THE ROLE OF THE LARGE CONDUCTANCE Ca^{2+}-ACTIVATED K^{+} CHANNEL IN ADENOSINE RECEPTOR-MEDIATED CYTOPROTECTION

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Proceedings and Publications

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- Fretwell & Dickenson. Role of Ca^{2+}-activated K^+ channels in adenosine A_1 receptor-mediated protection against hypoxia-induced cell death in myocardiac H9c2 cells – Fund & Clin Pharm EPHAR. 2008; P156

- Fretwell & Dickenson. Functional expression of the adenosine A_1 and κ-opioid receptor in myocardiac H9c2 cells – LifeSciences. 2007; Proc Life Sciences PC289

Abstract

The rat embryonic cardiomyoblast-derived H9c2 cell line is increasingly used for studies into cardioprotection, as these cells display similar properties to primary cardiomyocytes. Adenosine receptors are well known mediators of cardioprotection and trigger effectors such as the mitochondrial K_{ATP} channel – however, the role of the mitochondrial BK_{Ca} channel in adenosine receptor-mediated cardioprotection has not been investigated. GPCR assays provided evidence for functional expression of G_i-coupled adenosine A_1 and κ-opioid receptors, G_s-coupled β_2 adrenergic receptors and G_q-coupled UTP-binding P2Y purinergic receptors on H9c2 cells. Activation of the adenosine A_1 receptor with CPA (N(6)-cyclopentyladenosine) provided significant protection against hypoxia-induced cell death in these cells, as did opening of a BK_{Ca} channel with NS1619. The location of this BK_{Ca} channel was confirmed to be the mitochondria by the probing of subcellular fractions with BK_{Ca}-specific antibodies. Interestingly, CPA-induced protection against hypoxia was blocked by inhibition of the BK_{Ca} channel. In a model of hypoxia/reoxygenation in H9c2 cells both CPA and NS1619 significantly reduced cell death when used as postconditioning agents, and in both cases the protection was abolished by blockade of the BK_{Ca} channel. This data suggests for the first time that, in H9c2 cells, the BK_{Ca} channel is involved in A_1 receptor-mediated cytoprotection. To confirm this finding in a more physiologically relevant model – and validate the use of H9c2 cells as a model for cardioprotection –hypoxia/reoxygenation in isolated rat ventricle strips was investigated. It was discovered that blockade of the BK_{Ca} channel significantly attenuated protection afforded by hypoxia preconditioning and preconditioning triggered by activation of the adenosine A_1 and A_{2A} receptors.

For the first time, this report has shown an important role for the BK_{Ca} channel in adenosine receptor-mediated cytoprotection.

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Contents

1. Introduction

1.1. Cardiovascular disease

Cardiovascular disease is currently the leading cause of death in the developed world, with ischaemic heart disease accounting for over 94,000 deaths in the United Kingdom in 2006 alone (British Heart Foundation Statistics Database). Although heart disease is often thought to be an affliction of the elderly, since risk does increase with age, it is also the foremost cause of premature death in the UK in men and women (British Heart Foundation Statistics Database). Ischaemic heart disease, or coronary heart disease, is caused when a coronary artery becomes blocked - often as a result of a thrombus or atherosclerotic plaque. Blood flow to myocardial tissue is reduced leading to symptoms such as dyspnea, angina pectoris and myocardial infarction. As the populace increases in age, and risk factors such as obesity and diabetes become more common, the incidence of ischaemic heart disease will be raised – creating a substantial burden on the National Health Service. Research regarding the mechanisms involved in cardiac ischaemia and protection against the resultant tissue damage is plentiful, but as yet very few pharmacological agents have successfully transferred from producing positive experimental data (in cell and animal models) to encouraging clinical outcomes. Therefore, any insight into the complex pathways involved in ischaemia and ischaemia/reperfusion injury could help identify future, or potentiate existing, targets for therapy.

1.2. Ischaemia and ischaemia/reperfusion-induced cell death

Myocardial cells exhibit a variety of metabolic responses to ischaemia; the extent of the response depends on the degree of ischaemia experienced by the cells and so not all myocytes exhibit the same behaviour. Cellular responses to ischaemia include

myocardial stunning, hibernation and preconditioning. The phenomena of myocardial stunning has been described in a number of animal models including the isolated rabbit heart (Flameng *et al*, 1991) and dogs (Phillips & Ko, 2007), and has also been proven to occur in humans (Gerber *et al*, 1999). It describes the delayed return of myocardial contractile function following ischaemia, despite full restoration of blood flow, so allowing myocytes time to "repair" during a period of reduced energy requirement (Depre & Taegtmeyer, 2000). Alternatively, repeated stunning may lead to myocardial hibernation (Kim *et al*, 2003). This is often in response to chronic ischaemia/hypoxia and is a process where myocardial function is lessened in alignment with the reduced supply of oxygen and nutrients. These processes allow irreversible tissue damage to be avoided, so upon restoration of normal blood flow contractile function can eventually return. Another process that can occur in response to ischaemia is preconditioning, which enhances the resistance of the myocardium to irreversible damage and will be described later on in more detail (see section 1.4).

During ischaemia the reduced availability of oxygen and metabolic substrates is insufficient to support normal oxidative phosphorylation. This triggers the mitochondria to switch from aerobic to anaerobic respiration, so reducing formation of ATP and producing intracellular acidosis with accumulation of hydrogen ions (H^+) and lactate (see Figure 1.1, page 12). This leads to inhibition of the ATP-dependant sodium/potassium pump (Na^+/K^+-ATPase) and activation of the sodium/hydrogen exchanger (NHE) – both of these actions augmenting intracellular Na^+ accumulation. The increase in Na^+ activates the Na^+/Ca^{2+} exchanger, leading to intracellular accumulation of Ca^{2+}. Osmotic swelling occurs due to influx of Cl^- and H_2O. The reduction of ATP also reduces uptake of Ca^{2+} by the sarco/endoplasmic reticulum

Ca^{2+}-ATPase (SERCA), so further intracellular Ca^{2+} accumulation occurs. Ca^{2+}-dependant proteases are then activated, causing damage to the membranes and cytoskeleton. This, combined with the swelling of the cell, can lead to membrane rupture and oncotic necrosis. Oncosis, or "ischaemic cell death" (Schaper & Kostin, 2005), is a form of accidental cell death caused by failure of ion pumps. It is characterised by cell swelling, increased membrane permeability, blebbing and non-specific DNA degradation (Majno & Joris, 1995). In the literature the term "necrosis" is often used in place of oncotic necrosis, however, necrosis specifically refers to the changes that occur following any kind of cell death. This is characterised by further changes to the nucleus and cytoplasmic constituents and breakdown of the plasma membrane; up until this point it is thought that cells can be rescued, and the onset of necrosis represents irreversible cell death (for review see Trump & Berezesky, 1996). To remain consistent with the literature, from this point "necrosis" will be used to describe non-apoptotic cell death in this report.

Apoptosis is a distinct form of cell death and can be initiated by a cell's "internal clock" or an external signal (Majno & Joris, 1995). Unlike necrosis, it is characterised by cell shrinkage, nuclear condensation and specific nuclear cleavage, and budding of apoptotic bodies – these are then phagocytosed without stimulating an inflammatory response.

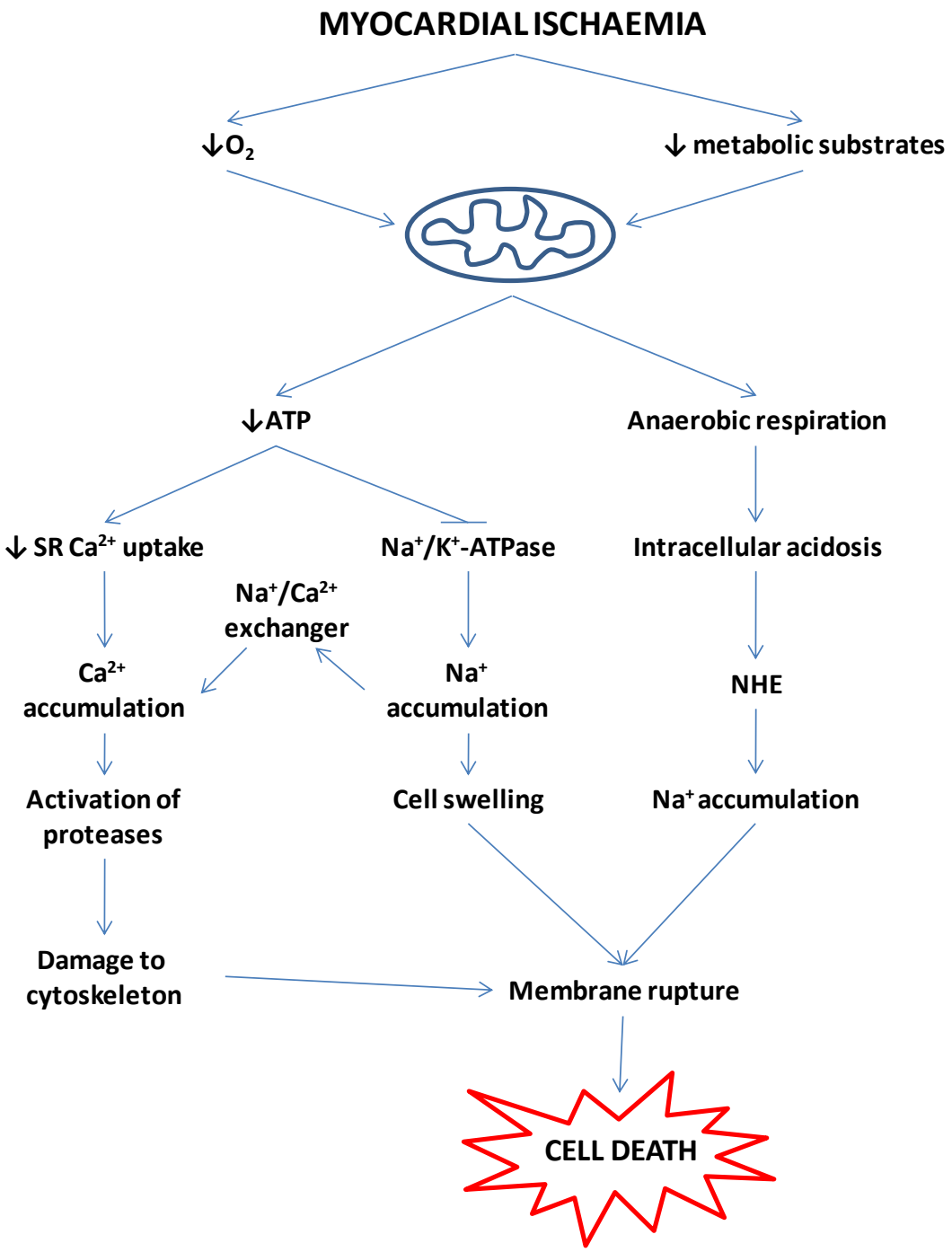


Fig.1.1: Proposed series of events leading from myocardial ischaemia to irreversible cell death. The reduced formation of ATP and intracellular acidosis caused by lack of oxygen disrupts ion transporters within the cell, leading to cell swelling and damage – resulting in irreversible necrosis.

It was originally thought that necrosis was the predominant form of cell death initiated by hypoxia/ischaemia, and there is evidence for and against a role for apoptosis. For example, hypoxia-induced apoptosis has been detected in neonatal and adult rat cardiomyocytes (Tanaka *et al*, 1994; de Moissac *et al*, 2000), but a study by Webster *et al* (1999) reported that apoptosis could only be measured when hypoxia was accompanied with reoxygenation or a shift in pH – and this was found to be the case in neonatal rat cardiomyocytes and the Langendorff-perfused mouse heart. Such discrepancies may arise due to the model used, different conditions for hypoxia and/or different methods of measuring apoptosis. It has been shown that different apoptotic pathways can be stimulated depending on culture conditions; one study utilising Jurcat cells (Malhotra *et al*, 2001) described caspase-3-dependent apoptosis following hypoxia in a glucose-free medium, but caspase-3-independant apoptosis following hypoxia in glucose-containing medium – suggesting that a caspase-independent death receptor pathway had been activated. Overall, the data regarding hypoxia-induced cell death is varied, but a there is a large body of evidence in favour of a role for apoptosis.

However, it is now believed that a combination of apoptosis and necrosis occurs – with apoptosis being the preferred pathway until ATP levels are too low to sustain the energy-dependent process, after which necrosis takes over (Tatsumi *et al*, 2003; Otani *et al*, 2006).

Initially the damage observed in myocardial tissue following an infarction (in the clinical setting and experimental models) was believed to be inflicted purely during the period of ischaemia. However, in 1987 Olafsson *et al* provided evidence that some injury occurs due to the reinstatement of blood flow during reperfusion. The Olafsson group used a canine model and reported that treatment with adenosine for

the first hour of reperfusion after a 90 minute period of ischaemia reduced infarct size and improved ventricular function compared to untreated controls (Olafsson *et al*, 1987). The observation that the outcome of the ischaemic insult could be improved by pharmacological treatment during reperfusion suggested that some of the damage was generated at this stage. Since this report it has been found that treatment with several pharmacological agents at the start of reperfusion can ease myocardial injury (for review see Gross & Auchampach, 2007).

Reperfusion is essential to "rescue" the ischaemic tissue, but it is now known to lead to further problems such as arrhythmia, decreased contractile function and irreversible cell death. During ischaemia neutrophils accumulate at the damaged tissue, and this process is accelerated upon reoxygenation (Hansen, 1995). Neutrophils release ROS, proteases and inflammatory mediators, which in turn recruit more neutrophils so amplifying the inflammatory-like response (Jordan *et al*, 1999). Alongside the inflammatory-like response, mitochondria are thought to be the main mediators of reperfusion injury (see Figure 1.2, page 15); it is postulated that the reintroduction of oxygen re-energises the mitochondrial respiration machinery, leading to excessive production of reactive oxygen species (ROS) (Korge *et al*, 2008). SERCA (Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase) is activated when ATP production proceeds, placing the sarcoplasmic reticulum under severe calcium overload. Calcium is released via ryanodine receptors then taken up again by SERCA – this leads to intracellular calcium oscillations and mechanical irregularities (Piper *et al*, 2006). The accumulation of calcium activates Ca^{2+}-dependant proteases, such as calpains, which degrade intracellular proteins and compromise the integrity of the cell membrane.

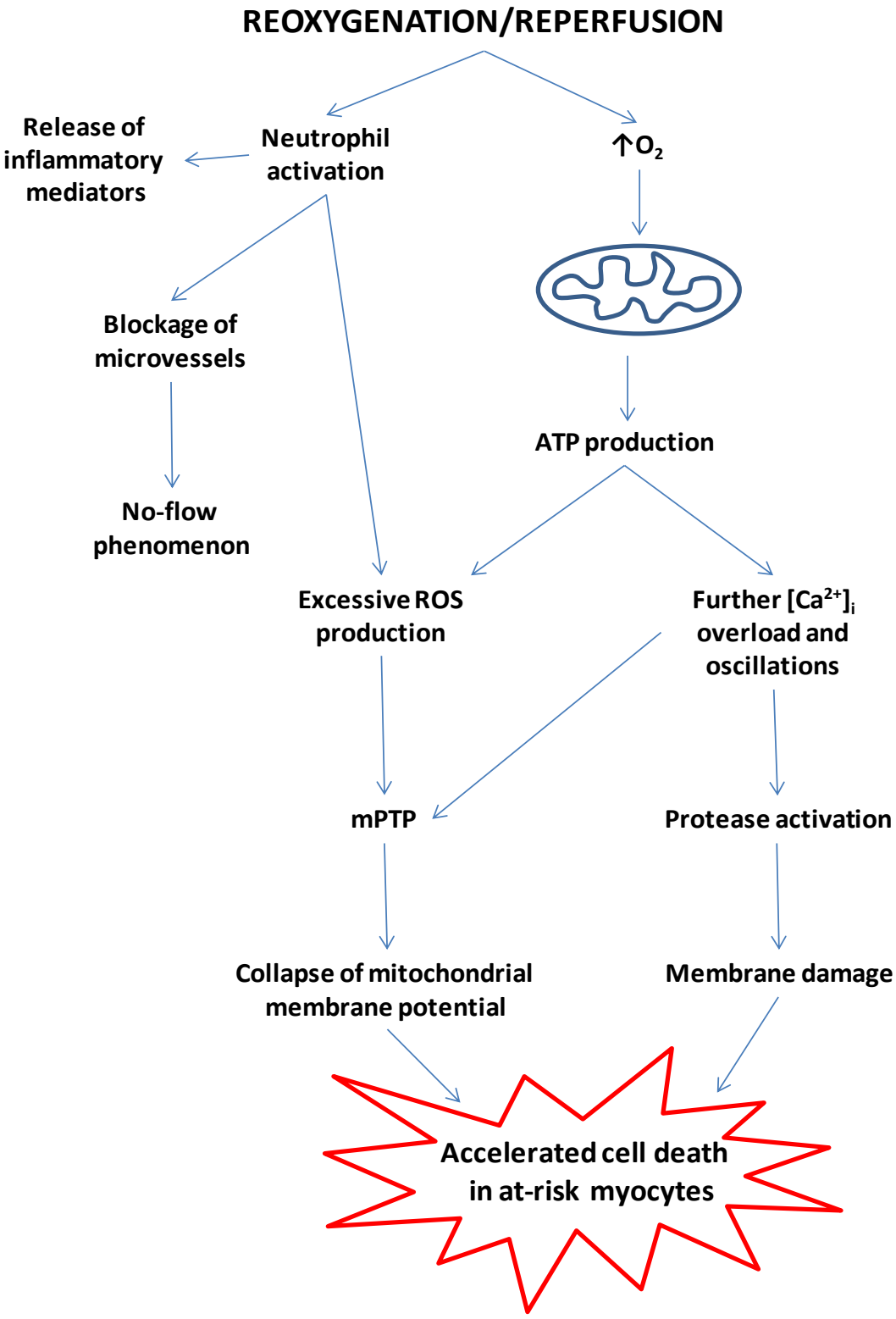


Figure 1.2: Proposed series of events leading from myocardial ischaemia/reperfusion to irreversible cell death. Upon reoxygenation the increase in oxygen and activation of neutrophils sets off a series of events. Ccommon features of both pathways are excessive ROS production and activation of the mPTP – eventually culminating in cell death.

1.3. A role for the mitochondria

Precise regulation of cytoplasmic potassium ion concentration is essential to maintain ion homeostasis and cell volume, and it is thought that K^+ homeostasis is the main regulator of mitochondrial matrix volume. Efflux of K^+ from the cell can lead to osmotic shrinkage, which stimulates mitochondrial release of cytochrome-c, activation of executioner caspase-3 and eventual cell death. This intrinsic apoptotic pathway is mediated by the mitochondria.

Mitochondria are responsible for generating cellular energy in the form of ATP via oxidative phoshorylation. Oxidative phosphorylation involves generation of a proton gradient across the inner mitochondrial membrane by the electron transport chain; inner membrane-bound ATP synthase then utilises the gradient to power the production of ATP from ADP and phosphate (P_i). If ATP synthesis is disrupted the whole cell is affected as ion homeostasis is disturbed, leading to a change in cell volume.

Several ion channels have been described that are located on the mitochondrial inner mitochondrial membrane, including two K^+ channels – the ATP-sensitive potassium channel (mitoK_{ATP}) and the large-conductance calcium-activated potassium channel (mitoBK_{Ca}). Both of these channels have been implicated in cardioprotection against ischaemia/reperfusion injury (for review see Nishida *et al*, 2009). The proton gradient across the mitochondrial membrane (formed by the mitochondrial electron transport chain) leads to a negative potential in the mitochondrial matrix compared to the cytosol, and this leads to Ca^{2+} influx into the mitochondria (Gunter & Pfeiffer, 1990). During ischaemia, when conditions favour Ca^{2+} influx, activation of K^+ channels partially depolarises the mitochondrial membrane so reducing the negative

gradient, and this attenuates the potentially damaging Ca^{2+} influx. This has been shown in an isolated cardiac mitochondrial preparation using the K_{ATP} openers diazoxide and pinacidil, and in intact cardiomyocytes with diazoxide (Holmuhamedov *et al*, 1999) and the BK_{Ca} channel opener NS1619 (Sato *et al*, 2005) – in each case treatment with the channel openers depolarised the mitochondrial membrane and reduced mitochondrial Ca^{2+} uptake.

It is thought that activation of the mitoK_{ATP} channel evokes cardioprotection through a pathway involving inhibition of the mitochondrial transition pore (Facundo *et al*, 2005; Krolikowski *et al*, 2005), however, the precise mechanisms by which mitoK_{ATP} channels mediate cardioprotection are debatable. The structure of mitoKATP channels is not fully known, but it is postulated that they are similar to the plasma membrane K_{ATP} channels and are composed of an inward rectifier potassium channel subunit (Kir) associated with a sulfonylurea receptor (SUR) (Mironova *et al*, 2004; O'Rourke, 2004). The cardioprotective role of the mitochondrial BK_{Ca} channel will be discussed later on (see section 1.7).

Not all mitochondrial channels exert such a cardioprotective effect. Also on the inner mitochondrial membrane is the mitochondrial permeability transition pore (mPTP), which is thought to be a key player in ischaemia/reperfusion injury. The mPTP consists of several proteins spanning the inner and outer mitochondrial membranes, so allowing the passage of solutes <1.5 kDa through the usually impermeable inner mitochondrial membrane. The structure of the mPTP is under debate, but the main components are proposed to be adenine nucleotide translocase (ANT), cyclophilin-D (CyP-D), mitochondrial phosphate carrier (PiC) and voltage-dependent anion channel (VDAC) – although recent evidence contrasts the original model for the mPTP where VDAC had a major role.

The mPTP opens in response to an increase in mitochondrial Ca^{2+}, facilitating the release of pro-apoptotic molecules into the cytosol (Rodriguez-Enriquez *et al*, 2004); though during periods of increased oxidative stress the mPTP is sensitised to Ca^{2+}, so increased Ca^{2+} is not actually required for pore opening (Halestrap *et al*, 1997). However, when ATP depletion occurs to an extent that is too low to support apoptosis the mPTP can also mediate necrosis (for review see Crompton, 1999; see Figure 1.3).

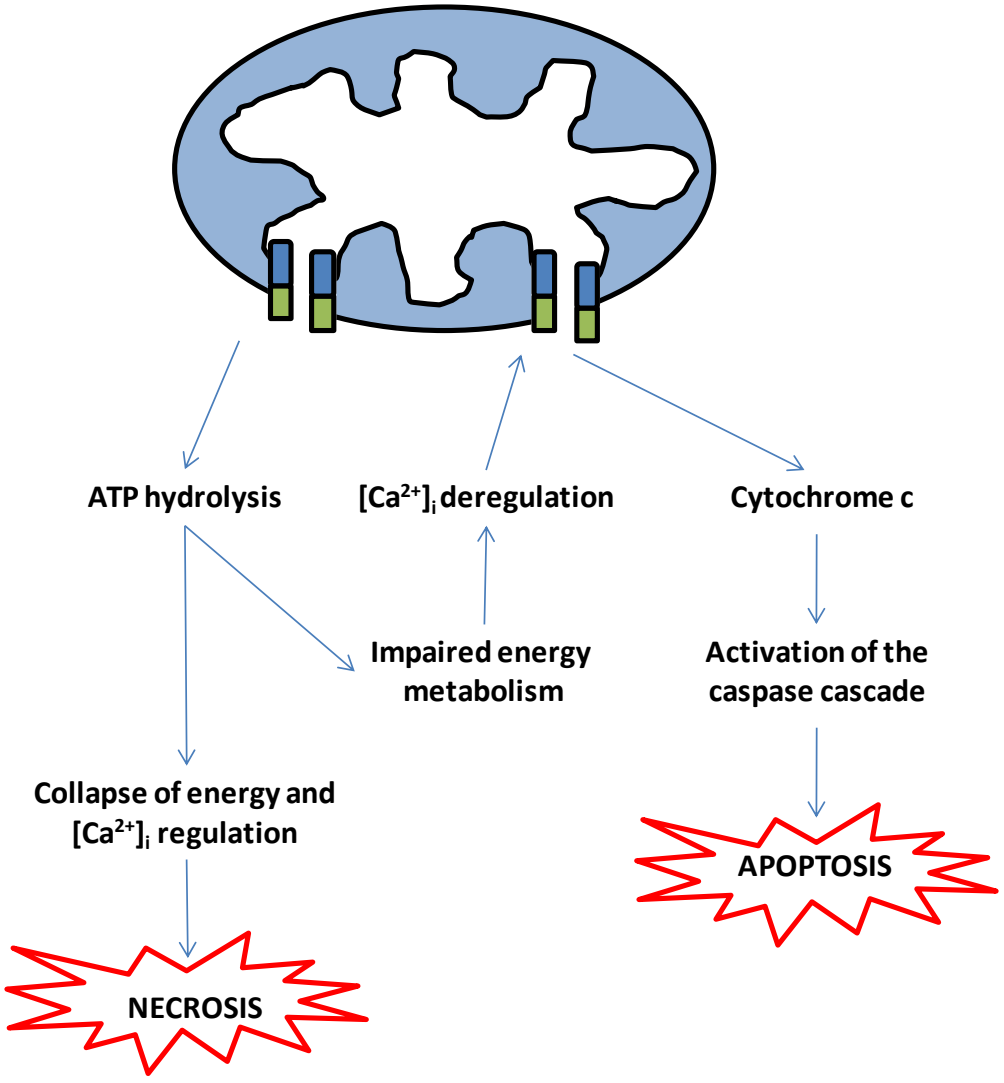


Figure 1.3: Regulation of cell death by the mPTP. Mitochondrial permeability transition can set off a series of events culminating in necrotic and/or apoptotic cell death. (Green/blue bars, open mPTP).

It has been suggested that mPTP is formed by a CyP-D-facilitated conformational change of the PiC, and that this is modulated by the conformational state of an associated ANT (Halestrap *et al*, 1997; for review see Halestrap, 2009). Evidence for these associated proteins arises from various reports. For example, it has been shown that livers from CyP-D knock-out mice are extremely resistant to Ca^{2+}-induced opening of the mPTP (Basso *et al*, 2005), pointing to an important role for this protein in mPTP formation. In 2008 it was discovered that PiC binds to CyP-D in a cyclosporine-sensitive manner (cyclosporine interacts with CyP-D, preventing pore formation), and that PiC can also associate with ANT – suggesting that PiC may also play a role in mPTP formation (Leung *et al*, 2008). The modulatory (i.e. not essential) role for the ANT arises from the evidence that livers from mice without ANT1 or ANT2 still present a permeability transition (Kokoszka *et al*, 2004). It is important to note, however, that only nucleotides transported by ANT (i.e. ATP and ADP) can inhibit mPTP opening, implicating ANT in regulation of the pore (Halestrap *et al*, 1997; the role of ANT is reviewed in Leung & Halestrap, 2008).

