - 10 μ g by intracardiac or IV injection) ...	50 - 78%	
or <i>in vitro</i> (0,1 - 0,2 μ g in perfusion medium) ...	25 - 40%	

Lipase activity, as determined by glycerol release, was present in both the crude mitochondrial and the post-mitochondrial supernatant fractions, acting on a variety of substrates (Table II). No attempt was made to characterise these enzymes in detail, although definite qualitative and quantitative differences between the mitochondrial and supernatant enzymes were observed. Using tributyrin as substrate, prior fasting of animals was found to lead to higher activities of both kinds of lipases, this activation being more pronounced in the mitochondrial fraction. Addition of NH₄Cl (20 mM) to the assay mixture gave similar results. Of particular interest was the stimulation of the mitochondrial but not of the supernatant lipase by heparin, a known activator of lipoprotein lipase.¹¹ This latter enzyme, predominantly present in the cytoplasm, is inhibited by the EDTA and sodium phosphate used in our assay, however, and is only weakly active at a pH below 7,0 in the absence of serum substrate. Furthermore, NaF, a potent inhibitor of adipose tissue and hepatic mitochondrial lipases¹² has no effect on lipoprotein lipase, whereas a definite inhibition was observed in both our enzyme fractions.

TABLE II. CHARACTERISATION OF CARDIAC LIPASES

	% of total activity in	
	Crude mitochondrial fraction	Post-mitochondrial supernatant fraction
(A) Substrate (10 mM)		
Monolein	55	45
Dipalmitin	30	70
Tributyrin	37	63
Triolein	35	65
(B) Modulation of lipase activity (using tributyrin as substrate)		
(i) NaF (20 mM)	74	71
(ii) Heparin (80 IU/ml)	125	100
(iii) NH ₄ Cl (20 mM)	168	138
(iv) cAMP - ATP - Mg - caffeine (<i>in vitro</i>)*	500	200
(v) Epinephrine:		
<i>in vivo</i> (25 µg IV)	500	200
or <i>in situ</i> (0.2 µg/ml perfusion medium)	190	140

* cAMP 2.5 µM, ATP 0.15 mM, MgCl₂ 20 mM, and caffeine (or theophylline) 2.5 mM.

Hormone sensitivity was ascertained by the *in vitro* incubation of enzyme fractions with cAMP, ATP, MgCl₂ and the phosphodiesterase inhibitor caffeine (or theophylline), by *in vivo* injection of epinephrine as described above, and by the *in situ* addition of catecholamine to the perfusion medium. Both lipase enzyme fractions responded to hormone stimulation by two- to threefold increased activities, and an activation ratio of 5 : 2 between mitochondrial and supernatant lipases was obtained in all three experiments.

The effect of ischaemia on glycogen phosphorylase and lipase activities of perfused rat hearts is illustrated in Figs 1 and 2. A consistent activation of glycogen phosphorylase was observed, and was most marked with a low-pressure perfusion lasting 20 minutes. The average stimulation under these conditions was 50-90% (9 experiments). Using triolein as substrate, both the mitochondrial and supernatant lipases in ischaemic tissue showed increased activities, giving results similar to those obtained *in vitro* and *in vivo* by lipase activation with epinephrine (Table II). With tributyrin as substrate, however, perfusion at low pressure enhanced the activity of the mitochondrial lipase only, while it inhibited the supernatant enzyme.

Before concluding that norepinephrine release mediated the observed enzyme activations during ischaemia, direct non-catecholamine-related influences (e.g. hypoxia) had to be examined. Fig. 3 illustrates the effect of low-pressure perfusion and phenoxybenzamine (PBA) on glycogen phosphorylase activation. When used in combination, the observed enzyme activation exceeded the sum of the activations produced by the two individual treatments,

