The role of Phosphodiesterase 4 in insulin- and phytocannabinoids-induced cardio-protection, and B-adrenergic cardiac damage.

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Thesis presented in fulfilment of the requirements for the degree MSc (in Medical Physiology) at Stellenbosch University.

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Declaration

By submitting this thesis, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2016

Abstract

Introduction: Cardiovascular disease (CVD) is one of the main non-communicable diseases globally contributing to deaths each year. Ischemic heart disease leading to myocardial infarction (or heart attack as it is commonly known) is one of the leading causes of death in the United States (Raven et al. 2008) and in South Africa (Stats SA, 2013). Much research is and has been done to understand the underlying factors that lead to this disease, but this study evaluates the role of phosphodiesterase 4 (PDE 4) in mediating damage or protection during an ischemic event. PDEs function in a coordinated manner under the instruction of various stimuli, mostly known to be from G-protein coupled receptors (GPCRs), in order to promote stimulus-specific cellular physiological effects. PDE4 was considered in this thesis because PDE4 exerts a dominant role in global cellular cAMP hydrolysis in rat cardiomyocytes (Johnson et al 2012) and is responsible for mediating β1- and β2-AR specific effects in normoxic cardiomyocytes (Mika et al 2014, Gregg et al 2010). This is through cAMP compartmentation (Nikolaev et al 2006), which might also be true for ischemia/reperfusion, but has never been evaluated.

Thus the PDEs expressed in cardiomyocytes might be responsible for the sensitization of cardiomyocytes to ischemic damage.

Aim: The aim was to find an alternative way to intervene during acute myocardial ischemia/infarction, through the GPCR pathways by evaluating the role of phosphodiesterase 4 (PDE 4) in mediating damage or protection during an ischemic event.

Methods: Cardiomyocytes were isolated from adult male Wistar rat hearts and cultured overnight on 100ug/ml laminin / entactin (L / E). Following overnight cultures at 5%CO2 and 37°C, experiments were done in modified medium X culture buffer (MMXCB) with different treatment conditions of rolipram (a PDE4 inhibitor), dobutamine (b1AR-agonist), formoterol (b2AR-agonist), isoproterenol (b1- and b2AR-agonist), HU210 (synthetic cannabinoid) and THC (delta-9 tetrahydrocannabinol) to compare adult rat cardiomyocytes (ARCM) attachment, contracture and viability. Cardiomyocytes cultured on L/E were subjected to 20 minutes of simulated ischemia [using MMXCB that contained 3mM 2-deoxyglucose (2DG) and 10mM Sodium dithionite (SDT)] with the different treatment drugs

administered during ischemia, followed by 15 minutes reperfusion. Cell viability was determined by staining cells with JC-1 and images of cells in a field view that was captured using fluorescence microscopy. The cells were analysed according to cell length, morphology and fluorescence intensity with the help of ImageJ Fiji (open source software at http://fiji.sc/Fiji).

Results: A Significant difference in the cell length parameter was observed between the normoxic untreated groups compared to the ischemic untreated groups and this indicated that the ischemic conditions were sufficient to result in hypercontracture induced by ischemia/reperfusion. Regardless of eliciting a proper ischemic insult, no treatments administered during ischemia could alter hyper contracture in the cardiomyocytes.

Mitochondrial viability, measured in red over green fluorescence intensity did not always portray a significant difference between the normoxic untreated and ischemic untreated groups. The mitochondrial viability did however increase significantly when 10nM HU210 was administered during ischemia compared to ischemia untreated group. 10uM THC increase the viability when compared to ischemia untreated. With the following experiments the results could not be obtained.

The rod-shaped ARCMs attachment as measured by percentage cell viability according to morphology, did show a significant decrease after ischemic stimulation, but no differences could be monitored when the different treatment conditions was compared as the percentage viability decreased greatly to levels of 20% and less.

Conclusion: This study suggests that B-AR pathways did not have an effect on cardiomyocyte hyper contracture during an severe ischemia insult, nor insulin. It did show that the cannabinoid-receptors increased mitochondrial viability during ischemia and does indeed play a role during ischemia/reperfusion. However, the exact mechanisms for these effects seen are unknown and need further investigation. PDE4 inhibition with and without different agonists also failed to alter hypercontracture. This lack of change in cardiomyocyte survival parameters in response to the different protective and damaging drugs is considered to be a result of the harsh ischemic conditions used. It is recommended to instead incorporate hypoxia with these treatments to evaluate the effects of PDE4 in the presence and absence of b-AR, cannabinoid and insulin signalling.

Opsomming

Inleiding: Kardiovaskulêre siekte is een van die grootste nie-oordraagbare siektes wat wêreldwyd bydra tot talle sterftes elke jaar. Iskemiese hartsiekte wat tot miokardiale infarksie (hartaanval, soos dit algemeen bekend staan) lei, is een van die grootste oorsake van sterftegevalle in die Verenigde State van Amerika (Raven et al. 2008) asook in Suid-Afrika (Stats SA, 2013). Baie navorsing is reeds en word tans gedoen om die onderliggende faktore wat tot hierdie siekte lei te verstaan. Hierdie studie evalueer die rol van die ensiem, fosfodiesterase 4 (PDE4) in miokardiale iskemie. Fosfodiesterases funksioneer in 'n gekoördineerde wyse in opdrag van verskeie stimuli, via G-protein gekoppelde reseptore (GPCRs), om sodoende stimulus-spesifieke sellulêre fisiologiese effekte teweeg te bring. Daar is op PDE-4 in hierdie tesis gefokus, vanweë sy dominante rol in die globale sellulêre cAMP hidrolise in rot kardiomiosiete (Johnson et al 2012). Dit is verantwoordelik vir die bemiddeling van βb1- en βb2-adrenergiese reseptor (AR) spesifieke effekte in normoksies kardiomiosiete (Mika et al 2014, Gregg et al 2010), deur middel van sikliese nukleotied (cAMP) kompartementalisering (Nikolaev et al 2006), wat moontlik ook vir iskemie / reperfusie geld, maar nog nie getoets is nie. Dit is dus moontlik dat die PDEs wat in kardiomiosiete voorkom, verantwoordelik is vir die sensitisering van kardiomiosiete tydens iskemie.

Doel: Om 'n alternatiewe manier te vind, deur middel van G-proteïen gekoppelde reseptore (GPCR's), te om die rol van PDE 4 in miokardiale beskadiging of beskerming tydens iskemie te evalueer.

Metodes: kardiomiosiete is uit volwasse manlike Wistar rotharte geïsoleer en oornag op 100ug / ml laminin / Entactin (L / E) gekweek. Na oornag inkubasie met 5% CO2 by 37 °C, is die selle in gemodifiseerde medium X kultuur buffer (MMXCB) blootgestel aan behandeling met verskillende middles soos rolipram, dobutamien, formoterol, isoproterenol, HU210 en THC en die effekte op volwasse rot kardiomiosiet (VRKM) vashegting, kontraktuur en lewensvatbaarheid vergelyk. Die kardiomiosiete gekweek op L / E, is aan 20 minute van gesimuleerde iskemie (MMXCB plus 3mM 2DG en 10mm SDT) blootgestel in teenwoordigheid van bogenoemde middels, met die verskillende behandeling dwelms toegedien tydens iskemie, gevolg deur 15 minute reperfusie. Die lewensvatbaarheid van selle is

bepaal deur kleuring met JC-1 en fluoressensie mikroskopie. Die selle is ontleed volgens lengte, morfologie en fluoressensie intensiteit met die hulp van Image J Fiji (Ope toegang tot sagteware deur http://fiji.sc/Fiji).

Resultate:'n Beduidende verskil in sellengte is gevind tussen die normoksiese onbehandelde groepe vergeleke met die iskemiese onbehandelde groepe wat toon dat die iskemiese toestande genoeg was om tot behoorlike beskadiging te lei, soos angedui deur die kontraktuur van die selle. Ongeag hierdie beskadiging, het geeneen van die ander middels toegedien tydens iskemie, enige effek op sellengte gehad nie.

Mitokondriale lewensvatbaarheid, is gemeet in rooi oor groen fluoressensie intensiteit en het nie altyd 'n beduidende verskil tussen die normoksiese onbehandelde en iskemiese onbehandelde groepe getoon nie. Die mitokondriale lewensvatbaarheid het egter aansienlik verhoog tydens toediening van 10nM HU210 tydens iskemie in vergelyking in vergelyking met die iskemie onbehandelde groep. Dieselfde is gevind toe isoproterenol in kombinasie met rolipram tydens iskemie toegedien is. Daarenteen het 10uM THC die mitokondriale lewensvatbaarheid grootliks verminder in vergelyking met onbehandelde iskemie. Laasgenoemde eksperiment kon egter nie by herhaling dieselfde resultate lewer nie.

Die staafvormige VRKMs vashegting is gemeet deur lewensvatbaarheid persentasie van sel soos aangedui deur morfologie, en het 'n beduidende afname na blootstelling aan iskemie getoon. Geen verskille is egter waargeneem tydens blootstelling aan die verskillende middels nie.

Gevolgtrekking: Hierdie studie dui daarop dat die beta-adrenergiese paaie nie 'n effek op kardiomiosiete hiperkontraksie tydens 'n iskemiese insident het nie. Dit het wel getoon dat kannaboïed-reseptor stimulasie, ‘n toename in mitokondriale lewensvatbaarheid tydens iskemie teweegbring en moontlik 'n rol tydens iskemie / reperfusie speel. Die presiese meganismes vir die effekte wat waargeneem was, is egter nog onbekend en benodig verdere ondersoek. PDE-4 inhibisie met en sonder verskillende agoniste het ook geen effek op kontraktuur gehad nie. Die gebrek aan verandering in kardiomiosiet oorlewingparameters in hul reaksie op die verskillende beskermende en skadelike middels, kan moontlik aan die erge iskemie waaraan die selle blootgestel is, toegeskryf word. Dit word aanbeveel om die selle aan hipoksie (liewer as iskemie) bloot te stel ten einde die effek van die middels te herevalueer.

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List of Abbreviations

2-AG:	2-arachidonylglycerol
2DG:	2-deoxyglucose
AC:	Adenyl cyclase
AEA:	arachidonoyl ethanolamide
AMI:	Acute myocardial infarction
ANOVA:	analysis of variance
ARCMs:	adult rat cardiomyocytes
ATP:	adenosine triphosphate
BBS:	Blebbistatin
Ca^{2+}:	Calcium
CaM:	Ca^{2+} / calmodulin
cAMP:	cyclic AMP
CB-1:	type-1 cannabinoid
CB-2:	type-2 cannabinoid
CBD:	cannibidiol
CBRs:	Cannabinoid receptors
cGMP:	cyclic GMP
CO^2:	Carbon dioxide
CVD:	cardiovascular disease
ECS:	endocannabinoid system
Gi:	G-inhibitory protein
GPCRs:	g-protein coupled receptors
Gs:	G-stimulatory protein
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I/R:	Ischemia/Reperfusion
IBMX:	inhibitor 3-isobutyl-1-methylxanthine
IHD:	ischemic heart disease
Ins	insulin
JC-1:	5, 5¢, 6, 6¢-tetrachloro-1, 1', 3', 3'-tetraethylbenzimidazolocarbocyanine iodide
L/E:	Laminin Entactin
LTCC:	L-type calcium channels

MI:	Myocardial Ischemia
mIU:	mili International Units
mM:	millimolar
MMXCB:	modified medium X culture buffer
MXCB:	medium X culture buffer
nM:	nanomolar
NO:	nitric oxide
O^2:	Oxygen
PBS:	phosphate buffered saline
PDEs:	Phosphodiesterases
PKA:	protein kinase A
PKG:	protein kinase G
PLB:	phospholamban
R/G:	red/green
RyR2:	ryanodine receptors
SDT:	Sodium dithionite (Sodium hydrosulfite)
SEM:	Standard error of the mean
SERCA:	sarcoplasmic- and endoplasmic reticulum calcium ATPase
SR:	sarcoplasmic reticulum
THC:	delta-9 tetrahydrocannabinol
VSMC:	vessel smooth muscle cells
WHO:	World Health Organisations
β-ARs:	beta-adrenergic receptors
µm:	micrometer
µM:	micromolar

Dedication

This dissertation is dedicated to:

Grandma Joey and **Grandpa Kalla Smith** and also to **Grandma Anna Naude**, thank you for believing in me and for all your encouragement, love and unconditional support throughout my studies.

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Chapter 1: Literature review

Introduction

Cardiovascular diseases are one of the main non-communicable diseases globally contributing to deaths each year (WHO, 2011). Much research is and has been done to understand the underlying factors that lead to this disease. Most cardiovascular diseases can be prevented or reduced by maintaining a healthy well balanced lifestyle. However there are still millions of people that suffer from some form of cardiovascular diseases (Raven et al, 2008). Most of these conditions are caused by arteriole problems for example; a blockage in an artery or an artery wall that has been ruptured. Heart attacks, Angina pectoris, Strokes and Atherosclerosis are all common forms of cardiovascular diseases (Raven et al. 2008).

Ischemic heart disease leading to myocardial infarction (or heart attack as it is commonly known) is one of the leading causes of death in the United States, and account for one-fifth of all deaths in the United States (Raven et al. 2008), a phenomenon which is also seen in white South Africans (accounts for 11.1% deaths in this population group) in 2013 (Stats SA, 2013). Myocardial infarction, results from an insufficient supply of blood to a certain small area or even a bigger area of the heart muscle. This causes the myocardial cells in these areas to die of oxygen and nutrient deprivation, waste and metabolite product accumulation and ion imbalances.

The myocardial cells are better known as contractile and non-contractile cardiomyocytes in the research field. Some of the cells are responsible for generating contractile force in the intact heart and others form the cardiac conduction system, which is responsible for the control of the rhythmic beating of the heart (Woodcock et al, 2005). In this thesis cardiomyocytes will refer to contractile cardiomyocytes isolated from the ventricles.

Physiology is the study of the functioning of living things (Sherwood, 2010). Maintaining homeostasis is essential for the survival and normal functioning of cells. Each cell has its own special activities that contribute as an integral part of a system to maintain homeostasis, and this is the foundation of physiology (Sherwood, 2010).

A brief but insightful physiological background will be given regarding the reason for the research project. This project was developed to gain answers to some of the many questions around the common cardiovascular event: Myocardial infarction (MI).

The aim is to find an alternative way to intervene during MI, through g-protein coupled receptor (GPCR) pathways, since the prevention or reduction of risk is already well researched. With the understanding of an ischemic event within the heart cells, this thesis also evaluates the role of phosphodiesterase 4 (PDE 4) in mediating damage or protection during an ischemic event.

Ischemic damage can be enhanced through beta-adrenergic receptor 1 (Spear et al, 2007), while protection can be elicited through beta-adrenergic receptor 2 (Chesley et al, 2000) as well as through cannabinoids (Shmist et al 2006,Durst et al, 2007) and insulin(Abdallah et al, 2006; Jonassen et al, 2001; Zhang et al, 2005). The usage of freshly isolated cardiomyocytes that were cultured for 1 day was incorporated during this investigation. A background of each of these components will be discussed and presented in the following pages.

Function of the heart

Normal condition

Normal function of the heart will briefly be discussed followed by how pathology occurs during ischemia and reperfusion therapy. The heart is one of the main components that compromise the circulatory system. Another two components are the blood vessels and blood. This system is responsible for the continuous movement of all body fluids (Young et al, 2010), example blood plasma, interstitial fluid and lymph. Its primary functions are the transportation of oxygen (O_2) and nutrients to the different tissues, and also the removal of carbon dioxide (CO_2) and metabolic waste products from the different tissues (Sherwood, 2010). It also plays a role in temperature regulation and the distribution of signalling molecules or cells. Thus in short the circulatory system contributes to maintaining homeostasis through the transportation of O_2, CO_2, waste, electrolytes and hormones from one area of the body to another (Sherwood, 2010).

Cardiomyocytes are the cells responsible for generating contractile force in the intact heart in response to electrical currents from the SA node. Specialized non-contractile cardiomyocytes that form the cardiac conduction system are responsible for control of rhythmic beating of the heart (Raven et al. 2008).

More than 40% of the cardiomyocyte cell volume contains mitochondria, the energy generating organelles. Mitochondria are very dependent on O_2 for energy production and this means the contractile cardiomyocytes depend on aerobic metabolism. The heart's primary fuel substrates are fatty acids and to a much lesser degree, glucose and lactate, but this is only during normal aerobic conditions. The heart is very adaptable and can thus shift into many metabolic pathways to gain nutrients. However pathologies arise when blood flow is restricted, this is not only due to insufficient nutrients, but also largely due to insufficient O_2 supply (Opie, 2004). This leads to cellular acidosis, ion imbalances, metabolite and waste product accumulation and cellular damage.

Ischemia and reperfusion pathologies

Ischemia, which is the deprivation of blood supply to an organ, has long been recognized as a critical factor in the clinical outcome of haemorrhagic shock, stroke, organ transplantation and myocardial infarction (Eltzschig et al, 2004).

As soon as the heart cells are challenged with a state of deprived O_2 the energy metabolism is switched to anaerobic metabolism (Raven et al. 2008), but heart cells cannot generate enough adenosine triphosphate (ATP) through anaerobic metabolism. This leads to a decrease in the energy levels within the heart cells, which in turn cause a build-up of ions such as calcium, hydrogen and sodium within the heart cells. The energy depletion and ion imbalances then lead to tissue damage.

The cellular effects caused by ischemia or hypoxia include; cellular acidosis, altered membrane potential, an alteration in the ion distribution, cellular swelling, cytoskeletal disorganization, decreased ATP and contracture (Eltzschig et al, 2004). The damage is worsened by an increased release of adrenaline and noradrenaline that cause beta-adrenergic receptor activation. This in turn favours cyclic AMP

production and thus activates the PKA-pathway, which is implicated in cell damage and death during ischemia.

Reperfusion, which refers to the re-establishment of blood flow in the ischemic tissue, enhances ischemic pathologies due to the rapid reintroduction of oxygen, ATP production and return to alkalinity of the intracellular fluid. The latter contribute to opening of the mitochondrial permeability transition pore, which dissipates the mitochondrial membrane potential and acts as a stimulus of apoptosis (Haunstetter et al, 1998). Hypercontracture is triggered by the excessive contractile activity of Ca^{2+} oscillations due to reperfusion. Hypercontracture also disrupts the cellular architecture leading to sarcolemmal rupture and cardiomyocyte death (Garcia-Dorado et al, 2014; Buja, 2005; Rodriguez-Sinovas et al, 2007).

Models of ischemia/reperfusion in cardiovascular research

In the cardiovascular research setting, ischemia/reperfusion is induced or simulated with a variety of methods to investigate the cause and effect of this disease state. Researchers try to find new methods of actions for intervention and prevention of this malfunction, such research is mainly conducted on animal hearts like rats and mice. These studies usually focus on whole heart/organ research or cellular research (Diaz et al, 2006).

Whole heart

In the event of conducting research on whole hearts, one of three protocols is used to induce ischemia: global, regional or low flow ischemia. Global ischemia is applied through deprivation of O_2 and nutrients throughout the entire heart by stopping delivery of oxygenated nutrient buffer to the heart (Ostádal et al, 1991).Regional ischemia is induced by blocking off the coronary artery with a silk suture, which then causes the deprivation of O_2 and nutrients in that area and then upon reperfusion causes myocardial infarction (Ostádal et al, 1991).

Low flow ischemia is induced when the fluid is pumped through the heart at a very slow but constant rate to thus simulate a partial blockage. Whole heart perfusions are usually elicited with the Langendorff's with/or working-heart perfusion techniques. Parameters that can be measured include total work heart mechanical

functions of functional recovery and tissue sections that can be probed for various signalling proteins (Webster et al, 2014).

Cardiomyocyte

Cellular research is very different to that done on whole hearts. Ischemia is simulated or induced through buffers or methods specified for specific cells; this would include: pelleting of cells and adding an oil layer on top, applying an ischemic buffer and/or a hypoxic gas.

