PHENYLEPHRINE PRECONDITIONING OF ISOLATED VENTRICULAR MYOCYTES INVOLVES MODULATION OF K_{ATP} CHANNELS THROUGH ACTIVATION OF SURVIVAL KINASES

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by

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Phenylephrine preconditioning of isolated ventricular myocytes involves modulation of K_{ATP} channels through activation of survival kinases

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Pretreatment with the α_1-adrenoceptor agonist phenylephrine has been shown to protect cardiac tissue from a subsequent period of ischaemia. This phenomenon, known as pharmacological preconditioning, decreases infarct size and increases the functional recovery of the intact heart. The role of protein kinases and ATP-sensitive potassium channels (K_{ATP}) in phenylephrine preconditioning was investigated in isolated ventricular myocytes using whole cell patch clamp, western blotting, fluorescence imaging and measurement of contractile activity. The role of PKC isoforms was examined using isoform-specific PKC activator and inhibitor peptides.

Preconditioning with phenylephrine increased the contractile recovery of isolated ventricular myocytes following simulated ischaemia and reperfusion, and this was also correlated with an improvement in calcium homeostasis during reperfusion. The protective effect of phenylephrine preconditioning on contractile recovery was abolished by inhibition of α_1-adrenoceptors, PKC or K_{ATP}. We observed a sustained activation of PKCε and δ in response to phenylephrine preconditioning, and identified a protective role for PKCε. PKCδ was required for phenylephrine preconditioning but also increased reperfusion injury. CaMKK, AMPK and p38 MAPK were all required for phenylephrine preconditioning, and activation of AMPK by phenylephrine preconditioning required PKCδ. However, both JNK and ERK appear not to be involved in the protective effect.

Inhibition of sarcolemmal K_{ATP} channels (*sarc*K_{ATP}) or mitochondrial K_{ATP} channels (*mito*K_{ATP}) prevented preconditioning. Peak *sarc*K_{ATP} current activated by metabolic inhibition was increased following phenylephrine preconditioning and inhibition of PKCδ, AMPK or p38 MAPK was sufficient to prevent the increase in current.

Increased *sarc*K_{ATP} current can enhance hyperpolarisation of the resting membrane potential, and may be responsible for the observed decrease in calcium loading and improved contractile recovery of phenylephrine preconditioned ventricular myocytes following ischaemia and reperfusion.

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Abbreviations

5-HD	5-hydroxydecanoic acid
ABC	ATP-binding cassette protein
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AK	adenylate kinase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANT	adenine nucleotide translocase
APD	action potential duration
ATP	adenosine triphosphate
BK_{Ca}	large conductance calcium-activated potassium channel
BSA	bovine serum albumin
CaMK II	Calmodulin-dependent kinase II
CaMKK	Calmodulin-dependent kinase kinase
CC	compound C
CFTR	cystic fibrosis transmembrane regulator
CHD	coronary heart disease
Chel	chelerythrine
CICR	calcium-induced calcium release
CK	creatine kinase
CON	Control
CSAID	Cytokine-suppressive anti-inflammatory drug
DAG	diacylglycerol
DNP	dinitrophenol
DTT	dithiothreitol
E-C coupling	excitation-contraction coupling
ECG	electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ERK	extracellular signal-regulated kinase
GDP	guanosine diphosphate

Glib	glibenclamide
GLUT	Glucose transporter protein
GPCR	G-protein coupled receptor
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IP_3	inositol 1, 4, 5-trisphosphate
IP_3R	IP_3 receptor
IPC	ischaemic preconditioning
JIP	JNK-interacting protein
JNK	c-Jun NH_2-terminal kinase
K_{ATP}	ATP-sensitive potassium channel
LDH	lactate dehydrogenase
LVDP	left ventricular developed pressure
LVEDP	left ventricular end diastolic pressure
MAPK	mitogen-activated protein kinase
MAPKAPK2	MAPK-activated protein kinase 2
MAPKKK	MAPK kinase kinase
MAPKK	MAPK kinase
MI	metabolic inhibition
MitoK_{ATP}	mitochondrial ATP-sensitive potassium channel
MPG	mercaptopropionyl glycine
MPTP	mitochondrial permeability transition pore
NADH	nicotinamide adenine dinucleotide
NBD	nucleotide binding domain
NCX	Sodium/calcium-exchanger
NHE	Sodium/hydrogen-exchanger
NO	nitric oxide
p38	p38 MAPK
PAGE	polyacrylamide gel electrophoresis
PD	PD98059
PDD	phorbol 12, 13-didecanoate
Phe	phenylephrine
PHE-PC	phenylephrine preconditioning
PKC	protein kinase C

PLC	phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonyl fluoride
Pra	prazosin
RACK	receptor for activated C kinase
RISK	reperfusion injury salvage kinase pathway
RMP	resting membrane potential
ROS	reactive oxygen species
RyR	ryanodine receptor
SAPK	stress-activated protein kinase
$SarcK_{ATP}$	sarcolemmal ATP-sensitive potassium channel
SB	SB202190
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SERCA	sarcoplasmic/endoplasmic reticulum calcium-ATPase
SF	substrate-free Tyrode
SP	SP600125
SR	sarcoplasmic reticulum
STO	STO-609
SUR	sulphonylurea receptor
SWOP	second window of protection
TAB1	TAK1-binding protein
TAK1	transforming growth factor β-activated kinase
TBST	Tris buffered saline 0.01% tween-20
TnC	Troponin C
TnI	Troponin I
T-tubules	transverse tubules
TUNEL	Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labelling
VDAC	Voltage activated anion channel
ψRACK	pseudoRACK

Contents

1 Introduction

1.1 Coronary Heart Disease

Coronary heart disease (CHD) is a disease of the blood vessels of the coronary circulation, most commonly due to a build up of fatty deposits within atherosclerotic plaques in the walls of blood vessels that cause narrowing of the vessels. Myocardial infarction or heart attack occurs when a narrowed region of a coronary artery is occluded preventing blood flow to a region of the heart (DeWood *et al.*, 1980). The inadequate perfusion of the tissue that ensues (myocardial ischaemia), causes a reduction in the levels of oxygen and metabolic substrates and accumulation of metabolites, leading to decreased cellular energy levels, increased intracellular pH and free radical production (Allen and Orchard, 1987). Ischaemia causes ionic dysregulation resulting in impaired contractile function, and prolonged ischaemia can cause death of cardiac myocytes. As the heart is required to maintain adequate perfusion of the tissues of the body, significant impairment of contractile function of the heart even for a period of a few minutes is potentially fatal and may result in significant morbidity to the individual. Myocardial ischaemia is also responsible for long term structural changes to the heart. These include fibrosis due to replacement of lost cells with fibroblasts and changes in the protein expression that may result in cardiac hypertrophy or heart failure (Giordano, 2005, Swynghedauw, 1999).

CHD represents a major cause of mortality. In 2005, an estimated 7.6 million deaths (or ~13% of total deaths) worldwide were caused by CHD (World Health Organisation, 2007). In the UK, 94,000 deaths (~16% of total deaths in 2006) were as a result of CHD (Allender *et al.*, 2008). CHD also results in significant morbidity; an estimated 1.4 million people in the UK have suffered a myocardial infarction and an estimated 1.98

million people in the UK have suffered from Angina pectoris, caused by narrowing of coronary vessels leading to painful transient ischaemic attacks (Allender *et al.*, 2008). There are also major economic costs associated with CHD; as it is estimated that CHD cost the UK health care system £3.2 billion in 2006, and that the total cost to the UK economy was £9 billion (Allender *et al.*, 2008).

1.2 Ischaemia and reperfusion

1.2.1 Ischaemia

Cardiac ischaemia is characterised by reduced coronary flow. This leads to insufficient perfusion of the heart tissue, and a consequent decrease in the delivery of O_2 and metabolic substrates, and decrease in extraction of waste products such as lactate and H^+ from the tissue. As the site of oxidative phosphorylation, the mitochondria play an important role in providing for the high energy demands of contracting ventricular myocytes. Some of the effects of ischaemia on energy levels, ionic homeostasis and contractile function are summarised in Figure 1.1.

The mitochondria are the site of the electron transport chain. Electrons are transferred from the flavoproteins $NADH/H^+$ and $FADH_2$, through the respiratory complexes of the electron transport chain, to the final electron acceptor O_2. The flow of electrons through the chain is linked to pumping of H^+ into the mitochondrial matrix, producing the proton gradient and mitochondrial membrane potential responsible for ATP synthesis by the F_1F_0 ATP synthase complex. O_2 acts as the final electron acceptor in the electron transport chain; therefore depletion of O_2 leads to inhibition of oxidative phosphorylation, dissipation of the proton gradient and mitochondrial membrane potential, and inhibition of ATP production (Hüttemann *et al.*, 2008). Furthermore, the mitochondria can actually consume ATP through reversal of the ATP-synthase under

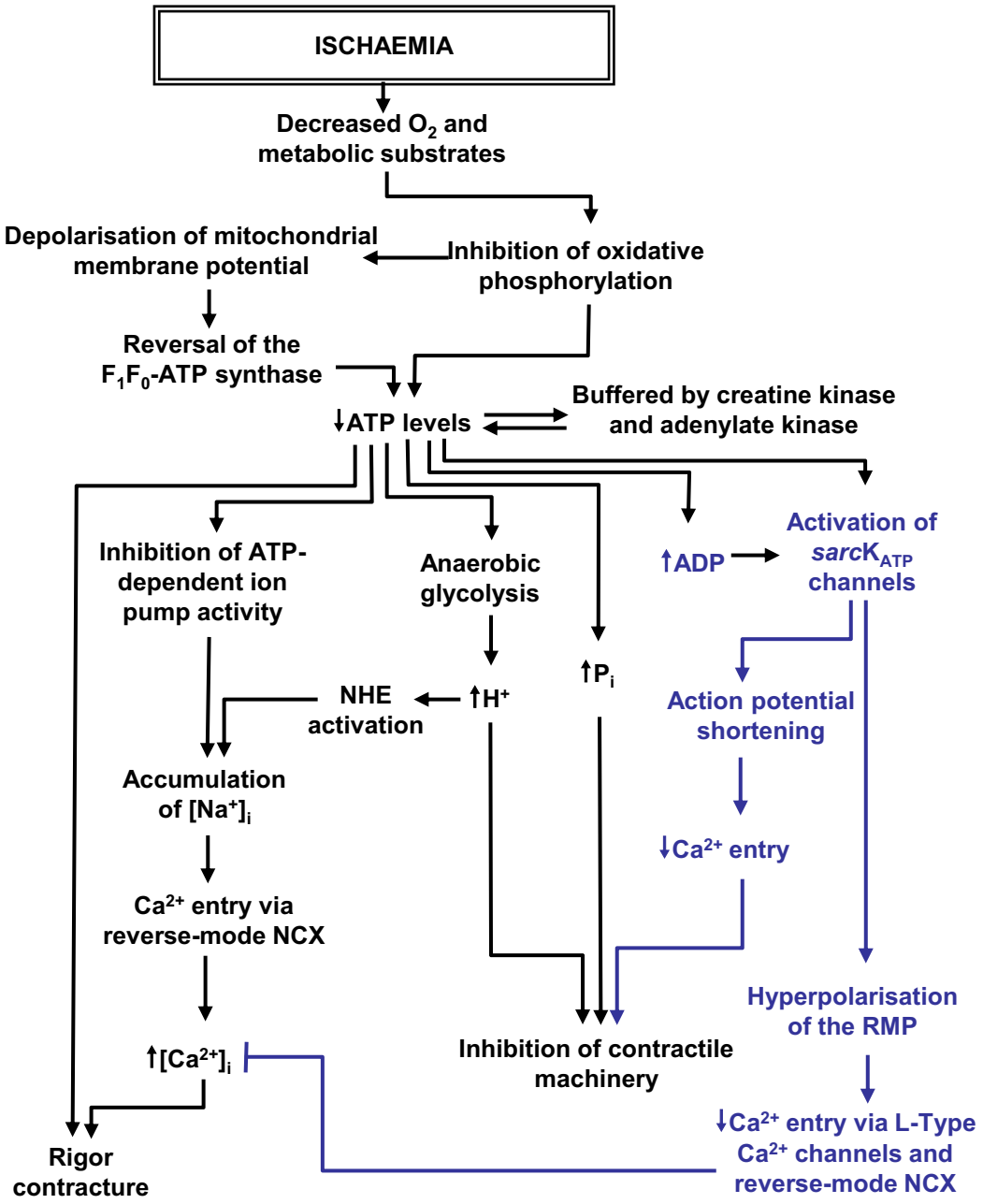

Figure 1.1. Effects of ischaemia on energy levels, ionic homeostasis and contractile function.

Ischaemia causes a decrease in delivery of O_2 and metabolic substrates to the heart. This causes inhibition of the electron transport chain followed by inhibition of the F_1F_0-ATPase, depolarisation of the mitochondrial membrane potential, and reversal of the F_1F_0-ATPase resulting in consumption of ATP. A decrease in ATP levels then leads to anaerobic glycolysis producing lactate and H^+, inhibits ATPases such as the Na^+/K^+-ATPase, and increases ADP and inorganic phosphate (P_i). Sarcolemmal ATP-sensitive K^+ channel (*sarc*K_{ATP}) opening and a consequent decrease in Ca^{2+} entry, as well as increased H^+ and P_i lead to inhibition of the contractile machinery. Inhibition of the Na^+/K^+-ATPase and activation of the NHE by H^+ leads to accumulation of Na^+, and subsequent Ca^{2+} entry via the NCX. Low ATP and high Ca^{2+} also lead to rigor contracture. Processes shown in blue are likely to be protective.

conditions where depolarisation of the mitochondrial membrane potential occurs, such as during cardiac ischaemia (Jennings *et al.*, 1991). Anaerobic glycolysis can produce ATP under these conditions resulting in a build up of lactate and H^+. However, this is not sufficient to meet the needs of the ventricular myocyte and some ATP is required for anaerobic respiration to take place, therefore this too is quickly prevented by the depletion of ATP.

The concentration of ATP within the cell is tightly controlled via several mechanisms. Adenylate kinase catalyses the reversible reaction of two molecules of ADP to form ATP and AMP (Carrasco *et al.*, 2001). The creatine kinase pathway allows the reversible transfer of high–energy phosphates from ATP to creatine to form phosphocreatine within areas of ATP production, primarily the mitochondria. Phosphocreatine is delivered to areas of energy utilisation, where the reverse transfer of phosphates results in regeneration of ATP from ADP (Wallimann *et al.*, 1998). During ischaemia, it would be expected that the ATP levels would fall rapidly, however ATP is buffered by creatine kinase, leading to a large reduction in phosphocreatine levels with a relatively small reduction in ATP (Allen and Orchard, 1987).

Adenylate kinase and creatine kinase physically associate with cardiac sarcolemmal ATP-sensitive K^+ channels (*sarc*K_{ATP}) (Carrasco *et al.*, 2001, Crawford *et al.*, 2002). *Sarc*K_{ATP} channels are functionally coupled to mitochondrial ATP production via the creatine kinase pathway (Sasaki *et al.*, 2001). ATP consumption brought about by mitochondrial depolarisation leads to a decrease in the concentration gradient driving delivery of phosphocreatine to the subsarcolemmal region, and a potential reversal of the direction of phosphocreatine shuttling, which then leads to rapid activation of *sarc*K_{ATP} channels. Knock out of creatine kinase increased the sensitivity of the *sarc*K_{ATP} channel to metabolic stress causing an increase in channel activation

(Abraham *et al.*, 2002). In addition, the protective effect of IPC on ATP turnover, phosphocreatine levels and creatine kinase activity following ischaemia and reperfusion was abolished by knock out of the Kir6.2 subunit of *sarc*K_{ATP} channels (Gumina *et al.*, 2003).

The cardiac action potential is the stimulus for contraction of the ventricular myocyte, and changes in the amplitude and duration of the action potential can alter the magnitude of contraction (Allen and Orchard, 1987). The resting membrane potential in cardiac myocytes is maintained by the inward-rectifier K^+ current I_{K1} (Kir2.1), in concert with ionic transporters. The action potential is initiated by depolarisation of sinoatrial node cells, and a wave of depolarisation then travels through the heart via gap junctions between myocytes (Berne and Levy, 2000). When the wave of depolarisation arrives at a myocyte, it activates voltage-gated Na^+ channels leading to a rapid influx of Na^+ that is responsible for the upstroke of the action potential (see Figure 1.2B). This influx is quickly curtailed by rapid inactivation of voltage-gated Na^+ channels and a decrease in driving force for Na^+ entry as the membrane potential approaches the equilibrium potential for Na^+. Depolarisation also results in a decrease in I_{K1}. Phase 1 repolarisation occurs due to inactivation of Na^+ channels, as well as activation of the K^+ current I_{to} in some cells. The plateau of the action potential is dependent on a balance between Ca^{2+} entry and K^+ efflux from the ventricular myocyte. Phase 3 repolarisation occurs through inactivation of voltage-gated Ca^{2+} channels and increased K^+ efflux via voltage-gated K^+ channels. As the action potential begins to repolarise I_{K1} is also increased, restoring the membrane potential to the resting level (Michael *et al.*, 2009).

Under resting conditions where intracellular Na^+ is low and the membrane potential is negative, the Na^+/Ca^{2+} exchanger (NCX) acts in forward mode causing influx of Na^+ and efflux of Ca^{2+}. Increased intracellular Na^+ levels and membrane depolarisation lead

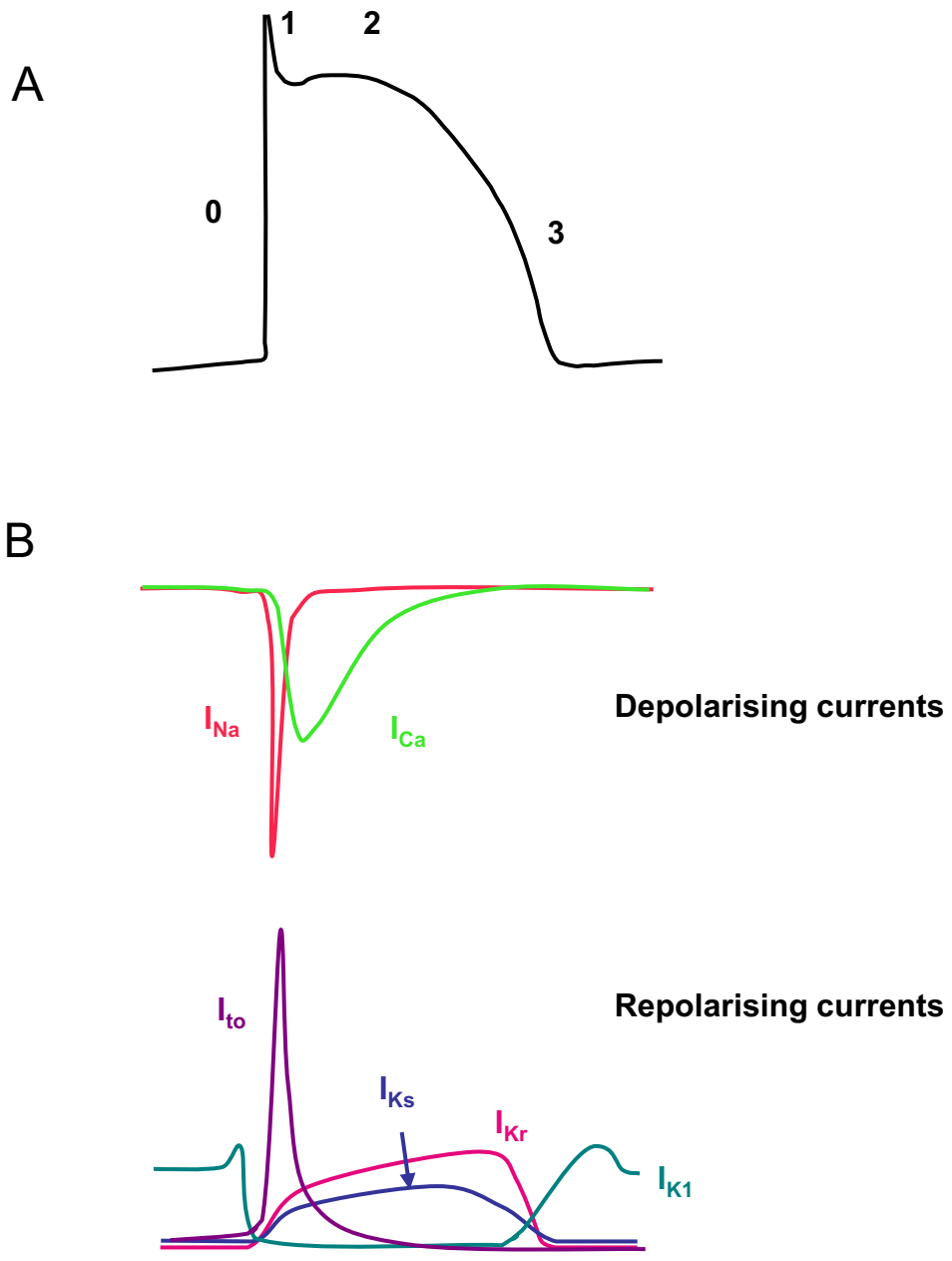

Figure 1.2. Currents underlying the cardiac action potential.

A. Diagram showing the cardiac action potential. The numbers denote the phases of the action potential. 0- upstroke of the action potential, 1- early repolarisation, 2- action potential plateau, 3- final repolarisation.

B. Schematic showing qualitative changes in depolarising Na^+ and Ca^+ currents, and repolarising K^+ currents during the action potential.

to a reversal of the NCX, which contributes to Ca^{2+} entry during the action potential plateau (Sher *et al.*, 2008, Zhang *et al.*, 2001), although the magnitude of Ca^{2+} entry and the timing of the reversal are still under debate. Ca^{2+} entering the myocyte during the plateau of the action potential is then able to activate ryanodine receptors (RyR) to trigger Ca^{2+}-induced Ca^{2+} release (CICR) from the stores in the sarcoplasmic reticulum (SR); allowing the large, rapid increase in intracellular Ca^{2+}, or systolic Ca^{2+} transient, that activates the contractile machinery of the ventricular myocyte (Eisner *et al.*, 2000).

Ca^{2+} must be removed from the cytosol to allow relaxation of myofilaments. Several mechanisms exist for removal of Ca^{2+} from the cytosol; the NCX and sarcolemmal Ca^{2+}-ATPase remove Ca^{2+} to the extracellular space, the mitochondrial Ca^{2+}-uniporter leads to mitochondrial Ca^{2+} uptake, and the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) removes cytosolic Ca^{2+} and refills the SR Ca^{2+} stores (Bers, 2002).

During hypoxia, the gating properties of voltage-gated Na^+ channels are altered leading to a persistent Na^+ current that shows impaired inactivation (Hammarstrom and Gage, 2002). This leads to a large influx of sodium during ischaemia. In addition, ATP-dependent ion transporters such as the Na^+/K^+-ATPase and SERCA are major consumers of ATP within the ventricular myocyte (Bers, 2008), so any decrease in ATP levels within the cell reduces the activity of these transporters and rapidly results in a disturbance of the ionic homeostasis. Na^+ entry through persistent Na^+ channels and decreased Na^+/K^+-ATPase activity causes accumulation of Na^+ within the cytosol which can result in reversal of the NCX and Ca^{2+} loading. Decreased intracellular pH, decreased ATP, and increased ADP levels can also inhibit the RyR (Xu *et al.*, 1996). Inhibition of the ATP-dependent SERCA prevents SR Ca^{2+} uptake leading to increased cytosolic Ca^{2+} levels and depleted SR Ca^{2+} stores (Eisner *et al.*, 2000). The depletion of SR Ca^{2+} reduces the amount of Ca^{2+} available for release from the SR via RyRs.

Decreased ATP levels can also affect K^+ channels. *Sarc*K_{ATP} channels are hetero-octameric channels capable of coupling metabolic status to cellular excitability. *sarc*K_{ATP} channels open under conditions of low ATP producing an outward K^+ current (Noma, 1983). It is well established that opening of *sarc*K_{ATP} channels during ischaemia is cardioprotective however the mechanisms involved in the cardioprotective effect of *sarc*K_{ATP} channel opening are still subject to debate and will be covered in section 1.6.

The contractile machinery has been estimated to be responsible for up to 60% of total ATPase activity (80% of the Ca^{2+}-activated ATPase activity) in the ventricular myocytes in the rat (Ebus and Stienen, 1996). Therefore actin-myosin cross-bridge cycling is not only inhibited by ATP depletion but also directly contributes to ATP depletion. The inhibition of ion transporters by ischaemia results in failure of the cardiac action potential and contractile failure. Shortly after contractile failure, inhibition of cross-bridge cycling causes cross-bridges to become locked in the shortened form and the development of rigor contracture (Hearse *et al.*, 1977).

1.2.2 Reperfusion

Reperfusion is necessary for the survival of ischaemic tissue; however, reperfusion may itself cause significant cellular injury. One commonly identified histological feature of hearts damaged by reperfusion injury is the presence of ‘contraction band necrosis’. Characterised by hypercontraction of sarcomeres and rupturing of the sarcolemmal membrane, contraction band necrosis is thought to occur through excessive activation of the contractile machinery during early reperfusion (Piper *et al.*, 2004, Altschuld *et al.*, 1985). In single cells this is seen as a hypercontracture of the cells. Some of the effects of reperfusion are summarised in Figure 1.3.

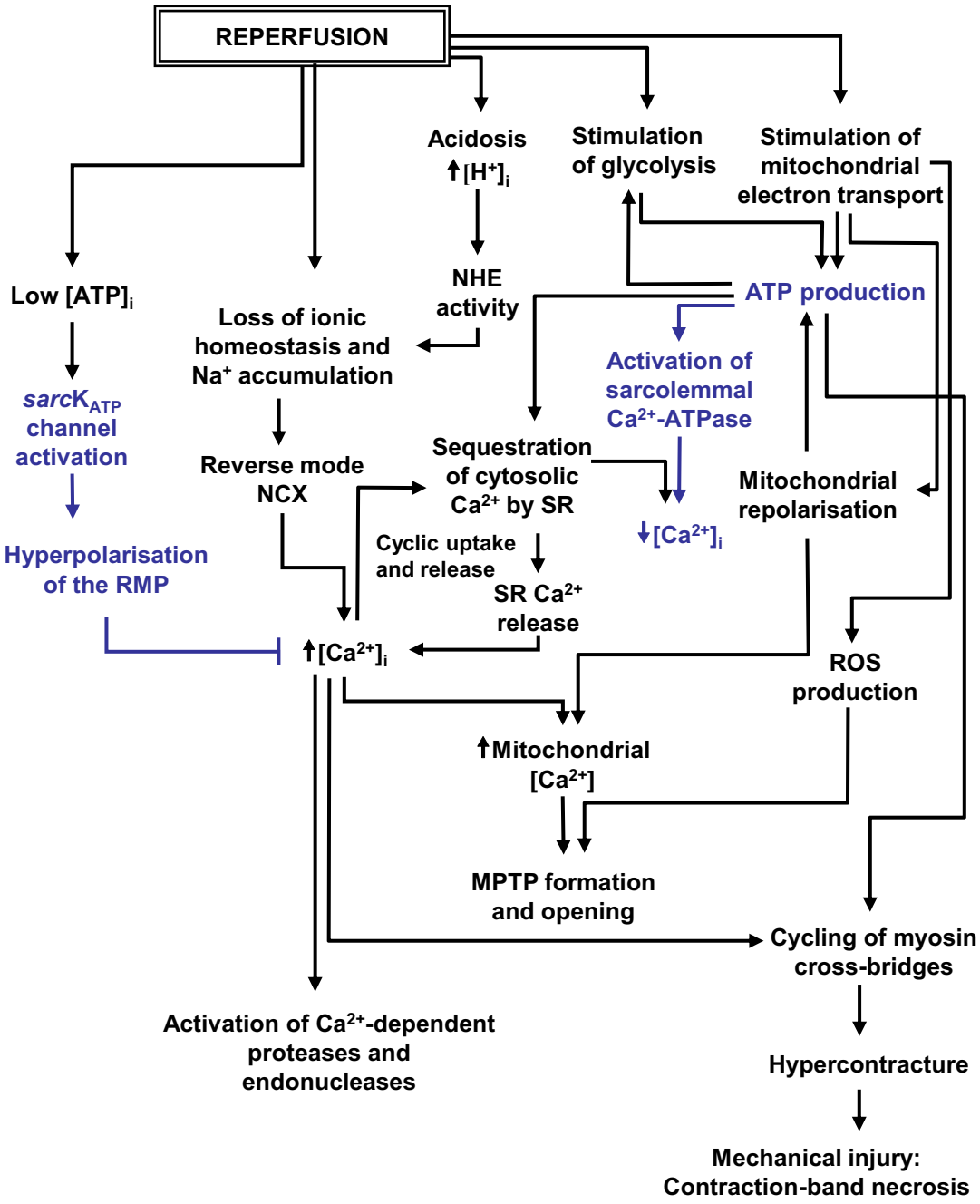

Figure 1.3. Consequences of reperfusion.

During ischaemia, the ATP levels are depleted, H^+ accumulates and the ionic homeostasis is disturbed causing accumulation of Na^+ and Ca^{2+}. Reperfusion leads to re-energisation of the mitochondria resulting in ATP production and mitochondrial repolarsation. Reverse-mode NCX and cyclic uptake and release of Ca^{2+} by the SR leads to increased intracellular Ca^{2+} ($[Ca^{2+}]_i$), which along with ROS production, low ATP and high inorganic phosphate levels causes opening of the mitochondrial permeability transition pore (MPTP). High $[Ca^{2+}]_i$ levels can also activate Ca^{2+} dependent enzymes such as proteases and endonucleases. Increasing ATP levels during ischaemia while $[Ca^{2+}]_i$ is still high leads to uncontrolled activation of the contractile machinery, resulting in hypercontracture and contraction-band necrosis. Processes in blue are likely to be protective.

During reperfusion, ischaemic tissue undergoes re-oxygenation, leading to re-initiation of mitochondrial electron transport and ATP production by oxidative phosphorylation in surviving cells. Although re-energisation of the mitochondria is needed for recovery of cellular function, it may activate several damaging pathways including Ca^{2+}-overload induced contracture and rigor contracture (Altschuld *et al.*, 1985, Vander Heide *et al.*, 1986), formation and opening of the mitochondrial permeability transition pore (MPTP) (Griffiths and Halestrap, 1995), and apoptotic cell death (Gottlieb *et al.*, 1994, Gottlieb and Engler, 1999). The timecourse of re-energisation of the cell may in fact alter the fate of a cell exposed to ischaemia-reperfusion. During ischaemia, the ATP levels of the cell are depleted and accumulation of Na^+ occurs within the cytosol. The increase in H^+ concentration that occurs during ischaemia also contributes to Na^+ accumulation, through the action of the Na^+/H^+ exchanger (NHE), which reduces cellular acidosis by extruding H^+ and pumping Na^+ into the cell (Avkiran *et al.*, 2001, Xiao and Allen, 1999). Some studies have shown the NHE to be inhibited by the high extracellular H^+ levels during ischaemia (Xiao and Allen, 1999), but other groups suggest that the NHE is active during ischaemia (Avkiran *et al.*, 2001, Murphy *et al.*, 1999). Na^+ accumulation and depolarisation of the cell membrane can result in uptake of Ca^{2+} via reverse mode NCX activity, leading to Ca^{2+}-loading of the myocytes (Schafer *et al.*, 2001).

Upon reperfusion, the cell may rapidly recommence ATP production, resulting in activation of ion transporters to restore the ionic balance within the cell. However, the recovering ATP levels also cause activation of the contractile machinery within the cell, often before the Ca^{2+} overload within the cell has been relieved, triggering hypercontracture and contraction-band necrosis through tearing of the sarcolemmal membrane (Ganote, 1983). Uptake of cytosolic Ca^{2+} into the SR and its subsequent

release due to opening of RyRs leads to rapidly oscillating Ca^{2+} levels that result in uncontrolled activation of the contractile machinery (Schafer *et al.*, 2001, Siegmund *et al.*, 1997). Disturbances in Ca^{2+} homeostasis during reperfusion can also result in arrhythmias (Takamatsu, 2008, Yellon and Downey, 2003).

Interventions that reduce the Ca^{2+}-loading of the cell during ischaemia and/or early reperfusion have been shown to improve recovery after metabolic inhibition (MI) and reperfusion (Rodrigo and Standen, 2005, Baczkó *et al.*, 2003). Inhibition of the NHE, although increasing cellular acidosis, increases survival of cardiac myocytes (Xiao and Allen, 1999, Baczkó *et al.*, 2008, Allen and Xiao, 2003) by preventing the Na^{+} accumulation that results in Ca^{2+} overload via activation of the NCX in reverse mode (Baczkó *et al.*, 2003, Inserte *et al.*, 2002, Takahashi *et al.*, 2003). Activation of *sarc*K_{ATP} channels during ischaemia and reperfusion also contributes to cell survival via a mechanism thought to involve a decrease in intracellular Ca^{2+}-loading (Baczkó *et al.*, 2004, Light *et al.*, 2001, Rainbow *et al.*, 2004). In support for this mechanism, Jovanovic found that co-transfection of *sarc*K_{ATP} channel subunits Kir6.2 and SUR2A (Jovanović *et al.*, 1998b) or Kir6.2 and SUR1 (Jovanović *et al.*, 1998a) into COS7 cells, conferred resistance to hypoxia-reoxygenation injury by reducing intracellular Ca^{2+}-loading.

Under conditions where the ATP production recovers more slowly upon reperfusion, the myocytes are at risk of rigor contracture. This is common after extended periods of ischaemia when the mitochondria are more likely to suffer damage and ATP production is reduced. Rigor contracture occurs when the ATP levels within the cell are low, but not zero. During ischaemia there is only a short period within which rigor contracture can develop (Hearse *et al.*, 1977, Altschuld *et al.*, 1985). During reperfusion, there may

be a much more extended period of low ATP, resulting in a more pronounced contracture than that seen during ischaemia (Piper *et al.*, 2006).

The mitochondria, as the site of ATP production are vital for cell survival. Under pathological conditions, these organelles may become the site of induction of necrotic and apoptotic cell death (Gustafsson and Gottlieb, 2008). One of the mechanisms responsible for mitochondria-induced cell death is opening of the MPTP. The molecular identity of the MPTP is still under some debate, though it seems clear that cyclophilin D is necessary for pore formation (Lim *et al.*, 2007), possibly in combination with the adenine nucleotide translocase (ANT) and voltage-activated anion channel (VDAC) (Lim *et al.*, 2007, Halestrap *et al.*, 2002, Hausenloy *et al.*, 2009). Under normal physiological conditions, the MPTP is kept closed, but is opened in response to a specific set of pathological conditions including mitochondrial depolarisation, mitochondrial Ca^{2+} overload, low adenine nucleotide levels, high phosphate levels, and ROS (Halestrap *et al.*, 1998). During ischaemia, the low pH within the cell inhibits pore opening. Upon reperfusion, the cell recovers its ability to produce ATP, resulting in production of ROS, repolarisation of the mitochondrial membrane potential, and accumulation of Ca^{2+} within the mitochondria through the action of the mitochondrial Ca^{2+} uniporter. Re-energisation of the cell leads to restoration of cellular pH to normal levels at which time all conditions necessary for pore opening have been met and MPTP formation and opening may be observed (Griffiths and Halestrap, 1995).

MPTP opening allows free movement of small ions and water, across the inner mitochondrial membrane, while large proteins >1.5 kDa remain within the mitochondrion and exert colloidal pressure leading to mitochondrial swelling (Halestrap *et al.*, 2004). Permeabilisation of the inner mitochondrial membrane causes dissipation

of the H^+ gradient across the inner mitochondrial membrane, which in turn results in reversal of the H^+ translocating F_1F_0-ATP synthase and hydrolysis of ATP.

Because the inner mitochondrial membrane is highly folded, MPTP-induced swelling of the mitochondria can also result in rupture of the outer mitochondrial membrane (Feldmann *et al.*, 2000). Rupture of the outer mitochondrial membrane also results in release of proteins such as cytochrome C, Smac/Diablo and apoptosis inducing factor from the inter-membrane space, which can induce apoptosis (Gustafsson and Gottlieb, 2008). It is important to note that pore opening is rapidly reversible through the removal of Ca^{2+}, therefore in cases where the cell is able to recover its Ca^{2+} homeostasis MPTP opening may only be transient (Kerr *et al.*, 1999). Transient pore opening may still be sufficient to damage the outer mitochondrial membrane and release cytochrome C, initiating apoptosis by activation of caspase 9 and in turn caspase 3.

Near to the site of infarction most cell death rapidly occurs through necrosis, but surrounding the necrotic tissue a region of apoptotic cell death is usually evident. Apoptosis is a form of programmed cell death which is necessary for development and maintenance of normal tissue architecture within the body. This process also contributes to cell death in pathological situations such as ischaemia-reperfusion. Apoptosis is a highly controlled, energy-dependent process characterised by cell shrinkage, condensation of chromatin, fragmentation of DNA and apoptotic body formation (Rizzuto *et al.*, 2003, Iliodromitis *et al.*, 2007). Apoptotic cell death has been observed during ischaemia and reperfusion. During ischaemia cell death is primarily necrotic, while reperfusion-induced cell death occurs via both necrosis and apoptosis (Gottlieb *et al.*, 1994, Gottlieb and Engler, 1999, Kajstura *et al.*, 1996, Eefting *et al.*, 2004). There are two major pathways leading to apoptosis known as the extrinsic and intrinsic pathways. The extrinsic pathway is activated by binding of ligands to cell-surface death

receptors, leading to recruitment of adaptor proteins such as procaspase-8 and Fas-associated death domain. This causes activation of initiators caspase 8 and 10, which can in turn activate effector caspases 3, and 7 (Gottlieb and Engler, 1999, Movassagh and Foo, 2008). The intrinsic pathway involves release of cytochrome C, Apoptosis Inducing Factor and endonuclease G from the inter-membrane space of the mitochondria. As stated above, this release may occur in response to MPTP opening, however it has also been suggested that proapoptotic members of the Bcl-2 family (Bax and Bak) may be inserted into the outer mitochondrial membrane where they could form a similar pore allowing release of pro-apoptotic proteins from the inter-membrane space (Korsmeyer *et al.*, 2000). As well as inducing necrotic cell death, Ca^{2+} plays a vital role in apoptotic cell death. Ca^{2+} is able to activate the Ca^{2+}-dependent phosphatase calcineurin, which dephosphorylates and activates the proapototic Bcl-2 protein BAD. Ca^{2+} also activates proteases including the calpains, a family of Ca^{2+} activated cysteine proteases, which are able to cleave proteins involved in apoptosis such as Bcl-$_{XL}$ and caspase-12 (Rizzuto *et al.*, 2003).

Reperfusion may also result in reversible myocardial dysfunction. The contractile recovery of the heart is affected by the survival rate of myocytes within the tissue and the amount of myocardial stunning observed (Yellon and Downey, 2003). Myocardial stunning involves a reversible contractile dysfunction that persists after reperfusion of the tissue following a sub-lethal ischaemic insult. The definition of stunning requires that normal or near-normal coronary flow is resumed and the injury must be fully reversible (Bolli and Marbán, 1999). In the first report of stunning by Heyndrickx *et al.* (1975), it was observed that contractility was depressed for more than 6 hours after a 15 minute coronary occlusion. There are two main hypotheses concerning the mechanism

of stunning involving the actions of ROS or Ca^{2+}, though the truth may be that the combination of these factors may be responsible (reviewed in Bolli and Marbán, 1999).

1.3 Preconditioning

1.3.1 Ischaemic preconditioning

A major breakthrough in the field of cardioprotection came in 1986 with the discovery of an intrinsic protective mechanism in the heart known as ischaemic preconditioning (IPC) by Murry *et al.* (1986). It was observed that four, five minute ischaemic episodes separated by five minutes of reperfusion were able to protect the canine heart from injury during a subsequent ischaemic insult or index ischaemia. Infarct size was decreased by 75% by IPC in a study using 40 minutes of index ischaemia followed by reperfusion; however IPC only served to delay cell death, because when the index ischaemia was extended to three hours the protection was lost. Although other studies had previously examined other cardioprotective strategies, the effects were not robust with a small magnitude of anti-infarct effect (10-20%) and poor reproducibility of protection (Yellon and Downey, 2003). In contrast, IPC produced a much greater limitation of infarct size and the effect could be replicated by others. The protective effect has been confirmed in all species studied (although some species differences were observed concerning the optimum duration of ischaemia and number of preconditioning cycles). The protective effect of preconditioning also appears to be reflected in improvements in contractile function, stunning, loss of cellular markers, and arrhythmias. Since this initial report on IPC, more than 3000 papers have been published on the subject; however the mechanisms involved are still not fully understood.

The protective effect of IPC develops immediately following the stimulus but is short-lived, with loss of protection occurring 1-4 hours after preconditioning depending on species and experimental differences (Murry *et al.*, 1991, Sack *et al.*, 1993). This classical or early preconditioning was described by Murry *et al.* (1986); additionally, there is also a delayed form of preconditioning, also known as second window of protection (SWOP) (Marber *et al.*, 1993, Kuzuya *et al.*, 1993), which develops around 24 hours after the preconditioning stimulus and lasts up to 72 hours. Although much more prolonged than classical preconditioning the protective effect of SWOP is less robust (Yellon and Downey, 2003). In addition to the post translational modifications that are responsible for classical preconditioning, studies with the protein synthesis inhibitor cycloheximide demonstrated that changes in gene expression are involved in the protection afforded by SWOP (Meldrum *et al.*, 1997b).

1.3.2 Mechanisms of preconditioning

Studies into the mechanisms of preconditioning led to the discovery that activation of the adenosine A_1 receptor triggered IPC in the rabbit heart (Liu *et al.*, 1991). It has now been established that ischaemic preconditioning is largely mediated by G-protein coupled receptor (GPCR) activation. Activation of adenosine A_1 and A_3, δ-opioid, bradykinin B_2, endothelin ET_1, and α_1-adrenergic receptors can induce preconditioning of the myocardium (Iliodromitis *et al.*, 2007, Cohen *et al.*, 2000), although ET_1 and AT_1 receptors do not appear to mediate IPC (Cohen *et al.*, 2000), and there is conflicting evidence as to whether the α_1-adrenoceptor is involved in triggering IPC (Banerjee *et al.*, 1993, Hu and Nattel, 1995, Bugge and Ytrehus, 1995, Thornton *et al.*, 1993). Because both adenosine A_1 and α_1-adrenergic receptor stimulation causes activation of protein kinase C (PKC), the observation that these receptors were able to induce preconditioning led to the hypothesis that preconditioning could be mediated by a

common signalling pathway involving PKC activation. The central role of PKC in IPC was shown simultaneously by two groups (Ytrehus *et al.*, 1994, Mitchell *et al.*, 1995), who demonstrated that inhibition of PKC could block IPC, and PKC activators were able to mimic the protective effect of preconditioning. As the IPC stimulus also results in generation of ROS, the role of ROS in triggering IPC has also been examined, leading to the finding that ROS generators can mimic the protective effect of IPC (Vanden Hoek *et al.*, 1998, Tritto *et al.*, 1997). Use of free radical scavengers is also able to inhibit IPC or to raise the threshold for IPC to occur (Vanden Hoek *et al.*, 1998, Baines *et al.*, 1997).

1.3.3 Models and end points used in preconditioning studies

Various experimental models have been used to examine the effects of ischaemia and reperfusion in the heart. Studies can be carried out 'in situ' in the anaesthetised animal (Hearse *et al.*, 1977, Murry *et al.*, 1986), which allows investigators to use an extensive reperfusion period that has been quoted as the 'gold standard' in the measurement of infarct size (Yellon and Downey, 2003). However, this method does require surgical expertise and several days follow-up during the reperfusion period. Isolated perfused hearts can also be used to examine the effect of ischaemia-reperfusion (Banerjee *et al.*, 1993, Inagaki *et al.*, 2003b). This model has the advantage that it is simpler than the in situ heart model; however the effects of nervous innervation and circulating hormones and factors are obviously lost. In these whole heart models, ischaemia is usually induced by one of two methods, no-flow ischaemia where the heart is subject to global ischaemia, or occlusion of a coronary artery to produce a regional ischaemia.

The infarct size expressed as a proportion of the area at risk measured by histological analysis after 4 days reperfusion was used in the original studies of IPC and is still viewed as the best end point for IPC studies (Yellon and Downey, 2003, Murry *et al.*,

1986). However, preconditioning is also able to improve the recovery of function of the heart (Landymore *et al.*, 1998, Shizukuda *et al.*, 1993) through an increase in myocyte survival, and/or a reduction in the amount of myocardial stunning. The effect of IPC on stunning is somewhat controversial, with some studies finding a protective effect (Landymore *et al.*, 1998, Shizukuda *et al.*, 1993), while others show no effect of IPC on stunning (Ovize *et al.*, 1992, Jenkins *et al.*, 1995). This may result from difficulty in determining the proportion of necrotic and stunned myocardium, and differences in the preconditioning protocols used, particularly as preconditioning protocols can themselves cause stunning (Bolli and Marbán, 1999, Bolli *et al.*, 1995). Measurements of haemodynamic function such as left ventricular developed pressure (LVDP), left ventricular end diastolic pressure (LVEDP) and contractility have been used to examine preconditioning, though these data should be interpreted with some caution due to the long lasting effects of stunning (Yellon and Downey, 2003, Banerjee *et al.*, 1993). Arrhythmias are also common following ischaemia-reperfusion, and some forms of preconditioning have been observed to exert an anti-arrhythmic effect (Banerjee *et al.*, 1993, Vegh *et al.*, 1990), however other studies show an increase in arrhythmias following preconditioning (Ovize *et al.*, 1995).

Isolated cells are also utilised for preconditioning studies for several reasons including the ability to visualise cells, the difficulty in carrying out electrophysiological and fluorescence measurements in the intact heart, the ability to examine the roles of ventricular myocytes in the absence of other cell types present within the heart, and a reduction in animal usage compared with whole heart studies (Diaz and Wilson, 2006). Isolated adult ventricular myocytes have been widely used to examine the effects of preconditioning on cell survival (Rodrigo and Samani, 2008, Chen *et al.*, 2001, Armstrong *et al.*, 1997), contractile function (Hudman and Standen, 2004, Eisner *et al.*,

1989), Ca^{2+} handling (Rodrigo and Standen, 2005, Baczkó *et al.*, 2003), ion channel activity (Sukhodub *et al.*, 2007, Rainbow *et al.*, 2005), membrane potential (Baczkó *et al.*, 2003, Sukhodub *et al.*, 2007) and the function of organelles including the mitochondria (Liu *et al.*, 1998, Hausenloy *et al.*, 2004) or SR (Xu *et al.*, 1996, Hohl *et al.*, 1992). Isolated ventricular myocytes are terminally differentiated and do not survive well in culture, therefore other cell types have been employed for molecular biological experiments. Neonatal cardiomyocytes (Engelbrecht *et al.*, 2004, Gray *et al.*, 1997), the rat embryonic ventricular cell line H9c2 (He *et al.*, 1999, Agnetti *et al.*, 2005), and the atrial tumour cell line HL-1 (Chaudary *et al.*, 2004, Fox *et al.*, 2005) have the advantage of growing in culture and have also been used in preconditioning studies, however these cell types have a morphology and protein expression profile more in common with neonatal hearts.

The lack of vasculature makes it impossible to achieve 'true ischaemia' in isolated or cultured cell preparations, so it is necessary to simulate ischaemia through the removal of metabolic substrates and induction of hypoxia (Chen *et al.*, 2001, Sukhodub *et al.*, 2007), or metabolic inhibition (Rodrigo and Standen, 2005, Baczkó *et al.*, 2004). Similarly, preconditioning must be induced using one of the above methods or through pharmacological interventions (Diaz and Wilson, 2006). However, Rodrigo's lab recently developed a method for producing ischaemic preconditioned isolated ventricular myocytes (Rodrigo and Samani, 2008). This method involves IPC in the isolated rat heart followed by dissociation of single cells and provides a model that can be used to examine the effects of 'true' IPC in isolated cells.

We used isolated adult rat ventricular myocytes that were preconditioned using phenylephrine. Myocytes were subject to metabolic inhibition and re-energisation with normal Tyrode solution to simulate ischaemia and reperfusion. The use of isolated

myocytes provides the capability to carry out electrophysiological and fluorescent measurements, and to directly compare the responses of control and preconditioned myocytes from the same heart. Although survival of heart tissue is generally thought to be the optimal end point for measurement of preconditioning, we have examined contractile recovery as an end point because this provides a measure of the proportion of myocytes that are viable and not stunned. It has been shown that the reduction in infarct size induced by phenylephrine preconditioning is correlated with an improvement in functional recovery in the heart (Banerjee *et al.*, 1993, Vasara *et al.*, 2002). In addition, contractile function is important for the heart to maintain perfusion of the body, and a significant decrease in contractile function over a sustained period caused by stunning could result in further injury due to inadequate perfusion of other organs.

1.3.4 Preconditioning of the human heart and clinical implications of IPC

Despite the powerful protection afforded by IPC, several factors have reduced its usefulness in the clinical setting. It is not possible to utilise IPC in treatment of acute myocardial infarction due to the necessity for intervention prior to an ischaemic episode. Cardioprotective strategies involving postconditioning of the heart, where the protective intervention is made during the reperfusion period following the index ischaemia, have been found to improve the outcome of patients after myocardial infarction (Ross *et al.*, 2005, Kloner and Rezkalla, 2006). The ethical issues involved in carrying out experimental procedures on humans, mean that much of the data concerning human preconditioning uses indirect endpoints such as a reduction in ST-elevation on the electrocardiogram (ECG), reduction in the number of episodes of angina, reduction in arrhythmias or increased patient survival (Iliodromitis *et al.*, 2007).

The protective effect of IPC has also been examined in surgical situations where it is known that myocardial ischaemia will occur. IPC has been successfully utilised in cardiac surgery such as coronary angioplasty and coronary artery bypass graft (Rezkalla and Kloner, 2007). However, the extra time required to carry out an IPC protocol prior to surgical procedures, and the potential for cross clamping of the vessel to result in embolus formation, reduce the attractiveness of IPC to the cardiac surgeon. Pharmacological interventions such as adenosine, glyceryl trinitrate or nicorandil have also been used in cardiac surgery as preconditioning or postconditioning agents. Despite some evidence suggesting favourable outcomes, the use of pharmacological preconditioning in cardiac surgery is still not commonplace. The short time course of preconditioning coupled with the fact that preconditioning cannot be reproduced by a second cycle has also decreased the use of preconditioning. It should be noted that the study of IPC has identified additional effects of commonly used drugs, such as the cardioprotective effects of ACE inhibitors, β-blockers and statins, or the undesirable anti-preconditioning effects of sulphonylureas (Cokkinos and Pantos, 2007). Therefore drug treatments can be tailored to improve cardioprotection in patients at risk of cardiovascular disease.

1.4 Phenylephrine preconditioning

1.4.1 Signalling by phenylephrine

Phenylephrine is an agonist at the G-protein coupled α_1-adrenoceptor. Three subtypes of α_1-adrenoceptor (α_{1A}, α_{1B} and α_{1D}) exist within the heart (for a review see Zhong and Minneman, 1999). The α_1-adrenoceptor primarily couples to the $G_{q/11}$ family of α G-protein subunits that activate PLC leading to formation of membrane-bound diacylglycerol (DAG) and soluble inositol 1, 4, 5-trisphosphate (IP_3) (Terzic *et al.*,

1993). IP_3 is able to bind to IP_3 receptors (IP_3R) on the SR, which stimulates the release of Ca^{2+} from the SR. A rise in intracellular Ca^{2+} together with DAG is able to activate PKC leading to phosphorylation of target proteins (an overview of this pathway is shown in Figure 1.4).

The α_1-adrenoceptor may also couple to a dihydropyridine-sensitive Ca^{2+} channel in some cell types possibly through direct activation of a G-protein coupled ion channel (Zhong and Minneman, 1999, Han *et al.*, 1987), and activation of phospholipase D by α_1-adrenoceptor stimulation has been observed in the heart (Clerk and Sugden, 1997) but the mechanisms responsible remain unclear. There is also the possibility that the G-protein $\beta\gamma$ subunits may underlie some α_1-adrenoceptor signalling mechanisms.

1.4.2 Phenylephrine preconditioning

α_1-adrenergic preconditioning of the heart was first described by Banerjee *et al.* (1993). It was shown that norepinephrine could precondition the isolated rat heart and the protection could be blocked by use of α_1-adrenoceptor antagonists or mimicked using the α_1-adrenoceptor agonist phenylephrine. Both norepinephrine and phenylephrine preconditioning decreased the prevalence of reperfusion arrhythmias and improved the functional recovery of hearts during the reperfusion period, as shown by improvements in LVDP, LVEDP and contractility. The α_{1B}-adrenoceptor subtype was found to be responsible for phenylephrine preconditioning as determined using subtype specific inhibitors (Vasara *et al.*, 2002).

It was later demonstrated that inhibition of PKC prevented phenylephrine preconditioning (Mitchell *et al.*, 1995). Immunofluorescence microscopy also showed that phenylephrine treatment resulted in translocation of PKCδ but not α or ε to the membrane suggesting that phenylephrine preconditioning may be mediated by the δ

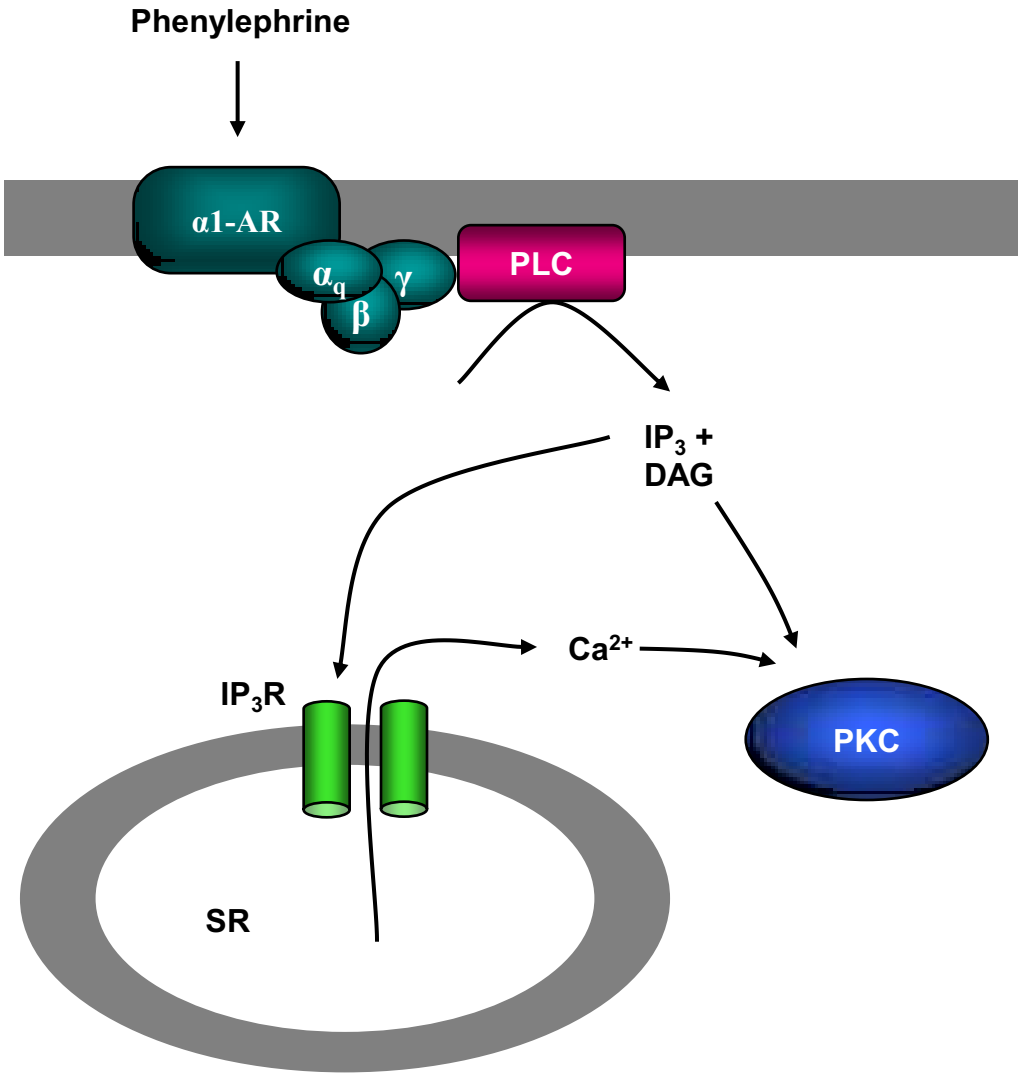

Figure 1.4. G_q-coupled signalling activated by phenylephrine.

Phenylephrine signalling via the G_q-coupled α_1-adrenoceptor. Phenylephrine activates the α_1-adrenoceptor leading to activation of PLC and formation of DAG and IP_3. Binding of IP_3 to the IP_3 receptor causes Ca^{2+} release from the SR. DAG and Ca^{2+} can then activate protein kinase C leading to phosphorylation of its substrates.

isoform of PKC (Mitchell *et al.*, 1995). In contrast to this finding, Gao and co-workers showed that PKCε translocated to the membrane fraction after phenylephrine treatment and that the effect was inhibited by the α_{1B}-adrenoceptor antagonist chloroethylclonidine (CEC) (Gao *et al.*, 2007). They also observed that inhibition of PKCε, using the specific inhibitor εV1-2, prevented phenylephrine preconditioning while it had no effect on the protection afforded by diazoxide preconditioning. The effect of phenylephrine preconditioning in the human myocardium was examined using human right atrial appendage by Loubani and Galiñanes (2002). This study concluded that ischaemic, diazoxide and phenylephrine preconditioning share a common signalling pathway that involves *mito*K_{ATP} channels, PKC and p38 MAPK (p38), although the role of specific PKC isoforms was not examined.