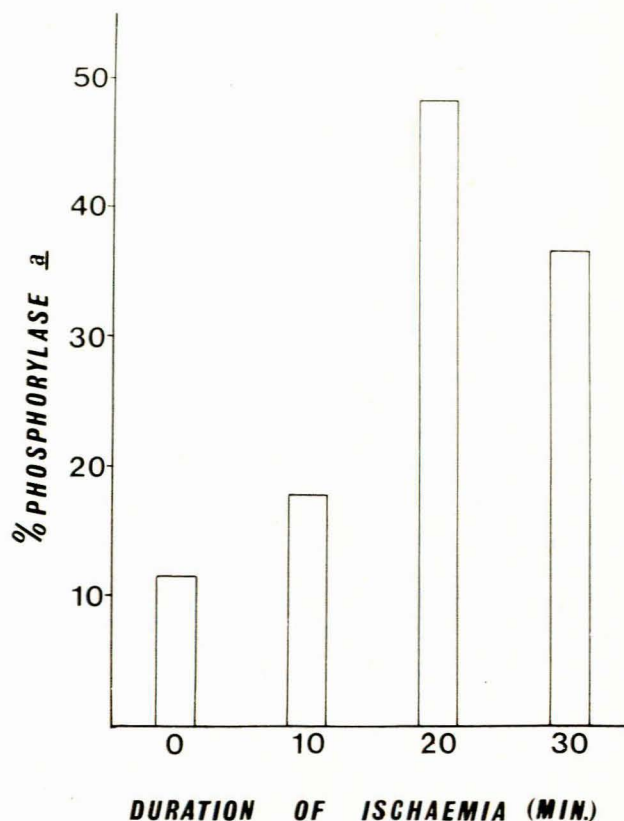


Fig. 1. The effect of ischaemia on cardiac glycogen phosphorylase. All perfusions were carried out for 30 minutes as described under 'Methods'. In the control experiments (0), the perfusion pressure during the entire 30 minutes was maintained at 70 mmHg. In the ischaemic cases, the duration of low-pressure perfusion varied from 10 to 30 minutes, as indicated.

suggesting a common mode of action, rather than independent actions causing additive changes.

Similar results were obtained in the case of lipase assays. Prolonged perfusion of rat hearts gradually depleted cardiac catecholamine stores. A marked ischaemic activation of phosphorylase was still observed after 35 minutes of perfusion but no stimulation was found after 75 minutes (Fig. 4). Similar results were obtained after *in vivo* catecholamine depletion by means of reserpine administration (Figs 5 and 6). Whereas activation of glycogen phosphorylase and lipase by low-pressure perfusion was observed in non-reserpinised animals, reserpine pretreatment completely abolished the stimulation by ischaemia. The inhibitory effect of ischaemia on the supernatant lipase with tributyrin as substrate (Fig. 2) was not significantly affected by reserpinisation, however (Fig. 6). The higher phosphorylase *a* and lipase values of reserpinised control hearts probably reflected catecholamine release by the drug, as illustrated by the stimulatory effect of 20 µg reserpine added to the perfusion system of a non-ischaemic control heart (Fig. 5).

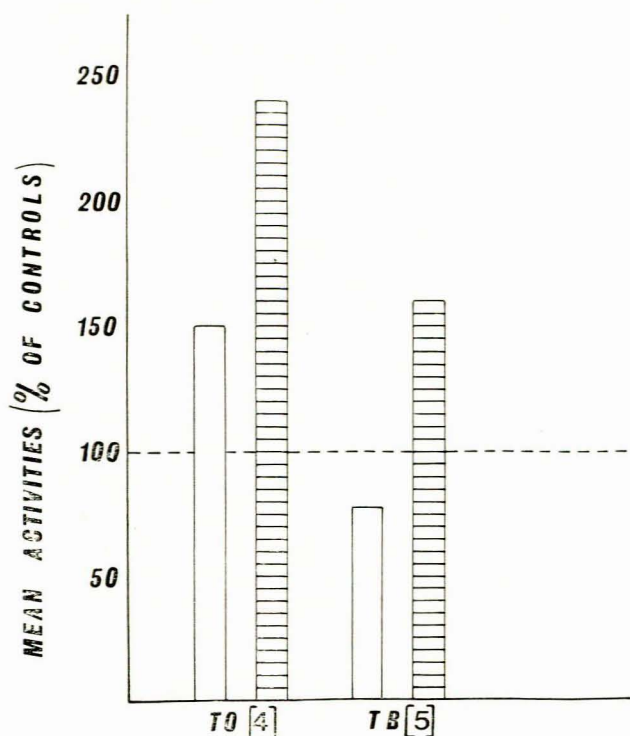


Fig. 2. The effect of ischaemia on cardiac hormone-sensitive lipases. Hearts were perfused at low pressure for 20 minutes, as described under 'Methods'. Lipase determinations were done using triolein (TO) and tributyrin (TB) as substrates, and were expressed as percentages of control values. Figures in parentheses indicate number of experiments.