VDAC was originally proposed to be an important part of the mPTP by Zoratti & Szabo in 1994, as it had been co-purified with ANT. The role of VDAC is controversial though, as more recently is has been shown that mitochondria from VDAC-null mice exhibited a Ca^{2+} - and oxidative stress-inducible permeability transition comparable to that observed in mitochondria from wild-type mice (Baines *et al*, 2007).

However, in comparison to this role in the initiation of cell death it has also been suggested that transient opening of this channel can have a cytoprotective effect (Hausenloy *et al*, 2004). Despite this, the mPTP appears to play a key role in regulation of ischaemia/reperfusion injury, and provides a good target for

cardioprotective therapies – and the potential modulation of the mPTP by the K_{ATP} channel, or BK_{Ca} channel, provides an exciting area of research.

It is proposed that during oxidative stress, while the energy state still favours apoptosis, the mPTP opens allowing influx of water and solutes into the mitochondria, increasing matrix volume and rupturing the outer membrane. This allows the release of cytochrome c from the intermembrane space, formation of the apoptosome (a complex of cytochrome c, APAF-1 and pro-caspase-9) and initiation of the caspase cascade – resulting in apoptotic cell death (see Figure 1.3). As oxidative stress increases, necrosis is favoured. Opening of the mPTP leads to ATP hydrolysis, so worsening energy metabolism and causing further Ca^{2+} deregulation. This feeds back to the mitochondria – initiating more mPTP opening and eventual loss of energy and Ca^{2+} regulation, culminating in necrosis (see Figure 1.3). However, although prolonged exposure is ultimately damaging, ischaemia can also have a beneficial effect.

1.4. Ischaemia as a trigger of cardioprotection

It is now well accepted that a brief period of ischaemia and reperfusion (I/R) can protect against a subsequent, prolonged I/R. This phenomenon, termed ischaemic preconditioning, has been demonstrated in various models including swine (Schott *et al*, 1990) and dog (Murry *et al*, 1986), as well as several in vitro models such as isolated rabbit cardiomyocytes (Armstrong *et al*, 1994). There is also evidence suggesting that ischaemic preconditioning can be beneficial when used in a clinical setting with coronary artery bypass graft surgery (CABG). For example, Wu *et al* (2002) found that brief periods of ischaemia before CABG, in patients with 3 vessel disease, significantly reduced the occurrence of ventricular fibrillation and

tachycardia after the operation. The same group (Laurikka *et al*, 2002) used the same preconditioning protocol in patients with 2 vessel disease and found that, following CABG, myocardial enzyme release was attenuated, heart rate was stabilised and recovery of stroke volume was increased compared to the control group.

The ischaemic preconditioning phenomenon first described by Murry *et al* (1986) is now described as "classical", or "early phase" preconditioning. This form of preconditioning refers to transient beneficial effects (1-2 hours) that develop within minutes of the cardioprotective stimulus, e.g. a brief period of sublethal ischaemia (see figure 1.4). It involves rapid modulation of pre-existing proteins within the cell, such as activation of GPCRs, protein kinases and ion channels, and provides protection in many models against ischaemia/reperfusion injury (for reviews see Sanada & Kitakaze, 2004; Bolli, 2007).

In 1993 two separate groups (Kuzuya *et al*, 1993; Marber *et al*, 1993) reported that some effects of myocardial protection could still be observed 24 hours after the initial preconditioning stimulus. This is now termed "late phase" or "second window" preconditioning, and describes long lasting effects (peak protection at 24-72 hours) that develop from 6 hours of the preconditioning stimulus. As the longer time span would suggest, the late phase of preconditioning arises from different signal transduction mechanisms to the early phase – although some commonalities do occur. A key feature of late phase preconditioning is up-regulation of cardioprotective genes and the synthesis of new proteins, e.g. inducible nitric oxide synthase (iNOS), which is a well known mediator of late phase preconditioning and is reported to provide protection via inhibition of mitochondrial swelling and inhibition of the mPTP (West *et al*, 2008). It has also been reported that anti-

apoptotic, prosurvival genes are also "switched on" and proteins synthesised during the late phase of preconditioning (Stein *et al*, 2007) – further strengthening the role of apoptosis in ischaemia/reperfusion injury. These two forms of ischaemic preconditioning can both reduce infarct size in various animal models (see Yellon & Downey, 2003, for comprehensive review), but in mice the early phase is reported to be more potent (Guo *et al*, 1998). However, the late phase of ischaemic preconditioning can also provide protection against myocardial stunning in conscious rabbits (Dawn *et al*, 1999). Figure 1.4 provides a basic outline of the phases involved in ischaemia/reperfusion injury and types of cardioprotection. The duration of ischaemia and reperfusion varies between experimental models, but the general outcomes are highlighted in the text.

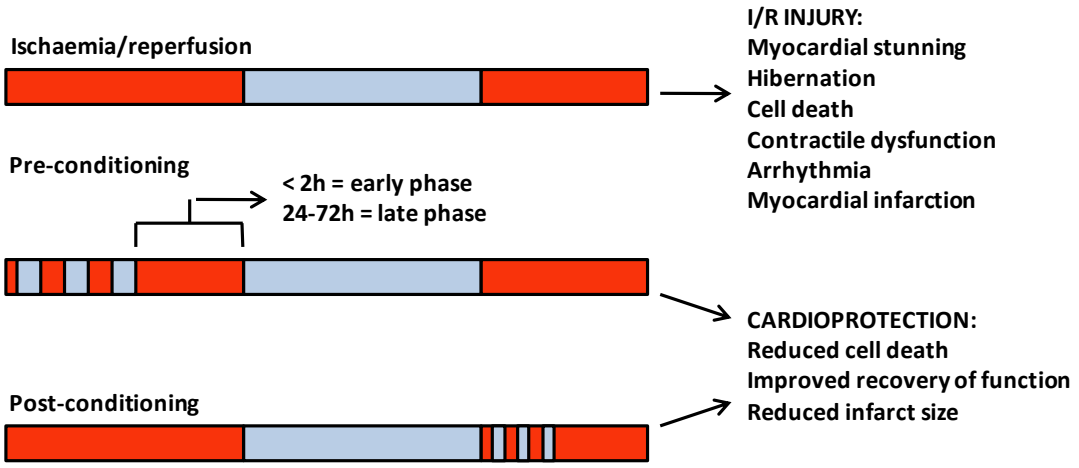


Figure 1.4: Graphical representation of ischaemia/reperfusion and pre/postconditioning. Ischaemia/reperfusion leads to ischaemia/reperfusion injury (I/R injury), which can be attenuated by ischaemic pre- or postconditioning. Areas of blue indicate ischaemia, red indicated normal perfusion. Diagram adapted from Ferdinandy *et al*, 2007.

Both the early and late phases of ischaemic preconditioning rely on signal transduction pathways to link the initial trigger of cardioprotection with the overall effectors, or mediators. During the preconditioning period of ischaemia autacoid

triggers such as adenosine (Headrick, 1996), opioid peptides (Pan *et al*, 2000), bradykinin (Romano *et al*, 2004) and catecholamines (Schömig, 1990) are released from the myocardial tissue. These are thought to stimulate specific G protein-coupled receptors (GPCRs) and protein kinases, which culminate in activation of mediators such as the mitochondrial ATP-dependent potassium (K_{ATP}) channel (for examples see Peart & Gross, 2003; Uchiyama *et al*, 2003). The precise signalling pathways involved in ischaemic preconditioning are still under debate, but extracellular signal-regulated kinase 1/2 (ERK1/2), protein kinase C (PKC), cAMP-dependent protein kinase (PKA), cGMP-dependant protein kinase (PKG), p38 mitogen-activated protein kinase (p38 MAPK) and nitric oxide (NO) all have well reported roles (Hausenloy & Yellon, 2006) (see Figure 1.5).

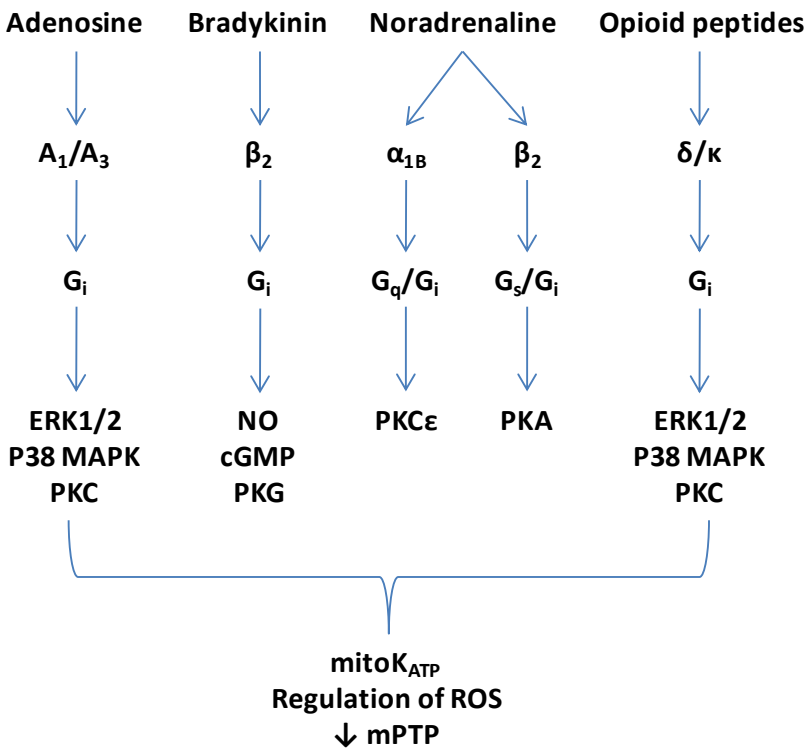


Figure 1.5: Proposed series of events following the release of autacoids during ischaemic preconditioning. Autacoids are released by myocardial cells and activate specific GPCR subtypes. The receptors bind to G proteins, which once activated can stimulate protein kinases and other effectors – culminating in activation of the mitochondrial K_{ATP} channel, ROS regulation and reduced mitochondrial permeability transition.

The MEK1/2-ERK1/2 and PI3K-PKB signalling pathways are key players in the "RISK" (Reperfusion Injury Salvage Kinase) pathway (Hausenloy & Yellon, 2004). Both of these pathways have been shown to be activated at the time of reperfusion following pharmacological preconditioning (Yang *et al*, 2004a & 2004b), leading to recruitment of downstream anti-apoptotic molecules (see Figure 1.6).

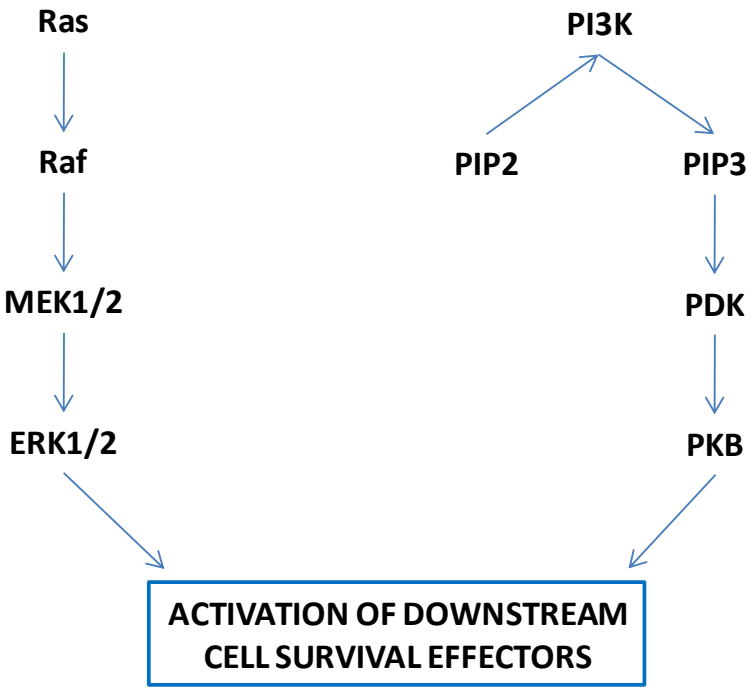


Figure 1.6: The basic steps leading to activation of ERK1/2 and PKB. (See main body of text for more detailed description.)

For initiation of the MEK/ERK1/2 pathway GTP-bound Ras recruits Raf to the plasma membrane, and then phosphorylates Raf. Active Raf then phosphorylates MAPK/ERK kinase 1 (MEK1) and MEK2. MEK1/2 phosphorylation leads to phosphorylation of ERK1/2, and then ERK1/2 can activate downstream targets. One anti-apoptotic pathway mediated by ERK1/2 activation is through the downstream activation of the protein kinase p90 ribosomal S6 kinase (p90RSK) (Herrera &

Sebolt-Leopold, 2002). p90RSK phosphorylates and inactivates the pro-apoptotic protein BAD, so inhibiting BAD-mediated cytochrome-c release and caspase activation (Downward, 1999).

The role of ERK1/2 in preconditioning and postconditioning is clear. For example, it has been shown that ERK1/2 phosphorylation is increased following a preconditioning stimulus in rat hearts in vitro (Hausenloy *et al*, 2005 & 2005a), and also in rat hearts in vivo – where a role for ERK1/2 was described in ischaemic preconditioning and preconditioning mediated by the δ-opioid receptor (Fryer *et al*, 2001). ERK1/2 phosphorylation is also increased in post-conditioned in vivo pig hearts (Schwartz & Lagranha, 2006) – pointing to recruitment of similar pathways for pre- and postconditioning. In fact, it has been reported that it is the first few minutes of reperfusion following the major ischaemic insult that is critical for both pre- and postconditioning (Hausenloy & Yellon, 2007a). It is known that activation of the adenosine A_1 receptor leads to ERK1/2 phosphorylation, but there is variation regarding the role of ERK1/2 in adenosine receptor-mediated cardioprotection. It was shown that the anti-apoptotic effect of adenosine in neonatal rat cardiomyocytes was mediated by the A_1 and A_3 receptors, and dependent on MEK/ERK1/2 activation (Germack & Dickenson, 2005). However, a study with isolated rat ventricle strips found that inhibition of MEK did not affect adenosine A_1, A_{2A} or A_3 receptor-mediated preconditioning, but did attenuate hypoxic preconditioning (Button *et al*, 2005). ERK1/2 is also proposed to be involved in mitoK_{ATP} channel signalling in isolated rabbit hearts (Naitoh et al, 2006).

The PI3K/PKB pathway is initiated by phosphorylation of PIP_2 by PI3K, generating PIP_3. PIP_3 recruits cytoplasmic PKB and PDK to the plasma membrane, where PDK activates PKB. Activated PKB then redistributes to the mitochondria (Miyamoto *et*

al, 2008) or nucleus (Shiraishi *et al*, 2004), and activates anti-apoptotic pathways. One such pathway is via inhibition of BAD (Datta *et al*, 1997), which is a mechanism triggered by both the PI3K/PKB and MEK/ERK1/2 pathways. PKB is also proposed to enhance the association of hexokinase with VDAC at the outer mitochondrial membrane, so preserving mitochondrial integrity and inhibiting opening of the mPTP (Gottlob *et al*, 2001). It has been shown that PDK1 (and, therefore, phosphorylation of PKB) is essential for ischaemic preconditioning, as the beneficial effect of ischaemic preconditioning is lost in hearts and cell models from PDK1-deficient mice (Budas *et al*, 2006). Phosphorylation of PKB is increased in various models of cardioprotection, including preconditioning of in vitro rat and rabbit hearts (Hausenloy *et al*, 2005 & 2005a; Solenkova *et al*, 2005), and postconditioning of in vivo pig hearts (Schwartz & Lagranha, 2006). Increased phosphorylation of PKB has also been described in the protection of isolated rat hearts by ischaemic postconditioning and low-pressure reperfusion, where inhibition of the mPTP is the proposed target (Bopassa *et al*, 2006). As with ERK1/2, activation of PKB via opening of the mitoK_{ATP} channel has also been reported (Ahmad *et al*, 2006), and the role of PKB phosphorylation in adenosine receptor-mediated cardioprotection is poorly understood. Qin *et al*, (2003) discovered that adenosine triggered preconditioning in isolated rabbit hearts by a PI3K-independent pathway, but another report (Solenkova *et al*, 2006), claimed that endogenous adenosine protects isolated rabbit hearts through phosphorylation of PKB during the early stage of reperfusion. In neonatal rat hearts, it was shown that PKB phosphorylation was involved in adenosine A_1 and A_3 receptor-mediated signalling, but not in A_1 and A_3 receptor-mediated cardioprotection (Germack *et al*, 2004).

It is reported that cross-talk occurs between the MEK1/2-ERK1/2 and PI3K-PKB pathways, and activation of both at the start of reperfusion is necessary to exert the protective preconditioning effect (Hausenloy *et al*, 2004; Solenkova *et al*, 2006). Taken as a whole, a role for the MEK/ERK1/2 and PI3K/PKB pathways in anti-apoptotic and cardioprotective signalling is clear, but further work is necessary to elucidate the role of these pathways in adenosine receptor-mediated cardioprotection.

The PI3K/PKB pathway can also activate endothelial nitric oxide synthesase (eNOS), which synthesises NO. NO has many roles in the cardiovascular system, including activation of the mitoK_{ATP} channel. NO activates soluble guanylyl cyclase, which then produces cGMP from GTP. cGMP then activates PKG, which phosphorylates a target on the outer mitochondrial membrane and (via an unknown intermediate step) activates PKC and consequentially the K_{ATP} channel (Cuong *et al*, 2006; Downey *et al*, 2008).

During ischaemia it is thought that there is in increase in cAMP which activates PKA – however the result of such activation is debatable. PKA activation prior to ischaemia has been reported to reduce infarct size in dogs (Sanada *et al*, 2004), but conversely blockade of PKA prior to ischaemia is reported to reduce infarct size and improve post-ischaemic recovery in isolated rat hearts (Makaula *et al*, 2005).

There is a large body of evidence suggesting PKC as a mediator of cardioprotection triggered by ischaemia and pharmacological agents, such as adenosine and the volatile anaesthetic isoflurane (Liu *et al*, 1999; Ludwig *et al*, 2004; Okada *et al*, 2005). There are ten isoforms of PKC, with the novel, Ca^{2+}-independent PKCδ and PKCε being dominant in murine heart with regards to protein expression and activity (Schreiber *et al*, 2001). However there are conflicting reports regarding the

expression of the different PKC isoforms; for example, in human ventricle it is reported that the Ca^{2+}-dependent PKCα, PKCβI and PKCβII are dominant, with PKCδ and PKCζ mainly expressed in the atria and PKCε and PKCλ expressed equally in both regions (Simonis *et al*, 2007). Therefore data from different animal models must be interpreted cautiously. Despite these discrepancies, PKCε is reported to be involved in cardioprotective signalling in several models as diverse as rabbit cardiomyocytes (Liu *et al*, 1999) and human myocardium (Sivaraman *et al*, 2009). Following a trigger, PKCε is activated and translocates within the cell to activate downstream effectors such the mitoK_{ATP} channel and the mPTP – where it exerts a protective effect by activation or inhibition of these proteins (for review see Budas & Mochly-Rosen, 2007). In contrast to the role for PKCε as a critical mediator of ischaemic pre- and postconditioning (Zatta et al, 2006), PKCδ has a role in mediating ischaemia/reperfusion-induced apoptosis Liu *et al*, 1999) – and it has been shown that inhibition of PKCδ at the time of reperfusion can improve recovery of function and reduce infarct size in murine hearts (Inagaki *et al*, 2003).

Overall, there is much evidence suggesting that protein kinases have an essential role in regulation of cardioprotective signalling, and due to the timing of activation it has been proposed that the RISK pathway has an important role in preconditioning and postconditioning (Hausenloy *et al*, 2005), although the exact mechanisms involved are unknown.

Ischaemic postconditioning is a more recently discovered cardioprotective phenomena (Zhao *et al*, 2003). This consists of brief, sublethal cycles of ischaemia/reperfusion after the prolonged ischaemic attack, and has since been observed in various experimental models, e.g. isolated rabbit hearts (Pinheiro *et al*, 2009) and isolated rat hearts (Penna *et al*, 2009).

Clarification of the signalling pathways involved in ischaemic pre- and postconditioning would provide scientists with potential targets for pharmacological treatments, so gaining the beneficial effects observed from these phenomena without the complexity of inducing ischaemia in patients. The pathways involved may be intricate, but it cannot be denied that GPCRs play an essential part in triggering the signal transduction pathways that eventually lead to cardioprotection.

1.5. G protein-coupled receptors

Currently, over 30% of prescription drugs target one or more GPCR, and it is thought that many more act through GPCRs indirectly (Vaidehi *et al*, 2009). GPCRs are a super-family of cell surface receptors characterised by seven trans-membrane spanning domains (7TM α-helices) with a long-chain lipid anchor to strengthen membrane association, an intracellular amino terminal and an extracellular carboxyl terminal (see Figure 1.7, page 30). They are widely distributed throughout mammalian cells and tissues, and are responsible for mediating intracellular responses to a range of extracellular signals. The binding of a ligand to the receptor sets off a cascade of events involving second messengers such as cyclic nucleotides, IP_3 and Ca^{2+}.

The structure of the light-sensitive rhodopsin receptor was the first to be determined (Palczewski *et al*, 2000), and the crystalline structure of this receptor provided a basis for understanding the structure and function of further GPCRs. The rhodopsin-like/Class A receptors form the largest subgroup of GPCRs, and they can be activated by an extensive range of ligands such as light (rhodopsin receptor), peptides (e.g. opioid receptors) and hormones (e.g. follicle stimulating hormone receptor).

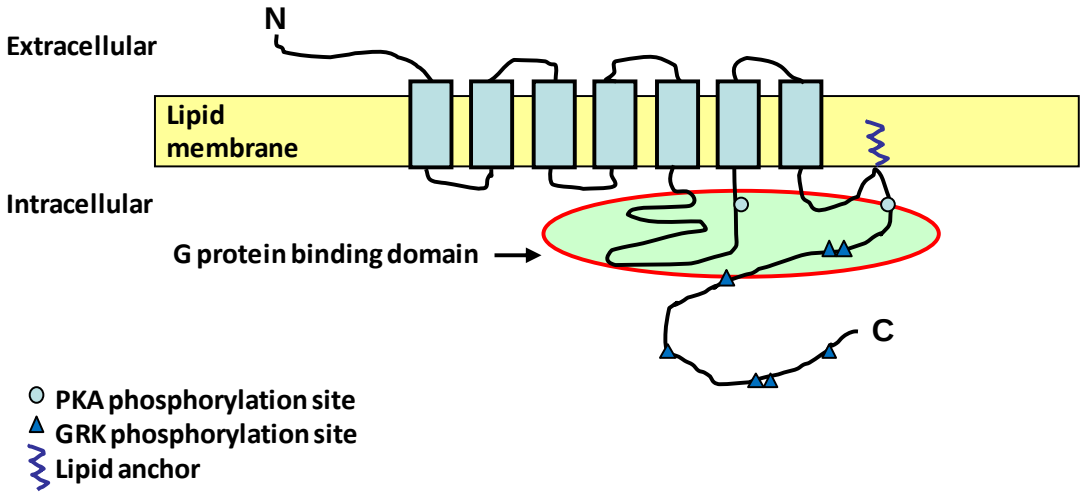


Figure 1.7: Schematic diagram showing the basic structure of a Class A GPCR. Class A GPCRs are activated when a ligand interacts with the 7TM domain, initiating a cascade of events culminating in an intracellular response.

Receptor activation occurs when a ligand binds to the receptor, creating a conformational change (see Figure 1.8 for overview, page 31). This allows the receptor to act as a guanine nucleotide exchange factor (GEF), catalysing the exchange of Gα-bound GDP for GTP. The heterotrimeric G protein dissociates into Gα-GTP and Gβγ subunits which then act upon downstream targets. The Gβγ subunit is generally thought to remain as a tightly associated heterodimer throughout the signalling process, however there is an exception to this rule. It has been shown that a Gβ subunit exists in the brain and retina that only associates with the γ-like domain of a regulator of G protein signalling (RGS), instead of a traditional Gγ protein (Cabrera *et al*, 1998; Witherow *et al*, 2000). Once dissociated from the Gα subunit, Gβγ is able to activate signalling enzymes such as phospholipase A_2 (PLA_2), or directly act upon ion channels such as L-type Ca^{2+} channels and G protein-coupled

inward rectifying K^+ channels (Jelsema & Axelrod, 1987; Viard, 1999; Hommers *et al*, 2003).

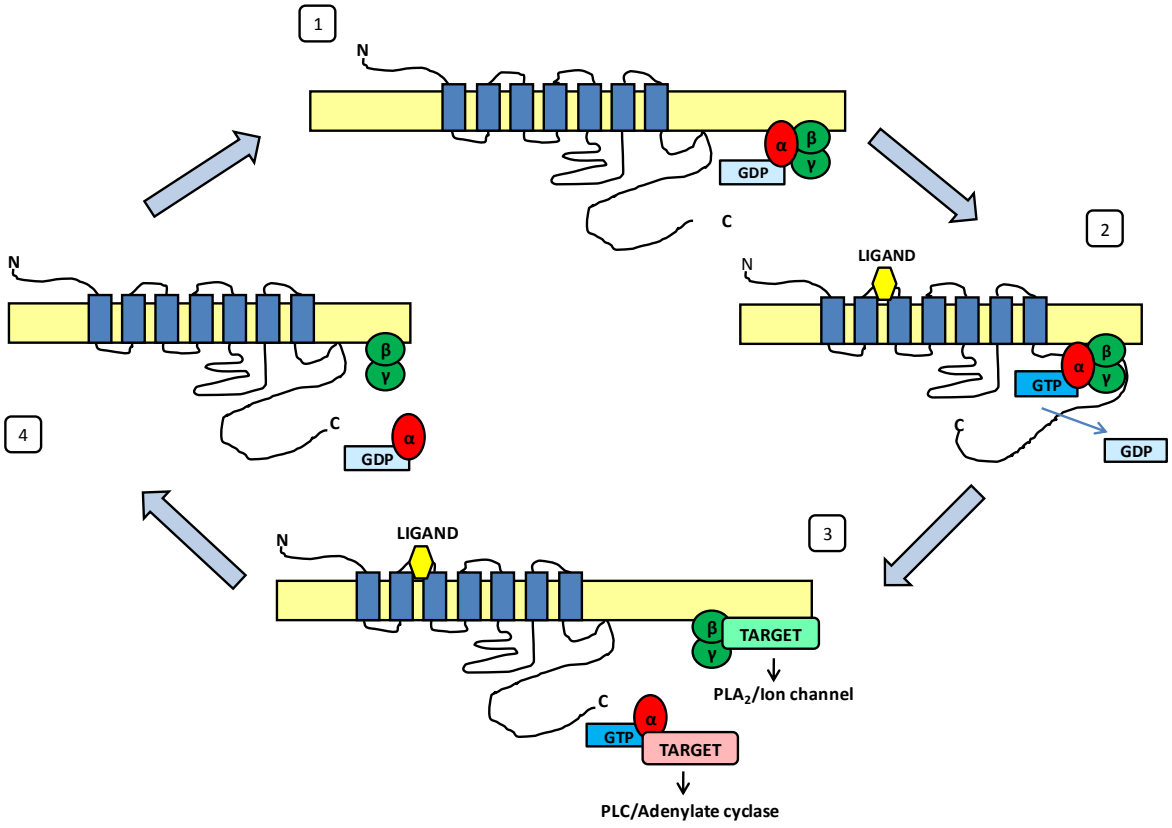


Figure 1.8: Conformational changes during GPCR activation. (1) In the unoccupied, resting state the inactive heterotrimeric G protein is bound to GDP. (2) Upon ligand binding a conformational change occurs in the receptor, exposing a catalytic component that exchanges GDP for GTP. (3) The active Gα-GTP subunit dissociates from the membrane-bound Gβγ subunit and both trigger downstream effectors and signalling pathways. (4) The signal is terminated by hydrolysis of GTP to GDP by intrinsic GTPase activity of the α-subunit, and the receptor/G protein complex returns to resting state.