1.5 Excitation-contraction (E-C) coupling in ventricular myocytes

1.5.1 E-C coupling

The physiological function of the heart relies on its ability to contract in response to the electrical excitation of the action potential (see Figure 1.5). Depolarisation of the myocyte during the action potential leads to activation of voltage-gated Ca^{2+} channels, Ca^{2+} entry, release of Ca^{2+} from the intracellular stores of the SR and activation of the Ca^{2+}-sensitive contractile machinery of the cell by a process known as E-C coupling (Bers, 2002). Ventricular myocytes contain large numbers of mitochondria in order to meet the huge energy demands of constant contraction. They also contain a network of sarcolemmal membrane invaginations known as transverse- or t-tubules that are found at the Z-line and allow rapid and synchronous Ca^{2+} entry and extrusion throughout the cell (Orchard and Brette, 2008). T-tubules also ensure that the entry of triggering Ca^{2+} through voltage-gated Ca^{2+} channels occurs close to the Ca^{2+}-activated RyRs that are

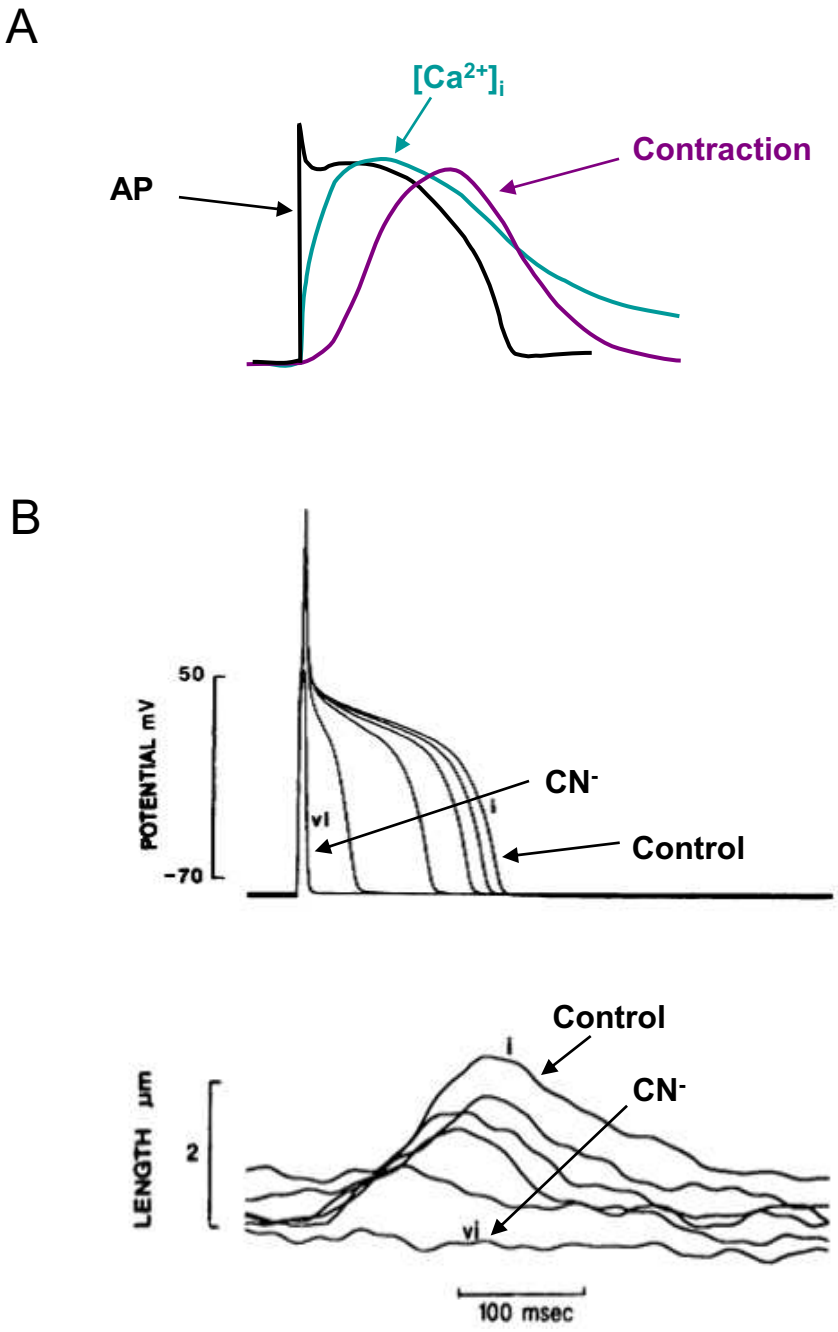

Figure 1.5. Cardiac action potential and contractile force.

A. Diagram showing the relationship between the action potential, the rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) or Ca^{2+} transient, and contraction in the ventricular myocyte, adapted from Bers (2002).

B. Action potential duration and cell shortening measured during metabolic inhibition with cyanide (CN^-) and 2-deoxyglucose, taken from Nichols *et al.* (1991).

present on the SR membrane, allowing a large, rapid release of Ca^{2+} from stores in the SR via CICR that is responsible for the Ca^{2+} transient. In small animals, which have a high heart rate, the t-tubule density is higher than in larger animals, suggesting that the t-tubular network helps to increase the speed of Ca^{2+} flux across the membrane.

The cardiac action potential travels as a wave of depolarisation through the myocardium. When it arrives at a myocyte, it causes activation of voltage-gated Na^{+} channels leading to depolarisation of the cell membrane (Berne and Levy, 2000). This depolarisation causes Ca^{2+} entry through voltage-gated Ca^{2+} channels, usually L-type Ca^{2+} channels, and is responsible for an increase in cytosolic Ca^{2+}, which activates RyRs triggering Ca^{2+} release from the SR through CICR. The major constituent of the Ca^{2+} transient varies between species with the SR providing 90-95% of the Ca^{2+} in the rat, while in the human ventricle only around 70% of the activating Ca^{2+} is released from the SR with around 25-28% entering through the L-type channel and reverse-mode NCX (Bers, 2008).

The action potential is the trigger for the increase in intracellular Ca^{2+}, therefore the force of contraction can be modulated by the amplitude or duration of the action potential and the size of the resulting Ca^{2+} transient, as well as through phosphorylation or modification of the contractile proteins (Bers, 2002). Ca^{2+} enters the cytosol during the plateau phase of the action potential; therefore an increase in the length or amplitude of the action potential plateau can increase the Ca^{2+} entry, and the magnitude of the resultant contraction. Conversely, shortening of the action potential duration (APD) occurs during ischaemia, or metabolic inhibition (Nichols *et al.,* 1991). A decrease in the length of the action potential, through an increase in the rate of phase 3 repolarisation, will decrease the length of the action potential plateau and reduce Ca^{2+} entry into the cytosol, resulting in a decrease in contractile force (see Figure 1.6).

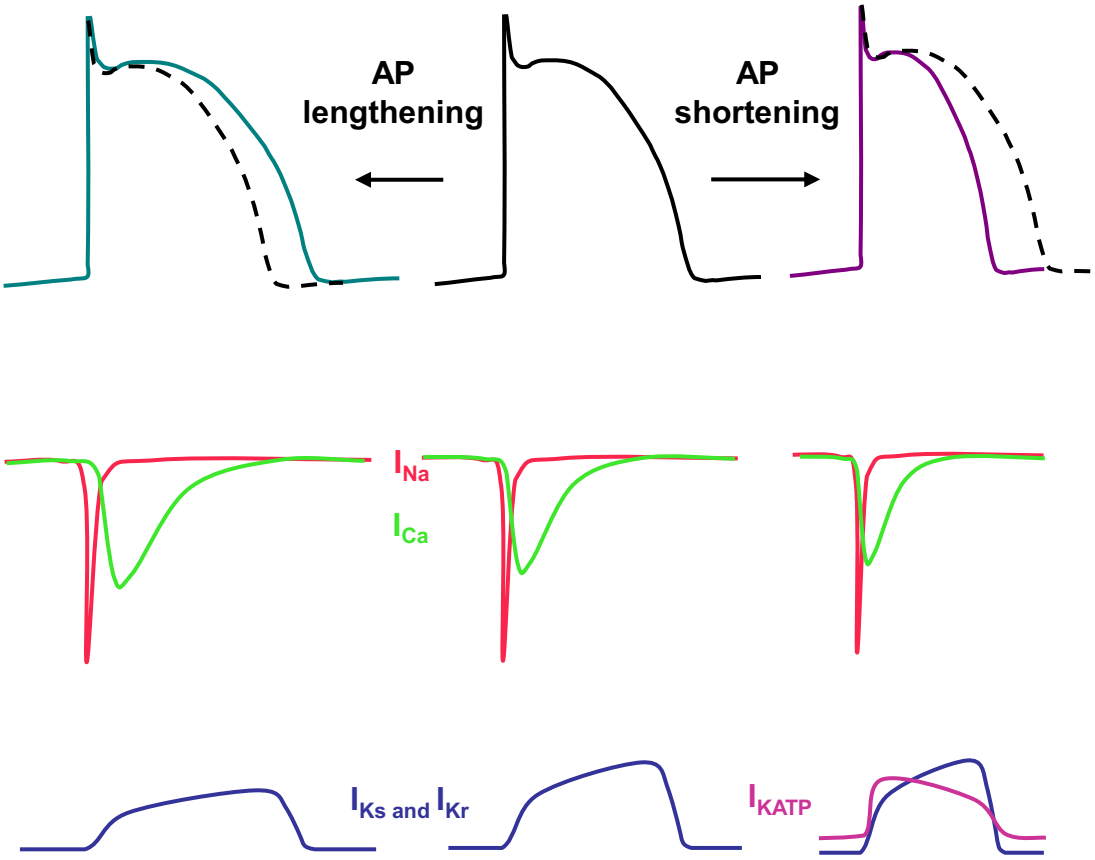

Figure 1.6. Changes in action potential duration.

Diagram showing how changes in depolarising or repolarising currents can alter action potential duration. The action potential duration can be altered by changes in the balance of Ca^{2+} and K^+ currents. An increase in Ca^{2+} current or decrease in K^+ currents can lead to lengthening of the action potential. Conversely, a decrease in Ca^{2+} current or an increase in repolarising K^+ current via activation of *sarc*K_{ATP} channels can shorten the action potential. *Sarc*K_{ATP} current profile based on experimental data from Weiss *et al.* (1992), and computer modelling by Ferrero *et al.* (1996) and Michailova *et al.* (2007).

Within the cardiac myocyte the major players in E-C coupling are clustered, with the L-type Ca^{2+} channels of the sarcolemmal or t-tubular membrane located in close proximity to the RyRs on the SR membrane. The Ca^{2+} that enters the cytosol during an action potential must also be removed from the cytosol in order for relaxation to occur. Ca^{2+} removal is achieved primarily via uptake into the SR by SERCA and removal from the cell by the NCX, with a small proportion (~1%) removed by the sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+}-uniporter (Bers, 2008).

The contractile machinery of myocardial cells is organised into sarcomeres made up of myosin containing thick filaments and actin containing thin filaments (Berne and Levy, 2000). Shortening of the sarcomere occurs through cycling of cross-bridges between adjacent thick and thin filaments leading to sliding of actin filaments along myosin filaments towards the Z line. The troponin complex is a regulatory protein complex of the thin filament made up of troponin C (TnC) troponin I (TnI) and tropomyosin (Bers, 2008). Binding of Ca^{2+} to TnC results in binding of TnI by TnC, allowing the myosin heads to interact with actin. Attachment of the myosin head to the actin filament produces cross-bridges that encourage Ca^{2+} binding and cross-bridge formation at neighbouring sites. Relaxation is thought to occur through Ca^{2+} dissociation from the myofilaments in response to Ca^{2+} removal from the cytosol or inhibitory phosphorylation of TnI.

1.5.2 Modulation of E-C coupling by ischaemia-reperfusion

Contractile failure: Contractile failure, rigor contracture and hypercontracture occur in response to ischaemia-reperfusion in the ventricular myocyte. Contractile failure normally occurs as a result of opening of *sarc*K_{ATP} channels. *Sarc*K_{ATP} channel opening increases the repolarising current and leads to shortening and eventual failure of the action potential. As Ca^{2+} entry during the action potential is the stimulus for contraction,

curtailment of the action potential results in impaired contraction (see Figure 1.6). Under conditions where the *sarc*K_{ATP} channels are inhibited, contractile failure is likely to occur through ionic dysregulation and depolarisation of the resting membrane potential (Rainbow *et al.*, 2004).

Rigor contracture: Rigor contracture during ischaemia occurs due to Ca^{2+}-independent actomyosin-ATPase activation and formation of rigor cross-bridges. Rigor contracture occurs when the ATP levels are low (10-100 μM) but not zero (Bremel and Weber, 1972, Nichols and Lederer, 1990b) and may also contribute to ATP depletion as ATP is consumed by the actomyosin-ATPase (Stapleton and Allshire, 1998).

Hypercontracture: Hypercontracture of ventricular myocytes during reperfusion causes mechanical damage and is characterised by a pathological shortening of the sarcomeres, resulting in distortion of the sarcomeric structure. In the intact heart, this may lead to separation of myocytes at the intercalated disks junctions, disruption of the sarcolemmal membrane and contraction band necrosis (Vander Heide *et al.*, 1986). Two different mechanisms can be respsonsible for hypercontracture upon reperfusion, Ca^{2+}-overload induced contracture and rigor contracture (Piper *et al.*, 2004). Loading of Ca^{2+} in to the cytosol of ventricular myocytes occurs during ischaemia and early reperfusion. Upon reperfusion, re-energisation of the mitochondria may lead to resynthesis of ATP. If the ATP levels rise rapidly, the contractile machinery of the myocyte may be activated before Ca^{2+} homeostasis has been restored. In this case, rapidly oscillating Ca^{2+} levels due to cyclic uptake and release of Ca^{2+} from the SR, lead to uncontrolled activation of the contractile machinery and hypercontracture of the cell (Piper *et al.*, 2004, Schafer *et al.*, 2001). Hypercontracture can also be caused by a Ca^{2+}-independent rigor contracture mechanism (Ladilov *et al.*, 2003). In cases where the resumption of mitochondrial ATP production during reperfusion is impaired, the myocyte may be exposed to the low ATP

levels that result in rigor contracture. During reperfusion, the ATP levels may be maintained within this window of low ATP for a much more prolonged period and the degree of rigor contracture that occurs during reperfusion may be much greater.

ROS: Ischaemia and reperfusion lead to production of ROS, which are able to modulate E-C coupling at several points. The ability of ROS to inhibit SERCA Ca^{2+} transport and ATPase activity was demonstrated using phorbol 12-myristate 13-acetate (PMA) activated leukocytes to produce ROS (Rowe *et al.*, 1983). Several forms of ROS appear to be responsible for this affect as H_2O_2 inhibited the Ca^{2+} transport without effect on the SERCA ATPase activity. Redox reactions are also able to stimulate the activity of the NCX, although the process appears to be more complex, requiring both a reducing and an oxidising agent such as $FeSO_4$ and dithiothreitol (DTT) or H_2O_2 that may induce thiol-disulphide interchange (Reeves *et al.*, 1986). Depression of the sarcolemmal Ca^{2+}-ATPase by ROS was observed by Kaneko *et al.* (1989a). Both ATP-dependent Ca^{2+} accumulation and Ca^{2+}-stimulated ATPase activity were decreased by superoxide, hydrogen peroxide and hydroxyl radicals. Lipid peroxidation was reported as the mechanism responsible for the depression in sarcolemmal Ca^{2+}-ATPase activity in this article; however, the same group observed that modification of sulfhydryl groups may also account for the depression in function (Kaneko *et al.*, 1989b).

ROS can also modulate ion channel activity during ischaemia and reperfusion. I_{K1} is significantly inhibited following exposure to ROS during ischaemia, which can lead to a depolarisation of the resting membrane potential (Jabr and Cole, 1993, Coetzee and Opie, 1992). Peak L-type Ca^{2+} current also appears to be decreased by ROS, although this may be associated with an increase in the time constant of inactivation of the current (Coetzee and Opie, 1992). The mechanism responsible for L-type Ca^{2+}

inhibition appears to be modification of cysteine groups within the channel by ROS (Fearon *et al.*, 1999, Zima and Blatter, 2006).

Ischaemia causes persistent Na^+ current activation (Hammarstrom and Gage, 2002, Saint, 2008). This current appears to result from modification of the gating properties of transient voltage-gated Na^+ channels during ischaemia. Because this current is resistant to inactivation, the amount of Na^+ entry through these channels is much larger than through rapidly inactivating channels and this contributes to depolarisation of the resting membrane potential and Na^+ loading during ischaemia. The effects of ischaemia on ion channels and transporters may result in a depolarisation of the ventricular myocyte during early ischaemia which can be arrhythmogenic (Jabr and Cole, 1993). However, later in ischaemia, depletion of ATP within the myocyte leads to opening of *sarc*K_{ATP} channels that causes hyperpolarisation of the resting membrane potential (see section 1.1 and 1.6).

pH: Changes in pH during ischaemia can also affect E-C coupling. Decreased cytosolic pH reduced the open probability of the RyR and decreased the affinity of the channel for ryanodine which appears to be correlated with channel opening (Xu *et al.*, 1996), resulting in a decrease in Ca^{2+} release from the SR for a given stimulus. Blanchard and Solaro (1984) showed that a decrease in pH resulted in reduction in Ca^{2+} binding to myofilaments and a decrease in Ca^{2+} sensitivity of the actomyosin-ATPase. A decrease in pH therefore results in a decrease in the contractile force generated (Fabiato and Fabiato, 1978, Steenbergen *et al.*, 1977).

Phosphate: Increased inorganic phosphate levels can also decrease the developed tension in skinned ventricular myocytes (Kentish, 1986). In another study it was shown that the decrease in tension is due to a decrease in Ca^{2+} sensitivity of the

actomyosin-ATPase, and the inhibitory effect of phosphate was reduced at lower ATP levels (Schmidt-Ott *et al.*, 1990).

1.5.3 Modulation of E-C coupling by phenylephrine

Because the contractile machinery is a major consumer of ATP within the myocyte, modulation of E-C coupling may be involved in preconditioning by protecting cellular ATP levels. Phenylephrine signalling is able to modulate several proteins involved in E-C coupling. Phenylephrine administration has biphasic effects on the L-type Ca^{2+} channel current. Zhang *et al.* (1998) observed a transient decrease in peak L-type current at around 2 minutes followed by a sustained potentiation of the current after 5-10 minutes of phenylephrine treatment. The unitary conductance of channels was not altered; however the open probability of the channel was increased through an increase in the number and length of openings. This effect could be blocked by the α_1-adrenoceptor antagonist prazosin or the PKC inhibitor chelerythrine. Therefore α_1-adrenoceptor dependent modulation of L-type Ca^{2+} current appears to be mediated by PKC. Phenylephrine treatment also increases NCX current in the forward and reverse modes without altering the reversal potential (Stengl *et al.*, 1998). This could compensate for the increase in Ca^{2+} entry via L-type channels under normoxic conditions, although it could lead to an increase in Ca^{2+} entry via reverse mode NCX activity during ischaemia-reperfusion.

Pyle *et al.* (2000) examined the effect of phenylephrine and opioid preconditioning on actomyosin-ATPase activity in the isolated rat heart. Preconditioning was associated with a decrease in actomyosin-ATPase activity suggesting that a decrease in energy utilisation due to decreased actin-myosin cross-bridge cycling may be responsible, at least in part, for the protective effect of phenylephrine preconditioning, by protecting ATP levels during ischaemia. An increase in the phosphorylation of TnI and C-protein

was also observed in this study. In contrast, no effect of phenylephrine preconditioning on C-protein or TnI was found in a study by Talosi *et al.* (1992). The conflicting results of these studies may be due to species differences as the study by Pyle used adult rats, while Talosi and co-workers used rabbits.

1.6 ATP-sensitive K^+ channels (K_{ATP})

The presence of ATP-sensitive potassium channels (*sarc*K_{ATP}) in the sarcolemmal membrane of ventricular myocytes was first described by Noma (Noma, 1983). The channels are inhibited by physiological concentrations of ATP, modulated by ADP and open upon ATP depletion leading to activation of an outward K^+ current. It was suggested that the *sarc*K_{ATP} channel played a cardioprotective role by coupling the membrane excitability to myocardial metabolism. Early results indeed confirmed this hypothesis with *sarc*K_{ATP} channel openers providing a protective effect in models of myocardial ischaemia (discussed in section 1.6.2). The *sarc*K_{ATP} channel was also implicated in the mechanism of IPC. However, the identification of a K_{ATP} channel within the mitochondrial membrane (*mito*K_{ATP}) has resulted in some dispute as to whether it is the *sarc*K_{ATP} channel or the *mito*K_{ATP} that is responsible for the protective effect of preconditioning (Hanley and Daut, 2005, Peart and Gross, 2002, Gross and Fryer, 1999); this will be discussed below in section 1.6.2-5.

1.6.1 Sarcolemmal K_{ATP} channels

SarcK_{ATP} channels open in response to a decrease in the cellular energy levels, allowing the cell to link membrane excitability to energetic state. The *sarc*K_{ATP} channel is a hetero-octameric complex comprised of four inward rectifying potassium channel (Kir6) pore-forming subunits and four regulatory sulphonylurea receptor (SUR) subunits, see Figure 1.7 (Seino and Miki, 2003). Two Kir6 subunits have been identified (Kir6.1 and

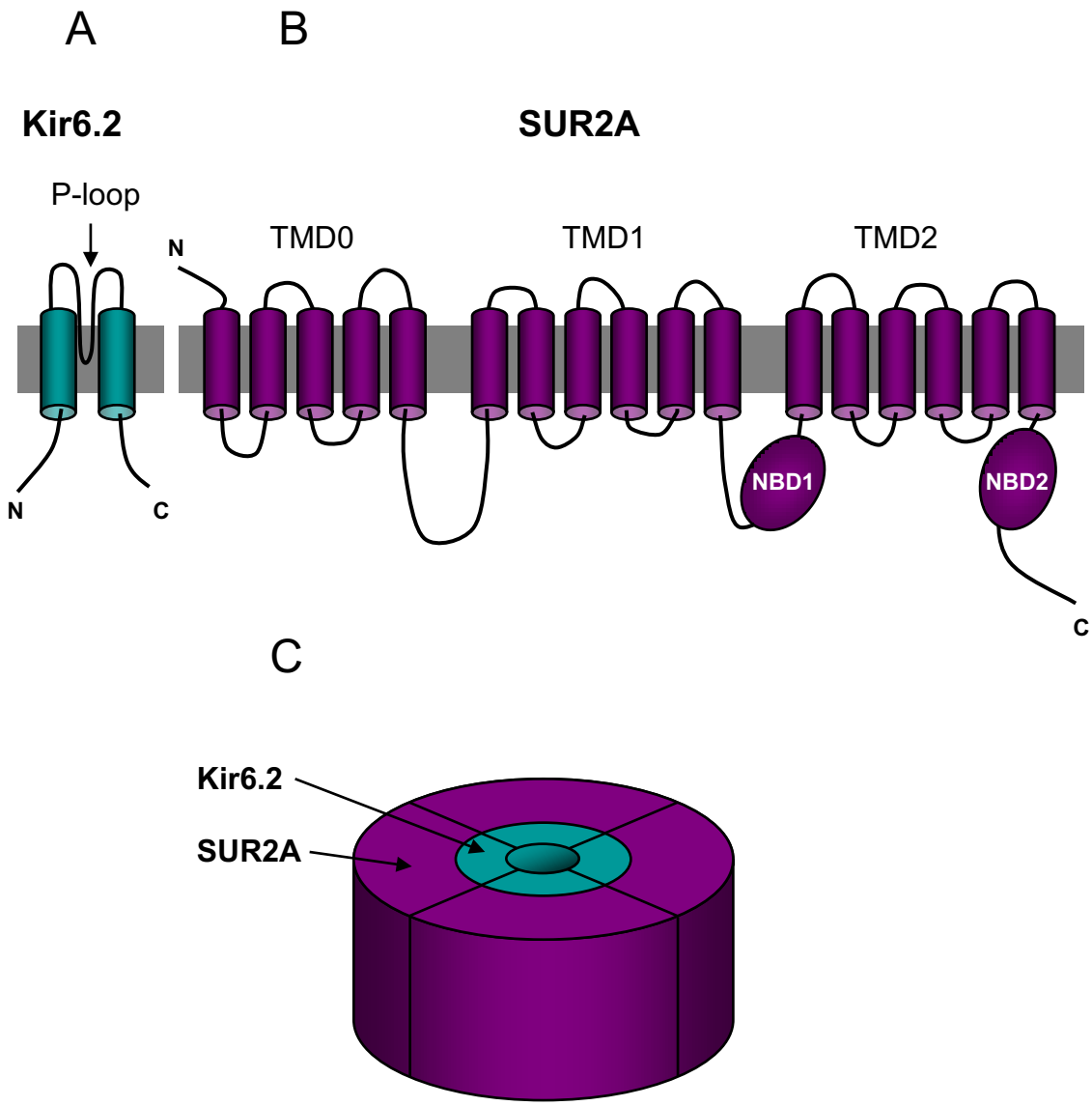

Figure 1.7. Structure of the cardiac sarcolemmal K_{ATP} channel.

A. Topology of the Kir6.2 pore forming subunit.

B. Topology of the sulphonylurea receptor SUR2A subunit.

C. Hetero-octameric structure of the cardiac sarcolemmal K_{ATP} channel comprised of four Kir6.2 subunits and four SUR2A subunits. Adapted from Seino and Miki (2003).

Kir6.2) which come together with SUR1, SUR2A or SUR2B to form functional channels; therefore different combination of subunits can form channels with different properties and regulation. The SUR is a member of the ABC protein family, which also contains the multidrug resistance-associated protein MRP1 transporter, and the ion channel cystic fibrosis transmembrane regulator (CFTR). Although not vital for pore formation, the SUR subunit is necessary for membrane targeting of the *sarc*K_{ATP} channel by masking the endoplasmic reticulum localisation signals that are present on both the Kir6 and SUR1 subunits. However, truncation of the final 36 amino acids of the C-terminus of Kir6.2 enables membrane expression of Kir6.2 tetramers in the absence of SUR (Tucker *et al.*, 1997). The *sarc*K_{ATP} channel in the sarcolemmal membrane of ventricular myocytes appears to be a complex of Kir6.2 and SUR2A (Inagaki *et al.*, 1996); but there is increasing evidence that other K_{ATP} subunits may be present within the ventricular myocyte, and SUR1/Kir6.2 appears to be the predominant *sarc*K_{ATP} channel composition within the atria (Flagg *et al.*, 2008). Singh *et al.* (2003) demonstrated using immunofluorescence microscopy that Kir6.2 and SUR2A immunofluorescence was localised to the sarcolemmal and mitochondrial membranes of ventricular myocytes, while Kir6.1 was localised in the mitochondria and sarcolemmal membrane of some cells, and SUR2B within the t-tubules. This suggests that *sarc*K_{ATP} channel subunits may be present within the mitochondria; however, their relationship to the putative *mito*K_{ATP} channel is unclear because the properties attributed to *mito*K_{ATP} appear to be different from those established for known *sarc*K_{ATP} subunit combinations (Hanley and Daut, 2005).

SarcK_{ATP} channels are inhibited by physiological concentrations of intracellular ATP, with a K_i for ATP of approximately 25 μM in inside out membrane patches (Lederer and Nichols, 1989). However, Mg-ADP and Mg-GDP have a stimulatory effect on the

channel, so that in the presence of 200 μM ADP, 50 μM GDP and 1 mM free Mg^{2+}, the K_i for ATP is increased to 100 μM (Nichols and Lederer, 1990a). In addition, the action of the adenylate kinase and creatine kinase pathways may even decrease ATP levels at the membrane during ischaemia (Carrasco *et al.*, 2001, Crawford *et al.*, 2002). Therefore channel opening occurs earlier in ischaemia than would be expected based on the observed depletion of ATP. In addition, phosphorylation of the *sarc*K_{ATP} channel by PKC may modulate channel opening during ischaemia, this will be discussed later in section 1.7.3.

Although it was originally assumed that the nucleotide sensitivity of the channel was conferred by the SUR subunit, it has since been determined that both the SUR and the Kir6 subunits are able to modulate channel function in response to nucleotide binding (Seino and Miki, 2003). A C-terminal truncated Kir6.2 subunit expressed in the absence of SUR was used to examine the effect of ATP on the Kir6.2 subunit (Tanabe *et al.*, 2000). A radiolabelled ATP analogue was able to radiolabel Kir6.2 in a Mg^{2+}-independent manner, and currents recorded from the truncated channel were inhibited by increasing concentration of the ATP analogue indicating that Kir6.2 contains the ATP binding site responsible for the inhibitory effect of ATP. The SUR contains two nucleotide binding domains (NBDs) that are responsible for activation of the channel by nucleotide diphosphates ADP and GDP. NBD2 also appears to possess ATPase activity (Seino and Miki, 2003, Alekseev *et al.*, 2005).

1.6.2 Role of *sarc*K_{ATP} channels in preconditioning

SarcK_{ATP} channels were proposed to play a protective role during ischaemia through an enhanced shortening of the action potential duration (APD) brought about by acceleration of phase 3 repolarisation, see Figure 1.5 and 1.6 (Noma, 1983). This would lead to a decrease in Ca^{2+} overload-induced cellular injury. The first studies into the role

of *sarc*K_{ATP} channels in cardioprotection appeared to confirm this theory (Gross and Auchampach, 1992, Lederer *et al.*, 1989). The role of K_{ATP} channels in preconditioning was first reported by Gross and Auchampach (1992). Inhibition of K_{ATP} channels using glibenclamide abolished the reduction in infarct size produced by IPC in their study, while a K^+ channel opener was able to mimic protection. Similarly, Yao and Gross (1994) observed that while administration of the K_{ATP} channel opener bimakalim or a 3 minute IPC stimulus alone had no effect on the infarct size, bimakalim was able to lower the threshold for IPC so that the combination of bimakalim and 3 minutes IPC limited the infarct size to a similar degree to 10 minutes of IPC alone. The protection afforded by a combination of bimakalim and IPC was associated with a marked increase in APD shortening suggesting that the *sarc*K_{ATP} channel may mediate preconditioning through shortening of the APD. Suzuki *et al.* (2002) demonstrated that knock-out of Kir6.2 in the mouse prevented the infarct reducing effect of IPC while having little effect on infarct size in the non-preconditioned heart. Knock out of Kir6.2 also prevented action potential shortening and increased the time to cessation of contraction. However, Grover *et al.* (1995) demonstrated that shortening of the APD was not required for the protective effect of K_{ATP} channel openers, because while APD shortening induced by the K_{ATP} channel opener cromakalim was reversed by the anti-arrhythmic drug dofetilide, it did not prevent the infarct limitation afforded by cromakalim. This suggests that APD shortening is not required for protection, which has been used as an argument in favour of the importance of *mito*K_{ATP} (see introduction to Garlid *et al.*, 1997), however *sarc*K_{ATP} activation may protect via another mechanism.

Another mechanism for the protective effect of *sarc*K_{ATP} channels involves a sustained hyperpolarisation of the resting membrane potential of the cell, which would decrease Na^+ and Ca^{2+} entry through voltage-gated ion channels. During ischaemia and

reperfusion, an increase in intracellular Na^+ moves the reversal potential of the NCX towards more negative potentials and depolarisation of the cell membrane leads to a reversal of the NCX and subsequent Ca^{2+} entry. Therefore, hyperpolarisation of the resting membrane potential through activation of *sarc*K_{ATP} channels would decrease the entry of Ca^{2+} through both voltage-gated Ca^{2+} channels and the reverse mode of the NCX.

Co-expression of Kir6.2 and SUR2A in the non-contractile COS-7 cell line, which does not normally express *sarc*K_{ATP} channels, produced *sarc*K_{ATP} current that could be activated by pinacidil and was inhibited by the sulphonylurea glibenclamide (glyburide) (Jovanović *et al.*, 1998b). Further, in cells co-expressing Kir6.2 and SUR2A, the K_{ATP} opener pinacidil reduced Ca^{2+}-loading during hypoxia-reoxygenation while transfection of either subunit alone had no effect on Ca^{2+} loading. The same group also demonstrated that co-expression of SUR1 and Kir6.2 produced a similar resistance to hypoxia-reoxygenation injury (Jovanović *et al.*, 1998a), which is of particular interest as it appears that the atrial *sarc*K_{ATP} channel may be made up of this subunit combination (Flagg *et al.*, 2008). Because the COS-7 cell line used for these experiments is non-contractile, early shortening of the APD cannot be responsible for the protection observed. In addition, Light *et al.* (2001) showed that *sarc*K_{ATP} channel inhibition increases Ca^{2+} loading during re-oxygenation, rather than during simulated ischaemia. The same group also showed that application of the *sarc*K_{ATP} channel openers pinacidil or P-1075 during reoxygenation was sufficient to hyperpolarise the resting membrane potential and decrease Ca^{2+} loading of ventricular myocytes (Baczkó *et al.*, 2004). These findings suggest that *sarc*K_{ATP} channels may induce protection through hyperpolarisation of the resting membrane potential and consequent decreases in Ca^{2+} loading.

While there is significant evidence that the *sarc*K_{ATP} channel is required for preconditioning of ventricular myocytes, the field remains divided concerning the precise role played *sarc*K_{ATP} channels in preconditioning.

1.6.3 Mitochondrial K_{ATP} channels

Several groups have reported the presence of K^+ or cation channels in the inner mitochondrial membrane that, upon opening, lead to an influx of K^+ into the mitochondrion. These have been termed *mito*K_{ATP} channels, however the properties of these channels vary between accounts and a structure for the channels has not been determined to date. *Mito*K_{ATP} channels were first described by Inoue *et al.* (1991), in giant mitoplasts obtained from rat liver mitochondria. They observed small conductance (9.7pS) channel openings that were inhibited by ATP in 31 out of 94 patches. These channels were not affected by the presence of ADP and inhibition by ATP did not require the presence of Mg^{2+}. However, the use of giant mitoplasts, which are produced by rupturing and reforming of the inner mitochondrial membrane, does provide the possibility that contamination with sarcolemmal membrane could occur (Hanley and Daut, 2005). Er *et al.* (2004) observed a K^+ channel in giant mitoplasts that was activated by testosterone or diazoxide, inhibited by glibenclamide or 5-HD, and had a conductance of 13 pS. Inhibition of the channel by Mg-ATP could be abolished by inhibition of the adenine nucleotide translocase (ANT) with carboxyatractyloside suggesting that ATP may block the channel from the matrix side. Paucek *et al.* (1992) also purified a 54 kDa mitochondrial K^+ channel with a single channel conductance of around 30 pS in 1 mM KCl. The opening of this channel was inhibited by ATP in the presence of Mg^{2+} or Ca^{2+} with a K_i for ATP of 39 μM.

Opening of *mito*K_{ATP} channels leads to an influx of K^+ into the mitochondrion that would be expected to dissipate the mitochondrial membrane potential, resulting in

uncoupling of electron transport from ATP synthesis, and oxidation of flavoproteins. These flavoproteins are fluorescent in their oxidised form and therefore measurement of the flavoprotein fluorescence or mitochondrial membrane potential can be used to examine the degree of mitochondrial uncoupling such as that induced by *mito*K_{ATP} opening (Hanley and Daut, 2005). Expression of dominant negative Kir6.1 and Kir6.2 constructs had no effect on the flavoprotein fluorescence observed in response to diazoxide treatment suggesting that these subunits are not present in *mito*K_{ATP} channels (Seharaseyon *et al.*, 2000).

It has been shown that the *mito*K_{ATP} openers diazoxide and pinacidil are protonophoric un-couplers, which dissipate the H^+ gradient within the mitochondria leading to an increase in state 4 respiration, a decrease in mitochondrial membrane potential, and decrease in ATP production similar to that observed for the uncoupling agent dinitrophenol (DNP) (Holmuhamedov *et al.*, 2004). Mitochondrial uncoupling can cause a decrease in mitochondrial membrane potential and increased flavoprotein oxidation independent of *mito*K_{ATP} channel opening (Rodrigo *et al.*, 2002), and uncoupling of the mitochondria with dinitrophenol (Holmuhamedov *et al.*, 2004, Rodrigo *et al.*, 2002) or the uncoupling proteins UCP1 or UCP2 (Teshima *et al.*, 2003, Bienengraeber *et al.*, 2003), appears to be cardioprotective. Additionally, several preconditioning stimuli have been shown to uncouple mitochondria (Ljubkovic *et al.*, 2007, Minners *et al.*, 2001), suggesting that *mito*K_{ATP} opening drugs may cause cardioprotection via a *mito*K_{ATP}-independent mechanism.

There is evidence for the presence of a large conductance Ca^{2+}-activated, K^+ channel (BK_{Ca}) and the voltage-gated K^+ channel Kv1.3 in mitochondrial inner membrane (Hanley and Daut, 2005). Application of the BK_{Ca} activator NS1619 resulted in an increase in flavoprotein fluorescence that was comparable with that induced by

diazoxide (Sato *et al.*, 2005). Activation of BK_{Ca} was also associated with a decrease in the mitochondrial membrane potential. This strengthens the theory that flavoprotein fluorescence may be a useful indicator of uncoupling and mitochondrial K^+ channel opening.

1.6.4 Role of *mito*K_{ATP} channels in preconditioning

Much of the work concerning the role of *mito*K_{ATP} channels in preconditioning comes from the use of the channel opener diazoxide, and the inhibitor 5-HD. As early as 1992, Auchampach and Gross (1992) observed that 5-HD was able to prevent ischaemic preconditioning of the canine heart, although the effect of 5-HD was not attributed to the *mito*K_{ATP} channel at the time. Garlid *et al.* (1997) demonstrated that diazoxide (30 μM) was able to protect rat hearts from lactate dehydrogenase (LDH) release induced by ischaemia-reperfusion injury, and protection by diazoxide could be inhibited by administration of glibenclamide or 5-HD. Diazoxide was able to activate *sarc*K_{ATP} channel currents with a $K_{1/2}$ value of 840 μM in their experiments; however the $K_{1/2}$ for activation of reconstituted *mito*K_{ATP} channels by diazoxide was 1,000 fold lower at 0.8 μM. Liu *et al.* (1998) observed that diazoxide induced a reversible oxidation of mitochondrial flavoproteins that could be inhibited by 5-HD. Diazoxide also protected isolated ventricular myocytes from ischaemia-reperfusion-induced cell death, suggesting that *mito*K_{ATP} channel opening may be cardioprotective. Lawrence *et al.* (2001) found that diazoxide was able to protect ventricular myocytes from MI-reperfusion injury; however, the flavoprotein fluorescence and mitochondrial membrane potential were not altered by diazoxide. This indicates that either the protective effect of diazoxide does not require *mito*K_{ATP} opening, or *mito*K_{ATP} channel opening is not well correlated with flavoprotein oxidation and depolarisation of the mitochondrial membrane potential.

The role of *mito*K_{ATP} channels in phenylephrine preconditioning has been examined using 5-HD in two studies (Loubani and Galiñanes, 2002, Cohen *et al.*, 2001). Cohen *et al.* demonstrated that phenylephrine preconditioning could be abolished by either 5-HD or the free radical scavenger *N*-2-mercaptopropionyl glycine (MPG) suggesting that phenylephrine preconditioning requires free-radicals and *mito*K_{ATP} activation. Similarly, Loubani and Galiñanes also found that 5-HD abolished phenylephrine preconditioning. Using PMA and anisomycin to activate PKC and p38 MAPK (p38), they further concluded that *mito*K_{ATP} channels are upstream of PKC and p38. However, these conclusions assume that 5-HD specifically blocks the *mito*K_{ATP} channel.

1.6.5 Specificity of pharmacological tools for examining *sarc*- and *mito*K_{ATP}

Activators: Although there is evidence that *sarc*- and *mito*K_{ATP} channels may be involved in preconditioning, a large part of this evidence comes from the use of purportedly specific activators such as diazoxide and nicorandil, and inhibitors such as glibenclamide, HMR1883, and 5-HD. However, these compounds also have non-specific effects that may be responsible for their actions in preconditioning. Diazoxide is able to activate the *sarc*K_{ATP} channel composed of Kir6.2 and SUR2A poorly under resting conditions, but ADP is able to significantly increase the responsiveness of the channel to diazoxide (D'hahan *et al.*, 1999). Therefore, it is possible given the high ADP levels during ischaemia, that diazoxide preconditioning could be mediated by *sarc*K_{ATP} channels. Diazoxide also has other actions including inhibition of succinate dehydrogenase, ATP-synthase and some nucleotide containing enzymes as well as causing mitochondrial uncoupling (Hanley and Daut, 2005). Nicorandil is reported to be a *mito*K_{ATP} selective drug, however it appears that nicorandil may be de-nitrated within cardiac mitochondria producing nitric oxide (NO) and this could be responsible for some of the effects of nicorandil (Sakai *et al.*, 2000).

Inhibitors: As well as inhibiting the *sarc*- and *mito*K_{ATP} channel, glibenclamide has also been reported to inhibit carnitine palmitoyltransferase resulting in a reduction in the rate of fatty acid oxidation (Hanley and Daut, 2005). Similarly, 5-HD can be metabolised by β-oxidation but the slow rate of the penultimate step leads to a reduction in the rate of β-oxidation. Glibenclamide is also able to block ABC transporters and Cl^- channels, and may inhibit Na^+/K^+-ATPase, Ca^{2+} channels, and uncouple rat liver mitochondria (Hanley and Daut, 2005, Lee and Lee, 2005).

Additionally, block of the *sarc*K_{ATP} channel by sulphonylureases such as glibenclamide and HMR1883 is reduced in the presence of high ADP levels leading to a potential underestimation of the importance of the *sarc*K_{ATP} channel (Rainbow *et al.*, 2005, Venkatesh *et al.*, 1991). It is important to take account of these non-specific effects of K_{ATP} activators and inhibitors when using these pharmacological tools to make conclusions concerning the role of *sarc*- and *mito*K_{ATP} channels.

1.7 Protein Kinase C (PKC)

1.7.1 PKC structure and function

PKC is a family made up of structurally related serine-threonine protein kinases that may be activated by lipid co-factors and Ca^{2+}. The kinase is made up of a conserved kinase domain, and a regulatory domain containing an inhibitory pseudosubstrate domain and the C1 and C2 domains responsible for lipid- and Ca^{2+}-dependence and membrane binding, see Figure 1.8 (Steinberg, 2008). The PKC family can be divided into three groups, the Ca^{2+} and DAG-sensitive conventional isoforms (α, β and γ), the Ca^{2+}-insensitive novel isoforms (η, ε, θ and δ) and the Ca^{2+} and DAG-insensitive atypical isoforms (λ, μ, ι and ζ) (Armstrong, 2004). Upon activation, PKC isoforms undergo translocation from the cytosol to various cellular components including the

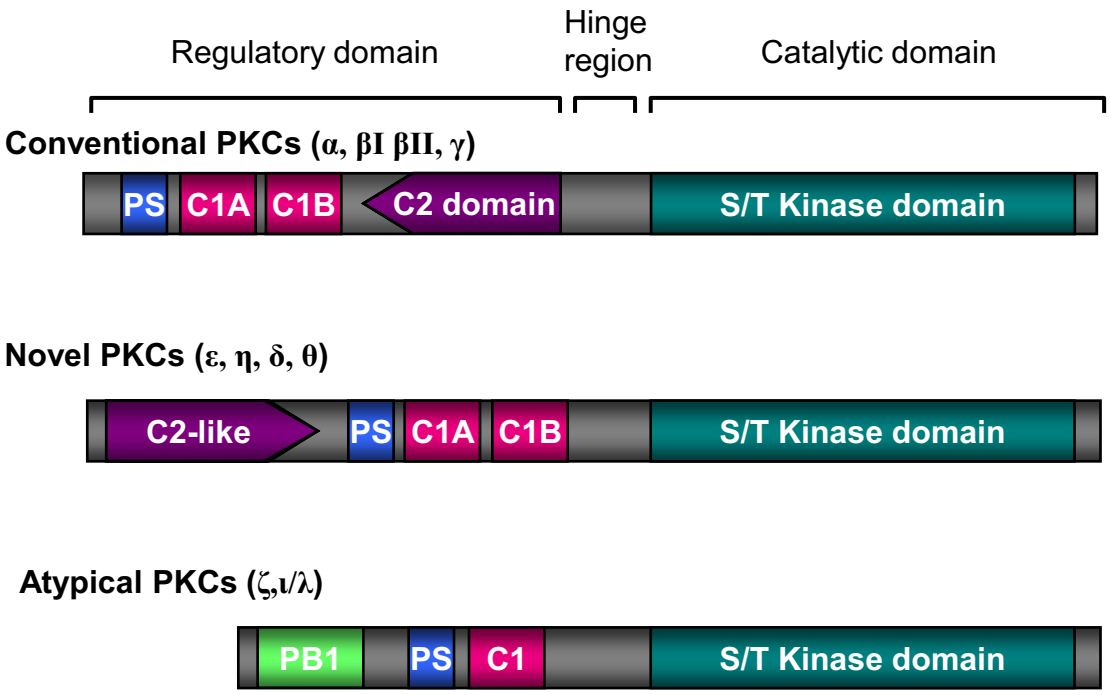

Figure 1.8. Domain structure of conventional, novel and atypical PKC isoforms.

Serine/threonine kinase domain (teal). Within the regulatory domain, the pseudosubstrate domain (PS; blue) is located NH_2-terminal to the C1 domain (pink). Tandem C1 domains in conventional and novel PKCs are responsible for DAG and PMA binding. Conventional PKCs possess a C2 domain (purple) which binds to lipids in a calcium-dependent fashion, while the C2 domain of novel PKCs lacks the calcium co-ordinating residues; the direction of the arrow demonstrates the orientation of the anti-parallel β-sheets that make up the C2 domain. Atypical PKCs possess a PB1 domain involved in protein-protein interactions (green). Adapted from Steinberg (2008) and Corbalán-García and Gómez-Fernández (2006).

sarcolemmal membrane (Mitchell *et al.*, 1995, Wang and Ashraf, 1998, Miyawaki *et al.*, 1996), mitochondria (Wang and Ashraf, 1999), cytoskeletal elements (Gray *et al.*, 1997, Disatnik *et al.*, 1994), golgi complex (Kang and Walker, 2005), perinuclear region (Gray *et al.*, 1997, Wang and Ashraf, 1998, Miyawaki *et al.*, 1996), nucleus (Mitchell *et al.*, 1995, Wang and Ashraf, 1998), intercalated discs (Gray *et al.*, 1997, Wang and Ashraf, 1998) and t-tubules (O-Uchi *et al.*, 2008).

The C1 domain of PKC is responsible for activation by lipid cofactors such as DAG, arachidonic acid and ceramide and the phorbol ester PMA (Steinberg, 2008). Both conventional and novel PKC isoforms contain tandem C1 domains while atypical PKCs contain a single C1 domain that lacks the arginine residues necessary for DAG and PMA binding. Interaction of positively charged residues in the C1 domain with anionic membrane phospholipids allows the hydrophobic C1 domain to penetrate the membrane and bind DAG. Some C1 domains such as the PKCα C1 are inaccessible in the resting state, becoming exposed during the conformational change caused by Ca^{2+}-dependent binding of the C2 domain to the membrane. The C2 domain is present in conventional and novel PKC isoforms and consists of eight anti-parallel β-sheets connected by membrane- and Ca^{2+}-binding loops. In conventional PKCs, Ca^{2+} ions are able to bind cooperatively to aspartate residues within the Ca^{2+}-binding loops; however, these aspartate residues are missing from novel PKC C2 domains resulting in their Ca^{2+}-independence. The orientation of the anti-parallel β-sheets in the C2 domains of conventional and novel PKCs is different, conventional PKCs show type I topology, while the C2 domain of novel PKCs has type II topology (Corbalán-García and Gómez-Fernández, 2006). The C2 domains of both conventional and novel PKCs also act as lipid binding domains (Steinberg, 2004).

As well as direct interaction with the membrane, the membrane localisation of activated PKC is maintained by binding to membrane-associated scaffold proteins known as Receptor for Activated C Kinase (RACKs) (Mochly-Rosen *et al.*, 1991). Each PKC contains a sequence that enables it to bind to its respective RACK, and a pseudo-RACK (ψRACK) sequence that mimics the PKC-binding site on the RACK located within the C2 domain. Interaction of these sites is thought to stabilise the C2 domain structure and this interaction must be disrupted for PKC activation to occur (Mochly-Rosen *et al.*, 1991, Schechtman *et al.*, 2004). PKCs also contain an autoinhibitory pseudosubstrate domain (House and Kemp, 1987). The sequence of this pseudosubstrate domain corresponds to that of a PKC substrate where the phosphorylatable residue is replaced by a non-phosphorylatable residue such as alanine. The pseudosubstrate interacts with the catalytic domain and maintains the enzyme in an inactive conformation under resting conditions. Upon activation, PKC undergoes a conformational change and the pseudosubstrate domain is reversibly released from the catalytic site allowing PKC to phosphorylate its substrates (Orr *et al.*, 1992).

PKCδ is also modulated by tyrosine phosphorylation of several residues that may alter the subcellular localisation and kinase activity of the enzyme as well as creating SH2 docking sites and inducing caspase cleavage of the enzyme (Steinberg, 2004).

1.7.2 PKC and preconditioning

Soon after the discovery that both adenosine receptor and α_1-adrenoceptor stimulation could evoke preconditioning, attention turned to the common downstream pathway activated by these interventions, particularly the activation of PKC. The role of PKC in preconditioning was identified concurrently by the groups of Downey and Banerjee in a series of papers in 1994 (Ytrehus *et al.*, 1994, Mitchell *et al.*, 1995, Liu *et al.*, 1994, Tsuchida *et al.*, 1994). It was observed that inhibition of PKC was sufficient to prevent

IPC and phenylephrine preconditioning, while activation of PKC using phorbol esters or DAG was able to mimic the cardioprotective effect of preconditioning. Although it was initially thought that PKC played a triggering role in IPC, Yang *et al.* (1997) showed that PKC inhibition during the preconditioning stimulus did not prevent IPC, while inhibition of PKC during the index ischaemia completely abolished the protective effect of IPC on infarct size. This suggests that PKC acts as a mediator of IPC during the index ischaemia rather than as a trigger for protection.

PKCε: The identification of the PKC isoforms responsible for preconditioning has proved more controversial and appears to be dependent on the preconditioning stimulus used and time point examined. PKCε translocates to the membrane in response to IPC or hypoxic preconditioning (Mitchell *et al.*, 1995, Gray *et al.*, 1997). Studies using the εV1-2 PKCε inhibitor peptide have shown that preconditioning can be abolished by inhibition of PKCε (Gray *et al.*, 1997). Similarly, activation of PKCε using the ψεRACK PKCε activator peptide prior to a hypoxic insult results in cardioprotection but the protection was lost when ψεRACK was administered only during reperfusion (Dorn *et al.*, 1999).

Mitchell *et al.* (1995) did not observe translocation of PKCε in response to phenylephrine preconditioning in the isolated rat heart, however PKCε translocation was observed by Tsouka *et al.* (2002) in isolated rat cardiac myocytes. Furthermore, Gao *et al.* (2007) observed that phenylephrine preconditioning increased translocation of PKCε to the membrane fraction during both the preischaemic period and after ischaemia-reperfusion in isolated ventricular myocytes, and PKCε inhibition using the εV1-2 inhibitor peptide abolished the protective effects of phenylephrine preconditioning on contractile recovery, ATP levels and cytochrome C release.

PKCδ: PKCδ also translocates to the membrane in response to IPC and phenylephrine preconditioning (Mitchell *et al.*, 1995, Tsouka *et al.*, 2002, Yoshida *et al.*, 1997), therefore it was proposed that PKCδ played a protective role in preconditioning, a hypothesis that was supported by the work of Meldrum *et al.* (1997a). A constitutively active PKCδ construct was also able to protect transfected neonatal ventricular myocytes from ischaemia-reperfusion injury (Zhao *et al.*, 1998). The development of PKCδ inhibitor (δV1-1) and activator (ψδRACK) peptides by Mochly-Rosen's group provided specific tools to examine the role of PKCδ (Chen *et al.*, 2001). Using these peptides it was found that activation of PKCδ increased ischaemia-reperfusion injury, while inhibition of PKCδ increased survival of isolated rat hearts and cardiac myocytes exposed to simulated ischaemia and reperfusion (Chen *et al.*, 2001). However, the same group also showed that activation of PKCδ 60 or 90 minutes before ischaemia reduced infarct size (Inagaki and Mochly-Rosen, 2005). The different effects observed in these studies may be due to differences in the time course of PKCδ activation. It was subsequently found that the damaging effect of PKCδ was mediated through increased reperfusion injury (Inagaki *et al.*, 2003b). Therefore, inhibition of PKCδ may be a useful strategy for postconditioning of the myocardium. In a 'first in human' trial of patients suffering from acute myocardial infarction, the δV1-1 peptide (KAI-9803) was found to cause a consistent, non-significant improvement in creatine kinase release and ST-segment elevation on the ECG (Bates *et al.*, 2008).

It is therefore possible that PKCδ may play a dual role in preconditioning, activating a protective pathway during the preconditioning stimulus and a damaging pathway during reperfusion. However the magnitude of the damaging effect of PKCδ during reperfusion and the strong protection obtained with PKCε activation means that the potential for a protective mechanism involving PKCδ has been somewhat ignored.

Conventional PKCs: The Ca^{2+}-dependent PKCα has also been implicated in preconditioning. Although translocation of PKCα has been observed in response to some forms of preconditioning in the heart, the effect is not reproducible in all studies and appears to be dependent on the preconditioning stimulus employed. Unsurprisingly, PKCα translocation has been observed in response to Ca^{2+} preconditioning (Miyawaki *et al.*, 1996) although it remains unclear whether this is important to protection or merely incidental. PKCα does not appear to translocate in response to IPC (Mitchell *et al.*, 1995, Tsouka *et al.*, 2002), and phenylephrine preconditioning resulted in translocation of PKCα in one study (Tsouka *et al.*, 2002) but another study found no effect (Mitchell *et al.*, 1995). PKCβ also seems to be activated by ischaemia-reperfusion within the heart. Knock out or inhibition of PKCβ reduced infarct size in mouse hearts, suggesting that PKCβ may increase ischaemia-reperfusion injury (Kong *et al.*, 2008).

1.7.3 PKC-dependent modulation of *sarc*K_{ATP} channel function

Because both PKC and *sarc*K_{ATP} channels have been implicated in the mechanism of preconditioning, further studies examined the effect of PKC modulation on *sarc*K_{ATP} channel activity. Hu *et al.* (1996) demonstrated that activation of PKC using the phorbol ester phorbol 12, 13-didecanoate (PDD), resulted in activation of *sarc*K_{ATP} current in ventricular myocytes dialysed with 400 μM ATP for 20 minutes prior to PDD application. This was associated with a change in the K_i for ATP from 260 μM to 601 μM in response to PDD treatment and an alteration in the Hill coefficient for ATP binding from 2.97 to 4.36. Therefore, it appeared that activation of PKC with PDD decreased the potency of ATP block of the channel while increasing the cooperativity of ATP binding. However, studies by Light *et al.* (2000, 1996) showed a decrease in the Hill coefficient and an increase in channel opening, which resulted in an excitatory effect on *sarc*K_{ATP} opening at higher ATP concentrations and an inhibitory effect at

lower ATP concentrations with a cross over at 110 μM. Enhancement of *sarc*K_{ATP} current by PKC was mediated through both the Kir6.2 and SUR2A subunits of the *sarc*K_{ATP} channel, and phosphorylation of Kir6.2 at Thr180 was demonstrated in response to PKC activation (Light *et al.*, 2000). The same group also showed that PMA treatment of tsA201 cells transfected with Kir6.2 and SUR2A decreased Ca^{2+} loading during reperfusion compared with non-transfected cells treated with PMA, or untreated cells expressing *sarc*K_{ATP} channels (Light *et al.*, 2001). This suggests that PKC is able to modulate *sarc*K_{ATP} channel activity leading to an improvement in Ca^{2+} homeostasis.

Measurements of APD_{90} and resting membrane potential in the presence and absence of glibenclamide suggest that *sarc*K_{ATP} channels open persistently during reperfusion in guinea pig right ventricular wall (Shigematsu *et al.*, 1995); however, reperfusion appears to result in rapid closure of the *sarc*K_{ATP} channel in isolated ventricular myocytes (Shigematsu and Arita, 1997). Ito *et al.* (2001) showed that activation of novel PKC isoforms resulted in persistent activation of *sarc*K_{ATP} channels during reperfusion that was augmented by combined activation of novel and conventional PKC isoforms, indicating that conventional and novel PKC isoforms may be responsible for persistent *sarc*K_{ATP} channel opening and hyperpolarisation of the resting membrane potential in the heart upon reperfusion.