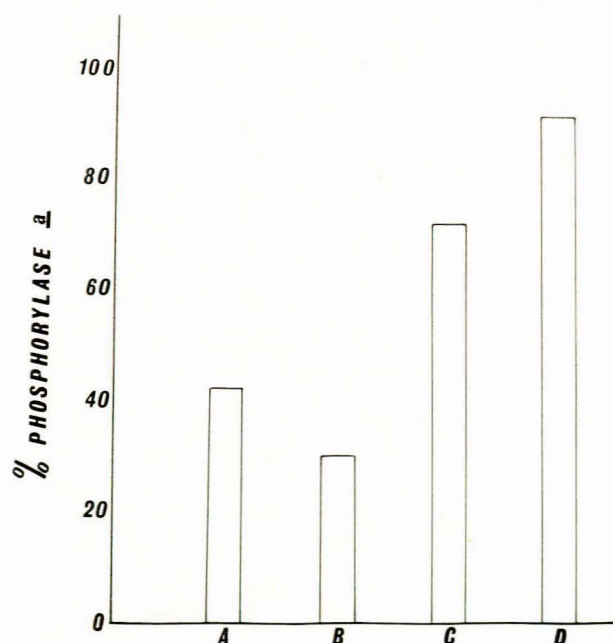


Fig. 3. The effect of phenoxybenzamine (PBA) and low-pressure perfusion on glycogen phosphorylase. PBA (20 μ M) added to perfusion medium immediately prior to perfusion carried out as described under 'Methods': (A) effect of low-pressure perfusion; (B) PBA effect; (C) theoretical sum of low-pressure perfusion + PBA; (D) observed effect of low-pressure perfusion + PBA.

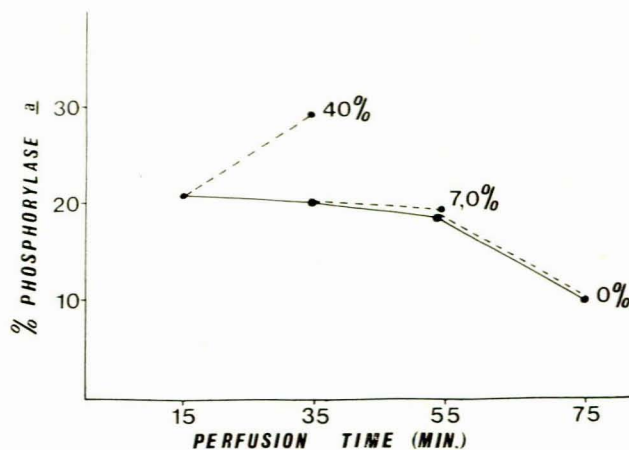


Fig. 4. The effect of prolonged perfusion on cardiac glycogen phosphorylase. Control hearts (o—o) were perfused for 15, 35, 55 and 75 minutes. Ischaemic hearts (o---o) were perfused for either 15, 35 or 55 minutes, followed in each case by a 20-minute period at low pressure. Figures indicate the percentage stimulation of glycogen phosphorylase activities after 35, 55 and 75 minutes of perfusion.

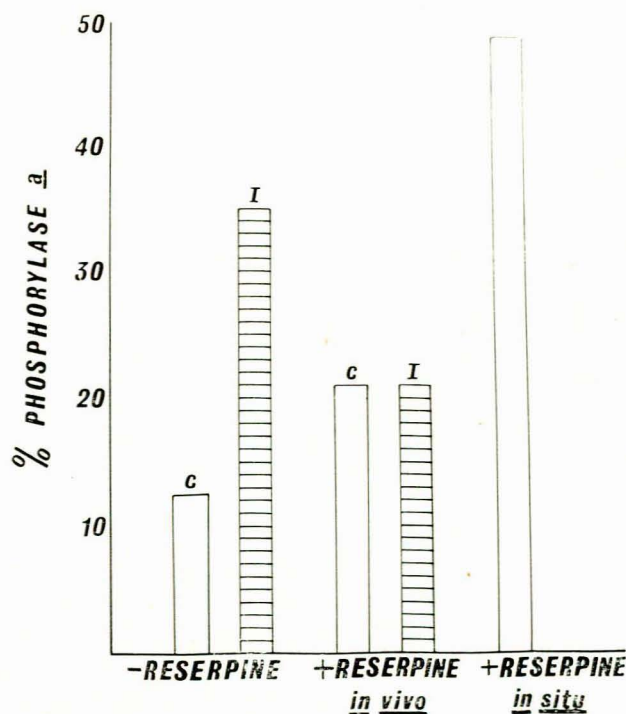


Fig. 5. The effect of reserpine *in vivo* (5 mg/kg intraperitoneally) and *in situ* (20 μ g added to perfusion medium) on glycogen phosphorylase activity in control (C) and ischaemic (I) hearts.