GPCRs are further classified by the associated G protein α subunit (see Figure 1.9, page 32).

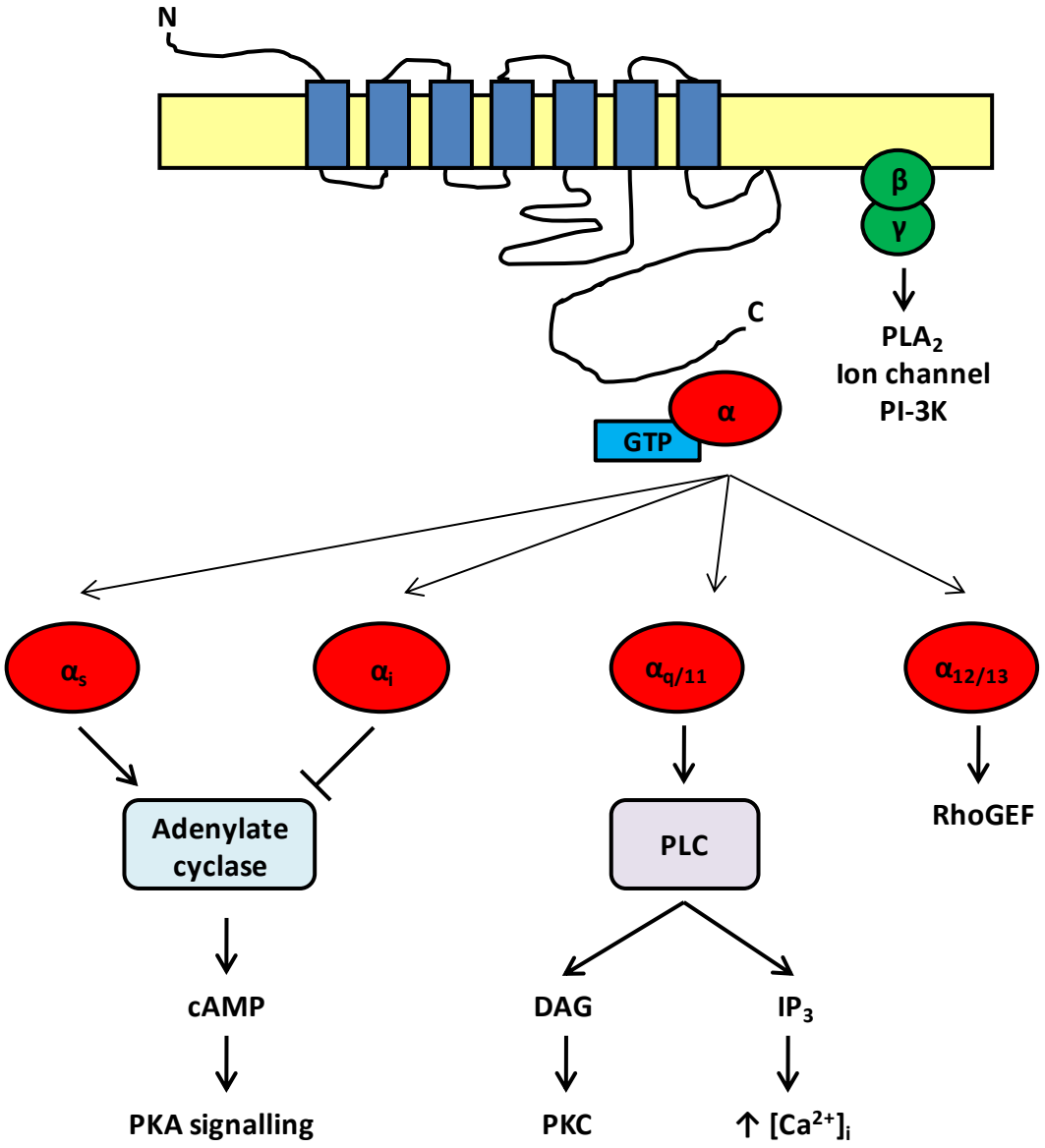


Figure 1.9: Signalling pathways mediated by the main classes of G protein. $G\alpha_s$ and $G\alpha_i$ proteins have respective stimulatory and inhibitory effects on adenylate cyclase and its downstream effectors. $G\alpha_{q/11}$ protein activates phospholipase C, leading to protein kinase C activation and calcium signalling. $G\alpha_{12/13}$ proteins act upon guanine nucleotide exchange factors of the Rho family.

The main subtypes are $G\alpha_s$, $G\alpha_{i/o}$ and $G\alpha_{q/11}$. $G\alpha_s$ proteins are so named because of the stimulatory effect of the subunit on the enzyme adenylate cyclase, which produces second messenger cAMP. $G\alpha_s$ proteins are selectively activated by cholera

toxin. The $G\alpha_{i/o}$ protein subunit, on the other hand, has an inhibitory effect on adenylate cyclase, and is sensitive to pertussis toxin. The $G\alpha_{q,11}$ proteins affect the phosphoinositide system by activating phospholipase C-β (PLCβ), which cleaves phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG then activates protein kinase C (PKC) while IP_3 stimulates the release of intracellular calcium. These proteins are also distinguished from $G\alpha_s$ and $G\alpha_{i/o}$ proteins as they are insensitive to both cholera toxin and pertussis toxin.

The G protein-mediated signal is terminated by hydrolysis of GTP to GDP and re-association of the inactive Gαβγ heterotrimer. The Gα subunit has innate GTPase activity, but this does not always terminate the signal fast enough. Regulators of G protein signalling (RGS) proteins enhance the speed of hydrolysis by acting as GTPase-activating proteins (GAPs), this is possible as they contain a RGS domain that binds to the Gα subunit and stimulates GTPase activity (for review see Hendriks-Balk *et al*, 2008).

There are many cardioprotective GPCRs expressed in the heart, including adenosine receptors, the signalling mechanisms of which are of interest with regard to finding pharmacological targets to protect against myocardial injury.

1.6. Adenosine receptors

Adenosine is a purine nucleotide released from myocardial tissue during periods of metabolic stress. It equilibrates the balance between cellular energy supply and demand, as well as having important roles in normal physiological regulation. Research has shown that adenosine is released during ischaemia or ischaemia/reperfusion, and it has been reported that the extracellular concentration of adenosine in the body can increase 100-fold up to 10 μM during hypoxia or

ischaemia (Schulte & Fredholme, 2003). This endogenous adenosine then exerts a cardioprotective effect against ischaemia/reperfusion-induced injury and cell death (Mubagwa & Flameng 2001; Fryer et al, 2002).

Four adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) belonging to the GPCR superfamily have been cloned and characterised (Fredholm *et al*, 2001). The A_1 and A_3 receptors are negatively coupled to adenylate cyclase via pertussis toxin-sensitive Gi/o proteins, therefore inhibit cAMP production (Fredholm *et al* 2001). It is well known that the A_1 and A_3 receptors are involved in cardioprotection (Mubagwa & Flameng 2001; Fryer, Auchampach & Gross 2002). Adenosine A_{2A} and A_{2B} receptors are positively coupled to adenylate cyclase via G_s proteins, and have high and low affinities for adenosine, respectively. All four of the adenosine receptor subtypes are expressed in the heart (see Auchamp & Bolli, 1999), but the presence of A_{2A} receptors on cardiomyocytes is debateable. Adenosine A_{2A} receptors are widespread throughout coronary vessels where activation causes potent vasodilation, but are reported to be absent from porcine cardiomyocytes (Hein *et al*, 2001). In 2002 it was reported that rat ventricular myocytes express A_{2A} receptors, but these receptors are not functional as no response was observed from selective agonism (Kilpatrick *et al*, 2002). However, in separate studies functional A_{2A} receptors have been described in adult and neonatal rat cardiomyocytes (Xu *et al*, 1996; Germack & Dickenson, 2004) and human atrial myocytes (Hove-Madsen *et al*, 2006).

Ischaemic preconditioning reduces infarct size in intact rabbits, and this protection has been shown to be abolished by infusion of different non-selective adenosine receptor antagonists prior to the preconditioning phase (Liu *et al*, 1991). This pointed to an important role for adenosine and one or more adenosine receptor subtypes in ischaemic preconditioning. This report also progressed on to show that

selective antagonism of the A_1 receptor blocked the protection afforded by ischaemic preconditioning. Further strengthening the role for the A_1 receptor in preconditioning, the same group (Thornton *et al*, 1992) demonstrated that infusion of a selective A_1 agonist prior to ischaemia reduced infarct size to the same extent as ischaemic preconditioning – but an A_{2A} agonist had no effect. It has also been shown that activation of the A_1 receptor is an effective trigger of the early and late phases of preconditioning in isolated hearts from normal and hypertensive rats (Hochhauser *et al*, 2007); in addition, this report showed that activation of the A_3 receptor was partially effective as a preconditioning trigger, particularly in the late-phase model. A place for the A_3 receptor in cardioprotection had also been described earlier by Auchampach *et al* (1997), who reported that activation of the A_3 receptor in conscious rabbits gave a level of protection equivalent to that obtained from ischaemic preconditioning. This report measured the effect of A_3 receptor activation on both myocardial stunning and infarct size, and claimed protection in both areas, interestingly without any hemodynamic changes. Activation of the A_1 receptor produces vasodilation, which is undesirable in the clinical setting (Mustafa *et al*, 2009). There are many studies assessing infarct size as a measure of cardioprotection, but fewer that look into myocardial function. One such study found that ischaemic preconditioning did not preserve myocardial function in dogs, but preconditioning with adenosine did (Phillips & Ko, 2007). However, this protection came at a cost as treatment with adenosine lead to an increased energy requirement of the heart and oxygen wasting.

The timing of pharmacological treatment can also affect the extent of protection gained, but findings do vary between models used. For example, infusion of adenosine at the time of reperfusion is effective at reducing infarct size in dogs

(Velasco *et al*, 1991), but no protection was observed in isolated rabbit hearts (Xu *et al*, 2001). It has been reported that activation of the A_{2B} receptor at the time of reperfusion is important for ischaemic preconditioning in rabbit hearts (Kuno *et al*, 2007), and also that postconditioning is dependent on activation of the A_{2B} receptor, but not A_1 or A_{2A} receptors (Philipp *et al*, 2006). These data suggest that the A_{2B} receptor is the main mediator of postconditioning, but the protective effect of ischaemic postconditioning is lost in A_1 receptor knock-out mice, suggesting an additional role for the A_1 receptor (Xi *et al*, 2008). Interestingly, the protection afforded by ischaemic preconditioning was absent in A_{2B} receptor knock-out mice – but mice lacking the A_1, A_{2A} or A_3 receptor had a preserved response (Eckle *et al*, 2007). It has been proposed that the release of adenosine during ischaemic preconditioning triggers activation of the A_1 and A_3 receptors, which (via a Gi protein and PKC-dependent pathway) activate the A_{2B} receptor. The A_{2B} receptor then mediates ischaemic preconditioning by targeting effecter proteins such as the KATP channel and the mPTP (Cohen & Downey, 2008).

There is a clear role for the adenosine A_1 and possibly A_{2B} and A_3 receptors in cardioprotection, however, the role of the A_{2A} receptor remains controversial. For example, in anesthetized rabbits there is evidence for and against adenosine A_{2A} receptor-mediated protection. Phillip *et al* (2006) described that postconditioning could be blocked with an antagonist at the A_{2B} receptor, but not the A_{2A} or A_1 receptors. Alternatively, Boucher *et al* (2004) described that activation of the A_{2A} receptor 5 minutes prior to (but not 5 minutes after) the onset of reperfusion successfully reduced infarct size.

Understanding the signal transduction mechanisms involved in adenosine receptor-mediated cardioprotection is an area of intense investigation, especially since

adenosine has shown promise in clinical trials (Bolli *et al* 2004). However, further work is needed before further clinical trials are considered. The Acute Myocardial Infarction Study of Adenosine Trials (AMISTAD and AMISTAD II) showed that adenosine was effective at reducing infarct size in humans, but there was no significant improvement of clinical outcome (Mahaffey *et al*, 1999; Ross *et al*, 2005). From the diverse range of data obtained from animal models it was perhaps unwise to invest such a large amount of time and money into clinical trials at this stage.

As mentioned previously, stimulation of the adenosine A_1 receptor has been found to lead to activation of signalling pathways involving several different protein kinases. PKC (Henry *et al*, 1996), ERK1/2 (Germack & Dickenson, 2005) and p38 MAPK (Dana *et al*, 2000) have all been implicated, and another commonly described feature of adenosine A_1 receptor-mediated cardioprotection is the mitochondrial K_{ATP} channel (e.g. Van Winkle *et al*, 1994; Heurteaux *et al*, 1995; Baxter & Yellon, 1999). The K_{ATP} channel is one of several cytoprotective channels expressed in the inner mitochondrial membrane, and has a well reported role in cardioprotection. More recently, however, a large conductance calcium-activated potassium channel (BK_{Ca} channel) located to the inner mitochondrial membrane has also been implicated in cardioprotection.

1.7. Large conductance calcium-activated potassium channels

There are two types of BK_{Ca} channel, one found on the inner mitochondrial membrane and the other on the cytoplasmic membrane. Mitochondrial BK_{Ca} channels are abundant in the brain and cardiac tissue, cytoplasmic BK_{Ca} channels are ubiquitously expressed throughout vascular beds in the membranes of smooth muscle cells (Korovkina & England, 2002). The exact structure of the BK_{Ca} channel is currently under debate, but it is thought that the channel consists of a tetramer of pore-forming α-subunits that alone constitute a fully functional channel (see Figure 1.10, page 39), with associated modulatory β-subunits (Yusifov *et al*, 2008). The β1 subunit is expressed in mammalian cardiac mitochondria (Ohya *et al*, 2005), and the β4 subunit is highly expressed throughout brain mitochondria (Piwonska *et al*, 2008) – however, although it is predominantly located to the brain, the β4 subunit has also been detected in cardiac tissue (Poulsen *et al*, 2009).

The α-subunits are proposed to form a C-terminal Ca^{2+} bowl, containing two Ca^{2+}-sensing domains (RCK, regulators of the conductance of potassium, domains), with the voltage-sensing domain located on the external side of the channel (Quin *et al*, 2006) (see Figure 1.11, page 39).

Xu *et al* (2002) first discovered the presence of this channel on the inner mitochondrial membrane of guinea pig ventricular cells, and reported that pharmacological activation of this channel provided protection against ischaemic injury. Since then, mitochondrial BK_{Ca} channels have been implicated in cardioprotective mechanisms in several different models including anesthetized dogs and isolated murine hearts (Shintani *et a*l, 2004; Wang *et al*, 2004).

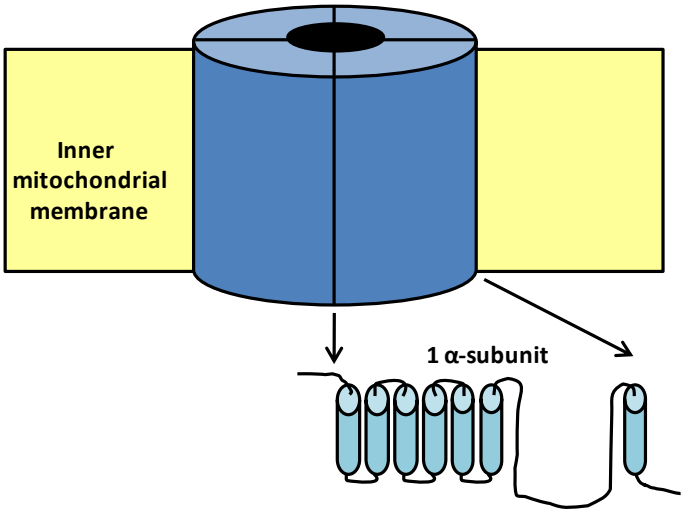


Figure 1.10: The BK_{Ca} channel is formed from a tetramer of α-subunits (dark blue). Each subunit consists of seven membrane spanning domains (see highlighted light blue section).

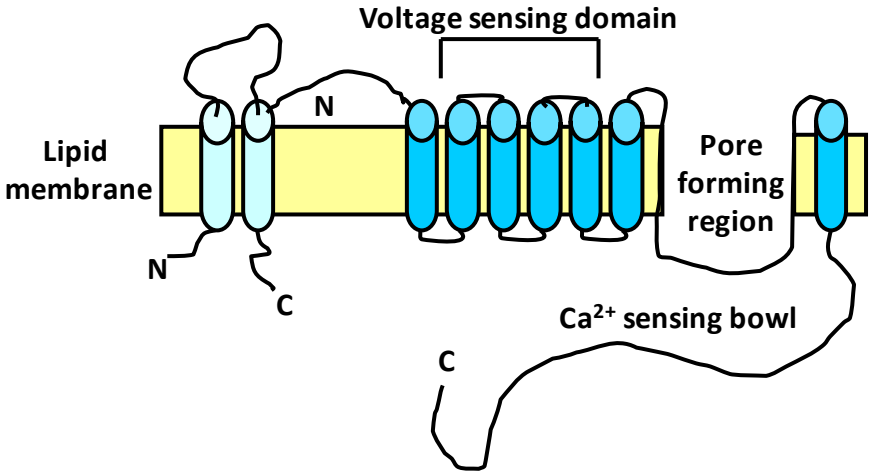


Figure 1.11: Proposed structure of the BKCa channel subunits. Each α-subunit (dark blue) has an internal Ca^{2+} sensing bowl and an external voltage sensing domain, and a pore is formed between the seventh and eight membrane spanning domains. One auxiliary β-subunit (light blue) can associate with the N terminal of an α-subunit.

On separate occasions Cao's group used rat ventricular myocytes and isolated perfused rat hearts to elaborate on the role of the mitochondrial BK_{Ca} channel, and

reported that this channel is responsible for triggering cardioprotection of ischaemic preconditioning (Cao *et al*, 2005a) and cardioprotection mediated by the κ-opioid receptor (Cao *et al*, 2005). In the latter study it was also deduced that PKC activation occurs upstream of the mitochondrial BK_{Ca} channel, and activation of the mitochondrial BK_{Ca} channel is proposed to lead to inhibition of the mPTP (Cao *et al*, 2005; Cheng *et al*, 2008). Several other studies have also implicated protein kinases in the signal transduction pathways leading to mitochondrial BK_{Ca} channel activation. In 2007, Gao *et al* reported that the cardioprotective isoflavone puerarin provides protection against ischaemia/reperfusion in isolated perfused rat hearts by activation of PKC and the mitochondrial BK_{Ca} channel. However, Sato *et al* (2005) claimed that activation of PKA enhanced NS1619 (BK_{Ca} channel opener)-induced flavoprotein oxidation, suggesting that a mechanistic link is present between the two signal transducers. However, no such augmentation was observed with PKC and the BK_{Ca} channel. Redel *et al* (2008) also found that PKA was involved in signal transduction leading up to BK_{Ca} channel activation, in this case in anaesthetic-induced preconditioning with desflurane. Activation of the BK_{Ca} channel is involved in preconditioning triggered by a variety of pharmacological agents including tumour necrosis factor-α, estradiol, the phosphodiesterase type 5 inhibitor sildenafil and the phosphodiesterase type 3 inhibitor cilostazol (Gao *et al*, 2005; Ohya *et al*, 2005; Wang *et al*, 2008; Fukasawa *et al*, 2008), and GPCR agonists such as (-)-U-50488 and adrenomedullin (Cao *et al*, 2005; Nishida *et al*, 2008). However, to date, the role of the BK_{Ca} channel in adenosine receptor-mediated signalling has yet to be investigated.

Overall, in simple terms, it can be presumed that pre- and postconditioning are triggered by stimulation of GPCRs, which leads to recruitment of cell survival

protein kinases, and culminates in activation of cytoprotective ion channels. However, the precise mechanisms are still under debate. Any information that could lead to elucidation of cardioprotective signalling would help in the search for effective pharmacological targets against ischaemia and ischaemia/reperfusion injury.

1.8. H9c2 cells and rat ventricle strips – models for studies into cardioprotection

The H9c2 cell line is derived from embryonic rat heart tissue (Kimes & Brandt, 1976), and is increasingly used as an in vitro model of cardiac tissue for studies into cardioprotection as these cells display similar morphological, electrophysiological and biochemical properties to primary cardiac cells (Hescheler *et al*, 1991). It has previously been shown that adenosine can protect against simulated ischaemia in H9c2 cells, although the subtype of adenosine receptor involved in such preconditioning and the type of cell death triggered by ischaemia were not reported (Nagarkitti & Sha'afi, 1998).

H9c2 cells are a useful model for investigating cardiac signal transduction, and also for assessing the cardioprotective potential of pharmacological agents from a cell viability perspective. It is always advisable to compare experimental results from more than one model as discrepancies often occur between different cell, tissue and animal models, so a comparison of data from more than one model gives a more rounded perspective and verification of results. To validate the results obtained from H9c2 cells in this report a model using isolated rat right ventricle strips was utilised.

Rat ventricle strips are a more physiologically relevant model for studies into cardioprotection than H9c2 cells, and are reported to exhibit a larger hypoxia-

induced mechanical impairment than strips from guinea pig or frog ventricles (Joseph *et al*, 2000) – therefore they are ideal for assessing the potential of pharmacological agents to protect against hypoxia. It has been shown that adenosine treatment does not provide any benefit to rat ventricle strips during hypoxia (Varela *et al*, 1999), but preconditioning with adenosine and agonists specific to the adenosine A_1, A_{2A} and A_3 receptors significantly increase the return of mechanical function during reperfusion (Button *et al*, 2005). However, the role of the BK_{Ca} channel in preconditioning of rat ventricle strips has not been investigated.

1.9. Aims of this project

The main aims of this project were:

1) To establish the functional expression of GPCRs on H9c2 cells, and assess the cytoprotective potential of such receptor activation

2) To investigate the role of the BK_{Ca} channel in adenosine A_1 receptor-mediated signalling in H9c2 cells

3) To validate the data obtained from H9c2 cells and research the role of the BK_{Ca} channel in adenosine receptor-mediated preconditioning in rat ventricle strips

2. Methods

2.1. Cell Culture

Rat embryonic cardiomyoblast-derived H9c2 cells (European Collection of Animal Cell Cultures, Salisbury, UK) were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Cells were maintained in a humidified incubator (95% air / 5% CO_2, 37°C) and grown to 70-80% confluency to avoid differentiation and formation of myotubes. Typically, cells were sub-cultured with a 1:5 split using trypsin (0.05% w/v)/EDTA (0.02% w/v). Cells for functional receptor expression assays were grown in 24 well plates (Sarstedt), cells for LDH assay were grown in 96 well plates (Sarstedt) and cells for caspase-3 analysis and western blot were grown in 60mm culture dishes (Greiner Bio-One).

2.2. Functional Expression of GPCRs:

i) Measurement of cAMP accumulation

Confluent H9c2 cells were incubated with 500 µl serum-free DMEM and 2 µCi [^{3}H]-adenine for 2 hours, then washed twice with Hank's Balanced Salt Solution (HBSS) to remove excess radioactivity. Cells were treated with the phosphodiesterase inhibitor rolipram (10 µM in 500 µl serum-free DMEM) and incubated for 15 minutes in a humidified incubator (95% air / 5% CO_2, 37ºC). Agonists were then added (1:10 dilution) and incubated for a further 15 minutes. When assessing G_i-coupled receptor activity, the agonists were added 5 minutes prior to a 10 minute incubation period with forskolin (10 µM). If required, cells were pre-treated with antagonists for 15 minutes prior to addition of rolipram. The experiments were

terminated by exchanging the media for 500 µl trichloroacetic acid (5% w/v) then plates were wrapped in foil and stored at -20ºC.

Samples were purified by gravitational column chromatography. Dowex columns were pre-washed with 5 ml HCl (1 M) and 20 ml distilled water, alumina columns were pre-washed with 20 ml imidazole (0.1 M). Well content was transferred to the dowex columns and washed through with 3 ml distilled water. The dowex columns were then placed on top of the alumina columns and 4 ml distilled water washed through both sets. 5 ml HCl (1 M) and 20 ml distilled water was then used to wash the dowex columns only, while 5 ml imidazole (0.1 M) was drained through the alumina columns and collected in scintillation vials. The cAMP had now been eluted from the alumina columns so they were washed with 20 ml imidazole (0.1 M). To the vials, 0.5 ml HCl (1 M) and 10 ml liquid scintillant was added (Packard Biosciences). Vials were shaken and activity read using a liquid scintillation counter (Packard Tri-Carb Liquid Scintillation Analyser). For reference, see Cordeaux *et al* (2000).

ii) Inositol trisphosphate Assay

Confluent H9c2 cells were pre-labelled with 3 µCi [^{3}H] *myo*-inositol in 500 µl serum-free DMEM for 24 hours. Excess radioactivity was then removed by two washes with HBSS, and then cells were incubated with LiCl (20 mM) in 500 µl serum-free DMEM for 30 minutes in a humidified incubator (95% air / 5% CO_2, 37ºC). Without removing the LiCl the cells were further incubated with G_q agonist for 30 minutes (1:10 dilution). Reactions were stopped by replacing the media with 1 ml termination mix (6 ml concentrated HCl in 500 ml dH_2O added to 500 ml methanol; pH 2-3).

Samples were purified by anion-exchange chromatography. HCl (10 ml; 1 M) was washed through the columns followed by 20 ml distilled water. Samples were then added to 4 ml neutralising solution (0.5 M NaOH, 25 mM Tris; pH 7) and run through the columns; 20 ml distilled water followed. 10 ml of ammonium formate (25 mM) was drained through then the columns were placed above scintillation vials. 3 ml HCl (1 M) was run through into the vials then 10 ml liquid scintillant was added before reading the radioactivity using a liquid scintillation counter (Packard Tri-Carb Liquid Scintillation Analyser). For reference, see White *et al* (1992).