Simulated ischemia

The buffers used to induce or simulate ischemia would contain normoxic buffer, where glucose and HEPES are reduced or omitted, pH reduced to 6.0-6.4,and supplemented with lactate, 2-deoxyglucose (2-DG) and Sodium hydrosulfite (Sodium dithionite) . 2-DG is a glucose analogue that cannot be metabolized; a very well-known inhibitor of glycolysis and a competitive inhibitor of glucose transport (Vijayaraghavan et al, 2006). It interferes with the cellular mechanisms by competing with glucose for the key enzymes associated with glycolysis (Lazlo et al, 1961). Sodium dithionite (Na_2S_2O4) is an O_2 scavenger (Egar et al 2014) and a powerful reducing agent. It is used to create anaerobic conditions in vitro (Archer et al, 1995). It removes all traces of O_2 in the buffer solutions (Bright et al, 1992).

Hypoxia conditions through pelleting

This is done by centrifuging freshly isolated cardiomyocytes into a pellet with only a thin layer of supernatant that is then covered by oil, which has proven to be an excellent model of simulated ischemia (Diaz et al, 2006).The reason behind this conclusion is that pelleting causes an increased metabolite build-up in an enclosed space. Metabolic inhibition occurs naturally through the build-up of lactate and H+ and exhaustion of available metabolic substrates, this then avoids using exogenous metabolic inhibitors (Diaz et al, 2006).

Hypoxia with gas

Severe hypoxia can be induced by using modular incubator chambers with nitrogen concentrations between 95% and 100%.This has been used to elicit hypoxia on the cultured cardiomyocytes in this study. The modular hypoxic incubator chamber has an air tight seal and the cardiomyocyte cultured plates are placed inside this chamber. The O_2 is then replaced by nitrogen gas to subject the cardiomyocytes to a hypoxic event. Additional modifications to the experimental buffer are sometimes

also made, for example the addition of lactate and 2-DG, as well as a reduction in pH to 6.0-6.4.
After simulating ischemia, reperfusion is replicated by adding well oxygenated media of normal ionic composition, normal physiological pH and proper temperature to the cells. Reperfusion contributes to myocardial cell damage through lethal necrosis of cardiomyocytes. This damaged caused by reperfusion is termed “reperfusion injury”. Reperfusion injury starts within the first few minutes of reperfusion in cardiomyocytes and then causes hyper contracture development in the cardiomyocytes (Abdallah et al, 2006).

Parameters that can be measured include cell length as an indication of contracture or hypercontracture, viability through cell morphology or cell stains for intracellular signalling or cell viability assessment for example mitochondrial viability.
Through understanding these pathways, one can develop new methods of intervention and treatment to reduce cell death during reperfusion.

Different cell death mechanisms during Ischemia

In acute myocardial ischemia of a proper and sufficient duration that causes substantial injury in all the models of ischemia research, the substantial majority of cell death (which is 90%) happens through oncosis (Diaz et al, 2006).Oncosis is derived from the Greek word “onkos”, which means swelling (Diaz et al, 2006). During oncosis there is cytoplasmic swelling with intracellular organelles swelling, causing increased cell membrane permeability, blebbing and ultimate cell membrane rupture. Necrosis is an example of oncosis, where cell membranes are ruptured due to unorganized cell death independant of ATP and allow hydrophilic dyes such as trypan blue to enter the necrotic cell cytosol. Consequently necrotic cell stain blue while uncompromised cells don’t take up the dye.

Some pathological conditions can cause cardiomyocytes to undergo apoptotic and necrotic responses. Signalling pathways involved in apoptotic responses in cardiomyocytes have been investigated in cell models in both the extrinsic and intrinsic pathways. These signalling responses are most readily observed, following

brief periods of ischemia in vivo or simulated ischemia in isolated cells (Woodcock, E.A. et al, 2005). Apoptosis is a well-organized cell death dependent on ATP, and thought to be enhanced by the availability of ATP during reperfusion (Dispersyn et al, 2001). In apoptosis, the cell cytosol shrinks; there is a marked chromatin condensation and the cells split into small apoptotic bodies that can be removed by neighbouring cells. Apoptotic cells can be detected by fluorescence intensity through JC-1 staining. This is used to test the mitochondrial membrane potential. If the JC-1 stain fluorescence red the cells are still viable, but as soon as the cells fluorescence green, the cells are in the apoptotic fate.

The use of cardiomyocytes in research

Experimentation with freshly isolated cardiac myocytes can allow us to gain knowledge of the cardiovascular system on a molecular basis. The isolation of a high quality yield of cardiac myocytes is the most important factor for a successful experiment. Isolated cardiomyocytes have been used for more than two decades in the research setting, to study the effect of ischemia. The enzymatic separation of cardiomyocytes can be done with a variety of different protocols. The key elements to isolating cardiomyocytes are using a Langendorff perfusion system with calcium-free media. This is a very delicate procedure and any changes in the collagenase, protease or trypsin activity or the time of perfusion can lead to a decrease or increase in the percentage viability yield of cardiomyocytes. Good cardiomyocyte isolation will yield 70% to 80% or even more rod-shaped viable cells with clear visible striations when viewed under the light microscope (Diaz et al, 2006).

The isolated cardiomyocytes can be cultured overnight or for a few days, which allows the possibility of genetic manipulation to precisely target the molecular machinery during ischemic events, and opens up exciting possibilities to gain further knowledge in this field. Uses for cardiomyocytes include studying cell volume regulation in ischemia; manipulating gene expression in cardiomyocytes to study ischemia and probing the role of mitochondria in ischemia in intact cardiomyocytes.

Advantages and disadvantages of isolated cardiomyocyte research

The most obvious advantage of cardiomyocyte research compared to whole heart research is the elimination of other cell types like endothelial cells and fibroblasts (Diaz et al, 2006). This means that the researchers can for instance only work with the mitochondria of the cardiomyocytes, since it is a pure collection of cardiomyocytes. Working with cardiomyocytes also gives the researcher the opportunity to study the cells visually much better through microscopy.

The disadvantage of using cardiomyocytes is losing the extracellular matrix and other neighbouring cells which results in not having the same response as in the entire heart for instance. The cardiomyocytes are also more prone to infections during experiments than in the whole heart experiments because they are kept for longer, so greater caution should be used during experimentation of cardiomyocytes.

B-Adrenergic receptors

Beta-adrenoceptors (β-AR), β_{1}-, β_{2}- and β_{3}-ARs are found in the heart, although β_1 is documented as the most prominent β-AR in the human heart (Brodde, et al 2006). The ratio of β1 to β2 adrenoceptors are about 4 to 1 in the ventricles (Brodde et al, 2006). Norepinephrine is a neurotransmitter that is released from the sympathetic adrenergic nerves, and binds to these receptors at the cardiomyocyte membrane (Thackeray et al 2012).The β_1 and β_2 –adrenergic receptors both work through the coupling of G-stimulatory (Gs) protein that activates adenylyl cyclase (AC), but β_2 along with β_3 can also couple to G-inhibitory (Gi) proteins.

As can be visualized by referring to figure 1, beta-adrenoceptors are coupled to Gs, which activate AC to increase cyclic AMP (cAMP).This then activate cAMP-dependent protein kinase A (PKA) that phosphorylates the L-type calcium channels, which increase calcium entry into the cardiomyocytes during action potentials. An increase in calcium entry leads to enhanced release of calcium by the sarcoplasmic reticulum. PKA phosphorylates sites on ryanodine receptor at the sarcoplasmic reticulum, which further enhances the release of calcium through the ryanodine receptors. This provides more calcium for binding the troponin-C, which enhances

contraction (inotropy). PKA can also phosphorylate myosin light chains; which contribute to the positive inotropic effect of beta-adrenoceptor stimulation. PKA-mediated phosphorylation of phospholamban lifts the inhibitory effect of phospholamban on sarcoplasmic- and endoplasmic reticulum calcium ATPase (SERCA). This allows SERCA to pump calcium faster back into the sarcoplasmic reticulum to enhance cardiomyocyte relaxation during diastole (lusitropy).The cardiac effects of β1- and β2-ARs result in positive inotropy, chronotropy (increased heart rate) and dromotropy (increased rate of conduction velocity), while only β1-ARs induces lusitropy. The coupling ofβ2- and β3-AR isoforms to Gi provides a balance in β-AR signalling by reducing the cAMP production (Thackerayet al. 2012), as illustrated below in Figure 1.1.

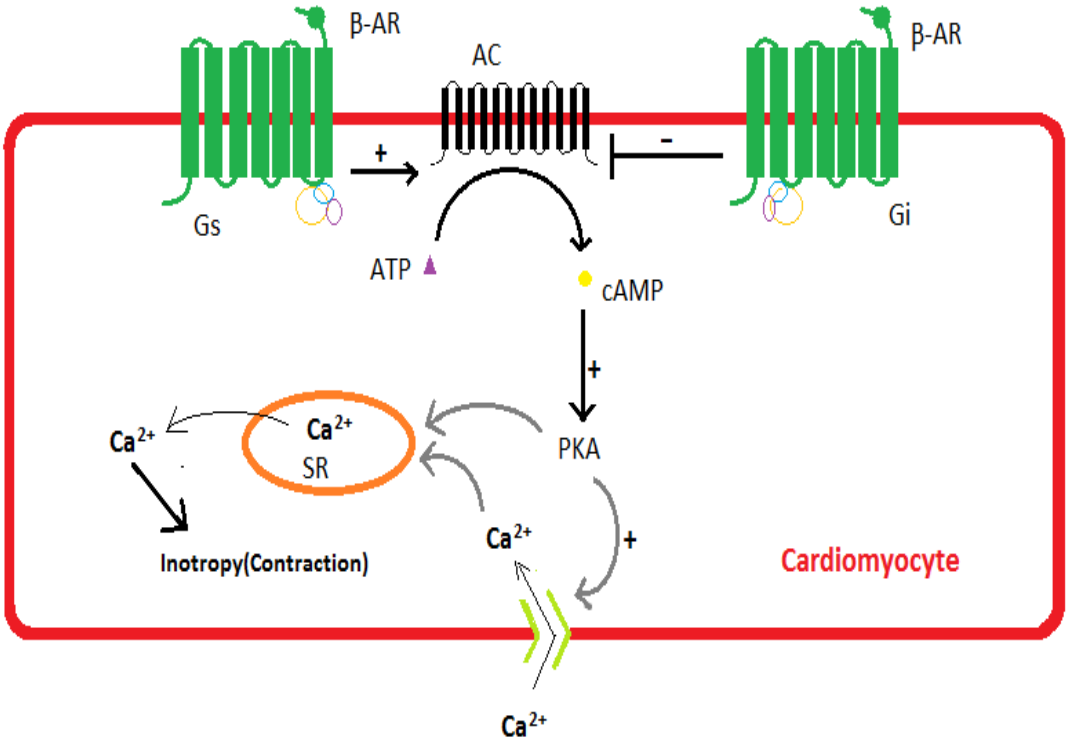

Figure 1.1: Beta-Signalling and cAMP, the signalling events after beta-adrenergic receptor activation

Abbreviations

B-AR:	beta-adrenergic receptor	AC:	Adenylyl Cyclase	Gs:	G-stimulatory protein
Gi:	G-inhibitory protein	PKA:	cAMP-dependant protein kinase A	SR:	Sarcoplasmic Reticulum
Ca^{2+}:	Calcium				

During an ischemic event overstimulation of B-AR occur, in response to a large and progressive release of the catecholamines from the adrenergic nerve terminals (Yu, Q. et al, 2008). This causes an enormous threshold activation of the β-adrenoceptors and a large release of cAMP, which can then further accelerate the ischemia induced

cell damage within the cardiomyocytes (Yu, Q. et al, 2008). This is partly because the induction of inotropy during ischemia promotes ATP consumption and intracellular calcium accumulation that promotes contracture and cell death.

In the research setting, β-adrenoceptor agonists and antagonists are used to study and identify the possible mechanism involved in the cAMP/PKA pathways. Dobutamine is used as a β1-AR agonist (Chou et al, 2012), formoterol is used as a β2-AR agonist (Jesinkey, 2014) and BRL37344 is a β3-agonist (García-Prieto et al 2014).Cardiac βAR stimulation in the rat cardiomyocytes, can affect apoptosis with β1-AR via the Gs-protein and induce pro-apoptotic effects, while the β2-AR via the Gi-protein, induces anti-apoptotic effects in the hypertrophic heart (Brodde et al, 2006) and in the ischemic heart (Spear et al, 2007;Chesley et al, 2000).

cAMP and cGMP signalling pathways

Cyclic AMP and cyclic GMP are second messengers involved in regulating a vast number of processes, such as cardiac function. cAMP influences growth, movement and differentiation in cells. cAMP also influences the vasculature: relaxation and contraction, movement, shape, proliferation and response (especially to vascular trauma and hypoxia) of the blood vessel smooth muscle cells (VSMC's). In cardiomyocytes this second messenger also influences the heart rate (chronotropic) and contractility (inotropic) actions as well as apoptosis and hypertrophy. Thus cAMP plays a regulating role in the strength and frequency of cardiac relaxation and contraction. The stimulation of adenylyl cyclases via GPCRs by catecholamines generates cAMP which mainly target PKA. Other targets for cAMP include: the exchange factor for Rap, Epac and cyclic nucleotide-gated ion channels (Bos, 2006).

The stimulation of guanylyl cyclases (GC) in response to natriuretic peptides and nitric oxides (NO) generates cGMP. In turn cGMP activates its downstream effectors, which include protein kinase G (PKG) and cyclic nucleotide-gated channels. cGMP also regulate inotropy and metabolic responses in cells.

By their generation and activation of downstream effectors, one can notice that cGMP and cAMP pathways can often have opposing influences on the cardiac function. There is cross-talk between these two signalling pathways through the

activity of cyclic nucleotide-degrading enzymes, which are better known as phoshodiesterases (PDEs). PDEs have been shown to play a role in directing the fate of cardiomyocytes in the context of anoxia, where ATP loss and contracture was enhanced by general inhibition of all PDEs with IBMX (Geisbuhler et al 2001).

Phosphodiesterases

PDE's are a class of enzymes that hydrolyse the cellular cyclic nucleotides cAMP and cGMP to their inactive form (Figure 1.2) and therefore play an essential role in the cyclic nucleotide cellular response pathways. As PDEs is the only enzymes controlling the degradation of cAMP, they are considered a very important component of the normal physiological function of most systems within the body, including in the heart. They are the key role players to ensure selective activation of different pathways in order to mediate selective GPCR functions in the heart. PDEs coordinate such physiological complexities through the compartmentalization of cyclic nucleotides within heart cells (Figure 1.3).

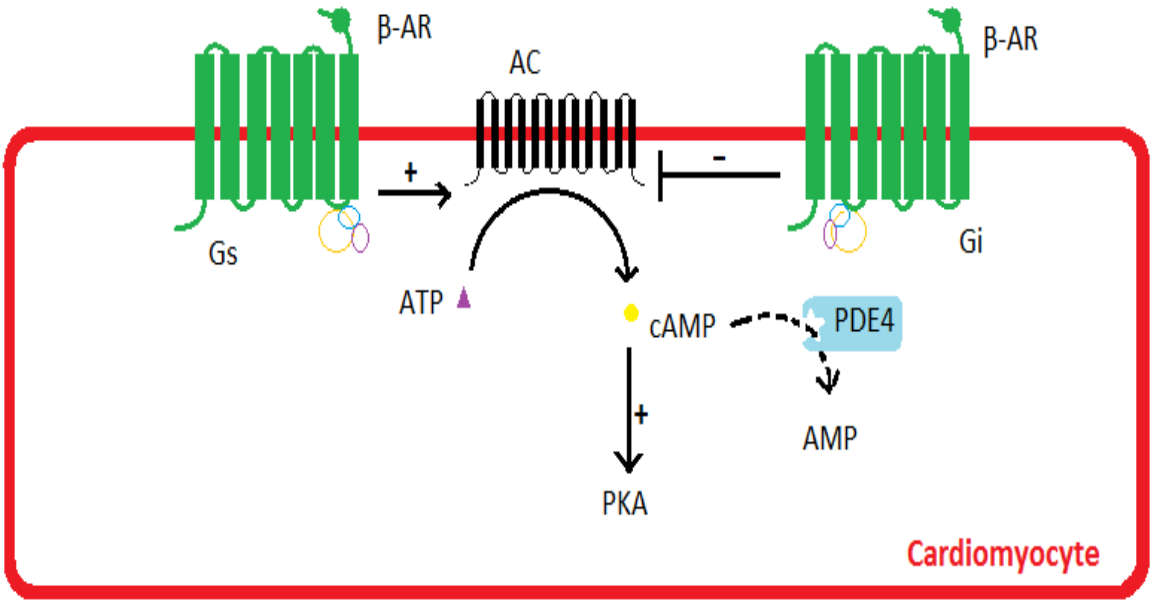

Figure 1.2cAMP breakdown: Showing cAMP breakdown with PDE4

Abbreviations

B-AR: beta-adrenergic receptor	AC: Adenylyl Cyclase	Gs: G-stimulatory protein
Gi: G-inhibitory protein	PKA: cAMP-dependant protein kinase A	PDE4: Phosphodiesterase 4

There are eleven families of PDE's identified (PDE1-PDE11), each with their own distinctive characteristics. They are found in different tissues, of which 7 families are found in the heart, while five PDE families have been studied in the heart. cAMP is hydrolyzed in the heart by four PDE's: PDE1, PDE2, PDE3 and PDE4. PDE1 is activated by Ca^{2+} / calmodulin (CaM) and can also hydrolyze cGMP. PDE2 is stimulated by cGMP and can also hydrolyze cGMP.PDE3 is inhibited by cGMP while PDE4 is specific for cAMP and insensitive to cGMP (Fischmeister et al. 2006). In mouse cardiomyocytes, it has been shown that PDE8 modulates cAMP signalling (Patrucco et al, 2010). PDE3 and PDE4 are the most abundant and major contributors of cAMP-hydrolysis in rodent cardiomyocytes (Richter et al, 2011).

PDE 4 in cardiac function

One of the main PDE's expressed in the heart is PDE4 (Fu et al, 2013), where it regulates global cAMP levels in cardiac cells. PDE4 is predominantly activated through β-AR stimulation and thereby shapes the compartments of cAMP produced byβ-AR and its physiological effects. The PDE4 family consist of 4 genes PDE4A, PDE4B, PDE4C and PDE4D that give rise to multiple isoforms. Only the PDE4A, PDE4B and PDE4D genes are expressed in cardiac tissue, and are localized to specific regions within the cardiomyocytes (Figure 1.3). These regions include the sarcoplasmic reticulum and Z-discs, where they are likely to influence cAMP and cGMP signalling to the end effectors of contractility (Roa, 2009).Different PDE4 isoforms control the PKA-mediated regulation of β1- and β2-ARs, and are said to be anchored to the L-type calcium channels (LTCC), cardiac ryanodine receptors (RyR2), the phospholamban / sarcoplasmic reticulum calcium ATPase (PLB/SERCA) complex and troponin I (Richter et al, 2011).