DISCUSSION

Processes involved in cardiac catecholamine metabolism constitute an important link between the maintenance of normal heart functions and the development of myocardial diseases. Thus catecholamines are of major importance in the regulation of myocardial energy production (glycolysis, lipolysis³) and energy utilisation (membrane Ca^{2+} fluxes;

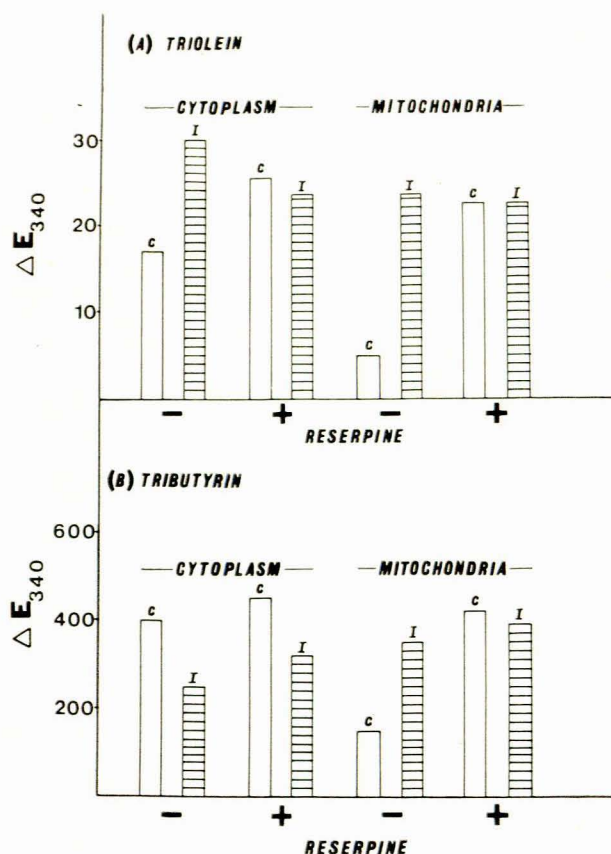


Fig. 6. The effect of reserpine pretreatment on lipase activities in control (C) and ischaemic (I) hearts.

thin filament interaction¹⁵). Excessive systemic liberation of catecholamines, however, as found for example in phaeochromocytoma, tetanus, and isoproterenol injection, causes a typical cardiomyopathy consisting of focal myocytolytic lesions.^{4,5} Increased levels of circulating catecholamines have also been found in patients with myocardial infarction¹⁴ and in experimental ischaemia,¹⁵ and the infarcted area itself has been implicated as the source of catecholamines which appear in the plasma.¹⁶

Using isolated rat hearts, we have been able to demonstrate effects caused by endogenous catecholamine release during low-pressure perfusions. These released amines have been detected by cellular effects rather than by direct chemical analysis. We have also found in the hearts that hormone-sensitive lipases, different from the well-known lipoprotein lipase, undergo catecholamine-mediated stimulation that is probably cAMP-dependent and thus similar to analogous enzymes in adipose tissue.¹⁷ However, all the effects of ischaemia on lipase activities in the isolated hearts could not be explained only on the basis of catecholamine release. In contrast to the *in vitro* stimulation by epinephrine of the supernatant lipase acting on tributyrin, inhibition of this enzyme was observed during ischaemia (Fig. 2). Furthermore, this inhibition was not affected by pre-perfusion reserpinisation, thus excluding catechola-

mine mediation. Considering that ischaemia may be accompanied both by catecholamine release and by hypoxia, it is possible that the potent antilipolytic effect of hypoxia¹² could have accounted for this apparent inhibition, raising the interesting possibility of differential lipase activation and selective free fatty acid liberation during ischaemia.