While it seems clear that PKC activation is able to cause a significant increase in *sarc*K_{ATP} current, the mechanisms responsible remain ambiguous. The different findings of these studies may arise from the different experimental models used or may be due to modification of different residues within this large and highly complex channel.

1.8 The mitogen-activated protein kinase (MAPK) family

1.8.1 MAPK

Mitogen-activated protein kinases (MAPK) are proline directed serine/threonine protein kinases that are activated by various mitogenic and stress signals. The three main MAPK families in mammalian cells, are the extracellular signal-regulated kinases 1/2 (ERK), Jun N-terminal kinases (JNK) and p38 MAPKs (p38). ERKs are mainly activated by mitogens, growth factors and physical stress; while JNKs, also known as stress-activated protein kinase (SAPKs), and p38s are activated by stresses such as ischaemia, metabolic stress, osmotic shock, UV, protein synthesis inhibition and cytokines but are relatively poorly activated by mitogenic stimuli (Kyriakis and Avruch, 2001, Petrich and Wang, 2004). MAPK exert effects on cellular behaviour through their ability to phosphorylate a wide variety of effector proteins and transcription factors.

The MAPKs ERK, JNK and p38 are activated by parallel three tier signalling modules that involve activation of a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK), which then phosphorylates the MAPK (Figure 1.9). This three tier module allows regulation of the pathway at many levels, amplification of the signal, and crosstalk between signalling pathways. MAPKKK activation generally requires three steps: recruitment to the membrane, homoligomerisation and phosphorylation by upstream kinases. For example, the activation of the MAPKKK Raf requires recruitment to the membrane by Ras, homoligomerisation, often as part of a complex containing additional regulatory proteins, and phosphorylation by upstream kinases such as p21-activated kinase. Raf then activates the dual specificity MAPKK MEK via serine phosphorylation in subdomain VIII, and MEK activates ERK via dual

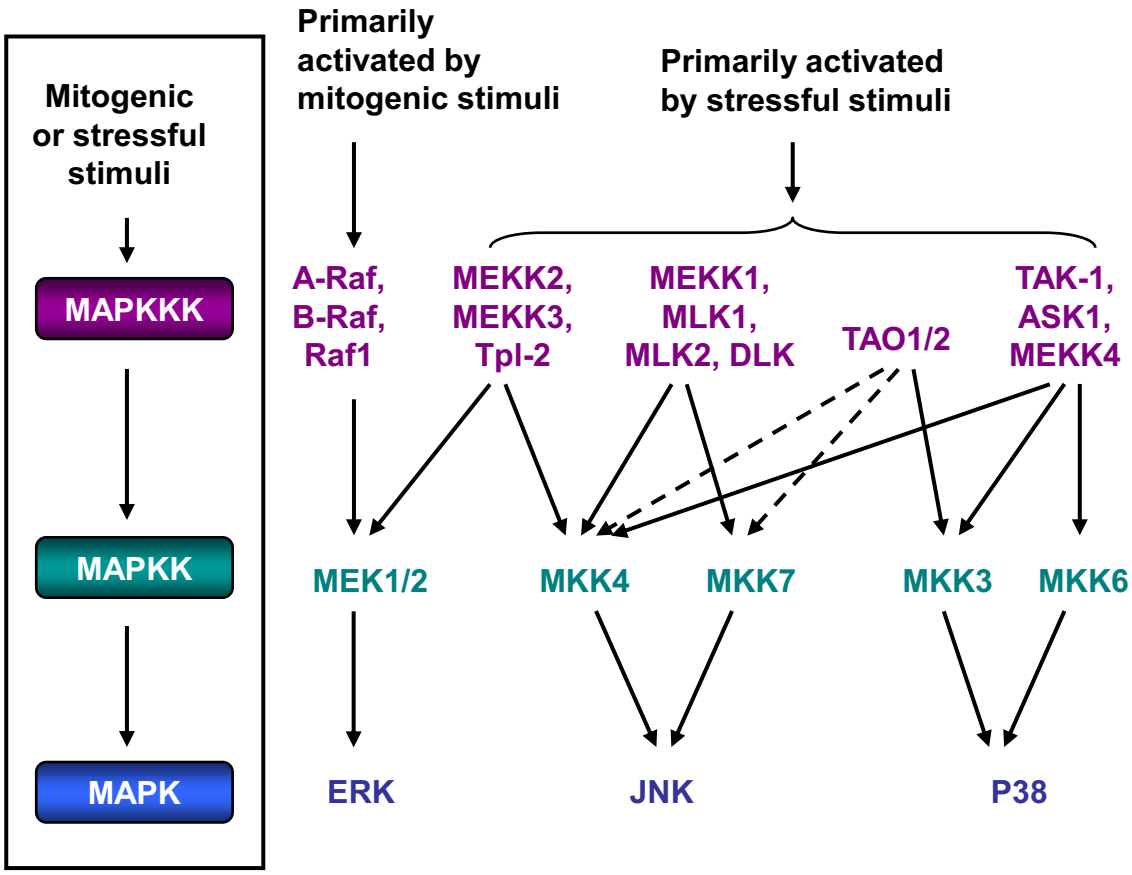

Figure 1.9. Three-tier signalling pathway responsible for MAPK activation.

Schematic of the MAPK three-tier signalling pathway, showing MAPKKK (purple), MAPKK (teal), and MAPK (blue). MAPK pathways responsible for activation of ERK, JNK and p38. Adapted from Kyriakis and Avruch (2001) and Roux and Blenis (2004).

phosphorylation of a Thr-X-Tyr motif in subdomains VII and VIII of ERK. Similar modes of activation exist for p38 and JNK (Kyriakis and Avruch, 2001).

Scaffolding proteins are important to the specificity of MAPK signalling. The concentrations of MAPK signalling module components at specific cellular locations are increased by these scaffolding proteins, thus allowing spatial control of signalling. Scaffold proteins can also prevent inappropriate activation of MAPK caused by extraneous signalling or cross-talk between pathways, since they bind specific kinases in a particular MAPK module. Examples of scaffolds include Kinase Suppressor of Ras (KSR) for the ERK signalling module (Claperon and Therrien, 2007), while JNK-interacting proteins (JIPs) scaffold the JNK pathway (Dickens *et al.*, 1997, Yasuda *et al.*, 1999). Intrinsic scaffolding properties may also be displayed by kinases, such as the MAPKKK MEKK1 which is able to scaffold components of the ERK and JNK signalling module and localise their signalling to cytoskeletal elements (Morrison and Davis, 2003).

1.8.2 ERK

ERK, also known as p44 and p42-MAPK, is strongly activated by mitogenic stimuli such as growth factors, phorbol ester treatment and serum and also activated to a lesser extent by GPCR activation, cytokines and various cellular stresses (Roux and Blenis, 2004). As noted above, activation of ERK is catalysed by dual phosphorylation of a Thr-Glu-Tyr motif within the activation loop of ERK by the MAPKKs MEK.

Upon activation, a proportion of active ERK accumulates in the nucleus so it is able to phosphorylate both cytosolic substrates and nuclear transcription factors resulting in a diverse range of signalling outcomes. ERK is able to phosphorylate the chaperone protein calnexin and cytoskeleton-associated proteins such as microfilaments and

paxillin. ERK can also phosphorylate the MAPK-activated kinases 90kDa ribosomal S6 kinases (Rsks), MAPK-interacting kinases (MNK1/2) and mitogen- and stress-activated kinases (MSK1/2), as well as transcription factors including Elk-1, MEF2, STAT3, c-Fos and NFAT (Kyriakis and Avruch, 2001, Roux and Blenis, 2004, Yoon and Seger, 2006).

1.8.3 Role of ERK in ischaemia-reperfusion and preconditioning

Ischaemia-reperfusion: Bogoyevitch *et al.* (1996) observed no activation of p42 or p44 ERK by ischaemia or ischaemia-reperfusion in the rat heart by in-gel MBP kinase assay. However, in other studies ERK activation was increased by reperfusion (Takeishi *et al.*, 2001, Knight and Buxton, 1996, Fryer *et al.*, 2001b). Inhibition of ERK using the MEK inhibitor PD98059 had no effect on infarct size in rat hearts subjected to ischaemia reperfusion (Fryer *et al.*, 2001b). Similarly, PD98059 had no effect on LDH release from isolated rabbit cardiac myocytes (Ping *et al.*, 1999a), implying that ERK is not essential for survival of ischaemia-reperfusion injury.

Preconditioning: While ERK may have little effect on ischaemia reperfusion injury, it appears to play a role in IPC. IPC stimuli have been shown to result in activation of ERK, primarily in the nuclear fraction (Ping *et al.*, 1999a, Mocanu *et al.*, 2002, Hausenloy *et al.*, 2005a, Hausenloy *et al.*, 2005a), with ERK activity returning to baseline levels during the subsequent index ischaemia (Fryer *et al.*, 2001b, Hausenloy *et al.*, 2005a). A second peak of ERK activity was observed during reperfusion (Fryer *et al.*, 2001b, Hausenloy *et al.*, 2005a), however one study showed no effect of IPC on ERK phosphorylation compared with non-preconditioned hearts (Takeishi *et al.*, 2001). The picture is made no clearer by the use of the inhibitor PD98059. Mocanu *et al.* (2002) observed that PD98059 had no effect on infarct size in IPC rat hearts. In contrast, others have shown that the protective effect of IPC on infarct size can be completely

abolished by PD98059 treatment (Fryer *et al.*, 2001b, Hausenloy *et al.*, 2005a), although PD98059 did not inhibit ERK activation within the nuclear fraction in response to IPC and δ-opioid preconditioning in the same study, implying that it may be the activation of cytosolic and not nuclear localised ERK that is important for preconditioning.

It now appears that reperfusion is the critical period for ERK-mediated protection from ischaemia-reperfusion injury. The infarct-limiting effect of IPC was lost when the MEK inhibitor U-0126 was administered from 5 minutes before reperfusion until 15 minutes after the onset of reperfusion (Solenkova *et al.*, 2006). However, if administration was delayed until 10 minutes after reperfusion, U-0126 did not block the protective effect of IPC on infarct size suggesting that ERK activation during early reperfusion is important for protection. Along with PI3K and Akt, ERK is a component of the reperfusion injury salvage kinase pathway (RISK) that confers protection from reperfusion injury through inhibition of apoptotic and necrotic cell death (Hausenloy *et al.*, 2005b). Inhibition of the MPTP appears to be an important consequence of RISK pathway activation.

Although the role of ERK in phenylephrine preconditioning of the heart has not been examined, ERK is phosphorylated in response to 5 and 15 minutes of acute phenylephrine treatment (Lazou *et al.*, 1998). This indicates that ERK could be involved in phenylephrine preconditioning.

In conclusion, ERK appears to be activated by IPC and may play a protective role in IPC, particularly during the reperfusion period when it may be involved in inhibition of the MPTP.

1.8.4 JNK

Unlike ERK, JNK is strongly activated by cellular stresses including heat shock, osmotic shock, UV, protein synthesis inhibition, DNA damage, oxidative stress and reperfusion injury and is weakly activated by mitogenic stimuli (Kyriakis and Avruch, 2001, Petrich and Wang, 2004). JNK is activated by phosphorylation of a conserved Thr-Pro-Tyr motif by the MAPKKs, MKK4/SEK1 and MKK7. The organisation and naming of the JNK family of proteins is complex; at least three genes code for JNK family members (JNK1, 2, and 3) while hnRNA splicing within the catalytic domain (producing α and βJNKs) and at the C-terminal (producing 46 kDa type 1 and 54 kDa type 2 JNKs) means that at least 10 different JNK proteins have been identified (Gupta *et al.*, 1996).

JNK is able to phosphorylate several components of the AP-1 transcription factor activating c-Jun (and JunD), ATF-2, MEF2A/C, and Elk-1/TCF (Kyriakis and Avruch, 2001), while JNK phosphorylation of NFAT4 and NFAT1c inhibits the Ca^{2+}-dependent nuclear localisation of these proteins (Chow *et al.*, 1997, Chow *et al.*, 2000). It also appears that JNK is involved in the regulation of apoptosis, playing a pro- or anti-apoptotic role dependent on the stimulus and cell type examined (Dhanasekaran and Reddy, 2008, Xia *et al.*, 1995). Transfection of a dominant negative form of JNK or MKK4 increased apoptosis, caspase 9 activation and DNA laddering in neonatal myocytes subjected to hypoxia-reoxgenation, while expression of a constitutively active form of upstream kinase MEKK1, decreased hypoxia-reoxgenation induced apoptosis (Dougherty *et al.*, 2002). In contrast, Aoki *et al.* (2002) showed that a dominant negative MKK4 reduced apoptotic cell death induced by oxidative stress (superoxide generation) in cultured adult rat ventricular myocytes. Some of the differences in these findings may be explained by the observations of Hreniuk *et al.* (2001), who

demonstrated that an anti-sense oligonucleotide directed against JNK1 but not JNK2 decreased reoxgenation-induced apoptotic cell death.

Although many well known JNK substrates are transcription factors, the important role of JNK in apoptosis indicates that phosphorylation of cytosolic substrates by JNK is important for regulation of cell death/survival.

1.8.5 Role of JNK in ischaemia-reperfusion and preconditioning

Ischaemia-reperfusion: Several studies have demonstrated that JNK is poorly activated by ischaemia (Bogoyevitch *et al.*, 1996, Yin *et al.*, 1997), although JNK activation during ischaemia was observed in one study (Takeishi *et al.*, 2001). Reperfusion results in a rapid and sustained activation of JNK (Bogoyevitch *et al.*, 1996, Knight and Buxton, 1996, Yin *et al.*, 1997), which can be abolished by Ca^{2+} depletion or perfusion of the free radical scavengers catalase and superoxide dismutase during ischaemia and reperfusion, suggesting that JNK activation may be due to ROS generated on reperfusion (Knight and Buxton, 1996). The effect of JNK inhibition during ischaemia-reperfusion has also been examined using antisense RNA against JNK1 or JNK2. Antisense RNA against JNK1 but not JNK2 decreased apoptosis in response to simulated ischaemia and reperfusion in neonatal cardiac myocytes (Hreniuk *et al.*, 2001). Kaiser *et al.* (2005) showed that knock out of JNK1 or JNK2 decreased infarct size in mouse hearts. However, the same study also showed a decrease in infarct size in the hearts of mice overexpressing MKK7, an upstream activator of JNK. Both knock out of JNK and overexpression of MKK7 decreased TUNEL within the infarcted regions of the hearts suggesting a decrease in apoptosis in both cases. This suggests that JNK can be either protective or damaging during ischaemia-reperfusion.

Preconditioning: The effect of IPC on activation of JNK is also contentious. JNK was activated by four or six cycles of IPC, and an increase in p46 JNK activity within the nuclear fraction and an increase in p54 JNK activity within the cytosolic fraction were observed (Ping *et al.*, 1999b, Sato *et al.*, 2000). Activation of JNK during the index ischaemia was observed in some IPC studies (Takeishi *et al.*, 2001, Iliodromitis *et al.*, 2002) but not others (Knight and Buxton, 1996, Fryer *et al.*, 2001a), while JNK activation during reperfusion was universally observed (Knight and Buxton, 1996, Sato *et al.*, 2000, Fryer *et al.*, 2001a).

The role of JNK in phenylephrine preconditioning has not been examined to date, however acute phenylephrine treatment is sufficient to activate JNK in the isolated rat heart (Lazou *et al.*, 1998). At present, the evidence suggests that JNK is activated by IPC and could play a protective role in preconditioning but further study of the role of JNK using more specific pharmacological or molecular tools is needed to determine the conditions required for the protective and injurious effects of JNK activation.

1.8.6 p38

Similarly to JNK, p38 can be activated in response to stressful stimuli such as osmotic shock, inflammatory cytokines, UV and γ-irradiation, toxins, protein synthesis inhibitors (i.e. anisomycin) and ischaemia-reperfusion (Kyriakis and Avruch, 2001, Petrich and Wang, 2004). The p38 family is made up of 4 isoforms, p38 α and β which are inhibited by CSAIDs (cytokine suppressive anti-inflammatory drugs), and γ and δ that are CSAID-insensitive. The major isoforms expressed in the heart are p38α and β (Roux and Blenis, 2004).

Activation of p38 is catalysed by dual phosphorylation of a conserved Thr-Gly-Tyr motif within the activation loop of p38 by MKK3 and MKK6. Although MKK6 is able

to activate all p38 isoforms, MKK3 preferentially activates the α isoform (Kyriakis and Avruch, 2001, Zarubin and Han, 2005). The JNK kinase MKK4 also possesses p38 kinase activity *in vitro* allowing the possibility of integration of these signalling pathways. Activation of p38 can also be achieved by an alternative mechanism involving TAB1 (TAK1-binding protein). TAB1 acts as a scaffold protein that binds p38 and promotes its auto-phosphorylation via a mechanism that requires AMPK activation/binding to TAB1 (Li *et al.*, 2005). Thus p38 can be activated by diverse stimuli and signalling mechanisms.

Differences in biological functions of the different p38 isoforms add complexity to their signalling. Knock out of p38α is developmentally lethal (Adams *et al.*, 2000), but overexpression studies have provided some understanding of the roles of p38 isoforms. Overexpression of MKK6 results in increased functional recovery from cardiac ischaemia (Martindale *et al.*, 2005). In contrast, overexpression of p38α, or MKK3, which primarily activates p38α has been observed to increase apoptosis under resting conditions (Wang *et al.*, 1998), although the role of MKK3 overexpression in ischaemia-reperfusion has not been examined.

p38 is able to phosphorylate a number of substrates including MNK 1 and 2, MSKs, p38 regulated/activated kinase (PRAK), MAPK-activated protein kinase 2 (MAPKAPK2) and the structurally related MAPKAPK3. Activation of p38 and downstream protein kinases leads in turn to phosphorylation of proteins including small heat shock protein HSP27, the scaffold protein 14-3-3ζ, 5-lipoxygenase, tyrosine hydroxylase, eukaryotic initiation factor eIF-4E, Akt, Bad, NHE and the transcription factors CREB, ATF1, STAT3, and NF-κB (Kyriakis and Avruch, 2001, Roux and Blenis, 2004).

1.8.7 Role of p38 in ischaemia-reperfusion and preconditioning

Ischaemia-reperfusion: Activation of p38 during ischaemia appears to be transient, with a peak of p38 phosphorylation after around 5-20 minutes, followed by a decrease in phosphorylation with prolonged ischaemia (Bogoyevitch *et al.*, 1996, Shimizu *et al.*, 1998, Ma *et al.*, 1999). A second peak of p38 phosphorylation is observed during reperfusion (Bogoyevitch *et al.*, 1996, Yin *et al.*, 1997, Ma *et al.*, 1999). Inhibition of p38 was also shown to improve post-ischaemic function in the isolated rat heart (Ma *et al.*, 1999), treatment with the p38 inhibitor SB203580 (10 μM) during ischaemia-reperfusion improved LVDP, and decreased CK loss, necrotic cell death and apoptotic cell death.

Preconditioning: Like the other MAPK, the role of p38 in preconditioning is controversial. IPC has been demonstrated to cause an increase in p38 phosphorylation during index ischaemia (Weinbrenner *et al.*, 1997), or early activation of p38 during index ischaemia (Takeishi *et al.*, 2001). Fryer *et al.* (2001a) observed no change in p38 phosphorylation within either the nuclear or cytosolic fractions in response to IPC suggesting that p38 activation is not modulated by preconditioning. However, SB203580 was able to abolish the protective effect of IPC on infarct size in this study therefore it is possible that the rapid dephosphorylation of p38 by phosphatases may have prevented detection of the phosphorylated protein. It also appears that the IPC stimulus is able to activate p38 (Sato *et al.*, 2000, Marais *et al.*, 2001), although in both of these studies a decrease in p38 activation was observed in response to the subsequent index ischaemia-reperfusion protocol. Although Sato *et al.* (2000) observed that inhibition of p38 with SB203580 abolished the protective effect of IPC on infarct size; it also decreased the infarct size in hearts subjected to ischaemia-reperfusion in the absence of preconditioning. In contrast, Marais *et al.* (2001) found no effect of

SB203580 on the viability of hypoxic preconditioned cardiac myocytes subjected to ischaemic pelleting. Interestingly, current evidence suggests that p38α may increase ischaemia-reperfusion damage, while activation of p38β is thought to be protective (Martindale *et al.*, 2005, Wang *et al.*, 1998, Saurin *et al.*, 2000). Therefore, it appears that p38 activation may result in activation of protective or damaging patways depending on the stimulus and the isoform of p38 activated.

1.8.8 Phenylephrine and p38

As well as activation by ischaemia, p38 is activated by acute phenylephrine treatment. Lazou *et al.* (1998) observed that treatment with 100 μM phenylephrine resulted in activation of p38 within 5 minutes and this activation was still maintained above basal levels after 45 minutes.

The requirement for p38 activation in phenylephrine preconditioning was identified by Loubani and Galiñanes (2002), using human atrial appendage. Phenylephrine preconditioning produced a similar level of protection to IPC in this study, and protection was abolished by the PKC inhibitor chelerythrine, the p38 inhibitor SB203580 or the *mito*K_{ATP} inhibitor 5-HD. They also suggested that there is only one signalling pathway involved in the protective effect of both ischaemic and pharmacological preconditioning. Using the *mito*K_{ATP} activator diazoxide, the PKC activator PMA and the p38 activator anisomycin they suggested that *mito*K_{ATP} lies upstream, and p38 lies downstream of PKC in preconditioning.

1.8.9 Activation of MAPK proteins by PKC: role in preconditioning

PKC is involved in activation of the MAPK (Bogoyevitch *et al.*, 1994). As increasing evidence suggested a role for both PKC and MAPK in preconditioning, focus turned to the identification of the PKC isoforms responsible for activation of each of the MAPK

cascades. The first PKC isoform to be examined was PKCε in two studies by Bolli's group (Ping *et al.*, 1999a, Ping *et al.*, 1999b). Overexpression of wild type PKCε in adult cardiac myocytes caused a significant increase in the activity of both isoforms of ERK. PKCε overexpression also significantly reduced LDH release induced by simulated ischaemia-reperfusion, and inhibition of ERK with PD98059 could block the reduction in LDH release (Ping *et al.*, 1999a). Overexpression of the same wild type PKCε construct was also sufficient to activate both JNK isoforms but had not effect on p38 activity (Ping *et al.*, 1999b). Subsequently, Heidkamp *et al.* (2001) found that overexpression of a constitutively active form of PKCε in neonatal rat ventricular myocytes caused a 7-fold increase in phosphorylation of ERK; while PKCδ overexpression did not significantly increase ERK phosphorylation suggesting that PKCε and not δ is responsible for activation of ERK. PKCε also induced a modest (4-fold) activation of JNK, however overexpression of PKCδ resulted in a marked increase in JNK phosphorylation with a 47-fold increase observed. Similarly, overexpression of PKCε did not significantly increase the phosphorylation of p38, while PKCδ overexpression led to a 15-fold increase in p38 phosphorylation. Taken together, these data indicate that ERK is preferentially activated by PKCε, while JNK and p38 are preferentially activated by PKCδ.

1.9 AMP-activated protein kinase (AMPK)

AMP-activated protein kinase (AMPK) is a kinase that is highly conserved across eukaryotic species and is comprised of α, β and γ subunits (see Figure 1.10). In lower organisms, it appears that the AMPK is required for cellular responses to starvation; however it has evolved in mammalian cells to regulate energy homeostasis at a whole body as well as cellular level. AMP is able to bind cooperatively to two Bateman domains on the γ-subunit of AMPK. The binding of AMP allosterically activates

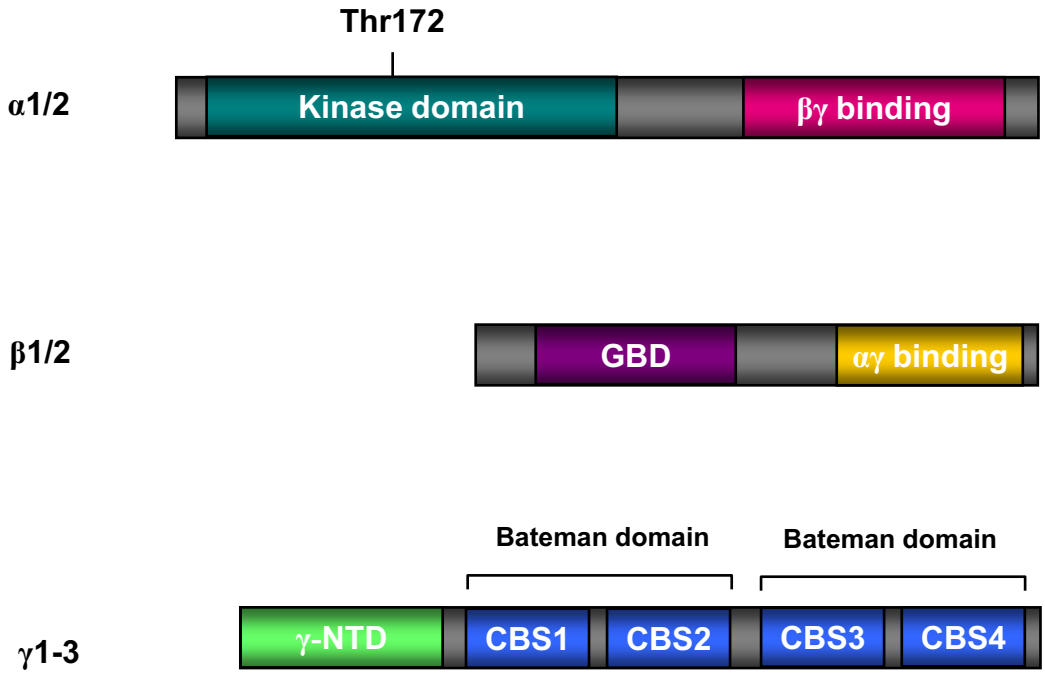

Figure 1.10. Structure of AMPK.

Domain structure of the α, β, and γ subunits of AMPK. Kinase domain (teal) showing the location of the Thr172 phosphorylation site responsible for AMPK activation. βγ-binding domain (pink). Glycogen binding domain (GBD; purple). αγ-binding domain (gold). Two pairs of CBS motifs 1-4 (blue) make up the two Bateman domains responsible for binding of AMP and ATP. The γ subunit also contains a variable length N-terminal domain (NTD; green). Adapted from Carling (2004) and Towler and Hardie (2007).

AMPK, and also induces a conformational change that may promote phosphorylation of the α-subunit at Thr172, or prevent dephosphorylation of this site by phosphatase PP2C (Suter *et al.*, 2006, Baron *et al.*, 2005). While the allosteric activation mediated by AMP-binding results in up to 10-fold activation of AMPK, phosphorylation at Thr172 can activate AMPK up to 1,000-fold (Suter *et al.*, 2006). AMPK may also be activated by a decrease in the phosphocreatine:creatine ratio within the cell (Ponticos *et al.*, 1998).

The LKB1:STRAD:MO25 complex is able to phosphorylate AMPK at Thr172 (Hawley *et al.*, 2003), and it appears that this complex is constitutively active. AMPK is also phosphorylated at Thr172 in a Ca^{2+}/calmodulin dependent manner by calmodulin-dependent protein kinase kinase (CaMKKα and β), particularly the β isoform (Towler and Hardie, 2007). It has been recently found that transforming growth factor β-activated kinase (TAK1) is able to activate AMPK in cell-free assays (Momcilovic *et al.*, 2006). TAK1 is a MAPKKK that acts upstream of both p38 and JNK. Co-expression of TAK1-binding protein (TAB1) increased the TAK1-dependent AMPK phosphorylation. This is of particular note when coupled with the fact that AMPK forms a complex with TAB1 and p38 MAPK that is able to promote activation of p38 MAPK via autophosphorylation in the ischaemic heart (Li *et al.*, 2005) and could potentially represent an alternative pathway for p38 MAPK activation. While LKB1 is ubiquitously expressed, CaMKK expression is more limited (Towler and Hardie, 2007). CaMKK expression has been demonstrated in the pig heart (Uemura *et al.*, 1998), suggesting that CaMKK could activate AMPK in the heart in response to Ca^{2+} signalling.

AMPK is able to directly phosphorylate several proteins involved in metabolism leading to a decrease in the activity of glycogen synthase, acetyl-CoA carboxylase, HMG-CoA reductase and hormone sensitive lipase and an increase in 6-phosphofructo-2-kinase

activity (Towler and Hardie, 2007). Glucose uptake is increased by AMPK activation through increases in the expression, activity and translocation of GLUT1 and 4 in several cell types (Li *et al.*, 2005, Pelletier *et al.*, 2005, Thong *et al.*, 2007). AMPK is also able to modulate transcription through phosphorylation of several transcriptional activators/regulators such as p300, TORC2 and HNF4-α. It has also been recently demonstrated that AMPK is able to directly phosphorylate the CFTR ion channel leading to a decrease in CFTR channel opening (Kongsuphol *et al.*, 2009) and also increases trafficking of the related *sarc*K_{ATP} channel to the membrane by an unknown mechanism (Sukhodub *et al.*, 2007).

1.9.1 Role of AMPK in preconditioning

Because AMPK is able to respond to metabolic stress by modulating energy homeostasis, it seemed likely that this kinase could be involved in the protection afforded by preconditioning. AMPK activation has been observed in isolated rat hearts after an IPC protocol (Nishino *et al.*, 2004). The observed AMPK activation was prevented by inhibition of PKC with chelerythrine or GF109203X indicating that IPC may activate AMPK through a PKC-dependent pathway. Khaliulin *et al.* (2007) also observed an increase in AMPK phosphorylation in the rat heart after an IPC protocol, and phosphorylation was further increased in the IPC group during the index ischaemia when compared with control hearts. However in this study, the protective effect of IPC on LVDP and LDH release was not blocked by the AMPK inhibitor compound C. The effect of overexpression of a dominant negative AMPKα_2 subunit on IPC has also been examined by Jovanovic's group (Sukhodub *et al.*, 2007). Overexpression of dominant negative AMPKα_2 subunit prevented IPC in isolated ventricular myocytes via a mechanism that does not involve protection of the mitochondrial membrane potential. IPC was associated with s*arc*K_{ATP} channel activation and increased recruitment of

Kir6.2 to the membrane in wild type myocytes but these effects were prevented by overexpression of dominant negative AMPKα_2. These findings indicate that AMPK likely to be involved in preconditioning and can modulate *sarc*K_{ATP} channel trafficking.

The role of AMPK in phenylephrine preconditioning has not been examined to date; however AMPK phosphorylation at Thr172 subsequent to acute phenylephrine treatment has been observed in H9c2 rat cardiac myoblasts. AMPKα_2 was also activated in the hearts of rats that received a 7-day infusion of phenylephrine, although the effects of chronic and acute exposure may differ (Xu *et al.*, 2007).

1.10 Overview of the project

The aims of the project were to examine the signalling pathway(s) that may be responsible for phenylephrine preconditioning of the heart. Experiments were carried out using isolated rat ventricular myocytes, to enable the fluorescent measurement of Ca^{2+} levels and electrophysiological measurement of *sarc*K_{ATP} channel current, as well as determination of kinase activation.

The initial part of the work, described in chapter 3, was aimed at characterising the effect of phenylephrine preconditioning on isolated ventricular myocytes, examining the effect of preconditioning on contractile function and Ca^{2+} handling following metabolic inhibition and reperfusion. Inhibitors were also used to assess the role of α_1-adrenoceptors, PKC and K_{ATP} channels in the signal transduction of phenylephrine preconditioning.

Having identified a requirement for PKC activation during phenylephrine preconditioning, isoform-specific Tat-conjugated PKC activator/inhibitor peptides were used to determine the identity of the PKC isoforms required for phenylephrine

preconditioning. Translocation of PKC isoforms in response to the phenylephrine preconditioning protocol was also confirmed by western blot.

The signal transduction of phenylephrine preconditioning downstream of PKC was investigated in chapter 5. The role of the MAPKs and AMPK in phenylephrine preconditioning was examined using protein kinase inhibitors, and western blot analysis was also used to assess whether the identified proteins were activated by PKC isoform(s) in response to phenylephrine preconditioning.

The final set of experiments, described in chapter 6, was designed to examine the role of K_{ATP} channels in phenylephrine preconditioning. Selective inhibitors were used to examine the role of the *sarc*K_{ATP} and *mito*K_{ATP} channel. Whole-cell patch clamp recordings were used to assess the effect of phenylephrine preconditioning on *sarc*K_{ATP} channel current, and the effect of protein kinase inhibitors on the *sarc*K_{ATP} currents was also examined.

2 Materials and methods

2.1 Isolation of adult rat ventricular myocytes

Adult male Wistar rats weighing 200-350 g were humanely killed by cervical dislocation. The heart was removed rapidly and placed into cold (nominally) Ca^{2+}-free Tyrode solution containing (in mM) 135 NaCl, 6 KCl, 0.33 NaH_2PO_4, 5 Na pyruvate, 10 glucose, 1 $MgCl_2$, 10 HEPES, pH was titrated to 7.4 with NaOH. The aorta was cannulated and the heart was retrogradely perfused via the aorta with Ca^{2+}-free Tyrode using a Langendorff apparatus for 6 minutes at a constant flow rate of 10 ml/min, at 37^{o}C, bubbled with O_2. Myocytes were isolated by enzymatic digestion by perfusion of Ca^{2+}-free Tyrode solution containing Type I collagenase from *Clostridium histolyticum* (approximately 0.4 units/ml) and protease type XIV from *Streptomyces griseus* (approximately 2.7 units/ml) and 0.05% bovine serum albumin (BSA) for 7 to 9 minutes. The perfusate during the first ninety seconds of enzyme perfusion was discarded, after which time the perfusate was collected and recycled. The heart was then perfused with normal Tyrode solution (Ca^{2+}-free Tyrode solution with the addition of 2 mM $CaCl_2$), for 3 minutes and the atria were dissected away. Individual myocytes were obtained by agitation of the ventricles in normal Tyrode solution at 37^{o}C in a shaking water bath. After approximately five minutes the heart was cut into two pieces, then the normal Tyrode containing the isolated cells was decanted into another flask and fresh normal Tyrode added to the remaining ventricular tissue. The solution was replaced at five minute intervals until six fractions of isolated myocytes were produced. The fractions were sieved (0.2 mm opening size) to remove any undigested tissue and placed in a test tube. Myocytes were washed twice by allowing myocytes to settle in a test tube for 10 minutes at room temperature (20-25^{o}C), then aspirating and replacing the Tyrode

solution. The isolation produced 70-90% viable rod-shaped myocytes. Isolated cells were stored in normal Tyrode at room temperature at a density of approximately 0.5 million cells/ml and used within 12 hours of isolation. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.2 Metabolic inhibition (MI) and re-energisation

Metabolic inhibition was achieved by superfusing myocytes with MI Tyrode, which contained 2 mM sodium cyanide and 1 mM iodoacetic acid in substrate-free Tyrode. Substrate-free (SF) Tyrode solution (normal Tyrode in which 10 mM sucrose and 5 mM NaCl replaced glucose and pyruvate respectively) contained (in mM) 140 NaCl, 6 KCl, 0.33 NaH_2PO_4, 10 sucrose, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, pH titrated to 7.4 with NaOH. Myocytes were superfused with MI Tyrode for 7 minutes and this was followed by 10 minutes of re-energisation, achieved by the removal of metabolic inhibitors and the addition of metabolic substrates glucose and pyruvate (normal Tyrode). We have used the term re-energisation, which we distinguish from reperfusion because the myocytes are not subject to true ischaemia in our study, and therefore cannot be reperfused.

2.3 Measurement of myocyte contractile response and morphology

Isolated ventricular myocytes were placed in a 300 μl perspex superfusion chamber (see Figure 2.1) on the stage of a Nikon inverted microscope and allowed to settle for approximately 5 minutes prior to superfusion. In the case of inhibitor experiments, the inhibitors were added to the bath while the myocytes settled for 10 minutes prior to superfusion, unless otherwise stated. The myocytes were continuously superfused with solutions at 4.5 ml/min at 35-37^{o}C during experiments. For the preconditioning protocol, phenylephrine was added to substrate-free Tyrode solution, myocytes were superfused with phenylephrine for 10 minutes followed by washout with normal Tyrode for 5

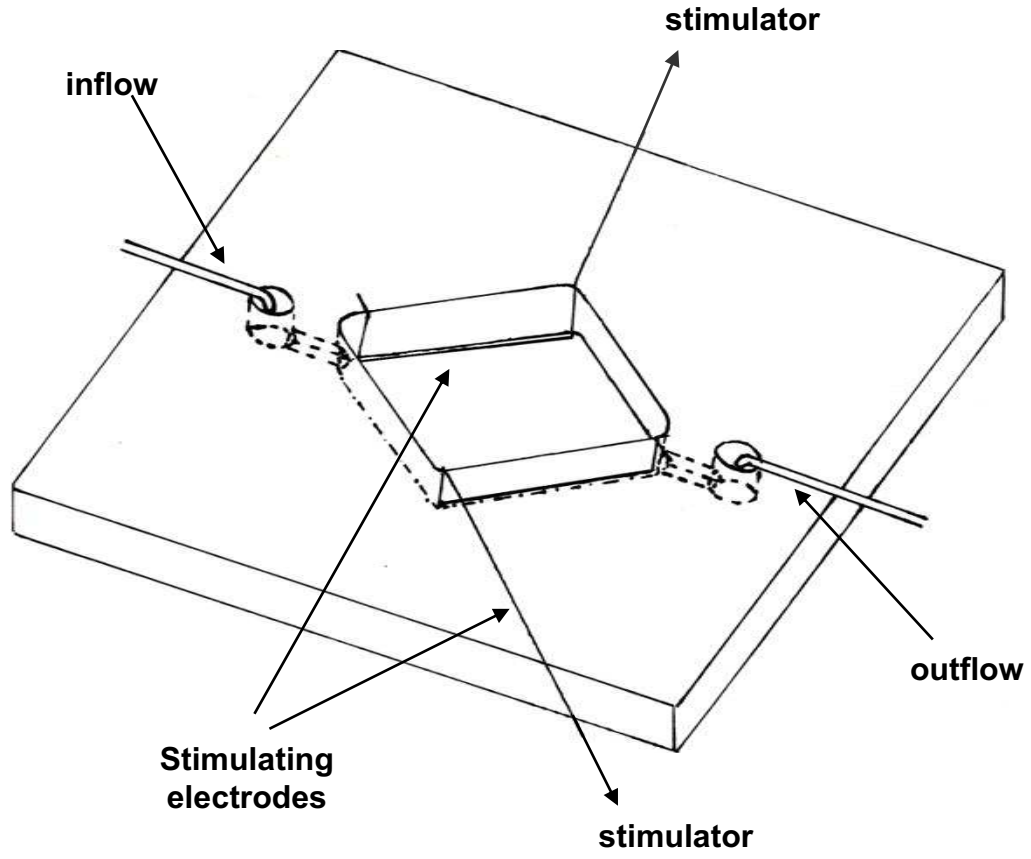

Figure 2.1. Diagram of the superfusion bath.

Diagram showing the superfusion bath used for contractile function, fluorescent imaging and patch-clamp experiments. Stimulating electrodes were placed parallel along two sides of the bath to allow electrical stimulation of the cells.

minutes prior to induction of MI (see Figure 2.2). Myocytes were stimulated to contract using electrical field stimulation (physiological stimulator, Farnell) at 1 Hz, with a pulse width of 5 ms at 150% of threshold voltage (approximately 15-30V), delivered to the bath using 0.25 mm platinum electrodes placed parallel along two sides of the diamond-shaped bath (see Figure 2.2). Myocytes were viewed with the aid of a charge-coupled device (CCD) camera and monitor. All experiments were recorded using a DVD recorder for later analysis. To determine recovery of contractile activity, healthy myocytes were identified and marked on the screen prior to MI. Those myocytes able to contract in time to field stimulation following 7 minutes of MI and 10 minutes of re-energisation and which stopped contracting when the stimulator was switched off, were judged to have recovered contractile function (see Figure 2.3). Myocytes that did not contract in time to field stimulation or contracted spontaneously were deemed not to have recovered. The time course of contractile failure, rigor contracture and contractile recovery of myocytes was determined by measuring the time from the onset of MI to the point when each myocyte stopped contracting to field stimulation, had completely shortened into rigor contracture and recovered contractile activity, respectively.

2.4 Measurement of intracellular calcium using the calcium-sensitive fluorescent dye Fura-2

Myocytes were loaded with fura-2 by incubation with 2.5 μM fura2-AM (acetoxymethyl ester) in normal Tyrode solution for 20 minutes at room temperature, then washed twice with Tyrode solution to remove any remaining extracellular dye. Myocytes were then left for 20 minutes to allow de-esterification of the intracellular dye. Fura-2 loaded myocytes were placed in a 300 μl perspex superfusion chamber (see

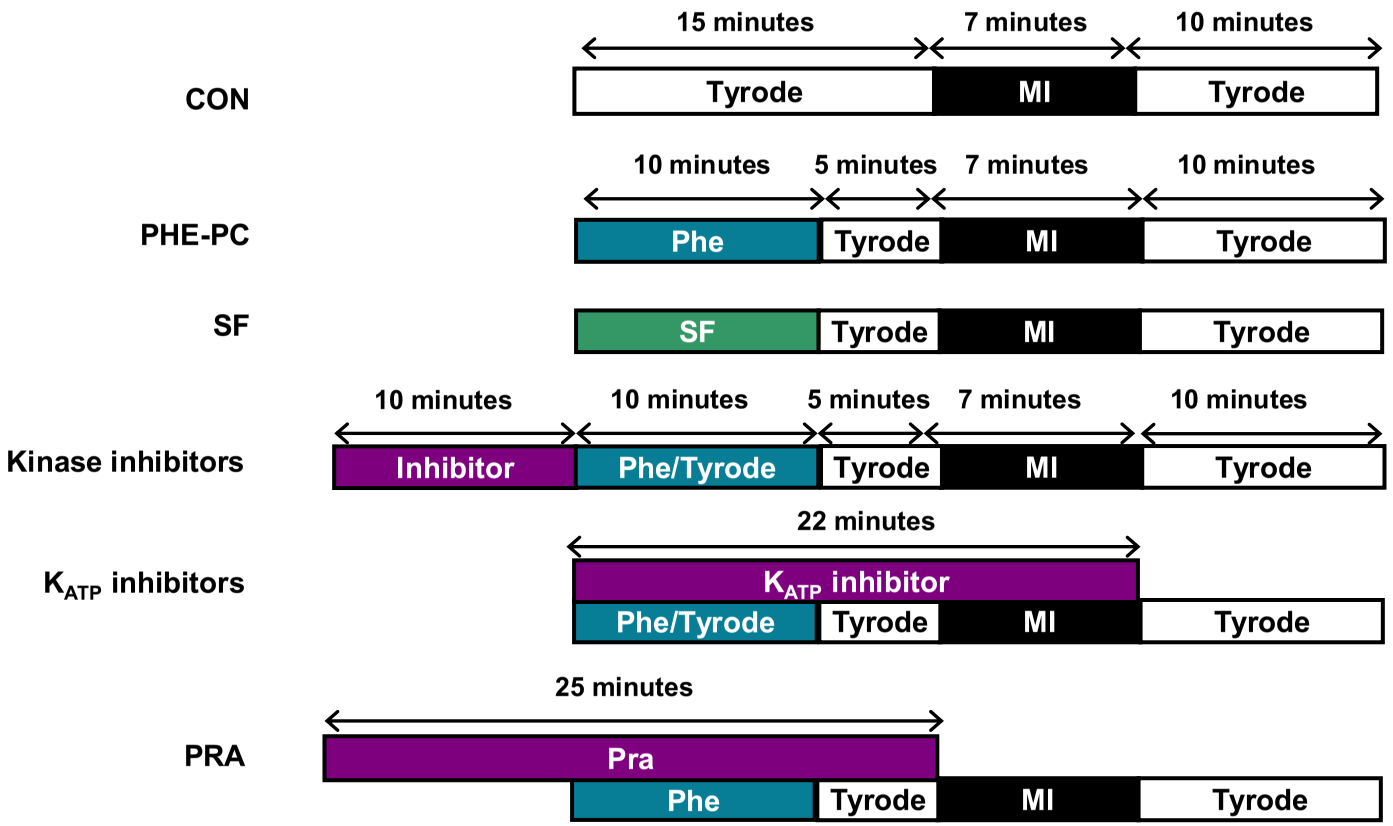

Figure 2.2. Protocol used for phenylephrine preconditioning, MI and re-energisation, and inhibitor treatments.

Ventricular myocytes were preconditioned with a 10 minute superfusion of μM phenylephrine (Phe) followed by 5 minutes wash out with normal Tyrode. Control myocytes were superfused with normal Tyrode for 15 minutes. Myocytes were then subject to 7 minutes of MI Tyrode (MI) and 10 minutes of re-energisation with normal Tyrode. Control (CON). Phe preconditioned (PHE-PC). Substrate free-Tyrode (SF). Prazosin (PRA). Details of inhibitors are given in the relevant results chapters.

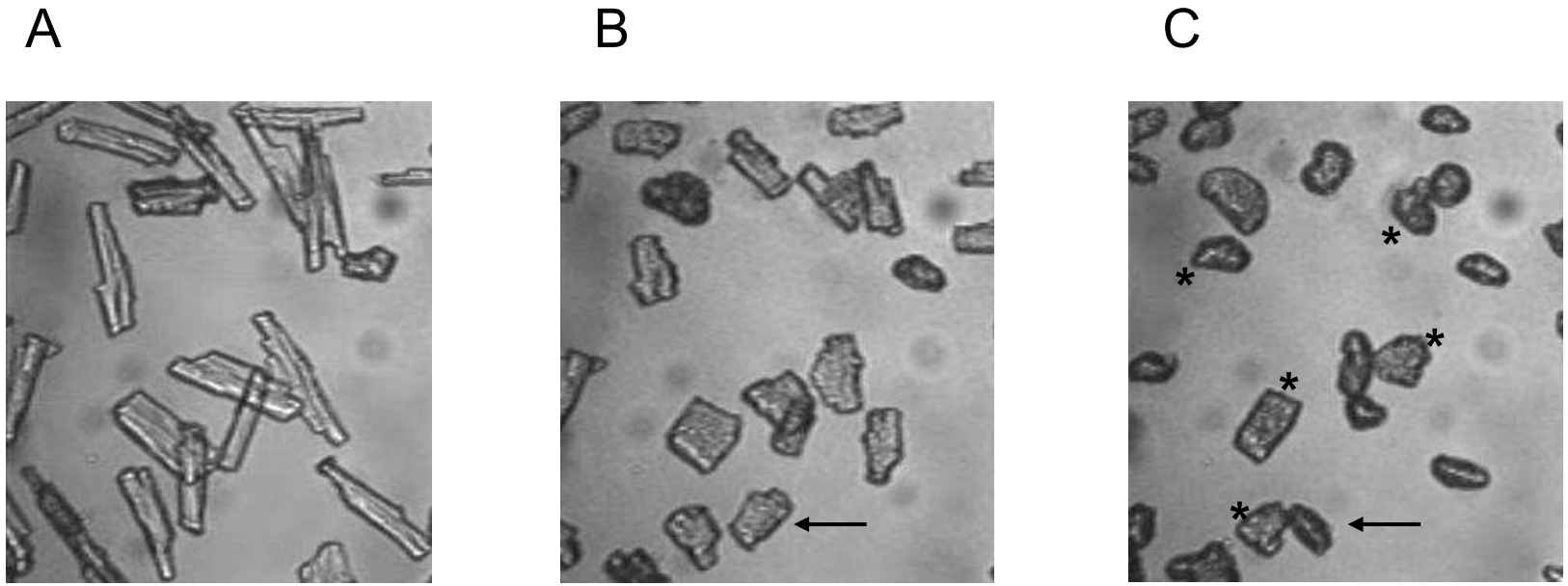

Figure 2.3. Cell morphology during metabolic inhibition and re-energisation of control myocytes.

A. Representative bright-field image of a field of control myocytes prior to metabolic inhibition (MI) taken from a video recording of a contractile function experiment.

B. Image showing the same field of control myocytes following 7 minutes of MI. Arrow shows a cell that had shortened into rigor contracture.

C. Image showing the same field of control myocytes following 10 minutes of re-energisation. Arrow shows a hypercontracted cell. Cells marked * recovered contractile activity.

Figure 2.1) on the stage of an inverted microscope (Nikon Eclipse TE200) and allowed to settle for approximately 5 minutes prior to superfusion. Myocytes were superfused at 4.5 ml/min at 35-37^{o}C and stimulated at 1 Hz (research stimulator, Harvard apparatus) as detailed above in section 2.3. Intracellular Ca^{2+} levels were measured from fields of cells containing 4-18 myocytes during preconditioning, MI and re-energisation with normal Tyrode (for details of the protocol see Figure 2.2), with a X 20 objective using a video imaging system (PTI). Fura-2 was excited alternately at 340 nm and 380 nm using a monochromator (PTI deltaRAM) and the emission at >520 nm was measured using a CCD camera (Photometrics Cascade:512B; Roper Scientific, Inc.). Data were sampled at 0.1 Hz. Rod-shaped myocytes contracting in response to field stimulation were identified prior to the start of the experiment and a region of interest was drawn around the cell (see Figure 2.4A and B). Any cells that had a resting diastolic fura-2 ratio > 1.20 prior to MI were also excluded from the analysis. Data analysis was performed using EasyRatioPro software (PTI).

2.5 Measurement of *sarc*K_{ATP} current using whole-cell patch clamp

An Axopatch 200B patch-clamp amplifier (Axon Instruments) was used in the whole-cell voltage clamp configuration. Cells were voltage clamped at -40 mV and stepped to 0 mV for 100 ms once every 10 seconds to measure *sarc*K_{ATP} currents. Data were sampled at 10 kHz using an analog-to-digital converter (ADC, Digidata 1440A) and stored to the hard disk of a computer for later analysis using PClamp 10 software (Axon Instruments). The amplifier was used to filter the output signal with a 2 kHz low pass filter. Junction potentials were zeroed after pipettes entered the bath. *Sarc*K_{ATP} currents were measured from the mean steady-state current during the final 10 ms of the 100 ms test pulse using Clampfit (PClamp 10, Axon instruments) and normalised to cell capacitance (pA/pF) to allow for variations in cell size.

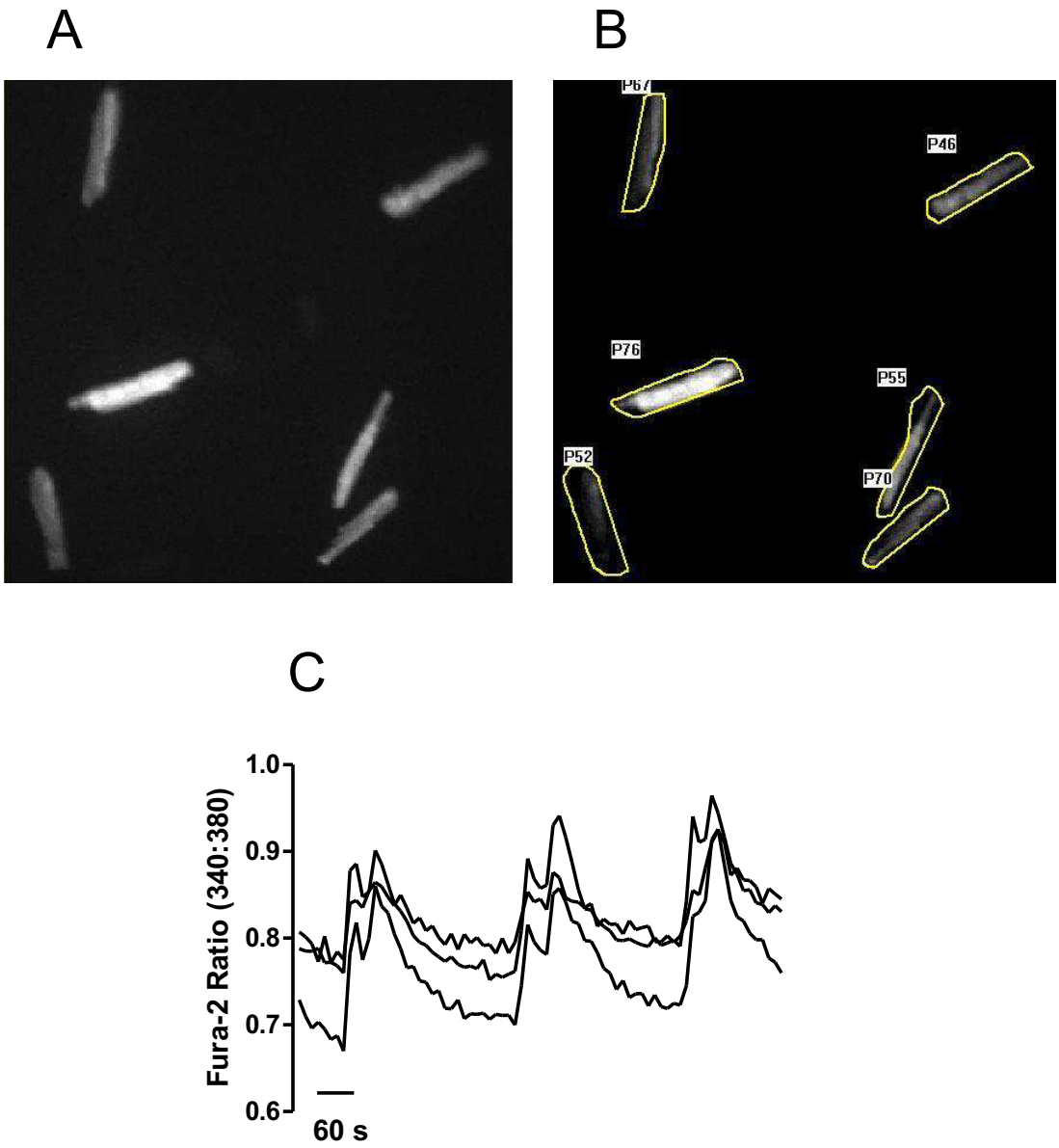

Figure 2.4. Measurement of intracellular calcium levels from fura-2 loaded ventricular myocytes.

A. Example fluorescent image of fura-2 loaded ventricular myocytes (excitation 380nm/emission >520nm). .

B. The same fluorescent image showing regions of interest drawn around myocytes.

C. An expanded region of a fura-2 trace showing the aliasing effect produced by slow sampling of rapid calcium transients, which resulted in recording of slowly oscillating calcium levels.

Thin wall filamented borosilicate glass (Harvard Apparatus; internal diameter (ID) 1.15 mm, external diameter OD 1.50 mm) was used to produce patch-style electrodes using a vertical two-stage puller (PC-10, Narishige). These electrodes were filled with an internal solution containing (in mM) 140 KCl, 5 EGTA, 0.4 MgATP, 0.1 NaGTP, and 10 HEPES, titrated to pH7.3 with KOH, and had resistances of 2-5 MΩ. Electrodes were discarded if not used within 2 hours of being pulled.

Isolated ventricular myocytes were placed in a perspex superfusion chamber (see Figure 2.1) on the stage of an inverted microscope (Nikon) and allowed to settle prior to superfusion. Myocytes were then preconditioned with phenylephrine or superfused with normal Tyrode in control experiments (protocols shown in Figure 2.2). Cadmium chloride (0.2 mM) to block Ca^{2+} currents and 4-aminopyridine (2 mM) to block voltage-gated K^{+} currents were added to all external solutions. Experiments were performed at room temperature.