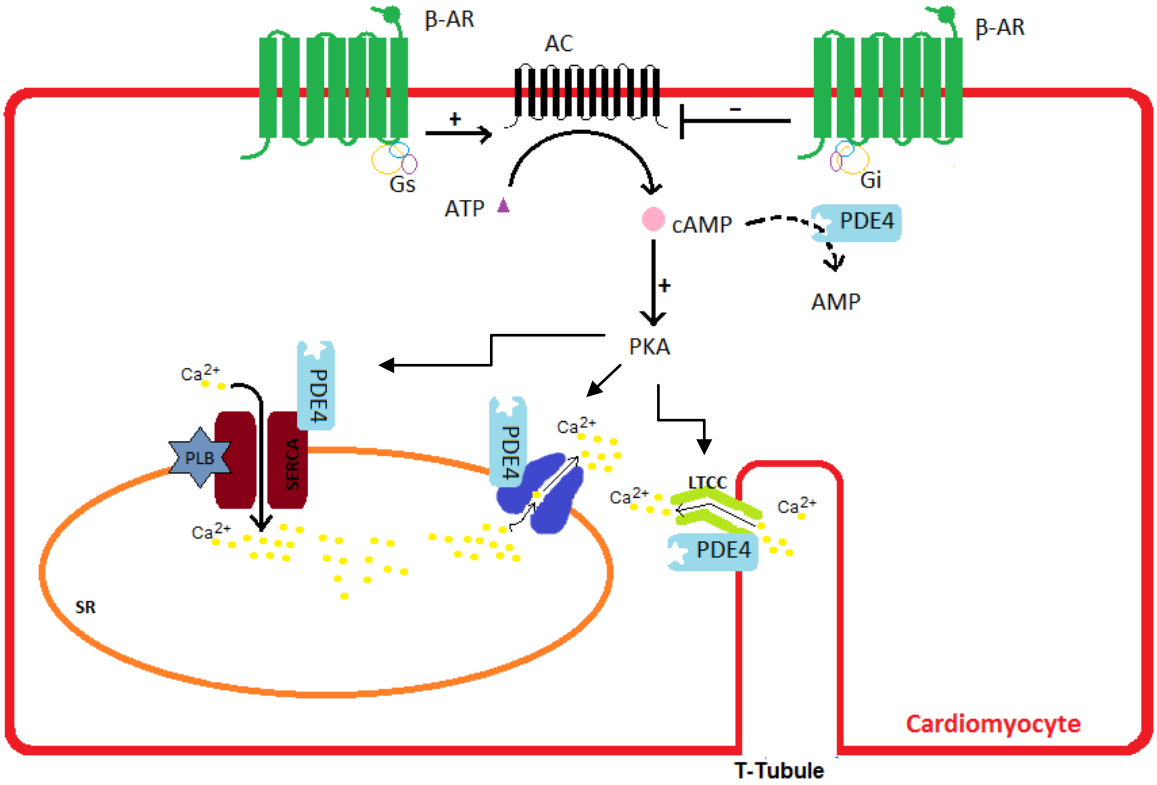

Figure 1.3: Localization of PDE4 in the cardiomyocyte.

Abbreviations

B-AR: beta-adrenergic receptor	AC: Adenylyl Cyclase	Gs: G-stimulatory protein
Gi: G-inhibitory protein	PKA: cAMP-dependant protein kinase A	PDE4: Phosphodiesterase 4
SR: Sarcoplasmic reticulum	Ca^{2+}: Calcium	PLB: Phospholamban
LTCC: L-type calcium channel	SERCA: sarcoplasmic- and endoplasmic reticulum calcium ATPase	

As early as the 1970's an interest arose regarding the cardiovascular effects of PDE inhibitor drugs for especially chronic heart failure (Feneck, 2007) and there is still significant interest in the potential of PDE inhibitors in the treatment of cardiomyopathy. Yuan James Rao and Lei XI stated that it seems PDE-inhibitors hold a great promise as clinically applicable agents. By this they say that it could improve cardiac performance and cell survival under critical situations. Ischemic heart attacks, heart failure and cardiopulmonary bypass surgery are some examples of the critical situations.

Although much research has been done on the possible therapeutic role of PDEs during cardiac pathology, most of the focus has been on hypertrophy and heart failure, while the role of PDEs in ischemia has been neglected.PDE4 is considered one of the most important key players in the control of cAMP compartmentalization from b-ARs, yet the role of PDE4 in b1-AR mediated damage and b2-AR induced protection has never been evaluated. The inhibition of PDE4 by roflumilast have

been proposed (Kwak et al 2007) to have potential for protecting cardiomyocytes during ischemia/reperfusion. However, these authors made this deduction based on a model of nitric oxide-induced apoptosis, and a closer model to ischemia/reperfusion (I/R) is necessary for such a deduction. There are no publications that evaluated the role of PDE4 during I/R, which is what was aimed by this thesis.

Insulin signalling in cardiomyocytes

Experimental and clinical studies have shown that the administration of insulin during reperfusion is cardio protective (Abdallah et al, 2006; Heng et al, 1977). Several studies and experiments have been done to determine whether insulin has a protective effect against myocardial reperfusion injury. In the late 1960s insulin was found to have protective properties when it was found to have a reduced-in-hospital mortality risk rate, when patients were treated with a glucose-insulin-potassium scheme (GIK), after experiencing an acute myocardial infarction (Sodi-Pallares et al, 1962). Dysfunction and defects of SERCA has been reported in ischemia/reperfusion myocardium (Yu et al, 2008), which led to decreased myocardial contractility and reduced removal of intracellular calcium. Yu et al (2008) found that insulin promoted SERCA activity and thereby increasing the re-uptake of Ca^{2+} through the SR. In a study by Rao et al (1997), they found with insulin treatment a reduction in extracellular lactate release after ischemia and after reperfusion. Insulin treatment improved the transition from anaerobic to aerobic metabolism, and resulted in improved cellular survival after ischemia/reperfusion (Rao et al, 1998).

Insulin-mediated glucose uptake is another important mechanism through which insulin induces cardio protection to the whole heart and cardiomyocytes (Heng et al, 1977). Insulin–mediated glucose uptake under normoxic conditions has been shown to be dependent on the activity of certain PDE3 and 4. In primary rat adipocytes the inhibition of PDE3B respectively with milrinone and OPC3911 reduced glucose uptake induced by insulin (Zmuda-Trzebiatowska et al 2005). PDE4 inhibition in whole heart perfusions was found to reduce insulin-mediated glucose uptake (Imahashi et al 2001). What make this especially interesting is that the insulin

receptor is a tyrosine kinase receptor and not a GPCR, yet it mediates its effects through PDEs. This might possibly be through the regulatory effects that kinases such as PKB, ERK and PKA have on PDEs (Omori, 2007). More noteworthy is the role that PDE4 plays in both b-AR signalling and insulin signalling, and therefore it is necessary to evaluate the role of PDE4 in insulin-mediated cardio protection.

Cannabinoids

Cannabis sativa L. has a long history as a medical plant and was fundamental in the discovery of the endocannabinoid system (ECS). Within our bodies, the ECS is considered a complex lipid signalling network, which comprises the classical cannabinoid receptors: type-1 cannabinoid (CB-1) receptor and type-2 cannabinoid (CB-2) receptor. The CB1 and CB2 receptors and endocannabinoid degrading enzymes are present in cardiovascular tissues (Patcher, 2008). In the ECS, different proteins play distinct roles in the control of numerous physiological and pathophysiological processes.

Endocannabinoids are cannabinoids that are naturally synthesized within the body. The natural two most studied endocannabinoids are anandamide also known as arachidonoyl ethanolamide (AEA) and 2-arachidonylglycerol (2-AG). Phytocannabinoids are cannabinoids which are natural plant-derived products and are capable of either directly interacting with the CB1/CB2 receptors or share chemical similarities with the endocannabinoids.CB1 and CB2 receptors are GPCRs that largely couple to Gi to produce cGMP and nitric oxide. The discovery of the cannabinoids as a whole came mainly from the marijuana plants which are also better known as *cannabis L sativa* plants. The psychoactive component, THC was one of the first studied cannabinoids.

Cardio protection by Endocannabinoids and Phytocannabinoids

Studies with endocannabinoids and phytocannabinoids proved that the cannabinoid system has potential therapeutic advantages for cardiovascular therapy. One example is found with Anandamide that was shown to have protective effects during ischemia, where it reduced the infarct sizes (Underdown et al, 2005).The psychoactive component delta-9 tetrahydrocannabinol (THC) and its non-

psychoactive isomer cannabidiol (CBD) have been found to protect the heart against ischemia/reperfusion injury (Shmist et al, 2006;Durst et al, 2007).

Commercial synthetic cannabinoids have also been created to study the possible beneficial effect of the cannabinoids. One of these synthetic components is HU210, with which this project took a small scope view.HU 210 behaves very similar to THC in its pharmacological effects (Ottani et al, 2001).In an ischemia/reperfusion rodent model, HU-210 decreased the incidence of ventricular arrhythmias in rats through the activation of CB2 receptors (Krylatov et al, 2001). HU210 also reduced LDH leakage from isolated rat hearts during reperfusion, indicating reduced tissue necrosis, which was interestingly associated with reduced tissue cAMP during reperfusion (Maslov et al 2013).

Given the fact that CB receptors are GPCRs and that they have been found to mediate their effects through cyclic nucleotides, it is imperative that the role of PDEs be investigated in cannabinoid-mediated cardio protection. In summary both endocannabinoids and phytocannabinoids have potential therapeutic applications in the cardiovascular setting especially in treatment of ischemia/reperfusion injury.

Hypothesis and strategy

We hypothesize that PDE4 plays a role in the development of ischemia/reperfusion damage and might therefore be considered for the clinical treatment of ischemia/reperfusion injury. This hypothesis is based on a number of observations; first and foremost, PDE activity has been shown to be necessary for protection of cardiomyocytes during anoxia (Geisbuhler et al 2001). These authors found that a general inhibition of PDEs with IBMX sensitized cardiomyocytes to ischemic damage, which was associated with enhanced ATP loss and an increased incidence of round injured cells (hypercontracture). PDEs function in a coordinated manner under the instruction of various stimuli, mostly known to be from GPCRs, in order to promote stimulus-specific cellular physiological effects. Thus any one or more of the known PDEs expressed in cardiomyocytes might be responsible for the sensitization of cardiomyocytes to ischemic damage, but PDE4 was considered in this thesis for

the following reasons. PDE4 exerts a dominant role in global cellular cAMP hydrolysis in rat cardiomyocytes (Johnson et al 2012). PDE4 is responsible for mediating b1- and b2-AR specific effects in normoxic cardiomyocytes (Mika et al 2014, Gregg et al 2010), through cAMP compartmentation (Nikolaev et al 2006), which might also be true for ischemia/reperfusion, but has never been evaluated.PDE4 activity has been found to be necessary for insulin-mediated glucose uptake in isolated perfused rat hearts, which might also be true during ischemia and thereby play a role in cardio protection by insulin. Finally, cannabinoids such as THC and HU210 induce cardio protection during ischemia through CB receptors. These are GPCRs that regulate cyclic nucleotides, possibly through PDEs, of which PDE4 was considered here.

AIM:

- To find an alternative way to intervene during acute myocardial ischemia/infarction, through the g-protein coupled receptor (GPCR) pathways by evaluating the role of phosphodiesterase 4 (PDE 4) in mediating damage or protection during an ischemic event.

OBJECTIVES:

1. To test the hypercontracture effects of the cardiomyocytes with the inhibition of PDE4 by rolipram during ischemia with the combined administration of b-AR agonists (dobutamine, folmoterol, isoproterenol), insulin and cannabinoids (THC and HU210).
2. To test the fluorescence intensity of the cardiomyocytes, by looking at the mitochondrial membrane potential for apoptosis, with the inhibition of PDE4 by rolipram during ischemia with the combined administration of b-AR agonists (dobutamine, folmoterol, isoproterenol), insulin and cannabinoids (THC and HU210).
3. To test the cell viability of the cardiomyocytes with the inhibition of PDE4 by rolipram during ischemia with the combined administration of b-AR agonists (dobutamine, folmoterol, isoproterenol), insulin and cannabinoids (THC and HU210).

Chapter 2: Materials and Methods

Rats

For this study, male Wistar rats with a weight ranging from 250-300g were used. Animals were housed in the Animal Facility at the University of Stellenbosch, Tygerberg Campus. The animals were kept on a 12 hour day/night cycle at a constant temperature of 22˚C and 40% humidity. All animals had free access to food (standard rat chow) and fresh clean water.

Ethical approval

The use of animals for this study was approved by the Ethical Committee of the Faculty of Health Sciences, Stellenbosch University. Project number SU-ACUM13-00018 was given to the study. This study conformed to the conditions described in the" Revised South African National Standard for the care and use of Animals for Scientific purposes" (South African Bureau of Standards, SANS 10386, 2008).

Chemicals

Rolipram, IBMX, isoproterenol, BRL-37344, HEPES, sodium pyruvate, sodium chloride (NaCl), 2, 3-butanedione monoxime (BDM),creatine, taurine, carnitine, M199 with Hank's salts, blebbistatin, protease IV, 2−deoxy-glucose and JC-1 were obtained from Sigma Aldrich. Bovine serum albumin (BSA) fraction V, BSA fatty acid free (FAF) were obtained from Roche, collagenase Type II from Worthington, insulin from Eli Lilly, laminin/entactin (L/E) and penicillin/streptomycin (pen/strep) from BD Biosciences. Sodium pentobarbital, D-glucose, calcium chloride, potassium chloride (KCl), disodium hydrogen phosphate (Na2HPO4), sodium dihydrogen phosphate (NaH2PO4), and magnesium sulphate (MgSO4) were obtained from Merck. THC and Hu210 were obtained from Cayman Chemicals. Formoterol was produced by TOCRIS and donated by Dr Salie.

The adult rat ventricular myocyte model

Coating of 96-well plates with laminin/entactin

96-well costar black, clear bottom, tissue culture plates were coated with laminin/entactin (L/E). The plates were coated by adding 5µl of 1ug/ml L/E (diluted in PBS, 1mM CaCl2) in the centre of the well and thereafter the plates were incubated overnight at 5% CO2 and 37˚C. After overnight incubation, the excessive L/E was removed with suction and the plates were washed with 100µl/well of PBS containing 1mM CaCl2, and allowed to dry in a laminar hood flow before plating the cells.

Isolation of Adult Rat Ventricular Cardiomyocytes

The cardiomyocyte isolation technique was based on a protocol published by Fischer et al, 1991 and reviewed by Louch et al, 2011. The rats were anesthetized by intra-peritoneal injection of 0.3ml sodium pentobarbital (euthanaze). After checking the pinch response to ensure the rat is fully unconscious, it was sterilized in 70% ethanol and dissected inside a laminar flow hood. The chest was opened to expose the heart by using sterilized scissors. The hearts were excised with a large portion of the aorta intact and arrested in ice cold (4°C) Buffer A that contained 0.5mM CaCl2. Buffer A contained in mM: KCl 6; Na2HPO4 1; NaH2PO4 0.2; MgSO4 1.4; NaCl 128; HEPES 10; D-glucose 11 and sodium pyruvate 2 (pH 7.4, 37°C, gassed with 95% O2 & 5% CO2).

Thereafter, the aorta was clamped to the catheter (cannula). The hearts were mounted with a silk suture via the aorta onto the cannula of a Langendorff apparatus, and retrogradely perfused with calcium free buffer (buffer A) to wash out the excessive blood from the coronary arteries.

After 5 minutes, the perfusion was switched to a digestion buffer (buffer B) containing 0.5% BSA fraction V, BSA (FAF), 440U/ml collagenase Type II, 0.2mg/ml protease IV and 18.0mM BDM added to buffer A. The first 10ml of the digestion buffer was discarded, while the rest was switched to recirculation and $CaCl_2$ was

added to raise the Ca^{2+} concentration to 0.1mM. After 10 minutes another Ca^{2+}-raise was done to increase the concentration to 0.2mM.The perfusion continued for another 10 -15 minutes until the heart was soft, swollen and soapy.

To ensure sufficient digestion, the bubble trap's air block was broken to test perfusion flow through digested heart with only gravitation. If the solution ran through easily, the heart was ready to be dissociated in buffer D.

Thus after complete digestion of the heart, the ventricles were cut off and placed in a petri dish with buffer D {2/3 of buffer C [1×buffer A, 0.5% BSA (FFA), 0.5% BSA fraction V, 9.0mM BDM] and 1/3 of buffer B, 0.3mM $CaCl_2$} .

Ventricular cardiomyocytes were separated and dissociated by moving the tissue with a tweezer, back and forth in the buffer. The cell suspension was then filtered through a 200µm nylon filter into a 50ml conical tube. The cells were sedimented for 10 minutes at room temperature and afterwards spun at 30× g for 2 minutes.

The supernatant was removed as it contained most dead cells, other cells and debris. Only the pellet of viable cells was kept. The pellet was resuspended in buffer E1 (buffer C with 0.6mM CaCl2) and sedimented for 10 minutes, followed by E2 (buffer C, 0.9mM CaCl2) and sedimented for 7 minutes and lastly resuspended in E3 (buffer C, 1.2mM CaCl2) and sedimented for 5 minutes. Calcium was thus re-introduced to the cells in this stepwise manner to a final concentration of 1.2mM. After sedimentation of 5 minutes, the supernatant was removed and the final pellet of calcium tolerant cells was resuspended in medium X culture buffer (MXCB) that contained 10µM blebbistatin (BBS) and 1.2mM $CaCl_2$. The cardiomyocytes were assessed for viability after this step. MXCB is a buffer modified from M199 in order to provide better survival and was used to culture the ventricular cardiomyocytes.Bebbistatin is an inhibitor of myosin 2, and is used to reduce contraction during the plating process of the cardiomyocytes.

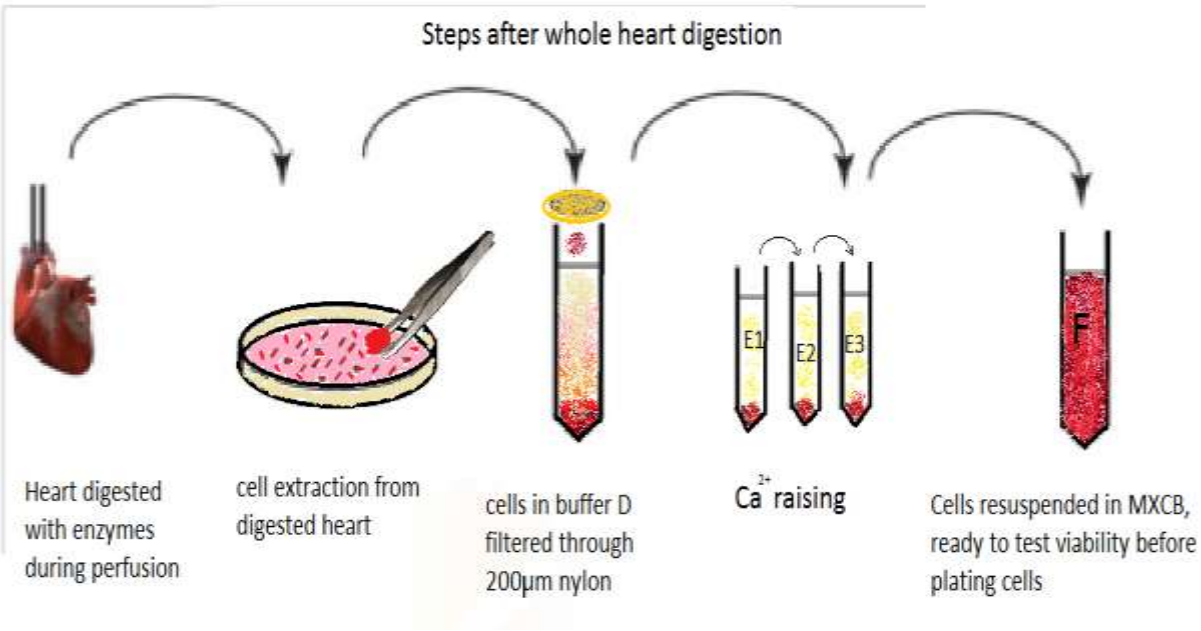

Figure 2.1: Steps of heart digestion

Assessment of viability

Viability of cardiomyocytes was assessed by filling 2 chambers of the improved nubauer haemocytometer with the final cell suspension (MXCB). Thereafter, the numbers of rod- and round-shaped cardiomyocytes were counted separately using a light microscope, and their percentages determined. Rod-shaped cells were considered viable (live), whereas round cells were considered non-viable (dead). The cardiomyocytes were diluted in MXCB, containing 10µM BBS and 1.2mM CaCl2, and seeded at a density of 2500-5000 rod shaped cells per well in 96-well culture plates, followed by overnight culture of the cells at 5%CO2 and 37˚C in the incubator. The experimental procedure with the different treatment groups during ischemia continued the following day and will be discussed in the experiments section.

Experimental procedures

The experimental procedure/timeline can be seen in figure 2.2A to figure 2.2C.

In figure 2.2A it indicates the experimental time line when no drugs are administered. This is the experimental procedure for the normal ischemic treatments without any treatments.
The normoxic treatment group also follows this experimental timeline. The only difference is instead of administering the ischemic wash buffer during the 20 minutes indicated; it is replaced with the normal CMCB wash buffer, without SDT and 2DG, and also without any drug treatments.