2.3. Protection against hypoxia- and hypoxia/reoxygenation-induced cell death: Experimental protocol

80% confluent H9c2 cells in glucose and serum-free DMEM were exposed to hypoxia (5% CO_2/0.5% O_2; 37°C) for 6 hours (NB. this time varied for time-course experiments) before performance of the appropriate cell viability assay. Normoxic incubation was used for controls.

Where pharmacological preconditioning was required, cells were incubated with GPCR agonist or potassium channel opener in 200 µl (when using microplates) / 2 ml (when using culture dishes) serum-free DMEM for 30 minutes; this medium was then discarded and replaced with 200 µl / 3 ml glucose and serum-free DMEM for the hypoxic/normoxic incubation. When required, cells were also treated with antagonist or potassium channel blocker for 15 minutes prior to the addition of agonist or channel opener.

For hypoxia-reoxygenation experiments, cells were exposed to hypoxia for 6 hours as before, and then reoxygenated for 18 hours in 200 μl / 3 ml DMEM containing glucose and 1% serum. Preconditioning occurred prior to hypoxia, postconditioning occurred at the start of reoxygenation – treatment protocol as before (see Figure 2.1 for summary)

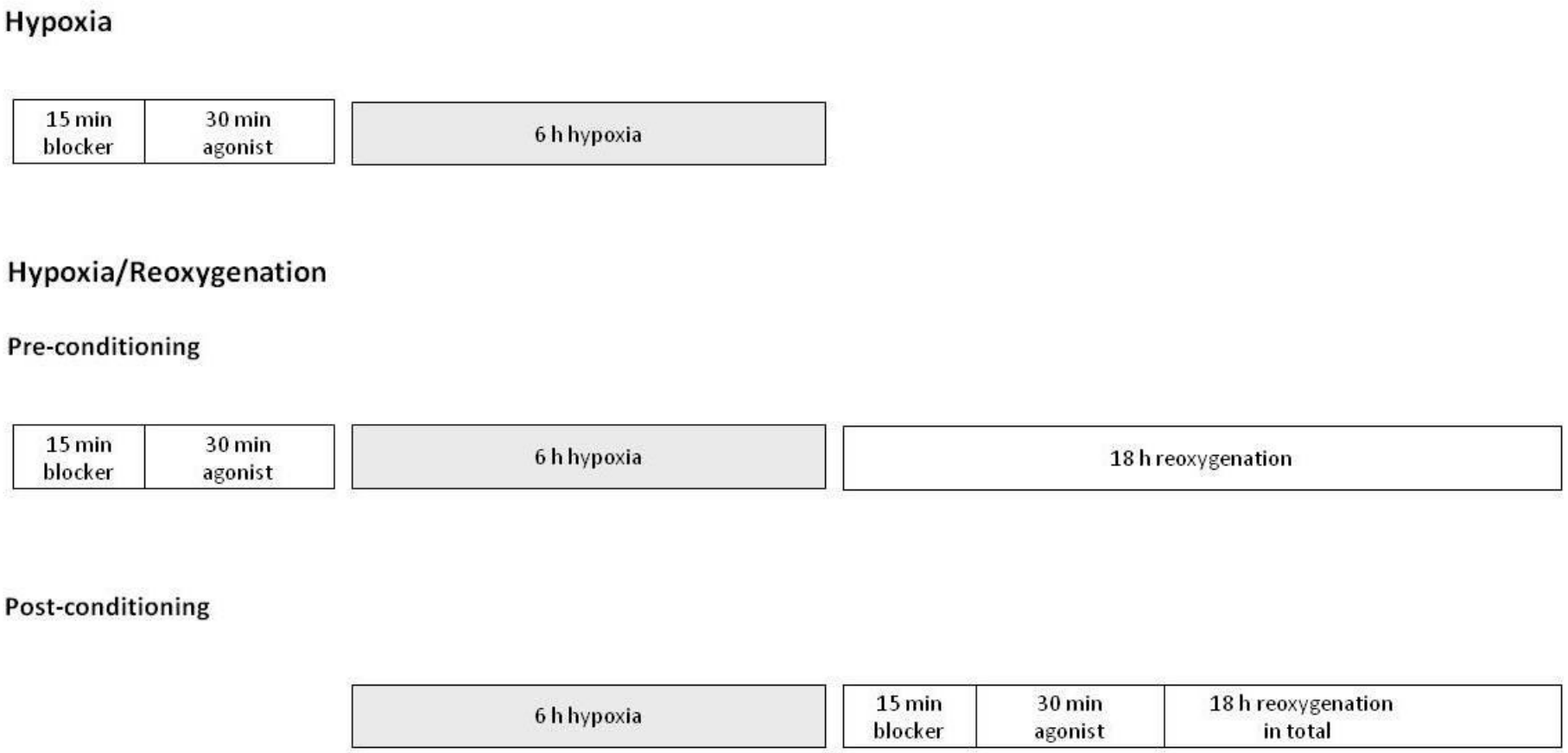


Figure 2.1: Protocol for hypoxia and hypoxia/reoxygenation experiments

i) MTT Assay

H9c2 cells were cultured in 96 well flat bottom plates, at a density of 5000 cells/well, for 24 hours in fully supplemented DMEM. Incubations were performed in 200ul glucose/serum-free DMEM, then cell viability measured by colorimetric assessment of the mitochondrial reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a coloured formazan product. Cells were incubated for 1 hour (37°C) in 0.5 mg/ml MTT. Solution was then aspirated away and replaced with 100 µl DMSO. Plates were shaken to solubilise the formazan product then absorbance read at 570nm using a standard plate reader (Model 680, Bio-Rad). Results expressed as percentage of basal MTT reduction.

ii) Lactate dehydrogenase (LDH) Assay

H9c2 cells were cultured in 96 well flat bottom plates, at a density of 5000 cells/well, for 24 hours in fully supplemented DMEM. Incubations were performed in 200 µl of the appropriate medium then cell death measured using the colorimetric CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). This kit allows LDH release to be measured via a coupled chemical reaction that leads to formation of a coloured formazan product. Firstly, LDH released from the cells catalyses the formation of pyruvate and NADH from lactate and NAD^+. The secondary reaction is catalysed by the dehydrogenase enzyme diaphorase (present in the substrate mix), and involves the formation of NAD^+ and a red formazan product from NADH and a tetrazolium salt. LDH release is proportional to the red formazan product, which is measured using a standard plate reader (Model 680, Bio-Rad).

Plates were centrifuged to compact any cell debris to the bottom of the wells (5 minutes at 300 g), and then 50 µl of supernatant was transferred to a non-sterile 96 well plate ("assay plate"; Greiner Bio-One). A further 60 µl of the supernatant was discarded before 10 µl of 10x lysis buffer (9% v/v Triton® X-100, provided with kit) was added to the remaining 90 µl. Following 45 minutes incubation at 37°C, the plates were centrifuged for 5 minutes at 300 g. As before, 50 µl of supernatant was added to the assay plate. Reconstituted assay buffer (50 µl; provided in the kit) was added to each well, and then the assay plates were wrapped in foil and agitated for 30 minutes at room temperature. To terminate the reactions, 50 µl of stop solution (1 M acetic acid, provided with kit) was dispensed into each well; absorbance was then read at 490nm.

LDH release was calculated as a percentage of total LDH, and results are presented as LDH release as a percentage of basal release (acquired from untreated controls).

iii) Caspase-3 Assay

Cells were cultured in 60 mm dishes, until confluent, in fully supplemented DMEM. Where appropriate, treatments were performed in 2 ml serum-free DMEM, this was replaced with 3 ml glucose/serum-free DMEM for the 6 hours incubation in hypoxia or normoxia. When the incubation period was over phosphate buffered saline (PBS) was used to wash the cells which were then trypsinised and centrifuged for 10 minutes at 300 g to obtain a cell pellet. Supernatant was discarded and pellets were stored at -20°C until required for the caspase-3 assay.

Cell pellets were resuspended in 120 µl lysis buffer (25 mM HEPES, 2.5 mM CHAPS, 2.5 mM DTT, pH 7.4) and incubated on ice for 20 minutes. Lysate was

then centrifuged for 10 minutes (4°C, 14000 rcf) and supernatant collected and stored on ice. Supernatant (30 µl) was added to a black microplate (Greiner Bio-One) along with 50 µl assay buffer (20 mM HEPES, 5 mM DTT, 0.1% CHAPS, 2 mM EDTA, pH 7.4) and 20 µl substrate (0.5 mM, Acetyl-Asp-Glu-Val-Asp-7-Amido-Methyl Coumarin). For blanks, assay buffer replaced supernatant.

Fluorescence was read over a 2 hour period resulting in a linear relationship between time and caspase-3 activity (FLUOstar Optima, BMG Labtech). In combination with the protein assay, caspase-3 activity per minute per µg protein (Ufluo/min/µg) was calculated and expressed as percentage of basal activity.

iv) DC Lowry Protocol – Protein Assay

Protein content of samples was measured using the DC Lowry Protocol (reagents from the Bio-Rad DC Protein Assay range) using bovine serum albumin standards (BSA). Standards were prepared with 2 mg/ml BSA to produce a calibration curve as below (table 2.1), to ensure that the relationship between protein content and absorbance was linear.

Table 2.1: BSA standard curve

Protein mg/ml	µl protein standard	µl dH2O
0.0	0	>10
0.2	20	180
0.4	40	160
0.5	50	150
0.8	80	120
1.0	100	100
1.5	150	50
2.0	>10	0

5 μl of standards and samples were added to a 96-well flat bottom plate in duplicate. 25 μl of reagent A' (comprising of 20 μl Reagent S to 1 ml Reagent A; BioRad) and 200 μl of Reagent B (BioRad) was added to each well and then plates were covered in foil and agitated for 15 minutes. Absorbance was read at 750nm using a standard plate reader (Model 680, Bio-Rad).

Protein content of the samples was obtained by manipulation of the equation of the standard curve; results expressed as μg protein/μl.

2.4. Western blot analysis of BK_{Ca} channel subunit expression and protein kinase phosphorylation

For the determination of BK_{Ca} channel subunit expression isolated mitochondrial, cytoplasmic and whole cell protein fractions (isolated by subcellular fractionation, see section 2.9) were mixed two parts to one part sample buffer (Cell Signalling Technology). For the assessment of protein kinase activation H9c2 cells were exposed to the conditions and treatments described previously and stated in the figure legends. Following stimulation cell supernatants were removed and the cells washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (100 μl; 150 mM NaCl, 50 mM Tris.HCl, 5 mM EDTA, 1% (v/v) IGEPAL CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS (sodium dodecyl sulphate), 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 0.1 mM phenylmethylsulphonylfluoride (PMSF), 10 μg/ml aprotinin and 5 μg/ml leupeptin). Cell lysates were cleared by centrifugation (5 minutes; 12,000 g; 4°C), the supernatant collected and mixed two parts to one part sample buffer – with an aliquot of the supernatant taken for analysis of protein content. Samples were boiled at 95ºC for 5 minutes and thoroughly vortexed to

ensure protein denaturation, and then stored at -20°C. Protein concentration was determined using Bio-Rad *DC* Protein assay as described above and protein samples separated by Sodium Dodecyl Sulphate/Polyacrylamide Gel Electrophoresis (SDS/PAGE) using a Bio-Rad Mini-Protean II system.

Equipment was set up according to the manufacturers' instructions and 20 ml of 12% acrylamide solution made per two gels (6.6 ml dH_2O, 8.0 ml 30% Acrylamide mix, 5.0 ml 1.5 M Tris buffer, 200 µl 10% SDS solution, 200 µl 10% ammonium persulphate (APS) solution, 20 µl TEMED) – APS and TEMED are responsible for initiating gel polymerisation so were added last. The unpolymerised acrylamide gel solution was pipetted between two glass plates held together in the BioRad equipment, with a gap at the top being left for dH_2O to ensure level setting of the gel. Once the gel had set, the water was poured off and replaced with stacking gel (for two gels: 4.1 ml dH_2O, 1.0 ml 30% Acrylamide mix, 750 µl 1.5 M Tris buffer, 60 µl SDS solution, 60 µl APS, 6 µl TEMED) and a plastic comb to form wells. The equipment was placed into the lower buffer chamber with electrophoresis buffer (24.8 mM Tris base, 192 mM Glycine, 3.5 mM SDS), ensuring that the buffer did not leak from between the two gels. Once the stacking gels had set the combs were removed and samples were loaded into the wells (20-30 µg/well depending on target protein). The circuit was then run for 45 minutes at 200 V.

Separated proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot system (1 hour at 100 V in 25 mM Tris base, 192 mM glycine and 20% MeOH). Following transfer, nitrocellulose membranes were stained with Poncea S (Sigma) and the appropriate band size identified and cut from the rest of the membrane. The desired strips of membrane were washed with the pH buffer Tris-

buffered saline (TBS) and blocked for 1 hour at room temperature in blocking buffer (TBS, 5% (w/v) skimmed milk powder, 0.1% Tween-20). Membranes were then incubated overnight at 4^{o}C in blocking buffer with the following primary antibodies: cytochrome *c* (Santa Cruz Biotechnology; 1:500); BK_{Ca} channel α subunit (Becton Dickinson; 1:250), BK_{Ca} channel β4 subunit (Sigma-Aldrich; 1:250), phospho-specific ERK1/2 (Thr^{202}/Tyr^{204}; Sigma Aldrich; 1:1000), and phospho-specific PKB (Ser^{473}; New England Biolabs (U.K.) Ltd; 1:500). The next day the primary antibody was removed and the membrane extensively washed three times for 10 minutes in TBS/Tween 20. Blots were then incubated for 1 hour at room temperature with the appropriate secondary antibody (1:1000) coupled to horseradish peroxidase (DAKO Ltd, Cambridge, UK) in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Little Chalfont, UK) and quantified by densitometry using GeneGenius BioImaging System (Syngene, Synoptics Ltd, UK). Replicate samples from each experiment were analysed on separate blots using total unphosphorylated ERK1/2 (New England Biolabs (U.K.) Ltd; 1:1000) and PKB (New England Biolabs (U.K.) Ltd; 1:1000) primary antibodies in order to confirm the uniformity of protein loading.

2.5. Subcellular fractionation

H9c2 cells were grown in 125 cm^2 culture flasks; to obtain enough protein 3 flasks were used per experiment. Once confluent cells were detached using trypsin and then centrifuged for 5 minutes at 300 g. The supernatant was discarded and the cell pellet resuspended in 1 ml PBS and centrifuged again (5 minutes, 300 g). This process was repeated, then the cell pellet was transferred to a Dounce tissue grinder

tube (Sigma) kept on ice using a Pasteur pipette and homogenised 10 times with grinder A and 10 times with grinder B (Sigma; each time consisting of one clockwise rotation of the grinder). The cell homogenate was resuspended in 500 µl of extraction buffer (10 mM HEPES, 1 mM EGTA, 200 mM mannitol, 1 mM sucrose) and transferred to a 2 ml centrifuge tube labelled "nucleus", then centrifuged for 5 minutes at 300 g (4°C). The supernatant was transferred to a 2 ml centrifuge tube labelled "mitochondria", with 100 µl being saved as total extract. The nuclear pellet was resuspended in 500 µl of extraction buffer and centrifuged for 10 minutes at 1000 g (4°C). The supernatant was again transferred to the mitochondria tube, and then the nuclear pellet was discarded. The mitochondrial fraction was centrifuged for 15 minutes at 10,000 g (4°C) then the supernatant was transferred to a 2 ml centrifuge labelled "cytoplasm". The mitochondrial pellet was resuspended in 500 µl extraction buffer and centrifuged for 10 minutes at 10,000 g (4°C). The supernatant was again transferred to the cytoplasmic extract tube and the mitochondrial pellet resuspended in 100 µl extraction buffer. All fractions were then stored at -20°C until required.

2.6. Right Ventricle Strip Preparation

All experiments were performed on adult Wistar rats of either sex which were executed by cervical dislocation in accordance with the Animals (Scientific Procedures) Act 1986. Hearts were rapidly removed and placed in ice-cold Krebs solution (119 mM NaCl, 25 mM $NaHCO_3$, 11.1 mM D-glucose, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.0 mM $MgSO_4$). One strip from the right ventricle wall (approximately 2 mm x 10 mm) was isolated per heart and then longitudinally attached to platinum electrodes and isometric force transducers. The strips were

placed under 2 g tension in a tissue bath (Harvard Single Heated Tissue Bath) containing 40 ml gassed Krebs (95% O_2-5%-CO_2, 37°C) and electrically stimulated at a frequency of 1 Hz (Ealing Dual Pulse Student Stimulator). Contractions were measured on a chart recorder (Harvard Student Oscillograph). All strips were equilibrated for 40 minutes in gassed Krebs (95% O_2-5% CO_2, 37°C) before drug challenge. Hypoxia was simulated by replacing the medium with gassed glucose-free Krebs (95% N_2-5% CO_2, 37°C). Normalisation of data controlled for variation of data due to ventricle thickness.

Experimental protocol:

Following the equilibration period, control strips were exposed to 30 minutes oxygenation followed by 30 minutes hypoxia and finally 30 minutes reoxygenation – this treatment protocol was previously established by members of my research group, therefore deemed appropriate for use with this current project (Button *et al*, 2005).

If appropriate, CPA or NS1619 was added to the medium for the final 2 minutes of oxygenation. When using DPCPX, Paxilline or Iberiotoxin the required drug was added at the start of oxygenation and remained there for the 30 minutes duration.

For hypoxic preconditioning, ventricle strips were equilibrated for 40 minutes as usual, and then exposed to a 10 minute preconditioning period of hypoxia followed by 20 minutes oxygenation, 30 minutes hypoxia and finally 30 minutes reoxygenation. When using iberiotoxin the drug was added after for the final 30 minutes of the equilibration. All drugs were washed out following treatment and no drugs were present during hypoxia or reoxygenation. See figure 2.2 for summary.

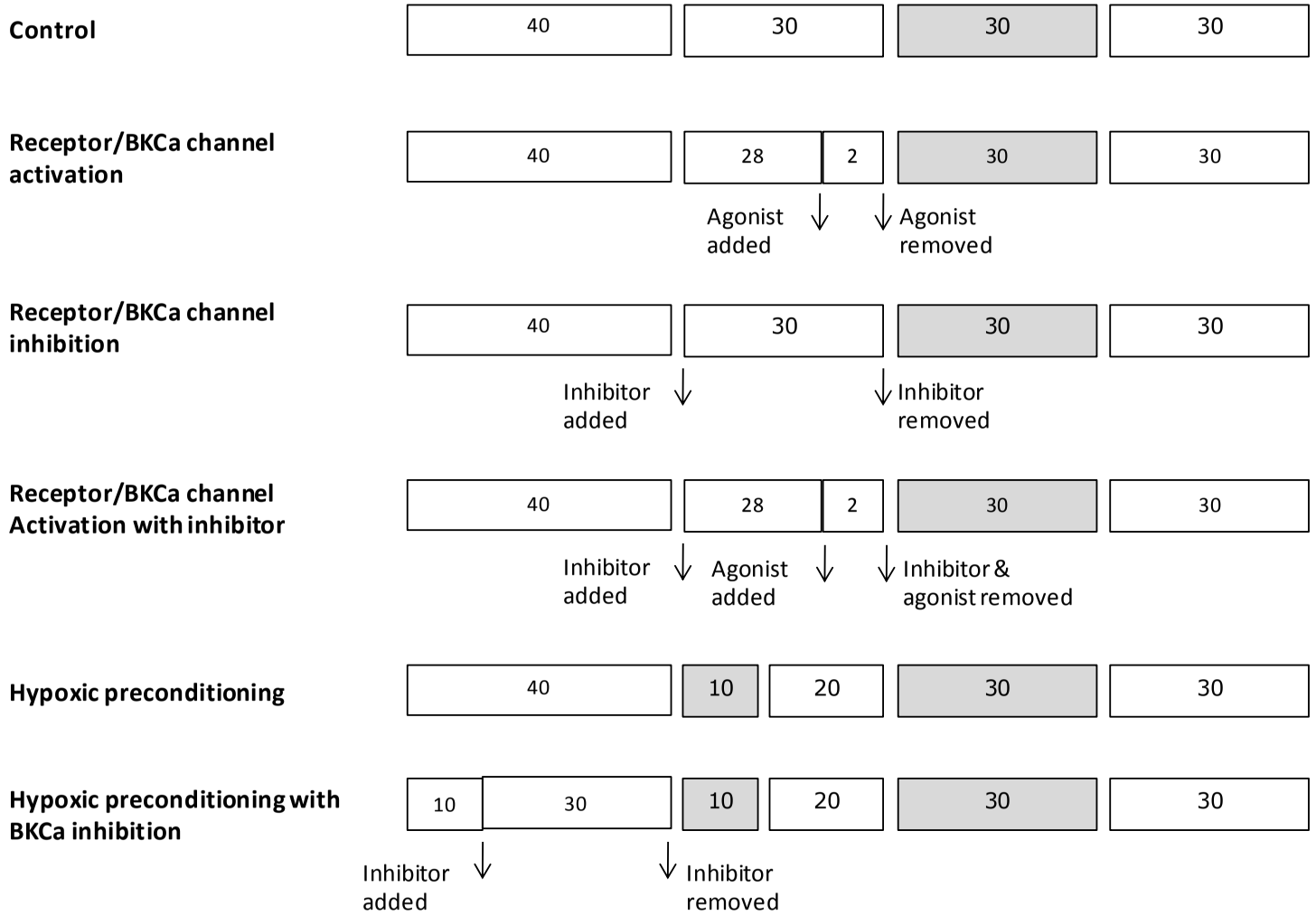


Figure 2.2: Protocol for rat ventricle hypoxia/reoxygenation experiments

2.7. Data analysis

GraphPad Prism 5 was used for all statistical analysis. The one-way ANOVA (analysis of variance) test was used to compare two or more sets of data, with the two-way ANOVA being used to compare grouped sets of data. Tukey's Multiple Comparison Post Test was used to further analyse the data to uncover where any significance lay. Significance was classed as $p<0.05$ and highlighted with an asterix; all data is presented as mean ± S.E.M.

2.8. Materials

Acetyl-Asp-Glu-Val-Asp-7-Amido-Methyl Coumarin, acetylcholine, 30% acrylamide mix, adenosine, ATP (adenosine triphosphate), baclofen, BSA (bovine serum albumin), $_{D}$-glucose, DPCPX (8-Cyclopentyl-1,3-dipropylxanthine), forskolin, histamine, imidazole, isoprenaline, lithium chloride, MRS1220 (N-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazo lin-5-yl]benzene acetamide), noradrenaline, NS1619 (1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one), PTX (pertussis toxic), rolipram, SDS (sodium dodecyl sulphate), TEMED (N,N,N',N'-Tetramethylethylenediamine), TCA (trichloroacetic acid), staurosporine, and UTP (uridine triphosphate) were all obtained from Sigma-Alrich.

(-)-U-50488 ((±)-U-50488 hydrochloride; trans-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cy clohexyl]benzeneacetamide hydrochloride), CGS (CGS 21680 hydrochloride; 4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H -purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride), CI-IB-MECA (2-CI-IB-MECA; 1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9- yl]-1-deoxy-N-

methyl-b-D-ribofuranuronamide), CPA (N^6-Cyclopentyladenosine), dobutamine (dobutamine hydrochloride; 4-[2-[[3-(4-Hydroxyphenyl)-1-methylpropyl]aminoethyl-1, 2-benzenediol hydrochloride], iberiotoxin, *nor*-BNI (*nor*-Binaltorphimine dihydrochloride; 17,17'-(Dicyclopropylmethyl)-6,6',7,7'-6,6'-imino- 7,7'-binorphinan-3,4',14,14'-tetrol dihydrochloride), paxilline, procaterol and SNC 80 ((+)-4-[(aR)-a-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3 methoxybenzyl]-N,N-diethylbenzamide) were all obtained from Tocris Biosciences.

All cell culture agents, unless stated, were obtained from Cambrex. Glucose-free DMEM was obtained from Invitrogen, and FBS (foetal bovine serum) was obtained from BioSera.

[^{3}H]-adenine was obtained from Amersham Biosciences; [3H]-myo-inositol was obtained from M.P Biomedicals.

Antibodies were obtained from the following suppliers: monoclonal BK_{Ca} channel α subunit from Beckon-Dickinson; polyclonal BK_{Ca} channel β4 subunit and monoclonal phospho-specific ERK1/2 (Thr^{202}/Tyr^{204}) from Sigma-Aldrich; polyclonal phospho-specific PKB (Ser^{473}), monoclonal total unphosphorylated ERK1/2 and polyclonal total unphosphorylated PKB from New England Biolabs (U.K.) Ltd; and monoclonal cytochrome *c* from Santa Cruz Biotechnology. All other chemicals were of analytical grade.

3. Functional GPCR studies

3.1. Functional expression of adenosine receptors on H9c2 cells

Adenosine is released from myocardial tissue during ischaemia or ischaemia/reperfusion (I/R), exerting cardioprotective effects against ischaemic injury/damage caused by reperfusion (Mubagwa & Flameng 2001; Fryer *et al*, 2002). Cardiomyocytes have been shown to express the adenosine A_1, A_{2A} and A_3 receptor subtypes (Germack & Dickenson, 2004; for review see Peart & Headrick, 2007), but the presence of functional adenosine receptors on H9c2 cells has not been reported. In this study GPCR expression on H9c2 cells has been investigated by measuring the effects of selective receptor agonists and antagonists on [^{3}H]-cAMP accumulation.

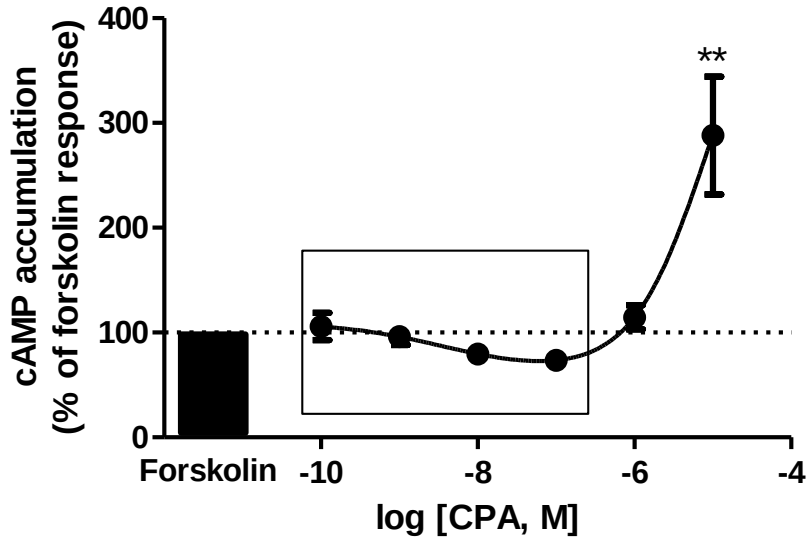
CPA, the selective A_1 receptor agonist, gave a modest inhibition of forskolin-induced cAMP accumulation (maximum inhibition occurring at 100 nM; 22.0 ± 4.2%; n=6; see Fig.3.1) but at higher concentrations produced a significant augmentation of cAMP-accumulation (maximum effect at 10 μM, 288 ± 56%, n=5, $p<0.01$). CGS 21680, the adenosine A_{2A} receptor agonist, did not increase cAMP accumulation (see Fig.3.2). CI-IB-MECA, the G_i-coupled A_3 receptor agonist (up to 1 μM), had no effect on forskolin-stimulated cAMP accumulation. However, 10 μM Cl-IB-MECA induced a significant increase in cAMP accumulation (179.6 ± 15% compared to forskolin response alone = 100%, n=5, $p<0.05$, see Fig.3.3).