2.6 Preparation of cytosolic and membrane protein fractions for measurement of PKC isozyme translocation

Ventricular myocytes (approximately 250,000 cells in 1 ml of Tyrode solution) were treated with phenylephrine (5 μM) or PMA (1 μM) in microcentrifuge tubes at 37^{o}C in a shaking water bath (protocol for treatment of myocytes is outlined in Figure 2.5A). At the time points indicated the myocytes were collected by centrifugation at 13000 x *g* for 15 seconds at room temperature. The supernatant was then carefully aspirated and the cell pellet was resuspended in 100 μl of ice-cold non-detergent lysis buffer containing (in mM) 10 β-glycerophosphate, 10 Tris-HCl, 2 EGTA, 2 EDTA, 1 Sodium orthovanadate, 1 benzamidine, 1 Dithiothreitol (DTT), 1 PMSF, and 10 μl of protease inhibitor cocktail (Sigma-Aldrich; P8340) was added to each ml of buffer. Cells were

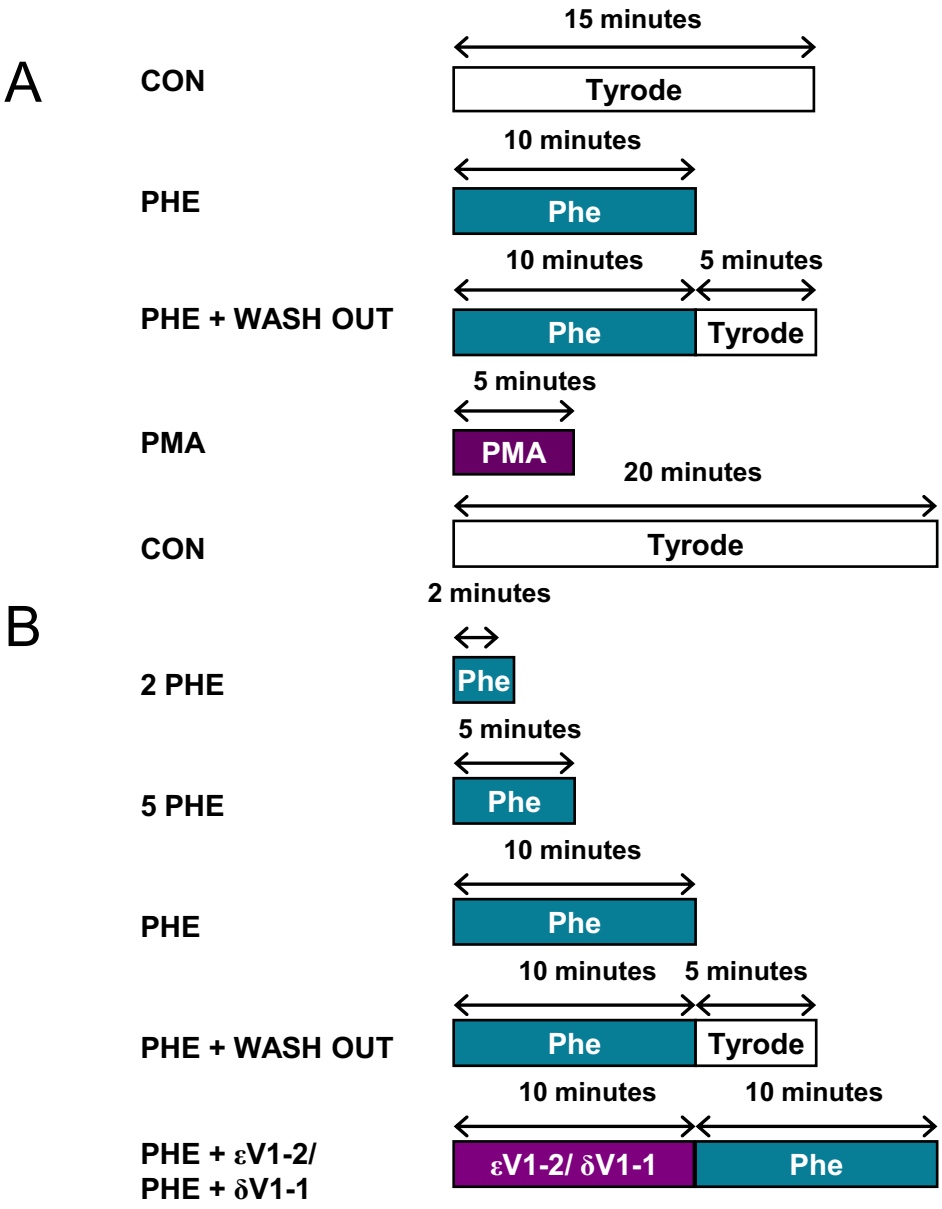

Figure 2.5. Protocols used for treatment of myocytes for western blotting.

A. Protocol used to investigate the translocation of PKCε and δ in response to treatment with 5 μM phenylephrine (Phe). Control, 15 minutes of normal Tyrode (CON). 10 minutes of Phe (PHE). 10 minutes of Phe followed by 5 minutes of washout with normal Tyrode (PHE + WASH OUT). 5 minutes of 1 μM PMA (PMA).

B. Protocol used to investigate phosphorylation of p38 MAPK and AMPK. Control, 20 minutes of normal Tyrode (CON). 2 minutes of Phe (2 PHE). 5 minutes of Phe (5 PHE). 10 minutes of Phe (PHE). 10 minutes of Phe followed by 5 minutes of washout with normal Tyrode (PHE + WASH OUT). 10 minutes of εV1-2 followed by 10 minutes of Phe (PHE + εV1-2). 10 minutes of δV1-1 followed by 10 minutes of Phe (PHE + δV1-1).

homogenised by hand using a glass homogeniser and the cell lysate removed to a microcentrifuge tube on ice for 30 minutes to ensure complete cell lysis had occured.

Cell lysates were then centrifuged in ultracentrifuge tubes at 98,000 x *g* for 30 minutes at 4^{o}C in an ultracentrifuge (Beckman Coulter) with a Beckman TLA100 rotor (protocol detailed in Figure 2.6). The supernatant containing the cytosolic protein fraction was retained. The membrane pellet was resuspended in 80 µl non-detergent lysis buffer and re-centrifuged at 98000 x *g* for 30 minutes at 4^{o}C to remove cytosolic contamination. The supernatant was discarded and the membrane pellet resuspended in 80 µl Triton lysis buffer (non-detergent buffer with the addition of 1% Triton X-100) and incubated on ice for 10 minutes to allow the detergent to solubilise the membranes. The membrane fraction was again centrifuged at 13,000 x *g* for 10 minutes at 4^{o}C and the supernatant containing the membrane protein fraction was retained. Cytosolic and membrane protein samples (50 µl) were added to 6x SDS-PAGE sample buffer (10 µl) and heated to 100^{o}C for 5 minutes prior to SDS-PAGE on 8% gels. 6x SDS-PAGE sample buffer contained (in mM) 600 dithiothreitol (DTT), 360 sodium dodecyl sulfate (SDS), 30% glycerol, 350 Tris pH 6.8 and a few grains of bromophenol blue to colour.

2.7 Preparation of protein samples for measurement of p38 and AMPK phosphorylation

Ventricular myocytes (approximately 250,000 cells in 1 ml Tyrode solution) were incubated with phenylephrine (5 µM) in the presence or absence of protein kinase inhibitors (εV1-2 and δV1-1, 100 nM) in microcentrifuge tubes (as outlined in Figure 2.5B). At time points indicated, the myocytes were collected by centrifugation at 13,000 x *g* for 15 seconds and the supernatant removed. The cell pellet was resuspended in 100 µl of ice cold Triton lysis buffer and incubated on ice for 10 minutes to disrupt the cell

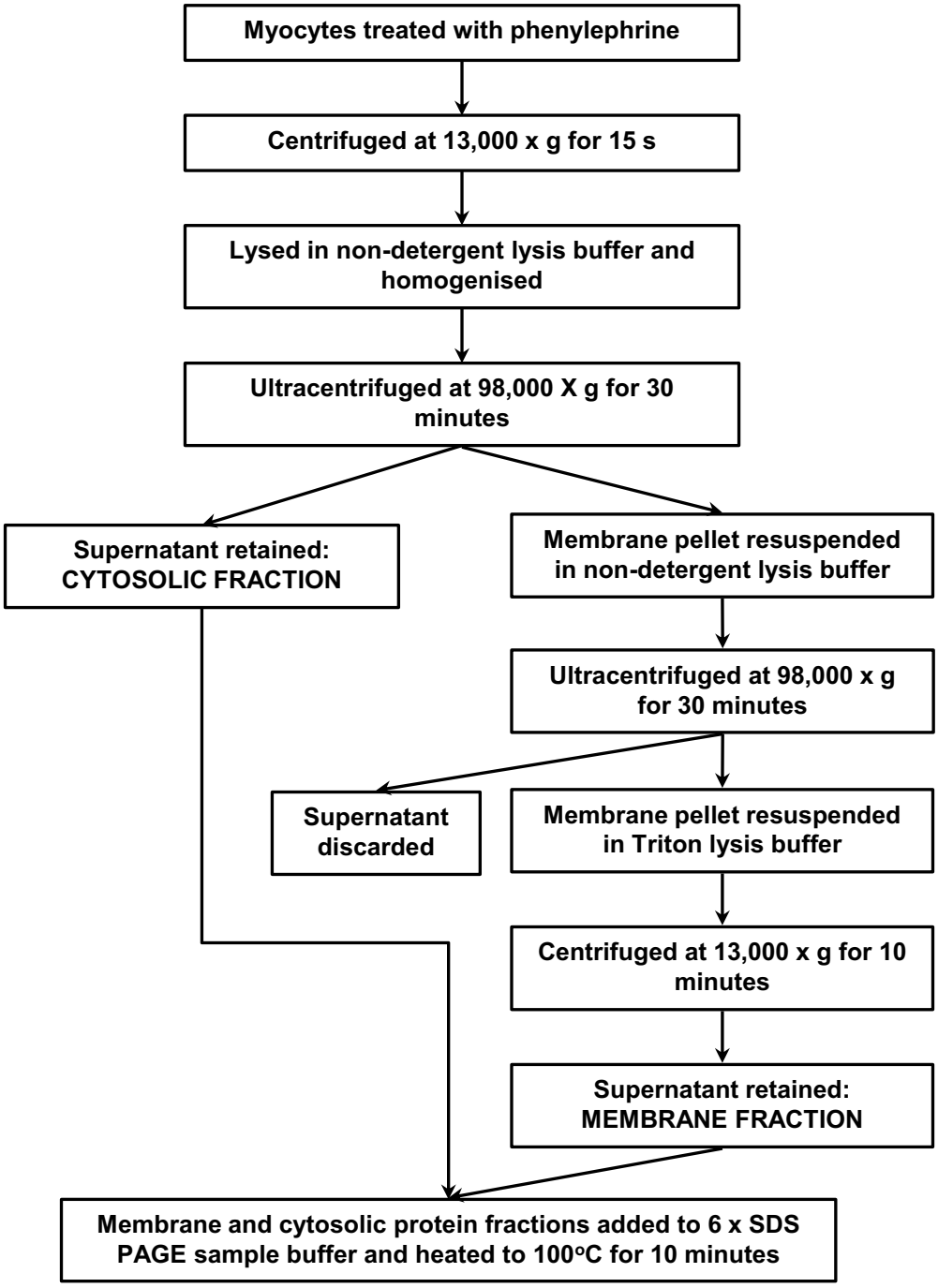

Figure 2.6. Protocol for separation of cytosolic and membrane fractions.
Flow diagram showing protocol used for preparation of membrane and cytosolic protein samples for western blot analysis of PKC translocation.

membranes. The lysates were then centrifuged at 13,000 x *g* for 10 minutes at 4°C and the supernatant was retained. Protein concentrations were calculated using a detergent compatible protein assay (DC Protein assay, BioRad) and samples were diluted with Triton lysis buffer to a concentration of 7.5 µg/µl. The diluted supernatant (60 µl) was added to a microcentrifuge tube containing 30 µl of 3x SDS-PAGE sample buffer and the samples were heated to 100°C for 5 minutes prior to SDS-PAGE on 10% gels. 3x SDS-PAGE sample buffer contained (in mM) 187.5 Tris HCl pH 6.8, 6% SDS, 30% glycerol, 150 DTT and bromophenol blue.

2.8 Western blot analysis

Proteins were separated along with pre-stained all blue Precision Plus protein standards (BioRad) on 8 or 10 % SDS-PAGE mini-gels (BioRad) at a constant voltage of 100 V for 100 minutes. Running buffer contained (in mM) 25 Tris, 192 glycine, 0.1% SDS. Gels and nitrocellulose membranes were soaked in transfer buffer containing (in mM) 25 Tris, 192 glycine, 0.01% SDS, and 10% methanol for 20 minutes prior to transfer. Proteins were then transferred to nitrocellulose membrane by semi-dry transfer at 15 V for 45 minutes. The membrane was rinsed in Tris Buffered Saline Tween-20 (TBST) containing (in mM) 50 Tris, 150 NaCl and 0.1% Tween-20. Membranes were blocked by incubation with TBST containing 5% non-fat dried milk for 1 hour with agitation then rinsed with TBST for 5 minutes. Primary antibodies were diluted in TBST containing either 5% non-fat dried milk or 5% BSA according to the manufacturer's directions (see Figure legends), and incubated overnight at 4°C with agitation. Membranes were washed four times for 5 minutes each in TBST. The membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody in TBST containing 5% non-fat dried milk for 1 hour at room temperature with agitation then washed six times for 5 minutes each. Bands were detected by incubation

of membranes with luminol working solution for 1 minute and exposure to x-ray film (SuperRX, Fujifilm). Luminol working solution contained 0.05% sodium luminol in 0.1M Tris pH 8.6 (1 ml), 18 mM para-coumaric acid in DMSO (10 µl), and 30% hydrogen peroxide (3.1 µl). Bands were quantified using a GeneGenius bioimaging system (Syngene) with GeneSnap and GeneTools software (Syngene). To strip blots, membranes were incubated with stripping buffer containing 0.1M glycine pH 2.8 with 1% glycine for 45 minutes at room temperature. Membranes were then blocked with 5% non-fat dried milk and re-probed with appropriate antibodies as stated in Figure legends.

2.9 Materials

Fura-2-AM was obtained from Molecular Probes (Invitrogen). The PKC activator PMA, AMPK inhibitor compound C, MEK inhibitor PD98059, p38 inhibitor SB202190, JNK inhibitor SP600125, and dithiothreitol were from Calbiochem (EMD Biosciences). Proteolytic enzymes were protease from *Streptomyces griseus* Type XIV and collagenase from *Clostridium histolyticum* Type I were both from Sigma-Aldrich. Nitrocellulose membrane (Schleicher & Schuell Bioscience) was obtained from Fisher Scientific. Anti-phospho (Thr180/Tyr182) p38 MAPK rabbit monoclonal antibody, anti-phospho Thr172 AMPKα rabbit polyclonal antibody and anti-total AMPKα rabbit polyclonal antibody (Cell Signalling Technology) were obtained from New England Biolabs (NEB UK). Anti-PKCε and anti-PKCδ rabbit polyclonal antibodies (Santa Cruz Biotechnology) were obtained from Autogen Bioclear. Anti-α-tubulin mouse monoclonal antibody, goat anti-rabbit IgG horseradish peroxidase conjugated antibody, goat anti-mouse IgG horseradish peroxidase conjugated antibody, rabbit anti-goat IgG horseradish peroxidase conjugated antibody and protease inhibitor cocktail were obtained from Sigma-Aldrich. X-ray film (Super RX, Fujifilm) was obtained from Fisher Scientific. PKC inhibitor peptides (see Table 4.1 for details of peptides) were

synthesised (Pepceuticals Ltd) and reversibly conjugated to Tat (47-57) peptide through formation of a disulphide bond between N-terminal cysteine residues, to produce inhibitor peptides which are rapidly released within the reducing intracellular environment (Begley *et al.*, 2004). Other laboratory compounds were obtained from Sigma-Aldrich or Fisher Scientific unless stated.

2.10 Analysis and Statistics

Data are presented as mean ± SEM. Data were analysed using Microsoft Excel and GraphPad Prism 5 (GraphPad, Inc.). Statistical significance was evaluated using either two-tailed, unpaired T-Test for comparison of data with two groups or one-way ANOVA followed by Tukey's post test using GraphPad Prism 5 for multiple comparisons. A P value of < 0.05 was considered significant. Data is presented as n = experiments, hearts, and cells; except for time to contractile failure and rigor data presented as n = cells, experiments, and hearts; or *sarc*K_{ATP} current data presented as n = experiments, and hearts.

3 Preconditioning of isolated ventricular myocytes by phenylephrine

3.1 Introduction

Preconditioning was first described by Murray *et al.* in 1986. It was observed that one or more short periods of ischaemia could protect the heart from a subsequent prolonged episode of potentially lethal ischaemia (Murry *et al.*, 1986). They demonstrated that the infarct size resulting from a 40 minute ischaemic insult was reduced by 75% after four 5 minute cycles of IPC. Since this initial report there have been many studies attempting to unravel the mechanisms of IPC and more than 3000 papers have been published on the subject. There is still much debate over the identities of the triggers, mediators and end effectors responsible for preconditioning, however the involvement of PKC, K_{ATP} channels and ROS has been well established (Yellon and Downey, 2003).

Studies into the mechanisms of preconditioning have revealed that many pharmacological agents can mimic the effect of IPC (Liu *et al.*, 1991, Banerjee *et al.*, 1993, Ytrehus *et al.*, 1994, Bugge and Ytrehus, 1996). The ability of the α_1-adrenergic agonist phenylephrine to protect the heart was first observed by Banjeree *et al.* (1993). They suggested that the protective effect of phenylephrine preconditioning was due to an improvement in myocardial ATP levels during reperfusion compared to non-preconditioned hearts.

Many of the studies on phenylephrine preconditioning to date have been carried out using the isolated perfused rat heart. Our study examines the effect of phenylephrine preconditioning in isolated ventricular myocytes. The use of isolated cells allows the determination of the loss of contractile function during MI and recovery following

re-energisation, whilst also making possible the measurement of intracellular Ca^{2+} and *sarc*K_{ATP} channel activity, parameters which cannot easily be determined in the whole heart.

The results in this chapter describe the effects of metabolic inhibition and re-energisation on isolated ventricular myocytes, and demonstrate a role for α_1-adrenoceptors, PKC and K_{ATP} channels in the protection afforded by phenylephrine preconditioning.

3.2 Results

3.2.1 The effect of phenylephrine preconditioning on the contractile function of isolated ventricular myocytes exposed to metabolic inhibition and re-energisation

The ability of phenylephrine to precondition the intact heart has been observed (Banerjee *et al.*, 1993, Tsuchida *et al.*, 1994), but only one study has examined phenylephrine preconditioning in isolated adult ventricular myocytes (Gao *et al.*, 2007). In this study, Gao *et al.* examined the effects of inhibition of α_1-adrenoceptor subtypes, PKCε, and *mito*K_{ATP} on contractile function and intracellular Ca^{2+} in phenylephrine preconditioned ventricular myocytes subject to simulated-ischaemia and reperfusion. We set out to characterise the effect of phenylephrine preconditioning on contractile function in isolated ventricular myocytes subject to metabolic inhibition (MI) with 2mM sodium cyanide and 1mM iodoacetic acid, followed by re-energisation.

To determine the effect of phenylephrine preconditioning on contractile function, isolated ventricular myocytes were preconditioned with 5 μM phenylephrine in substrate-free Tyrode for 10 minutes followed by 5 minutes of normal Tyrode (protocols described in Figure 3.1). In control experiments, myocytes were superfused

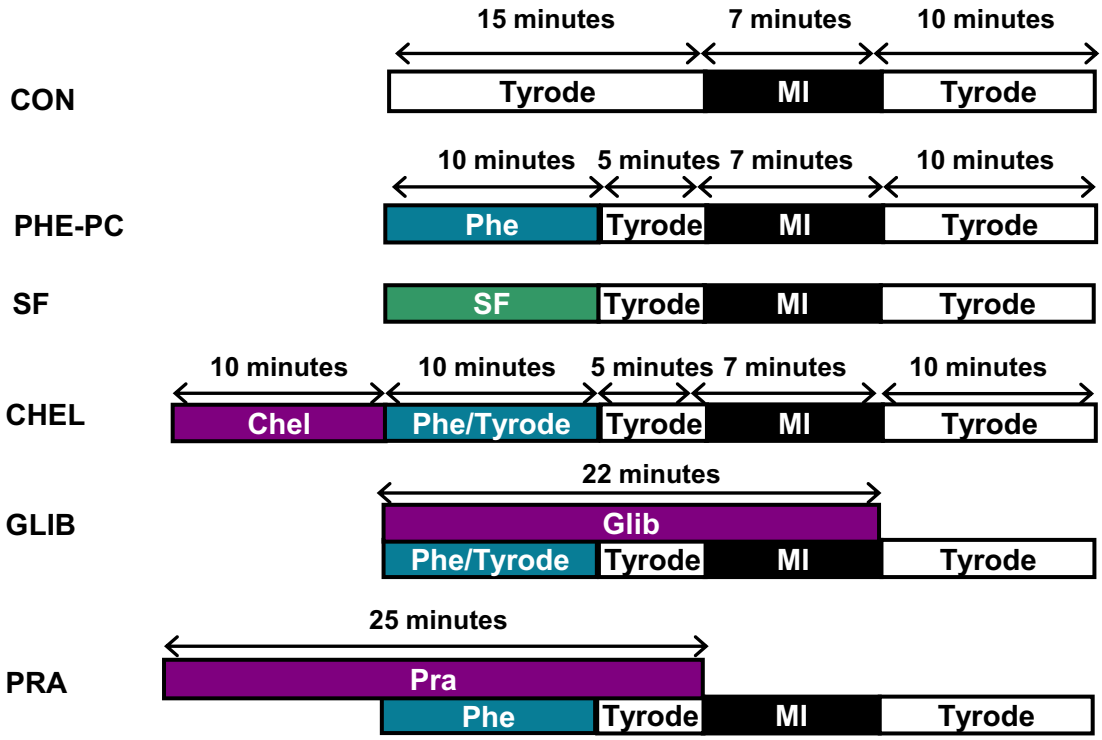

Figure 3.1. Protocol used for phenylephrine preconditioning, metabolic inhibition and re-energisation, and inhibitor treatments.

Ventricular myocytes were preconditioned with 10 minutes of 5 μM phenylephrine (Phe) followed by 5 minutes wash out with normal Tyrode (Tyrode). Control myocytes were superfused with normal Tyrode for 15 minutes. Substrate-free Tyrode treated myocytes were superfused with substrate-free Tyrode for 10 minutes followed by 5 minutes wash out with normal Tyrode. Myocytes were then subject to 7 minutes of metabolic inhibition with MI Tyrode (MI) and 10 minutes of re-energisation with normal Tyrode. Control (CON). Phe preconditioned (PHE-PC). Substrate free-Tyrode (SF). Chelerythrine (CHEL). Glibenclamide (GLIB). Prazosin (PRA).

with normal Tyrode for 15 minutes. Myocytes were then exposed to 7 minutes of metabolic inhibition with MI Tyrode, followed by 10 minutes of re-energisation with normal Tyrode. The cells were field stimulated to contract at 1 Hz at 150% of threshold voltage (approximately 20-30 V). The percentage of cells (from a field of 10-50 cells) able to contract in response to electrical field stimulation was measured at one minute intervals during MI and re-energisation. Representative images from control myocytes taken prior to MI, at the end of MI and following re-energisation are shown in Figure 2.3. As some preconditioning stimuli have been reported to alter the time to contractile failure and rigor contracture, these were also examined.

The effect of MI and re-energisation on the contractile function of control and phenylephrine preconditioned ventricular myocytes is shown in Figure 3.2A. During MI, there was a time-dependent decrease in the percentage of cells contracting, with contraction failing in all cells within 5 minutes of application of MI. There was no significant difference in the time course of contractile failure between control and phenylephrine preconditioned myocytes, with 18.6 ± 10.4% (n = 4, 4, and 109 for experiments, hearts and cells respectively) of control myocytes and 9.1 ± 4.0% (n = 4, 4, and 119) of phenylephrine preconditioned myocytes able to contract after 4 minutes of MI (P = NS).

We also examined the effect of phenylephrine preconditioning on the mean time to contractile failure and rigor contracture in our system. Phenylephrine preconditioning increased the mean time to contractile failure compared with control myocytes (Figure 3.3A and B), from 165 ± 4 seconds (n = 102, 3, and 3 for cells, experiments, and hearts) in control myocytes to 171 ± 6 seconds in phenylephrine preconditioned myocytes (n = 69, 3, and 3; $P < 0.05$; Figure 3.3A). During MI, rigor contracture caused rod-shaped myocytes to shorten into rectangular cells (representative image shown Figure 2.4 B);

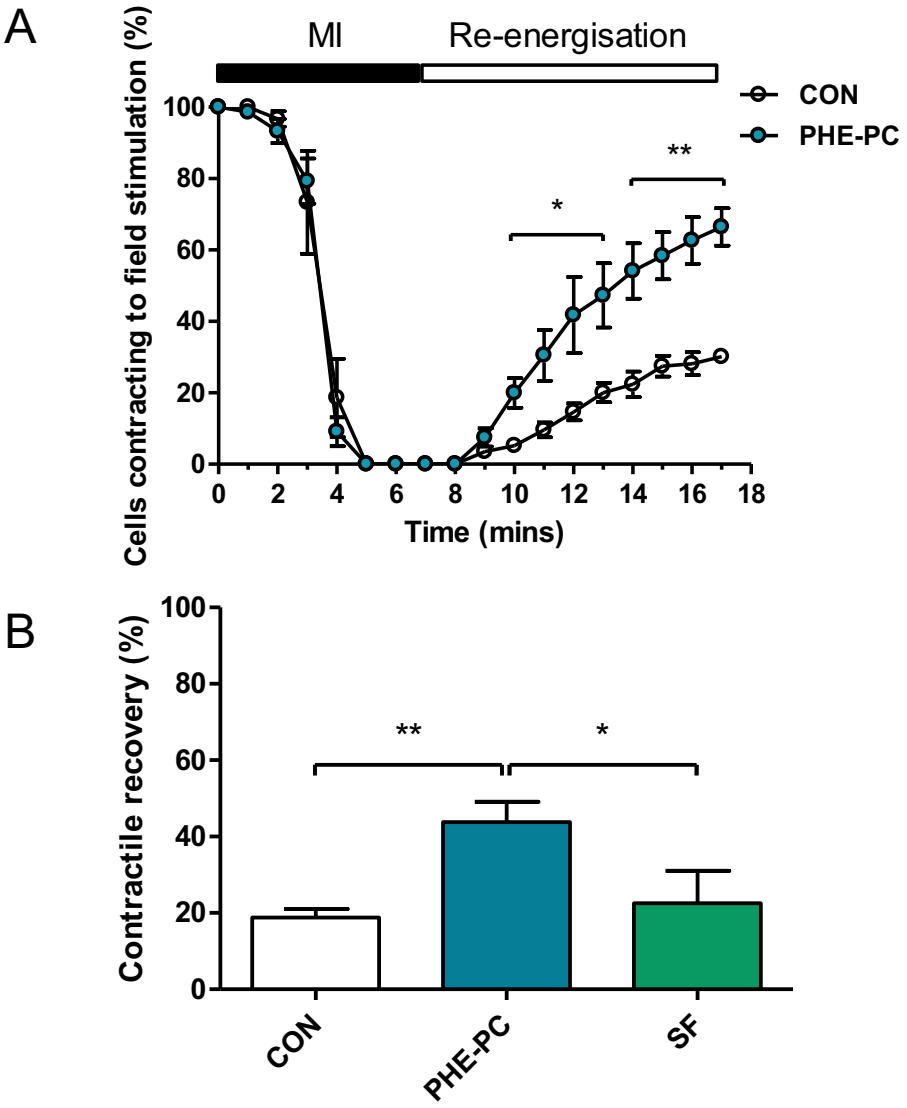

Figure 3.2. Contractile activity of phenylephrine preconditioned ventricular myocytes during metabolic inhibition and re-energisation.

A. Time course showing percentage of control (CON) and phenylephrine preconditioned (PHE-PC) ventricular myocytes able to contract in response to field stimulation at one minute intervals during metabolic inhibition (7 minutes) and re-energisation (10 minutes). CON (open circles, n = 4, 4, and 109 for experiments, hearts and cells respectively). PHE-PC (blue circles, n = 4, 4, and 119). *P < 0.05, ** P < 0.01.

B. Contractile recovery following 7 minutes of MI and 10 minutes of re-energisation. Ventricular myocytes were superfused with 5 μM phenylephrine in substrate-free Tyrode (PHE-PC), or substrate-free Tyrode (SF) for 10 minutes followed by 5 minutes of washout with normal Tyrode, or superfused with normal Tyrode for 15 minutes (CON), prior to metabolic inhibition (MI) and re-energisation. CON (open bar, n = 8, 5, and 221 for experiments, hearts and cells respectively). PHE-PC (blue bar, n = 5, 5, and 168). SF (green bar, n = 5, 5, and 121) myocytes. *P < 0.05, ** P < 0.01.

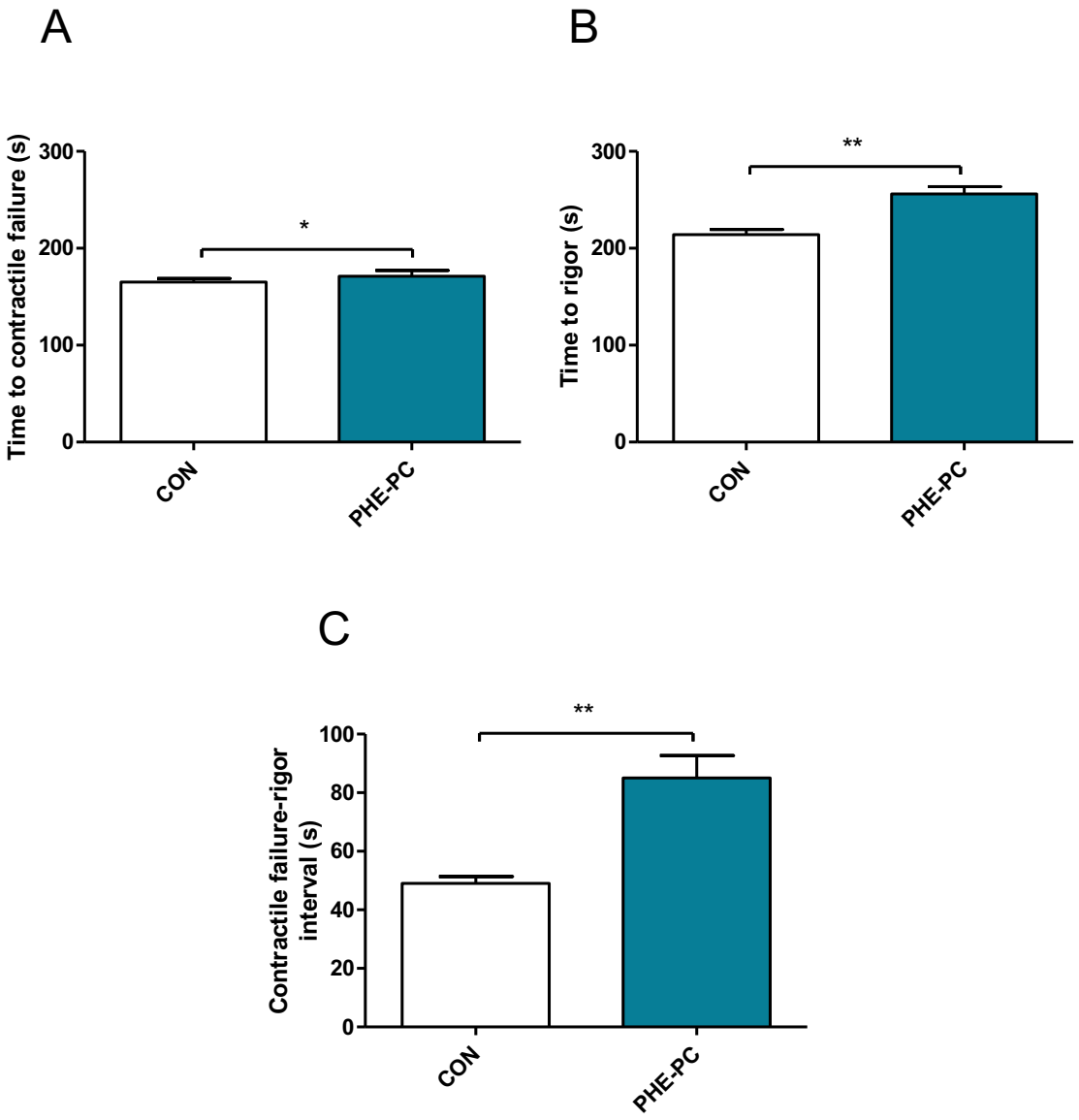

Figure 3.3. Effect of phenylephrine preconditioning on time to contractile failure and rigor contracture.

A. Bar chart showing mean time to contractile failure in control (CON) and phenylephrine preconditioned (PHE-PC) myocytes. CON (open bars, n = 102, 3, and 3 for cells, experiments, and hearts respectively). PHE-PC (blue bars, n = 69, 3, and 3).*$P < 0.05$.

B. Bar chart showing mean time to rigor contracture in CON and PHE-PC myocytes. **$P < 0.01$.

C. Bar chart showing the interval between contractile failure and rigor contracture in CON and PHE-PC myocytes. **$P < 0.01$.

the peak of this shortening was taken as the time to rigor contracture in our experiments. The mean time to rigor contracture was also increased from 214 ± 5 seconds (n = 102, 3, and 3) in control myocytes to 256 ± 8 seconds (n = 69, 3, and 3) by phenylephrine preconditioning ($P < 0.01$; Figure 3.3B). The interval between contractile failure and rigor was also significantly increased following phenylephrine preconditioning ($P < 0.01$; Figure 3.3C).

Re-energisation resulted in a time dependent recovery of contractile activity in response to electrical field stimulation, but with significantly more phenylephrine preconditioned myocytes recovering than control (Figure 3.2A). After three minutes of re-energisation the difference was already significant, 20.0 ± 4.1% (n = 4, 4, and 119 for experiments, hearts and, cells respectively) of phenylephrine preconditioned myocytes had recovered contractile function compared with just 5.2 ± 1.1% (n = 4, 4, and 109) of control myocytes ($P < 0.05$). Following 10 minutes of re-energisation, 30.1 ± 1.9% (n = 4, 4, and 109) of control myocytes recovered contractile function, and phenylephrine preconditioning increased the contractile recovery of isolated ventricular myocytes to 66.4 ± 5.2% (n = 4, 4, and 119; $P < 0.01$).

Pyruvate is able to act as a free radical scavenger and could potentially inhibit any free radical-mediated preconditioning mechanism (Dobsak *et al.*, 1999). As the normal Tyrode contained 5 mM pyruvate, phenylephrine was prepared in substrate-free Tyrode. Therefore, we examined the effect of treatment with substrate-free Tyrode on the contractile recovery of ventricular myocytes, to ensure that the preconditioning achieved with phenylephrine was due to a specific adrenergic response and not simply the removal of the metabolic substrates glucose and pyruvate from the preconditioning solution. The contractile recovery of substrate-free Tyrode treated myocytes following 10 minutes of re-energisation with normal Tyrode was 22.6 ± 8.5% (n = 5, 5, and 121),

which was not significantly different from the 18.8 ± 2.3% (n = 8, 5, and 221) of control myocytes that recovered contractile activity (P = NS; Figure 3.2B), but was significantly lower than the 43.8 ± 5.4% (n = 5, 5, and 168) of phenylephrine preconditioned myocytes that recovered contractile activity following re-energisation (P < 0.05). This indicates that the protective effect of phenylephrine preconditioning is mediated by phenylephrine signalling and not a consequence of substrate-free Tyrode treatment.

3.2.2 Phenylephrine preconditioning requires activation of the α_1-adrenoceptor

Since phenylephrine is an α_1-adrenoceptor agonist, it is likely that the preconditioning effect of phenylephrine is mediated via the G_q-coupled α_1-adrenoceptor. To test this, we investigated the effect of the α_1-adrenoceptor antagonist prazosin on the ability of phenylephrine to precondition isolated ventricular myocytes.

To examine the role of α_1-adrenoceptors in phenylephrine preconditioning, isolated ventricular myocytes were treated with the α_1-adrenoceptor antagonist prazosin, 10 minutes prior to the preconditioning protocol, and continuing until the start of MI Tyrode, to ensure complete α_1-adrenoceptor blockade. Following concentration-response experiments we used 10 μM prazosin in our study. As shown in Figure 3.4, treatment with prazosin abolished the protective effect of phenylephrine preconditioning, reducing the contractile recovery from 56.7 ± 3.7% (n = 4, 4 and 159) to 29.3 ± 8.3% (n = 5, 4 and 200; P < 0.05), which was not significantly different to that of control myocytes (23.6 ± 3.7%, n = 4, 4, and 175; P = NS) suggesting that phenylephrine does indeed signal via the α_1-adrenoceptor to produce preconditioning.

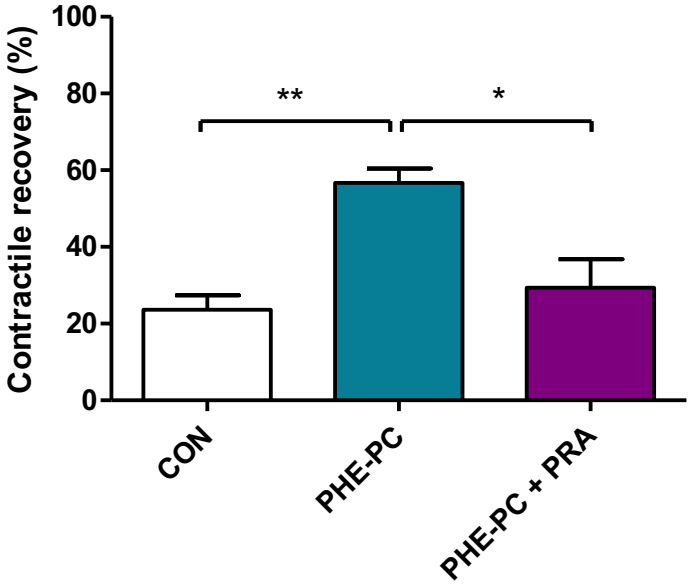

Figure 3.4. α_1-adrenoceptor stimulation is required for phenylephrine preconditioning.

Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 4, 4, and 175 for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 4, 4, and 159). Myocytes preconditioned with phenylephrine in the presence of the α_1-adrenoceptor antagonist prazosin (10 μM) (PHE-PC + PRA; purple bar, n = 5, 4, and 200). * $P < 0.05$, ** $P < 0.01$.

3.2.3 Protein Kinase C is required for preconditioning by phenylephrine

Activation of the α_1-adrenoceptor leads to increases in the levels of the second messengers diacylglycerol (DAG) and Ca^{2+} that can activate PKC. It has been established that activation of PKC is required for phenylephrine preconditioning of isolated rat hearts (Mitchell *et al.*, 1995, Tsuchida *et al.*, 1994). Because of the known involvement of PKC in phenylephrine preconditioning, we set out to confirm the role of PKC in phenylephrine preconditioning of ventricular myocytes.

The role of PKC in preconditioning was examined by pretreatment of ventricular myocytes with the non-selective PKC inhibitor chelerythrine (5μM) for 10 minutes prior to preconditioning. The contractile recovery of phenylephrine preconditioned ventricular myocytes was reduced from 43.8 ± 5.4% (n = 5, 5, and 168) to 14.2 ± 5.4% (n = 4, 4, and 115) by pretreatment with chelerythrine prior to preconditioning ($P < 0.01$; Figure 3.5). Chelerythrine did not significantly affect the contractile recovery of non-preconditioned myocytes when compared to control myocytes; with a percentage contractile recovery of 18.8 ± 2.3% (n = 8, 5, and 221) of control myocytes and 22.2 ± 1.0% (n = 4, 4, 120) of chelerythrine treated myocytes (P = NS). This suggests that PKC activation is necessary for phenylephrine preconditioning. However, chelerythrine treatment alone did not affect the recovery of non-preconditioned ventricular myocytes after MI and re-energisation, suggesting that PKC is not tonically active in these cells.

We also examined the effect of chelerythrine on the mean time to contractile failure and rigor contracture during MI. Chelerythrine decreased the time to contractile failure during MI in both control and phenylephrine preconditioned myocytes (Figure 3.6A), from 189 ± 6 seconds (n = 148, 5 and 4 for cells, experiments, and hearts respectively) in control myocytes to 122 ± 4 seconds (n = 124, 4, and 4) in control myocytes treated with chelerythrine ($P < 0.01$); and from 181 ± 4 seconds (n = 86, 4, and 4) in

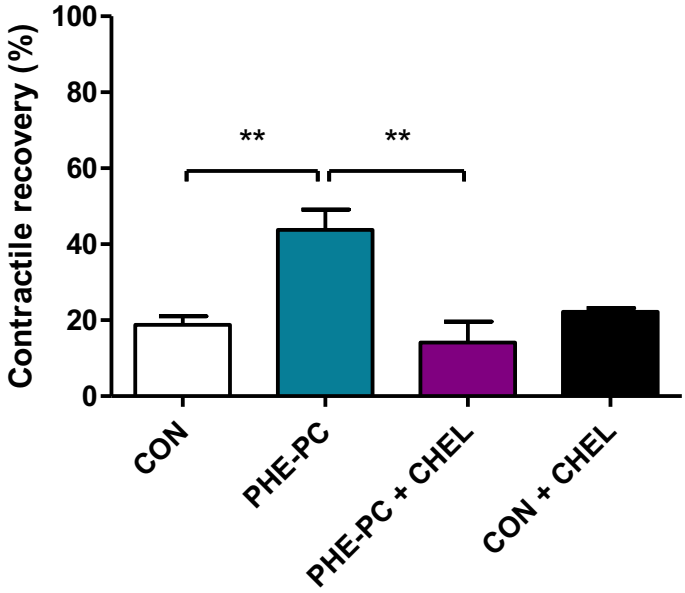

Figure 3.5. Protein Kinase C activation is required for phenylephrine preconditioning.

Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 8, 5, and 221 for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 5, 5, and 168). Phenylephrine preconditioned myocytes pretreated with chelerythrine (5 μM) for 10 minutes prior to preconditioning (PHE-PC + CHEL; purple bar, n = 4, 4, and 115). Control myocytes pretreated with chelerythrine (CON + CHEL; black bar, n = 4, 4, 120). ** $P < 0.01$.

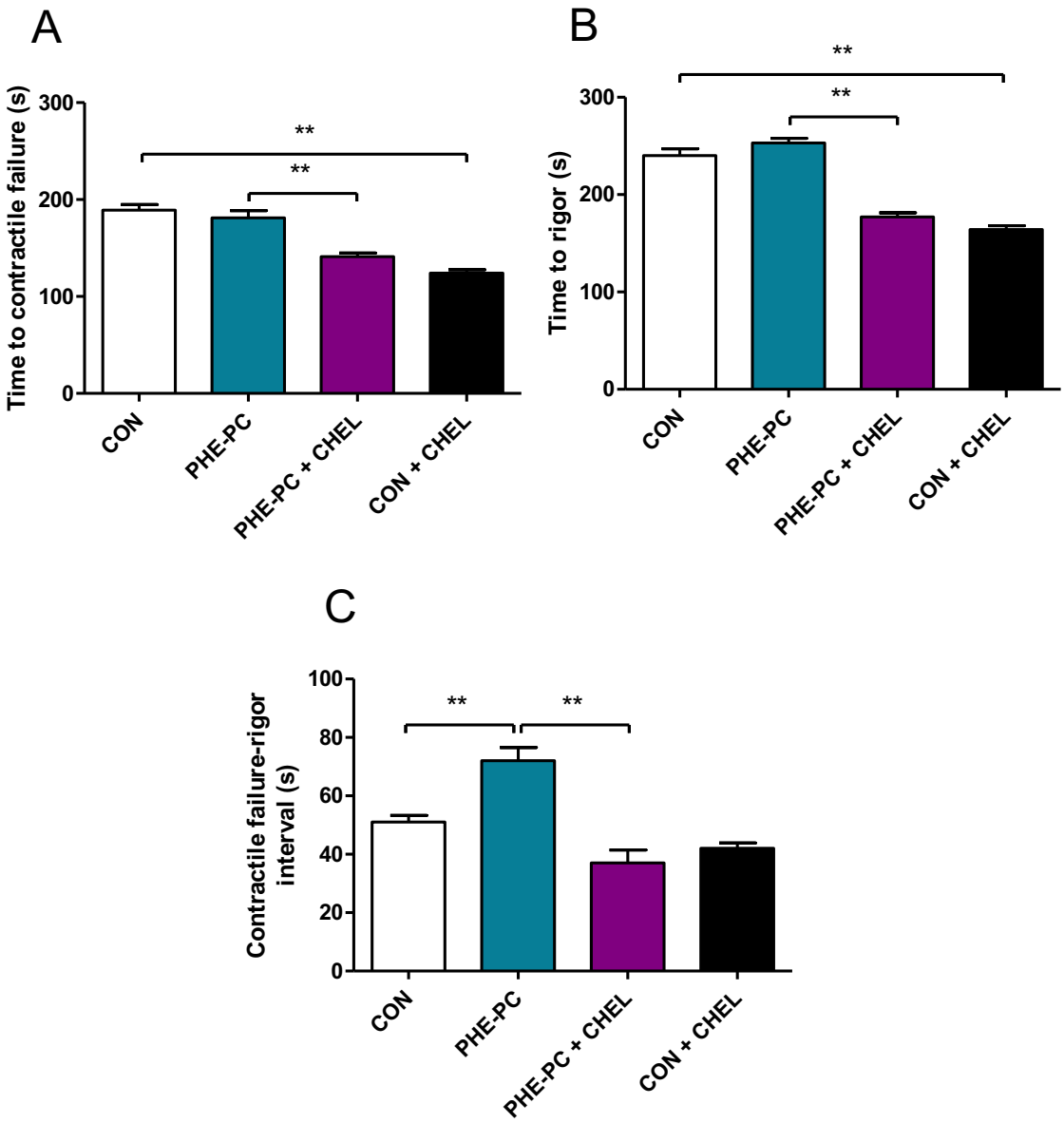

Figure 3.6. Effect of chelerythrine on time to contractile failure and rigor contracture.

A. Mean time to contractile failure in control myocytes (CON; open bars, n = 148, 5 and 4 for cells and experiments, and hearts respectively), phenylephrine preconditioned myocytes (PHE-PC; blue bars, n = 86, 4, and 4), phenylephrine preconditioned myocytes treated with 5 μM chelerythrine (PHE-PC + CHEL; purple bars, n = 98, 4, and 4) and control myocytes treated with chelerythrine (CON + CHEL; black bars, n = 124, 4, and 4). **P < 0.01.

B. Mean time to rigor contracture in CON, PHE-PC, PHE-PC + CHEL, and CON + CHEL myocytes. **P < 0.01.

C. Interval between the onset of contractile failure and rigor contracture in CON, PHE-PC, PHE-PC + CHEL, and CON + CHEL myocytes. ** P < 0.01.

phenylephrine preconditioned myocytes to 141 ± 4 seconds (n = 98, 4, and 4) in phenylephrine preconditioned myocytes pretreated with chelerythrine ($P < 0.01$). Similarly, the mean time to rigor contracture was decreased from 240 ± 7 seconds (n = 148, 5 and 4) in control myocytes to 164 ± 4 seconds (n = 124, 4, and 4) in control myocytes pretreated with chelerythrine ($P < 0.01$; Figure 3.6B); and from 253 ± 5 seconds (n = 86, 4, and 4) in phenylephrine preconditioned myocytes to 177 ± 4 seconds (n = 98, 4, and 4) in phenylephrine preconditioned myocytes pretreated with chelerythrine ($P < 0.01$). We also examined the effect of chelerythrine preconditioning on the time interval between contractile failure and rigor contracture (Figure 3.6C). Phenylephrine preconditioning resulted in a significant increase in the interval between contractile failure and rigor contracture, from 51 ± 2 seconds in control myocytes (n = 148, 5 and 4) to 72 ± 5 seconds in phenylephrine preconditioned myocytes (n = 86, 4, and 4; $P < 0.01$) that was abolished by pretreatment with chelerythrine prior to phenylephrine preconditioning (37 ± 4 seconds; n = 124, 4, and 4; $P < 0.01$). This suggests that PKC activation alters the time course of contractile failure and rigor contracture during MI, and PKC has additional effects on these parameters following phenylephrine preconditioning.

3.2.4 K_{ATP} channel activation is required for phenylephrine preconditioning

It is proposed that the hyperpolarisation of the cell membrane that occurs in response to *sarc*K_{ATP} channel opening in the heart could limit Ca^{2+} entry and eventual Ca^{2+} overload (Baczkó *et al.*, 2004, Jovanović *et al.*, 1998b). The ability of *sarc*K_{ATP} channel activation to hyperpolarise the membrane potential has been documented, and the *sarc*K_{ATP} channel appears to be necessary for preconditioning to occur (Gross and Auchampach, 1992). However, the precise role of *sarc*K_{ATP} channel opening in IPC

remains controversial, while the *mito*K_{ATP} channel has also been implicated in IPC (see Garlid *et al.*, 1997, and Chapter 1).

Activation of the *mito*K_{ATP} channel appears to be necessary for phenylephrine preconditioning (Loubani and Galiñanes, 2002, Cohen *et al.*, 2001). In contrast, nothing is known about the involvement of the *sarc*K_{ATP} channel in phenylephrine preconditioning. Therefore, we set out to test the role of K_{ATP} channels in phenylephrine preconditioning using the non-selective K_{ATP} channel inhibitor glibenclamide. Glibenclamide (10 μM) was applied from the beginning of preconditioning and continued until the end of MI. Figure 3.7 shows that glibenclamide inhibited phenylephrine preconditioning and reduced the percentage contractile recovery to that seen in control myocytes. The percentage recovery of contractile activity of phenylephrine preconditioned ventricular myocytes was 43.8 ± 5.4% (n = 5, 5 and 168 for experiments, hearts, and cells), which was reduced to 8.2 ± 6.9% (n = 4, 4, and 83) by glibenclamide treatment ($P < 0.01$). To determine whether glibenclamide was having an effect independent of phenylephrine preconditioning, we also treated control myocytes with glibenclamide (10 μM). We observed that 18.8 ± 2.3% (n = 8, 5, 221) of control myocytes recovered contractile activity and this was reduced to 3.2 ± 2.2% (n = 4, 4, and 94) in the presence of glibenclamide alone but this did not reach significance (P = NS). These data suggest that K_{ATP} channel activation is necessary for phenylephrine preconditioning of isolated ventricular myocytes. K_{ATP} channels may also be involved in the recovery of myocardial function in the absence of preconditioning since glibenclamide slightly reduced contractile recovery in non-preconditioned cells.

SarcK_{ATP} channel opening is thought to be responsible for action potential shortening during ischaemia (Lederer *et al.*, 1989). This causes early failure of the action potential,

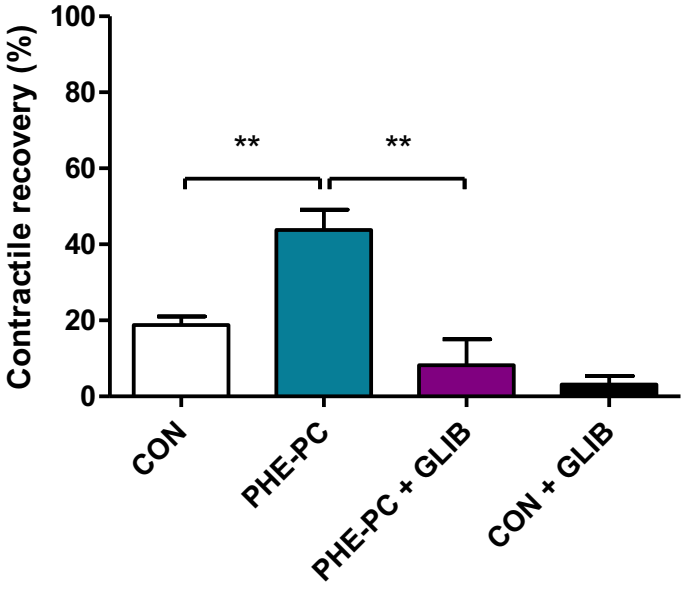

Figure 3.7. Glibenclamide prevents phenylephrine preconditioning.

Percentage of myocytes contracting to field stimulation following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 8, 5, and 221 for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 5, 5, and 168). Phenylephrine preconditioned myocytes treated with 10 μM glibenclamide during preconditioning and MI (PHE-PC + GLIB; purple bar, n = 4, 4, and 83). Control myocytes treated with glibenclamide (CON + GLIB; black bar, n = 4, 4, and 94). ** $P < 0.01$.

which protects the ATP levels of the cell and may lead to less Ca^{2+} influx during ischaemia. We suspected that inhibition of *sarc*K_{ATP} channels with glibenclamide would prevent or delay action potential shortening, resulting in a delay of contractile failure during MI. Inhibition of *sarc*K_{ATP} channels might also cause rigor contracture to occur earlier, as the later onset of contractile failure would deplete ATP more rapidly within the micro-domains containing the contractile elements. To test this, we examined the effect of glibenclamide on the time to contractile failure and rigor after application of MI, in control and phenylephrine preconditioned myocytes. Time to onset of contractile failure was not significantly delayed in response to glibenclamide treatment; the time to contractile failure was 165 ± 4 seconds (n = 102, 3, and 3 for cells, experiments, and hearts respectively) in control myocytes, 173 ± 4 seconds (n = 109, 4, and 4) in control myocytes treated with glibenclamide, 171 ± 6 seconds (n = 69, 3, and 3) in phenylephrine preconditioned myocytes, and 160 ± 5 seconds (n = 86, 4, 4) in phenylephrine preconditioned myocytes treated with glibenclamide (all P = NS; Figure 3.8A). However, glibenclamide significantly reduced the mean time to rigor contracture in both control and phenylephrine preconditioned myocytes (Figure 3.8B). The mean time to development of rigor contracture was 214 ± 4 seconds (n = 102, 3, and 3) in control myocytes, and 184 ± 4 seconds (n = 109, 4, and 4) in control myocytes treated with glibenclamide ($P < 0.01$). Following phenylephrine preconditioning, the mean time to rigor contracture was 256 ± 8 seconds (n = 69, 3, and 3) in phenylephrine preconditioned myocytes, and 171 ± 5 seconds (n = 86, 4, and 4) in phenylephrine preconditioned myocytes treated with glibenclamide ($P < 0.01$). The interval between contractile failure and rigor was also significantly decreased by glibenclamide (in the presence and absence of phenylephrine preconditioning) compared with control and phenylephrine preconditioned ventricular myocytes ($P < 0.01$; Figure 3.8C). This

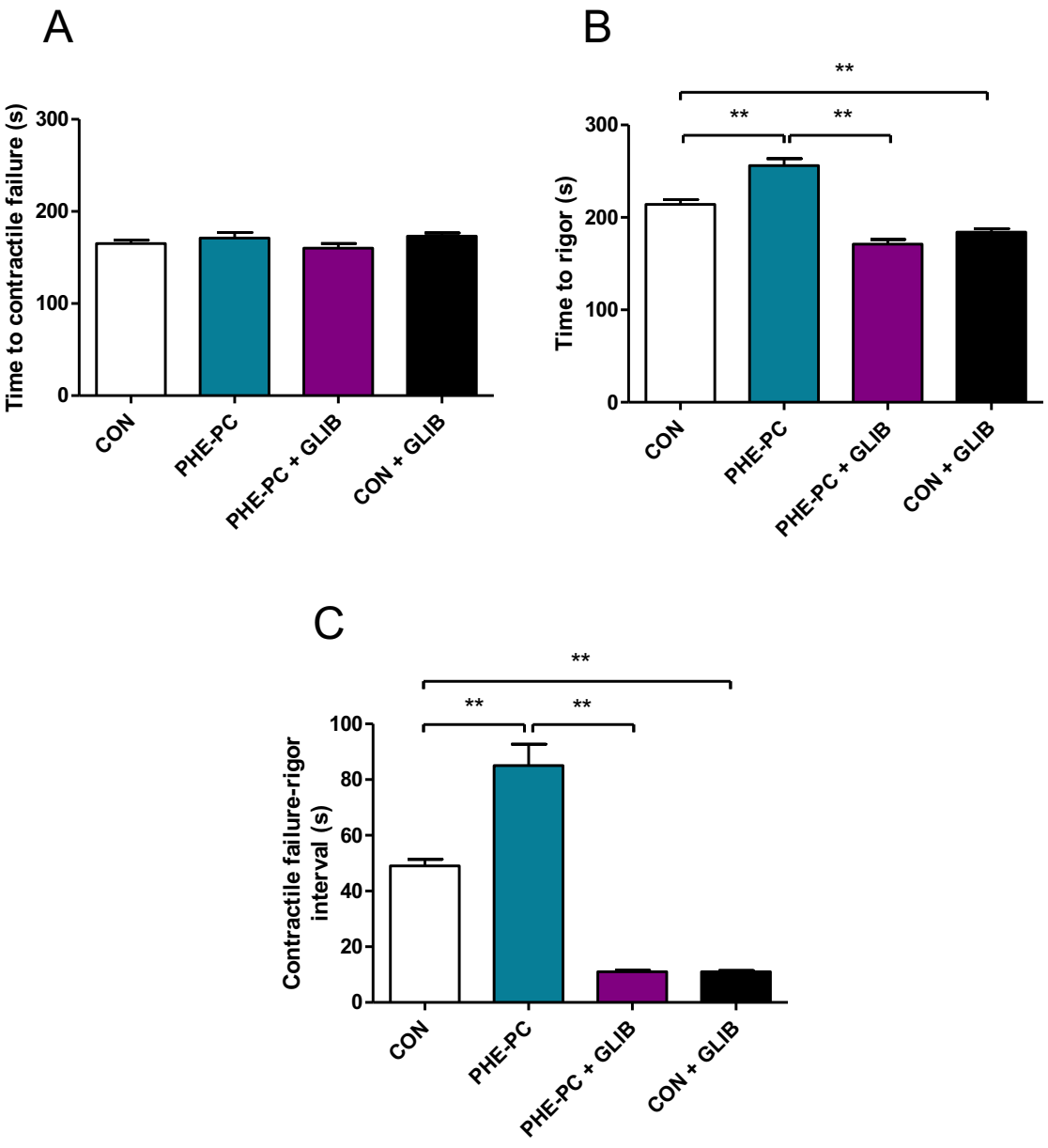

Figure 3.8. Effect of glibenclamide on time to contractile failure and rigor contracture.

A. Mean time to contractile failure in control myocytes (CON; open bars, n = 102, 3 and 3 for cells and experiments, and hearts respectively), phenylephrine preconditioned myocytes (PHE-PC; blue bars, n = 69, 3, and 3), phenylephrine preconditioned myocytes treated with 10 μM glibenclamide (PHE-PC + GLIB; purple bars, n = 86, 4, and 4) and control myocytes treated with glibenclamide (CON + GLIB; black bars, n = 109, 4, and 4). P = NS.