In figure 2.2B it indicates the administration of drugs prior to ischemia and throughout the 20 minutes simulated ischemia. There are treatments which were administered during ischemia and reperfusion. So for this, the administration time of drugs would shift from during ischemia and onwards till end of the reperfusion stage.

In figure 2.2C it indicates when the drugs are only administered during ischemia. It is thus the same timeline as in figure 2.2A but the only difference is the drug treatments which were administered during the 20 minutes of simulated ischemia.

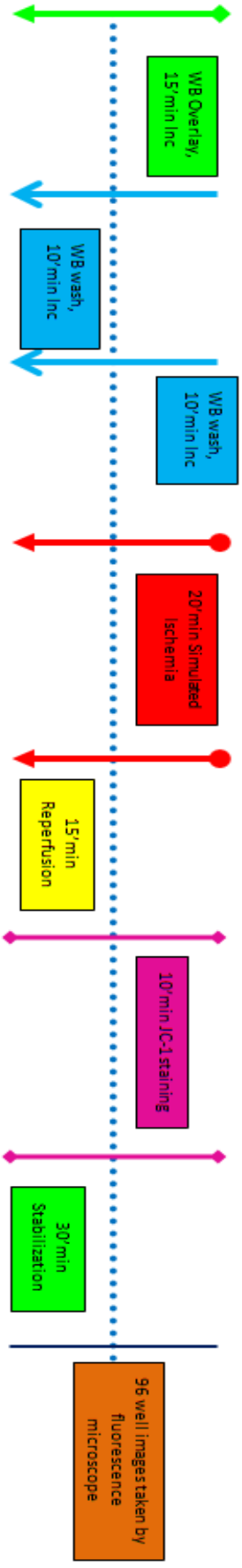

Figure 2.2A: Experiment line with no drug treatments.

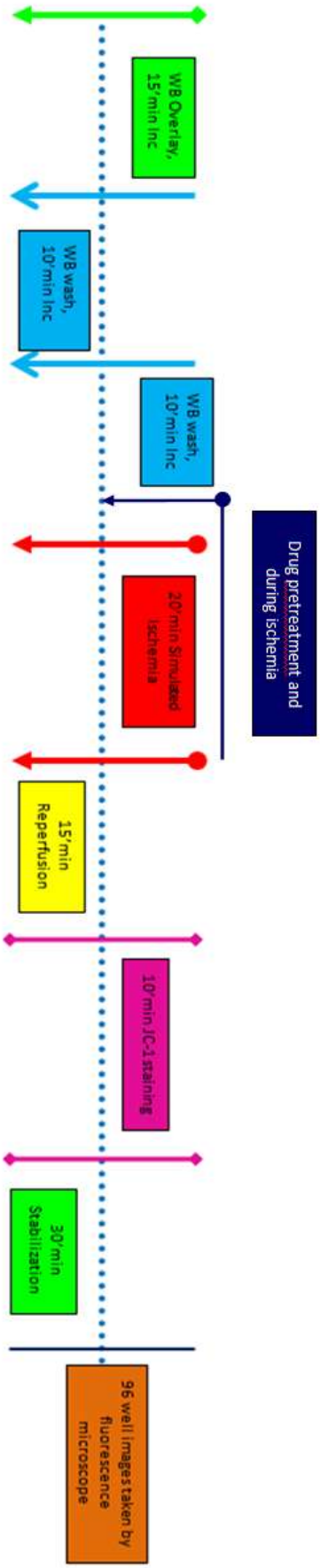

Figure 2.2B: Experimental time line with drug treatments prior to ischemia and during ischemia.

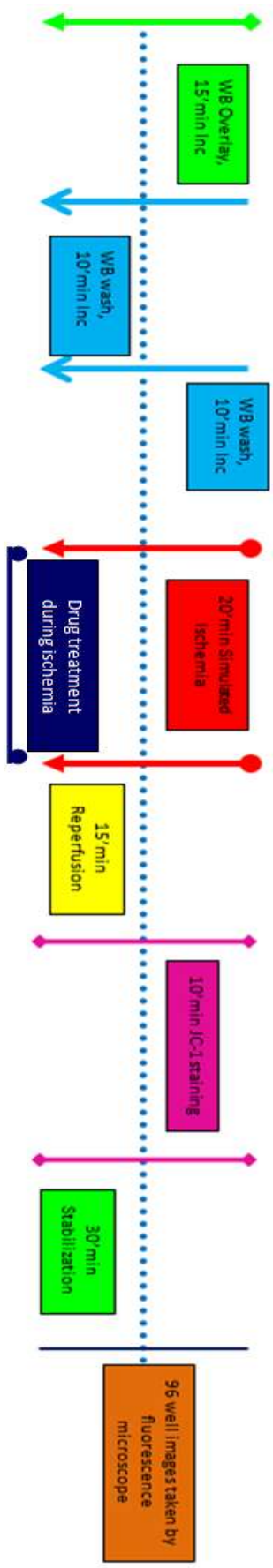

Figure 2.2C: Experimental time line with drug treatments only administered during ischemia.

The overnight cultured cardiomyocytes in 96 well plates were subjected to simulated ischemia/reperfusion, with different treatment conditions added to the ischemic buffer. Do note that the 96 well plates were used in order to set up a high throughput system and compare more conditions per experiment. As can be seen in the figure 2.2 above, the cardiomyocytes received a growth medium by adding 100µl per well of the freshly prepared wash buffer (modified MXCB - BBS) on top of the overnight MXCB (+BBS). After overlaying the culture plates, they were incubated for 15 minutes @ 37˚C.

After the 15 minutes of incubation, the media in the wells was discarded and another 100µl/well of fresh MMXCB (-BBS) was added to the wells and incubated for 10minutes at 37˚C. This was again repeated and incubated for another 10minutes (total feeding time= 35 minutes)

Control cells were incubated in MMXCB, while ischemia was simulated by incubating cardiomyocytes for 20minutes at 37˚C with ischemic wash buffer [with 10mM sodium hydrosulphite (SDT) and 3mM 2−deoxyglucose (2−DG), (−HEPES), pH 6.4]. After 20 minutes, the cardiomyocytes were washed twice with 200µl MMXCB and reperfused with 100µl MMXCB for 15 minutes at 37˚C. The time and concentrations used to elicit ischemia (mimic ischemia) was determined by another colleague (Labuschagne, SW, 2015).

During the ischemic event, the different drugs were administered during ischemia in separate wells. The conditions had Ischemic buffer with 10uM rolipram, 10uM Dobutamine, 10uM and 10nM HU210, or combination of the Dobutamine with rolipram or HU210 and rolipram, given only during the ischemic period.

Thereafter, the cells were stained with 3.8mM JC-1 for 10 minutes at 37˚C and washed twice with 100µl of MMXCB. A stabilization period of 30 minutes was given after staining and followed by fluorescence microscopy [excitation: 490nm; emission: 590nm and 530 for JC-1 aggregates (red) and monomers (green) respectively]. Images were captured, analysed, and data expressed as red/green fluorescence ratio as a live index. Increased red fluorescence indicated increased viability. And decreased red fluorescence indicated increased cell death. The cell length and the total number of cells were measured as well.

The dissipation of the mitochondrial electrochemical membrane potential gradient is known as an early event in apoptosis. In normal cells, due to the membrane potential gradient, the dye concentrates in the mitochondrial matrix, where it forms red fluorescent aggregates.
Any event that dissipates the mitochondrial membrane potential (like an ischemic event) prevents the accumulation of the JC-1 dye in the mitochondria and thus, the dye is dispersed throughout the entire cell leading to a shift from red to green fluorescence (JC-1 monomers).

Microphotograph Analysis

By using a fluorescence microscope the captured images of cells were taken. The captured images represents one field view per well at 10× objectives. The analysis was done by measuring the captured images of cells. The cell count, cell length and fluorescence analyses were determined directly from the microphotographs.

For the cell count the total numbers of ARCMs (rod and round) were counted. The rod shaped and round shaped cells (hyper-contracted cells) were counted in each well for all the different conditions within 3 to 8 hearts and compared.

For the cell length, the length in each cell was measured using the Image J, FIJI software program. The average length of all the cells per well (per condition) was determined and compared within the different amount of hearts.

For fluorescence analysis, red (R) and green (G) fluorescence in each cell were measured using the Image J, FIJI software program. The ratio of Red and green fluorescence was calculated for each cell, and the averages per well was determined in each condition and within the different amount of hearts.

All of the photos are taken after reperfusion and JC-1 staining and thus all parameters: cell viability, R/G ratio and cell length are assessed after reperfusion.

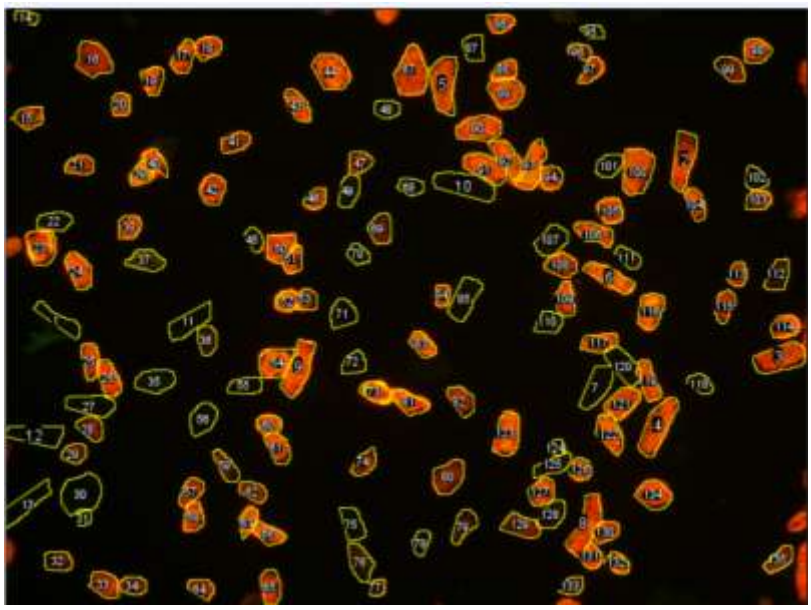

Figure 2.3: Cell analyses through Image J Fiji

cAMP ASSAY

Adult rat cardiomyocytes cultured overnight at 2500 cells/well in duplicate for 2 rats, were assessed during normoxia for cAMP responses to the following drugs: 10uM rolipram, 100nM isoproterenol, 100uM IBMX, 10nM rolipram with 100nM isoproterenol, 100uM IBMX with 100nM isoproterenol. The cardiomyocytes were stimulated with these drugs for 10 minutes at 37C. Thereafter the stimulation buffer was removed and the cell permeabilized with 80ul/well of somatic cell releasing reagent (FL-SAR) from SIGMA. After 5 min incubation at room temperature, the somatic cell releasing reagent was harvested from the cells and duplicate treatments merged per treatment per heart. 50ul of each sample was assayed for cAMP according to the manufacturer's protocol, using the cAMP enzyme immunoassay kit (CA201) from SIGMA.

Statistical Analysis

All values are expressed as the mean ± standard error of the mean (SEM). Graph Pad Prism (version 6.0) was used to compare results, using one-way analysis of variance (ANOVA) and Bonferonni was used as the post hoc test. Probability values of less than 0.05 ($p < 0.05$) were considered significantly different.

Chapter 3: Results

The drugs used during these experiments included dobutamine and isoproterenol which was expected to exacerbate damage with B1 stimulation during ischemia, insulin administered as a protective treatment during ischemia.

Formoterol, a b2AR-agonsit, BRL-37344 as a b3AR-agonist and THC (main component of marijuana and a phytocannabiniod) as well as HU210 a synthetic cannabinoid (also influencing the CB-receptors) was used to elicit protection with b2AR, b3AR and cannabinoid receptor stimulation during ischemia.

The 3-isobutyl-1-methylxanthine (IBMX) is a non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine used in the total cAMP measurement experiment.

Rolipram is the drug used to inhibit PDE4 and was combined with the different drug treatments to evaluate if this would elicit protective effects or worsen the pathology caused by the ischemic event.

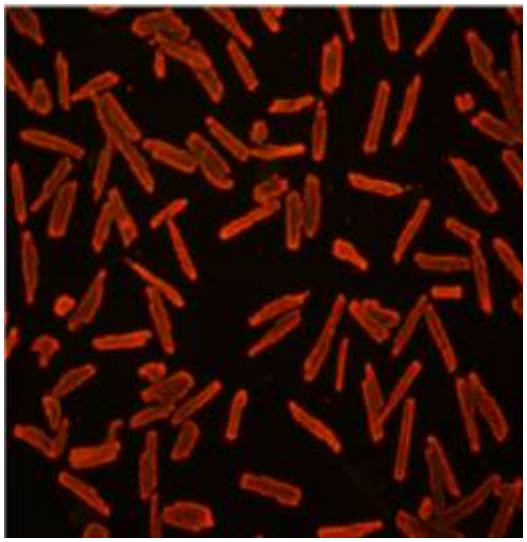

Figure 3.1 photo of the normoxia cells from experiment.

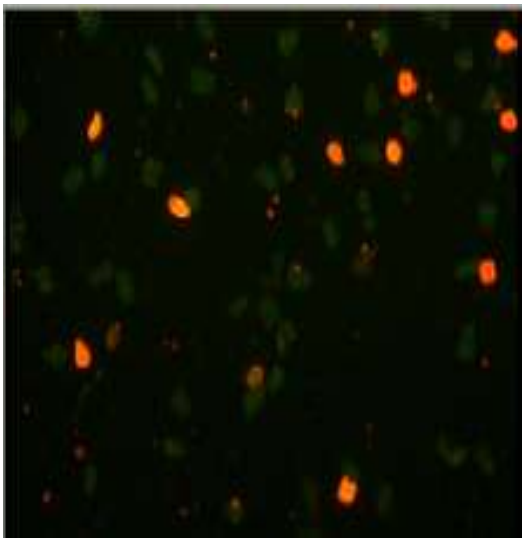

Figure 3.2: photo of the ischemia cells from experiment

Effect of insulin and PDE4 inhibition on ischemia/reperfusion injury

Figure 3.3A shows the average cell lengths of cardiomyocytes at the end of ischemia/reperfusion, when treated during ischemia with 0.3mIU insulin and rolipram (1, 10 and 50uM) alone and in combination. A significant difference ($p \leq 0.01$) was found between the ischemic untreated (71.4±2.0um)and the normoxic untreated (115.9±1.2um) cells. This indicates a significant development of hypercontracture induced in the cardiomyocytes by simulated ischemia/reperfusion. Hypercontracture was not altered by any of the treatments, given that the results didn't show any improvement in cell length with any of the treatments applied during ischemia (range: 63.9±2.2um - 75.3±9.0um).

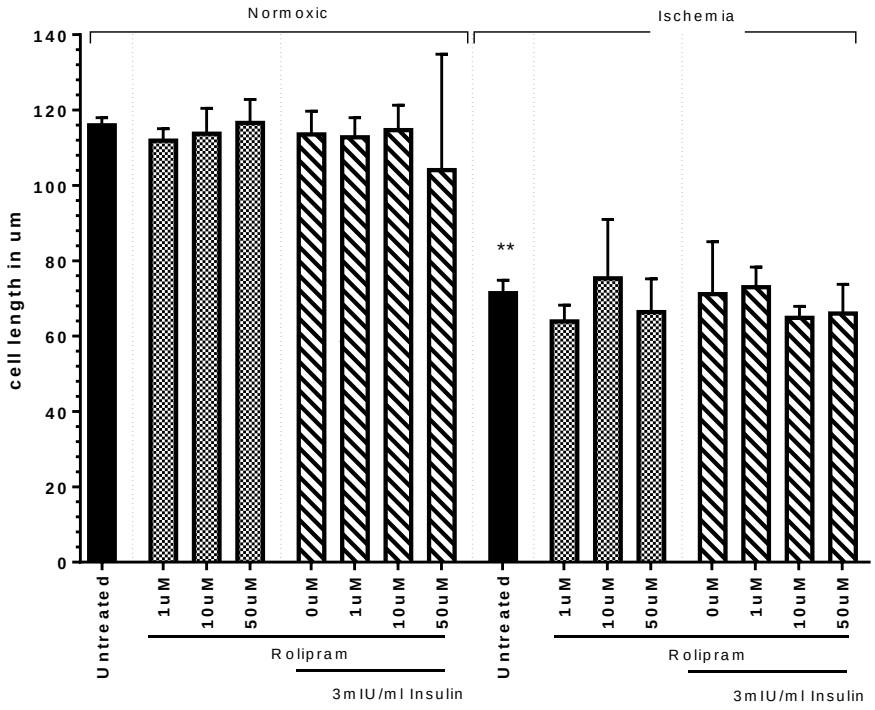

Figure 3.3A: Graph of cell lengths in micrometre (um) as an indication of hypercontracture after ischemia/reperfusion. Comparing different concentrations of rolipram administered during ischemia and normoxic conditions, with and without insulin. N=3, with **= $p \leq 0.01$compared to normoxia untreated.

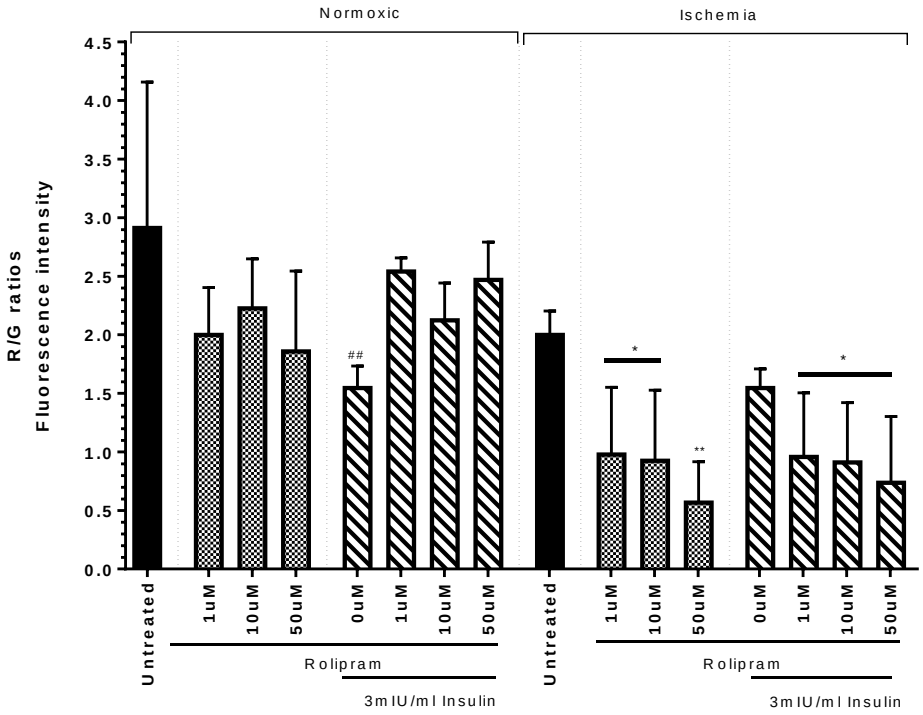

Figure 3.3B: Graph of red over green (R/G) fluorescence intensity ratio evaluating cell viability through mitochondrial membrane potential. Comparing different concentrations of rolipram administered during ischemia and normoxic conditions, with and without insulin. N=3 (*=p≤ 0.05 and **= p ≤ 0.01 compared to ischemia untreated. (##=p ≤ 0.01 compared to normoxia untreated).

In figure 3.3B the red / green (R/G) fluorescence ratio was plotted and no significant difference was observed between the untreated normoxic group and untreated ischemia group. Insulin administration during normoxia with no rolipram (1.55± 0.11) significantly (p ≤ 0.01) reduced the ratio of R/G compared to the normoxic untreated group. Compared to untreated ischemia (1.99 ± 0.12), rolipram treatment alone reduced R/G at concentrations of 1uM (0.98 ± 0.33, p ≤0.05), 10uM (0.92 ± 0.35, p ≤0.05) and 50uM (0.57 ± 0.20, p ≤0.01). Similar results were also found when 3mIU insulin was combined with rolipram at 1uM, 10uM and 100uM.