The inhibition afforded by CPA was reversed by pertussis toxin (PTX), a specific blocker of G_i protein (119.1 ± 4% of forskolin response in the presence of PTX; n=6; see Fig.3.4), and by the adenosine A_1 receptor antagonist DPCPX (107.4 ± 7% of forskolin response in the presence of DPCPX; n=4; see Fig.3.4).

The augmentation of cAMP accumulation induced by CI-IB-MECA was blocked by the adenosine A_1 receptor antagonist DPCPX (112.2 ± 7% of forskolin response in the presence of DPCPX; n=4; see Fig.3.5) but not significantly by the adenosine A_3 receptor antagonist MRS 1220 (130.8 ± 11% of forskolin response in the presence of MRS 1220; n=4; see Fig.3.5).

These data suggest that H9c2 cells functionally express G_i-coupled adenosine A_1 receptors, but not adenosine A_{2A} or A_3 receptors, and that at high agonist concentrations the adenosine A_1 receptor can also couple to G_s protein.

(a)



(b)

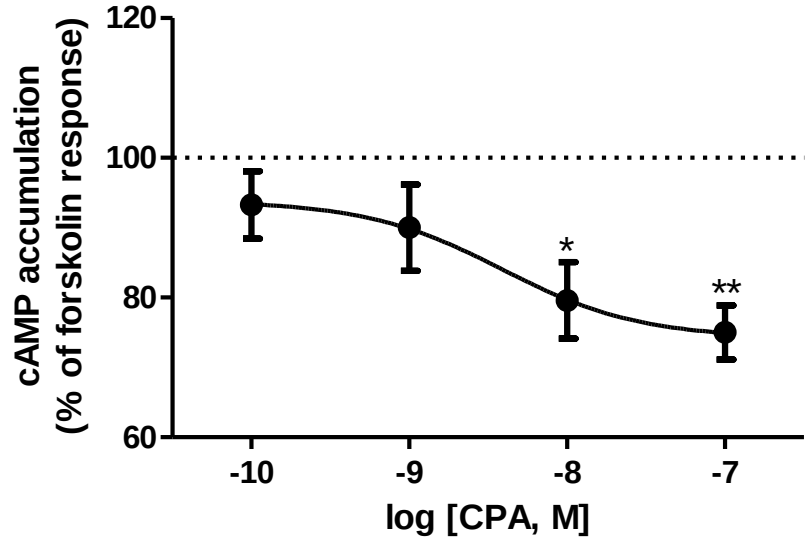


Figure 3.1: CPA-mediated effects on forskolin-induced cAMP accumulation in H9c2 cells. Cells were pre-treated with the indicated concentrations of the selective adenosine A_1 receptor agonist CPA for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin response (=100%). Each point represents mean ± S.E.M. of 5 separate experiments each performed in triplicate. (b) is an enlargement of the frame in (a). * $p<0.05$ vs. forskolin alone response; ** $p<0.01$ vs. forskolin alone response.

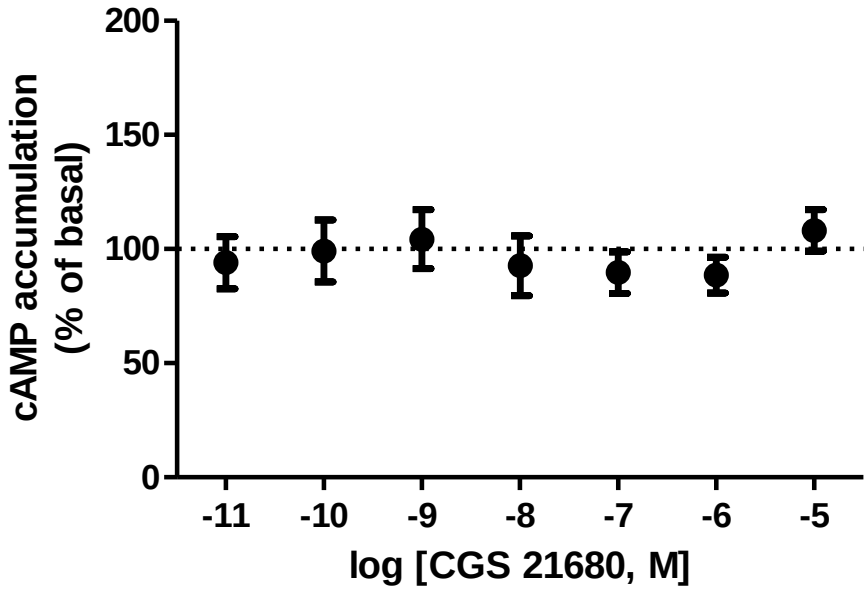


Figure 3.2: CGS 21680-mediated cAMP accumulation in H9c2 cells. Cells were treated with the indicated concentrations of the selective adenosine A_{2A} receptor agonist CGS 21680 hydrochloride for 15 minutes. Data expressed as percentage of basal cAMP accumulation (=100%). Each point represents mean ± S.E.M of 6 separate experiments each performed in triplicate.

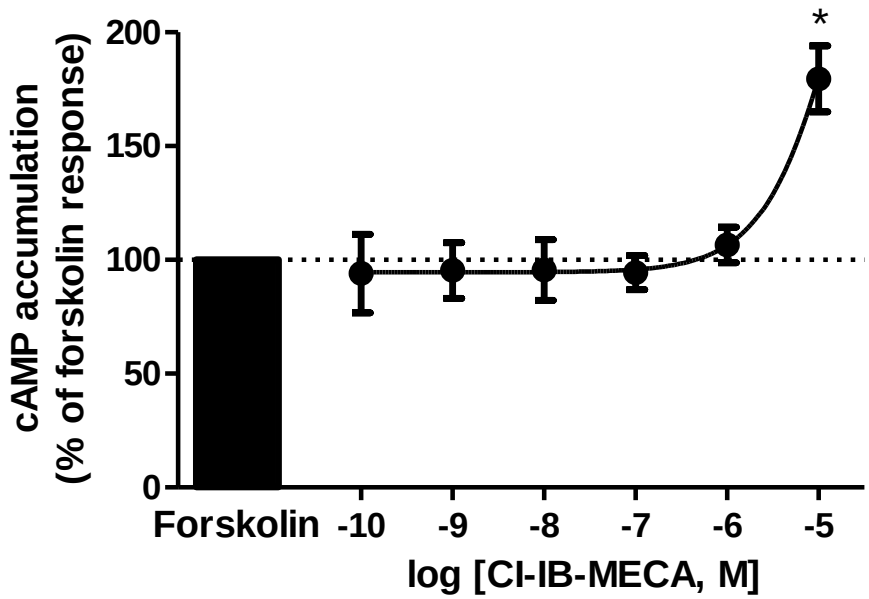
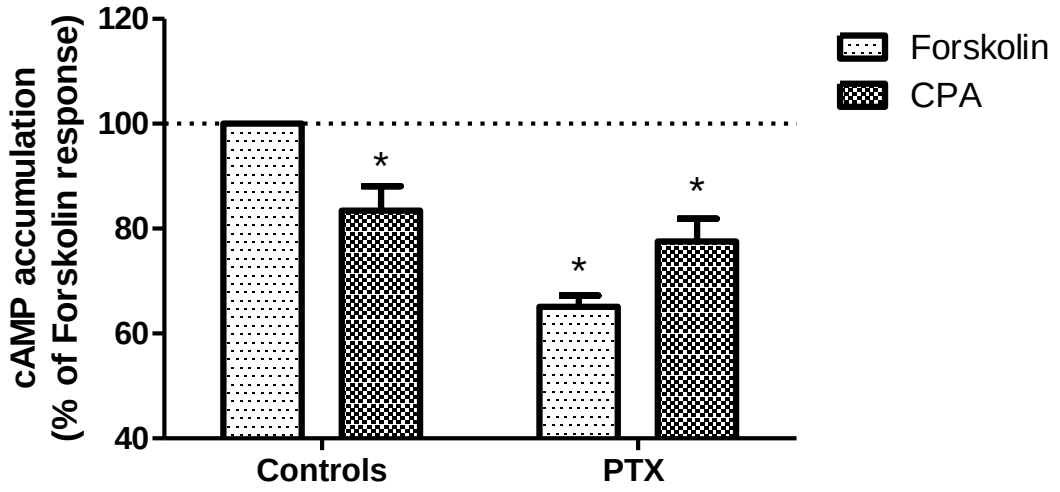


Figure 3.3: CI-IB-MECA-mediated effects on forskolin-induced cAMP accumulation in H9c2 cells. Cells were pre-treated with the indicated concentrations of the adenosine A_3 receptor agonist CI-IB-MECA for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin response (=100%). Each point represents mean ± S.E.M. of 5 separate experiments each performed in triplicate. * $p<0.05$ vs. forskolin alone response.

(a)



(b)

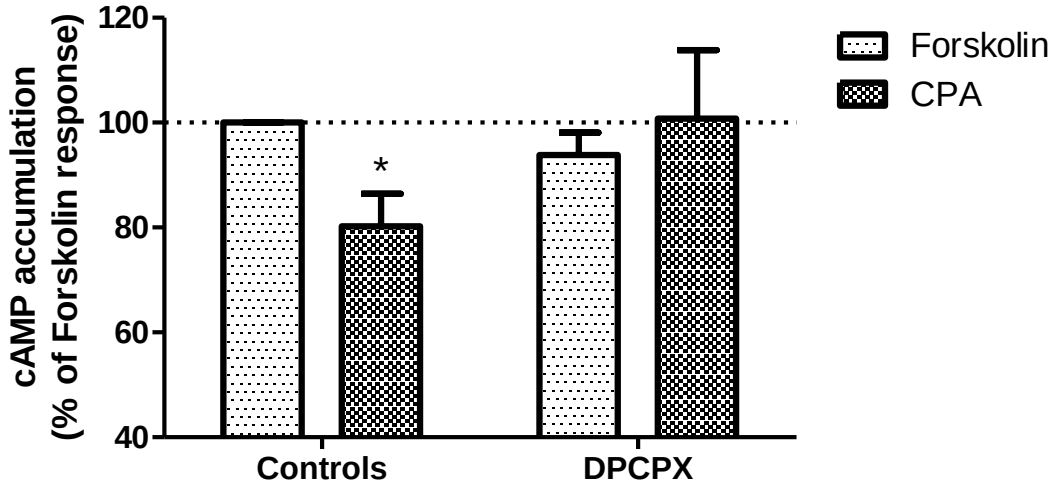
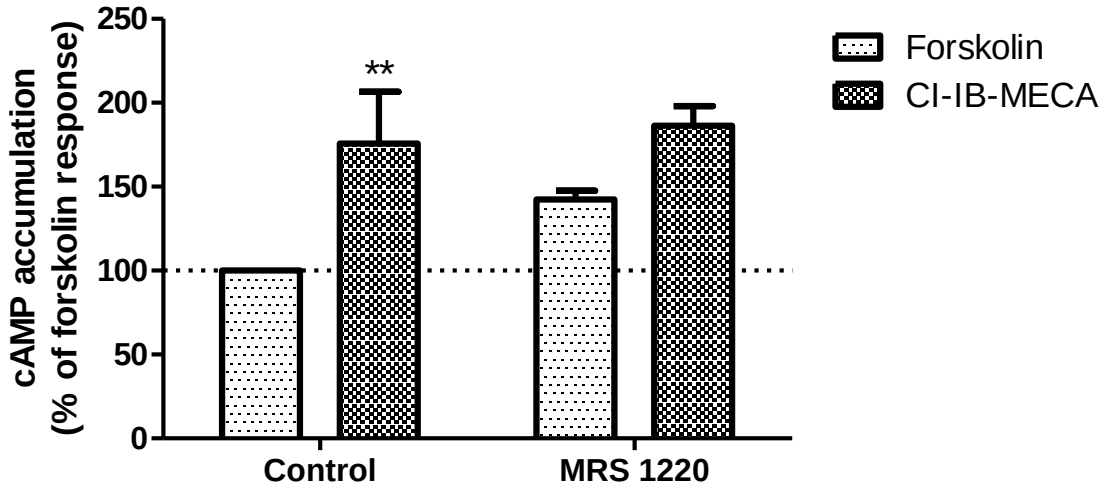


Figure 3.4: The effect of pertussis toxin (PTX) and DPCPX on CPA-induced inhibition of forskolin-mediated cAMP accumulation in H9c2 cells. (a) Control and PTX-treated cells (100 ng/ml; 16 hours) were pre-treated with 100 nM CPA for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. (b) Control and DPCPX-treated cells (10 μM) were pre-treated with 100 nM CPA for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin control response (=100%). Each point represents mean ± S.E.M. of 4 separate experiments each performed in triplicate. * $p<0.05$ vs. forskolin alone response.

(a)



(b)

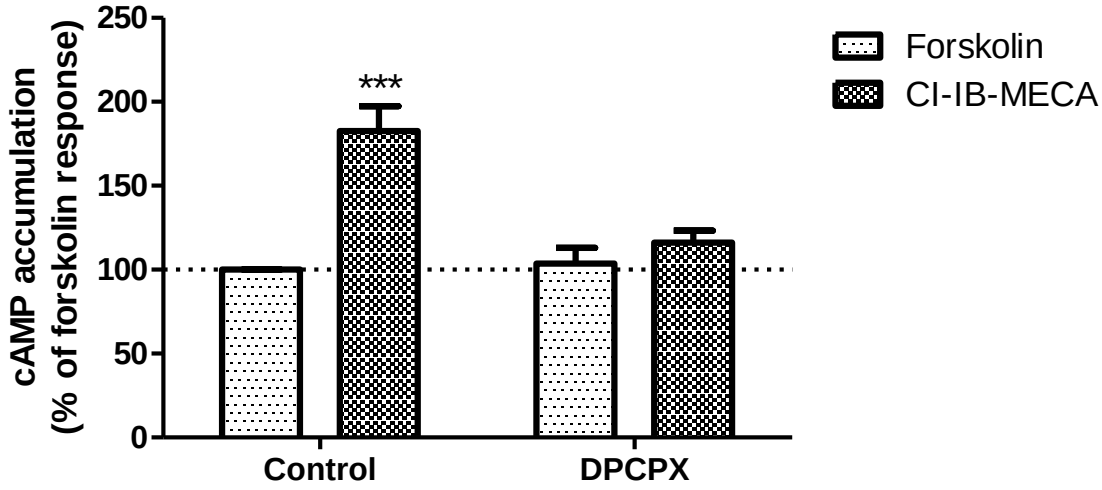


Figure 3.5: The effect of MRS 1220 and DPCPX on CI-IB-MECA-induced augmentation of forskolin-mediated cAMP accumulation. (a) Control and MRS 1220-treated cells (10 μM) were pre-treated with 10 μM CI-IB-MECA for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. (b) Control and DPCPX-treated cells (10 μM) were pre-treated with 10 μM CI-IB-MECA for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin control response (=100%). Each point represents mean ± S.E.M. of 4 separate experiments each performed in triplicate. *** $p<0.001$ vs. forskolin alone response.

3.2. Functional expression of opioid receptors on H9c2 cells

It has been shown that during periods of stress (e.g. cardiac ischaemia) the synthesis and release of some opioid peptides are increased, leading to modulation of response and cytoprotection (Clement-Jones *et al* 1980; Falcone *et al* 1993). The receptors are widely distributed throughout the central nervous system and periphery; it has been reported that the κ- and δ-opioid receptors are the dominant subtypes present in rat heart (Zimlichman *et al*, 1996), and there is supporting data for the involvement of both receptors in cardioprotection (Valtchanova-Matchouganska & Ojewole, 2003). The presence of functional κ- and δ-opioid receptors on H9c2 cells has not been reported, so this study investigated this by measuring the effects of selective receptor agonists and antagonists on [^{3}H] - cAMP accumulation.

The δ-opioid receptor agonist SNC 80 had no effect on forskolin-induced cAMP accumulation (see Fig.3.6), whereas the κ-opioid agonist (-)-U-50488 induced a robust inhibition (maximal inhibition occurring at 1 μM, 25.5 ± 7.4%, n=4, $p<0.05$, see Fig.3.7). The G_i-protein blocker PTX attenuated (-)-U-50488-mediated inhibition of cAMP accumulation (15 ± 2.6% reduction of forskolin response in the presence of PTX compared to 27.5 ± 4.1% reduction of forskolin response seen in controls, n=4, see Fig.3.8a) and the selective kappa-opioid receptor antagonist *nor*-Binaltorphimine completely reversed (-)-U-50488-mediated inhibition of cAMP accumulation (112.4 ± 11.4% of forskolin response in the presence of *nor*-BIN, n=4, see Fig.3.8b).

Overall, these data suggest that H9c2 cells functionally express G_i-coupled κ-opioid receptors, but not δ-opioid receptors.

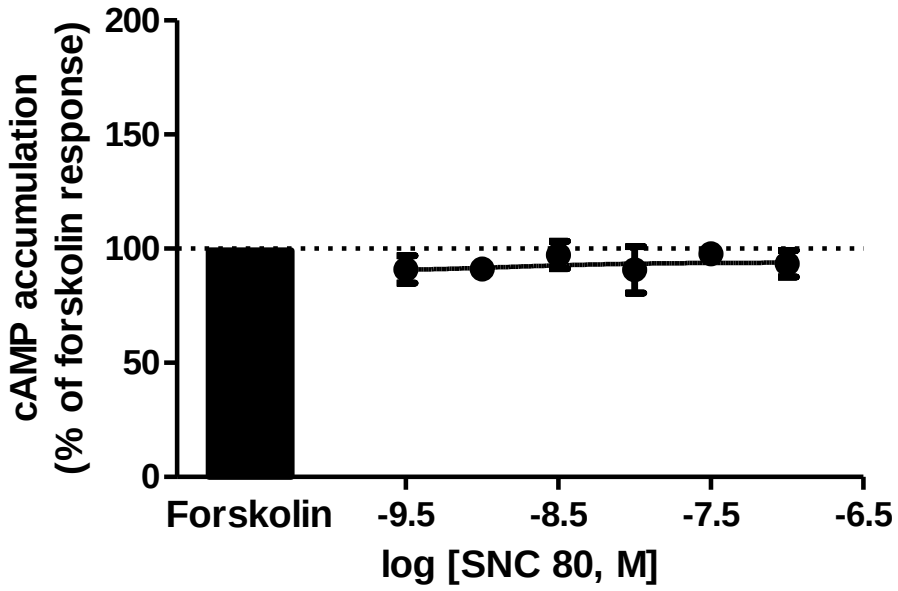


Figure 3.6: SNC 80-mediated effect on forskolin-induced cAMP accumulation in H9c2 cells. Cells were pre-treated with the indicated concentrations of the selective δ-opioid receptor agonist SNC 80 for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin response (=100%). Each point represents mean ± S.E.M. of 4-6 separate experiments each performed in triplicate.

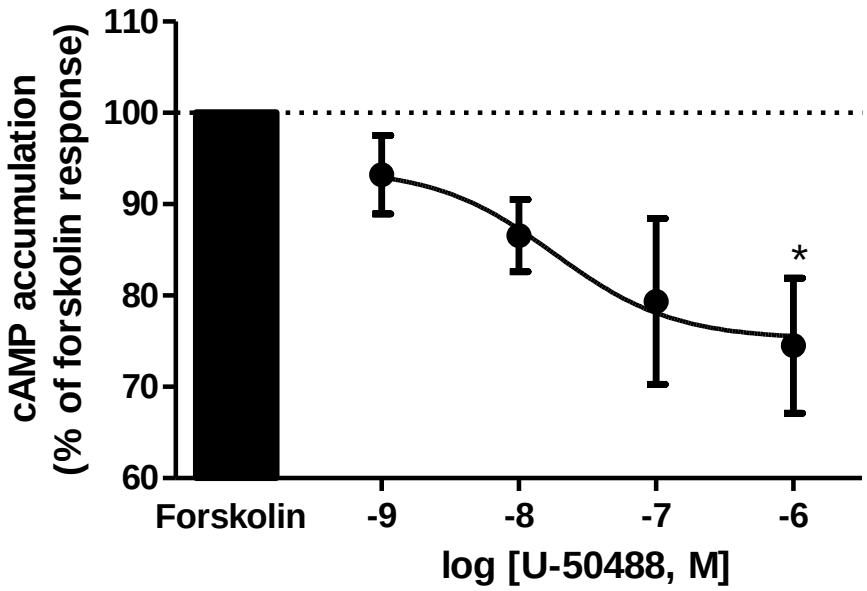
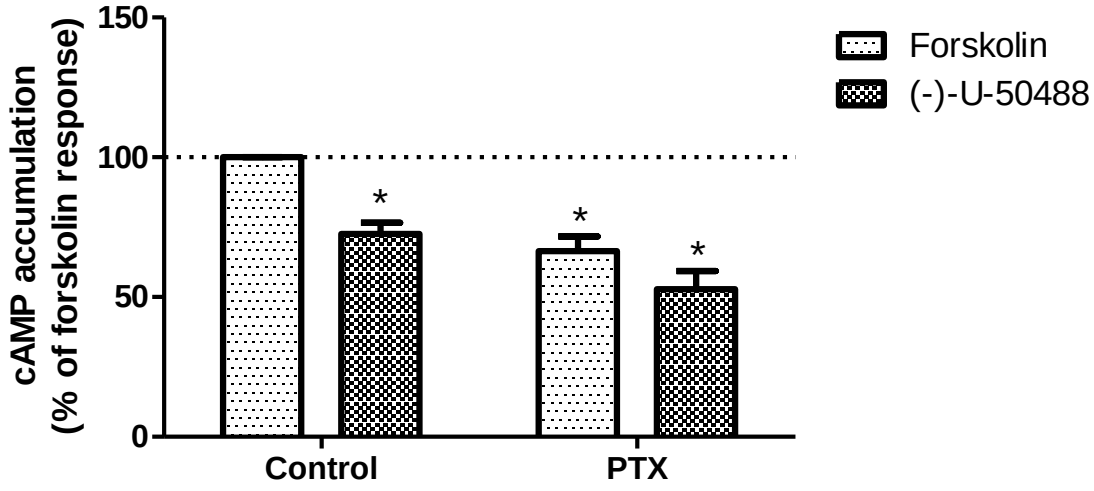


Figure 3.7: (-)-U-50488-mediated effect on forskolin-induced cAMP accumulation in H9c2 cells. Cells were pre-treated with the stated concentrations of the selective κ-opioid receptors agonist for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin response (=100%). Each point represents mean ± S.E.M of 5 separate experiments each performed in triplicate. * $p<0.05$ vs. forskolin alone response.

(a)



(b)

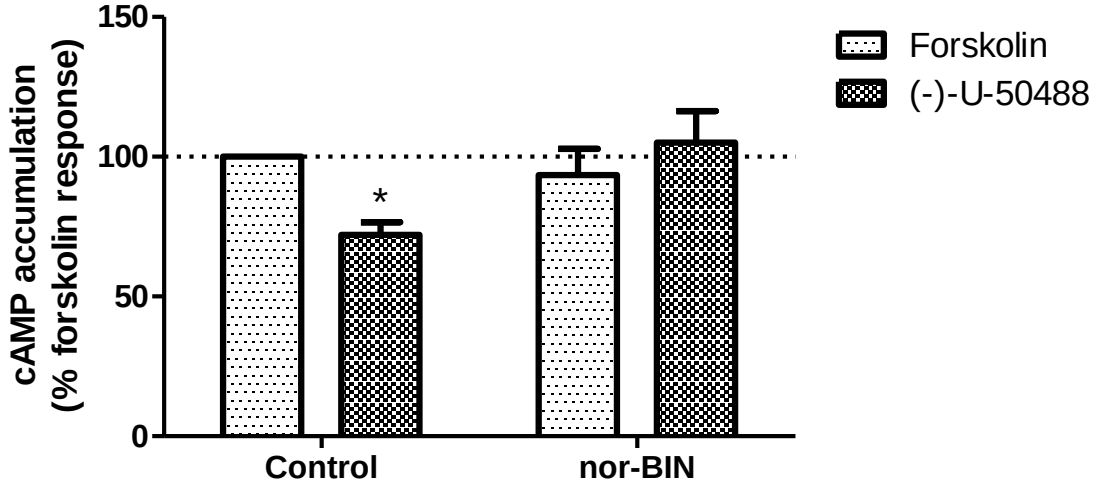


Figure 3.8: The effects of PTX and nor-BIN on (-)-U-50488-mediated inhibition of forskolin-induced cAMP accumulation in H9c2 cells. (a) Control and PTX-treated cells (100 ng/ml; 16 hours) were pre-treated with 1 μM (-)-U-50488 for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. (b) Control and nor-BIN-treated cells (10 μM) were pre-treated with 1 μM (-)-U-50488 for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin control response (=100%). Each point represents mean ± S.E.M. of 5 separate experiments each performed in triplicate. * $p<0.05$ vs. forskolin alone response.

3.3. Functional expression of beta-adrenergic receptors on H9c2 cells

During ischaemia it is well accepted that catecholamines, such as noradrenaline, are released and consequential activation of G protein-coupled adrenergic receptors occurs. The presence of both the β_1 and β_2 subtypes has been demonstrated on H9c2 cells using radioligand binding studies (Dangel *et a*l, 1996) and using antibody imaging (Ianoul *et al*, 2005). In this study, the presence of functional beta-adrenergic receptors on H9c2 cells was investigated by measuring the effects of selective receptor agonists and on [^{3}H]-cAMP accumulation.

The non-selective β-adrenergic receptor agonist isoprenaline stimulated a concentration-dependent increase in cAMP accumulation (maximal effect occurring at 100 nM; 622 ± 117% basal response; n = 4; see Fig.3.9). The selective β_1 agonist dobutamine stimulated cAMP accumulation to an extent (10 μM induced 174 ± 57% basal response; n=4; see Fig.3.10), however, a larger response was seen from the selective β_2 agonist procaterol (1 μM induced 203 ± 29% basal response, n=4; 10 μM induced 598 ± 150% basal response; n=4; see Fig.3.11).