B. Mean time to rigor contracture in CON, PHE-PC, PHE-PC + GLIB, and CON + GLIB myocytes. **P < 0.01.

C. Interval between the onset of contractile failure and rigor contracture in CON, PHE-PC, PHE-PC + GLIB, and CON + GLIB myocytes. ** P < 0.01.

suggests that inhibition of K_{ATP} channels with glibenclamide may cause a change in energy utilisation during MI resulting in increased energy depletion compared with untreated myocytes.

3.2.5 Phenylephrine preconditioning improves calcium handling in cardiac myocytes exposed to metabolic inhibition and re-energisation

IPC and pharmacological preconditioning of ventricular myocytes improves their ability to handle Ca^{2+} both during MI and on re-energisation (Rodrigo and Samani, 2008, Rodrigo *et al.*, 2002, Rodrigo *et al.*, 2004). Uncontrolled rises in intracellular Ca^{2+} can activate damaging Ca^{2+}-dependent processes such as Ca^{2+}-activated enzymes (proteases, phospholipases, endonucleases and phosphatases), MPTP formation and opening and Ca^{2+} overload-induced contracture, resulting in cellular injury and death through both necrotic and apoptotic mechanisms (Piper *et al.*, 2004, Dong *et al.*, 2006), as well as leading to reversible contractile dysfunction or stunning (Bolli and Marbán, 1999).

To test whether phenylephrine preconditioning improves Ca^{2+}-handling in ventricular myocytes during MI and re-energisation, we measured Ca^{2+} levels of isolated ventricular myocytes field stimulated at 1 Hz, using the ratiometric Ca^{2+}-sensitive dye Fura-2. Due to the slow sampling rate of 0.1 Hz used in the experiments, it was not possible to measure individual Ca^{2+} transients. However, the aliasing effect produced by slow sampling of rapid transients did result in recording of slowly oscillating Ca^{2+} levels that were dependent on stimulation, (see Figure 2.4C) that made it possible to determine whether Ca^{2+} transients were evoked in response to field stimulation, providing an indicator of contractile function in the cells (Rodrigo and Samani, 2008).

Representative fura-2 traces from control and phenylephrine preconditioning experiments are shown in Figure 3.9A and B, and mean Ca^{2+} data are shown in Figure

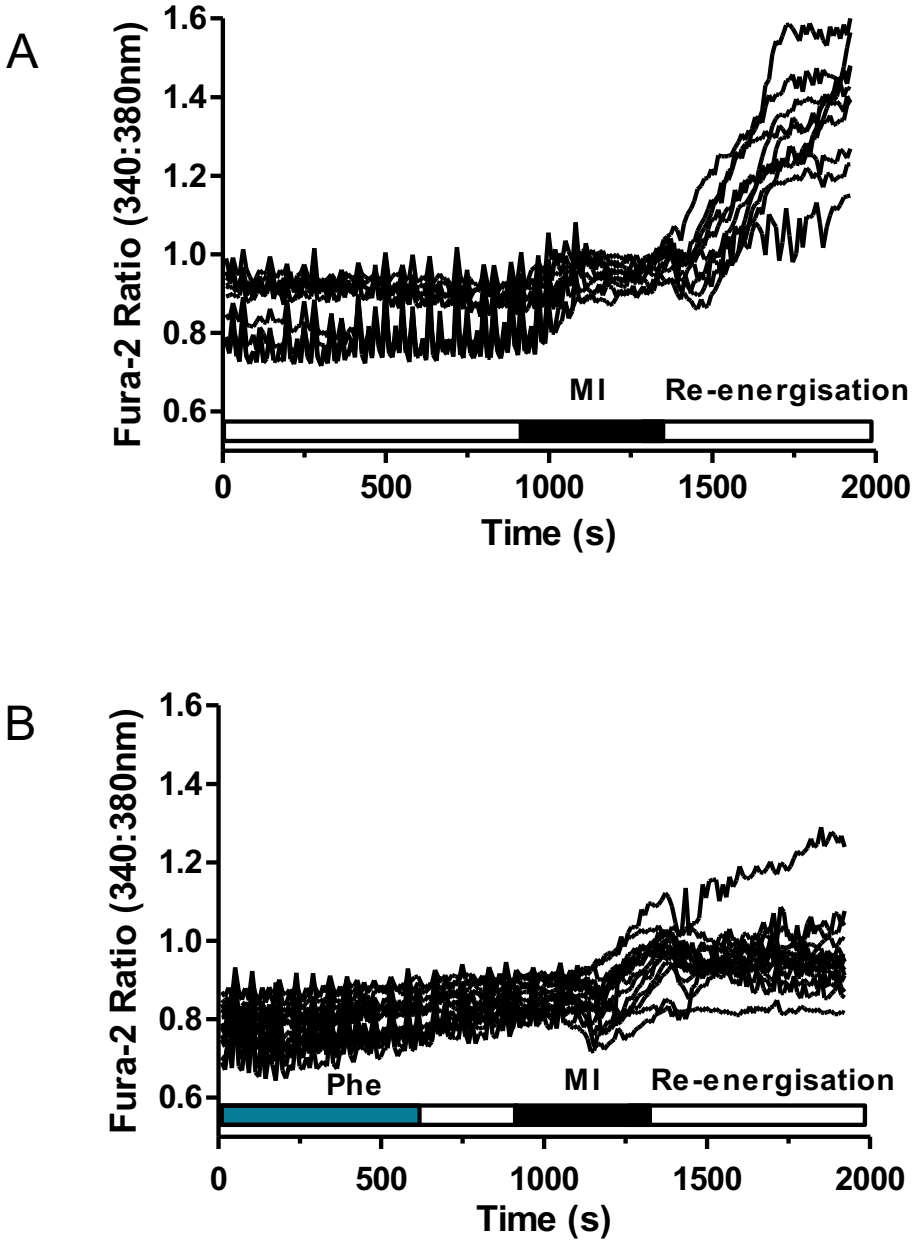

Figure 3.9. Effect of phenylephrine preconditioning on intracellular calcium levels of isolated ventricular myocytes during metabolic inhibition and re-energisation.

A. Typical trace from a field of control myocytes showing effect of 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation on the fura-2 ratio.

B. Typical fura-2 trace from a field of phenylephrine preconditioned myocytes exposed to 7 minutes of MI and 10 minutes of re-energisation.

3.10. The mean fura-2 ratio was measured at the start of the preconditioning with phenylephrine, at the end of wash out of phenylephrine, at the end of 7 minutes of MI and after 10 minutes of re-energisation with normal Tyrode. In the case of control experiments, measurements were taken at the start of the experiment and after 15 minutes of superfusion with normal Tyrode prior to MI. The initial Ca^{2+} levels of control and phenylephrine preconditioned ventricular myocytes were not different and were not significantly altered by preconditioning with phenylephrine (P = NS; Figure 3.10 A and B). Metabolic inhibition resulted in a slight increase in the fura-2 ratio of cells; the fura-2 ratio of control myocytes increased from 0.82 ± 0.04 to 1.02 ± 0.08 (n = 4, 3 and 32, for experiments, hearts, and cells respectively), while the fura-2 ratio of phenylephrine preconditioned myocytes increased from 0.79 ± 0.02 to 0.95 ± 0.03 (n = 5, 3, and 57) at the end of 7 minutes of MI (both P = NS). Upon re-energisation with normal Tyrode, the fura-2 ratios of both control and phenylephrine preconditioned myocytes initially declined (within ~1-2 minutes). With continued superfusion of normal Tyrode, the fura-2 ratio increased in some cells, while other cells maintained low fura-2 ratios. The mean fura-2 ratio at the end of re-energisation was significantly higher than at the start of the experiment in both control and phenylephrine preconditioned myocytes ($P < 0.01$). The mean fura-2 ratio of ventricular myocytes in control experiments was 1.38 ± 0.07 at the end of re-energisation, which was significantly higher than the mean ratio of 1.13 ± 0.07 in phenylephrine preconditioned myocytes ($P < 0.05$). In addition, the protection of Ca^{2+} homeostasis induced by phenylephrine preconditioning appeared to occur mainly during the re-energisation period. There was a significant increase in fura-2 ratio of control myocytes between the end of MI and end of the re-energisation ($P < 0.01$); but the increase in the fura-2 ratio

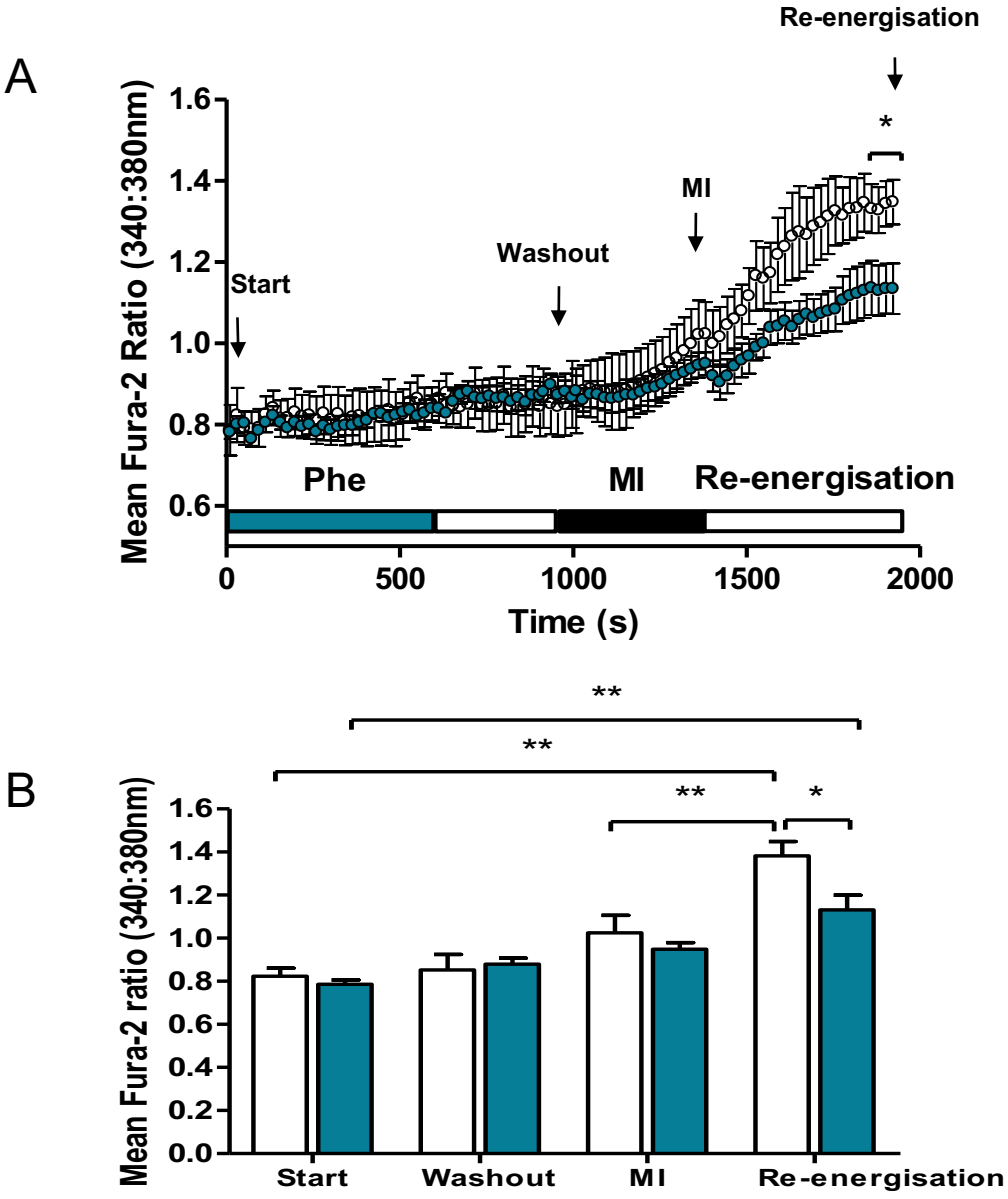

Figure 3.10. Effect of phenylephrine preconditioning on calcium handling during metabolic inhibition and re-energisation.

A. Line graph showing the mean fura-2 fluorescence during preconditioning, metabolic inhibition and re-energisation. Arrows above indicate time points examined in 3.10 B. control (CON; open bars, n = 4, 3, and 32, for experiments, hearts, and cells respectively). Phenylephrine preconditioned (PHE-PC; blue bars, n = 5, 3 and 57). * $P < 0.05$ vs. CON.

B. Bar chart of the mean fura-2 ratio in CON and PHE-PC myocytes, recorded at the start of superfusion with phenylephrine (start), at the end of wash out of phenylephrine (wash out), at the end of 7 minutes of metabolic inhibition (MI) and after 10 minutes of re-energisation with normal Tyrode (re-energisation). In the case of control myocytes data shown for the start of the experiment and after 15 minutes superfusion with normal Tyrode prior to MI. * $P < 0.05$, ** $P < 0.01$.

of phenylephrine preconditioned myocytes over the same period was not significant (P = NS).

We examined the proportion of myocytes that could contract in response to field stimulation following MI and re-energisation in these experiments, determined by observation of cells under bright-field illumination. We found that 19.2 ± 8.4% (n = 4, 4, and 32) of control myocytes and 50.9 ± 8.5% (n = 5, 3, and 57) of phenylephrine preconditioned myocytes recovered contractile activity ($P < 0.05$; Figure 3.11A). Both control and phenylephrine preconditioned myocytes that were able to contract in response to field stimulation following MI and re-energisation, generally had a fura-2 ratio of less than 1.00. We chose a value that was 25% above this (a ratio of 1.20) to represent myocytes that were able to maintain Ca^{2+} homeostasis, and therefore examined the proportion of myocytes that had a fura-2 ratio of less than 1.20 following 10 minutes of re-energisation to represent cells with low Ca^{2+} levels. The percentage of control myocytes with a fura-2 ratio below 1.20 at the end of re-energisation was 32.8 ± 13.9% (n = 4, 4, and 32; Figure 3.11B) and this was increased by preconditioning with phenylephrine to 74.5 ± 9.4% (n = 5, 3, and 57), indicating that significantly more phenylephrine preconditioned ventricular myocytes were able to maintain low Ca^{2+} levels after MI and re-energisation ($P < 0.05$). The proportion of cells recovering contractile activity and low Ca^{2+} levels were well correlated, suggesting that the phenylephrine preconditioning-induced improvement in Ca^{2+} handling may be associated with the improvement in contractile function in isolated ventricular myocytes following MI and re-energisation.

3.3 Summary

Isolated ventricular myocytes were used in this study to facilitate measurement of the contractile function and intracellular Ca^{2+} level, which is problematic in the whole heart.

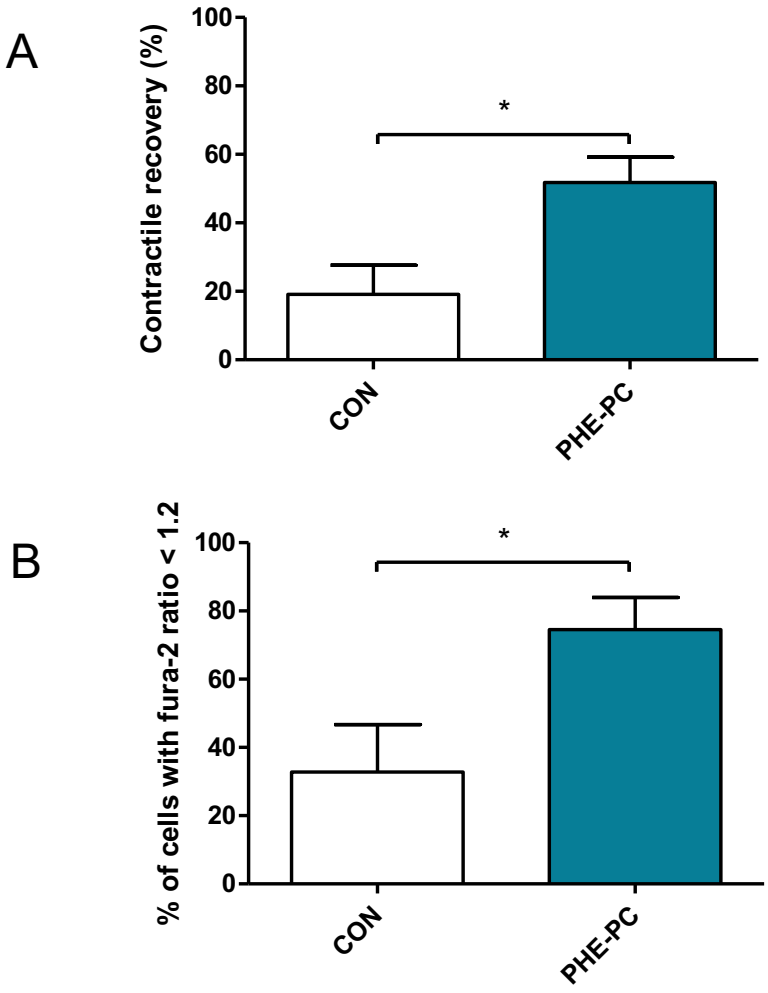

Figure 3.11. Proportion of fura-2 loaded myocytes recovering low calcium levels and contractile activity following MI and re-energisation.

A. Bar chart showing the contractile recovery of fura-2 loaded control (CON) and phenylephrine preconditioned (PHE-PC) myocytes, observed under bright-field illumination following MI and re-energisation. (CON; open bars, n = 4, 3, and 32, for experiments, hearts, and cells respectively). Phenylephrine preconditioned (PHE-PC; blue bars, n = 5, 3 and 57). * $P < 0.05$.

B. Bar chart showing the percentage of the same CON and PHE-PC myocytes, with a fura-2 ratio below 1.20 following 7 minutes of MI and 10 minutes of re-energisation. * $P < 0.05$.

The results in this chapter demonstrate the ability of phenylephrine to precondition isolated ventricular myocytes, protecting them from the damaging effects of MI re-energisation injury. We found that phenylephrine preconditioning increased the percentage of myocytes that recovered contractile activity as well as preventing the rise in intracellular Ca^{2+} that occurred during re-energisation and this may be causal.

The increase in contractile recovery produced by phenylephrine preconditioning was prevented by pretreatment with the α_1-adrenoceptor antagonist prazosin, suggesting that phenylephrine preconditioning acts through stimulation of the G_q-coupled α_1-adrenoreceptor. In addition, we examined the role of K_{ATP} channel activation in phenylephrine preconditioning using the non-selective K_{ATP} channel inhibitor glibenclamide. Application of glibenclamide was sufficient to block the protective effects of phenylephrine preconditioning on contractile recovery in our experiments suggesting that K_{ATP} channel activation is necessary for phenylephrine preconditioning. However, glibenclamide does not allow us to determine the specific roles of *sarc*- and *mito*K_{ATP} channels in phenylephrine preconditioning. Phenylephrine preconditioning was also abolished by inhibition of PKC using the PKC inhibitor chelerythrine. In the next chapter we will further investigate the role of PKC in phenylephrine preconditioning, examining the roles of specific PKC isoforms.

4 The role of Protein Kinase C in phenylephrine preconditioning

4.1 Introduction

In the previous chapter, we demonstrated the involvement of PKC in phenylephrine preconditioning using the non-selective PKC inhibitor chelerythrine (Section 3.2.3). Inhibition of PKC with chelerythrine resulted in a loss of the protective effect of phenylephrine preconditioning without effect on the contractile recovery of non-preconditioned ventricular myocytes. However, chelerythrine is a non-selective PKC inhibitor and more specific tools are required examine the role of individual PKC isoforms in phenylephrine preconditioning. Therefore we set out to investigate which PKC isoforms are involved in the protection afforded by phenylephrine using ε, δ and conventional PKC-specific inhibitor and activator peptides (see Table 4.1).

4.2 PKC

PKC isoforms are divided into the conventional Ca^{2+} and DAG sensitive (PKC α, β, γ), novel Ca^{2+}-independent (PKC η, δ, ε, θ) and atypical DAG-independent isoforms (PKC λ, μ, ζ, ι) (for a review see Steinberg, 2008), of which PKC α, ε and δ have all been observed to translocate during preconditioning with various stimuli (Mitchell *et al.*, 1995, Gray *et al.*, 1997, Tsouka *et al.*, 2002). While it appears that PKCε exerts a cardioprotective effect during ischaemia-reperfusion (Gray *et al.*, 1997, Dorn *et al.*, 1999, Inagaki and Mochly-Rosen, 2005), the effects of PKCδ and PKCα are less well defined.

Peptide name	Amino acids	Sequence	Effect of peptide	Reference
βC2-4	PKCβ 218-226	SLNPEWNET	conventional PKC inhibitor	(Ron *et al.*, 1995)
αC2-4	PKCα 218-226	SLNPQWNET	PKCα inhibitor	(Hu *et al.*, 2000)
εV1-2	PKCε 14-21	EAVSLKPT	PKCε inhibitor	(Johnson *et al.*, 1996)
ψεRACK	PKCε 85-92	HDAPIGYD	PKCε activator	(Dorn *et al.*, 1999)
δV1-1	PKCδ 8-17	SFNSYELGSL	PKCδ inhibitor	(Chen *et al.*, 2001)
ψδRACK	PKCδ 74-81	MRAAEDPM	PKCδ activator	(Chen *et al.*, 2001)
Tat	Tat 47-57	YGRKKRRQRRR	Cell-permeable carrier peptide	(Begley *et al.*, 2004).

Table 4.1 PKC modulator peptides
Sequences of PKC inhibitor and activator peptides used in experiments. Peptides were reversibly conjugated to the Tat carrier peptide through formation of a disulphide bond between free cysteines at the N-terminus (Begley *et al.*, 2004).

4.2.1 PKC inhibitor and activator peptides

Early studies examining the role of PKC in preconditioning were hindered by the lack of isoform-specific PKC inhibitors and activators. Although some progress was made through the examination of PKC translocation or use of poly-pharmacology to isolate the role of a subset of PKC isoforms, the results of these early studies may at times have been misleading. However, the discovery of the receptors for activated C Kinase (RACKs) and the specific regulatory regions of PKC proteins that mediate PKC interactions with RACKs, enabled Mochly-Rosen's group to develop isoform-specific PKC activator and inhibitor peptides (Mochly-Rosen *et al.*, 1991, Schechtman *et al.*, 2004). PKCs are able to bind to RACKs through interaction of unique RACK-binding sequences present on each PKC isoform with the corresponding sequences present on the RACKs. PKCs also contain a ψRACK sequence that mimics the PKC-binding site on the RACK and is able to interact with the RACK-binding site, maintaining the PKC in an inactive conformation under resting conditions (Schechtman *et al.*, 2004). Short peptide sequences that mimic the RACK-binding sequence of PKCs have been developed that prevent PKC activation (Chen *et al.*, 2001, Johnson *et al.*, 1996, Ron *et al.*, 1995, Hu *et al.*, 2000), while sequences that mimic the ψRACK sequence facilitate PKC activation and translocation (Chen *et al.*, 2001, Dorn *et al.*, 1999).

Tat is an 86 amino acid protein derived from the HIV-1 virus. Truncated forms of the Tat protein, containing the Tat basic domain, are efficiently taken up by cells and can act as carrier peptides (Vives *et al.*, 1997). The PKC-modulating peptides can be reversibly-conjugated to Tat through formation of a disulphide bond, which is rapidly reduced within the cell releasing the free PKC-modulating peptide allowing intracellular delivery of the peptides, see Table 4.1 (Begley *et al.*, 2004).

4.3 Results

4.3.1 Inhibition of conventional PKC isoforms does not affect the ability of phenylephrine to precondition isolated ventricular myocytes.

Phenylephrine activates α_1-adrenoceptors and through subsequent phospholipase C (PLC) activation, can increase the levels of lipid cofactors (such as DAG) and cytosolic Ca^{2+} that are necessary for conventional PKC activation. PKCα translocation has previously been observed in response to phenylephrine preconditioning in one study (Tsouka *et al.*, 2002), however another found no effect on PKCα (Mitchell *et al.*, 1995). In addition, infarct size was reduced by knock out of PKCβ, suggesting that PKCβ may increase ischaemia-reperfusion injury (Kong *et al.*, 2008). Therefore, the role of the Ca^{2+}-dependent conventional PKC isoforms in phenylephrine preconditioning of isolated ventricular myocytes was examined using the Tat-conjugated conventional PKC inhibitor peptide βC2-4.

To assess the role of conventional PKC isoforms in phenylephrine preconditioning, conventional PKC isoforms (α, β, and γ) were inhibited by incubating ventricular myocytes with the βC2-4 peptide (100 nM) for 10 minutes prior to phenylephrine preconditioning and measurement of the contractile recovery following MI and re-energisation (protocol is detailed in Figure 4.1A). We chose this concentration of βC2-4 as it has been previously shown that addition of 100 nM βC2-4 to the patch pipette allows dialysis of sufficient inhibitor into the cell suggesting that extracellular application of the same concentration of a cell-permeant form of the peptide should result in inhibition of conventional PKC isoforms (Hu *et al.*, 2000). As shown in Figure 4.2, 69.9 ± 2.8% (n = 4, 4, and 184, for experiments, hearts, and cells respectively) of phenylephrine preconditioned ventricular myocytes recovered contractile function

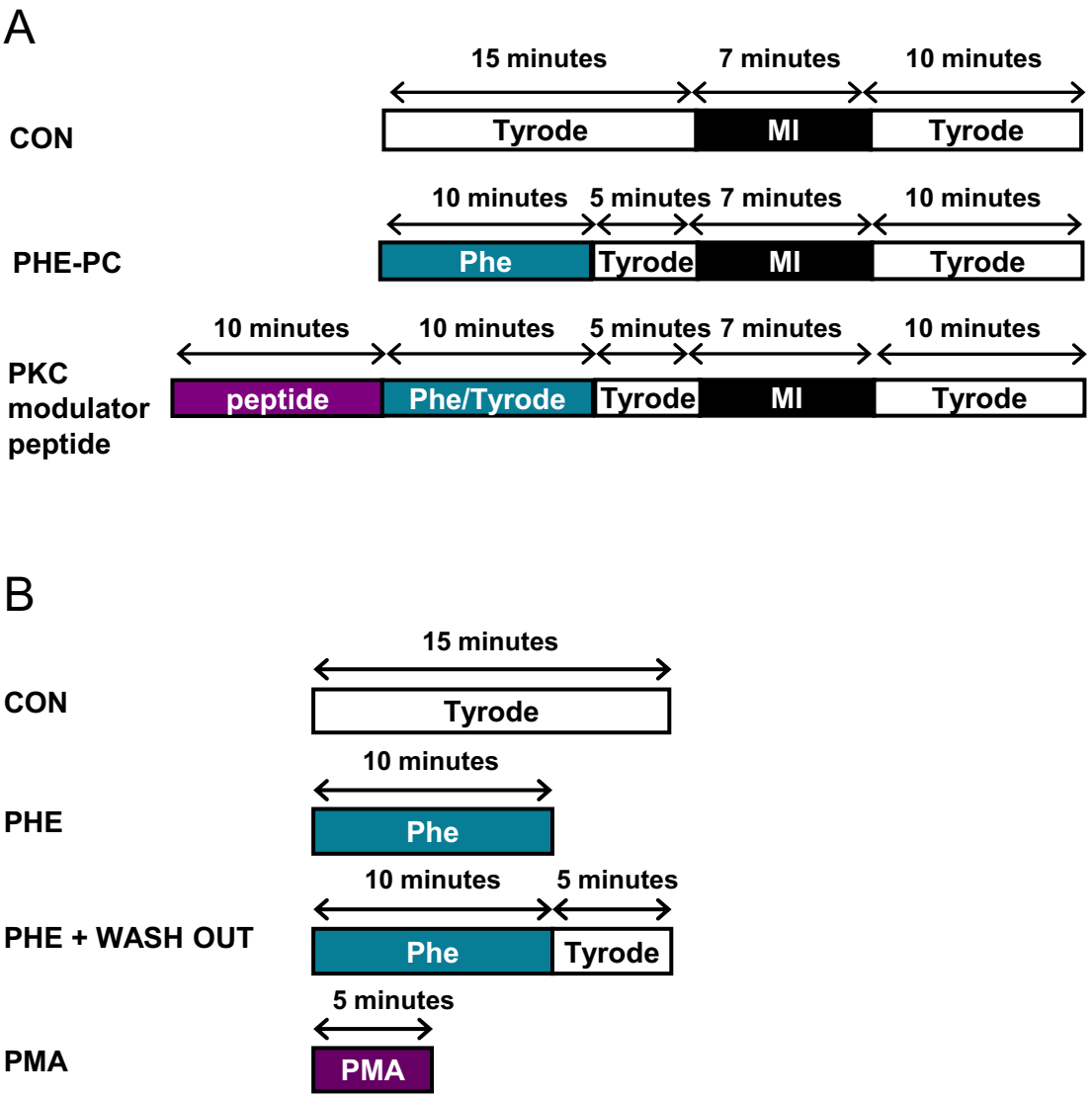

Figure 4.1 Protocols used for phenylephrine preconditioning, metabolic inhibition and re-energisation, inhibitor/activator treatments and western blot experiments.

A. Ventricular myocytes were preconditioned with a 10 minute superfusion of phenylephrine (Phe) followed by 5 minutes of wash out with normal Tyrode. Control myocytes were superfused with normal Tyrode for 15 minutes. Myocytes were then subject to 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control (CON). Phe preconditioned (PHE-PC). PKC modulator peptides, see Table 4.1.

B. Protocol used to investigate the translocation of PKCε and δ in response to treatment with 5 μM phenylephrine (Phe). Control, 15 minutes of normal Tyrode (CON). 10 minutes of Phe (PHE). 10 minutes of Phe followed by 5 minutes of washout with normal Tyrode (PHE + WASH OUT). 5 minutes of 1 μM PMA (PMA).

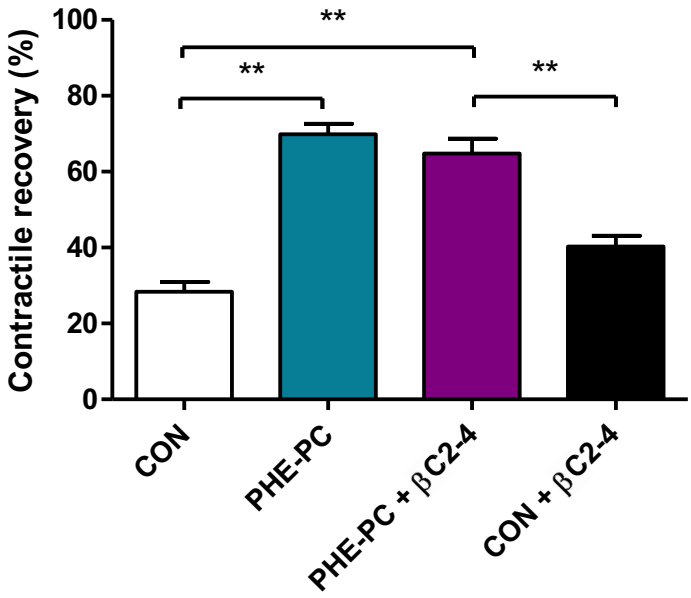

Figure 4.2. Inhibition of conventional PKC isoforms has no effect on phenylephrine preconditioning.

Percentage contractile recovery after 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 4, 4, 160, for experiments, hearts, and cells). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 4, 4, and 184). Phenylephrine preconditioned myocytes treated with the conventional PKC inhibitor peptide βC2-4 (100 nM) for 10 minutes (PHE-PC + βC2-4; purple bar, n = 4, 4, and 159). Control myocytes pretreated with βC2-4 (CON + βC2-4; black bar, n = 4, 4, and 157). ** $P < 0.01$.

compared to 28.4 ± 2.6% (n = 4, 4, 160) of control myocytes (P < 0.01). This enhanced contractile recovery was unaffected by pretreatment with the βC2-4 inhibitor peptide (64.8 ± 4.0%; n = 4, 4, and 159; P = NS), however the βC2-4 peptide did increase the percentage of control myocytes that recovered contractile activity to 40.3 ± 2.8% (n = 4, 4, and 157), but this did not reach significance (P = NS). This suggests that conventional isoforms of PKC are not involved in phenylephrine preconditioning of isolated ventricular myocytes, but activation of conventional PKC isoforms may increase MI re-energisation injury in non-preconditioned myocytes.

4.3.2 Effect of the Tat-conjugated αC2-4 PKCα inhibitor peptide

We assessed the effect of the Tat-conjugated αC2-4 peptide, an inhibitor of PKCα, on the contractile function of ventricular myocytes. We have shown in the previous set of experiments that inhibition of conventional PKC isoforms (α, β and γ) did not block phenylephrine preconditioning, therefore examined the effect of the Tat-conjugated αC2-4 peptide on contractile recovery as a control for the Tat-peptide. We used the αC2-4 peptide at 100 nM, a concentration that has previously been shown to be effective in inhibiting Kv channel modulation in isolated mesenteric smooth muscle in our laboratory (Rainbow *et al.*, 2009). The αC2-4 peptide had no significant effect on the recovery of phenylephrine preconditioned myocytes, 57.2 ± 1.5% (n = 5, 5, and 140) of phenylephrine preconditioned myocytes and 58.7 ± 3.7% (n = 4, 4, and 97) of myocytes treated with αC2-4 prior to phenylephrine preconditioning recovered contractile activity following MI and re-energisation (P = NS; Figure 4.3). The contractile recovery of ventricular myocytes treated with αC2-4 alone was 38.6 ± 4.5% (n = 4, 4, and 99), which was not different from control myocytes 31.0 ± 2.1% (n = 5, 5, and 132; P = NS). Here, we show that a Tat-congugated PKCα specific inhibitor peptide αC2-4, had no effect on the recovery of contractile activity in control or

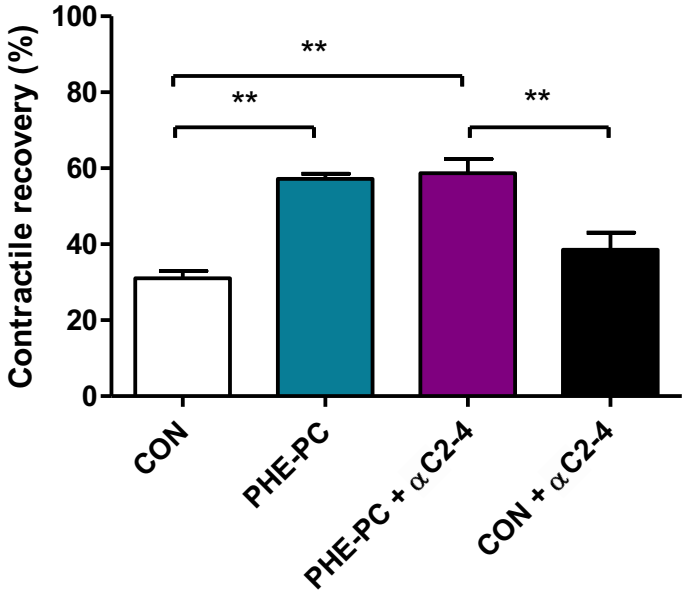

Figure 4.3. Effect of the α2-4 Tat-conjugated PKCα inhibitor peptide

Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 5, 5, and 132, for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 5, 5, and 140). Phenylephrine preconditioned myocytes pretreated with the α2-4 Tat-conjugated peptide (100 nM) for 10 minutes (PHE-PC + αC2-4; purple bar, n = 4, 4, and 97). Control myocytes pretreated with the α2-4 peptide (CON + αC2-4; black bar, n = 4, 4, and 99). ** $P < 0.01$.

phenylephrine preconditioned myocytes. This suggests that the Tat-peptide itself, reversibly-conjugated to a specific PKCα inhibitor peptide, has no effect on the contractile recovery of myocytes.

4.3.3 Activation of PKCε by phenylephrine

PKCε is a DAG-dependent and Ca^{2+}-independent novel PKC isoform that has been implicated in IPC (Gray *et al.*, 1997). Mitchell *et al.* (1995) did not observe translocation of PKCε in response to phenylephrine preconditioning in the whole heart. In contrast, Gao *et al.* (2007) observed translocation of PKCε to the membrane fraction of isolated ventricular myocytes after both the phenylephrine preconditioning stimulus and the subsequent simulated-ischaemia and reperfusion. We set out to examine whether PKCε was activated by the phenylephrine preconditioning stimulus in ventricular myocytes.

To test if PKCε translocates to the membrane in response to phenylephrine preconditioning during the time course for the preconditioning protocol, ventricular myocytes were treated with phenylephrine (5 μM) for 10 minutes or with phenylephrine (5 μM) for 10 minutes followed by 5 minutes of washout with normal Tyrode. Control myocytes were incubated in normal Tyrode for 15 minutes, and myocytes were exposed to the phorbol ester PMA (1 μM) for 5 minutes as a positive control (protocol for treatment of cells is described in Figure 4.1B). As shown in Figure 4.4, the percentage of PKCε in the membrane was increased from 29.8 ± 7.0% to 64.4 ± 6.6% after phenylephrine treatment ($n = 4$ experiments; $P < 0.05$), indicating that significant activation of PKCε occurs in response to acute phenylephrine treatment. After five minutes washout of phenylephrine, the percentage of PKCε in the membrane was 69.5 ± 7.1% ($n = 4$ experiments; $P < 0.01$). These results indicate that PKCε is activated within 10 minutes of phenylephrine treatment, and PKCε protein remains in the membrane

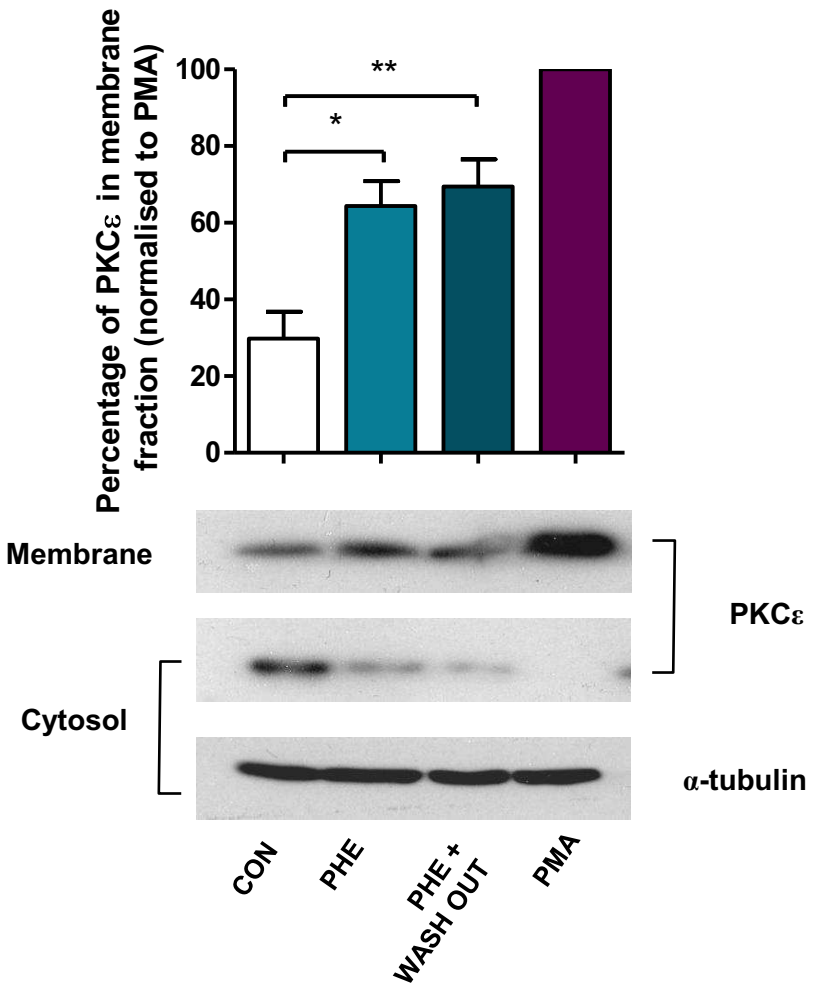

Figure 4.4. Phenylephrine preconditioning activates PKCε.

Western blot analysis showing the amount of PKCε in the cytosolic and membrane fractions of myocytes treated with phenylephrine or PMA. Anti-PKCε rabbit antibodies (Santa Cruz Biotechnology) were diluted 1 : 500 and anti-α-tubulin mouse antibodies (Sigma-Aldrich) were diluted 1 : 2,000. Bar chart shows the percentage of PKCε in the membrane fraction in response to phenylephrine preconditioning. Myocytes were treated with normal Tyrode for 15 minutes (CON; open bar), 5 μM phenylephrine for 10 minutes (PHE; blue bar), phenylephrine followed by 5 minutes wash out with normal Tyrode (PHE + WASH OUT; dark blue bar) or 1 μM PMA for 5 minutes (PMA; purple bar, n = 4, and 4, for experiments, and hearts). * $P < 0.05$ ** $P < 0.01$.

fraction at the end of the preconditioning protocol; therefore PKCε may act as a trigger or mediator of phenylephrine preconditioning.

4.3.4 Inhibition of PKCε prevents phenylephrine preconditioning

In the previous experiment, we observed translocation of PKCε to the membrane fraction in response to the phenylephrine preconditioning protocol. Furthermore, it has recently been observed that inhibition of PKCε using the εV1-2 inhibitor peptide prevented the protective effect of phenylephrine preconditioning on recovery of Ca^{2+} transients, contractile function and ATP levels in isolated ventricular myocytes (Gao *et al.*, 2007). Therefore, we examined the role of PKCε in phenylephrine preconditioning of isolated ventricular myocytes using the Tat-conjugated PKCε inhibitor peptide εV1-2.

To assess the role of PKCε in phenylephrine preconditioning, myocytes were incubated with the εV1-2 peptide (100 nM) for 10 minutes prior to phenylephrine preconditioning. This concentration of the εV1-2 peptide has been shown to be effective in inhibiting Kv channel modulation in our laboratory (Rainbow *et al.*, 2009). The εV1-2 inhibitor abolished protection by phenylephrine in isolated ventricular myocytes (Figure 4.5). The contractile recovery of ventricular myocytes was significantly decreased from 56.7 ± 3.7% (n = 4, 4, and 159, for experiments, hearts, and cells) in phenylephrine preconditioned myocytes to 27.3 ± 5.9% (n = 4, 4, and 136) by pretreatment with the εV1-2 inhibitor ($P < 0.05$), which was not significantly different to control experiments where 23.6 ± 3.7% (n = 4, 4 and 151) of control myocytes recovered contractile activity (P = NS). In addition, the presence of εV1-2 had no effect on the contractile recovery of control myocytes subjected to MI and re-energisation, which was 27.2 ± 3.5% (n = 4, 4, and 175; P = NS). These results suggest that PKCε is involved in phenylephrine preconditioning. However, PKCε inhibition had no effect in non-preconditioned

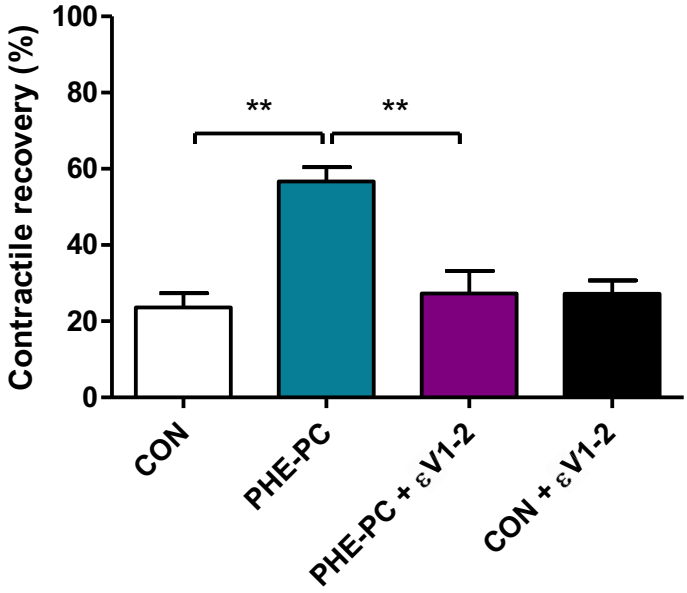

Figure 4.5. Inhibition of PKCε prevents phenylephrine preconditioning.

Percentage of myocytes contracting to field stimulation following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 4, 4, and 175 for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 4, 4, and 159). Phenylephrine preconditioned myocytes pretreated with εV1-2 inhibitor peptide (100 nM) for 10 minutes (PHE-PC + εV1-2; purple bar, n = 4, 4, and 136). Control myocytes pretreated with εV1-2 (CON + εV1-2; black bar, n = 4, 4, and 151). ** $P < 0.01$. n = 4 experiments.

myocytes, suggesting PKCε activation may not be tonically active in isolated ventricular myocytes.

4.3.5 Activation of PKCε is sufficient to protect ventricular myocytes and the protection is not additive with phenylephrine preconditioning

We have demonstrated that PKCε translocates to the membrane in response to phenylephrine treatment and that inhibition of PKCε abolishes phenylephrine preconditioning. We next investigated the effect of PKCε activation on the contractile recovery of myocytes exposed to MI and re-energisation, using the Tat-conjugated ψεRACK activator peptide. In addition, we sought to determine whether the effect of phenylephrine preconditioning was enhanced by PKCε activation by pretreating myocytes with the ψεRACK prior to preconditioning.

To test the effect of PKCε activation on the contractile recovery of ventricular myocytes, myocytes were pretreated with the ψεRACK peptide (100nM) for 10 minutes prior to phenylephrine preconditioning. This concentration of the ψεRACK peptide has previously been shown to activate PKCε and improve functional recovery in the isolated rat heart subject to ischaemia and reperfusion (Inagaki *et al.*, 2003b). The contractile recovery of phenylephrine preconditioned myocytes treated with the ψεRACK peptide was 62.2 ± 5.4% (n = 4, 4, and 146), which was not significantly different from phenylephrine preconditioned myocytes, of which 69.9 ± 2.8% (n = 4, 4, 184) recovered contractile activity following MI and re-energisation (P = NS; Figure 4.6). Treatment of control ventricular myocytes with the ψεRACK peptide, increased contractile recovery to 57.9 ± 5.1% (n = 4, 4, and 137), which was significantly higher than that of control myocytes (28.4 ± 2.6%; n = 4, 4, and 160; $P < 0.01$), but not different from either phenylephrine preconditioned myocytes or myocytes treated with ψεRACK alone (both P = NS). PKCε activation is therefore sufficient to protect ventricular myocytes from

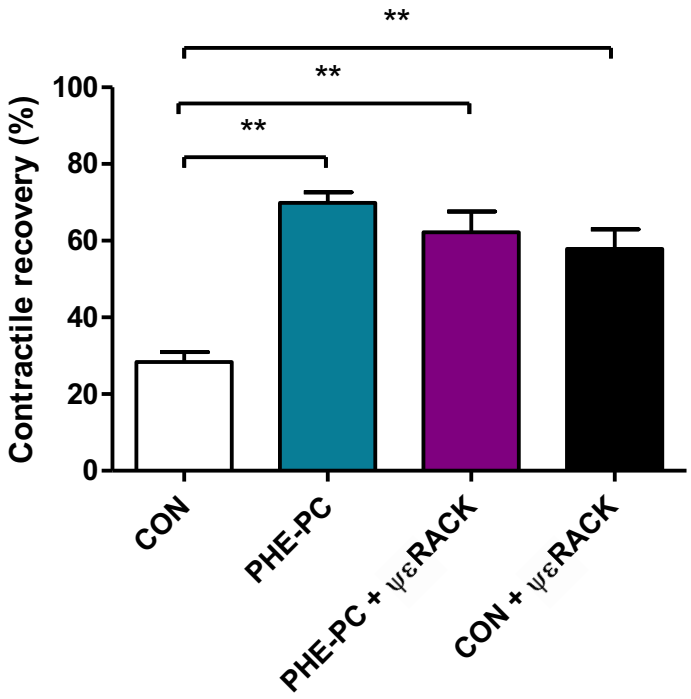

Figure 4.6. Activation of PKCε protects cells from metabolic inhibition re-energisation induced injury.

Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 4, 4, 160, for experiments, hearts, and cells). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 4, 4, and 184). phenylephrine preconditioned myocytes pretreated with ψε RACK activator peptide (100 nM) for 10 minutes (PHE-PC + ψεRACK; purple bar, n = 4, 4, and 146). Control myocytes pretreated with ψεRACK (CON + ψεRACK; black bar, n = 4, 4, and 137). ** $P < 0.01$.

the damaging effects of MI re-energisation injury and the effect is not enhanced by phenylephrine preconditioning, suggesting a common mechanism of protection involving PKCε.

4.3.6 Activation of PKCδ by phenylephrine

PKCδ is a DAG-dependent, Ca^{2+}-independent novel PKC isoform that has been observed to translocate to the membrane in response to phenylephrine preconditioning in isolated ventricular myocytes (Tsouka *et al.*, 2002) and isolated hearts (Mitchell *et al.*, 1995), though its role in preconditioning is still under debate. We set out to examine whether PKCδ was activated in response to the phenylephrine preconditioning stimulus in isolated ventricular myocytes.

To test if PKCδ translocates to the membrane in response to phenylephrine preconditioning, ventricular myocytes were treated with phenylephrine (5 μM) for 10 minutes or with phenylephrine (5 μM) for 10 minutes followed by 5 minutes of washout with normal Tyrode. Control myocytes were incubated in normal Tyrode for 15 minutes and the phorbol ester PMA (1 μM) for 5 minutes was used as a positive control (protocol is described in Figure 4.1B). PKCδ was translocated to the membrane following 10 minutes of phenylephrine treatment, as shown in Figure 4.7. The percentage of PKCδ in the membrane was increased from 48.0 ± 7.7% to 88.9 ± 6.1% in response to 10 minutes of phenylephrine treatment (n = 5 experiments; $P < 0.01$). After five minutes washout of phenylephrine, PKCδ remained translocated to the membrane, with 91.0 ± 7.9% of the PKCδ in the membrane fraction (n = 5 experiments, $P < 0.01$). The observation that PKCδ translocates to the membrane in response to phenylephrine treatment indicates that PKCδ may be involved in the protection of ventricular myocytes by phenylephrine preconditioning.

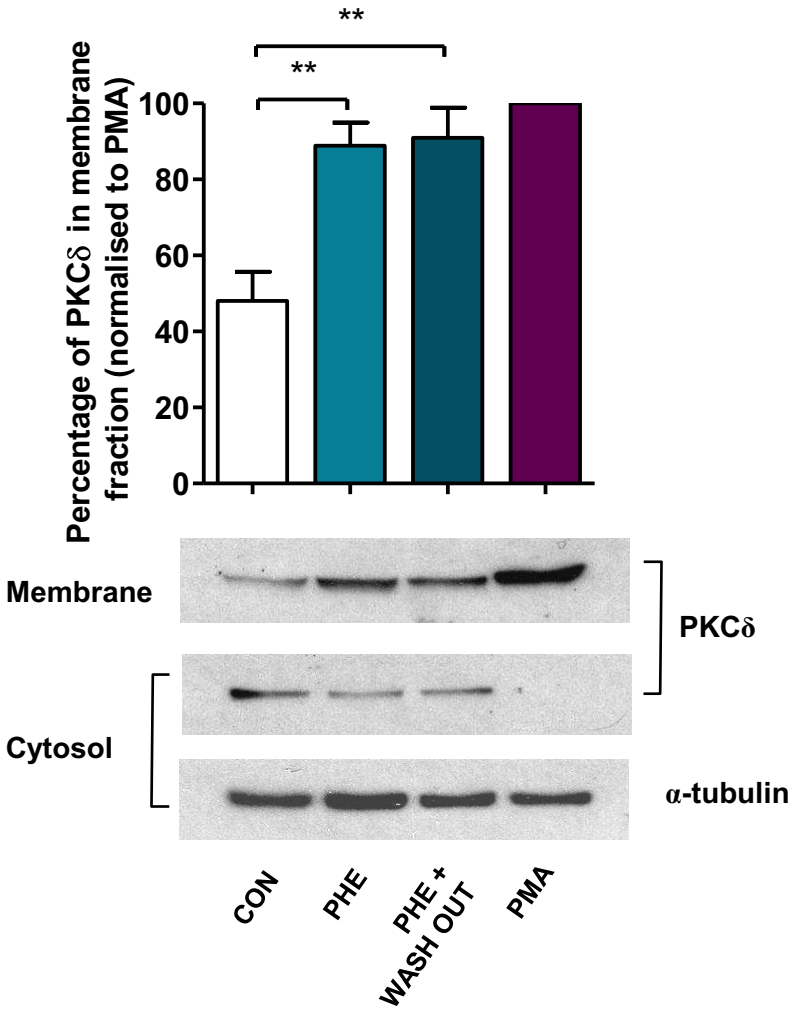

Figure 4.7. Phenylephrine preconditioning activates PKCδ.

Western blot analysis showing the amount of PKCδ in the cytosolic and membrane fractions of myocytes treated with phenylephrine or PMA. Anti-PKCδ rabbit antibodies (Santa Cruz Biotechnology) were diluted 1 : 500 and anti-α-tubulin mouse antibodies (Sigma-Aldrich) were diluted 1 : 2,000. Bar chart shows the percentage of PKCδ in the membrane fraction in response to phenylephrine preconditioning. Myocytes were treated with normal Tyrode for 15 minutes (CON; open bar), 5 μM phenylephrine for 10 minutes (PHE; blue bar), phenylephrine followed by 5 minutes wash out with normal Tyrode (PHE + WASH OUT; dark blue bar) or 5 minutes of PMA (1 μM; purple bar, n = 4, and 4, for experiments, and hearts). ** $P < 0.01$.

4.3.7 Inhibition of PKCδ increases protection of myocytes exposed to metabolic inhibition and re-energisation

In the previous experiment we demonstrated that PKCδ is activated during the phenylephrine preconditioning protocol. However, the role of PKCδ in preconditioning is controversial as it has been implicated in mediating both protection (Meldrum *et al.*, 1997a, Zhao *et al.*, 1998) and reperfusion injury (Inagaki *et al.*, 2003b, Chen *et al.*, 2001). We therefore investigated the role of PKCδ in phenylephrine preconditioning of isolated ventricular myocytes using the Tat-conjugated PKCδ inhibitor peptide, δV1-1.

To examine the role of PKCδ in phenylephrine preconditioning, isolated ventricular myocytes were pretreated with the δV1-1 Tat-conjugated inhibitor peptide (100nM) for 10 minutes prior to preconditioning. We chose this concentration of δV1-1 because it has been previously shown to decrease PKCδ-mediated reperfusion injury within the heart (Chen *et al.*, 2001, Inagaki *et al.*, 2003a). As shown in Figure 4.8, treatment of myocytes with the δV1-1 inhibitor peptide alone resulted in an increase in the contractile recovery when compared to control myocytes, from 31.0 ± 1.9% (n = 5, 5, and 132, for experiments, hearts, and cells respectively) in control myocytes to 51.5 ± 4.6% (n = 4, 4, and 79) in control myocytes treated with δV1-1 ($P < 0.01$). In response to phenylephrine preconditioning following treatment with δV1-1, 66.3 ± 2.3% (n = 4, 4, and 98) of myocytes recovered contractile function, which was not significantly different from the contractile recovery of phenylephrine preconditioned myocytes (57.2 ± 1.3%; n = 5, 5, and 140; P = NS). However, this level of protection was significantly higher than in the presence of δV1-1 alone ($P < 0.01$). This suggests that PKCδ may be involved in MI re-energisation injury.

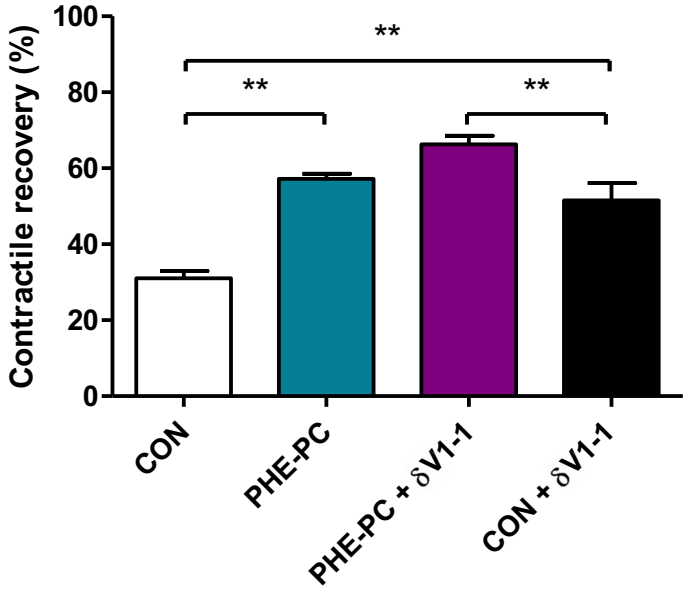

Figure 4.8. Inhibition of PKCδ protects cardiac myocytes from metabolic inhibition re-energisation injury.

Percentage contractile recovery after 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON, open bar, n = 5, 5, and 132, for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 5, 5, and 140). Phenylephrine preconditioned myocytes pretreated with the δV1-1 inhibitor peptide (100 nM) for 10 minutes (PHE-PC + δV1-1; purple bar, n = 4, 4, and 98). Control myocytes pretreated with δV1-1 (CON + δV1-1; black bar, n = 4, 4, and 79) ** $P < 0.01$.

4.3.8 Activation of PKCδ prevents preconditioning by phenylephrine

Since PKCδ was activated in response to phenylephrine preconditioning and inhibition of PKCδ increased recovery of contractile activity, we suspected that activation of PKCδ would decrease survival in both phenylephrine preconditioned myocytes and those treated with ψδRACK alone. We therefore examined the effect of activating PKCδ on the contractile recovery of isolated ventricular myocytes using the Tat-conjugated PKCδ activator peptide ψδRACK.