Increased catecholamine release during ischaemia may have serious effects on heart functions. Although glycolysis and glycogen depletion have been implicated in the pathogenesis of ventricular fibrillation,¹⁸ the deleterious effects of catecholamine excess probably relate to lipid and Ca^{2+} metabolism. An important unresolved question is whether increased plasma or tissue free fatty acid concentrations, or both, are good or bad either for the normal or for the ischaemic myocardium. There is a variety of mechanisms by which increased tissue fatty acids could adversely affect myocardial functions.^{12,15-20} Such fatty acids may be acylated to form long-chain acyl CoA esters, or they may react with intracellular cations to form soaps. Possible results of this are non-specific detergent effects on membranes and enzymes, while an effective reduction in ionic Ca^{2+} at crucial cellular sites could affect Ca^{2+} -dependent contraction events.

Free fatty acids also influence mitochondrial oxidative phosphorylation,²¹ while they could conceivably cause lability of other types of membranes. The significant role of free fatty acids has further been emphasised by the beneficial effect of nicotinic acid, administered as an inhibitor of lipolysis, on the myocytolytic lesions induced

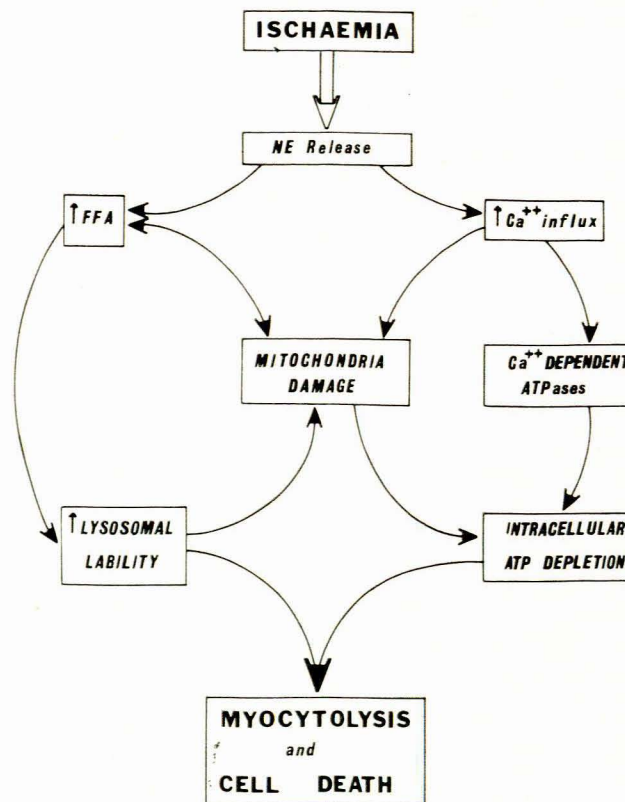


Fig. 7. Proposed mechanisms of catecholamine-mediated myocardial cell damage during ischaemia.

by isoproterenol.²² The *in vivo* observations supporting this general hypothesis include a positive correlation between plasma free fatty acid concentrations and the development of postinfarction arrhythmias, left ventricular failure and hypotension. The role of endogenous cardiac fatty acid production in these processes remains to be investigated.

The other ill-effects of catecholamine release relate to the Ca^{2+} metabolism of the heart. Cardiac microsomes contain intrinsic cAMP-dependent protein kinase(s) and phosphatases, which contribute to the regulation of Ca^{2+} fluxes. It is postulated that the increased transmembrane Ca^{2+} influx induced by sympathomimetic amines could cause: (a) excessive activation of Ca^{2+} -dependent intracellular ATPases and/or (b) mitochondrial calcification, swelling and functional deterioration resulting in a massive deficiency of cardiac high-energy phosphates, which could lead to structural damage as observed in ischaemia.^{13,23} Experimental support for this hypothesis comes from the beneficial effect on these lesions of calcitonin and EDTA (decreasing extracellular Ca^{++} supply), Verapamil (restricting transmembrane Ca^{++} conductivity), and Mg^{++} or K^{+} salts (inhibiting intramitochondrial Ca^{++} deposition).²³ All these mechanisms are schematically represented in Fig. 7, although the quantitative importance of the different elements needs to be explored in detail, since they may be important in the pathophysiology of cardiac ischaemia and of its serious complications.

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