These data suggest that the G_s-coupled β_2 adrenergic receptor could be present on H9c2 cells, but the presence of a functional β_1 adrenergic receptor is less clear.

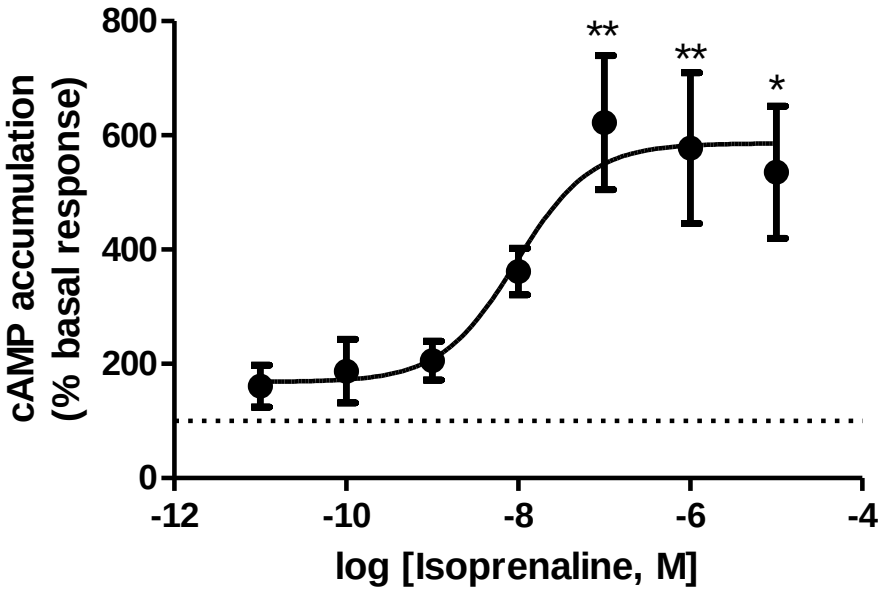


Figure 3.9: The effect of isoprenaline on cAMP accumulation in H9c2 cells. Cells were treated with the indicated concentrations of isoprenaline for 10 minutes. Data expressed as the percentage of basal response (=100%). Each point represents mean ± S.E.M of 4 separate experiments each performed in triplicate. * $p<0.05$ vs. basal response, ** $p<0.01$ vs. basal response.

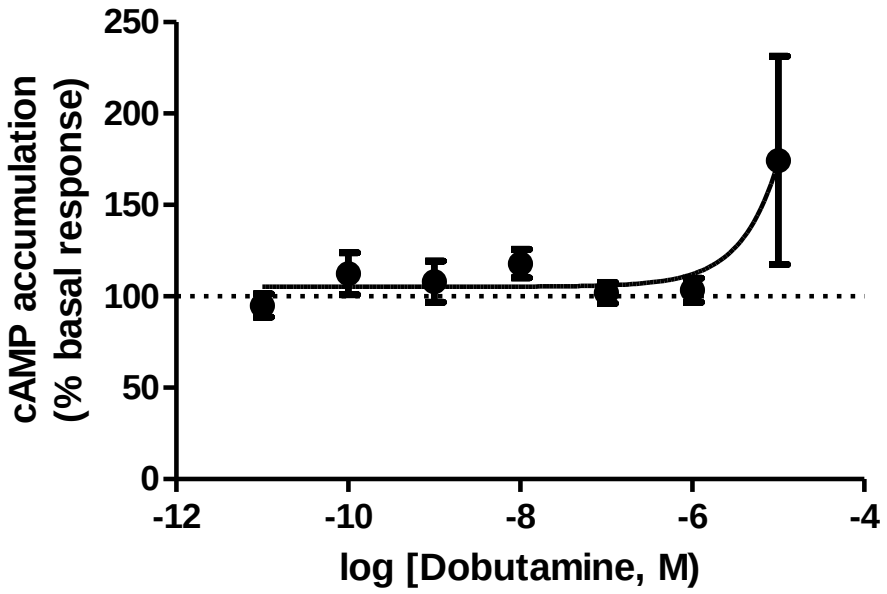


Figure 3.10: The effect of dobutamine on cAMP accumulation in H9c2 cells. Cells were treated with the indicated concentrations of the β_1 receptor agonist dobutamine for 10 minutes. Data expressed as the percentage of basal response (=100%). Each point represents mean ± S.E.M of 4 separate experiments each performed in triplicate.

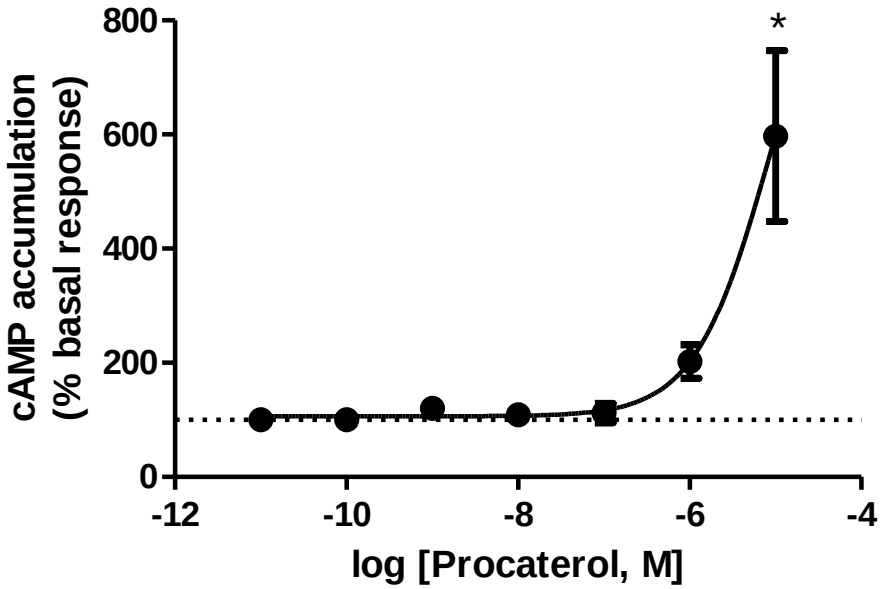


Figure 3.11: The effect of procaterol on cAMP accumulation in H9c2 cells. Cells were treated with the indicated concentrations of the β_2 receptor agonist procaterol for 10 minutes. Data expressed as the percentage of basal response (=100%). Each point represents mean ± S.E.M of 4 separate experiments each performed in triplicate. * $p<0.05$ vs. basal response.

3.4: Functional expression of other G_i and G_q protein-coupled receptors on H9c2 cells

The presence of a selection of other GPCRs on H9c2 cells was also investigated as, to date, an extensive functional study has not been reported.

Firstly, the effects of baclofen and acetylcholine on forskolin-induced cAMP accumulation were tested. Baclofen is an agonist at the G_i-coupled $GABA_B$ receptor, which has been found to be expressed in mammalian heart and exert a cardioprotective effect during ischaemia (Lorente *et al*, 2000). In H9c2 cells this agent provided some inhibition of cAMP accumulation, but not to a statistically significant level (maximal inhibition occurring at 10 nM, 83 ± 9% of forskolin response = 100%, n=4, $p>0.05$, see fig.3.12a). Therefore, functional expression of this receptor on H9c2 cells is debatable.

There are several subtypes of acetylcholine receptor, and the G_i-coupled muscarinic M_2 subtype is known to have parasympathetic effects in the heart. However, no significant effect on cAMP accumulation was observed from acetylcholine suggesting that the muscarinic M_2 receptor is not functionally expressed on H9c2 cells (see fig.3.12).

It was also decided to test a range of agonists at G_q-coupled receptors by measuring the accumulation of radiolabelled inositol trisphosphate (IP_3) following receptor stimulation. As previously mentioned, mammalian cardiomyocytes express G_i-coupled M_2 receptors, but there is also evidence supporting the expression of G_q-coupled M_1 receptors (Sharma *et al*, 1997). Acetylcholine did stimulate an increase in IP_3 accumulation in H9c2 cells, but not significantly implying that the G_q-coupled

muscarinic receptors may not be functionally expressed (100 µM, 132 ± 12% of control = 100%, n=6, p>0.05, see fig.3.13).

Histamine H_1 receptors predominantly couple to G_q protein, but there is evidence stating that the H_2 receptor can also bind G_q protein (Kühn *et al*, 1996). Both of these subtypes have been found to have a role in the mediation of the effects of histamine in the heart (Flynn *et al*, 1979), but this study found no evidence for functional expression of a histamine receptor on H9c2 cells (100 µM, 94 ± 8% of control = 100%, n=6, p>0.05, see fig. 3.13).

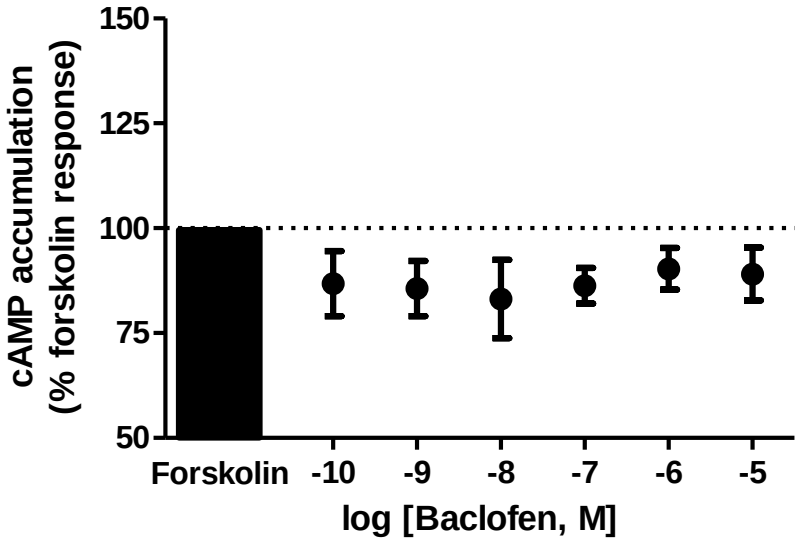
As mentioned previously, catecholamines such as noradrenaline are released from cardiac tissue during ischaemia; it has been found that G_q-coupled α_{1A}, α_{1B} and α_{1D}-adrenergic receptors are all expressed in rat heart, with the α_{1B} receptor being the predominant subtype and mainly responsible for the cardioprotective effects of α-adrenergic receptor agonism (Gao *et al*, 2007). Treatment of H9c2 cells with noradrenaline did result in a sizeable increase in IP_3 accumulation – but this was not statistically significant (100 µM, 150 ± 15% of control = 100%, n=4, p>0.05, see fig. 3.13).

Cardiac ischaemia stimulates the release of ATP and UTP, which then activate purinergic P2Y receptors. The expression of $P2Y_4$ receptors, which preferentially bind UTP, is low in the heart but the expression of $P2Y_2$ (equal affinity for ATP and UTP) and $P2Y_{11}$ (preferentially bind ATP) is high (Erlinge & Burnstock, 2008). Although ATP did not elicit an increase in IP_3 accumulation in H9c2 cells (100 µM, 103 ± 15% of control = 100%, n=6, p>0.05, see fig.3.13), UTP gave a sizeable response (100 µM, 199 ± 15% of control = 100%, n=4, p<0.01, see fig.3.13). This

suggests that one or more of the UTP-binding receptors are functionally expressed on H9c2 cells.

In conclusion, these data provide evidence for the functional expression of G_i-coupled adenosine A_1 and κ-opioid receptors, G_s-coupled β_2 adrenergic receptors and G_q-coupled UTP-binding P2Y purinergic receptors on H9c2 cells.

(a)



(b)

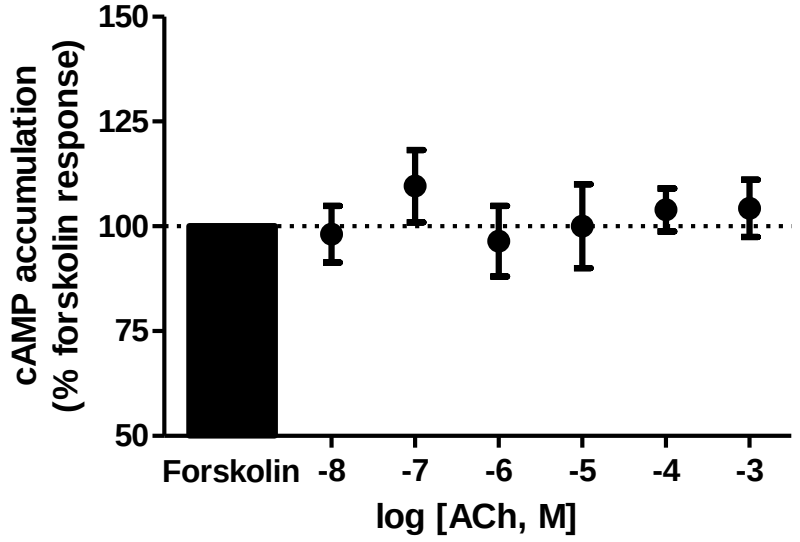


Figure 3.12: The effects of baclofen and acetylcholine of forskolin-induced cAMP accumulation in H9c2 cells. Cells were pre-treated with the indicated concentrations of (a) the $GABA_B$ receptor agonist baclofen or (b) the muscarinic receptor agonist ACh for 5 minutes prior to the addition of 10 μM forskolin for a further 10 minutes. Data expressed as the percentage of forskolin response (=100%). Each point represents mean ± S.E.M. of 4 separate experiments each performed in triplicate.

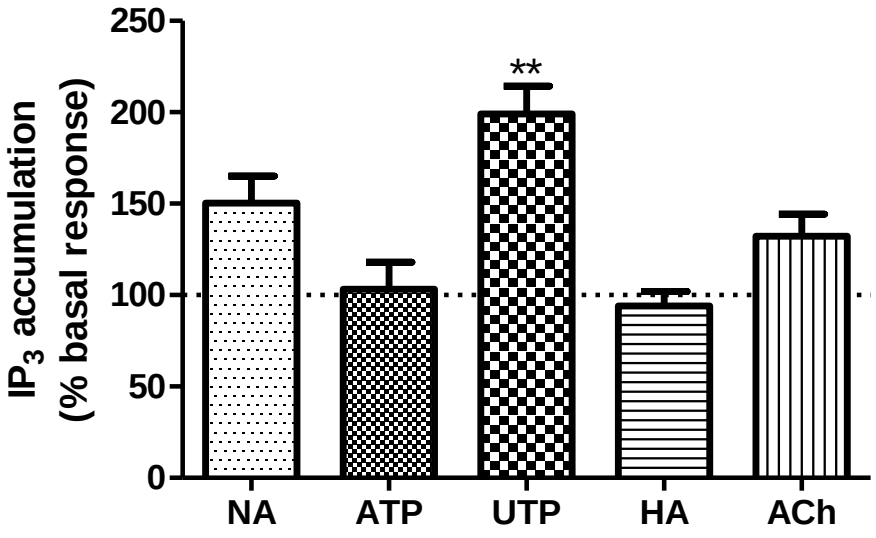


Figure 3.13: The effects of selected agonists on inositol phosphate accumulation in H9c2 cells. Cells were incubated with the selected agonists (noradrenaline (NA), ATP, UTP, histamine (HA), ACh all at 100 μM) for 30 minutes. Data expressed as the percentage of basal response (=100%). Each point represents mean ± S.E.M. of 4 separate experiments each performed in triplicate.

4. Cardioprotective potential of functionally expressed receptors on H9c2 cells

4.1. Determining the effects of GPCR agonists against hypoxia-induced cell death in H9c2 cells

As mentioned in the previous chapter, all of the GPCRs found to be functionally expressed on H9c2 cells have been implicated in cardioprotective mechanisms so it was important to assess such properties in this cell line. Initially it was necessary to investigate the effect of hypoxia upon H9c2 cells, allowing the ideal length of time in hypoxia to be chosen for further experiments involving pharmacological preconditioning.

Cell death was measured following 1, 2, 4, 6, 8 and 24 hours in hypoxia (0.5% O_2) using assays to measure MTT reduction, LDH release and caspase-3 activity (see fig.4.1). 6 hours of hypoxia was chosen for further experiments as it produced a large response in all of the assays (MTT reduction was decreased to 33 ± 8% of control, n=4, $p<0.01$; LDH release was increased to 352 ± 101% of control, n=4; caspase-3 activity was increased to 235 ± 19% of control, n=4, $p<0.05$) and allowed a whole experiment with preconditioning and viability assay to be completed in a working day.

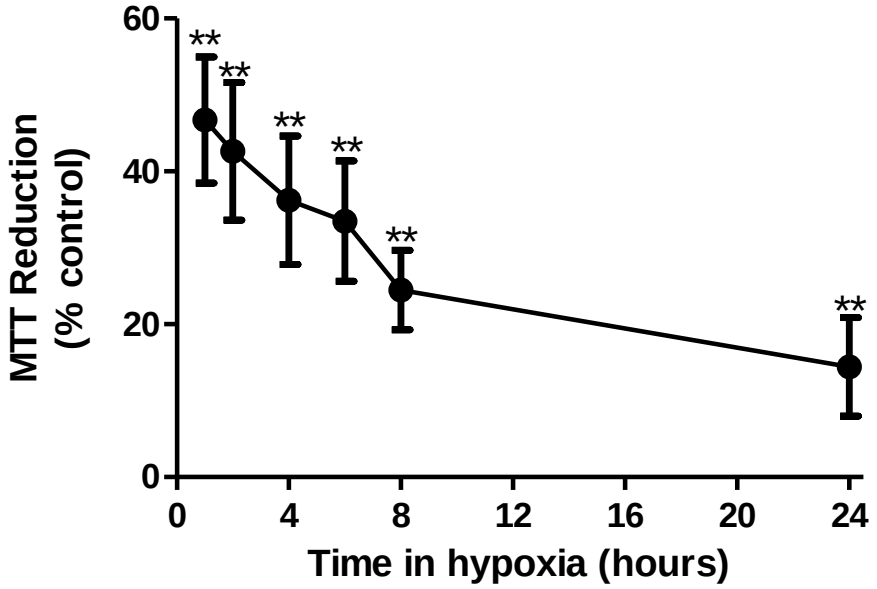
The adenosine A_1 receptor agonist CPA (100 nM) was the only drug to increase MTT reduction during hypoxia (123 ± 24% compared to 65 ± 13% = hypoxic control, n=4, $p<0.05$, see fig.4.2a). Interestingly, the β_2 adrenergic receptor agonist procaterol (10 μM) significantly increased MTT reduction during normoxia (164 ± 34% compared to normoxic control = 100%, n=4, $p<0.01$).

LDH release during hypoxia was significantly reduced by CPA (100 nM, 102 ± 10% compared to 161 ± 10% = hypoxic control, n=7, $p<0.01$, see fig.4.2b) and the κ-opioid receptor agonist (-)-U-50488 (100 nM, 112 ± 9% compared to 161 ± 10% = hypoxic control, n=7, $p<0.05$). None of the drugs had a significant effect during normoxia.

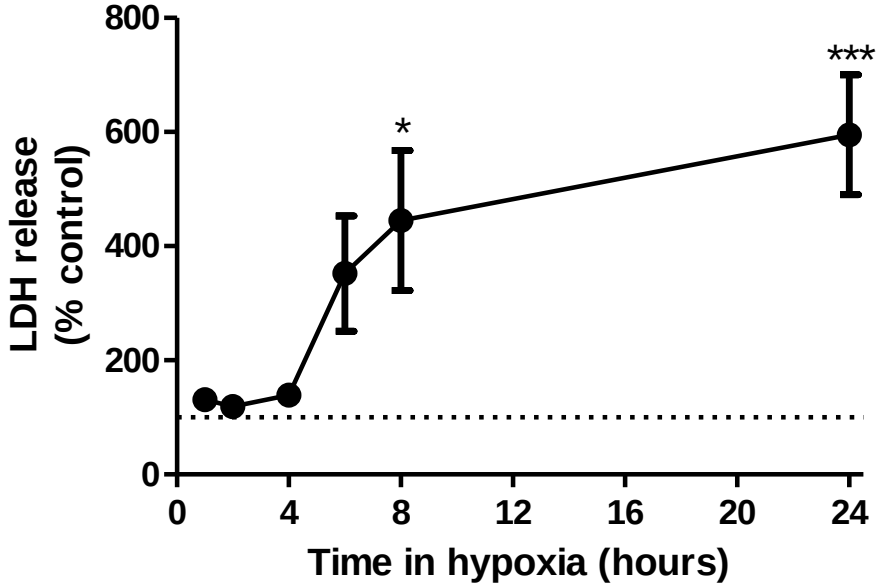
CPA also significantly reduced hypoxia-induced caspase-3 activity (100 nM, 103 ± 14% compared to 174 ± 11% = hypoxic control, n=5, $p<0.05$, see fig.4.2c), as did the non-selective beta-adrenergic receptor agonist isoprenaline (10 μM, 99 ± 15% compared to 174 ± 11% = hypoxic control, n=5, $p<0.05$). As before, none of the drugs had a significant effect during normoxia.

Overall, the most promising target for protection against hypoxia-induced cell death in H9c2 cells was the adenosine A_1 receptor, which attenuated cell death according to all three parameters measured. Stimulation of the beta-adrenergic receptors also appeared to be beneficial, as did activation of the κ-opioid receptor. Importantly, these data show that i) it is possible to stimulate a preconditioning response in H9c2 cells, and ii) the protective properties of activation of the adenosine A_1 receptor observed in cardiac myocytes (Safran *et al*, 2001) can be reproduced in H9c2 cells.

(a)


MTT Reduction
(% control)
60
40
20
0
**
**
**
**
**
**
0
4
8
12
16
20
24
Time in hypoxia (hours)

(b)


LDH release
(% control)
800
600
400
200
0
*
***
0
4
8
12
16
20
24
Time in hypoxia (hours)

(c)

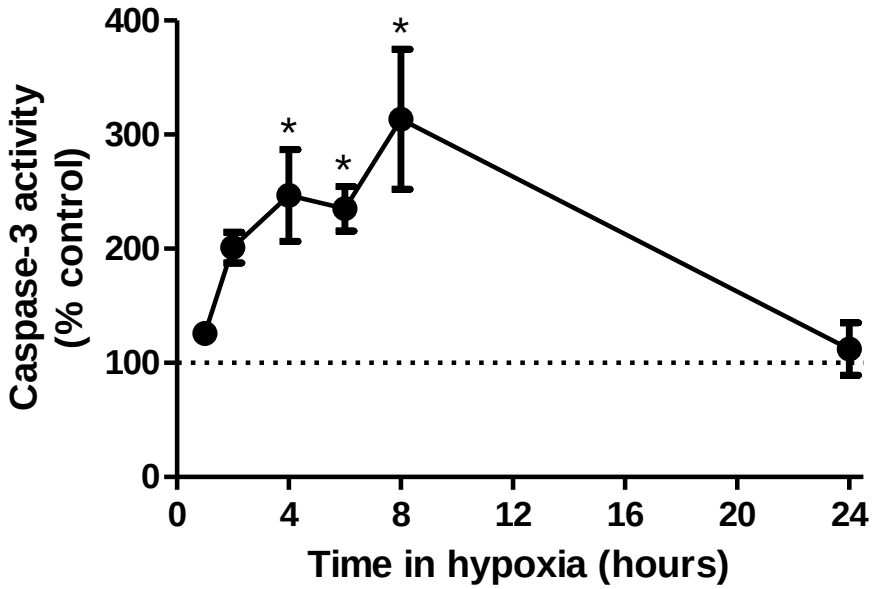
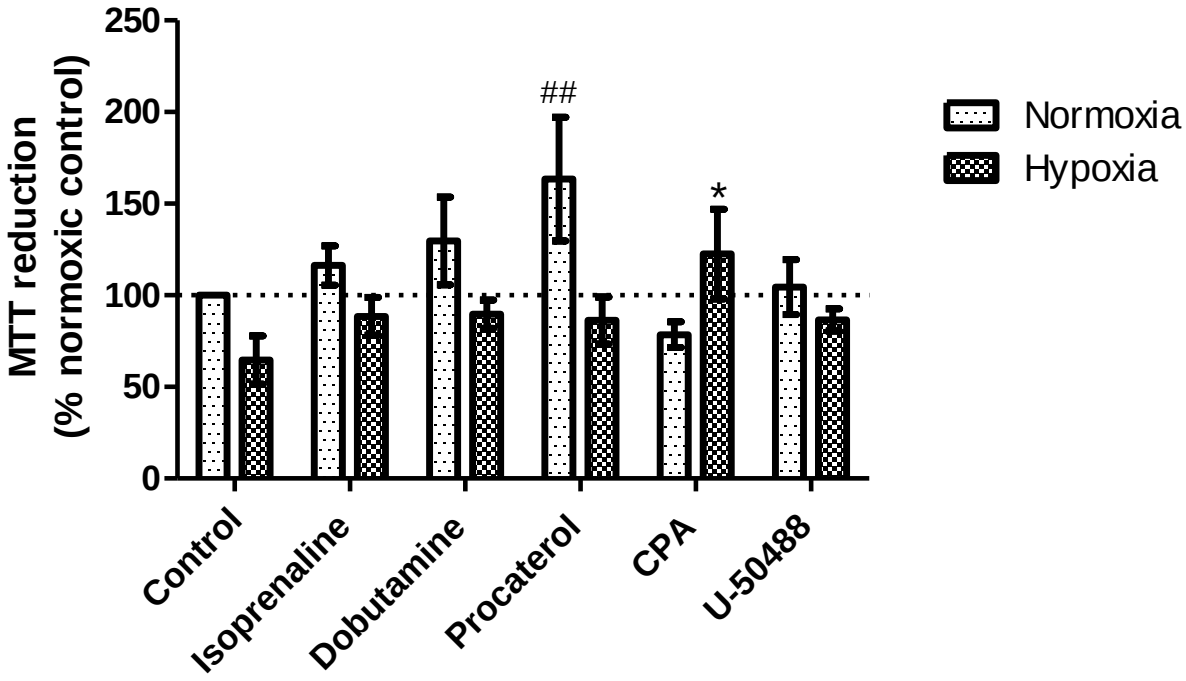
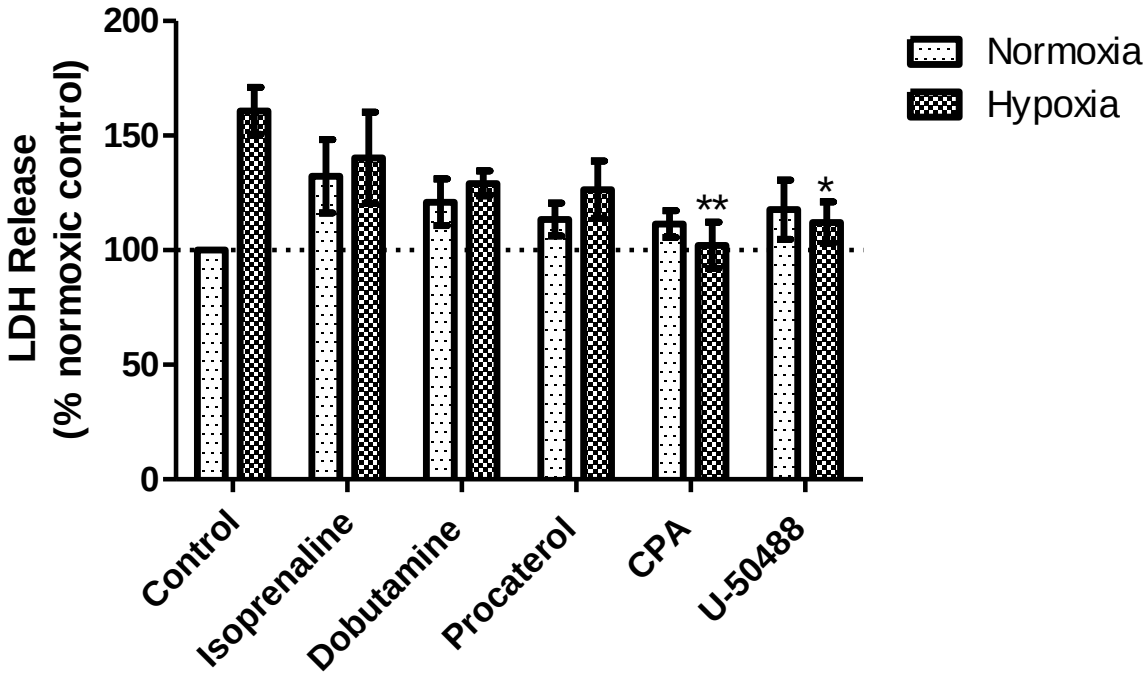


Figure 4.1: Effect of hypoxia on H9c2 cell viability over time. Cells were exposed to hypoxia for the stated times then cell viability was assessed via (a) MTT reduction, (b) LDH release and (c) caspase-3 activity. Data expressed as the percentage of control response (=100%). Each point represents mean ± S.E.M. of 4-6 separate experiments; for each experiment the mean was taken from 2 (caspase-3) or 6 (MTT and LDH) replicates. * $p<0.05$ vs. control; ** $p<0.01$ vs. control; *** $p<0.001$ vs. control.