To test the effect of PKCδ activation on the contractile recovery of phenylephrine preconditioned ventricular myocytes exposed to MI and re-energisation, myocytes were pretreated with the ψδRACK peptide (100 nM) for 10 minutes prior to preconditioning. This concentration of ψδRACK was chosen as it has been shown to increase PKCδ-mediated reperfusion injury (Chen *et al.*, 2001). As demonstrated in Figure 4.9, activation of PKCδ inhibited preconditioning by phenylephrine. The contractile recovery was reduced from 62.0 ± 3.7% (n = 3, 3, and 97) in phenylephrine preconditioned myocytes to 49.7 ± 3.1% (n = 3, 3, and 84) in phenylephrine preconditioned myocytes pretreated with ψδRACK ($P < 0.05$). However, pretreatment with ψδRACK alone increased contractile recovery of ventricular myocytes, from 30.4 ± 0.2% (n = 3, 3, and 112) in control myocytes to 43.9 ± 0.6% (n = 3, 3, and 109) in control myocytes treated with ψδRACK ($P < 0.05$). These results suggest that although activation of PKCδ does partially block phenylephrine preconditioning, there may also be a protective role of PKCδ in MI re-energisation injury.

4.4 Summary

In this chapter, the role of specific PKC isoforms in phenylephrine preconditioning of isolated ventricular myocytes was investigated using isoform specific peptides (see

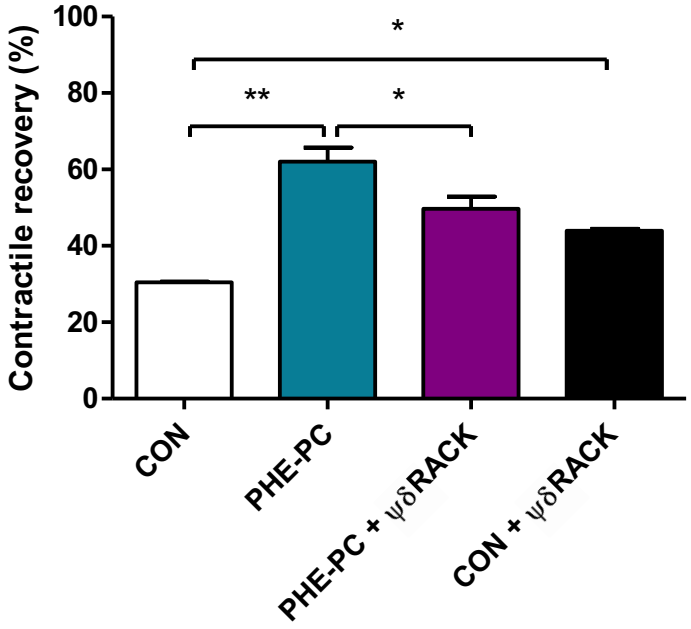

Figure 4.9. Effect of Activating PKCδ on phenylephrine preconditioning of isolated cardiac myocytes.

Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 3, 3, and 112, for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 3, 3, and 97). Phenylephrine preconditioned myocytes pretreated with ψδRACK activator peptide (100 nM) for 10 minutes (PHE-PC + ψδRACK; purple bar, n = 3, 3, and 84). Control myocytes pretreated with ψδRACK (CON + ψδRACK; black bar, n = 3, 3, and 109). * $P < 0.05$, ** $P < 0.01$.

Table 4.1). These experiments demonstrated that inhibition of the conventional isoforms of PKC (α, β and γ) had no effect on phenylephrine preconditioning of isolated ventricular myocytes, indicating conventional PKCs do not play a role in phenylephrine preconditioning. Translocation of PKCε to the membrane fraction was observed in response to the phenylephrine preconditioning protocol. PKCε inhibition was found to abolish the protective effect of phenylephrine preconditioning on contractile recovery of ventricular myocytes, while the PKCε activator peptide protected myocytes to a similar degree to phenylephrine preconditioning. PKCδ also translocated to the membrane in response to acute phenylephrine treatment. Paradoxically, while PKCδ activation partially inhibited phenylephrine preconditioning, either activation or inhibition of PKCδ increased the recovery of non-preconditioned myocytes. Therefore, it appears that phenylephrine preconditioning activates both PKCε and δ which have differing effects on the contractile recovery of ventricular myocytes. In the next chapter we will examine components of the phenylephrine preconditioning signalling pathway downstream of PKC.

5 The role of MAPK and AMPK in phenylephrine preconditioning

5.1 Introduction

As we have shown in chapter 4, PKCε, and possibly δ, are involved in the signal transduction of phenylephrine preconditioning (Section 4.3.3-8). We next examined potential downstream components of the signalling pathway(s). Both AMPK and proteins of the MAPK family can be activated in response to acute phenylephrine treatment (Lazou *et al.*, 1998, Xu *et al.*, 2007). Additionally, these kinases may be involved in the protection afforded by IPC (Sukhodub *et al.*, 2007, Fryer *et al.*, 2001b, Fryer *et al.*, 2001a). We therefore set out to investigate the role of AMPK and the MAPK in phenylephrine preconditioning of isolated ventricular myocytes.

5.2 MAPK family

The MAPK superfamily is divided into the ERKs, JNKs and p38 MAPKs (p38). There is conflicting evidence for involvement of MAPK in preconditioning, with differences in the timecourse of activation and the effect of inhibition of MAPK in various studies, for a review see Armstrong (2004). ERK has also been implicated in protection from reperfusion injury as part of the reperfusion injury salvage kinase (RISK) pathway (Hausenloy *et al.*, 2005b).

Lazou *et al.* (1998) observed a pronounced (12-fold) activation of p38 along with a smaller activation of ERK and JNK within 5 minutes of perfusion of 100 μM phenylephrine in an isolated perfused rat heart model. This suggests that activation of MAPK isoforms in response to the phenylephrine preconditioning stimulus could be involved in protection of cardiac myocytes. p38 appears to be involved in reperfusion

injury and cardioprotection, possibly dependent on the p38 isoform activated. Beneficial and damaging effects of p38 are reviewed in Bassi *et al.* (2008). It has previously been shown that p38 activation is necessary for activation of phenylephrine preconditioning (Loubani and Galiñanes, 2002).

5.3 AMPK

AMPK is activated by disturbances in cellular energy homeostasis and is able to regulate energy metabolism and utilisation at a cellular and system-wide level (Hardie, 2008). Binding of AMP to AMPK at two cooperative Bateman domains, results in allosteric activation of the kinase, as well as promoting phosphorylation at Thr172 within the activation loop of the catalytic domain of the α-subunit. ATP is also able to bind antagonistically to the Bateman domains, therefore making the kinase highly sensitive to changes in the AMP:ATP ratio of the cell (Towler and Hardie, 2007). It has recently been recognised that AMPK activation is important for IPC (Sukhodub *et al.*, 2007, Nishino *et al.*, 2004, Khaliulin *et al.*, 2007). It has also been observed that AMPK can be activated by phenylephrine treatment in H9c2 cells and the rat heart (Xu *et al.*, 2007).

5.4 Results

5.4.1 Inhibition of ERK increases the contractile recovery of cardiac myocytes exposed to metabolic inhibition and re-energisation.

Previous studies into the role of ERK in preconditioning have had mixed results. Several studies suggested a protective role for ERK in IPC (Fryer *et al.*, 2001b, Solenkova *et al.*, 2006). In contrast, others demonstrated that ERK inhibition had no effect on infarct size in rat hearts subject to IPC (Mocanu *et al.*, 2002), and no difference in ERK phosphorylation was observed following IPC (Takeishi *et al.*, 2001).

The role of ERK in phenylephrine preconditioning has not been examined, therefore we set out to determine the effect of ERK inhibition on the protection afforded by phenylephrine preconditioning in isolated ventricular myocytes subject to MI and re-energisation with normal Tyrode.

To assess the role of ERK in phenylephrine preconditioning of ventricular myocytes, ERK activation was prevented by pretreatment of ventricular myocytes with PD98059 (50 μM) for 10 minutes prior to preconditioning with phenylephrine (protocols are described in Figure 5.1A). PD98059 prevents activation of ERK by inhibiting the upstream kinase MEK. As shown in Figure 5.2, pretreatment of myocytes with PD98059 did not significantly affect the recovery of contractile activity in phenylephrine preconditioned myocytes; 64.08 ± 2.3% (n = 6, 6, and 143, for experiments, hearts, and cells respectively) of phenylephrine preconditioned myocytes and 62.8 ± 5.2% (n = 4, 4, and 88) of phenylephrine preconditioned myocytes pretreated with PD98059 recovered contractile activity following MI and re-energisation (P = NS). The recovery of contractile activity of control myocytes was 27.9 ± 2.0% (n = 6, 6, and 148), which was increased to 59.1 ± 2.8%% (n = 4, 4, and 95) by pretreatment of control myocytes with PD98059 ($P < 0.01$). These results suggest that ERK does not have a role in phenylephrine preconditioning but may play a damaging role during MI re-energisation in non-preconditioned myocytes.

5.4.2 Inhibition of JNK increases the contractile recovery of cardiac myocytes exposed to MI and re-energisation

Although translocation of JNK is observed in response to IPC (Ping *et al.*, 1999b, Sato *et al.*, 2000), there is little direct evidence for a role of JNK in preconditioning. Either knock out of JNK or overexpression of an upstream kinase MKK7 (and consequent increase in JNK activation) resulted in a decrease in infarct size in the hearts of

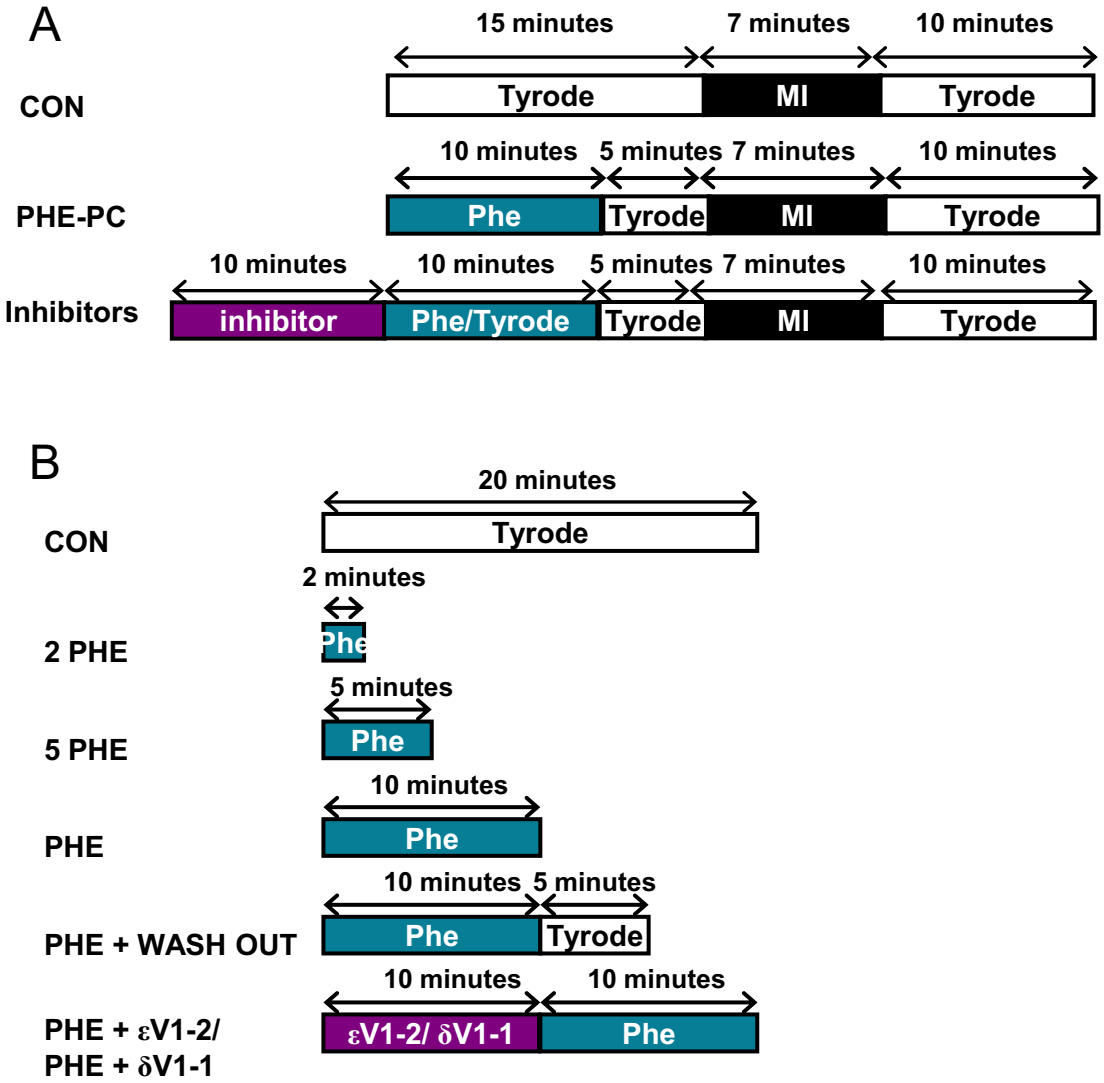

Figure 5.1. Protocols used for phenylephrine preconditioning, metabolic inhibition re-energisation, inhibitor treatments and western blot experiments

A. Ventricular myocytes were preconditioned with 10 minutes of phenylephrine (Phe) followed by 5 minutes of washout with normal Tyrode. Control myocytes were superfused with normal Tyrode for 15 minutes. Myocytes were then subject to 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control (CON). Phe preconditioned (PHE-PC). Inhibitors-MEK inhibitor PD98059, JNK inhibitor SP600125, p38 inhibitor SB202190, AMPK inhibitor compound C, CaMKK inhibitor STO-609.

B. Protocol used to investigate phosphorylation of p38 MAPK and AMPK. Control, 20 minutes of normal Tyrode (CON). 2 minutes of Phe (2 PHE). 5 minutes of Phe (5 PHE). 10 minutes of Phe (PHE). 10 minutes of Phe followed by 5 minutes of washout with normal Tyrode (PHE + WASH OUT). 10 minutes of PKCε inhibitor εV1-2 followed by 10 minutes of Phe (PHE + εV1-2). 10 minutes of PKCδ inhibitor δV1-1 followed by 10 minutes of Phe (PHE + δV1-1).

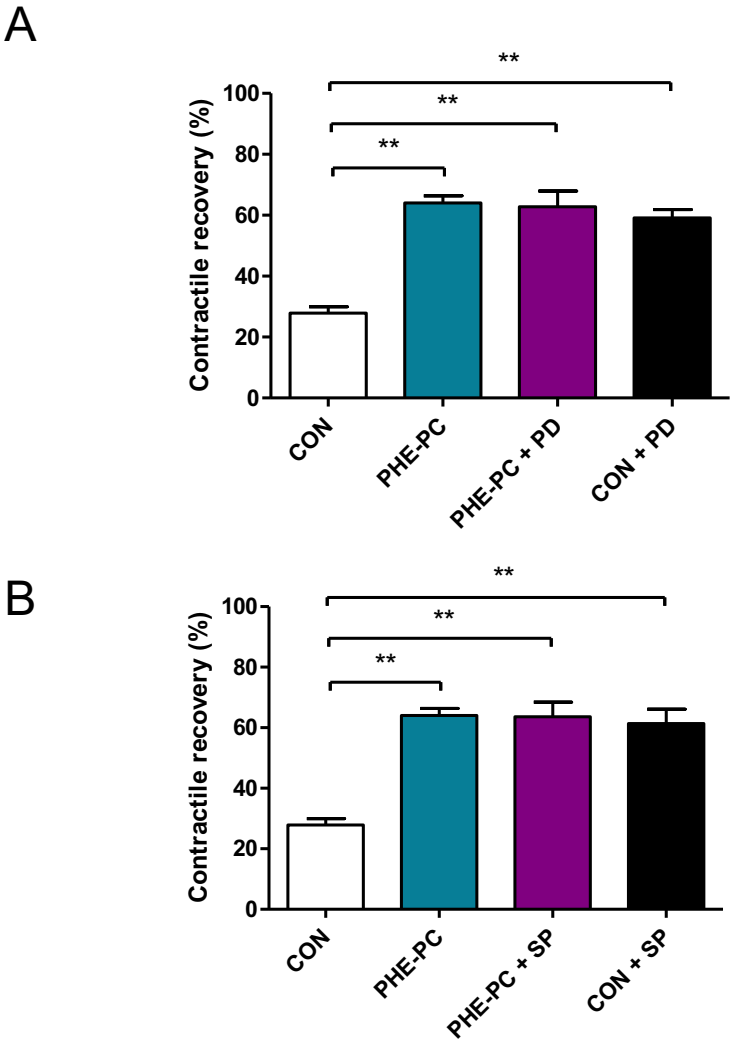

Figure 5.2. Effect of inhibition of ERK and JNK on contractile recovery of phenylephrine pretreated myocytes.

A. Percentage contractile recovery after 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 6, 6, and 148, for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 6, 6, and 143). Phenylephrine preconditioned myocytes pretreated with the MEK inhibitor PD98059 (50 μM) for 10 minutes (PHE-PC + PD; purple bar, n = 4, 4, and 88). Control myocytes pretreated with PD98059 (CON + PD; black bar, n = 4, 4, and 95). ** $P < 0.01$.

B. Percentage contractile recovery following 7 minutes of MI and 10 minutes of re-energisation. CON (open bar, n = 6, 6, and 148, for experiments, hearts and cells respectively). PHE-PC (blue bar, n = 6, 6, and 143). Phenylephrine preconditioned myocytes pretreated with the JNK inhibitor SP600125 (25 μM) for 10 minutes (PHE-PC + SP; purple bar, n= 4, 4, and 101). Control myocytes pretreated with SP 600125 (CON + SP; black bar, n = 4, 4, and 107). ** $P < 0.01$.

transgenic mice (Kaiser *et al.*, 2005), suggesting that JNK may play both beneficial and damaging roles in ischaemia-reperfusion. The role of JNK in phenylephrine preconditioning has not been examined; although a modest 2-fold activation of JNK was observed by Lazou *et al.* (1998) in response to acute phenylephrine treatment. We therefore examined the role of JNK in phenylephrine preconditioning of isolated ventricular myocyte subject to MI and re-energisation with normal Tyrode.

The role of JNK in phenylephrine preconditioning was assessed by pretreatment of ventricular myocytes with the JNK inhibitor SP600125 (25 μM) for 10 minutes prior to phenylephrine preconditioning. As shown in Figure 5.2, the contractile recovery of phenylephrine preconditioned myocytes pretreated with SP600125 was 63.7 ± 4.8% (n = 4, 4, and 101) which was not different from the contractile recovery of phenylephrine preconditioned myocytes (61.8 ± 2.9%; n = 6, 6, and 143; P = NS) but was significantly higher than that of control cells, which was 30.7 ± 1.5% (n = 6, 6, and 148). The contractile recovery of control myocytes pretreated with SP600125 was significantly higher than control cells (61.4 ± 4.7%; n = 4, 4, and 107; $P < 0.01$), and was not different from that seen in phenylephrine preconditioned myocytes or preconditioned myocytes pretreated with SP600125 (P = NS). These data suggest JNK is not a mediator of the protective effect of phenylephrine preconditioning, and that JNK may play a damaging role during MI re-energisation in non-preconditioned myocytes.

5.4.3 Inhibition of p38 prevents preconditioning by phenylephrine

While the role of p38 in IPC remains under debate, it has been previously demonstrated that administration of the p38 antagonist SB203580 abolished phenylephrine preconditioning of human atrial appendages (Loubani and Galiñanes, 2002). Therefore, we examined the effect of inhibition of p38 on phenylephrine preconditioning of isolated ventricular myocytes, subject to MI and re-energisation.

To assess the role of p38 in phenylephrine preconditioning, isolated ventricular myocytes were pretreated with the p38 inhibitor SB202190 (10 μM) for 10 minutes prior to phenylephrine preconditioning. Pretreatment of myocytes with SB202190 inhibited the protective effect of phenylephrine preconditioning on contractile recovery (Figure 5.3); 58.6 ± 2.8% (n = 5, 5, and 277) of phenylephrine preconditioned myocytes recovered contractile activity, and this was reduced to 21.7 ± 10.1% (n = 4, 4, and 110) in phenylephrine preconditioned myocytes pretreated with SB202190 ($P < 0.05$), which was not significantly different to control myocytes, of which 25.3 ± 3.4% recovered contractile activity (n = 5, 5, 163; P = NS). Pretreatment of control myocytes with SB202190 did not affect the contractile recovery compared with control myocytes, 27.5 ± 7.6% (n = 3, 3, and 76) control myocytes pretreated with SB202190 recovered contractile function (P = NS). This suggests that p38 activation is necessary for preconditioning by phenylephrine.

5.4.4 p38 is not activated by phenylephrine preconditioning during the pre-ischaemic period

Activation of p38 is catalysed by dual phosphorylation at Thr180 and Tyr182 by upstream MAPKKs. Acute phenylephrine treatment (100 μM) has previously been observed to activate p38 within 5 minutes in isolated perfused hearts using both in-gel kinase assays and measurement of dual phosphorylation levels (Lazou *et al.*, 1998). Therefore, we examined the phosphorylation of p38 in isolated ventricular myocytes in response to our phenylephrine preconditioning protocol.

To assess the activation of p38 by phenylephrine preconditioning we carried out western blotting and densitometry to determine the ratio of phosphorylated p38 to total protein (using total AMPKα as a measure of total protein due to poor binding of our

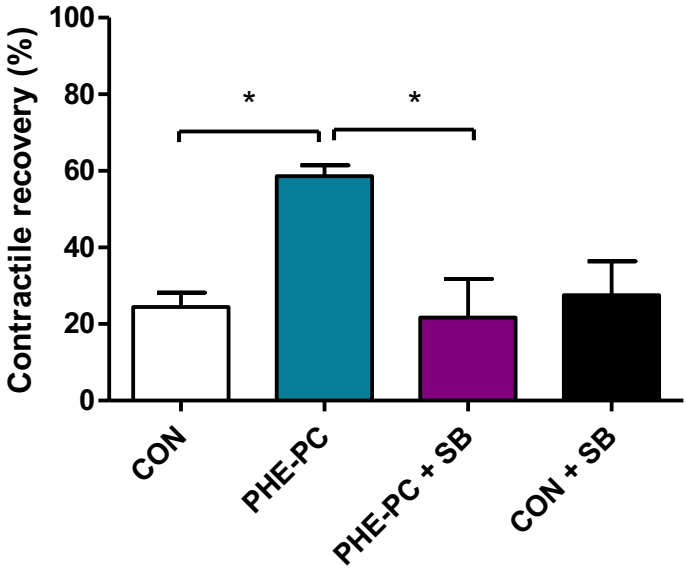

Figure 5.3. Inhibition of p38 inhibits preconditioning by phenylephrine.
Percentage recovery of contractile function following 7 minutes of MI and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 5, 5, and 163 for experiments, hearts, and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 5, 5, and 277). Phenylephrine preconditioned myocytes pretreated with the p38 inhibitor SB202190 (10 μM) for 10 minutes (PHE-PC + SB; purple bar, n = 4, 4, and 110). Control myocytes pretreated with SB202190 (CON + SB; black bar, n = 3, 3, and 76). * $P < 0.05$.

total p38 antibody). The time course of p38 phosphorylation is shown in Figure 5.4A and B. The phosphorylation of p38 was not significantly affected by phenylephrine treatment at any time point examined, and the phosphorylation of p38 was reduced to 0.4 ± 0.2 fold after 10 minutes of phenylephrine treatment (n = 3 experiments; P = NS). Similarly, inhibition of PKCε and δ had no effect on the phosphorylation of p38 after 10 minutes of phenylephrine treatment (P = NS; Figure 5.4A and C). These results suggest that p38 is not activated during the phenylephrine preconditioning protocol, but phenylephrine preconditioning was abolished by p38 inhibition suggesting that p38 is activated by phenylephrine preconditioning. We did not examine p38 activation during MI and re-energisation due to protein loss, therefore cannot rule out the possibility that activation of p38 occurs during this period.

5.4.5 Inhibition of AMPK prevents preconditioning by phenylephrine

AMPK is able to modulate metabolic pathways to reduce cellular energy expenditure in response to increases in the AMP:ATP ratio. Due to its role in conservation of cellular energy reserves, AMPK was identified as a potential target for the effects of preconditioning. Three recent studies have demonstrated a role for AMPK in IPC (Sukhodub *et al.*, 2007, Nishino *et al.*, 2004, Khaliulin *et al.*, 2007). Therefore we examined the role of AMPK in phenylephrine preconditioning of isolated ventricular myocytes subjected to MI and re-energisation with normal Tyrode.

To investigate the role of AMPK in phenylephrine preconditioning, ventricular myocytes were incubated with the AMPK inhibitor compound C (10 μM) for 10 minutes prior to preconditioning. Inhibition of AMPK caused a decrease in the contractile recovery of phenylephrine preconditioned myocytes, as shown in Figure 5.5. The contractile recovery of phenylephrine preconditioned myocytes was 58.4 ± 2.9% (n = 5, 5, and 277, for experiments, hearts, and cells respectively) and this was reduced to

A

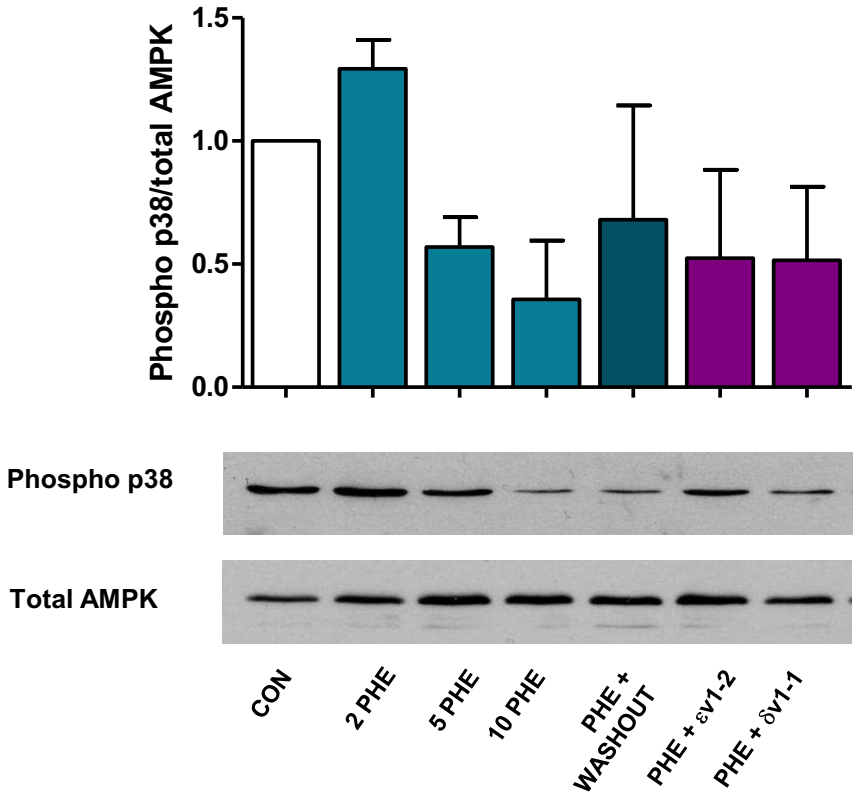

Figure 5.4. p38 phosphorylation is not changed after phenylephrine preconditioning

Western blot analysis of p38 phosphorylation at Thr180/Tyr182 following phenylephrine treatment. Anti-phospho p38 rabbit antibodies and anti-total AMPKα rabbit antibodies (CST) were diluted 1 : 500. Bar chart shows quantitation of p38 phosphorylation in response to phenylephrine treatment (ratio of phospho p38 to total AMPK). Myocytes were treated with normal Tyrode for 15 minutes (CON, open bar), 5 μM phenylephrine for 2, 5 or 10 minutes (2 PHE, 5 PHE, and PHE; blue bars), phenylephrine for 10 minutes followed by washout with normal Tyrode for 5 minutes (PHE + WASH OUT; dark blue bar), or pretreated with 100 nM εV1-2 (PHE + εV1-2) or 100 nM δV1-1 (PHE + δV1-1) prior to phenylephrine treatment (purple bars; n = 3, and 3 for experiments, and hearts).

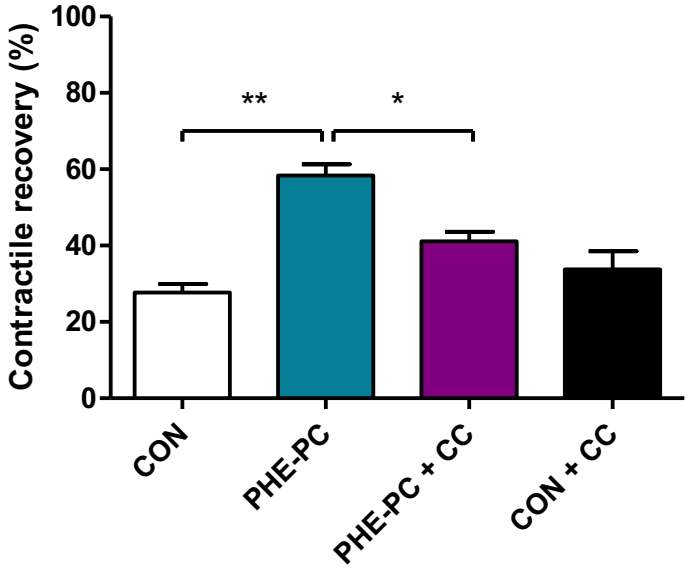

Figure 5.5. Inhibition of AMPK with Compound C (CC) inhibits phenylephrine preconditioning.

Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 5, 5, and 163 for experiments, hearts and cells). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 5, 5, and 277). Phenylephrine preconditioned myocytes pretreated with the AMPK inhibitor compound C (10 μM) for 10 minutes (PHE-PC + CC; purple bar, n = 4, 4, and 128). Control myocytes pretreated with compound C (CON + CC; black bar, n = 4, 4, and 155). *$P < 0.05$, ** $P < 0.01$.

41.1 ± 2.5% (n = 4, 4, and 128) in phenylephrine preconditioned myocytes pretreated with compound C ($P < 0.05$). The contractile recovery of phenylephrine preconditioned myocytes pretreated with compound C, and control myocytes pretreated with compound C (33.8 ± 4.8%; n = 4, 4, and 155) were not significantly different from the recovery of contractile activity in control myocytes of 27.7 ± 2.6% (n = 5, 5, and 163; both P = NS). These results suggest that AMPK activation is required for phenylephrine preconditioning of ventricular myocytes but is not involved in recovery from MI re-energisation injury.

5.4.6 Activation of AMPK by phenylephrine

As well as allosteric activation by AMP, AMPK can be activated by phosphorylation of the α-subunit at Thr172. Phosphorylation of AMPKα at Thr172 is catalysed by the upstream kinases LKB1 or CaMKKβ and to a lesser extent CaMKKα (Towler and Hardie, 2007). Phosphorylation of this residue is facilitated by AMP binding and can activate the kinase 100-fold compared to the 10-fold activation induced by AMP binding, so phosphorylation of Thr172 can be used as an indicator of AMPK activity.

To assess the activation of AMPK by phenylephrine preconditioning, we carried out western blotting and densitometry to determine the ratio of AMPKα phosphorylated at Thr172 to total AMPKα (phospho AMPKα/total AMPKα). The time course of phosphorylation of AMPKα at Thr172 in response to acute phenylephrine treatment is shown in Figure 5.6A and B. The phosphorylation of AMPK was unaffected by acute phenylephrine treatment for 2 or 5 minutes. A 10 minute incubation of ventricular myocytes with 5 μM phenylephrine resulted in a significant (2.3 ± 0.3 fold) increase in the phosphorylation of AMPK (n = 4 experiments; $P < 0.05$). Following 10 minutes of phenylephrine and 5 minutes washout of phenylephrine with normal Tyrode this phosphorylation had decreased to 0.5 ± 0.2 fold (n = 4 experiments; $P < 0.01$ vs. 10

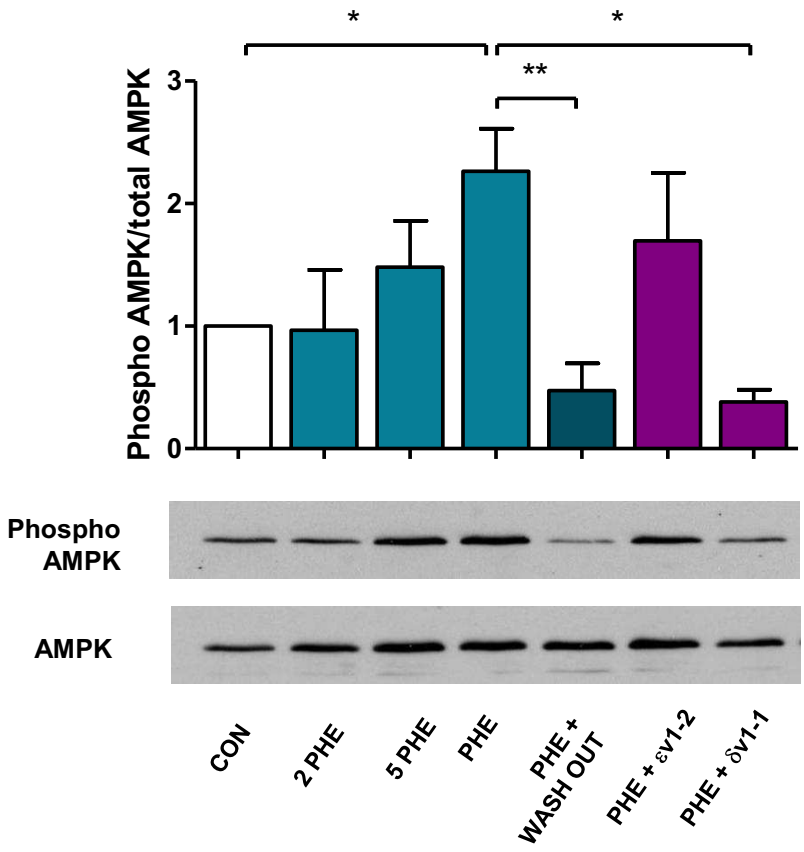

Figure 5.6. AMPK phosphorylation after acute phenylephrine treatment is abolished by inhibition of PKCδ.

Western blot analysis showing AMPKα phosphorylation at Thr172 following phenylephrine treatment. Anti-phospho AMPKα Thr172 rabbit antibodies and anti-total AMPKα rabbit antibodies (CST) were diluted 1 : 500. Bar chart shows quantitation of AMPKα phosphorylation in response to phenylephrine treatment (ratio of phospho AMPK to total AMPK). Myocytes were treated with normal Tyrode for 15 minutes (CON; open bar), phenylephrine for 2, 5 or 10 minutes (2 PHE, 5 PHE, and PHE; blue bars), phenylephrine for 10 minutes followed by 5 minutes of washout with normal Tyrode (PHE + WASH OUT; dark blue bar), or pretreated with 100 nM εV1-2 (PHE + εV1-2) or 100 nM δV1-1 (PHE + δV1-1) prior to phenylephrine treatment (purple bars, n = 4, and 4 for experiments, and hearts). * $P < 0.05$, ** $P < 0.01$.

minutes phenylephrine; P = NS vs. control). These results indicate that AMPK is transiently activated by the phenylephrine preconditioning protocol.

5.4.7 PKCδ, is involved in activation of AMPK by phenylephrine preconditioning

Nishino *et al.* (2004) observed an increase in AMPK activity after an IPC stimulus that was prevented by inhibition of PKC with chelerythrine. It therefore seemed likely that PKC may be upstream of AMPK in the signalling pathway responsible for IPC. We investigated whether PKCε and PKCδ were upstream of AMPK in the signalling pathway of phenylephrine preconditioning.

To assess the role of PKCε and δ in activation of AMPK by phenylephrine preconditioning, ventricular myocytes were pretreated with the inhibitors εV1-2 (100 nM) and δV1-1 (100 nM) for 10 minutes prior to a 10 minute treatment with phenylephrine (5 μM). Pretreatment with εV1-2 reduced the phosphorylation from 2.3 ± 0.3 fold in phenylephrine treated myocytes to 1.7 ± 0.6 fold in myocytes treated with εV1-2 and phenylephrine, but this did not reach significance (n = 4 experiments; P = NS; Figure 5.6A and C). It should be noted that in one experiment, the phosphorylation of AMPK following εV1-2 and phenylephrine treatment was only 0.01 fold, however this did not reach significance using Grubb's outlier test therefore the result was included in the analysis. Inhibition of PKCδ using δV1-1 completely abolished phenylephrine-dependent AMPK phosphorylation (0.4 ± 0.1 fold; n = 4 experiments; P < 0.05 vs. phenylephrine; P = NS vs. control). These results suggest that PKCδ is upstream of AMPK in the signalling pathway responsible for phenylephrine preconditioning however, it is not known whether PKCδ directly modulates AMPK.

5.4.8 CaMKK activation is required for phenylephrine preconditioning

CaMKK is a Ca^{2+}/calmodulin-dependent protein kinase that can modulate AMPK activity by phosphorylation of Thr172 on the AMPK α-subunit (Towler and Hardie, 2007). Phenylephrine acts via G_q-coupled α_1-adrenoceptors which can increase intracellular Ca^{2+} levels through activation of IP_3R (Zhong and Minneman, 1999); therefore we suspected that CaMKK may be responsible for the increased phosphorylation of AMPKα in response to phenylephrine treatment.

To examine the role of CaMKK in phenylephrine preconditioning, ventricular myocytes were pretreated with the CaMKK inhibitor STO-609 (25 μM) for 10 minutes prior to phenylephrine preconditioning. As shown in Figure 5.7, inhibition of CaMKK caused a partial inhibition of phenylephrine preconditioning, reducing the contractile recovery of myocytes from 59.7 ± 3.5% (n = 4, 4, and 133, for experiments, hearts, and cells respectively) in phenylephrine preconditioned myocytes to 43.2 ± 1.5% (n = 4, 4, and 169) in phenylephrine preconditioned myocytes pretreated with STO-609 ($P < 0.05$). Treatment of control myocytes with STO-609 caused a small but significant increase in recovery of contractile activity, from 31.1 ± 0.7% (n = 4, 4, and 148) in control myocytes to 44.9 ± 5.2% (n = 4, 4, and 143) in control myocytes treated with STO-609 ($P < 0.05$). These results suggest that CaMKK may be the relevant AMPK kinase in phenylephrine preconditioning; however, it appears that CaMKK activation may also contribute to ischaemia-reperfusion injury.

5.5 Summary

In this chapter we examined the role of MAPKs and AMPK in phenylephrine preconditioning of isolated ventricular myocytes. We used inhibitors of AMPK and MAPKs to examine the role of these kinases in phenylephrine preconditioning. Our

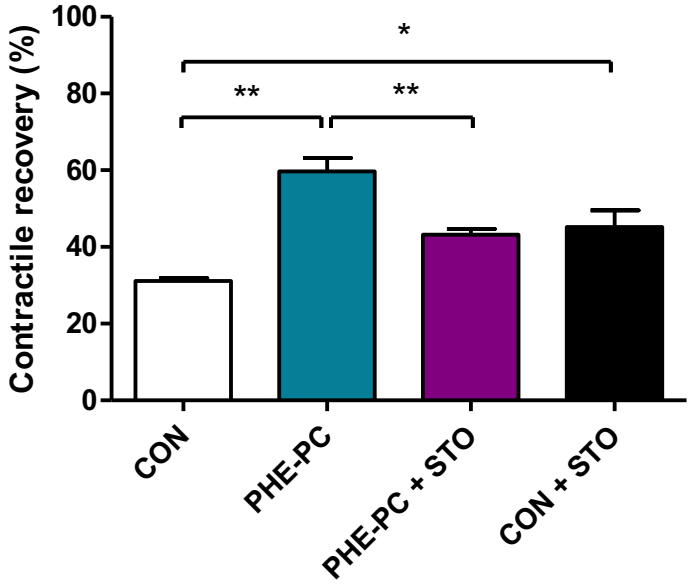

Figure 5.7. Inhibition of CaMKK decreases phenylephrine preconditioning.

Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 4, 4, and 148, for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 4, 4, and 133). phenylephrine preconditioned myocytes pretreated with the CaMKK inhibitor STO-609 (25 μM) for 10 minutes (PHE-PC + STO; purple bar, n = 4, 4, and 169). Control myocytes pretreated with STO-609 (CON + STO; black bar, n = 4, 4, and 143). *P < 0.05, ** P < 0.01.

study found no effect of inhibiting MEK/ERK or JNK on the contractile recovery of phenylephrine preconditioned ventricular myocytes suggesting that these enzymes do not mediate the protective effect of phenylephrine preconditioning. In contrast, inhibition of p38 was able to completely abolish the increase in contractile recovery induced by phenylephrine preconditioning, therefore it appears that p38 is involved in the signal transduction of phenylephrine preconditioning. However, we did not observe activation of p38 during the pre-ischaemic period in our study. Inhibition of AMPK or CaMKK also prevented phenylephrine preconditioning and AMPK activation required PKCδ. It therefore seems likely that PKCδ and CaMKK act upstream of AMPK in the signal transduction of phenylephrine preconditioning and p38 may also be involved in this pathway. In the next chapter we will examine the role of the K_{ATP} channel in phenylephrine preconditioning, going on to demonstrate a role for PKCδ, AMPK and p38 in the modulation of the *sarc*K_{ATP} channel by phenylephrine preconditioning.

6 The role of K_{ATP} channels in phenylephrine preconditioning

6.1 Introduction

In chapter 3 it was demonstrated that phenylephrine preconditioning could be completely abolished by inhibition of K_{ATP} channels with the non-selective K_{ATP} channel inhibitor glibenclamide (Section 3.2.4). However, it has been reported that glibenclamide is able to inhibit both the cardiac *sarc*K_{ATP} channel and the *mito*K_{ATP} channel. In order to clarify the roles of *sarc*K_{ATP} and *mito*K_{ATP} channels in phenylephrine preconditioning, we have employed HMR1883 and 5-hydroxydecanoate, specific inhibitors of the *sarc*- and *mito*K_{ATP} channels.

6.2 *Sarc*K_{ATP} channels

SarcK_{ATP} channels were first described by Noma in 1983, as a K^+ channel that was inhibited by ATP levels above 1mM (Noma, 1983). A decrease in cellular ATP levels results in activation of the *sarc*K_{ATP} channel leading to an increase in K^+ conductance and hyperpolarisation of the cell membrane. It has therefore been proposed that the protective effect of *sarc*K_{ATP} channel activation is secondary to hyperpolarisation of the cell membrane and decreased Ca^{2+} entry (Baczkó *et al.*, 2004, Lederer *et al.*, 1989). Gross *et al.* (1992) demonstrated that the K_{ATP} channel blocker glibenclamide abolished the protective effect of IPC on infarct size, while a K^+ channel opener (RP 52891) was able to mimic the protection of IPC. Later studies also suggested a protective role for the *sarc*K_{ATP} channel in IPC (Jovanović *et al.*, 1998b, Seino and Miki, 2003, Liu *et al.*, 1996, Haruna *et al.*, 1998). However, the role of the *sarc*K_{ATP} channel in

preconditioning was brought into question by the discovery of a mitochondrial K_{ATP} channel (for details see Introduction and below).

6.3 *Mito*K_{ATP} channels

It was initially thought that it was *sarc*K_{ATP} channels that were important to the protection afforded by preconditioning. However, the identification of the *mito*K_{ATP} channel and the discovery that *mito*K_{ATP} channel inhibitors prevent preconditioning, while openers mimic preconditioning, resulted in much debate concerning the role of these channels (Garlid *et al.*, 1997). Although the structure of these channels is still unknown, channel modulators, primarily the channel opener diazoxide and the channel blocker 5-HD, have been extensively used to examine the roles of the *mito*K_{ATP} channel in preconditioning (Reviewed in Hanley and Daut, 2005, Peart and Gross, 2002).

6.4 Results

6.4.1 HMR1883 prevents phenylephrine preconditioning

As stated above, glibenclamide is able to block both the *sarc*- and *mito*K_{ATP} channels. We have shown that glibenclamide abolished by phenylephrine preconditioning, therefore the role of the *sarc*K_{ATP} channel was further studied using the *sarc*K_{ATP} channel specific inhibitor HMR1883 (Gögelein *et al.*, 1998).

In order to assess the role of the *sarc*K_{ATP} channel in phenylephrine preconditioning, ventricular myocytes were treated with HMR1883 during phenylephrine treatment, washout, and metabolic inhibition, followed by 10 minutes of re-energisation with normal Tyrode (protocol described in Figure 6.1). The recovery of contractile activity was decreased from 63.2 ± 5.3% (n = 5, 5, and 164, for experiments, hearts, and cells respectively) in phenylephrine preconditioned myocytes, to 43.6 ± 5.4% (n = 5, 5, and 128; $P < 0.05$; Figure 6.2) in myocytes preconditioned with phenylephrine in the

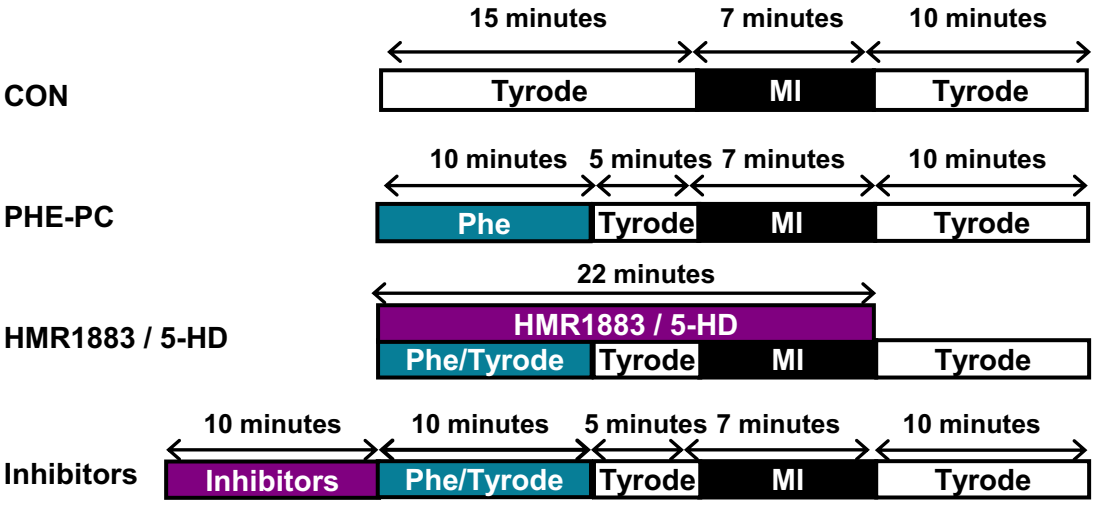

Figure 6.1. Protocol used for phenylephrine preconditioning, metabolic inhibition re-energisation and inhibitor treatments

Ventricular myocytes were preconditioned by superfusion with phenylephrine (Phe) for 10 minutes followed by 5 minutes of washout with normal Tyrode. Control myocytes were superfused with normal Tyrode for 15 minutes. Myocytes were then subject to 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control (CON). Phe preconditioned (PHE-PC). *Sarc*K_{ATP} inhibitor HMR1883, *mito*K_{ATP} inhibitor 5-hydroxydecanoic acid (5-HD). HMR1883. Inhibitors- AMPK inhibitor compound C, PKCδ inhibitor δV1-1, p38 inhibitor SB202190.

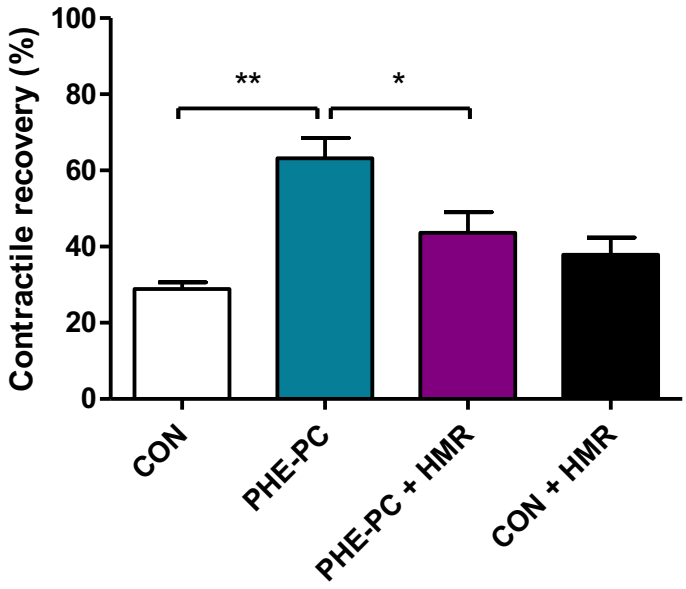

Figure 6.2. Effect of HMR 1883 on phenylephrine preconditioning.
Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 5, 5, and 141, for experiments, hearts and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 5, 5, and 164). Phenylephrine preconditioned myocytes treated with 10 μM HMR 1883 (PHE-PC + HMR; purple bar, n = 5, 5, and 128). Control myocytes treated with HMR1883 (CON + HMR; black bar, n = 4, 4, and 88). * $P < 0.05$, ** $P < 0.01$.

presence of HMR1883 (10 μM). Although the contractile recovery of myocytes treated with HMR1883 prior to phenylephrine preconditioning was higher than that of control myocytes (28.8 ± 1.7%; n = 5, 5, and 141), this did not reach statistical significance (P = NS). These results suggest that *sarc*K_{ATP} channel activation is required for phenylephrine preconditioning.

In section 3.2.4 we showed that glibenclamide treatment decreased the time to rigor contracture and the interval between contractile failure and rigor, therefore we examined whether HMR1883 caused similar changes. Similar to glibenclamide, HMR1883 had no effect on the mean time to contractile failure (P = NS; Figure 6.3A). The mean time to rigor contracture was significantly decreased by HMR1883 in both control and phenylephrine preconditioned myocytes (Figure 6.3B). The mean time to rigor contracture was 236 ± 4 seconds (n = 97, 4, and 4, for cells, experiments and hearts respectively) in control myocytes, which was reduced to 205 ± 4 seconds (n = 88, 4, and 4) in control myocytes treated with HMR1883 (P < 0.01). Similarly, the mean time to rigor contracture was 250 ± 3 seconds (n = 99 cells, 4, and 4) in phenylephrine preconditioned myocytes and 232 ± 4 seconds (n = 83, 4, and 4) in phenylephrine preconditioned myocytes treated with HMR 1883 (P < 0.01). HMR1883 also reduced the interval between contractile failure and rigor contracture from 45 ± 2 seconds (n = 100, 4, and 4) in phenylephrine preconditioned myocytes to 21 ± 1 seconds (n = 84, 4, and 4) in phenylephrine preconditioned myocytes treated with HMR1883 (P < 0.01), and from 37 ± 1 seconds (n = 98, 4, and 4) in control myocytes to 21 ± 1 seconds (n = 89, 4, and 4) in control myocytes treated with HMR1883 (P < 0.01; Figure 6.3C). This suggests that *sarc*K_{ATP} channel opening alters the time to onset of rigor contracture, however similar to glibenclamide; the effect appears to be independent of changes in APD shortening and contractile failure.

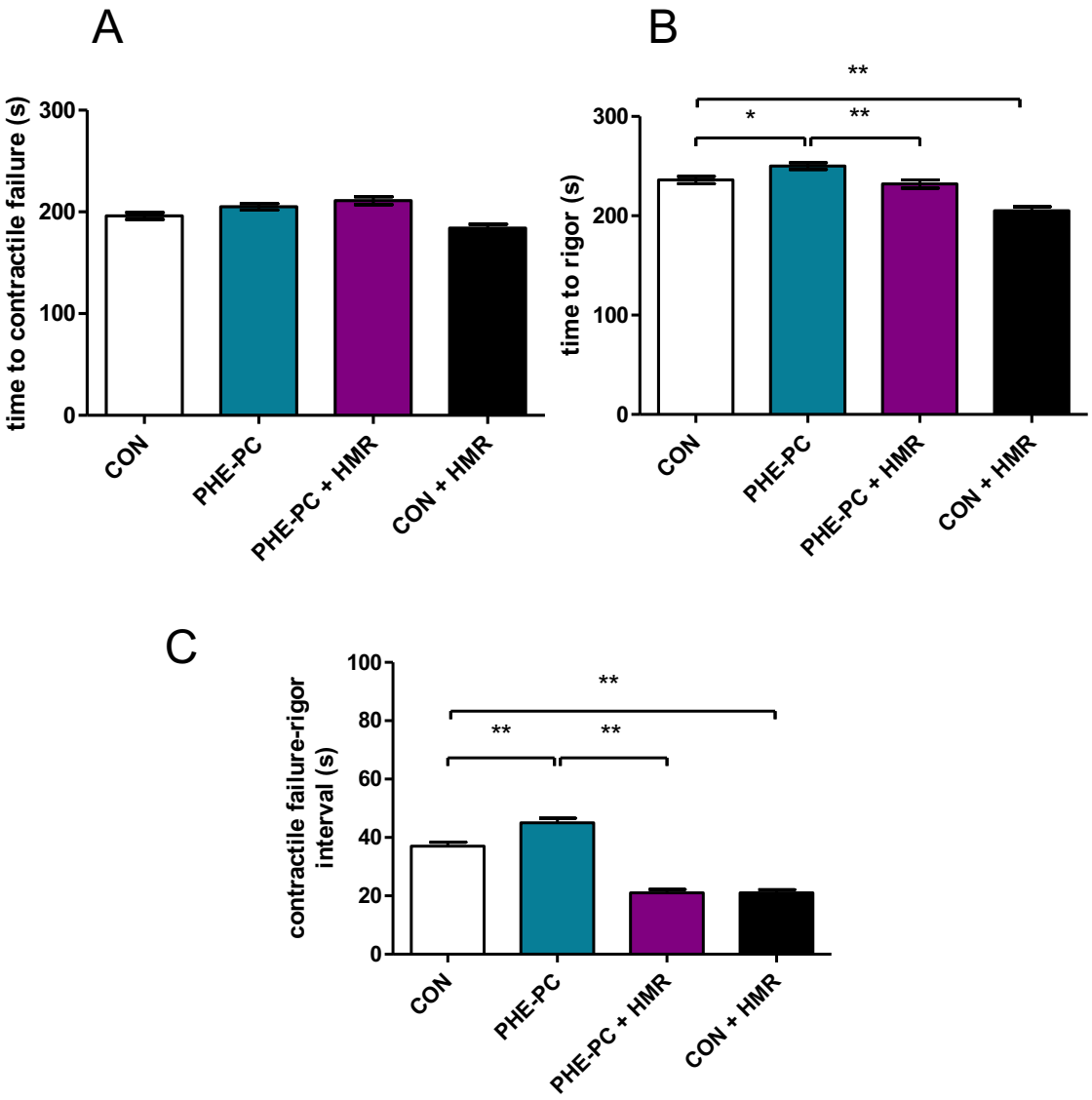

Figure 6.3. Effect of HMR 1883 on time to contractile failure and rigor contracture.

A. Bar chart showing mean time to contractile failure in control (CON) and phenylephrine preconditioned (PHE-PC) myocytes, and phenylephrine preconditioned myocytes treated with 10 μM HMR 1883 (PHE-PC + HMR), and control myocytes treated with HMR 1883 (CON + HMR). CON (open bars, n = 97, 4, and 4, for cells, experiments, and hearts respectively). PHE-PC (blue bars, n = 99, 4, and 4). PHE-PC + HMR (purple bars, n = 83, 4, and 4). CON + HMR (black bars, n =88, 4, and 4,). P = NS.

B. Bar chart showing mean time to rigor contracture in CON, PHE-PC, PHE-PC + HMR, and CON + HMR myocytes. *P < 0.05, **P < 0.01.

C. Bar chart showing interval between the onset of contractile failure and rigor contracture, in CON, PHE-PC, PHE-PC + HMR, and CON + HMR myocytes. ** P < 0.01.

6.4.2 5-HD inhibits phenylephrine preconditioning

As well as *sarc*K_{ATP} channels, glibenclamide is reported to inhibit the putative *mito*K_{ATP} channels. *Mito*K_{ATP} channels have been reported to act upstream of PKC and p38 in the signalling pathway of phenylephrine preconditioning (Loubani and Galiñanes, 2002, Cohen *et al.*, 2001). Therefore, we examined the role of *mito*K_{ATP} channels using the selective *mito*K_{ATP} channel inhibitor 5-HD.

To assess the role of the *mito*K_{ATP} channel in phenylephrine preconditioning, 5-HD (0.5 mM) was administered during preconditioning and MI (protocol as shown in Figure 6.1). As shown in Figure 6.4, 5-HD abolished phenylephrine preconditioning of isolated ventricular myocytes. The contractile recovery was reduced from 54.1 ± 1.7% (n = 3, 3, and 84, for experiments, hearts and cells respectively) in phenylephrine preconditioned myocytes to 34.8 ± 6.0% (n = 3, 3, 90) in myocytes preconditioned with phenylephrine in the presence of 5-HD ($P < 0.05$), which was not significantly different from the recovery of contractile activity in control myocytes (25.9 ± 5.2%; n = 3, 3, and 70; P = NS) or myocytes treated with 5-HD alone (31.6 ± 0.9%; n = 3, 3, and 79; P = NS). This suggests that *mito*K_{ATP} channels may be involved in phenylephrine preconditioning.