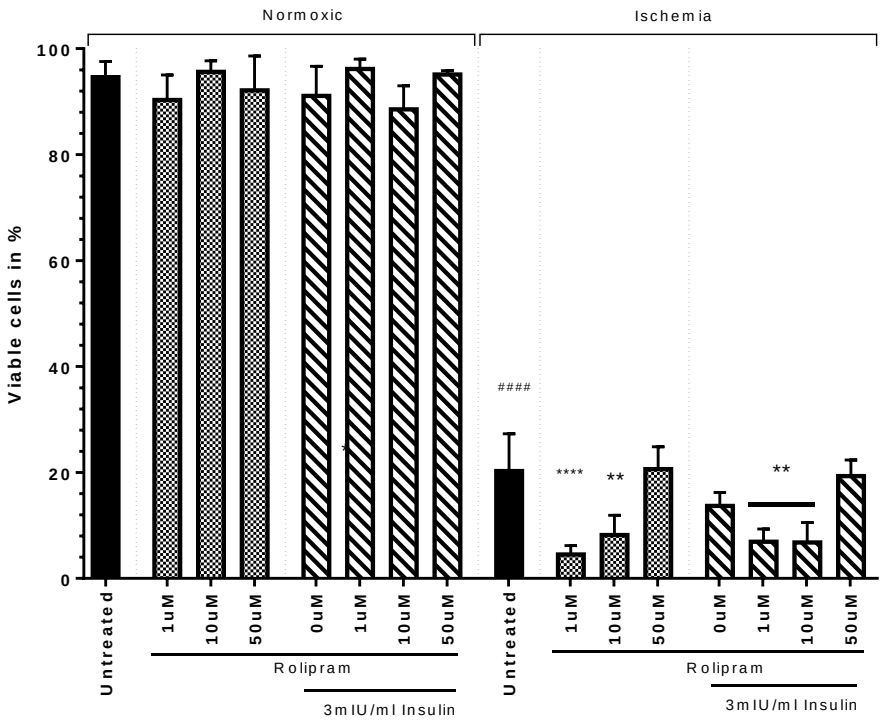

Figure 3.3C: Graph of the percentage viable cells, comparing the effect of different concentrations of rolipram administered during ischemia and normoxic conditions respectively, with and without insulin. N=3. (**= p ≤ 0.01 compared to ischemia untreated; ****=.p ≤ 0.0001 compared to ischemia untreated and ####=.p ≤ 0.0001 compared to normoxic untreated).

As shown in figure 3.3C, the percentage viable cells was significantly reduced (p≤0.0001) by the ischemic untreated condition (20.3±4.1%) compared to the normoxic untreated condition (94.6±1.7%). This indicates that a proper ischemic insult was achieved to study the effects of the different drugs. When rolipram concentrations in ischemia were compared to untreated ischemia, a significant decrease in viability was found with the concentrations of 1uM (4.52 ± 0.96%, p≤0.0001) and 10uM (8.20 ± 2.15%, p≤0.01) rolipram. Similar results were found when rolipram was administered with insulin during ischemia. Considering these findings the 10uM concentration was chosen to use throughout the project as this is the concentration also used in most literature (Bethke, T. et al, 1992; Georget, M. et al, 2003; De Arcangelis, V. et al, 2008; Qvigstad, E. et al, 2010; Beca, S. et al, 2011; Leroy, J. et al 2011; Parks, R.J. et al, 2014). The insulin treatment with 3IU/ml did not show a protective effect, which is in contrast to the literature. No differences were found between the normoxic conditions when compared to the normoxic untreated.

Effect of B1 receptor stimulation on ischemia/ reperfusion injury

In figure 3.4A the average cell lengths obtained after ischemia/reperfusion, in the presence and absence of dobutamine were compared. A significant difference ($p \leq 0.05$) was found with the ischemic untreated 0uM Dobutamine (60.8±1.8um) compared to normoxic untreated 0uM Dobutamine (112.3±0.8um). This indicates that the ischaemic conditions were enough to result in a proper insult which resulted in a decrease in cell length, which indicates hyper contracture. The results did not show a significant improvement or further damaging effect during ischemia with the different dobutamine concentrations. The mean length range for the different treatments during ischemia was between 59.0±1.5um and 64.3±3.7um.

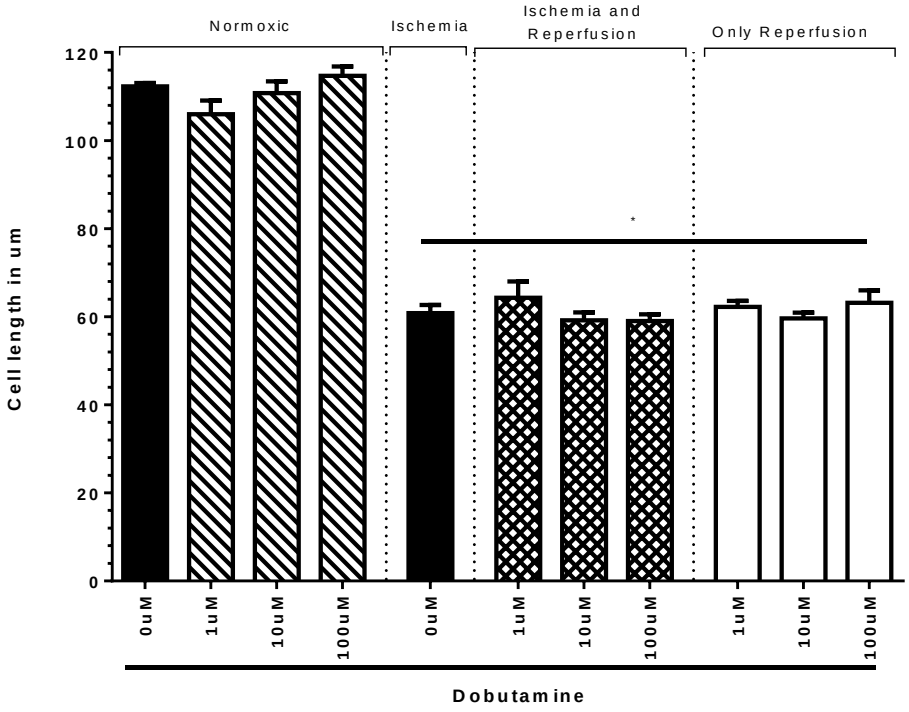

Figure 3.4A: Graph of cell lengths in micrometre (um) as an indicator of contracture. Comparing different concentrations of dobutamine administered during ischemia/reperfusion, reperfusion only and during normoxic conditions. N=4. (*= $p \leq 0.05$ compared to normoxia untreated).

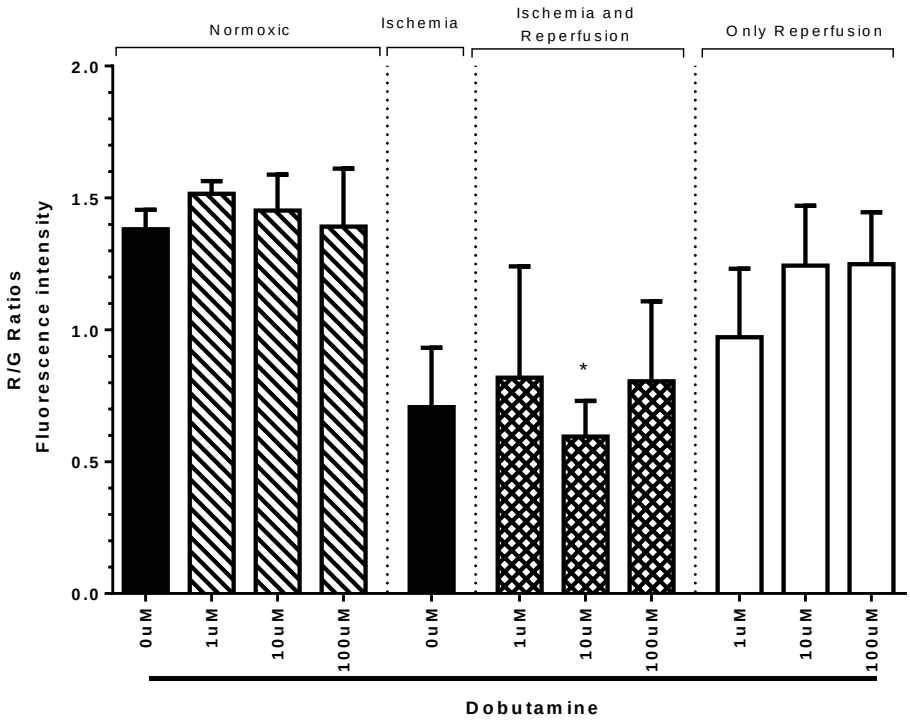

Figure 3.4B: Graph of red over green (R/G) ratio of fluorescence intensity ratio as an indicator of mitochondria function and cell viability. Comparing different concentrations of dobutamine during ischemia/reperfusion, reperfusion only and during normoxic conditions. N=4. (*= $p \leq 0.05$ compared to normoxia untreated).

In figure 3.4B, no significant differences were found with R/G ratio when the normoxic untreated (1.3±0.1) was compared to the ischemia untreated (0.7±0.2). A significant difference ($p \leq 0.05$) was however found between the normoxic untreated 0uM Dobutamine when compared with the Dobutamine 10uM administered during ischemia and reperfusion (0.6±0.1).

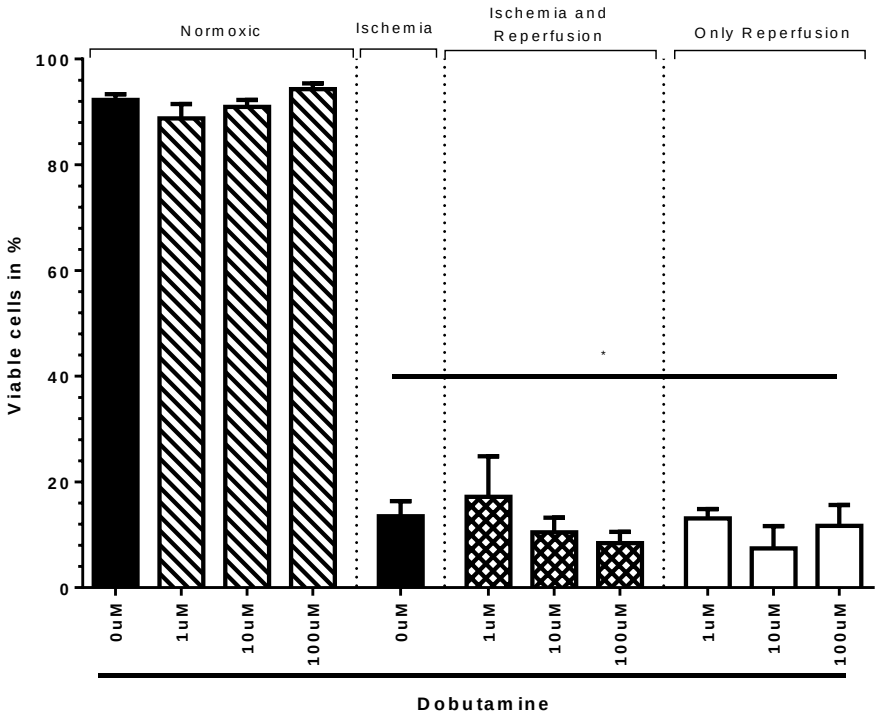

Figure 3.4C: Graph of total viable cells in percentage, comparing different concentrations of dobutamine during ischemia/reperfusion, reperfusion only and during normoxic conditions. N=4. (*= p ≤ 0.05 compared to normoxia untreated).

In figure 3.4C a significant difference of p ≤ of 0.05 and smaller was found when comparing the percentage cell viability of ischemic conditions to normoxic untreated 0uM Dobutamine. The mean percentage viability of the normoxic untreated 0uM Dobutamine group was 92.2±1.1% and the ischemic untreated 0uM Dobutamine group was 13.5±2.8%. None of the treatments in the ischemic conditions or during reperfusion altered cell viability (7.4±4.2% to 17.2±7.6%). There were also no significant differences between the normoxic untreated 0uM compared to the other normoxic treatment concentrations of Dobutamine.

Effects of Isoproterenol on ischemia/reperfusion injury

Figure 3.5A shows cell lengths compared for different concentrations of isoproterenol administered during normoxic conditions only, during ischemia and reperfusion, and lastly administered only during reperfusion. A significant difference ($p \leq 0.05$) was found when the different ischemia conditions were compared to the normoxic untreated (0uM Isoproterenol). The latter had a mean cell length of 109.9±1.7um and the ischemia untreated showed a mean cell length of 68.7±3.2um. Cell lengths for isoproterenol treatments during ischemia/reperfusion and reperfusion ranged from 62.7±2.8um to 67.2±4.2um, Isoproterenol had no effects on cell length when administered to cardiomyocytes under normoxic conditions.

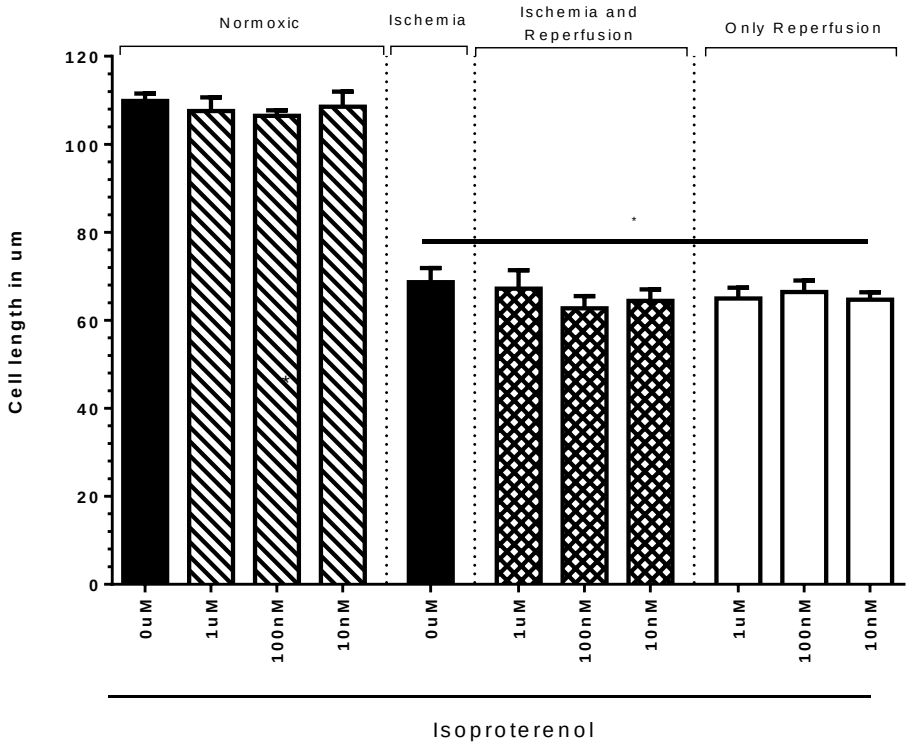

Figure 3.5A: Graph of cell lengths in micrometre (um), comparing different concentrations of isoproterenol administered during ischemia/reperfusion, reperfusion only and only during normoxic conditions. N=4. (*= $p \leq 0.05$ compared to normoxic untreated 0uM).

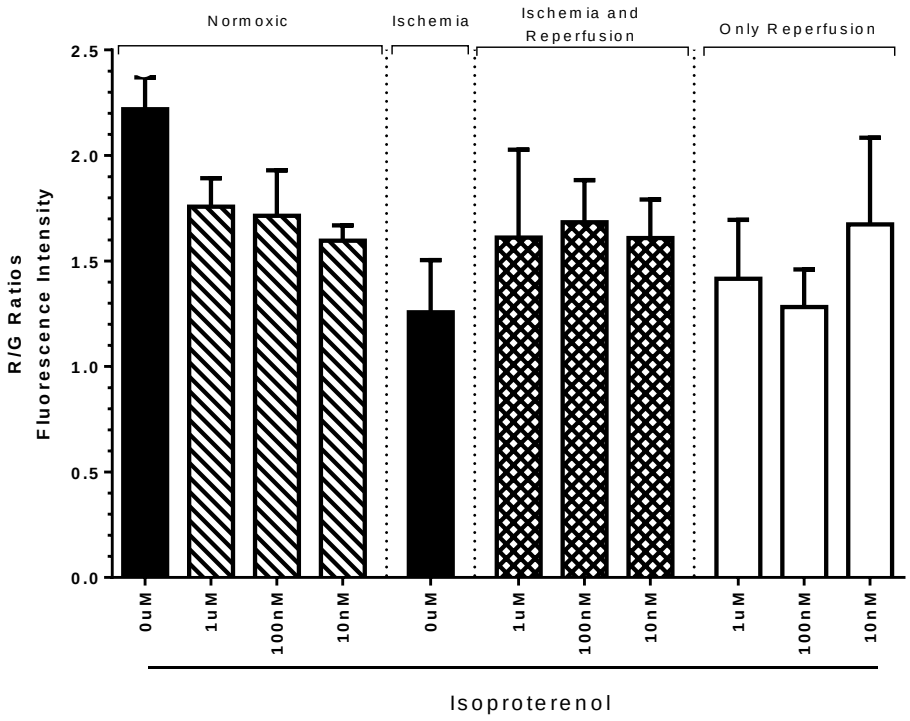

Figure 3.5B: Graph of red over green (R/G) fluorescence intensity ratio as a measure of mitochondrial function and cell viability. Comparing different concentrations of isoproterenol during ischemia/reperfusion, reperfusion only and during normoxic conditions. N=4

In Figure 3.5B, the R/G fluorescence ratio was studied to assess the cell viability after ischemia/reperfusion. Comparison between the different concentrations of isoproterenol during ischemia/reperfusion, reperfusion and also during normoxic conditions showed no differences. The normoxic untreated group had a mean fluorescence intensity of 2.2±0.2 and the ischemia untreated 0uM Isoproterenol had a mean fluorescence intensity of 1.3±0.2. The normoxic treatments had no significant differences when compared to the normoxic untreated group.

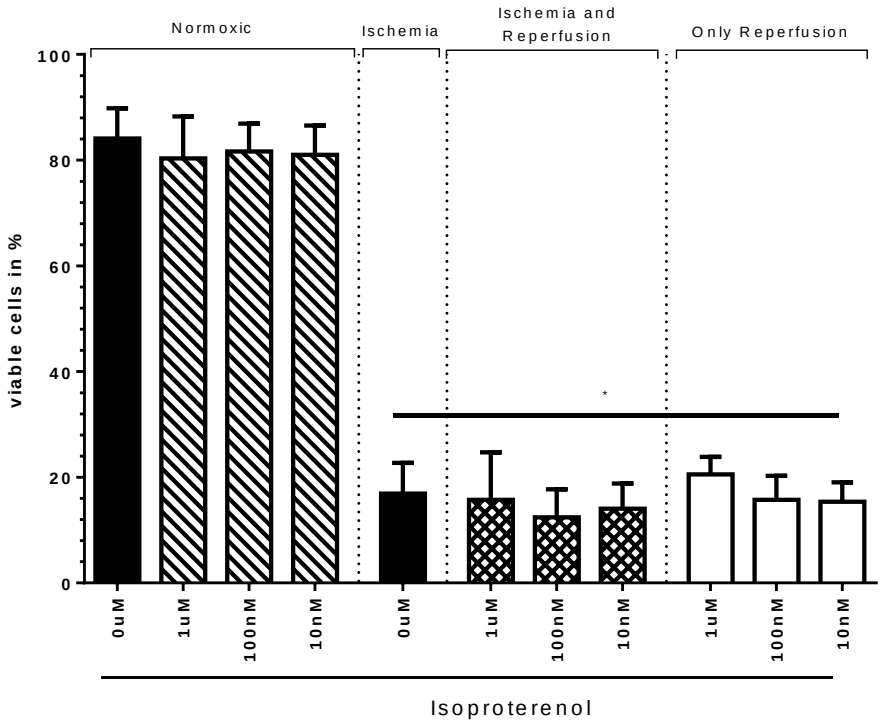

Figure 3.5C: Graph of total viable cells in percentage Comparing different concentrations of isoproterenol during ischemia/reperfusion, reperfusion only and during normoxic conditions. N=4 and p. (*= p ≤ 0.05 compared to normoxia untreated).