(a)


MTT reduction
(% normoxic control)
250
200
150
100
50
0
##
*
Control
Isoprenaline
Dobutamine
Procaterol
CPA
U-50488
Normoxia
Hypoxia

(b)


LDH Release
(% normoxic control)
200
150
100
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**
*
Control
Isoprenaline
Dobutamine
Procaterol
CPA
U-50488
Normoxia
Hypoxia

(c)

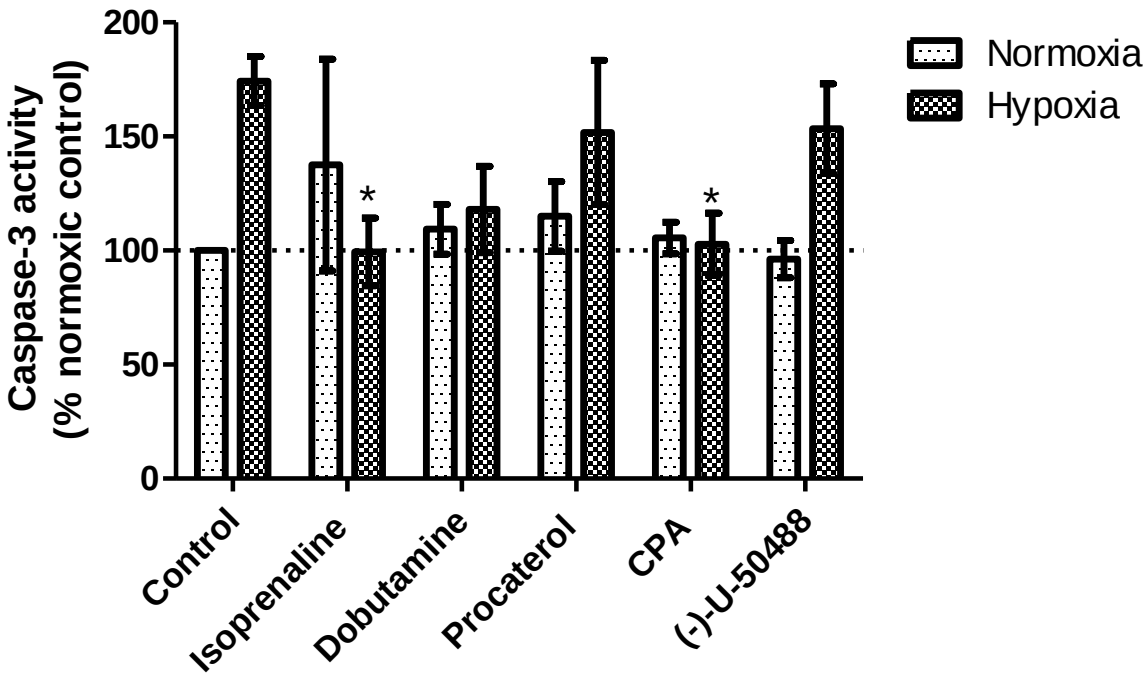


Figure 4.2: Effects of GPCR agonists on hypoxia-induced cell death in H9c2 cells. Cells were exposed the stated agonist (Concentrations as follows: 10 μM isoprenaline, 10 μM dobutamine, 10 μM procaterol, 100 nM CPA, 100 nM (-)-U-50488) for 30 minutes prior to 6 hours hypoxia. Cell viability was measured by (a) MTT reduction, (b) LDH release and (c) caspase-3 activity. Data expressed as the percentage of Normoxic control response (=100%). Each point represents mean ± S.E.M. of 4-6 separate experiments; for each experiment the mean was taken from 2 (caspase-3) or 6 (MTT and LDH) replicates. ## $p<0.01$ vs. normoxic control; * $p<0.05$ vs. hypoxic control; ** $p<0.01$ vs. hypoxic control.

4.2: Establishing a role for the ATP-sensitive potassium channel (K_{ATP}) and the large-conductance calcium-activated potassium channel (BK_{Ca} channel)

K_{ATP} channels have a widely accepted role in cardioprotection and ischaemic preconditioning (for review see Gross & Peart, 2003). Sarcolemmal K_{ATP} (sarcoK_{ATP}) channels were the first to be described, with evidence for a mitochondrial K_{ATP} (mitoK_{ATP}) emerging at a later date. Garlid *et al* (1997) were the first to publish data showing a link between the mitoK_{ATP} channel and cardioprotection using diazoxide and rat hearts; therefore this study postulated that diazoxide would have a protective effect against hypoxia in the cardiac H9c2 cell line.

Diazoxide significantly reduced hypoxia-induced LDH release (123 ± 3% compared to 156 ± 9% = hypoxic control, n=5, $p<0.01$, see fig.4.3), and this effect was reversed by co-treatment with the K_{ATP} channel blocker 5-HD (153 ± 6% compared to 156 ± 9% = hypoxic control, n=5, see fig.4.3). This data supports the presence of a K_{ATP} channel in H9c2 cells, and as diazoxide is a potent opener of cardiac mitoK_{ATP} channels but a weak sarcoK_{ATP} opener it is highly likely that the channel in question is located to the mitochondria . However, as much evidence already exists regarding the role of the K_{ATP} channel in cardioprotection and its link with GPCRs, for the remainder of this study it was decided to proceed purely with research in to the Ca^{2+}-activated K^+ channel.

Siemen et al (1999) first described the presence of a Ca^{2+}-activated K^+ channel on the inner mitochondrial membrane of human glioma LN229 cells, and since this discovery the activation of mitoBK_{Ca} channels has been implicated in

cardioprotection against ischemia and reperfusion injury in various models including isolated perfused guinea pig hearts (Xu *et al*, 2002), anesthetised dogs (Shintani *et al*, 2004) and isolated guinea pig ventricular myocytes (Sato *et al*, 2005). It was, therefore, postulated that activation of these channels would also elicit a protective effect against hypoxia-induced cell death in H9c2 cells.

The BK_{Ca} channel activator, NS1619 (10 µM), significantly decreased LDH release during hypoxia (111 ± 10% compared to 161 ± 12% = hypoxic control, n=7, $p<0.05$, see fig.4.4) and this was attenuated by the selective BK_{Ca} channel blockers paxilline (1 µM, 151 ± 15% compared to 161 ± 12% = hypoxic control, n=7) and iberiotoxin (10 nM, 141 ± 13% compared to 161 ± 12% = hypoxic control, n=5). NS1619 (10 µM) also considerably reduced caspase-3 activity during hypoxia (126 ± 11% compared to 195 ± 16% = hypoxia control, n=5, $p<0.05$, see fig.4.5), and this was completely reversed by both paxilline (207 ± 26% compared to 195 ± 16% = hypoxia control, n=5) and iberiotoxin (196 ± 59% compared to 195 ± 16% = hypoxia control, n=5).

To confirm that the channel in question was the mitochondrial BK_{Ca} channel, mitochondria from H9c2 cells were collected and probed with antibodies for the BK_{Ca}-α and β4 subunits and the mitochondrial marker cytochrome *c* (see fig.4.6). Bands for the BK_{Ca}-α and β4 subunits were clearly observed in the mitochondrial fraction, but not in the cytoplasmic fraction or total extract. Cytochrome *c* was abundant in the mitochondrial fraction but absent from the cytoplasmic fraction, confirming the purity of the fractions.

In conclusion, these data show that NS1619 is activating the mitochondrial BK_{Ca} channel, and that this channel has cytoprotective potential in H9c2 cells.

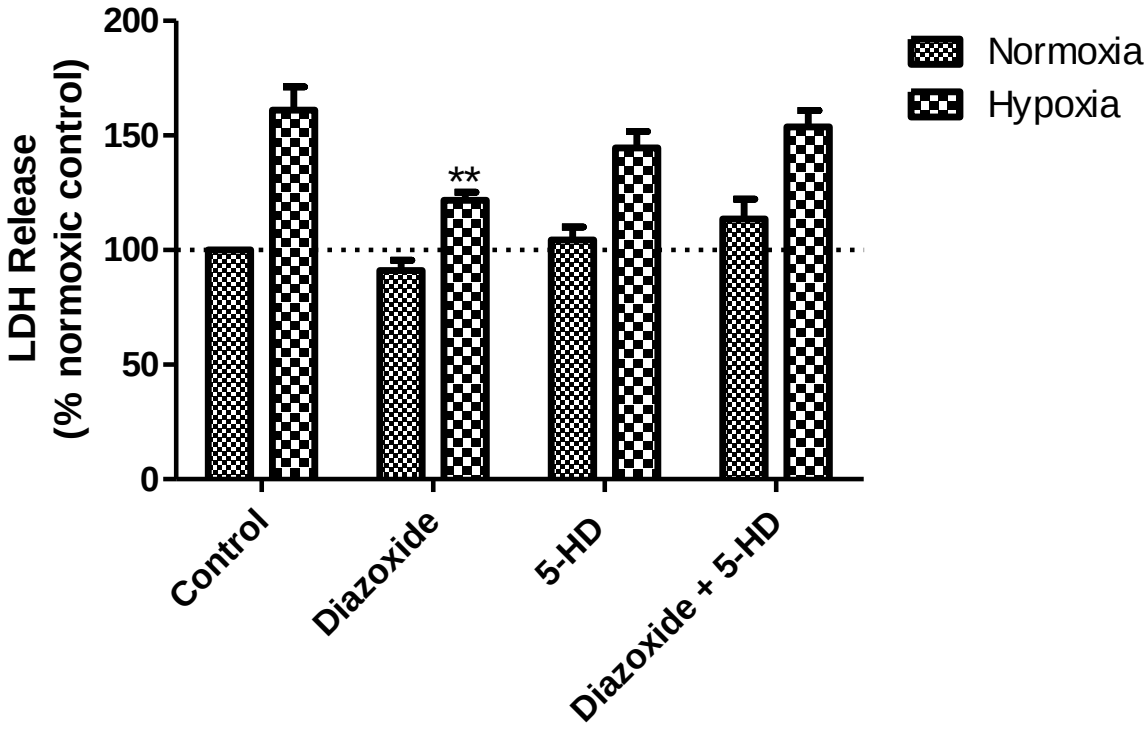


Figure 4.3: Establishing a role for the K_{ATP} channel in protection against hypoxia-induced cell death in H9c2 cells. Cells were exposed to the channel blocker 5-HD (100 μM) for 45 minutes, with the addition of diazoxide (30 μM) for the final 30 minutes, prior to 6 hours hypoxia. Cell viability was assessed via LDH release. Data expressed as the percentage of normoxic control response (=100%). Each point represents mean ± S.E.M. of 5 separate experiments; for each experiment the mean was taken from 6 replicates. ** $p<0.01$ vs. normoxic control.

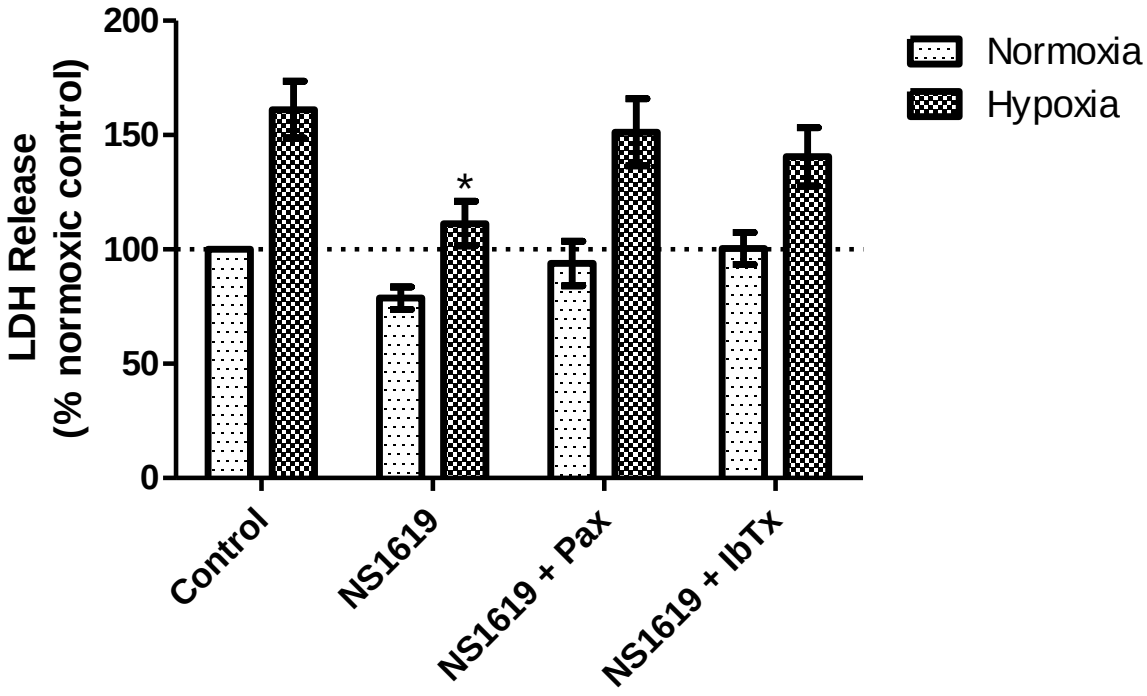


Figure 4.4: Establishing a role for the BK_{Ca} channel in protection against hypoxia-induced LDH release in H9c2 cells. Cells were exposed to the channel blockers paxilline (1 μM) or iberiotoxin (10 nM) for 45 minutes, with the addition of NS1619 (10 μM) for the final 30 minutes, prior to 6 hours hypoxia. Cell viability was assessed via LDH release. Data expressed as the percentage of normoxic control response (=100%). Each point represents mean ± S.E.M. of 5-7 separate experiments; for each experiment the mean was taken from 6 replicates. * $p<0.05$ vs. normoxic control.

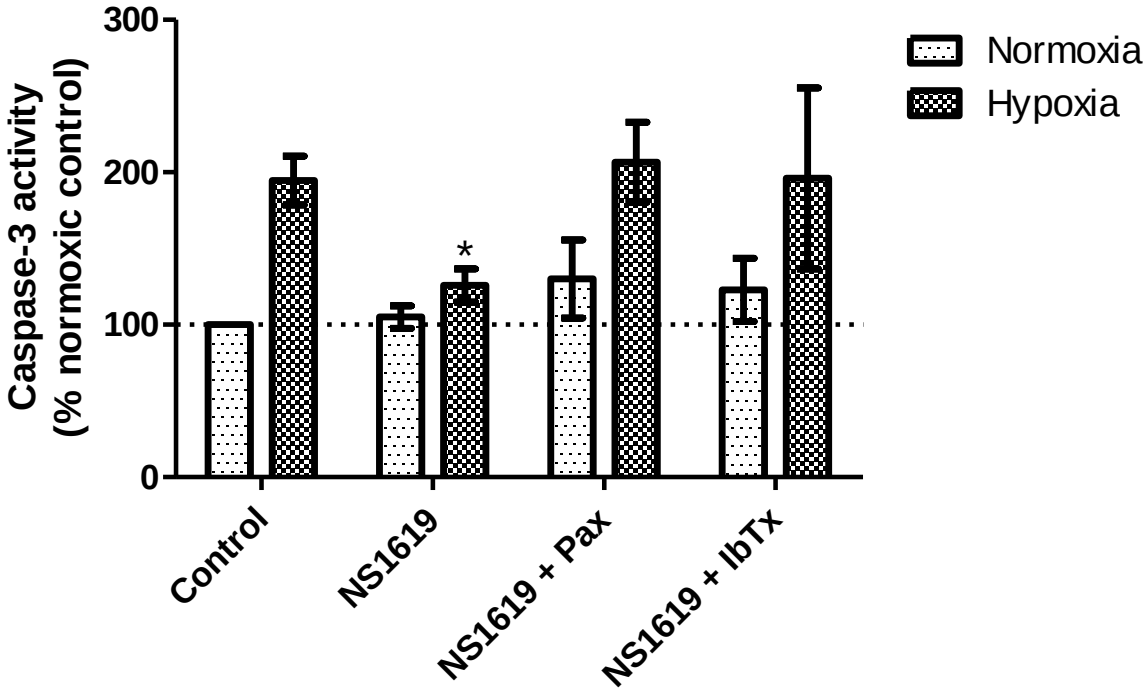


Figure 4.5: Establishing a role for the BK_{Ca} channel in protection against hypoxia-induced caspase-3 activation in H9c2 cells. Cells were exposed to the channel blockers paxilline (1 μM) or iberiotoxin (10 nM) for 45 minutes, with the addition of NS1619 (10 μM) for the final 30 minutes, prior to 6 hours hypoxia. Cell viability was assessed via caspase-3 activity. Data expressed as the percentage of normoxic control response (=100%). Each point represents mean ± S.E.M. of 5-7 separate experiments; for each experiment the mean was taken from 2 replicates. * $p<0.05$ vs. normoxic control.

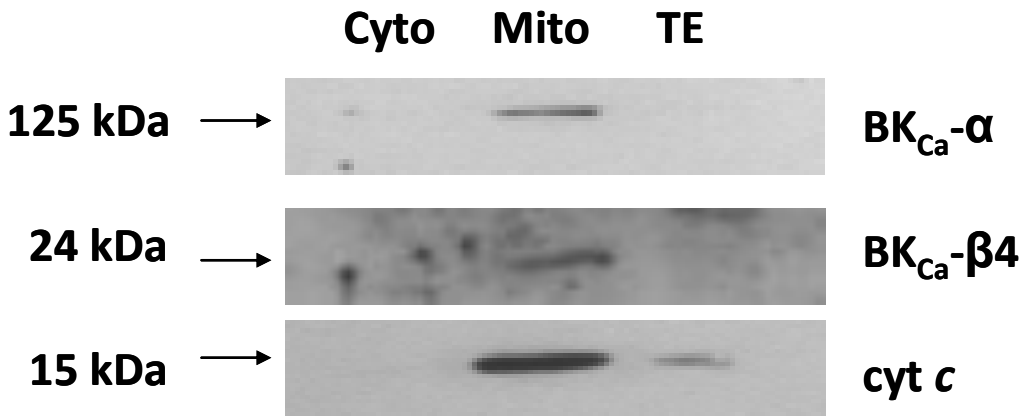


Figure 4.6: Establishing the presence of the mitochondrial BK_{Ca} channel subunits in H9c2 cells. Mitochondria were extracted from H9c2 cells by differential centrifugation and probed with antibodies specific to the BK_{Ca}-α and BK_{Ca}-β4 subunits (30 μg protein was loaded per lane). Cyto represents the cytoplasmic fraction; TE represents total extract. Cytochrome *c* was used to confirm purity of the mitochondrial fraction. Blots are representative of 4 separate experiments.

4.3: Determining a link between the adenosine A_1 receptor and the BK_{Ca} channel

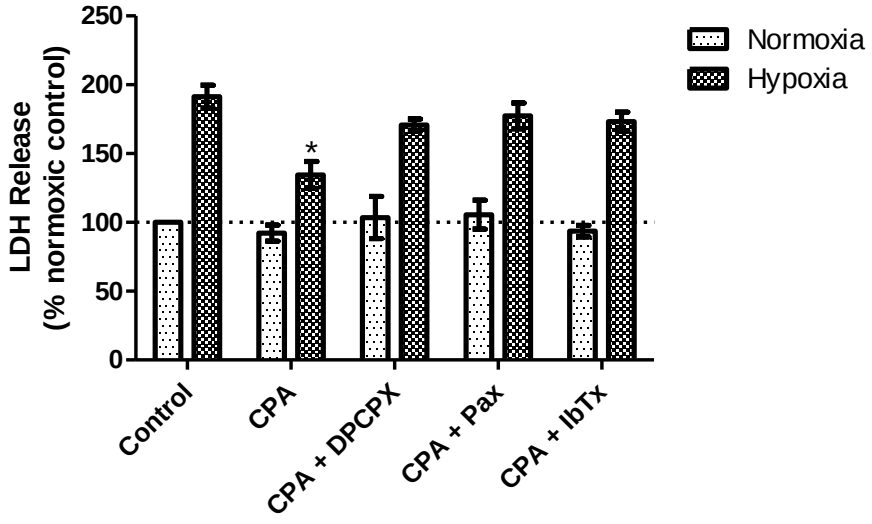
There is some evidence that BK_{Ca} channels have a role in cardioprotection induced by some GPCR agonists including the κ-opioid receptor agonist (-)-U-50488 (Cao *et al*, 2005). However, to date, the role of the BK_{Ca} channel in adenosine receptor-induced cardioprotection has yet to be investigated. The adenosine A_1 receptor activates similar G_i protein-modulated pathways to the κ-opioid receptor, so it was hypothesized that the BK_{Ca} channel may also feature in cardioprotection induced by this receptor.

As before, activation of the adenosine A_1 receptor with CPA reduced LDH release during hypoxia (100 nM, 135 ± 10% compared to 191 ± 8% = hypoxic control, n=6, $p<0.05$, see fig.4.7a). This effect was attenuated by the A_1 receptor antagonist DPCPX (10 μM, 171 ± 5% compared to 191 ± 8% = hypoxic control, n=6), and interestingly, also by the BK_{Ca} channel blockers paxilline (1 μM, 177 ± 9% compared to 191 ± 8% = hypoxic control, n=6) and iberiotoxin (10 nM, 173 ± 7% compared to 191 ± 8% = hypoxic control, n=6).

This was also the case when assessing caspase-3 activity. CPA reduced caspase-3 activity during hypoxia (128 ± 12% compared to 184 ± 16% = hypoxic control, n=5, $p<0.05$, see fig.4.7b), and this was prevented by both paxilline (174 ± 17% compared to 184 ± 16% = hypoxic control, n=5) and iberiotoxin (181 ± 30% compared to 184 ± 16% = hypoxic control, n=5).

None of the blockers affected LDH release or caspase-3 activity when used alone (data not shown). These data have, for the first time, uncovered a role for the BK_{Ca} channel in adenosine A_1 receptor-induced protection against hypoxia.

(a)



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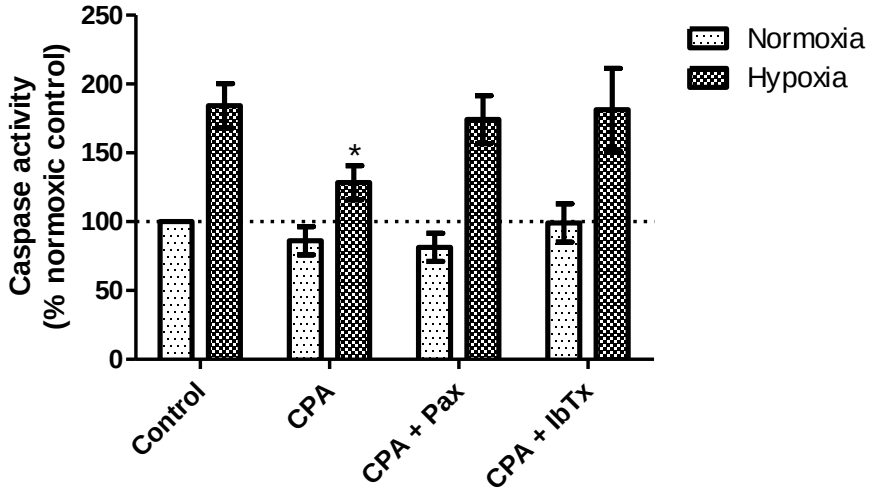


Figure 4.7: The effect of BKCa channel blockers on CPA-mediated protection against hypoxia-induced cell death in H9c2 cells. Cells were exposed to paxilline (1 μM) or iberiotoxin (10 nM) for 45 minutes, with the addition of CPA (100 nM) for the final 30 minutes prior to 6 hours hypoxia. Cell viability was assessed via (a) LDH release or (b) caspase-3 activity. Data expressed as the percentage of normoxic control response (=100%). Each point represents mean ± S.E.M. of 5-6 separate experiments; for each experiment the mean was taken from 2 (caspase-3) or 6 (LDH) replicates. * $p<0.05$ vs. hypoxic control.

5. The role of protein kinases ERK1/2 and PKB in adenosine A_1 receptor and BK_{Ca} channel-mediated cytoprotection

Several protein kinases have been implicated in ischaemic pre- and postconditioning, including ERK1/2 and PKB. Both of these are pro-survival kinases included in the RISK pathway (Reperfusion Injury Salvage Kinase), and have been associated with the cardioprotective effects of K_{ATP} channels (de Toit *et al*, 2008; Grossini *et al*, 2009; for extensive review see Hausenloy & Yellon, 2007). In addition, there is existing data stating that PKB can be activated by the BK_{Ca} channel opener NS1619 (100 μM) in neurones (Gáspár *et al*, 2008). Both kinases have been documented to form part of the cardioprotective signalling cascades initiated by activation of adenosine receptors (Germack *et al*, 2004; Downey *et al*, 2008), so when taking this knowledge into account it was postulated that ERK1/2 and PKB are involved in signal transduction mediated by the adenosine A_1 receptor and BK_{Ca} channel.