6.4.3 The effect of phenylephrine preconditioning on characteristics of whole-cell *sarc*K_{ATP} current

We have shown that the protective effect of phenylephrine preconditioning on contractile recovery was inhibited by treatment with the *sarc*K_{ATP} channel blockers glibenclamide (section 3.2.4/Figure 3.7) and HMR1883 (section 6.4.1/Figure 6.2). It has been reported that hypoxic preconditioning (Budas *et al.*, 2004) and pharmacological preconditioning with PMA or adenosine (Liu *et al.*, 1996) lead to a decrease in the time to activation of *sarc*K_{ATP} current, and/or an increase in the size of the resultant *sarc*K_{ATP}

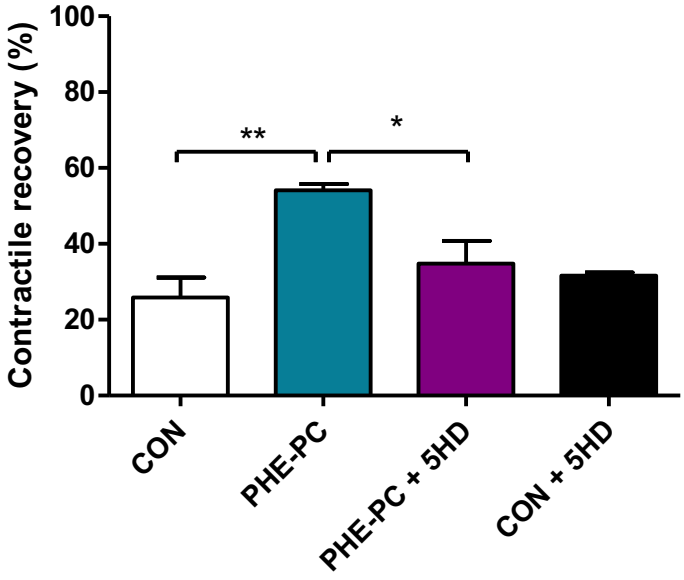

Figure 6.4. Effect of 5-hydroxydecanoic acid (5-HD) administration on phenylephrine preconditioning.
Percentage contractile recovery following 7 minutes of metabolic inhibition (MI) and 10 minutes of re-energisation. Control myocytes (CON; open bar, n = 3, 3, and 70, for experiments, hearts, and cells respectively). Phenylephrine preconditioned myocytes (PHE-PC; blue bar, n = 3, 3, and 84). Phenylephrine preconditioned myocytes treated with 0.5 mM 5-HD (PHE-PC + 5-HD; purple bar, n = 3, 3, and 90). Control myocytes treated with 5-HD (CON + 5-HD; black bar, n = 3, 3, and 79). * $P < 0.05$, ** $P < 0.01$.

current (Liu *et al.*, 1996, Budas *et al.*, 2004). Because PKC activation by preconditioning can modulate *sarc*K_{ATP} channel properties, we examined the effect of phenylephrine preconditioning on the whole-cell *sarc*K_{ATP} current evoked by MI.

To investigate the effect of phenylephrine preconditioning on whole-cell *sarc*K_{ATP} current, we measured the *sarc*K_{ATP} currents activated during superfusion of MI Tyrode, in voltage-clamped control and phenylephrine preconditioned ventricular myocytes using the whole-cell patch clamp technique. Very little outward current was passed at 0 mV prior to MI as shown in Figure 6.5A and Figure 6.6A. During MI, *sarc*K_{ATP} currents were activated after a delay, resulting in a rapid increase in the outward K^+ current. The peak current density at 0 mV was 31.6 ± 2.7 pA/pF (n = 6, and 5, for cells, and hearts respectively) in control myocytes, and was significantly increased in phenylephrine preconditioned myocytes to 46.0 ± 5.0 pA/pF (n = 6, and 5; $P < 0.05$; Figure 6.5B).

The effect of phenylephrine preconditioning on the time to activation and peak amplitude of the *sarc*K_{ATP} current was also examined. The time to activation of *sarc*K_{ATP} current (234 ± 16 seconds in control (n = 8, and 4) and 226 ± 28 seconds phenylephrine preconditioned myocytes respectively (n = 8, and 4); Figure 6.6B) and time to peak current (304 ± 18 seconds and 313 ± 31 seconds; Figure 6.6C) were not significantly altered by phenylephrine preconditioning. This suggests that the protective effect of *sarc*K_{ATP} channels in phenylephrine preconditioning occurs through an increase in the magnitude of *sarc*K_{ATP} current and is not due to a change in the time course of channel activation.

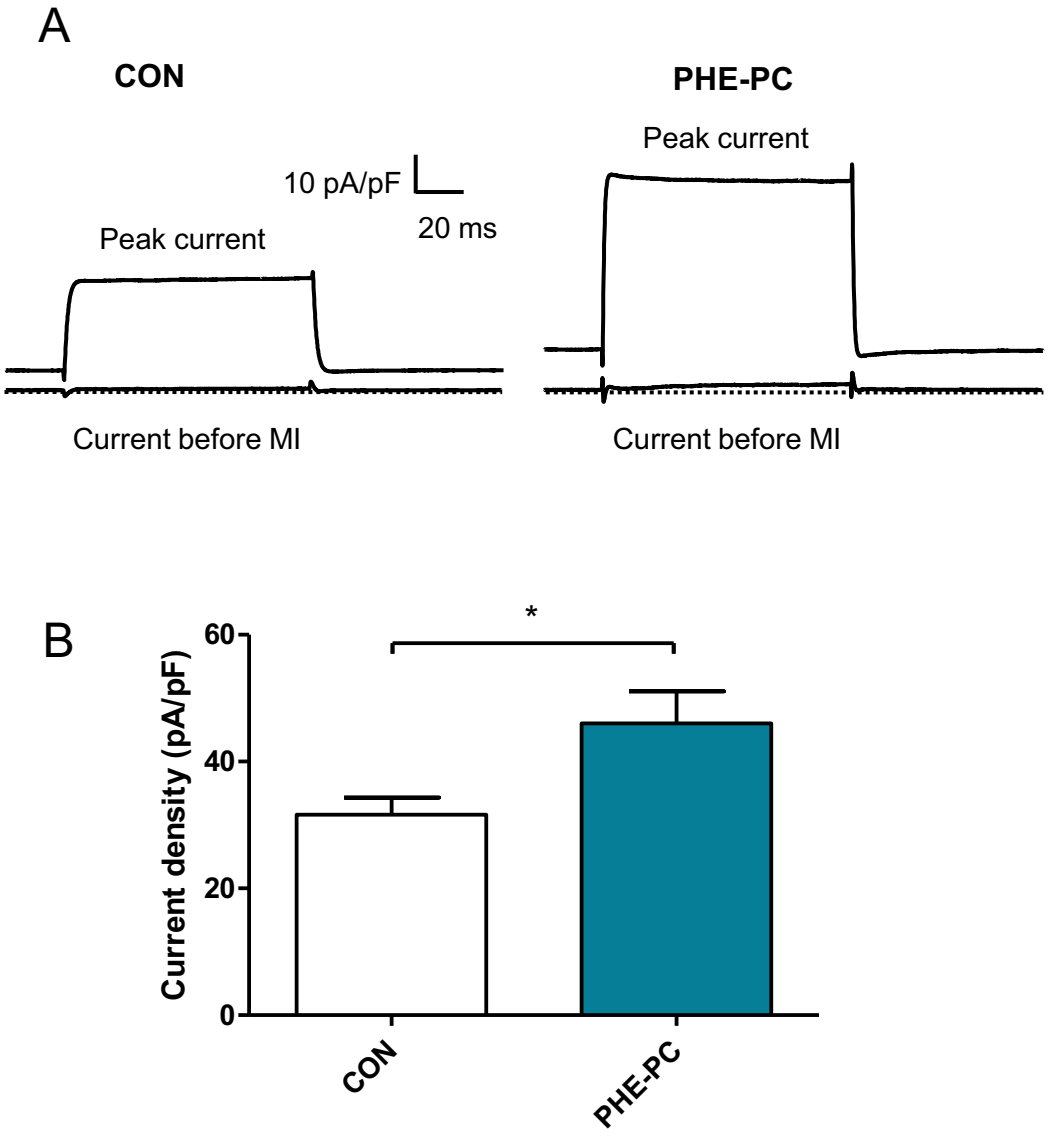

Figure 6.5. Phenylephrine preconditioning increases maximal *sarc*K_{ATP} current density.

A. Typical whole cell membrane current recorded during metabolic inhibition with MI Tyrode from a control (CON) myocyte and PHE-PC myocyte. Myocytes were voltage clamped at -40 mV and stepped to 0 mV for 100 ms every 10 seconds. Dashed line indicates zero current line.

B. Bar chart showing the mean peak *sarc*K_{ATP} current density at 0 mV recorded during superfusion of MI Tyrode in CON and PHE-PC myocytes. CON (open bar, n = 6, and 5 for cells and hearts respectively). PHE-PC (blue bar, n = 6, and 5). * $P < 0.05$.

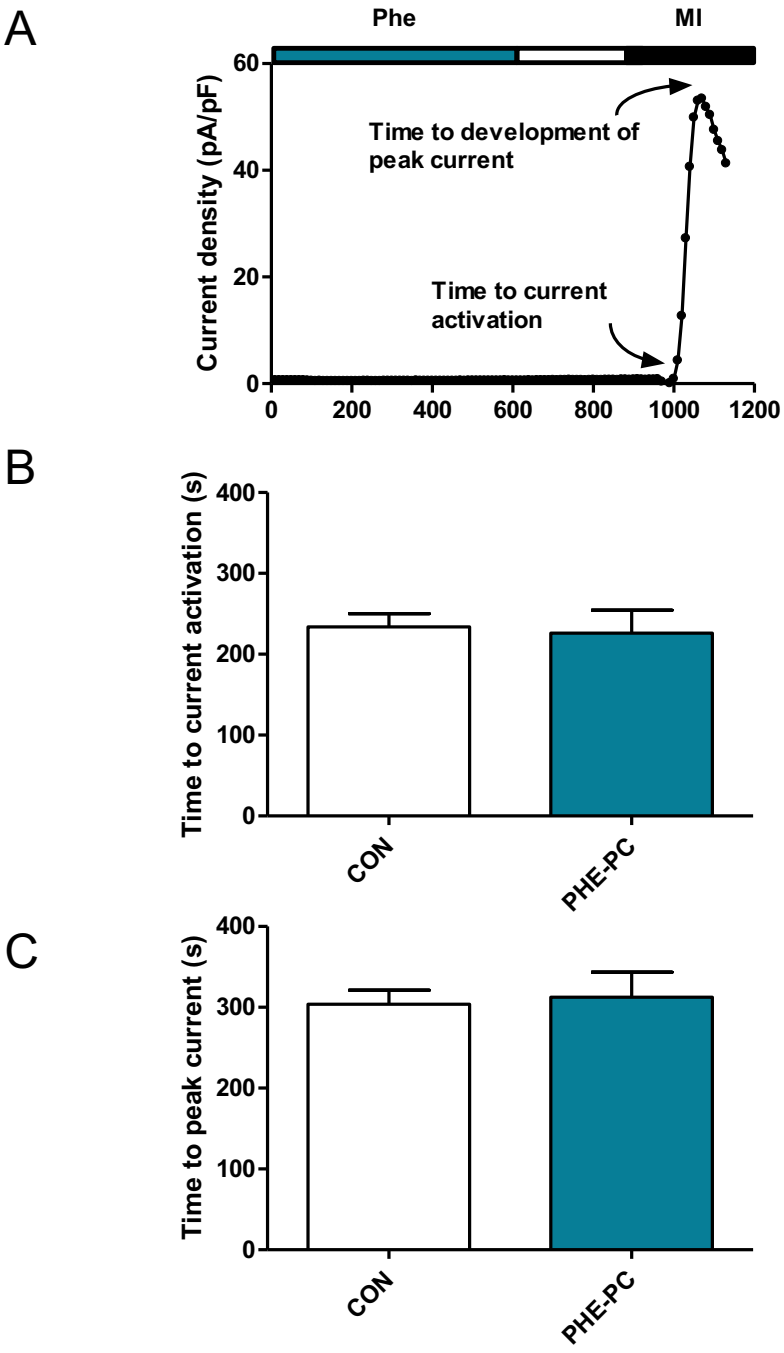

Figure 6.6. Phenylephrine preconditioning does not alter the time to activation and peak amplitude of *sarc*K_{ATP} current.

A. Line graph showing the time course of whole-cell current density recorded during preconditioning and superfusion of MI Tyrode (as Figure 6.4) from a typical phenylephrine preconditioned (PHE-PC) myocyte.

B. Bar chart showing the mean time to activation of *sarc*K_{ATP} current during superfusion of MI Tyrode in control (CON) and PHE-PC myocytes.

C. Bar chart showing the mean time to peak *sarc*K_{ATP} current during superfusion of MI Tyrode in CON and PHE-PC myocytes. CON (open bars, n = 8, and 4, for cells, and hearts). PHE-PC (blue bars, n = 8, and 4). P = NS.

6.4.4 Inhibition of AMPK abolishes modulation of *sarc*K_{ATP} current by phenylephrine preconditioning

Sukhodub *et al.* (2007) found that *sarc*K_{ATP} channels were recruited to the membrane in response to hypoxic preconditioning, and this increase was prevented in transgenic mice expressing a dominant-negative form of AMPK. As we previously identified a protective role for AMPK in phenylephrine preconditioning (section 5.4.5/Figure 5.5), we investigated the effect of AMPK inhibition on *sarc*K_{ATP} current amplitude in phenylephrine preconditioned myocytes.

To assess the effect of AMPK inhibition on the phenylephrine preconditioning-induced increase in MI-activated *sarc*K_{ATP} current, isolated ventricular myocytes were treated with the AMPK inhibitor compound C (10 μM) for 10 minutes prior to phenylephrine preconditioning and exposure to MI. Compound C treatment completely abolished the increase in peak *sarc*K_{ATP} current density activated by MI in phenylephrine preconditioned myocytes (Figure 6.7). The peak current density measured during MI was reduced from 46.0 ± 5.0 pA/pF (n = 6, and 5) in phenylephrine preconditioned myocytes, to 31.3 ± 3.0 pA/pF (n = 7, and 5) in myocytes treated with compound C prior to preconditioning ($P < 0.05$), which was not significantly different from the current density in control myocytes (31.6 ± 2.7 pA/pF; n = 6, and 5; P = NS). Therefore, inhibition of AMPK is sufficient to prevent the modulation of *sarc*K_{ATP} current by phenylephrine preconditioning.

The mean time to current activation was 211 ± 5 seconds (n = 8, and 7) in control myocytes, 219 ± 29 seconds (n = 8, and 6) in phenylephrine preconditioned myocytes and 210 ± 18 seconds (n = 7, and 5) in phenylephrine preconditioned myocytes pretreated with compound C (P = NS). The mean time to peak current was also not significantly different in compound C pretreated myocytes, 283 ± 8 seconds (n = 8, and

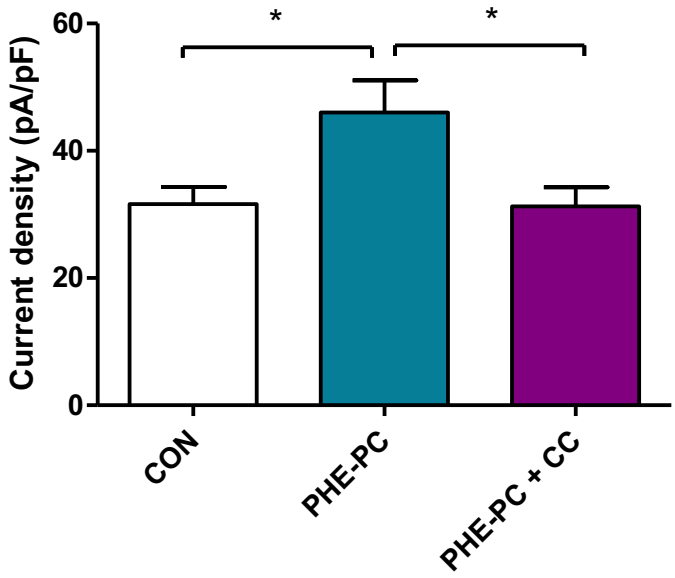

Figure 6.7. Inhibition of AMPK prevents the increase in peak *sarc*K_{ATP} current density induced by phenylephrine preconditioning.

Bar chart showing the mean peak *sarc*K_{ATP} current density recorded in MI Tyrode (as per Figure 6.4) in control (CON) and phenylephrine preconditioned (PHE-PC) myocytes and phenylephrine preconditioned myocytes treated with the AMPK inhibitor Compound C (10 μM) for 10 minutes prior to preconditioning (PHE-PC + CC). CON (open bar, n = 6, and 5, for, cells and hearts respectively). PHE-PC (blue bar, n = 6, and 5). PHE-PC + CC (purple bar, n = 7, and 5). * $P < 0.05$.

7) in control myocytes, 305 ± 32 seconds (n = 8, and 6) in phenylephrine preconditioned myocytes, and 271 ± 21 seconds (n = 7, and 5) in phenylephrine preconditioned myocytes pretreated with compound C (P = NS).

6.4.5 Inhibition of PKCδ prevents modulation of s*arc*K_{ATP} current by phenylephrine preconditioning

We previously observed that inhibition of PKCδ prevented activation of AMPK by phenylephrine preconditioning (see section 5.4.7/Figure 5.6). We have also demonstrated that inhibition of AMPK prevented modulation of *sarc*K_{ATP} channel current by phenylephrine (see section 6.4.4/Figure 6.7); therefore we surmised that PKCδ may be acting upstream of AMPK in the modulation of *sarc*K_{ATP} current by phenylephrine preconditioning.

To examine the role of PKCδ in the modulation of *sarc*K_{ATP} current by phenylephrine preconditioning, isolated ventricular myocytes were pretreated with the PKCδ inhibitor δV1-1 (100 nM) for 10 minutes prior to patching and phenylephrine preconditioning. Pretreatment with the δV1-1 peptide reduced the peak current density from 49.4 ± 4.3 pA/pF (n = 8, and 6) in phenylephrine preconditioned ventricular myocytes, to 36.1 ± 3.8 pA/pF (n = 8, and 4) in phenylephrine preconditioned myocytes treated with δV1-1 ($P < 0.05$; Figure 6.8), which was not significantly different from the current density of 31.3 ± 2.3 pA/pF recorded from control myocytes (n = 8, and 6; P = NS). This suggests that PKCδ is responsible for mediating the increase in peak *sarc*K_{ATP} current that is induced by phenylephrine preconditioning.

The mean time to current activation was 211 ± 5 seconds (n = 8, and 7) in control myocytes, 219 ± 29 seconds (n = 8, and 6) in phenylephrine preconditioned myocytes and 225 ± 16 seconds (n = 9, and 5) in phenylephrine preconditioned myocytes

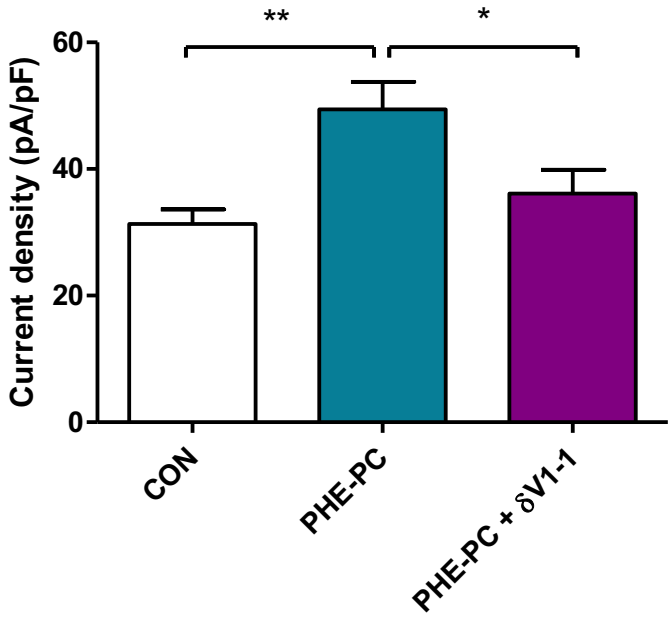

Figure 6.8. Inhibition of PKCδ prevents the increase in peak *sarc*K_{ATP} current density induced by phenylephrine preconditioning.

Bar chart showing the mean peak *sarc*K_{ATP} current density recorded in MI Tyrode (as per figure 6.4) in control (CON) and phenylephrine preconditioned (PHE-PC) myocytes, and phenylephrine preconditioned myocytes treated with the PKCδ inhibitor δV1-1 (100 nM) for 10 minutes prior to preconditioning (PHE-PC + δV1-1). CON (open bar, n = 8, and 6, for cells, and hearts). PHE-PC (blue bar, n = 8, and 6). PHE-PC + δV1-1 (purple bar, n = 8, and 4). * $P < 0.05$, ** $P < 0.01$.

pretreated with δV1-1 (P = NS). The mean time to peak current was also not significantly different in δV1-1 pretreated myocytes, 283 ± 8 seconds (n = 8, and 6) in control myocytes, 305 ± 32 seconds (n = 8, and 6) in phenylephrine preconditioned myocytes, and 305 ± 21 seconds (n = 9, and 5) in phenylephrine preconditioned myocytes pretreated with δV1-1 (P = NS).

6.4.6 Inhibition of p38 prevents modulation of the *sarc*K_{ATP} channel by phenylephrine preconditioning

Both we (section 5.4.3/Figure 5.3) and Loubani and Galiñanes (2002) found that activation of p38 is required for phenylephrine preconditioning. Activation of p38 usually occurs through phosphorylation by upstream MAPKKs MKK3 and MKK6; however, it has recently been found that p38 can also be activated by an alternative mechanism involving binding of p38 and AMPK to the scaffold protein TAB-1, which promotes the autophosphorylation of p38 (Li *et al.*, 2005). We therefore examined that possibility that p38 acts downstream of AMPK to modulate *sarc*K_{ATP} current.

In order to investigate the role of p38 in the phenylephrine preconditioning-induced increase in *sarc*K_{ATP} current, isolated ventricular myocytes were pretreated with the p38 inhibitor SB202190 (10 μM) for 10 minutes prior to patching, preconditioning and exposure to MI. As shown in Figure 6.9, the current density was reduced from 46.5 ± 4.5 pA/pF (n = 5, and 5) in phenylephrine preconditioned myocytes to 28.3 ± 2.0 pA/pF (n = 4, and 2) in myocytes pretreated with SB202190 prior to preconditioning ($P < 0.05$), which was not significantly different from the current activated in control myocytes, 32.0 ± 3.3 pA/pF (n = 5, and 5; P = NS). This finding indicates that p38 is involved in the modulation of *sarc*K_{ATP} current by phenylephrine preconditioning.

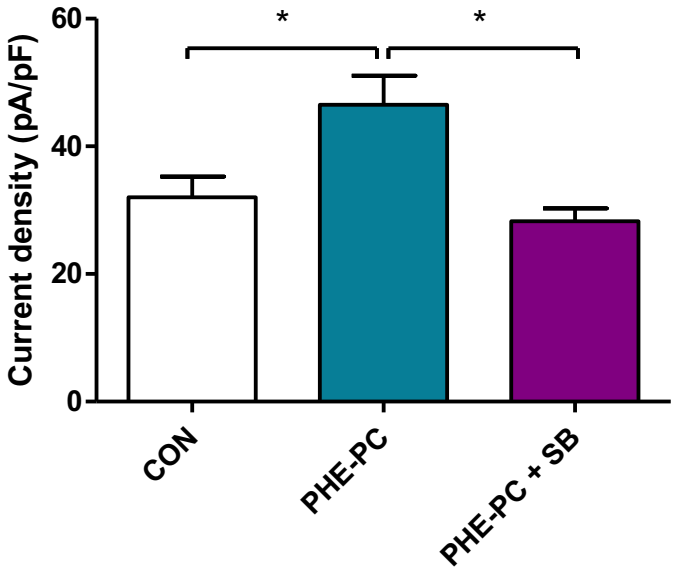

Figure 6.9. Inhibition of p38 abolished the increase in peak *sarc*K_{ATP} current density induced by phenylephrine preconditioning

Bar chart showing the mean peak *sarc*K_{ATP} current density recorded in MI Tyrode (as per Figure 6.4) in control (CON) and phenylephrine preconditioned (PHE-PC) myocytes, and phenylephrine preconditioned myocytes treated with the p38 inhibitor SB202190 (10 μM) for 10 minutes prior to preconditioning (PHE-PC + SB). CON (open bar, n = 5, and 5, for cells and hearts respectively). PHE-PC (blue bar, n = 5, and 5). PHE-PC + SB (purple bar, n = 4, and 2). * $P < 0.05$.

The mean time to current activation was 212 ± 27 seconds (n = 5, and 5) in control myocytes, 206 ± 53 seconds (n = 5, and 5) in phenylephrine preconditioned myocytes and 200 ± 22 seconds (n = 5, and 3) in phenylephrine preconditioned myocytes pretreated with SB202190 (P = NS). The mean time to peak current was also not significantly different following pre-treatment with SB202190, 280 ± 32 seconds (n = 5, and 5) in control myocytes, 265 ± 65 seconds (n = 5, and 5) in phenylephrine preconditioned myocytes, and 260 ± 33 seconds (n = 5, and 3) in phenylephrine preconditioned myocytes pretreated with SB202190 (P = NS).

6.4.7 Glibenclamide reduces *sarc*K_{ATP} current density

Our group and others have previously shown that metabolic inhibition results in the activation of *sarc*K_{ATP} channels, causing shortening of the action potential and/or hyperpolarisation of the resting membrane potential (Baczkó *et al.*, 2004, Sukhodub *et al.*, 2007, Rainbow *et al.*, 2005, Rodrigo *et al.*, 2004). Sulphonylurea drugs are able to bind to the SUR subunit of the *sarc*K_{ATP} channel and inhibit channel opening. In order to confirm that the *sarc*K_{ATP} channel was responsible for the outward current observed in our experiments, the sulphonylurea drug glibenclamide was used to inhibit *sarc*K_{ATP} current in non-preconditioned control myocytes. Glibenclamide (10 μM) was applied simultaneously with MI in these experiments. The current density activated by MI was reduced from 32.9 ± 1.2 pA/pF (n = 3, and 3) in control myocytes to 17.0 ± 2.0 pA/pF in control myocytes treated with glibenclamide (n = 3, and 2) (Figure 6.10). This suggests that the channel observed in our experiments is the cardiac *sarc*K_{ATP} channel.

6.5 Summary

In this chapter we have examined the role of K_{ATP} channels in phenylephrine preconditioning of isolated ventricular myocytes. Using pharmacological tools we have

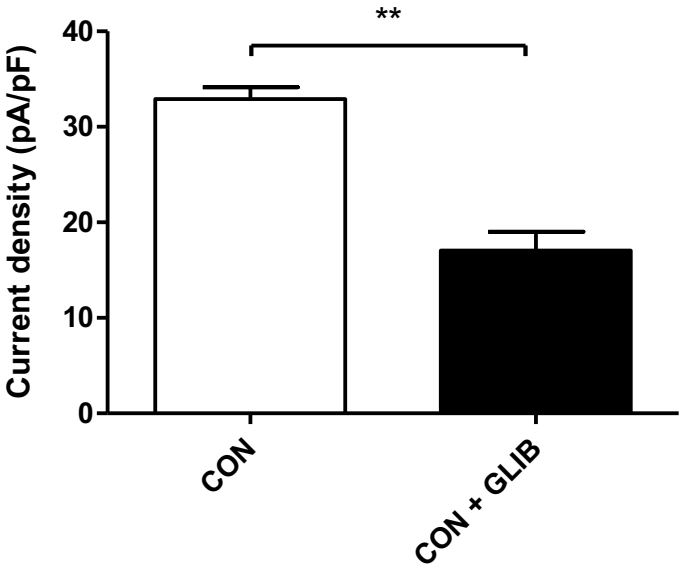

Figure 6.10. Inhibition of *sarc*K_{ATP} channel with glibenclamide reduces peak current density.

Bar chart showing the mean peak *sarc*K_{ATP} current density recorded from non-preconditioned myocytes (as per figure 6.4) during superfusion with MI Tyrode alone (CON) or applied simultaneously with 10 μM glibenclamide (CON + GLIB). CON (open bar, n = 3, and 3 for cells, and hearts respectively). CON + GLIB (black bar, n = 3, and 2). ** $P < 0.01$.

demonstrated a role for both the *sarc*- and *mito*K_{ATP} channel in phenylephrine preconditioning of isolated ventricular myocytes. Furthermore, phenylephrine preconditioning resulted in an increase in the peak *sarc*K_{ATP} current density activated by MI, but did not alter the time to current activation or time to peak current. The increase in current density was prevented by inhibition of PKCδ, AMPK and p38MAPK. It is therefore concluded that the protective effect of phenylephrine preconditioning is mediated via activation of PKCδ, AMPK and p38MAPK resulting in modulation of *sarc*K_{ATP} channels.

7 Discussion

The aims of this study were to examine the cardioprotective mechanisms involved in phenylephrine preconditioning in isolated ventricular myocytes. Using the α_1-adrenergic agonist prazosin, we showed that phenylephrine preconditioning is mediated via specific activation of the α_1-adrenoceptor. The role of PKC in preconditioning has been well documented, but there is still argument over the isoforms involved. Therefore, we examined the role of several PKC isoforms in phenylephrine preconditioning. We showed sustained activation of PKCε and δ in response to phenylephrine preconditioning and identified a protective role for PKCε and both protective and damaging effects of PKCδ, while conventional PKC isoforms appear not to play a part in phenylephrine preconditioning of ventricular myocytes. In order to identify downstream targets of PKC we studied the MAPK family, CaMKK and AMPK. We showed that inhibition of AMPK, p38, and CaMKK, prevented phenylephrine preconditioning and discovered that AMPK phosphorylation in response to phenylephrine required PKCδ activation. *Sarc*- and *mito*K_{ATP} channels also appear to be required for phenylephrine preconditioning because preconditioning was prevented by HMR1883 and 5-HD.

We have shown for the first time that phenylephrine preconditioning modulates *sarc*K_{ATP} current activated by MI. Phenylephrine preconditioning increased the peak *sarc*K_{ATP} current amplitude in ventricular myocytes compared with control, but did not alter the time course of current activation. This suggests that phenylephrine preconditioning evokes cardioprotection by increasing *sarc*K_{ATP} current density during ischaemia-reperfusion; and theorise that this protection occurs via an increase in hyperpolarisation of the cell membrane, a decrease in Ca^{2+} entry through voltage-gated

Ca^{2+} channels and reverse-mode NCX, and a consequent decrease in Ca^{2+} loading of ventricular myocytes. Additionally, we showed that inhibition of PKCδ, AMPK or p38 prevented the increase in peak *sarc*K_{ATP} current suggesting that these kinases are involved in transduction of the cardioprotective signalling from the α_1-adrenoceptor to the *sarc*K_{ATP} channel. This is the first study to show involvement of p38 in regulation of the *sarc*K_{ATP} channel.

7.1 Could phenylephrine be a useful pharmacological tool for protection of the heart from ischaemia-reperfusion injury?

Although IPC is able to provide a large degree of protection from myocardial ischaemia-reperfusion injury (Murry *et al.*, 1986), in the case of myocardial infarction it is not possible to use IPC in a therapeutic setting due to the need to precondition before the ischaemic event. IPC has been used to reduce damage during cardiac surgery which involves a planned ischaemic episode. However, the introduction of modern stents, the time required for the IPC protocol and the potential risk of clot formation have all reduced the use of IPC (Rezkalla and Kloner, 2007). The field has moved towards the study of pharmacological preconditioning, which may allow elucidation of common mechanisms involved in ischaemic and pharmacological preconditioning, and the potential for development of new therapies for coronary heart disease. Examination of pharmacological preconditioning has also identified cardioprotective effects of a range of commonly used drugs, allowing doctors to tailor drug treatment by using cardioprotective drugs and excluding those with an unfavourable profile (Rezkalla and Kloner, 2007, Cokkinos and Pantos, 2007).

In this study, we have demonstrated that phenylephrine preconditioning increases the recovery of contractile activity in ventricular myocytes exposed to

ischaemia-reperfusion injury simulated by metabolic inhibition and re-energisation (section 3.2.1/Figure 3.2). This is in agreement with previous findings in whole hearts (Mitchell *et al.*, 1995, Tsuchida *et al.*, 1994, Tosaki *et al.*, 1995, Cleveland *et al.*, 1997) and isolated ventricular myocytes exposed to hypoxia (Gao *et al.*, 2007). A similar ability of phenylephrine preconditioning to reduce infarct size has also been reported in several studies (Vasara *et al.*, 2002, Cohen *et al.*, 2001, Tsuchida *et al.*, 1994, Hale and Kloner, 1994). The recovery of ventricular function is dependent on the viability of the myocytes and the proportion of stunned myocytes. Therefore, it appears that the improvement in myocardial post-ischaemic function following phenylephrine preconditioning may be related to an increase in cell survival. Although infarct size remains the favoured end-point for preconditioning studies, we examined contractile recovery as an end point in our studies, because this provided a measure of the proportion of functional myocytes (those that were viable and not stunned). Stunning is a reversible process (Bolli, 1990), however a loss of contractility within significant portions of the heart over a sustained period is still likely to result in mortality or significant morbidity to the individual. Therefore, improvement in the recovery of mechanical function in ventricular tissue following ischaemia-reperfusion is an important outcome of preconditioning.

The contractile recovery of control and phenylephrine preconditioned myocytes did vary between data sets. This is likely to be due to differences in the quality of ventricular myocyte isolations, or variations in bath temperature depending on environmental conditions, although this was thermostatically controlled. The contractile recovery in control experiments ranged from 18.8 ± 2.3% to 31.1 ± 0.7%. Following phenylephrine preconditioning the contractile recovery ranged from 43.8 ± 5.4% to 69.9 ± 2.8%, which was 1.85-2.46 fold higher in phenylephrine preconditioning datasets than

in the respective controls. Cell isolations were not used if the recovery in control experiments was greater than 33% because this provided a large window for the protection. In addition, when the recovery was higher than this level, the cells often appeared to be preconditioned because the protection was not augmented by phenylephrine preconditioning.

7.1.1 Phenylephrine preconditioning alters the time course of contractile failure and rigor contracture during metabolic inhibition: possible effect on cellular energy homeostasis

The time to contractile failure during ischaemia or MI is dependent on the degree of APD shortening due to opening of *sarc*K_{ATP} channels, maintenance of ionic homeostasis, ATP levels and the sensitivity of the contractile machinery to Ca^{2+}. A reduction in the APD and its eventual failure should decrease the Ca^{2+} influx during MI and improve Ca^{2+} homeostasis (Light *et al.*, 2001, Rainbow *et al.*, 2004). It has been shown that diazoxide preconditioning decreases the time to contractile arrest in isolated ventricular myocytes (Rodrigo *et al.*, 2004). Early opening of *sarc*K_{ATP} channels during MI leads to premature APD shortening and failure resulting in early contractile failure. Furthermore, because the contractile machinery is one of the major consumers of ATP in the ventricular myocyte, an additional benefit of early failure of contraction could be maintenance of cellular ATP levels during MI.

In contrast, preconditioning with PMA in isolated ventricular myocytes (Hudman and Standen, 2004) and IPC in whole hearts (Mitani *et al.*, 1994), have been observed to increase the time to contractile failure. The effect of phenylephrine preconditioning on time to contractile failure has not been examined previously but Rehring *et al.* (1997), observed that IPC and adenosine preconditioning decreased time to electrical arrest

during global ischaemia in the isolated rat heart, while phenylephrine preconditioning did not significantly change the time to electrical arrest when compared to non-preconditioned hearts.

We observed a small but significant increase in the mean time to contractile failure following phenylephrine preconditioning in one set of experiments (8 seconds, section 3.2.1/Figure 3.3), although this did not reach significance in the second set of experiments (7 seconds; section 3.2.3/Figure 3.6). The biological significance of this small change is not clear. In addition, we did not observe any change in the time to activation or peak *sarc*K_{ATP} following phenylephrine preconditioning, suggesting that phenylephrine preconditioning does not increase shortening of the APD. This supports the finding that phenylephrine preconditioning does not significantly alter the time to contractile failure.

Ischaemic rigor contracture occurs due to a decrease in ATP levels during an ischaemic insult (Hearse *et al.*, 1977, Altschuld *et al.*, 1985). Although IPC increases cell survival and ATP levels during reperfusion (Banerjee *et al.*, 1993, Wolfe *et al.*, 1993), it has been reported to cause a paradoxical increase in the magnitude of contracture and a decrease in the time to ischaemic rigor contracture (Kolocassides *et al.*, 1996, Hearse and Sutherland, 1999). It was proposed that the exacerbation of ischaemic contracture may result from lower ATP levels in the IPC group due to the antecedent ischaemia. However, α-adrenergic stimulation with norepinephrine or phenylephrine produced a similar decrease in the time to onset of ischaemic contracture (Vasara *et al.*, 2002, Hearse and Sutherland, 1999), despite findings that phenylephrine preconditioning protects ATP levels during early ischaemia (Gao *et al.*, 2007, Rehring *et al.*, 1997), suggesting that the exacerbation of contracture may be linked to the protective mechanism of preconditioning. Unexpectedly, the mean time to rigor contracture was

significantly increased in our studies (see section 3.2.1/Figure 3.3B and section 6.4.1/Figure 6.3B). This is in contrast with the previous findings that norepinephrine (Hearse and Sutherland, 1999) or phenylephrine (Vasara *et al.*, 2002) preconditioning significantly reduced time to ischaemic contracture in isolated rat hearts.

It is possible that the difference between our results and previous findings are due to the use of isolated myocytes in our study. These myocytes lack the cell-cell communication via gap junctions which occurs in the whole heart. The gap junction protein Connexin 43 is important for preconditioning in the heart (Schulz *et al.*, 2003, Schwanke *et al.*, 2002), although it has recently been suggested that protection associated with Connexin 43 may not be related to cell-cell communication (Li *et al.*, 2004, Boengler *et al.*, 2009). Unlike in the heart, the isolated myocytes used in our study were unloaded and were electrically stimulated at above threshold voltage. Unloaded myocytes have to do less work than those in the heart, therefore the difference between our study and those in the whole heart may result from a change in energy utilisation. In addition, in our study the myocytes were continuously superfused with MI Tyrode to induce metabolic inhibition. Superfusion will allow removal of metabolites, while inhibition of glycolysis will prevent build up of lactate and H^+. In contrast, in the heart ischaemia results in a build up of metabolites, and these may be involved in the early entry into ischaemic rigor contracture.

We propose that the increased time to rigor contracture in our study may result from modulation of contractile proteins, because phenylephrine preconditioning increases phosphorylation of TnI and C protein and reduces Ca^{2+}-dependent actomyosin-ATPase activity in a PKC-dependent manner during ischaemia (Pyle *et al.*, 2000, Pyle *et al.*, 2002). These effects of phenylephrine might be expected to slow cross-bridge cycling during ischaemia and reduce the degree of ATP depletion caused by contraction of the

myocyte, therefore increasing the time to ATP depletion and the onset of rigor contracture. In agreement with this hypothesis, it has been previously shown that the ATP levels during ischaemia were significantly increased following phenylephrine preconditioning, when compared with non-preconditioned controls in isolated rat ventricular myocytes (Gao *et al.*, 2007) or isolated rat hearts (Rehring *et al.*, 1997). Taken together, the findings of Gao, Pyle, and our own study suggest that an increase in ischaemic ATP levels due to slowing of cross-bridge cycling could be responsible for the increased time to rigor contracture following phenylephrine preconditioning.

7.2 Phenylephrine preconditioning is mediated via the α_1-adrenoceptor

There is conflicting evidence concerning the role of the α_1-adrenoceptor in IPC. It appears that norepinephrine is released during ischaemic episodes (Carlsson *et al.*, 1985) and could act as a trigger for IPC. In agreement with this hypothesis, it has been shown that inhibition of α_1-adrenoceptors prevents improvements in functional recovery (Banerjee *et al.*, 1993, Hu and Nattel, 1995), tissue creatine kinase activity (Cleveland *et al.*, 1997), and infarct size (Kitakaze *et al.*, 1994, Kariya *et al.*, 1997) induced by IPC. Other groups found no effect of α_1-adrenoceptor inhibition on IPC (Bugge and Ytrehus, 1995, Thornton *et al.*, 1993, Vasara *et al.*, 2002). Despite the conflicting evidence concerning the involvement of the α_1-adrenoceptor in IPC, pharmacological activation of the α_1-adrenoceptor is protective (Banerjee *et al.*, 1993, Tsuchida *et al.*, 1994, Hale and Kloner, 1994, Hearse and Sutherland, 1999).

Phenylephrine is an agonist at the α_1-adrenoceptor, so we examined whether the preconditioning effect of phenylephrine was transduced via specific activation of the α_1-adrenoceptor. Pyruvate is an effective free-radical scavenger (Dobsak *et al.*, 1999)

that has been shown to increase the threshold for preconditioning to occur (Rodrigo *et al.*, 2002, Sargent *et al.*, 1994), therefore phenylephrine was prepared in substrate-free Tyrode solution. We examined the effect of substrate-free Tyrode superfusion prior to MI and re-energisation, to ensure that the protective effect of phenylephrine preconditioning was due to specific activation of the α_1-adrenoceptor and not removal of metabolic substrates from the Tyrode solution. Pretreatment of myocytes with substrate-free Tyrode alone did not affect the contractile recovery of myocytes compared to control myocytes in our study (section 3.2.1/Figure 3.2), suggesting that the protective effect of phenylephrine preconditioning is mediated via an adrenergic response. Furthermore, in agreement with previous studies (Banerjee *et al.*, 1993, Vasara *et al.*, 2002), we demonstrated that the α_1-adrenoceptor antagonist prazosin abolished the protective effect of phenylephrine preconditioning on contractile recovery, indicating that phenylephrine preconditioning is mediated by activation of the α_1-adrenoceptor (section 3.2.2/Figure 3.4).

7.3 Phenylephrine preconditioning improves Ca^{2+} handling during re-energisation

Re-energisation of ventricular myocytes following a prolonged period of metabolic inhibition results in a significant degree of cytosolic Ca^{2+} loading (Rodrigo and Standen, 2005, Baczkó *et al.*, 2003). IPC can decrease Ca^{2+} loading (Rodrigo and Samani, 2008, An *et al.*, 2001), and interventions that decrease Ca^{2+} loading have been shown to improve the recovery of ventricular myocytes (Rodrigo and Standen, 2005, Baczkó *et al.*, 2008, Baczkó *et al.*, 2004). High free Ca^{2+} levels in ventricular myocytes contribute to cellular injury following ischaemia-reperfusion, through hypercontracture of myocytes and contraction-band necrosis (Piper *et al.*, 2004, Altschuld *et al.*, 1985), opening of the MPTP (Griffiths and Halestrap, 1995, Halestrap *et al.*, 2004), and

activation of Ca^{2+}-dependent proteases and other pro-apoptotic proteins (Rizzuto *et al.*, 2003, Dong *et al.*, 2006). Phenylephrine preconditioning has also been observed to decrease Ca^{2+} loading in isolated ventricular myocytes (Gao *et al.*, 2007) and isolated perfused rat hearts (Tosaki *et al.*, 1995). Similarly, we also observed that phenylephrine preconditioning significantly reduced Ca^{2+} loading following re-energisation. Phenylephrine preconditioning did not cause a significant reduction of Ca^{2+} homeostasis during MI in our study when compared with control, although it is possible that a significant improvement of intracellular free Ca^{2+} levels may have been observed if the length of MI had been extended.

Similarly, other groups have shown that hypoxic and pharmacological preconditioning can improve Ca^{2+} homeostasis particularly through a reduction in Ca^{2+} loading during reperfusion (Baczkó *et al.*, 2004, Light *et al.*, 2001). They also showed that the improvement in Ca^{2+} homeostasis during reperfusion appears to be mediated by *sarc*K_{ATP} channel, because activation of *sarc*K_{ATP} channels using pinacidil or P-1075 during reperfusion improved Ca^{2+} levels, while inhibition of *sarc*K_{ATP} channels with HMR1098 increased Ca^{2+} loading (Baczkó *et al.*, 2004). In addition, PMA preconditioning in *sarc*K_{ATP} deficient tsA201 cells, or expression of *sarc*K_{ATP} channel subunits Kir6.2/SUR2A did not prevent Ca^{2+} loading during reperfusion, however PMA preconditioning of tsA201 cells expressing *sarc*K_{ATP} channels protected Ca^{2+} levels during reperfusion (Light *et al.*, 2001). This also suggests that the expression of *sarc*K_{ATP} channels is not sufficient to protect from Ca^{2+} overload, and that preconditioning-induced modulation of *sarc*K_{ATP} channels is required for protection of Ca^{2+} homeostasis. This fits with our results, because we found that control myocytes (which express *sarc*K_{ATP} channels) were not protected from Ca^{2+} loading during

re-energisation, but Ca^{2+} homeostasis was maintained during the re-energisation period in phenylephrine preconditioned myocytes where the *sarc*K_{ATP} current was increased.

The proportion of myocytes able to maintain low Ca^{2+} levels following MI and re-energisation in our study, was increased from 32.8 ± 13.9% in control myocytes to 74.5 ± 9.4% in phenylephrine preconditioned myocytes; which correlated well with the proportion of myocytes recovering contractile activity, 19.2 ± 8.4% in control myocytes and 50.9 ± 8.5% in phenylephrine preconditioned myocytes (see section 3.2.5/Figure 3.11). Therefore, it appears that the contractile recovery of ventricular myocytes in our study is related to Ca^{2+} handling during re-energisation. As well as increasing cell death, high intracellular Ca^{2+} levels during the reperfusion period can also lead to reversible stunning of the myocardium (reviewed in Bolli and Marbán, 1999), therefore it is possible that some myocytes did not recover contractile function in our experiments due to stunning.

7.4 PKC isoforms play multiple roles in phenylephrine preconditioning-induced protection against simulated ischaemia-reperfusion injury

Many forms of preconditioning are receptor-mediated, and converge on activation of a common downstream pathway involving activation of PKC (Cohen *et al.*, 2000). The central role of PKC in preconditioning was identified in a series of studies of ischaemic and pharmacological preconditioning in 1994 (Ytrehus *et al.*, 1994, Mitchell *et al.*, 1995, Liu *et al.*, 1994, Tsuchida *et al.*, 1994). PKC activation is also central to phenylephrine preconditioning, inhibition of PKC abolished the protective effect of phenylephrine preconditioning on infarct size (Tsuchida *et al.*, 1994), CK release and MTT reduction (Loubani and Galiñanes, 2002, Cleveland *et al.*, 1997), and functional recovery

(Mitchell *et al.*, 1995, Cleveland *et al.*, 1997). In accordance with these earlier findings, we found that inhibition of PKC with chelerythrine completely abolished the protective effect of phenylephrine preconditioning on contractile recovery (section 3.2.3/Figure 3.5). These results indicate that activation of PKC is required for phenylephrine preconditioning of the myocardium at the level of single myocytes, and also suggests that our model is an appropriate one for examination of phenylephrine preconditioning because our results correlate well with previous findings in whole hearts.

7.4.1 The protective effect of PKC involves regulation of energy reserves: evidence from contractile failure and ischaemic contracture studies

We have shown that phenylephrine preconditioning increased the mean time to contractile failure and rigor contracture during MI (section 7.1.1), and suggest that this reflects an improvement in the management of cellular energy levels, which may impact on cell survival. PKC activation is required for phenylephrine preconditioning, so we thought it likely that PKC may be involved in the increase in time to contractile failure and rigor contracture.

We found that inhibition of PKC with chelerythrine significantly reduced the mean time to contractile failure in phenylephrine preconditioned myocytes (section 3.2.3/figure 3.6A). However, PKC also reduced the time to contractile failure in non-preconditioned myocytes. This data suggests that PKC activation may be involved in regulating energy levels during ischaemia, in phenylephrine preconditioned and non-preconditioned myocytes. Inhibition of PKC may lead to impaired energy homeostasis during MI, followed by early activation of *sarc*K_{ATP} channels and/or ionic dysregulation, resulting in a decrease in the time to contractile failure.

Although a decrease in the time to contractile failure often results in a decrease in energy utilisation and a consequent increase in time to ischaemic contracture, we found that chelerythrine reduced the mean time to rigor contracture in both control and phenylephrine preconditioned myocytes (section 3.2.3/Figure 3.6B). Rigor contracture occurs due to locking of actin-myosin cross-bridges in the shortened form. Ischaemic rigor contracture occurs as a result of a fall in ATP levels during ischaemia, and the process is enhanced by increased intracellular Ca^{2+} concentrations (Hearse *et al.*, 1977). A decrease in the time to rigor contraction may therefore signify a decrease in the time to ATP depletion during ischaemia.

We have shown that the time interval between contractile failure and rigor contracture was increased in phenylephrine preconditioned myocytes compared to control myocytes (section 3.2.1/Figure 3.3C). Chelerythrine pretreatment prior to phenylephrine preconditioning reduced the time interval between contractile failure and rigor contracture to a value not different from that observed in control myocytes (section 3.2.3/Figure 3.6C). This suggests that as well as increasing the time to contractile failure and rigor contracture during ischaemia, activation of PKC may have additional effects on the time course of contractile failure and rigor contracture in phenylephrine preconditioned myocytes resulting from protection of energy levels. In accordance with this, Gao *et al.* (2007) showed that phenylephrine preconditioning improved ATP levels during ischaemia. The improvement in ATP levels was abolished by the PKCε inhibitor εV1-2, suggesting that PKCε could be responsible for the protection of ATP levels during ischaemia.

A possible mechanism for PKC-dependent regulation of ATP levels is the reduction in ATP consumption by the contractile machinery following PKC activation. The ability of phenylephrine preconditioning to cause a 20% reduction in actomyosin ATP

utilisation via a PKC-dependent mechanism in ischaemic rat hearts has been demonstrated (Pyle *et al.*, 2000, Pyle *et al.*, 2002). Furthermore, Blunt *et al.* (2005) showed that a similar reduction in actomyosin ATP utilisation (28%), improved ATP levels during ischaemia and increased the time to ischaemic rigor contracture in the hearts of mice treated with propylthiouracil or with a point mutation in Troponin T. Their study also showed that the reduction in ATPase activity was sufficient to cause a significant improvement in myocardial functional recovery.

A second mechanism for a PKC-dependent increase in ATP levels involves stimulation of ATP production. Activation of PKC can stimulate glucose uptake via GLUT-4 (Thong *et al.*, 2007). It is thought that glucose levels can limit the rate of glycolysis during ischaemia; therefore an increase in glucose uptake may increase the ATP levels within the heart during ischaemia (Manchester *et al.*, 1994). PKC, particularly the ε isoform, also appears to be involved in regulation of the *mito*K_{ATP} channel. Opening of *mito*K_{ATP} channels has been shown to increase mitochondrial volume resulting in stimulation of mitochondrial respiration (Lim *et al.*, 2002). Phosphorylation of cytochrome C oxidase subunit IV by PKCε has been observed to increase cytochrome C oxidase activity following hypoxic preconditioning in neonatal cardiac myocytes (Ogbi and Johnson, 2006). This would be likely to increase the rate of electron flow through the electron transport chain and consequently increase the proton-motive force and ATP production. However, cytochrome C oxidase would be inhibited by the cyanide used for metabolic inhibition in our study; therefore stimulation of cytochrome C oxidase is unlikely to be responsible for the change in time to contractile failure and rigor. It is possible that the mechanisms described above may be responsible for the PKC and preconditioning-dependent improvement in ATP levels during ischaemia, leading to a delay in contractile failure and rigor contracture.

7.4.2 Which PKC isoforms are responsible for phenylephrine preconditioning?

Conventional PKCs: The requirement for PKC activation in preconditioning is well established, however the isoforms responsible for the protective effect of IPC and phenylephrine preconditioning are less well defined. Of the conventional PKC isoforms only PKCα has been implicated in IPC. Translocation and hence activation, of PKCα was not observed in response to IPC in several studies in isolated rat and rabbit hearts and adult rat cardiac myocytes (Mitchell *et al.*, 1995, Tsouka *et al.*, 2002, Ping *et al.*, 1997). In contrast, Yoshida *et al.* (1997) did observe PKCα translocation during IPC in the isolated rat heart. This may be related to the observation that IPC results in only a transient translocation of PKCα in the rat heart (Kawamura *et al.*, 1998).

Similarly, mixed results exist concerning the role of PKCα in phenylephrine preconditioning. Mitchell *et al.* (1995) reported no translocation of PKCα in response to phenylephrine preconditioning in the rat heart, while Tsouka *et al.* (2002) observed translocation of PKCα in adult rat cardiac myocytes. A constitutively active form of PKCα decreased LDH release but did not significantly decrease ischaemic cell death in neonatal myocytes (Zhao *et al.*, 1998). In contrast, another conventional isoform PKCβII has been implicated in ischaemia-reperfusion injury in the heart (Kong *et al.*, 2008). The evidence suggests that although PKCα may play a role in preconditioning it is unlikely to be important to the protection. Sustained translocation of PKC following the preconditioning stimulus appears to be important for protection (Yang *et al.*, 1997); therefore the transient nature of PKCα translocation suggests that this isoform is unlikely to act as a mediator of preconditioning. It also seems likely that PKCβ is not involved in preconditioning because this isoform increases ischaemia-reperfusion injury.

We observed that inhibition of conventional PKC isoforms did not affect the contractile recovery of phenylephrine preconditioned ventricular myocytes (section 4.3.1/Figure 4.2), suggesting that the conventional PKC isoforms do not mediate the protective effect of phenylephrine preconditioning, and the translocation of PKCα observed in some previous studies may be coincidental.

PKCε: A vital role for PKCε in the transduction of IPC has been demonstrated (Gray *et al.*, 1997), and similarly PKCε activation appears to be required for the improvements in Ca^{2+} handling, contractile function and ATP levels that were afforded by phenylephrine preconditioning (Gao *et al.*, 2007). In our study, inhibition of PKCε by pretreating myocytes with the PKCε inhibitor εV1-2 was sufficient to abolish the protective effect of phenylephrine preconditioning on contractile recovery of ventricular myocytes (section 4.3.4/Figure 4.5), suggesting that PKCε activation is indeed required for phenylephrine preconditioning. We also observed that activation of PKCε using the ψεRACK peptide was sufficient to protect ventricular myocytes from MI re-energisation injury and this protection was not augmented by phenylephrine preconditioning (section 4.3.5/Figure 4.6). Our results indicate that phenylephrine preconditioning and treatment with the ψεRACK peptide may share a common mechanism of protection involving the activation of PKCε.

Examination of the time course of PKCε activation following phenylephrine treatment, showed that PKCε translocates to the membrane fraction in response to phenylephrine treatment suggesting that PKCε is activated by the preconditioning stimulus of phenylephrine preconditioning (section 4.3.3/Figure 4.4), which is in agreement with previous reports (Gao *et al.*, 2007, Tsouka *et al.*, 2002). In addition, a proportion of the PKCε remained in the membrane fraction after 5 minutes of washout of the phenylephrine, a time point equivalent to the onset of MI in our experiments, indicating

that PKCε undergoes a sustained translocation in response to phenylephrine preconditioning and may act as a mediator of preconditioning during the subsequent index ischaemia. The role of PKC as a mediator rather than a trigger of preconditioning is supported by the results of Yang *et al.* (1997), who showed that inhibition of PKC with staurosporine during the IPC stimulus does not prevent protection; however inhibition of PKC during the subsequent index ischaemia abolished the protective effect of IPC. This suggests that the sustained translocation of PKCε to the membrane fraction is required for the protective effect of phenylephrine preconditioning.

Several possible mechanisms have been proposed for the cardioprotective effect of PKCε activation. PKCε has been shown to translocate to the mitochondria following IPC (Ohnuma *et al.*, 2002) and there is increasing evidence that mitochondrial targets of PKCε may be central to preconditioning (reviewed in Budas and Mochly-Rosen, 2007). The gap junctional protein Connexin 43 can be phosphorylated by PKCε (Doble *et al.*, 2000). Connexin 43 is required for IPC (Schulz *et al.*, 2003, Schwanke *et al.*, 2002), although there is still debate concerning the mechanism responsible for the protective effect of Connexin 43 in IPC because it appears that gap junction formation may not be necessary for preconditioning (Li *et al.*, 2004, Boengler *et al.*, 2005). It has been demonstrated that Connexin 43 is present in subsarcolemmal mitochondria and the concentration of Connexin 43 in mitochondria is increased following IPC, suggesting that a mitochondrial role of Connexin 43 could be responsible for preconditioning (Boengler *et al.*, 2009).

It has been shown that PKCε co-immunoprecipitates with the VDAC, ANT and hexokinase II putative components of the MPTP (Baines *et al.*, 2003). PKCε is able to phosphorylate VDAC and possibly the ANT, and has been shown to modulate the opening of the MPTP during ischaemia-reperfusion. However, the VDAC and ANT

appear not to be essential for MPTP formation (Baines *et al.*, 2007, Kokoszka *et al.*, 2004). PKCε is also involved in modulation of the *mito*K_{ATP} channel (Jaburek *et al.*, 2006, Kim *et al.*, 2006, Hassouna *et al.*, 2004). There is evidence that the ANT could be part of the *mito*K_{ATP} channel (Ardehali *et al.*, 2004, Kopustinskiene *et al.*, 2003), therefore phosphorylation of the ANT by PKCε may be responsible for *mito*K_{ATP} opening, which has in turn been suggested to reduce MPTP opening during the subsequent reperfusion period (Lim *et al.*, 2007, Costa *et al.*, 2006). Opening of *mito*K_{ATP} channels also leads to mitochondrial swelling, which can increase the rate of mitochondrial respiration and increase ATP production (Lim *et al.*, 2002).