Figure 3.5C shows the percentage viable cells compared between the different concentrations of isoproterenol during ischemia/reperfusion, reperfusion only and during normoxic conditions. All the ischemia treatments showed a significant difference ($p \leq 0.05$) compared to normoxic untreated (84.1±5.7%) The ischemia untreated 0uM had a viability of 16.9±5.8%, indicating that cell viability reduced by 67.2% after ischemia/reperfusion. No significant differences were observed when the different treatments were administered during ischemia/reperfusion and reperfusion only, compared to the ischemia untreated 0uM group.

Effects of seven different treatments in ischemia with and without the combined administration of 10uM rolipram.

In Figure 3.6A the cell lengths were plotted to evaluate cardiomyocyte hypercontracture after ischemia/reperfusion. A comparison between the different treatments (100nM isoproterenol, 10uM formoterol, 10uM dobutamine, 10nM HU210) applied during ischemia, with and without 10uM Rolipram was done. A significant difference (p≤0.0001) was observed between the ischemia untreated (58.2±3.3um) compared to the normoxic untreated group (104.7±6.7um). A similar degree of hypercontracture was found for all the other treatments given during ischemia, which indicates a significant ischemic insult was induced. HU210 was also tested in this experiment, comparing administration during ischemia only, and during ischemia/reperfusion with untreated ischemia, and no protection was found. The mean cell length range for the treatments during ischemia was between 57.6±1.7um and 69.1±4.7um.

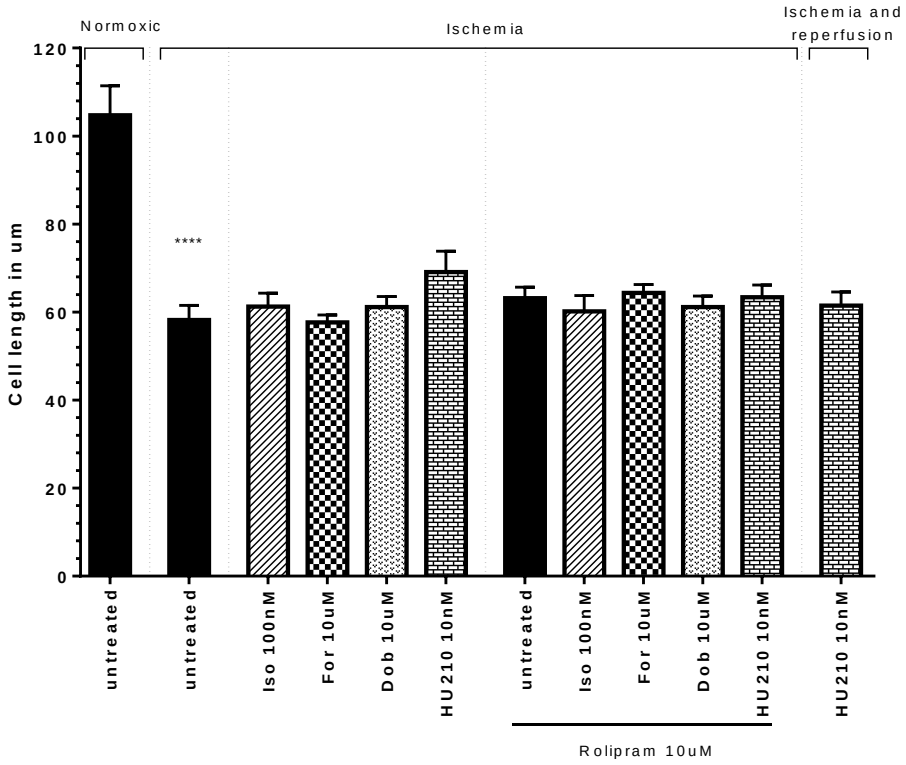

Figure 3.6A Graph of cell lengths in micrometre (um), evaluating cardiomyocyte hypercontracture. Comparing different treatments: 100nM isoproterenol (Iso), 10uM formoterol (For), 10uM dobutamine (Dob), and 10nM HU210 during ischemia, with and without 10uM Rolipram. N=4 (****=p≤0.0001compared to untreated normoxic control).

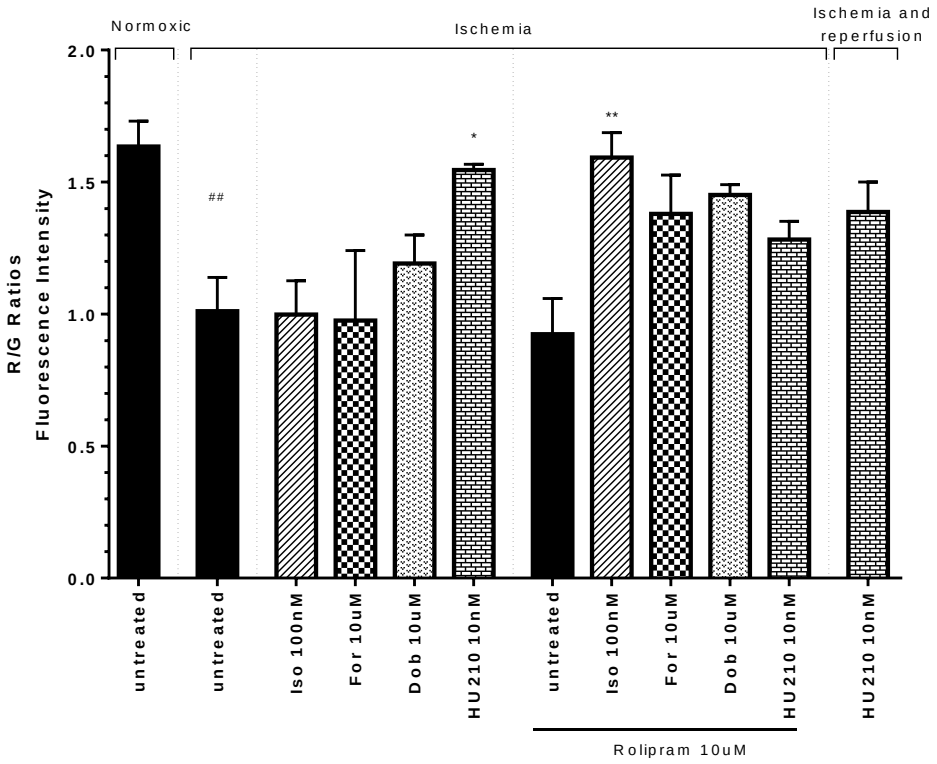

Figure 3.6B Graph of red over green (R/G) fluorescence intensity as an indicator of mitochondrial viability. Comparing different treatments: 100nM isoproterenol (Iso), 10uM formoterol (For), 10uM dobutamine (Dob), and 10nM HU210 during ischemia, with and without 10uM Rolipram. N=4 (*=p≤0.05 compared to ischemia untreated, **=p≤0.01 compared to ischemia untreated and ## = p≤0.05 compared to normoxia untreated).

The graph of R/G fluorescence ratio (figure3.6B) shows a significantly ($p \leq 0.05$) higher mitochondrial viability for ischemia administered HU210 (1.5±0.0) compared to ischemia untreated (1.0±0.1). A difference ($p \leq 0.01$) was observed between the normoxic untreated (1.6±0.0) and ischemia untreated (1.0±0.1), indicating a significant insult due to ischemic/reperfusion. The isoproterenol treatment during ischemia (1.0±0.1) and rolipram treatment during ischemia (0.9±0.1) were similar to ischemia untreated, but when combined during ischemia, isoproterenol and rolipram combined treatment significantly ($p \leq 0.01$) improved fluorescence intensity (1.6±0.1).

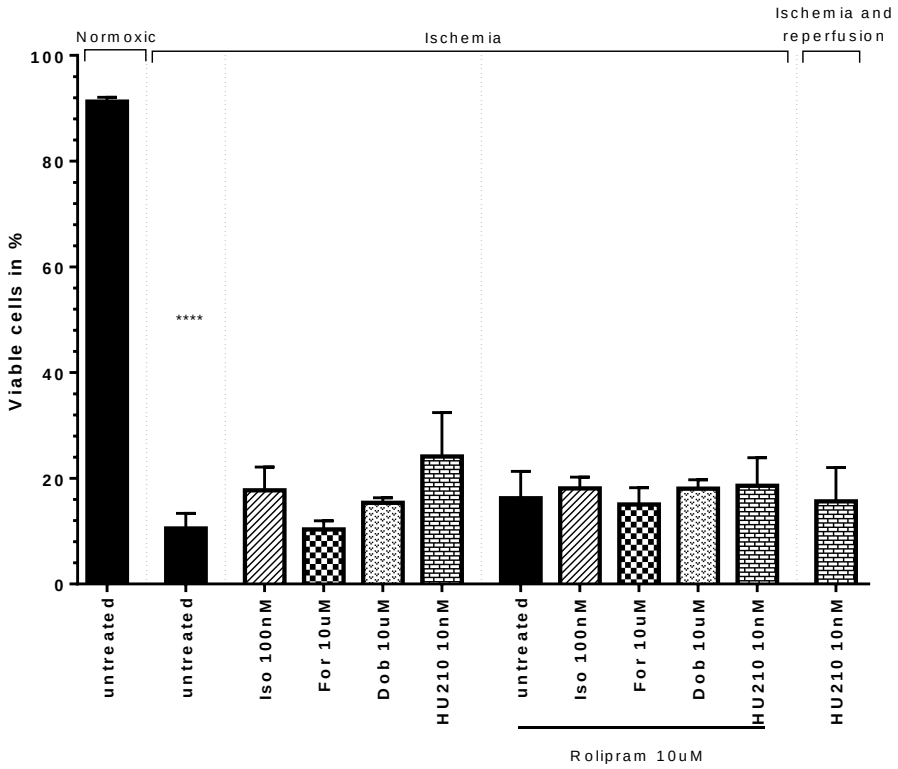

Figure 3.6C Graph of total viable cells over total viable and non-viable cells in percentage. Comparing different treatments: 100nM isoproterenol (Iso), 10uM formoterol (For), 10uM dobutamine (Dob), and 10nM HU210 during ischemia, with and without 10uM Rolipram. N=4 (****=p≤0.0001 compared to control).

Figure 3.6C shows that ischemia untreated had a mean percentage viability of 10.5±2.8% and the normoxic untreated had a mean percentage of 91.3±0.8%. This indicates a significant ($p \leq 0.0001$) loss of viable cells during the ischemia/reperfusion insult. No other differences were observed with any of the treatments applied during ischemia or for HU210 during ischemia/reperfusion.

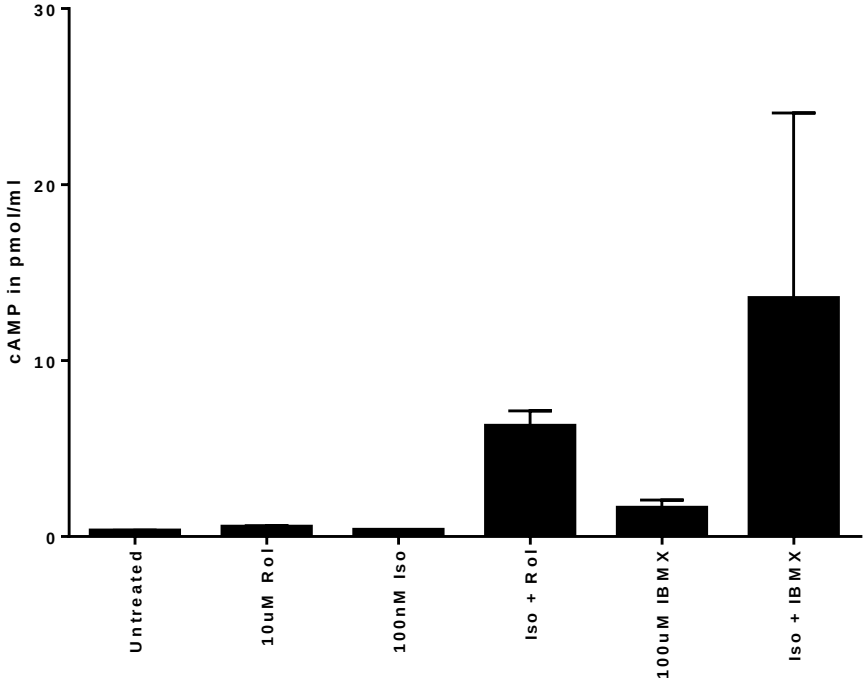

Figure 3.6D: cAMP measurement. Cultured adult cardiomyocytes were incubated for 10 minutes with 10uM rolipram (Rol), 100nM Isoproterenol (Iso), 100nM isoproterenol with 10uM rolipram, 100uM IBMX, and 100nM isoproterenol + 100uM IBMX, followed by total cAMP measurement. n=2

The data in this graph (figure3.6D) shows that cAMP production at the basal level is very low in the untreated cardiomyocytes (0.33 ± 0.02pmol/ml), and remained so even in the presence of PDE4 inhibition with rolipram (0.56 ± 0.05pmol/ml), while showing a slight increase by total PDE inhibition with IBMX (1.626 ± 0.3225). Unfortunately no statistical analysis could be done because only an n-value of 2 was used in this experiment. The production of cAMP by 100nM Isoproterenol (0.38 ± 0.02) was not visible in the absence of PDE inhibition, but increased 16 fold when combined with PDE4 inhibition (Iso + Rol: 6.38 ± 0.61pmol/ml) and 35 fold with total PDEs inhibition (Iso + IBMX: 13.54 ± 7.446pmol/ml).

Effects of 10uM rolipram administered at different time points prior to ischemia.

Figure 3.7A shows a comparison of cell length between 10uM formoterol and 10uM dobutamine treatment during ischemia respectively, with and without 10uM rolipram, where rolipram was added from 5minutes prior to ischemia. A significant difference with a p≤0.0001 was observed between the ischemia untreated (76.0±1.9um) compared to the normoxic untreated group(114.2±3.0um). This indicates a proper ischemic insult. No significant differences were observed in the other ischemia treated groups compared to ischemia untreated.

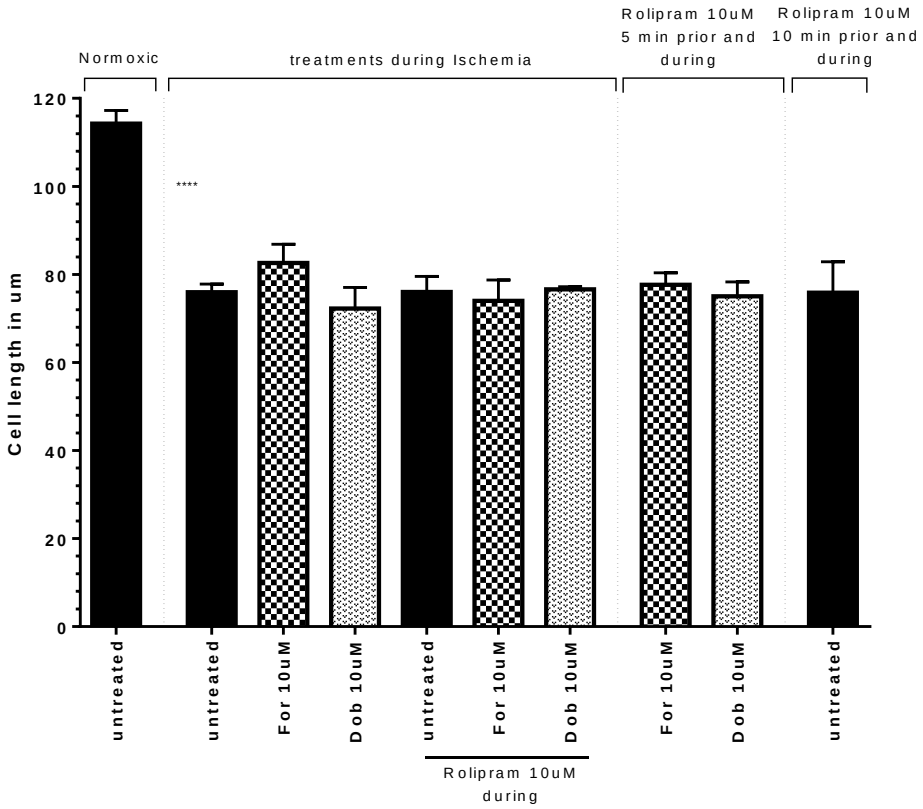

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Figure 3.7A. Graph of cell lengths in micrometre (um), evaluating cardiomyocyte hypercontracture. Comparing 10uM of dobutamine (Dob) and formoterol (For) during ischemia, with and without different administrations treatment of 10uM rolipram. N=4. (****=p≤0.0001 compared to normoxic untreated).

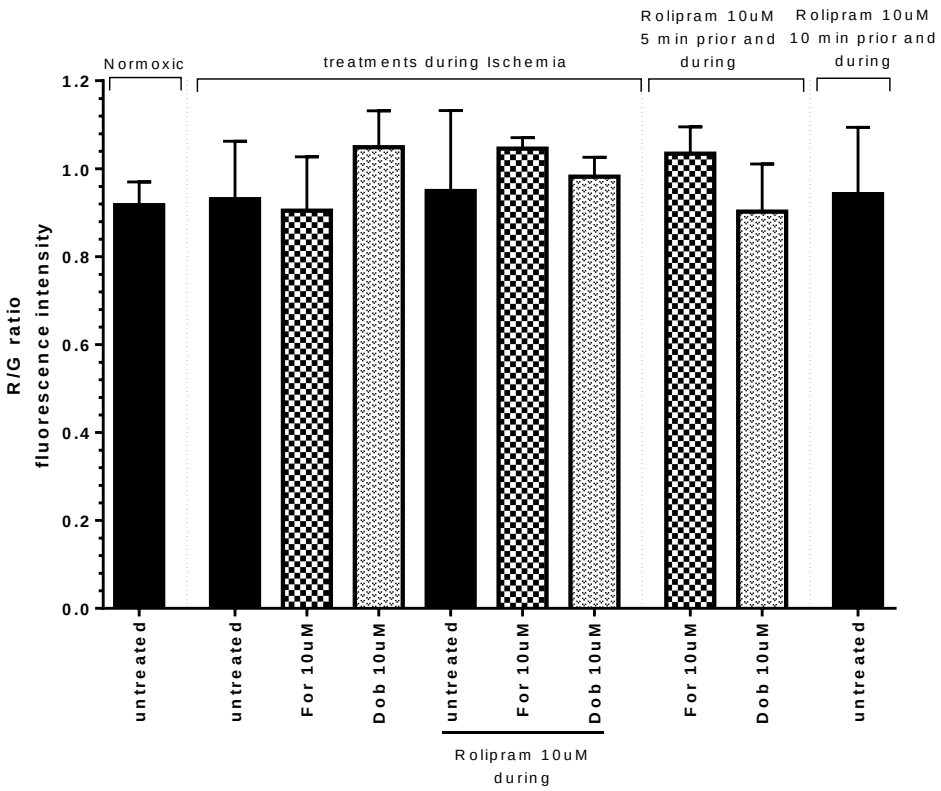

Figure 3.7B. Graph of red over green (R/G) fluorescence intensity ratio. Comparing 10uM of dobutamine (Dob) and 10uM formoterol (For) during ischemia conditions with and without different administration treatments of 10uM rolipram. N=4.

In Figure 3.7B, the R/G fluorescence ratio was studied to evaluate cell viability after ischemia/reperfusion. 10uM formoterol and 10uM dobutamine administered during ischemia with different administered time points of rolipram 10uM, did not show a significant difference. No differences were found between the normoxic untreated group (0.9±0.05) and the ischemia untreated (0.9±0.1), or any of the treatment groups (0.9±0.0 and 1.0±0.1)

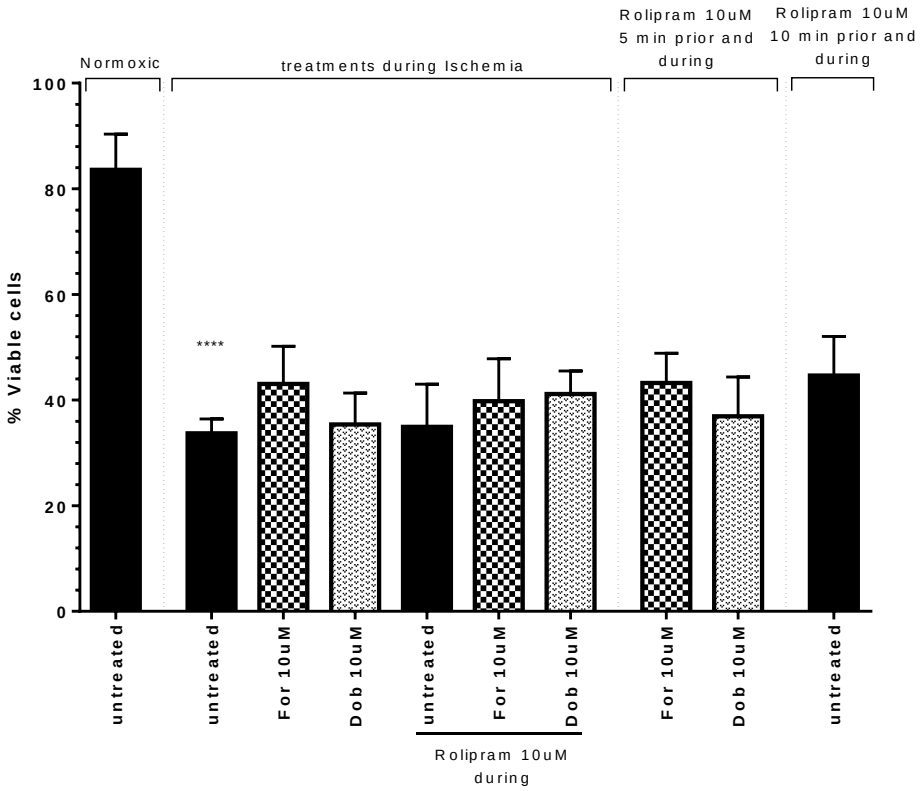

Figure 3.7C. Graph of cell viability in percentage. Comparing 10uM of dobutamine (Dob) and 10uM formoterol (For) during ischemia conditions with and without different administration treatments of 10uM rolipram. N=4 (****=p≤0.0001 compared to control).

In figure 3.7C shows that the mean percentage viability for the ischemia untreated group (33.7±2.7%) was significantly ($p \leq 0.0001$) reduced compared to the normoxic untreated group (83.6±6.8%). None of the treatments during ischemia altered percentage cell viability (34.3±6.6% to 46.1±9.5%) when compared to the ischemia untreated group.

Cannabinoids and b-AR agonists in combination with and without 10uM rolipram administered 5minutes prior to and during ischemia.

In Figure 3.8A cell lengths in um show cardiomyocyte hypercontracture compared between the different treatments during ischemia, with and without 10uM Rolipram administered from 5min pre-treatment and pertreatment ischemia. A significant difference ($p \leq 0.0001$) was observed between the ischemia untreated group (70.8±4.2um) and the normoxic untreated group (95.6±0.6um). 100nM isoproterenol without 10uM rolipram during ischemia had a mean cell length of 59.7±2.7um, which was significantly shorter than that of the ischemia untreated group. 10uM BRL-37344 treatment without 10uM rolipram during ischemia (57.4±0.2um) had a significantly ($p \leq 0.01$) shorter cell length compared to ischemia untreated. This was also true for HU210 administration when rolipram was administered from 5 min before and during ischemia (57.3±2.3um, $p \leq 0.01$) compared to the ischemia untreated group.

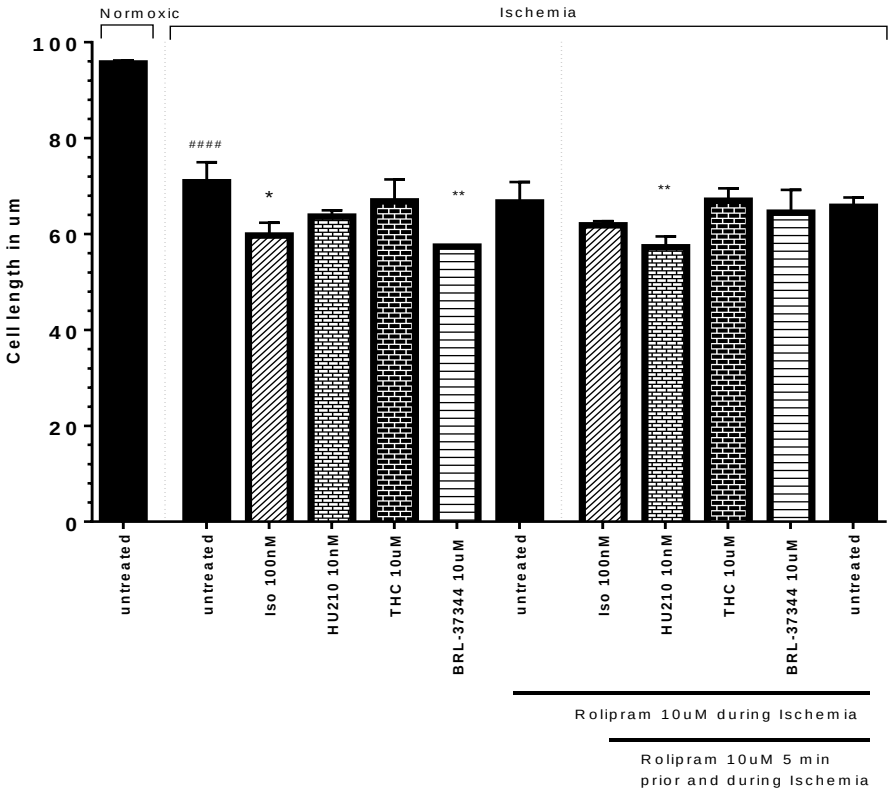

Figure 3.8A Graph of cell lengths in micrometre (um), evaluating cell hypercontracture. Comparing different treatments (100nM isoproterenol (Iso), 10nM HU210, 10uM THC, and 10uM BRL-37344) during ischemia, with and without 10uM Rolipram administered from 5 min prior and during ischemia. N=4 (*=p≤0.05 compared to ischemia untreated, **=p≤0.01 compared to ischemia untreated and ####=p≤0.0001compared to normoxic untreated).

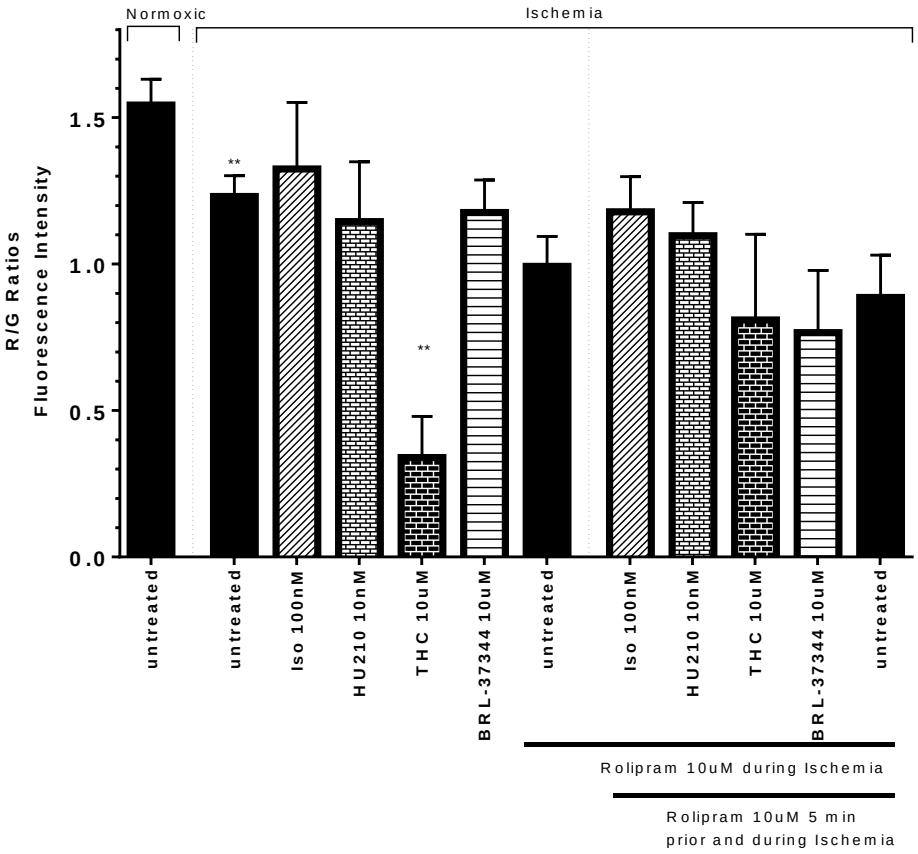

Figure 3.8B Graph of red over green (R/G) fluorescence intensity ratio. Comparing different treatments (100nM isoproterenol (Iso), 10nM HU210, 10uM THC, and 10uM BRL-37344) during ischemia, with and without 10uM Rolipram administered from 5 min prior and during ischemia. N=4 (**=p≤0.01 compared to ischemia untreated).

In Figure 3.8B the R/G fluorescence intensity was measured for the different treatments (100nM isoproterenol, 10nM HU210, 10uM THC, and 10uM BRL-37344) during ischemia, with and without 10uM Rolipram administered 5 minutes prior and throughout ischemia. R/G fluorescence ratio did not show a significant decrease when the ischemia untreated group (1.2±0.1) was compared to the normoxic untreated group with a fluorescence intensity of 1.5±0.1. A significant difference ($p \leq 0.01$) was however found between when 10uM THC treatment during ischemia without rolipram (0.3±0.1) was compared to the ischemia untreated group (1.2±0.1).

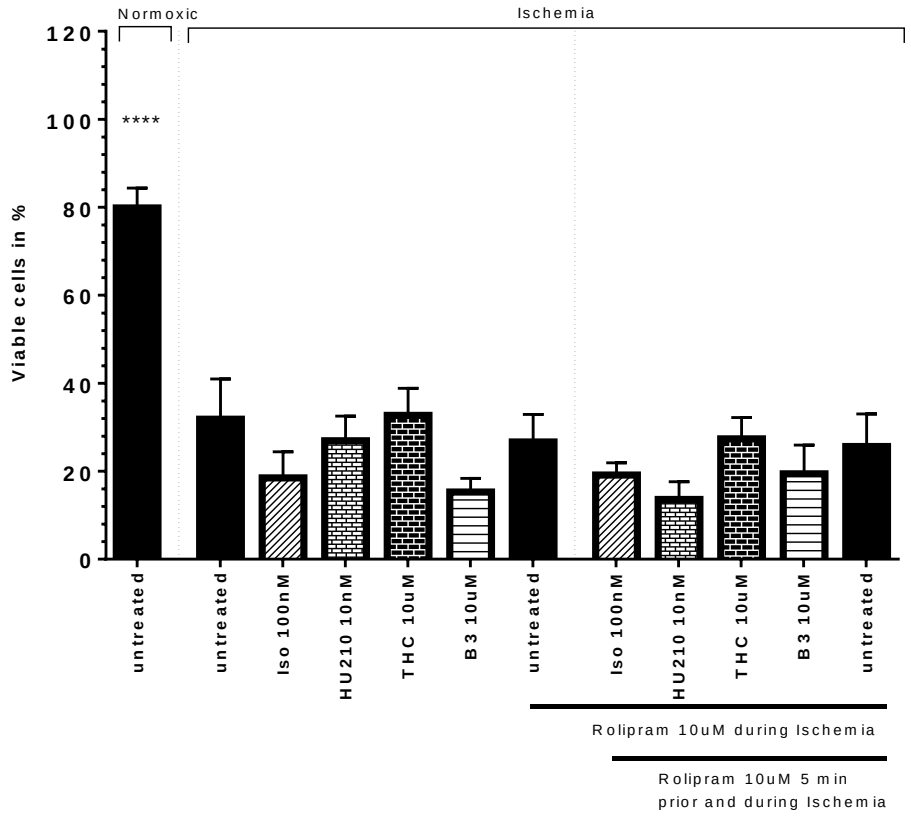

Figure 3.8C Graph of cell viability in percentage. Comparing different treatments (100nM isoproterenol (Iso), 10nM HU210, 10uM THC, and 10uM BRL-37344) during ischemia, with and without 10uM Rolipram administered from 5 min prior and during ischemia. N=4 (**=p≤0.0001 compared to ischemia untreated).

In Figure 3.8C cell viability in percentage (total rod over total cells) show cardiomyocyte viable cell attachment compared between the different treatments during ischemia, with and without 10uM Rolipram administered from 5min pre-treatment and pertreatment ischemia. A significant difference ($p \leq 0.0001$) was observed between the ischemia untreated group (31.9±1.3%) and the normoxic untreated group (79.8±4.5%). No further differences were observed in the other groups compared to ischemia, which might also be due to a very potent ischemic treatment.

Effects of cannabinoids and b-AR agonists with rolipram during ischemia only.

In figure 3.9A, the average cell lengths were compared to one another in 10uM Dobutamine (Dob), 10uM formoterol (For), 100nM isoproterenol (Iso), 10uM BRL-37344, 10uM THC, and 10nM HU210. A significant difference with a p-value ≤ 0.0001 was found between the ischemic untreated condition (58.8±1.6um) when compared to normoxic untreated condition (95.9±2.3). This indicates a proper insult with the ischemic conditions applied. There are seven different treatment conditions during ischemia that all received a combination of rolipram 10uM administration during ischemia and also without the rolipram administration. The results did not show a significant improvement or further damaging effect during ischemia with the other different treatments when compared to ischemia untreated, except for the THC 10uM without the rolipram 10uM administration. The significant difference between these two groups had a p-value≤0.05 with mean cell lengths of Ischemia untreated 58.8±1.6um and THC 10uM without rolipram 10uM, 65.7±2.

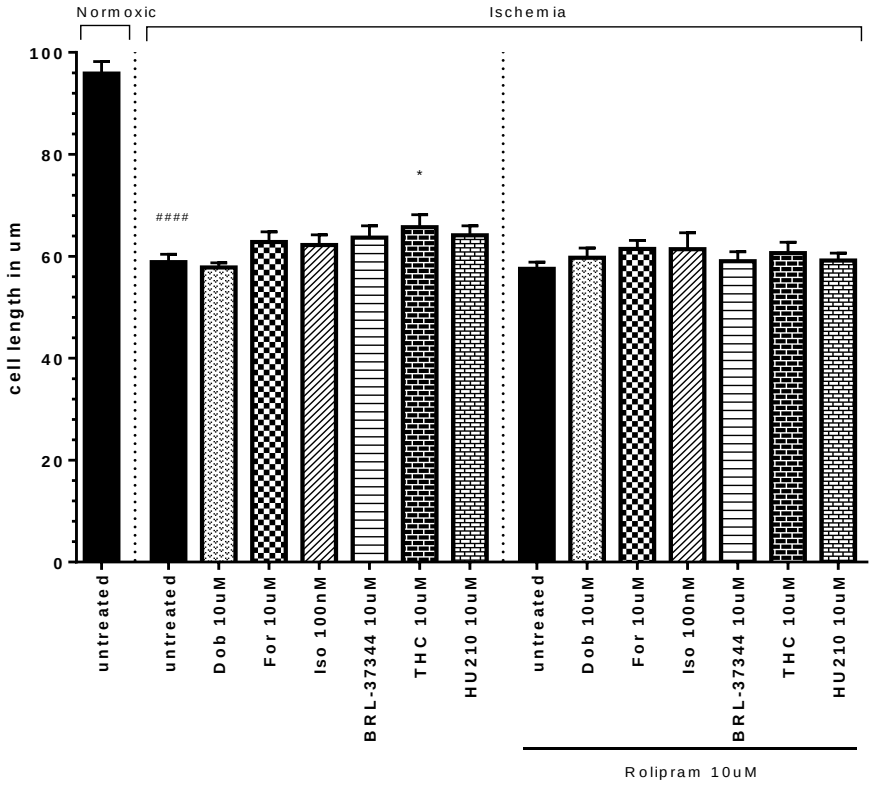

Figure 3.9A Graph of cell lengths in um, resembling cell contracture. Comparing different treatments (10uM Dobutamine (Dob), 10uM formoterol (For), 100nM isoproterenol (Iso), 10uM BRL-37344, 10uM THC, and 10nM HU210) during ischemia, with and without 10uM Rolipram administered during ischemia. N=7 (*=p≤0.05 compared to ischemia untreated and ****=p≤0.0001compared to normoxic untreated).

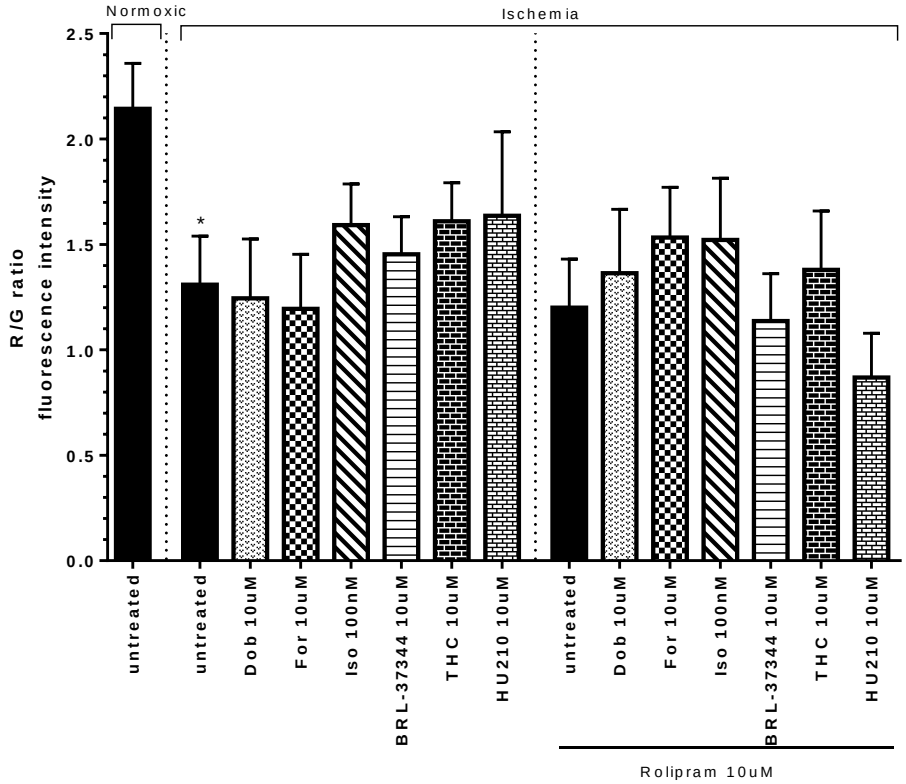

Figure 3.9B Graph of red over green fluorescence intensity. Comparing different treatments (10uM Dobutamine (Dob), 10uM formoterol (For), 100nM isoproterenol (Iso), 10uM BRL-37344, 10uM THC, and 10nM HU210) during ischemia, with and without 10uM Rolipram administered during ischemia. N=7 (*=p≤0.05 compared to normoxic untreated).

In figure 3.9B, the R/G fluorescence intensity was measured and the different treatments during ischemia with and without rolipram 10uM administration were compared. A significant difference was found between the ischemic untreated group (1.3±0.2) compared to the normoxic untreated group (2.1±0.2) with a p-value≤0.05. The HU210 that received the rolipram combination during ischemia with a mean fluorescence of 0.9±0.2 had a lowering effect on fluorescence intensity when compared to the normoxic untreated group (2.1±0.2). The formoterol given during ischemia without rolipram (1.2±0.3) also showed a significant difference when compared to the untreated normoxic conditions with a p-value≤ 0.05. The same significant difference was found for B3 (1.1±0.2) and untreated (1.2±0.2) treatments in combination with rolipram when compared to normoxic untreated groups (2.1±0.2). The range for the ischemic conditions varied between 0.9±0.2 and 1.6±0.4.