As well as modulation of *mito*K_{ATP} channels, it is possible that PKCε may be able to modulate *sarc*K_{ATP} channels. Aizawa *et al.* (2004) showed that activation of PKCε can prime the *sarc*K_{ATP} channel to open in response to a subsequent exposure to isoflurane. Such priming of the channel by PKCε could induce early activation of *sarc*K_{ATP} channels during ischaemia resulting in protection. The same group also examined the effect of PKCε and δ on channel opening following isoflurane preconditioning (Marinovic *et al.*, 2005). Pretreatment of myocytes the PKCε activator KAE1-1 increased *sarc*K_{ATP} current activated by pinacidil in their study, however, inhibitor studies suggest that PKCδ and not ε, is responsible for the increase in current following isoflurane preconditioning. Although pre-treatment with the PKCε activator can modulate *sarc*K_{ATP} channels, the effect has not been observed following any other form of preconditioning, therefore the physiological relevance of the mechanism is unknown.

Cytochrome C oxidase subunit IV is phosphorylated by PKCε, resulting in an increase in cytochrome C oxidase activity (Ogbi and Johnson, 2006). Increased cytochrome C oxidase activity is likely to enhance ATP production which may improve cardiac ATP levels during ischaemia. However, while this mechanism may be relevant to hearts/cells

subject to true ischaemia or hypoxia, cytochrome C oxidase is inhibited by cyanide (Wainio and Greenless, 1960), therefore this mechanism is unlikely to be involved in the protective effect of PKCε in our study.

PKCδ: The role of PKCδ in ischaemia-reperfusion injury and preconditioning is particularly ambiguous. Inhibition of PKCδ with the PKCδ inhibitor peptide δV1-1, has been shown to protect the heart from the damaging effects of ischaemia-reperfusion through a reduction in reperfusion injury (Inagaki *et al.*, 2003b, Chen *et al.*, 2001, Sivaraman *et al.*, 2009), and the same peptide (also known as KAI-9803) caused a consistent, non-significant improvement in creatine kinase and ST-segment elevation when administered following myocardial infarction in a 'first in human' study (Bates *et al.*, 2008). In contrast, overexpression of a constitutively active form of PKCδ has been observed to increase the survival of neonatal cardiac myocytes subjected to simulated ischaemia and re-energisation (Zhao *et al.*, 1998). IPC also increased infarct size in PKCδ null mice (Mayr *et al.*, 2004), and infarct size was reduced in mouse hearts treated with the ψδRACK PKCδ activator peptide 60 minutes prior to ischaemia (Inagaki and Mochly-Rosen, 2005), suggesting that PKCδ activation can be protective.

We observed translocation of PKCδ to the membrane following phenylephrine preconditioning that was maintained following wash out of phenylephrine; indicating that PKCδ remains activated by phenylephrine preconditioning at a time equivalent to the start of the index ischaemia, which is the critical period for PKC activation in preconditioning (Yang *et al.*, 1997). In agreement with results obtained by Mochly-Rosen's group (Inagaki *et al.*, 2003b, Chen *et al.*, 2001), inhibition of PKCδ increased the contractile recovery of ventricular myocytes exposed to MI and re-energisation and slightly increased recovery of contractile activity in phenylephrine preconditioned myocytes, suggesting that PKCδ may increase cellular injury during MI

and re-energisation. A PKCδ activator peptide (ψδRACK) also partially blocked the phenylephrine preconditioning-induced increase in contractile recovery, but unexpectedly activation of PKCδ in non-preconditioned myocytes resulted in an increase in contractile recovery.

These results suggest that PKCδ increases injury in ventricular myocytes exposed to MI and re-energisation, likely through the earlier identified increase in reperfusion injury (Inagaki *et al.*, 2003b, Chen *et al.*, 2001); however, PKCδ may also protect myocytes from MI re-energisation injury. We suggest that the outcome of PKCδ activation is dependent on the time course of activation during phenylephrine preconditioning. Activation of PKCδ during the preconditioning period appears to be cardioprotective (Inagaki and Mochly-Rosen, 2005). We propose that PKCδ activation protects the heart during preconditioning through an increase in *sarc*K_{ATP} current density, see Marinovic *et al.* (2005), section 6.4.5/Figure 6.8, and further discussion in section 7.8.2 onwards. In addition, it has been shown that PKCδ may be upstream (Inagaki and Mochly-Rosen, 2005) or downstream (Rybin *et al.*, 2007) of PKCε in some signalling pathways, which could allow integration of the signalling by these kinases.

It appears that PKCδ may not be responsible for the energy sparing effects of preconditioning because although PKCδ is able to phosphorylate metabolic proteins such as the F_1F_0-ATPase, pyruvate dehydrogenase, malate dehydrogenase and NADH dehydrogenase following IPC (Mayr *et al.*, 2004), it has been shown that PKCδ does not protect the cellular energy levels during ischaemia, and activation of PKCδ may in fact contribute to the reduction in ATP levels during ischaemia-reperfusion (Mayr *et al.*, 2004, Fryer *et al.*, 2002, Nguyen *et al.*, 2008).

Activation of PKCδ during the reperfusion period increases reperfusion injury (Inagaki *et al.*, 2003b, Chen *et al.*, 2001). Modulation of the metabolic proteins detailed above

could be involved in the PKCδ-dependent reperfusion injury by enhancing ATP depletion during ischaemia-reperfusion. Apoptosis is also increased by PKCδ following ischaemia-reperfusion (Inagaki *et al.*, 2003a, Reyland, 2007). Tyrosine phosphorylation and caspase cleavage of PKCδ appear to be required for induction of apoptosis by PKCδ (Steinberg, 2004, Reyland, 2007, DeVries-Seimon *et al.*, 2007).

In order to determine the time course of the protective and damaging effects of PKCδ activation it would be necessary to carry out further experiments. The effect of PKCδ activation and inhibition during the MI, and re-energisation phases could be examined using the δV1-1 and ψδRACK peptides.

7.5 Are Mitogen-activated protein kinases ERK, JNK and p38 involved in phenylephrine preconditioning?

7.5.1 Inhibition of ERK does not affect phenylephrine preconditioning

ERK is activated by mitogenic and stressful stimuli in the ventricular myocyte and can be activated downstream of PKCε (Ping *et al.*, 1999a). It has been previously shown that U-0126 or PD98059 (which inhibit MEK, the kinase upstream of ERK), prevented IPC in the isolated rat or rabbit heart (Fryer *et al.*, 2001b, Solenkova *et al.*, 2006). Phenylephrine treatment has been shown to activate ERK in the isolated perfused rat heart (Lazou *et al.*, 1998), however the role of ERK in phenylephrine preconditioning has not been studied previously.

Our data demonstrate that the MEK inhibitor PD98059 did not affect the contractile recovery of phenylephrine preconditioned myocytes, and protected control myocytes from MI re-energisation injury to a similar level to that seen with phenylephrine preconditioning (section 5.4.1/Figure 5.2A). This suggests that ERK may increase injury following MI and re-energisation, which is in contrast to the protective role

previously identified for ERK in IPC (Fryer *et al.*, 2001b, Solenkova *et al.*, 2006). Reperfusion appears to be the critical time for the protective effect of ERK in IPC (Solenkova *et al.*, 2006), therefore it is possible that we failed to see a protective effect of ERK because we administered the MEK inhibitor prior to preconditioning rather than during the re-energisation period. However, ERK inhibition protected control myocytes from MI re-energisation injury, suggesting that ERK activation during MI re-energisation increases cellular injury.

We suggest that the damaging effect of ERK in phenylephrine preconditioning observed in our study may be due to modulation of NHE activity by ERK during MI re-energisation. Moor *et al.* (2001) determined that both ERK and a downstream kinase p90RSK are able to phosphorylate the NHE increasing NHE activity during simulated ischaemia-reperfusion in neonatal ventricular myocytes. Stimulation of NHE activity leads to Na^+ accumulation, NCX activation and Ca^{2+} loading of myocytes (Schafer *et al.*, 2001, Baczkó *et al.*, 2003, Baczkó *et al.*, 2008, Allen and Xiao, 2003), and inhibition of ERK or NHE-1 decreased Ca^{2+} overload induced by H_2O_2 in neonatal ventricular myocytes (Rothstein *et al.*, 2002). Therefore ERK-mediated NHE activation during MI and re-energisation may increase necrotic cell death and/or stunning of ventricular myocytes by increasing Ca^{2+} loading.

7.5.2 Inhibition of JNK does not affect phenylephrine preconditioning

JNK is a member of the MAPK family that is activated primarily by stressful stimuli. JNK can be activated by IPC (Ping *et al.*, 1999b, Sato *et al.*, 2000) and acute phenylephrine treatment (Lazou *et al.*, 1998), but the role of JNK in phenylephrine preconditioning has not been examined. Similarly to ERK, our data demonstrated that JNK did not affect the contractile recovery of phenylephrine preconditioned myocytes, however the contractile recovery of control myocytes subject to MI and re-energisation

was increased by pretreatment with the JNK inhibitor SP600125 (section 5.4.2/Figure 5.2B). There was no significant difference between the contractile recovery of phenylephrine preconditioned myocytes, SP600125 treated control myocytes and phenylephrine preconditioned myocytes treated with SP600125. We therefore propose that JNK is not involved in phenylephrine preconditioning. It is possible that inhibition of JNK could have two independent effects, inhibiting phenylephrine preconditioning, while increasing the survival of ventricular myocytes via a different mechanism.

In agreement with our data, inhibition of JNK through over-expression of JNK interacting protein 1 or a dominant negative form of the JNK upstream kinase (JNKK2) decreased cell death in H9c2 cells subject to simulated ischaemia-reperfusion (He *et al.*, 1999). Contrary to this, JNK inhibition increased both the total and apoptotic cell death in neonatal myocytes subjected to simulated ischaemia-reperfusion (Engelbrecht *et al.*, 2004), though the observed differences could be due to differences in the experimental systems used. It has been established that JNK has both a pro-apoptotic and anti-apoptotic role and JNK signalling may be influenced by the time course of activation and parallel activation of pro-apoptotic or pro-survival pathways, reviewed in Dhanasekaran and Reddy (2008). JNK may be cardioprotective in some forms of preconditioning depending on the balance of protective and damaging pathways activated but it appears that JNK is not involved in the protection afforded by phenylephrine preconditioning in our study.

7.5.3 p38 is required for phenylephrine preconditioning of isolated ventricular myocytes

p38 is also activated primarily by stressful stimuli and is able to modulate proteins implicated in ischaemic and pharmacological preconditioning. Activation of p38 results in the phosphorylation of MAPKAPK2 (Nagy *et al.*, 2007), which in turn

phosphorylates effector proteins such as αB crystallin (Eaton *et al.*, 2001) and the related small heat shock protein HSP27 (Weber *et al.*, 2005) resulting in cytoskeletal re-arrangement and formation of F-actin (Dalle-Donne *et al.*, 2001). p38 (particularly p38β) co-localisation with Connexin 43 following IPC has been observed to correlate with a reduction in infarct size, suggesting that p38 may modulate Connexin 43 function in response to IPC (Schwanke *et al.*, 2002). Inhibition of p38 has also been observed to increase cardiac myocyte apoptosis following IPC (Nagy *et al.*, 2007).

We found that inhibition of p38 with SB202190 completely abolished the protective effect of phenylephrine preconditioning on the contractile recovery of ventricular myocytes (section 5.4.3/Figure 5.3). This result is supported by a previous study in the human myocardium by Loubani and Galiñanes (2002), in which they demonstrated that phenylephrine preconditioning was inhibited by the p38 inhibitor SB203580. Using of activators and inhibitors of *mito*K_{ATP} channels, PKC and p38 the authors also suggested that p38 activation occurs downstream of *mito*K_{ATP} and PKC.

The majority of the p38 present in the heart is p38α and β, which are both inhibited by the CSAIDs SB202190 and SB203580 used in these studies, suggesting that the α and/or β isoforms mediate the protective effects observed. Overexpression of MKK6 which activates both p38α and β decreased the infarct size in hearts subjected to ischaemia-reperfusion (Martindale *et al.*, 2005). However, overexpression of MKK3, alone or in combination with p38α increased neonatal cardiomyocyte cell death, while overexpression of p38β and MKK3 increased the survival of cells to control levels (Wang *et al.*, 1998). Therefore, it appears that activation of p38α may be injurious and activation of p38β may be protective. Taken together with our findings, these data suggest that activation of p38β may be responsible for phenylephrine preconditioning of isolated ventricular myocytes. Furthermore, in this study we have identified a novel

mechanism for p38-dependent cardioprotection, involving modulation of the *sarc*K_{ATP} channel (section 6.4.6/Figure 6.9, and further discussion in section 7.8.2).

Although we examined whether p38 was activated in response to the phenylephrine preconditioning stimulus, we did not observe a consistent activation of p38 following the phenylephrine preconditioning stimulus (section 5.4.4/Figure 5.4). This may be due to a high basal activity of p38 or dephosphorylation of p38 due to phosphatase activity. It is also possible that p38 is not activated during the preconditioning stimulus but later during the index ischaemia. Previous studies showed that p38 was poorly activated by an IPC stimulus (Eaton *et al.*, 2001), but activation was increased during the subsequent index ischaemia (Takeishi *et al.*, 2001, Eaton *et al.*, 2001). Mocanu *et al.* (2000) showed that treatment with SB203580 during the preconditioning stimulus did not affect the protection afforded by IPC, however protection was lost when the inhibitor was administered during the first 15 minutes of the index ischaemia. In addition, Loubani and Galiñanes (2002) placed p38 downstream of PKC in phenylephrine preconditioning, therefore we propose that similar to PKC, the index ischaemia is likely to be the critical period for p38 activation (Yang *et al.*, 1997, Mocanu *et al.*, 2000). We did not examine p38 phosphorylation during the index ischaemia because loss of cytosolic protein due to cell lysis resulted in highly variable results.

7.6 AMPK is required for phenylephrine preconditioning

AMPK can be activated by an increase in the AMP:ATP ratio resulting in a decrease in ATP-consuming pathways and up-regulation ATP generating pathways (Hardie, 2008). AMPK therefore represents a potential target for the protective effect afforded by preconditioning. Several studies have demonstrated that AMPK activation is necessary for IPC (Sukhodub *et al.*, 2007, Nishino *et al.*, 2004, Khaliulin *et al.*, 2007), however AMPK activation during preconditioning may be specific to IPC because of the

perturbations in the AMP:ATP ratio brought about during the preconditioning ischaemia. The role of AMPK in phenylephrine preconditioning has not been investigated to date. Our data show that inhibition of AMPK with compound C abolished phenylephrine preconditioning (section 5.4.5/Figure 5.5), indicating that AMPK activation is required for phenylephrine preconditioning. Additionally, we found that the phenylephrine preconditioning stimulus increased AMPKα phosphorylation at Thr172 by 2.4 ± 0.4 fold, which returned to control levels following washout with normal Tyrode (section 5.4.6/Figure 5.6A and B). Xu *et al.* (2007) showed a similar activation (1.5 fold) of AMPK in response to a 10 minute phenylephrine treatment in H9c2 cells. These results indicate that phenylephrine preconditioning does activate AMPK, however the relevance of AMPK phosphorylation prior to the index ischaemia depends upon where AMPK fits in the signalling pathway.

Nishino *et al.* (2004) observed that PKC inhibition abolished the increase in AMPK activity induced by IPC and therefore placed PKC upstream of AMPK. This finding led us to investigate the identity of the PKC isoform responsible for the phenylephrine-dependent phosphorylation of AMPK. Inhibition of PKCδ reduced AMPK phosphorylation to control levels in our study, while inhibition of PKCε resulted in a partial inhibition of AMPK phosphorylation, however this inhibition did not reach statistical significance (section 5.4.7/Figure 5.6A and C). We propose that PKCδ is involved in the activation of AMPK during phenylephrine preconditioning although it is not clear whether PKCδ can directly activate AMPK or its upstream kinases. As mentioned above for p38, if PKC is upstream of AMPK, then the index ischaemia is likely to be the important period for AMPK activation. The consequences of transient activation of AMPK prior to MI are not certain, but this may prime the kinase for later activation, cause translocation of AMPK or binding to scaffold and/or substrate proteins.

Further experiments examining the activation of AMPK during MI (and it's PKC-dependence) are required to support this hypothesis, however we observed a loss of protein during MI due to cell lysis, and so examined time points prior to MI. Administration of compound C during the MI and/or re-energisation periods could also be used to determine the crtitical period for AMPK activation in phenylephrine preconditioning.

AMPK can be activated via phosphorylation of the α-subunit at Thr172 by the upstream kinases LKB1 and CaMKK. Phenylephrine treatment increases intracellular Ca^{2+} levels through activation of the G_q coupled α_1-adrenoceptor, therefore we thought it likely that the Ca^{2+}-activated kinase CaMKK could be responsible for AMPK activation in our study. We found that inhibition of CaMKK blocked the increase in contractile recovery afforded by phenylephrine preconditioning (section 5.4.8/Figure 5.7), to a similar degree as inhibition of AMPK. This suggests that CaMKK may be the relevant AMPK kinase in phenylephrine preconditioning.

As well as activating AMPK, CaMKK is able to activate the calmodulin-dependent kinases or CaMKs (Uemura *et al.*, 1998). Studies using the CaMK II inhibitor KN-93 have indicated that CaMK II may play a protective role in IPC (Benter *et al.*, 2005, Osada *et al.*, 2000), whereas transgenic expression of a CaMK II specific inhibitor peptide AC3-I, decreased infarct size in IPC and control hearts and increased *sarc*K_{ATP} channel current through an increase in surface expression of Kir6.2 (Li *et al.*, 2007) suggesting that CaMK II activation during ischaemia may be injurious (Liu *et al.*, 2001). While we propose that the protective effect of CaMKK is likely to be mediated by AMPK, we cannot rule out the possibility that the inhibition of phenylephrine preconditioning produced by the CaMKK inhibitor STO-609 is a result of inhibition of CaMK II and not AMPK. However, it seems that CaMK II may oppose the effects of

phenylephrine preconditioning, suggesting that CaMK II activation is unlikely to be responsible for the protective effects observed with phenylephrine preconditioning.

7.7 Phenylephrine preconditioning requires activation of both *sarc*K_{ATP} and *mito*K_{ATP} channels

It was initially proposed that opening of *sarc*K_{ATP} channels could be responsible for cardioprotection through shortening of the APD and a decrease in Ca^{2+} loading via voltage-gated Ca^{2+} channels (Noma, 1983, Lederer *et al.*, 1989). While it was found that *sarc*K_{ATP} channels were responsible for APD shortening (Yao and Gross, 1994), and involved in preconditioning (Jovanović *et al.*, 1998b, Seino and Miki, 2003, Yao and Gross, 1994, Suzuki *et al.*, 2002), it was later shown that APD shortening is not required for preconditioning (Grover *et al.*, 1995). The characterisation of a *mito*K_{ATP} channel, added to the controversy concerning the role of *sarc*K_{ATP} channels in IPC, because the K_{ATP} channel blocker glibenclamide is able to block both the *sarc*- and *mito*K_{ATP} channels. There is now evidence for the involvement of both the *sarc*K_{ATP} (Jovanović *et al.*, 1998b, Suzuki *et al.*, 2002) and *mito*K_{ATP} channel (Liu *et al.*, 1998, Garlid *et al.*, 1997) in preconditioning.

The time course of *mito*- and *sarc*K_{ATP} activation in whole hearts subject to IPC enhanced by adenosine perfusion was determined by Toyoda *et al.* (2000). In their study, preconditioning was abolished by treatment with 5-HD during the index ischaemia but not in the reperfusion period, while HMR1883 prevented improvement in post-ischaemic function, but not infarct size, induced by preconditioning when applied during ischaemia and/or reperfusion. Similarly, Light *et al.* (2001), observed that 5-HD increased intracellular Ca^{2+} during hypoxia but not reperfusion, while HMR1883 had little effect on intracellular Ca^{2+} levels during hypoxia but increased Ca^{2+} overload

following reperfusion. Therefore, it appears that *mito*K_{ATP} channels are activated during ischaemia, while *sarc*K_{ATP} channels are activated during both the ischaemia and reperfusion periods.

The effect of glibenclamide on phenylephrine preconditioning has not been examined previously. We found that glibenclamide completely abolished the protective effect of phenylephrine preconditioning on contractile recovery, suggesting that *mito*- and/or *sarc*K_{ATP} channels are involved in the protection afforded by phenylephrine preconditioning (section 3.2.4/Figure 3.7). Glibenclamide also slightly reduced the contractile recovery of non-preconditioned myocytes compare to control, suggesting that activation of *sarc*- and/or *mito*K_{ATP} channels are involved in protection from MI and re-energisation injury even in the absence of preconditioning. Glibenclamide does also have K_{ATP}-independent effects including inhibition of carnitine palmitoyltransferase, ABC transporters and Cl^- channels, Ca^{2+} channels and the Na^+/K^+-ATPase (Hanley and Daut, 2005, Lee and Lee, 2005), which may also be responsible for the reduced recovery in the presence of glibenclamide.

In order to determine whether the *sarc*K_{ATP} channel was involved in phenylephrine preconditioning, we examined the effect of inhibition of *sarc*K_{ATP} channels with HMR1883 on the contractile recovery of myocytes subject to MI and re-energisation. HMR1883 reduced the recovery of contractile activity in phenylephrine preconditioned myocytes in our study (section 6.4.1/Figure 6.2). This is the first observation that *sarc*K_{ATP} channels are involved in the protective effect of phenylephrine preconditioning. While HMR1883 decreased the recovery of phenylephrine preconditioned myocytes, we also observed that the recovery of phenylephrine preconditioned myocytes treated with HMR1883 was marginally higher than in control myocytes, although this did not reach statistical significance. Previous studies have

shown that IPC was not completely blocked by HMR1883, or its sodium salt HMR1098 (Fryer *et al.*, 2000, Jung *et al.*, 2000). These results may be explained by the observation that block of *sarc*K_{ATP} current by HMR1098 is antagonised by increasing ADP concentrations, so that HMR 1098 is unable to block *sarc*K_{ATP} current evoked by MI due to the increased MgADP concentrations (Rainbow *et al.*, 2005). In fact, while some previous studies did not observe a significant inhibition of IPC using HMR1883 in anaesthetised rats or rabbits (Fryer *et al.*, 2000, Jung *et al.*, 2000), phenylephrine preconditioning of isolated ventricular myocytes was inhibited by HMR1883 in our study.

We also observed that inhibition of *mito*K_{ATP} channels with 5-HD blocked phenylephrine preconditioning (section 6.4.2/Figure 6.4). This is in agreement with previous studies that found phenylephrine preconditioning was inhibited by 5-HD in isolated rabbit hearts (Cohen *et al.*, 2001), human atrial appendage (Loubani and Galiñanes, 2002), and isolated rat ventricular myocytes (Gao *et al.*, 2007). Gao *et al.* (2007) also demonstrated that the increase in ATP levels, and decrease in Ca^{2+} levels and cytochrome c release afforded by phenylephrine preconditioning were abolished by 5-HD.

These results suggest that both *sarc*- and *mito*K_{ATP} channels are involved in phenylephrine preconditioning. However, from this data it is not possible to determine whether *sarc*- and *mito*K_{ATP} channels are activated by the same signalling pathway. Evidence from previous studies suggests that *sarc*- and *mito*K_{ATP} channels may act via parallel pathways (Light *et al.*, 2001, Kong *et al.*, 2001, Sanada *et al.*, 2001). However, Suzuki *et al.* (2003) demonstrated that the cardioprotective effect of diazoxide was abolished in hearts from Kir6.2 knock out mice or wild type mice treated with HMR1883 but not 5-HD. Furthermore, diazoxide caused APD shortening during

ischaemia in wild type mouse hearts in their study, suggesting that diazoxide can activate the *sarc*K_{ATP} channel and shorten the APD. While the authors, citing D'hahan *et al.* (1999) suggest that diazoxide directly activates *sarc*K_{ATP} channels, these results may also indicate that *mito*K_{ATP} channels act upstream of *sarc*K_{ATP}. In order to test whether *mito*K_{ATP} are upstream of *sarc*K_{ATP} channels, we could examine the effect of combined treatment of phenylephrine preconditioned myocytes with both 5-HD and HMR1883. In addition, because phenylephrine preconditioning increased *sarc*K_{ATP} current density, we could test the effect of *mito*K_{ATP} inhibition on *sarc*K_{ATP} current density activated by MI.

Our findings indicate that PKCδ, AMPK and p38 are upstream of *sarc*K_{ATP} channels, and when taken together with previous observations that *mito*K_{ATP} channels act upstream of PKC and p38 (Loubani and Galiñanes, 2002), this suggests that *mito*K_{ATP} channels could be upstream of *sarc*K_{ATP} channels in phenylephrine preconditioning. Additionally, treatment with either HMR1883 or 5-HD was sufficient to inhibit preconditioning in our study suggesting that both *mito*- and *sarc*K_{ATP} channels could be acting via the same pathway.

7.7.1 The effect of glibenclamide and HMR1883 on time to contractile failure and rigor contracture

Activation of *sarc*K_{ATP} channels by metabolic inhibition is known to result in curtailment of the action potential leading to eventual failure of electrical, and therefore mechanical activity (Lederer *et al.*, 1989, Nakaya *et al.*, 1991). Therefore, the time to contractile failure during MI is an appropriate indicator of *sarc*K_{ATP} channel activation and is a useful tool to determine this from groups of cells. Knockout of Kir6.2, or treatment of wild-type mouse hearts with the *sarc*K_{ATP} specific inhibitor HMR1098 but not the *mito*K_{ATP} inhibitor 5-HD, has been found to increase the time to contractile

arrest during ischaemia (Suzuki *et al.*, 2002). Similarly, expression of a C-terminal fragment of SUR2A that reduced functional *sarc*K_{ATP} expression, prevented APD shortening and increased the time to contractile failure in ventricular myocytes (Rainbow *et al.*, 2004). In contrast, we observed that glibenclamide (section 3.2.4/Figure 3.8A) or HMR1883 (section 6.4.1/Figure 6.3A) treatment did not significantly affect the mean time to contractile failure in isolated ventricular myocytes exposed to MI.

Ischaemic rigor contracture occurs due to inhibition of cross-bridge cycling and rigor bond formation at low, 10-100 μM, but not zero ATP levels, and correlates well with the depletion of ATP levels within the ventricular myocyte (Hearse *et al.*, 1977, Bremel and Weber, 1972, Stapleton and Allshire, 1998). Therefore, the time to ischaemic rigor contracture provides an indicator of ATP depletion during ischaemia. The time to onset of rigor contracture can also be modulated by the degree of Ca^{2+} loading (Hearse *et al.*, 1977). Suzuki *et al.* (2002), observed a decrease in time to ischaemic rigor contracture in Kir6.2 knock out hearts or hearts treated with HMR1098. This was attributed to Ca^{2+} loading and ATP depletion caused by an increase in the time to cessation of contraction. Similarly, we observed a significant decrease in time to rigor contracture in myocytes treated with glibenclamide (section 3.2.4/Figure 3.8B) or HMR1883 (section 6.4.1/Figure 6.3B) during MI but this was not associated with any change to time to contractile failure, suggesting another mechanism may be responsible for the early onset of rigor contracture. Therefore, we think it likely that treatment of ventricular myocytes with glibenclamide or HMR1883 causes an early decline in ATP levels, possibly in combination with Ca^{2+} accumulation, in a manner that is independent of APD shortening, leading to a decrease in the time to rigor contracture. Additionally, the decrease in mean time to rigor contracture was observed in myocytes treated with either

glibenclamide or HMR1883 indicating that inhibition of the *sarc*K_{ATP} channel may be responsible for this effect.

7.8 Modulation of *sarc*K_{ATP} current by phenylephrine preconditioning

Whilst the failure of phenylephrine preconditioning to alter the time to contractile failure suggests that early *sarc*K_{ATP} channel activation leading to electrical failure was not evident, protection against MI-reperfusion injury could still be mediated via *sarc*K_{ATP} channel activation, through a strong and sustained hyperpolarisation of the membrane potential working to reduce Ca^{2+}-loading (Baczkó *et al.*, 2004, Light *et al.*, 2001, Jovanović *et al.*, 1998b). Hypoxic preconditioning has been shown to increase the magnitude of *sarc*K_{ATP} current and decrease the time to current activation (Budas *et al.*, 2004, Liu *et al.*, 2001). In addition, pharmacological preconditioning using adenosine or PMA increased *sarc*K_{ATP} current (Light *et al.*, 2000, Liu *et al.*, 1996, Hu *et al.*, 1999, Hu *et al.*, 2003). Our study is the first to investigate the effect of phenylephrine preconditioning on the *sarc*K_{ATP} current. We found that phenylephrine preconditioning increased the peak *sarc*K_{ATP} current activated by MI (section 6.4.3/Figure 6.5C). Previous studies have reported that the preconditioning-induced increase in *sarc*K_{ATP} current is PKC-dependent and may involve a change in the gating of the channel by ATP (Light *et al.*, 2000, Hu *et al.*, 1999), or an increase in surface expression of *sarc*K_{ATP} channels (Sukhodub *et al.*, 2007, Budas *et al.*, 2004). From our results it is not possible to determine whether the increase in current is due to a change in translocation or gating of the *sarc*K_{ATP} channel. The observed increase in *sarc*K_{ATP} current density together with the finding that the *sarc*K_{ATP} channel inhibitor HMR1883 abolishes phenylephrine preconditioning, indicate that phenylephrine preconditioning may protect

isolated ventricular myocytes via an increase in *sarc*K_{ATP} channel current density, and not early failure of the action potential.

Unlike hypoxic preconditioning, phenylephrine preconditioning did not significantly alter the time to current activation or time to development of peak current (section 6.4.3/Figure 6.6). This is consistent with our finding that the time to contractile failure was only increased by 8 and 7 seconds following phenylephrine preconditioning (section 3.2.1/Figure 3.3 and section 3.2.3/Figure 3.6). Additionally, Rehring *et al.* (1997) showed that although adenosine and IPC decreased the time to action potential failure in the isolated rat heart (measured from the final QRS complex of the ECG), phenylephrine preconditioning did not significantly alter the time to failure of the action potential. Taken together, these observations suggest that phenylephrine preconditioning has little effect on the time course of *sarc*K_{ATP} activation and APD shortening during the index ischaemia and that an increase in APD shortening is unlikely to underlie the protective effect of phenylephrine preconditioning.

7.8.1 Incomplete block of *sarc*K_{ATP} current by glibenclamide is sufficient to prevent phenylephrine preconditioning

While it was expected that treatment with 10 μM glibenclamide applied simultaneously with MI would completely abolish *sarc*K_{ATP} current activation, we observed that glibenclamide only partially blocked *sarc*K_{ATP} current in our study (section 6.4.7/Figure 6.10). The decrease in peak current density observed in our study equates to a 48% decrease, which is similar to previously observed levels of MI-activated *sarc*K_{ATP} current inhibition by glibenclamide of 46% and 40% (Rainbow *et al.*, 2005, Krause *et al.*, 1995). The relief of glibenclamide block of the *sarc*K_{ATP} channel during MI is due to an antagonistic effect of ADP on sulphonylurea block of the *sarc*K_{ATP} channel

(Venkatesh *et al.*, 1991, Ripoll *et al.*, 1993). Mg^{2+} is required for the reversal of sulphonylurea block of the *sarc*K_{ATP} channel by ADP (Ripoll *et al.*, 1993), and this may explain the difference between our results and those of a previous study from our group, which found that glibenclamide block of *sarc*K_{ATP} channels in inside-out patches was not reversed by ADP in the absence of Mg^{2+} (Lawrence *et al.*, 2002). In contrast, we carried out whole-cell recordings so it is likely that the Mg^{2+} levels within ventricular myocytes are sufficiently high, and our pipette solution contained 0.4 mM Mg-ATP, suggesting that Mg^{2+} would not limit ADP-dependent reversal of sulphonylurea block of *sarc*K_{ATP} channels.

The ability of ADP to prevent binding of the sulphonylurea HMR1883 to the *sarc*K_{ATP} channel has also complicated investigations into the role of the *sarc*K_{ATP} channel in preconditioning. HMR1883 did not block preconditioning in some studies, and this may be due to incomplete inhibition of *sarc*K_{ATP} channels or a lack of involvement of the *sarc*K_{ATP} channel in preconditioning (Fryer *et al.*, 2000, Jung *et al.*, 2000). In fact, Rainbow *et al.* (2005) observed only a 6% decrease in *sarc*K_{ATP} current density in ventricular myocytes when HMR1098 was applied simultaneously with metabolic inhibition, under conditions similar to those used in our study.

Based on our results it appears that a 50% inhibition of *sarc*K_{ATP} current may be sufficient to completely abolish preconditioning. Phenylephrine preconditioning induced a 46% increase in peak *sarc*K_{ATP} current density, therefore a 50% inhibition of this current would be sufficient to reduce the current to baseline levels and inhibit the protection afforded by phenylephrine preconditioning-induced *sarc*K_{ATP} channel modulation.

7.8.2 Are PKCδ, AMPK and p38 involved in phenylephrine preconditioning-induced modulation of *sarc*K_{ATP} current?

PKCδ: Several studies have demonstrated that PKC is able to modulate *sarc*K_{ATP} channel function (Light *et al.*, 2000, Ito *et al.*, 2001, Liu *et al.*, 1996, Marinovic *et al.*, 2005, Hu *et al.*, 1999), but there has been little study of the isoform(s) of PKC responsible for modulation of the *sarc*K_{ATP} channel. The increase in amplitude of the MI-induced *sarc*K_{ATP} current in phenylephrine preconditioned myocytes in our study could be abolished by a specific PKCδ inhibitor peptide (section 6.4.5/Figure 6.8). These results demonstrate that PKCδ is responsible for the increase in *sarc*K_{ATP} current density induced by phenylephrine preconditioning (see Figure 7.1 for proposed signalling pathway). Similarly, Marinovic *et al.* (2005) found that isoflurane preconditioning increased the *sarc*K_{ATP} current activated upon subsequent application of pinacidil and the effect could be abolished by inhibition of PKCδ, but not PKCε. In the same study, it was also shown that activation of PKCε or PKCδ using the specific activators KAD1-1 and KAE1-1 resulted in a similar increase in peak current to that observed following isoflurane. This suggests that PKCδ may be required for modulation of *sarc*K_{ATP} current in two different forms of pharmacological preconditioning suggesting a common mechanism of cardioprotection. We did not examine the effect of PKCε inhibition on the phenylephrine preconditioning-induced increase in *sarc*K_{ATP} current density.

Inhibition PKCδ prior to phenylephrine preconditioning abolished the increase in current density, and PKCδ but not PKCε inhibition, prevented activation of the downstream kinase AMPK in our study. Therefore, we feel it is unlikely that PKCε is involved in modulation of *sarc*K_{ATP} channel via this mechanism. However, it is possible that PKCε may be involved in the modulation of *sarc*K_{ATP} channels. To test this

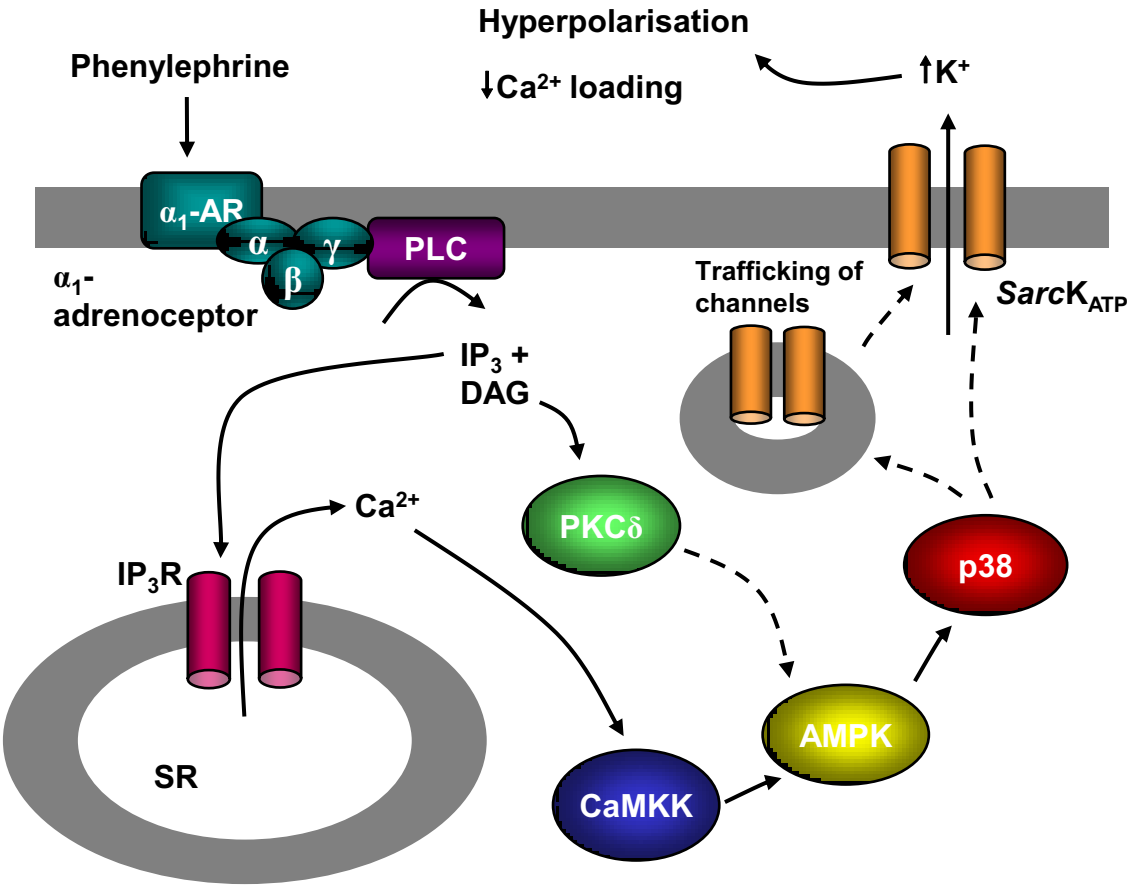

Figure 7.1. Proposed signalling pathway responsible for modulation of *sarc*K_{ATP} by phenylephrine preconditioning.

Diagram showing proposed pathway responsible for modulation of *sarc*K_{ATP} channels by phenylephrine preconditioning. Activation of the Gq-coupled α_1-adrenoceptor leads to activation of phospholipase C, and production of IP_3 and DAG. DAG is able to activate the Ca^{2+}-independent PKCδ, while binding of IP_3 to IP_3 receptors on the SR causes release of Ca^{2+} from intracellular stores that can activate CaMKK. These kinases are then able to activate AMPK and p38, leading to modulation of *sarc*K_{ATP} channel activity through an increase in trafficking of channels to the sarcolemmal membrane and/or modulation of the gating properties of the channel. Dashed lines may indicate indirect effects.

possibility, it would be necessary to investigate the effect of pretreatment with the PKCε inhibitor εV1-2 prior to phenylephrine preconditioning on the MI-activated *sarc*K_{ATP} current.

AMPK: A recent study by Jovanovic's group showed that AMPK mediates IPC through an increase in trafficking of the *sarc*K_{ATP} channel to the sarcolemmal membrane (Sukhodub *et al.*, 2007). We have shown that PKCδ acts upstream of AMPK in phenylephrine preconditioning (section 5.4.7/Figure 5.6), and that PKCδ is involved in the modulation of *sarc*K_{ATP} current by phenylephrine preconditioning (section 6.4.5/Figure 6.8). Therefore, we thought it likely that AMPK was also involved in mediating the phenylephrine preconditioning-induced increase in the *sarc*K_{ATP} current activated by MI. We found that this was indeed the case, inhibition of AMPK using compound C was sufficient to prevent the increase in *sarc*K_{ATP} current caused by phenylephrine preconditioning (section 6.4.4/Figure 6.7). We suggest that the increase in *sarc*K_{ATP} current induced by phenylephrine preconditioning may be mediated by a PKCδ- and AMPK-dependent increase in *sarc*K_{ATP} current (see Figure 7.1 for proposed pathway). The increased current density may occur through a translocation of channels to the sarcolemmal membrane similar to the mechanism observed for IPC by Sukhodub *et al.* (2007). A change in the affinity of the *sarc*K_{ATP} channel for ATP similar to that observed by Hu *et al.* (1999) and Light *et al.* (2000) may also occur, because previous studies have shown that ATP levels during ischaemia were significantly higher following phenylephrine preconditioning than in control rat hearts (Rehring *et al.*, 1997), or myocytes (Gao *et al.*, 2007). If the affinity of the *sarc*K_{ATP} channel for ATP were unchanged, this would result in a significant delay in *sarc*K_{ATP} channel opening; however we observed no difference in time to *sarc*K_{ATP} channel activation following phenylephrine preconditioning.

p38: Activation of p38 is required for phenylephrine preconditioning of ventricular myocytes (section 5.4.3/Figure 5.3). In addition, p38 appears to be activated down stream of PKCδ (Heidkamp *et al.*, 2001) and AMPK (Li *et al.*, 2005) in the heart. Therefore we thought it likely that p38 may be involved in modulating *sarc*K_{ATP} channel activity following phenylephrine preconditioning. We found that inhibition of p38 using SB202190 completely abolished the increase in *sarc*K_{ATP} current induced by phenylephrine preconditioning. This is the first finding indicating that the cardioprotective effect of p38 may be mediated via *sarc*K_{ATP} channels. Furthermore, we suggest that p38 may be acting downstream of PKCδ and AMPK to increase *sarc*K_{ATP} current (see Figure 7.1 for proposed pathway).

This hypothesis is strengthened by the presence of a similar signalling pathway involved in regulation of glucose transporter-4 (GLUT-4) translocation. PKC activation is required for the activation of AMPK by IPC in the heart (Nishino *et al.*, 2004) and GLUT-4 translocation to the sarcolemmal membrane was enhanced by activation of PKC or AMPK using the phorbol ester PMA or AMPK activator AICAR (Thong *et al.*, 2007). p38 is required for the AICAR, hypoxia or DNP stimulated increase in glucose uptake and GLUT-4 translocation (Pelletier *et al.*, 2005, Li *et al.*, 2005). Additionally, phenylephrine treatment has also been shown to increase GLUT-4 translocation to the sarcolemmal membrane (Egert *et al.*, 1999). It is therefore possible that the increase in GLUT-4 and *sarc*K_{ATP} channel activity induced by phenylephrine preconditioning may occur through similar mechanisms involving activation of PKCδ, AMPK and p38 and subsequent translocation of *sarc*K_{ATP} channels and GLUT-4 to the sarcolemmal membrane.

Both GLUT-4 and the *sarc*K_{ATP} channel subunit Kir6.2 have been shown to co-immunoprecipitate with Caveolin-3 in adult ventricular myocytes (Garg *et al.*, 2009,

Koneru *et al.*, 2007), indicating that these proteins could be present within the same intracellular vesicles or even physically associated. Das *et al.* (2008) showed that the allegedly protective p38β isoform can associate with Caveolin-3 under resting conditions in the rat heart, where it is suggested that p38β is inactive. They also showed that the amount of p38β associated with Caveolin-3 is reduced by IPC and increased by ischaemia-reperfusion suggesting that IPC releases activated p38β, which can then phosphorylate target proteins within the local environment. It appears that p38β may be present within the same microdomains as GLUT-4 and *sarc*K_{ATP} channels, so p38β may be the isoform responsible for inducing the translocation of *sarc*K_{ATP} and GLUT-4. Co-localisation of *sarc*K_{ATP} and GLUT-4 could also have an important physiological role, as GLUT-4 is responsible for glucose uptake in muscles, so association of these proteins could allow efficient coupling of cellular energy levels and membrane excitability. It has already been shown that two other important regulators of cellular energy homeostasis, adenylate kinase and creatine kinase, physically associate with *sarc*K_{ATP} channels allowing tight coupling of the energetic balance of the cell with membrane excitability (Carrasco *et al.*, 2001, Crawford *et al.*, 2002), suggesting that association of metabolic regulators with *sarc*K_{ATP} channels is a physiologically relevant phenomenon.

Translocation of GLUT-4 in response to phenylephrine preconditioning could also contribute to the protective effects of PKCδ, AMPK and p38 shown in our study. Increased membrane expression of GLUT-4 leads to increased glucose uptake during ischaemia and reperfusion. GLUT-4 knockout hearts showed a lower functional recovery and decreased ATP levels after ischaemia and reperfusion, particularly in fasted animals (Tian and Abel, 2001). However, the presence of pyruvate in our storage

and reperfusion solutions will reduce the reliance of the cells on glucose during MI and re-energisation, possibly limiting any protective effect of GLUT-4 translocation.

7.9 Possible effects of phenylephrine preconditioning on ATP levels

Phenylephrine preconditioning has been shown to improve ATP levels during ischaemia (Gao *et al.*, 2007, Rehring *et al.*, 1997) and reperfusion (Banerjee *et al.*, 1993, Gao *et al.*, 2007). Phenylephrine preconditioning reduces actomyosin-ATPase activity and slows cross-bridge cycling leading to a decrease in ATP utilisation during ischaemia (Pyle *et al.*, 2000). Opening of *mito*K_{ATP} channels causes an increase in mitochondrial volume and activation of PKCε also increases the activity of cytochrome C oxidase, both of which are likely to increase mitochondrial ATP production. GLUT-4 translocation to the membrane is also induced by phenylephrine preconditioning (Egert *et al.*, 1999); likely via activation of PKC, AMPK and p38, leading to an increase in glucose uptake into ventricular myocytes during ischaemia. AMPK also increases the activity of the glycolytic enzyme phosphofructokinase 1, which can increase glycolytic flux, and therefore ATP levels (Arad *et al.*, 2007).

7.10 How does *sarc*K_{ATP} channel modulation induce protection?

Peak *sarc*K_{ATP} current density was increased by phenylephrine preconditioning in our study (section 6.4.3/Figure 6.5). While it appears that an increase in *sarc*K_{ATP} current is cardioprotective, the mechanisms responsible for the protective effects of *sarc*K_{ATP} channel opening are still under debate. It was initially thought that *sarc*K_{ATP} channel opening induced protection by shortening the APD through an acceleration of phase-3 repolarisation leading to a decrease in Ca^{2+} entry through voltage-gated Ca^{2+} channels during ischaemia. However, Grover *et al.* (1995) observed that preconditioning could still be evoked in the absence of APD shortening, suggesting that APD shortening is not

sufficient to induce preconditioning and that another mechanism underlies the protection observed.

The independence of APD shortening and *sarc*K_{ATP}-mediated protection is supported by the findings of Jovanovic. In the non-contractile COS-7 cell line co-transfected with *sarc*K_{ATP} channel subunits (Kir6.2/SUR2A or Kir6.2/SUR1), pinacidil was able to protect cells from Ca^{2+} overload during hypoxia-reoxygenation (Jovanović *et al.*, 1998a, Jovanović *et al.*, 1998b). Because this cell line is non-excitable, APD shortening cannot be responsible for the protective effect of *sarc*K_{ATP} channels in this study.

Although the protective effect of phenylephrine preconditioning was prevented by inhibition of *sarc*K_{ATP} channels in our study, we did not observe a change in time to contractile failure or *sarc*K_{ATP} channel activation following phenylephrine preconditioning. Similarly, Rehring *et al.* (1997) showed that the time to electrical arrest in the heart was not altered by phenylephrine preconditioning. These findings suggest that *sarc*K_{ATP} channels do mediate preconditioning but APD shortening is not required for the protection. Furthermore, Light *et al.* (2001) showed that although pharmacological preconditioning improved Ca^{2+} handling during the ischaemia and reperfusion periods, inhibition of *sarc*K_{ATP} channels with HMR1883 increased Ca^{2+} loading during reperfusion, but did not significantly affect Ca^{2+} levels during ischaemia. It is important to note that HMR1883 is less effective at blocking *sarc*K_{ATP} channels during ischaemia; however, these results do suggest that the protection afforded by *sarc*K_{ATP} channels may occur during both ischaemia and reperfusion. Similarly, we found that phenylephrine preconditioning had only a small effect on Ca^{2+} levels during ischaemia but significantly improved Ca^{2+} levels during re-energisation (see Figure 7.2). This adds weight to the theory that *sarc*K_{ATP} induced protection may occur through an inhibition of Ca^{2+} loading, primarily during reperfusion. Ito *et al.* (2001), provided

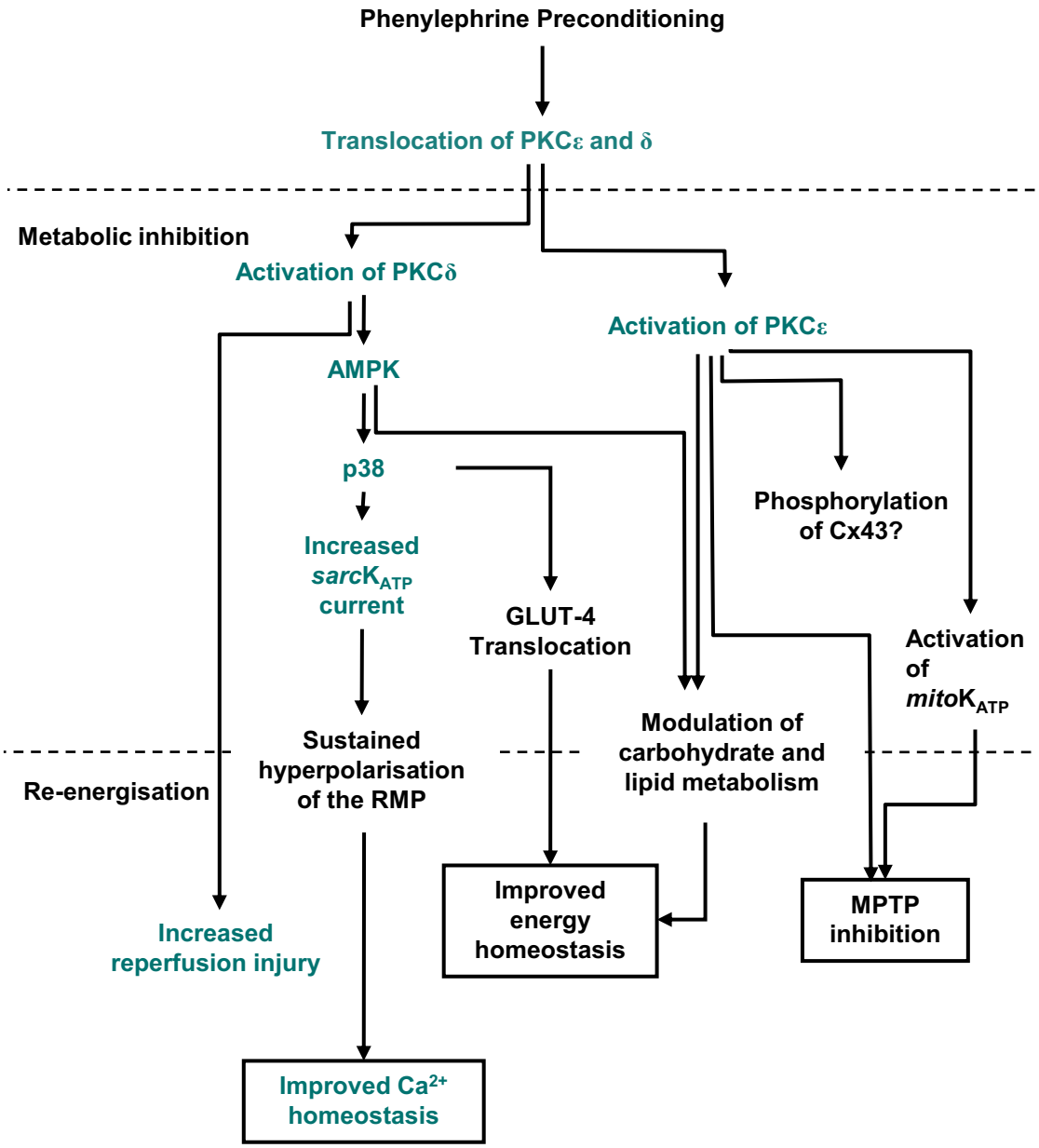

Figure 7.2. Diagram showing effects of phenylephrine preconditioning during metabolic inhibition and re-energisation.

Phenylephrine preconditioning causes activation of the α_1-adrenoceptor and consquent activation and translocation of PKCδ and ε to the membrane fraction. This translocation is maintained following preconditioning. During ischaemia, PKCδ is able to modulate *sarc*K$_{ATP}$ channels leading to an increase in current density, via a pathway that appears to require activation of AMPK and p38. This results in sustained hyperpolarisation of the membrane and a decrease in Ca^{2+} loading. Activation of PKCδ during reperfusion can also increase reperfusion injury. PKCε also shows a sustained translocation following phenylephrine preconditioning. PKCε appears to phosphorylate a number of cytosolic and mitochondrial targets leading to inhibition of the MPTP and improvements in energy homeostasis. Processes shown in teal have been shown in our study.

evidence that activation of conventional and novel PKC isoforms contribute to persistent *sarc*K_{ATP} channel opening during reperfusion, suggesting that PKC activation may modulate channel activity during reperfusion. We surmise that the increase in *sarc*K_{ATP} current induced by phenylephrine preconditioning, may protect ventricular myocytes from ischaemia-reperfusion injury through an increase in *sarc*K_{ATP} current density during ischaemia and a persistent hyperpolarisation of the cell membrane leading to a decrease in Ca^{2+} loading during both ischaemia and reperfusion.

Phenylephrine preconditioning has been demonstrated to reduce Ca^{2+} loading following ischaemia-reperfusion (see section 3.2.5, Gao *et al.*, 2007, Tosaki *et al.*, 1995). Ca^{2+} loading during ischaemia and reperfusion occurs largely as a result of Na^+ accumulation and reverse-mode NCX activity. Phenylephrine preconditioning also reduces Na^+ accumulation during ischaemia and reperfusion (Tosaki *et al.*, 1995). Increased ATP levels in phenylephrine preconditioned myocytes during ischaemia and reperfusion (Gao *et al.*, 2007, Rehring *et al.*, 1997), would be likely to maintain the activity of the Na^+/K^+-ATPase during ischaemia, and enhanced hyperpolarisation of the cell membrane via activation of s*arc*K_{ATP} channels would decrease Na^+ entry through the inactivation-resistant persistent voltage-gated Na^+ channels (Hammarstrom and Gage, 2002). Phenylephrine preconditioning also decreases H^+ accumulation during ischaemia (Rehring *et al.*, 1998, Vasara *et al.*, 2003), most likely due to reduced ATP utilisation and a consequent decrease in the rate of anaerobic respiration. NHE activation by the high intracellular H^+ concentration will cause Na^+ influx and H^+ efflux. The NHE may be inhibited during ischaemia due to extracellular acidosis (Xiao and Allen, 1999), although this is debated and may be dependent on the length of ischaemia and degree of extracellular acidification (Avkiran *et al.*, 2001, Murphy *et al.*, 1999). NHE activity and the associated Na^+-loading during reperfusion, is likely to be lower in phenylephrine

preconditioned myocytes due to a decrease in H^+ accumulation during the preceding ischaemia.

Na^+ accumulation during ischaemia-reperfusion results in efflux of Na^+ and influx of Ca^{2+} through reverse mode NCX activity, and cyclical release and uptake of Ca^{2+} from the SR (Schafer *et al.*, 2001). The reversal potential of the NCX is positive (~20 mV) under resting conditions, so Na^+ enters the cell and Ca^{2+} is removed (Stengl *et al.*, 1998). However increased intracellular Na^+ levels during ischaemia move the equilibrium towards more negative potentials meaning that the NCX reverses direction and Ca^{2+} enters the cell (Baczkó *et al.*, 2003). Hyperpolarisation of the cell membrane during ischaemia and reperfusion through the activation of *sarc*K_{ATP} channels, should increase the time to NCX reversal and reduce Ca^{2+} entry. In addition, decreased Ca^{2+} entry through voltage-gated Ca^{2+} channels, due to hyperpolarisation of the cell membrane by *sarc*K_{ATP} channels also probably contributes to the improved Ca^{2+} homeostasis.

We think it is likely that an increase in *sarc*K_{ATP} current density results in sustained hyperpolarisation of the sarcolemmal membrane that reduces Ca^{2+} loading of ventricular myocytes, and this contributes to the protective effect of phenylephrine preconditioning.

7.11 Conclusions

We have demonstrated that phenylephrine preconditioning acts via α_1-adrenoceptors to activate both PKCε and δ. While PKCε plays a protective role in phenylephrine preconditioning, PKCδ appears to have both a damaging role brought about by an increase in reperfusion injury, and a protective role during the preconditioning period and index ischaemia. The protective effect of PKCδ appears to involve activation of AMPK and p38 leading to an increase in *sarc*K_{ATP} current. Phenylephrine preconditioning also improves Ca^{2+} handling during re-energisation and we hypothesise

that the modulation of *sarc*K_{ATP} current is responsible for the decrease in Ca^{2+} loading during re-energisation.

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