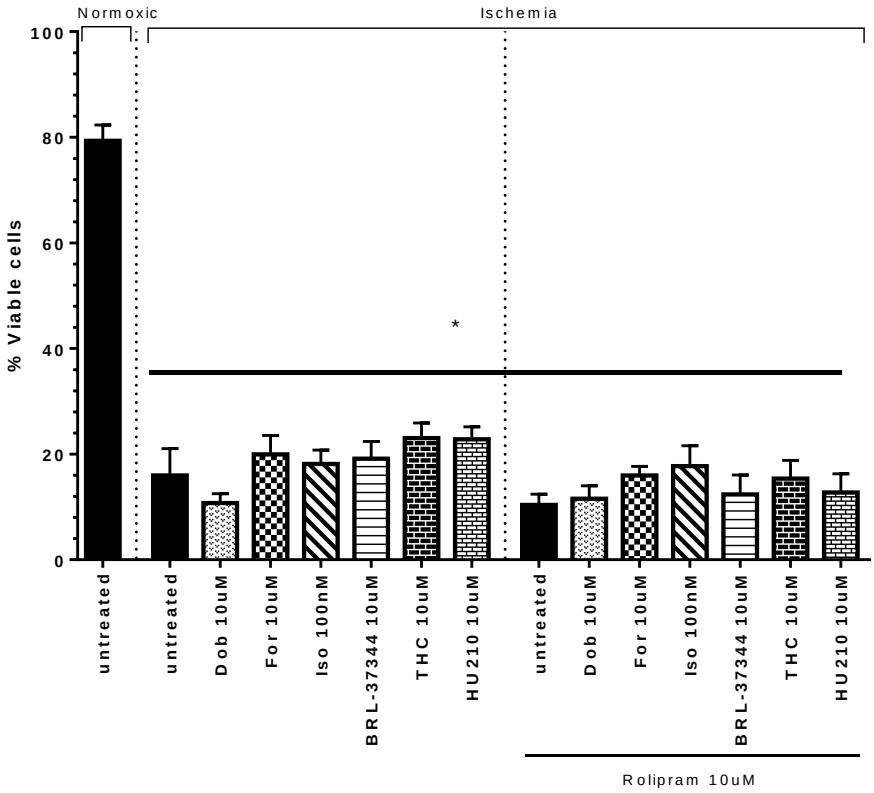

Figure 3.9C Graph of cell viability in percentage. Comparing different treatments (10uM Dobutamine (Dob), 10uM formoterol (For), 100nM isoproterenol (Iso), 10uM BRL-37344, 10uM THC, and 10nM HU210) during ischemia, with and without 10uM Rolipram administered during ischemia. N=7 (*=p≤0.05 compared to normoxic untreated).

Figure 3.9C shows the percentage viability of the total viable cells over total viable and non-viable cells. It compares the different treatments during ischemia with and without rolipram 10uM administration. The only significant difference through a one-way ANOVA variance test had a p-value≤0.05 and was found between the normoxic untreated (79.3±3.0%) and all the ischemic groups (ischemia untreated 16.0±5.1%). This shows the sufficient knockdown of our ischemia, but unfortunately no significant differences were found between the different ischemia conditions when compared to ischemia untreated. The mean percentage range for the different ischemia conditions varied between 10.4±2.1% and 23.0±2.9.

Chapter 4: Discussion

Isolation procedure of the cardiomyocytes

Most work on PDE expression in the cardiovascular setting made use of whole heart or tissue, this allowed for the possibility that some PDE activity came from vascular cells or fibroblasts, instead of only cardiomyocytes (Maurice et al, 2003; Johnson et al, 2012). Using cultured isolated adult cardiomyocytes allows the evaluation of PDEs in a pure cell population, and was therefore used in this study.

Drug treatments in normoxic conditions

The drug treatments administered during ischemia were also tested in normoxic conditions to determine whether the drug had any influence on the cardiomyocytes in the absence of ischemia. No differences were found between the normoxic drug treatments when compared to the normoxic untreated group. Due to the absence of drug effects during normoxia, normoxic drug controls were omitted from later experiments in order to maximize on the number of ischemic test groups per experiment. In addition, some of the experimental treatments (formoterol, THC, HU210 and BRL-37344) administered during ischemia without the indication of normoxic treatments, were tested by other colleagues and they found no significant differences with untreated normoxia (A Botha, SW Labuschagne and E Samodien). DMSO and ethanol vehicle controls were also tested by colleagues and were found to have no effects during normoxia or ischemia/reperfusion (data not shown).

Ischemic insult compared to viable control cells

Normoxia and ischemia/reperfusion treated cardiomyocytes were subjected to the same number of washes, in order to normalize for loss of cells through cell washes. The cell washing did not affect the percentage cell viability which ranged between 79.3±2.97% and 94.6±1.7% for the normoxic untreated through all the different experiments during the study. When the cardiomyocytes were treated with ischemia buffer, the percentage viability decreased to a range of 10.5±2.8% to 33.7±2.7%.

This shows a proper ischemic insult, as the percentage viability decreased, while the non-viable round shaped cells increased as can be seen in figure 3.1 and figure 3.2.

The cell lengths also showed a proper insult from ischemia, where cell lengths of normoxic untreated groups (95.59±0.6382um to 115.93±1.174 um) were significantly reduced to 58.20±3.330um to 75.95±1.866um by ischemia. The reduction in cell length resembles hypercontracture of the rod shaped cells to round shaped cells.

Non-protective effect of Insulin treatment during ischemia

The results also highlighted that the Insulin treatment did not show any protection (figure 3.3A, B and C) as predicted, in contrast with literature (Abdallah et al, 2006; Jonassen et al, 2001; Zhang et al, 2005). A possible explanation for the absence of protection with insulin in this study might be due to the low n-value, given that only 3 male wistar rats were used. With later experiments, another lab colleague did however find cardiac protective effects form insulin as described in literature (Labuschagne SW, 2015). It is also possible that the ischemic conditions used, 3mM 2DG and 10mM SDT, were too harsh, given that most of the ischemic untreated groups had a percentage viability of less than 20%. A severe ischemic insult could possibly have blocked of the insulin's tyrosine receptors due to a very low pH. The insulin protection should therefore be tested again for a shorter period of ischemic time with the same chemical ischemia, or under a longer ischemic time with a lower dose of chemical ischemia. Insulin experiments were discontinued and not tested in combination with PDE4 inhibition. No answer came from this thesis with regards to the involvement of PDE4 in insulin-mediated cardio protection, and therefore it should be re-investigated in the future with a greater n-value and milder ischemic conditions or under hypoxic conditions.

Absence of protection with b2AR, b3AR and cannabinoid receptor stimulation during ischemia

The 10uM concentration formoterol used in this project was decided based on a dose response of formoterol (10nM, 10uM and 100uM) treatment during ischemia, on survival parameters at the end of ischemia/reperfusion done by Labuschagne, SW, 2015 with the same ischemic regime as in this project. Both 10uM and 100uM formoterol was found to reduce hypercontracture and increase cell viability, but 10uM was chosen for this study because it was closer to concentrations (1nM-1uM) used in the literature (Salie et al 2011, Somvanshi et al 2014). However, no cardio protection was found with 10uM formoterol in this study, with no change in hypercontracture or cell viability compared to untreated ischemia/reperfusion (figures 3.6, 3.7 and 3.9). Under the harsh ischemic conditions used in this thesis, which reduced percentage cell survival to10.5±2.8 % (in figure 3.6C), 33.7±2.7% (figure3.7C) and 16.0±5.1% in the 'Effects of cannabinoids and b-AR agonists with rolipram during ischemia only' experiment (figure 3.9C). Maybe if 100uM formoterol was chosen, it would have exerted a cardio protective effect.

The 10uM concentration of BRL-37344 (b3AR agonist) chosen for this study is based on literature where 7uM BRL-37344 protected cardiomyocytes from injury in a model of hypoxia/reperfusion (García-Prieto et al 2014). BRL-37344 treatment without 10uM rolipram during ischemia (57.4±0.2um) had a significantly ($p \leq 0.01$) shorter cell length compared to ischemia untreated (70.8±4.2um) in figure 3.8A. No further changes in cardiomyocyte survival parameters were seen with 10uM BRL-37344 in the last experiment (figure 3.9) with BRL-37344, which was also in contrasts with the literature that implicates stimulation of this receptor with cardio protection (Aragón et al 2011,García-Prieto et al 2014). The absence of protection with yet another cardio protective agent strongly suggest a common problem, which yet again might be a function of the huge loss in viable cells, given that the range of viable cells at the end of ischemia/reperfusion was between 10.4±2.1% and 23.0±2.9 in figure 3.9C.

The 10uM concentration THC used in this project was decided based on a dose response of THC treatment during ischemia, on survival parameters at the end of ischemia/reperfusion done by another lab colleague (Dr. Samodien, E) also with the same ischemic regime as this project. it was found to increase cell viability and decrease cell contracture. However in this study, 10uM THC did not show a protection or an increase in cell length in the experiment done, as can be seen in figure 3.8A. It did however correlate with Dr E Samodien's findings when it decreased the total R/G fluorescence intensity in figure 3.8B. In the following experiment (figure 3.9A) it did have a significant increase in cell lengths but the R/G fluorescence intensity did not show any differences (figure 3.9B). These fluctuations might yet again be a function of the huge loss in viable cells, given that the range of viable cells at the end of ischemia/reperfusion was between 10.4±2.1% and 23.0±2.9%.

The 10nM HU210 concentration used in this project was also decided based on a dose response of HU210 treatment during ischemia, on survival parameters at the end of ischemia/reperfusion done by Dr. E Samodien. The mitochondrial viability (figure3.6B) showed that the HU210 administered during ischemia had a significantly ($p \leq 0.05$) increased value ischemia (1.5±0.0) when compared to ischemia untreated (1.0±0.1), but this could not be seen in the following experiments (figures 3.8 and figures 3.9) for any of the parameters (hypercontracture, mitochondrial membrane potential and cell viability). This is yet again in contrast with the literature found for HU210 in ischemia/reperfusion (Krylatovet al, 2001).

Absence of damage exacerbation with B1 stimulation during ischemia

A dose range of 1uM, 10uM and 100uM dobutamine was administered during normoxia and ischemia and showed no effect on any of the survival parameters (figure 3.4). In view of this result a concentration of 10uM dobutamine was chosen given that 1uM dobutamine was used by Wang et al (2014) to pre-treat rats in vivo before ischemia/reperfusion. Incidentally in this study dobutamine exerted cardio protection in the form of reduced infarct size. On the other hand Pantos et al (2006) found that dobutamine treatment during reperfusion enhanced functional recovery.

One study that implicated b1AR in ischemia/reperfusion damage did not make use of dobutmine or any b-AR agonist, but instead used a b1AR antagonist to subtract b1AR stimulation during ischemia against a background of norepinephrine in the whole heart (Spear et al 2007). However, the absence of any enhancement of ischemic injury was confirmed in the laboratory by other lab colleagues in their studies, which shows consistency and confidence in the data. It is therefore possible that the ischemic insult exerted maximum damage, making it impossible for dobutamine to enhance hypercontracture or cell death. We decided not to use 1uM to determine if a higher concentration would elicit the damaging outcome as expected but still did not see any changes.

Isoproterenol during normoxic treatments did not show a difference in the concentrations tested, and a decision was made to use the 100nM concentration as this is what literature suggests as well (Zhou et al, 2001;Leroy et al, 2011). The 100nM isoproterenol administered during ischemia only showed a significant difference when compared to untreated ischemia, for mitochondrial membrane potential in figure3.6B, but not in cell length and cell viability. The experiment in figure 3.9 did not produce the same results. Like with the dobutamine, this result was confirmed in the laboratory in the studies of other lab colleagues, and might also not enhance cell damage because maximum damage might have already been achieved with the harsh ischemic conditions used. Yet again in these experiments the average percentage viable cells for all ischemic treatments were 16.9±5.8% (figure 3.5C), 10.5±2.8% (figure 3.6C) and 16.0±5.1% (figure 3.9C).

In figure3.6D a screening experiment was done to measure the cAMP production at the basal level in the untreated cardiomyocytes. This showed low basal levels of cAMP and remained so even in the presence of PDE4 inhibition with rolipram. A slight increase by total PDE inhibition with IBMX was monitored but no statistical analysis was done due to a low n-value of 2. 100nM Isoproterenol did not indicate a production of cAMP but did however increased 16 fold when combined with PDE4 inhibition (Iso + Rol) and 35 fold with total PDEs inhibition (Iso + IBMX). This shows that the absence of survival changes in the presence versus absence of these drugs is not due to faulty drugs.

No effect of Rolipram on cardiomyocytes

For this project the 10uM rolipram concentration was chosen as literature also used 10uM rolipram in their experiments (Bethke et al, 1992; Georget et al, 2003; De Arcangelis et al, 2008; Qvigstad et al, 2010; Beca et al, 2011; Leroy et al 2011; Parks et al, 2014). As most of these experiments were done with the cardiac tissue or whole heart perfusions at normal physiological functions. For the project we hoped to see a further damaging effect when rolipram was applied, this would have indicated a protective mechanism in which PDE4 does play a role during protection against ischemia. But the results would still determine whether PDE4 inhibtion during an ischeamic event would contribute to further damage or protection. Unfortunately rolipram did not show any differences during the normoxia treatments (figure 3.3). Even with rolipram ischemic pre-treatment and pertreatment combined, there was no effect on the poor cardiomyocyte survival (figure 3.3; 3.6; 3.7; 3.8 and 3.9) found. With all the treatments given and the combination therapy treatment of the different treatments with rolipram, only one treatment (10nM HU210 combined with rolipram) showed a significant decrease in cell hypercontracture (figure 3.8A), but this could not be replicated again with the following experiment (figure 3.9A).

The graph of R/G fluorescence ratio (figure3.6B) showed a significant increase with a $p \leq 0.01$ in mitochondrial viability when 100nM Isoproterenol combined with rolipram during ischemia was compared to the ischemia untreated group. The separate treatments of isoproterenol and rolipram during ischemia did not show any differences when compared to ischemia untreated, but when combined during ischemia, isoproterenol and rolipram significantly ($p \leq 0.01$) improved mitochondrial viability. This could not be supported by the following experiments in figures 3.8 and figures 3.9.

Chapter 5: Recommendations and conclusion

Pitfalls in this study and recommendations for future work

The ischemic conditions were considered successful in inducing sufficient ischemia/reperfusion damage because untreated ischemia/reperfusion consistently reduced cell length significantly to show hypercontracture and reduced cell survival percentages approximately 4 fold to 20%. Yet, all the cardio protective treatments failed to elicit protection while damage could not be enhanced by b1AR stimulation with dobutamine or isoproterenol. Similar results were found by another MSc student (Botha A) who could not consistently find protection with insulin and formoterol, while b1AR stimulation with dobutamine and isoproterenol failed to enhance ischemia/reperfusion injury (data not shown). Collectively this data might suggest that there may have been a fundamental flaw in the simulated ischemia/reperfusion model used, which might be the ischemic conditions that were too harsh for any of the drugs to alter survival or damage in the cardiomyocytes.

The ischemic conditions of 3mM 2DG and 10mM SDT treatment for 20 minutes were chosen based on simulated ischemia/reperfusion times and doses optimized by an MSc student in the laboratory (Labuschagne SW, 2015), who found cardio protection with insulin, formoterol and BRL-37344 and no changes in survival parameters with isoproterenol and dobutamine (data not shown). The ischemic conditions might be dependent on the researcher, and should therefore be optimized for each researcher. Not all researchers will handle the cells in an identical manner, and this might result in different total numbers of cells remaining attached to the culture surface at the end of reperfusion. With more cells remaining behind at the end of the experiments for microscopy evaluation, a more accurate assessment can be made of the drug effects.

The main rationale for keeping the ischemic doses and times identical for all students in the laboratory was so that all the work formed part of one main project that investigates the role PDEs in cardio protection and damage, which was thought, would make the data comparable. Instead all students did not consistently find the same data, except for the lack of effects with dobutamine and isoproterenol. Thus as

long as each researcher get to the final end point of an ischeamic event one should consider to optimize the protocol of mimicking the ischemia accordingly.

It might have been more expedient to have used a shorter time of ischemia for the ischemic chemical conditions used in this thesis. Alternatively, given that SDT reduce oxygen levels in solution (Bright et al, 1992; Egar et al 2014), SDT rather than 2DG should be reduced and ischemia optimized for a longer duration. This would ensure that the development of pathology occur slower during ischemia, which could allow proper evaluation of the drugs tested here. SDT should specifically be considered, given that it is largely responsible for the ischemic effect due to its ability to reduce oxygen in solution (Bright et al, 1992; Egar et al 2014).

Hypoxia should also be considered instead of chemical induced ischemia, given that hypoxia is more commonly used in the literature (Diaz et al, 2006), while only a few publications made use of SDT. Search terms with sodium dithionite+cardiomyocytes in Pubmed delivered only 7 publications, while an additional 2 were found with the search terms sodium hydrosulfite, while hypoxia+cardiomyocytes delivers 2200 publications. A low frequency of publications on the use of SDT might indicate that it is not a method that works well, which might be the change in the potency of SDT over time from the time point that it is prepared in solution. The latter observation was made during the project, where SDT would rapidly bleach phenol red in the M199 based experimental buffer when freshly made SDT was added, while this bleaching effect occurred slower when SDT was used later in the day. Thus, it seems that SDT might be very unstable and its use from the point of preparation to use on the cells should preferably be kept constant in future experiments. Yet, if this drug is unstable it would be more appropriate to use hypoxia instead of simulated ischemia, especially because in a hypoxia model pathologies develop very slowly, normally over a time frame of hours (Kang et al, 2000).

Interestingly, in experiments where cardiomyocytes were preloaded with fluo4 and JC-1 to measure intracellular calcium and MPTP opening respectively, these fluorescent labels were bleached at the end of ischemia (data not shown). This is similar to the bleaching effect that SDT has on phenol red in the M199 based experimental medium used, when SDT was add to prepare ischemic buffer. SDT therefore limits the use of fluorescent markers during ischemia, which prevents the

measurement of some critical markers such as intracellular calcium, pH and nitric oxide. In this regard, a model of hypoxia would also be more appropriate for the measure of these parameters given that the fluorescent labels should be preserved, given their use in hypoxia (Lu et al, 2010; Perry et al, 2011; Sun et al, 2004; Xiao et al, 2011).

Looking at the fluorescence intensity results, it may appear to be inconsistent, possible reason for this might be due to the JC-1 staining. The red fluorescence of the stain on the cells fades quickly and is thus important to capture the images as quickly as possible. Our experiments were performed in sterile black costar 96-well plates. This means 96 photos needed to be taken, which takes time. This might be a possible reason for the inconsistencies found. Also the JC-1 stain is very light sensitive (precaution was used not to disrupt the staining) and it might not have been properly dissolved. This is possible explanations but every experiment was performed with caution not to alter the results obtained.

For future research, it would be interesting to test PDE4's role in ischemia by still investigating the cellular level (cardiomyocytes) through cardiomyocytes contraction and apoptosis, but cAMP measurements can also be taken, looking at the pH during ischemia as well as the calcium levels. Glucose uptake can also be measured. Finally this investigation can also look at whole heart perfusions with global and regional ischemia experiments.

Conclusion

Rolipram was used to inhibit PDE4 to determine whether a difference in survival parameters would occur when combined with b1AR, b2AR, b3AR, insulin, THC and HU210 signalling pathways. 10uM THC, 10nM HU210 and 100nM isoproterenol did indicate differences but could not be supported with follow up experiments. In this view, no difference in protective effects could be elicited, nor could pathology be worsened with any of the drug treatments. This is thought to be a consequence of the extremely harsh ischemic conditions used. For the final conclusion of this study: PDE4 may not be able to alter protection or damaging pathways in an isolated cardiomyocyte model of severe ischemia.

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