However, in this study it was found that 10 μM NS1619 did not induce phosphorylation of ERK1/2 (see fig.5.1) or PKB (see fig.5.2) in H9c2 cells; conversely, 100 μM significantly inhibited the activation of both protein kinases. ERK1/2 was maximally inhibited following 15 minutes treatment (39 ± 9% of control; n=5; $p<0.05$; see fig.5.3), after which levels increased over time and phosphorylation was actually raised above control following 180 minutes treatment (144 ± 25% of control; n=5; $p>0.05$). Maximum inhibition of PKB phosphorylation occurred following 60 minutes treatment with 100 μM NS1619 (32 ± 15% of control; n=4; $p<0.05$; see fig.5.4), after which the level of activation gradually increased but did not reach that of the control.

It was postulated that NS1619 may activate ERK1/2 and PKB during periods of stress, such as hypoxia. However, when H9c2 cells were pre-treated with NS1619 (10 μM) then subjected to hypoxia for 1, 2, 4, or 6 hours no phosphorylation of either protein was observed (see figs.5.5 and 5.6).

It has previously been shown that CPA can activate ERK1/2 and PKB in both cardiomyocyes and the $DDT_{(1)}$MF-2 hamster vas deferens smooth muscle cell line (Germack & Dickenson, 2000; Germack *et al*, 2004). In this study it was also found that CPA activated ERK1/2 in H9c2 cells in a transient manner, with maximal phosphorylation occurring with 5 minutes treatment (264 ± 26% of control; n=4; $p<0.001$; see fig.5.7). However, activation of PKB was not observed (see fig.5.8).

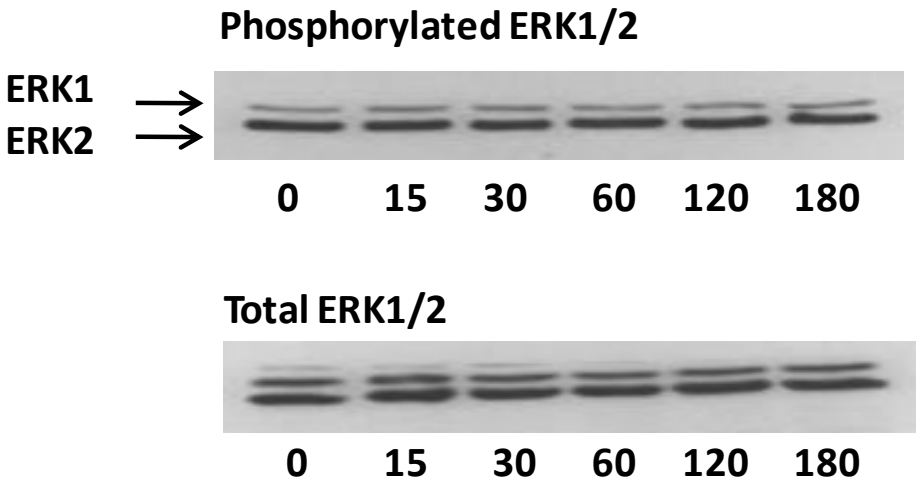
Earlier results have shown that by blocking the BK_{Ca} channel CPA-mediated protection against hypoxia-induced cell death in H9c2 cells can be attenuated. It was decided to investigate whether ERK1/2 or PKB are involved in this process as it has been shown that ERK1/2 is phosphorylated following activation of the adenosine A_1 receptor, and is well documented that both ERK1/2 and PKB are implicated in cytoprotective pathways. However, in this model CPA (100 nM)-induced ERK1/2 phosphorylation was not blocked by paxilline (1 μM; 277 ± 75% of control compared to 227 ± 48% of control for CPA alone; n=4; see fig.5.9) or iberiotoxin (10 nM; 223 ± 26% of control compared to 227 ± 48% of control for CPA alone; n=4; see fig.5.9). No phosphorylation of PKB was observed (see fig.5.10).

From these data it appears that ERK1/2 and PKB are not activated upstream of BK_{Ca} channel-mediated signal transduction since no phosphorylation of either protein kinase was detected. It is important to note, however, that compartmentalisation of these activated proteins has been described (Chuderland *et al*, 2008; Gonzalez &

McGraw, 2009). It is therefore possible that if this is the case in H9c2 cells, by using whole cell lysates the phosphorylated proteins may have been diluted to a level undetectable by the method used.

The sizeable phosphorylation of ERK1/2 by stimulation of the adenosine A_1 receptor further confirms earlier data relating to the functional expression of this receptor on H9c2 cells – and although ERK1/2 does not seem to be activated upstream of adenosine A_1 receptor/BK_{Ca} channel signalling it is possible that it is an intermediate step in this pathway.

(a)



(b)

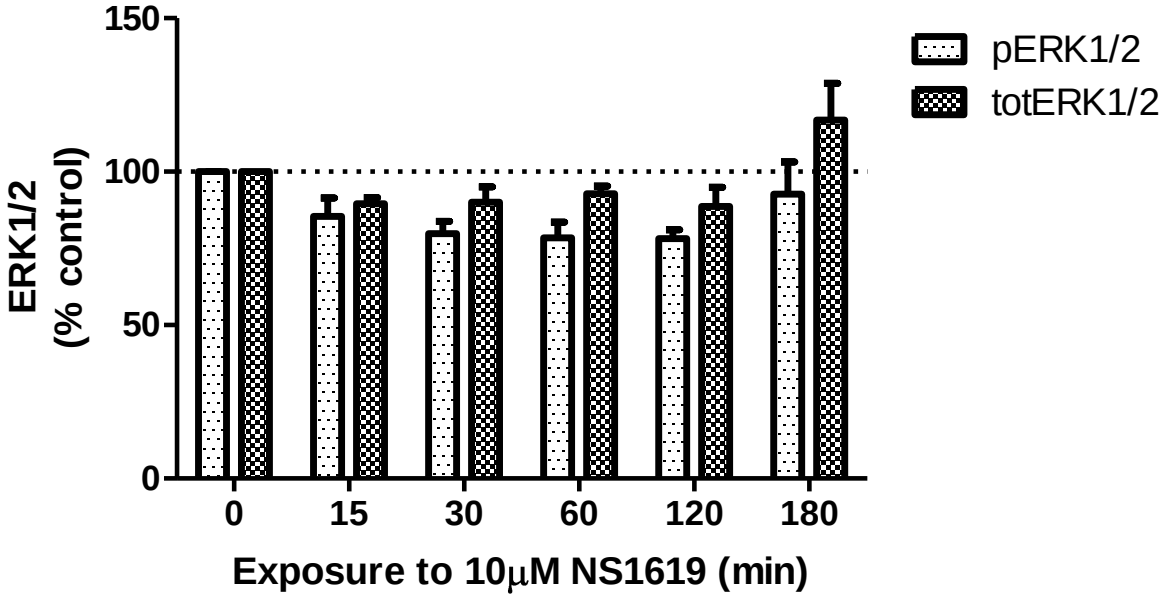
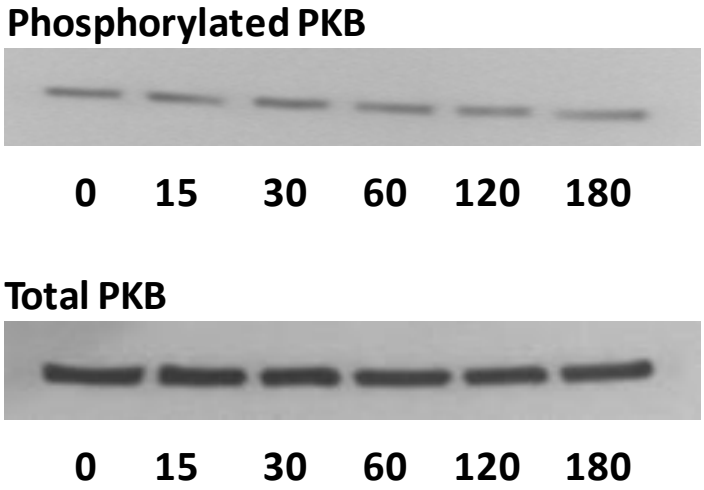


Figure 5.1: The effect of 10 μM NS1619 on phosphorylation of ERK1/2 in H9c2 cells. Cells were exposed to NS1619 for the indicated periods of time then cell lysates were collected and probed for phosphorylated ERK1/2 and total ERK1/2. (a) Representative blot with exposure time (in minutes) underneath. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 6 separate experiments.

(a)



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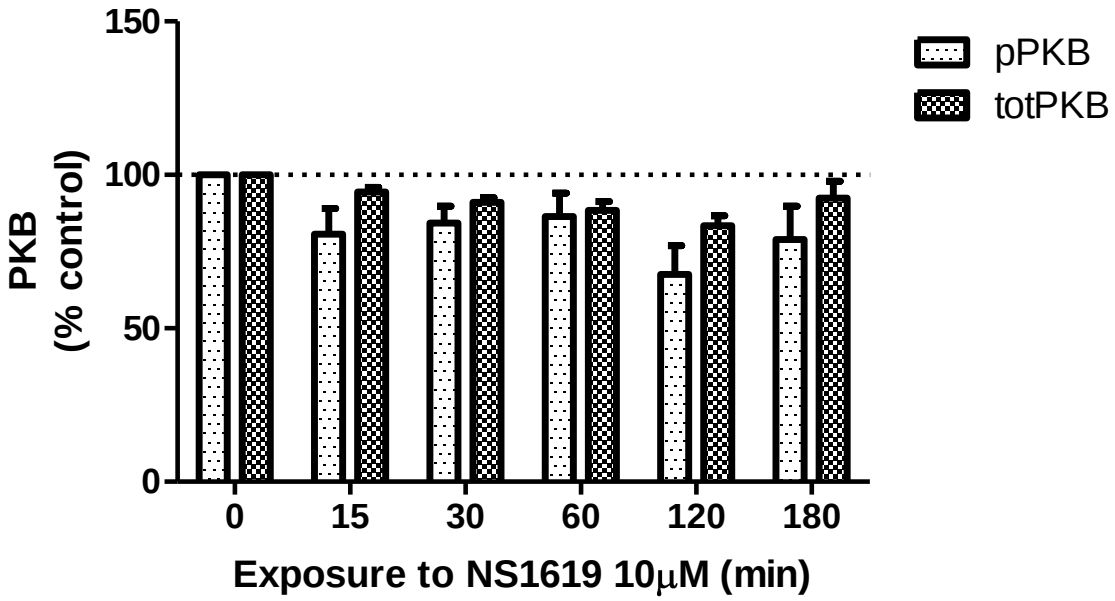
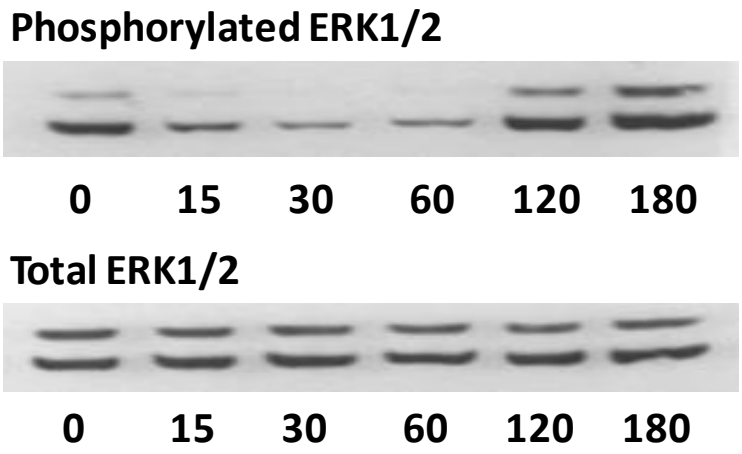


Figure 5.2: The effect of 10 μM NS1619 on phosphorylation of PKB in H9c2 cells. Cells were exposed to NS1619 for the indicated periods of time then cell lysates were collected and probed for phosphorylated PKB (Ser 473) and total PKB. (a) Representative blot with exposure time (in minutes) underneath. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 6 separate experiments.

(a)



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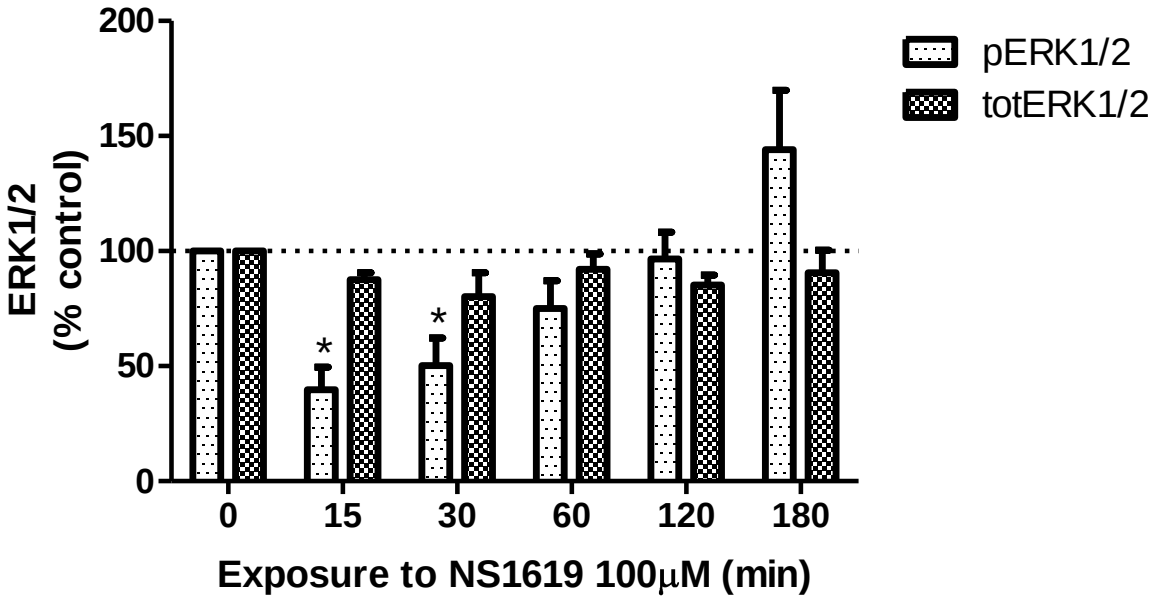
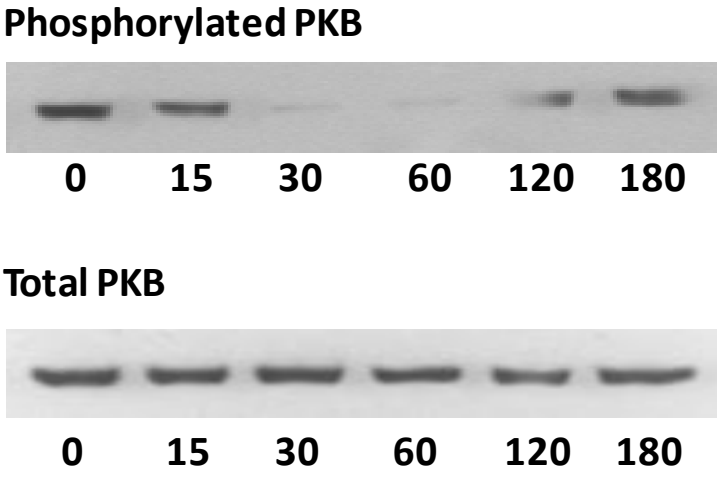


Figure 5.3: The effect of 100 μM NS1619 on phosphorylation of ERK1/2 in H9c2 cells. Cells were exposed to NS1619 for the indicated periods of time then cell lysates were collected and probed for phosphorylated ERK1/2 and total ERK1/2. (a) Representative blot with exposure time (in minutes) underneath. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 5 separate experiments. * $p<0.05$ vs. untreated pERK1/2 control.

(a)



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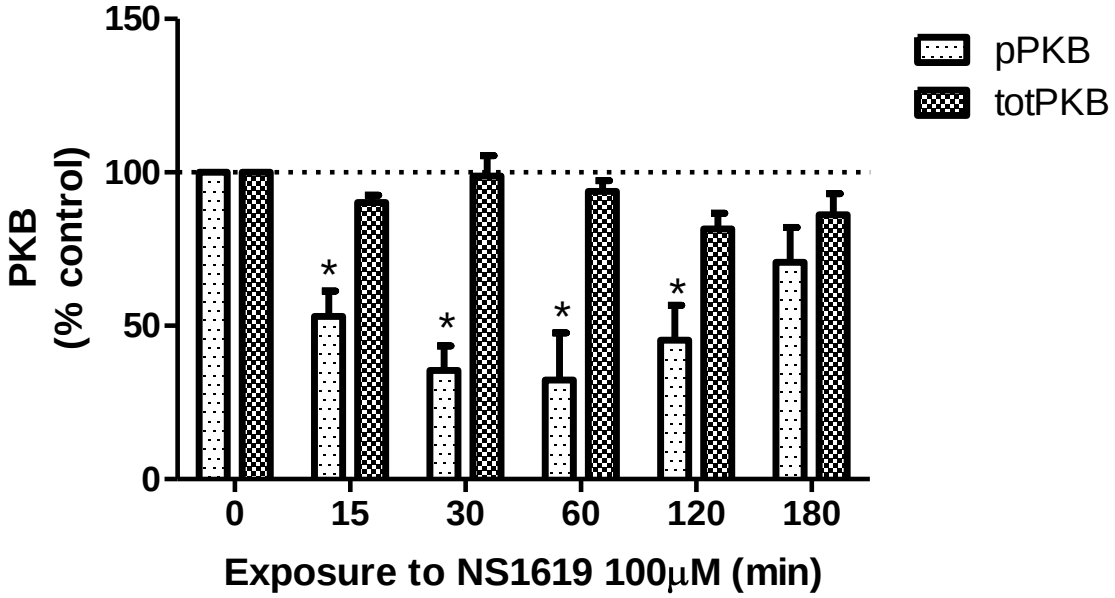
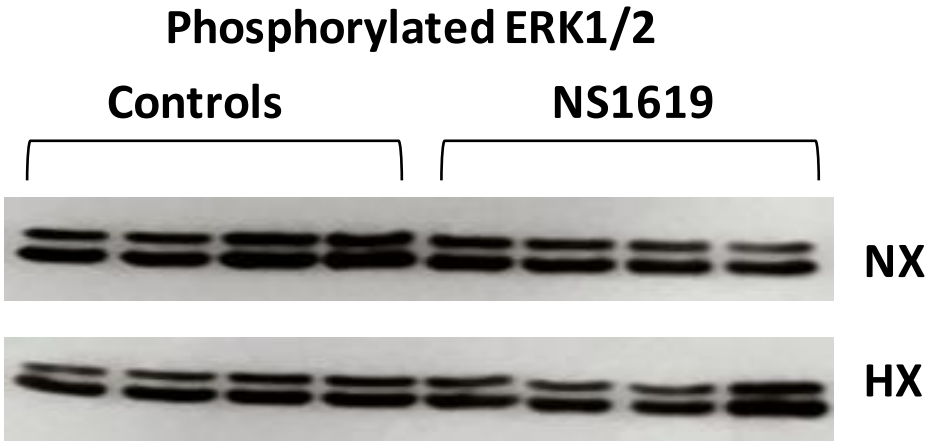
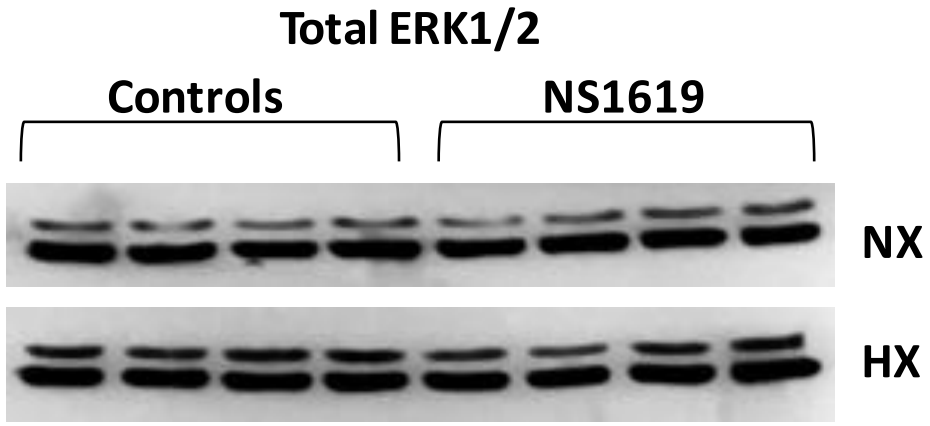


Figure 5.4: The effect of 100 μM NS1619 on phosphorylation of PKB in H9c2 cells. Cells were exposed to NS1619 for the indicated periods of time then cell lysates were collected and probed for phosphorylated PKB (Ser 473) and total PKB. (a) Representative blot with exposure time (in minutes) underneath. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 4 separate experiments. *$p<0.05$ vs. untreated pPKB control.

(a)


Phosphorylated ERK1/2
Controls
NS1619
NX
HX

(b)


Total ERK1/2
Controls
NS1619
NX
HX

(c)

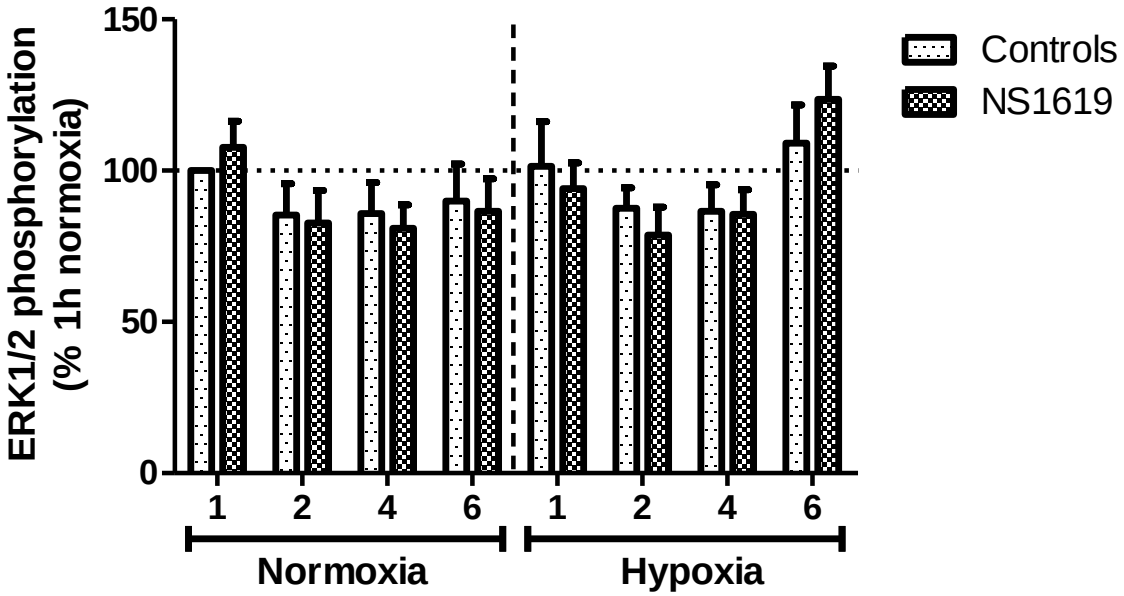
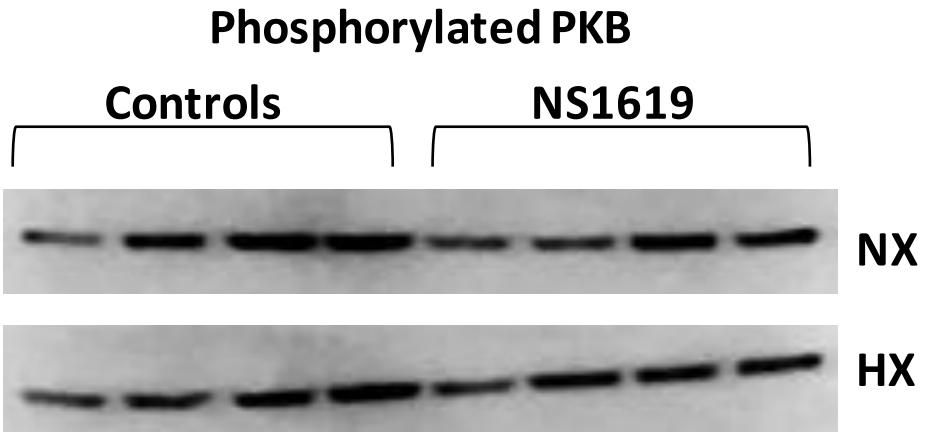
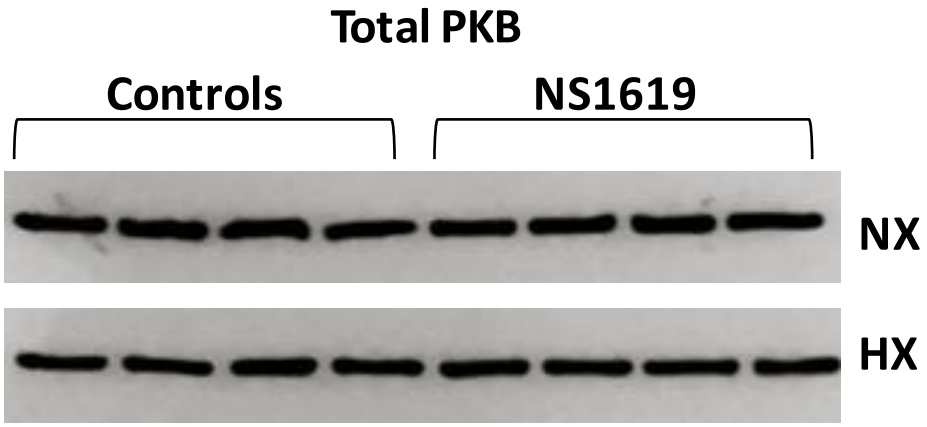


Figure 5.5: The effect of NS1619 on ERK1/2 phosphorylation during hypoxia. H9c2 cells were treated with 10 μM NS1619 (or serum-free DMEM for controls) for 30 minutes prior to hypoxic/normoxic incubation for the indicated amount of time (hours; see graph). Cell lysates were then collected and probed for phosphorylated ERK1/2 and total ERK1/2. (a) Representative blot for phosphorylated ERK1/2 (b) Representative blot for total ERK1/2 (c) Data expressed as percentage of 1 hour untreated normoxic control, total ERK1/2 not shown. Each bar represents mean ± S.E.M. of 6 separate experiments.

(a)


Phosphorylated PKB
Controls
NS1619
NX
HX

(b)


Total PKB
Controls
NS1619
NX
HX

(c)

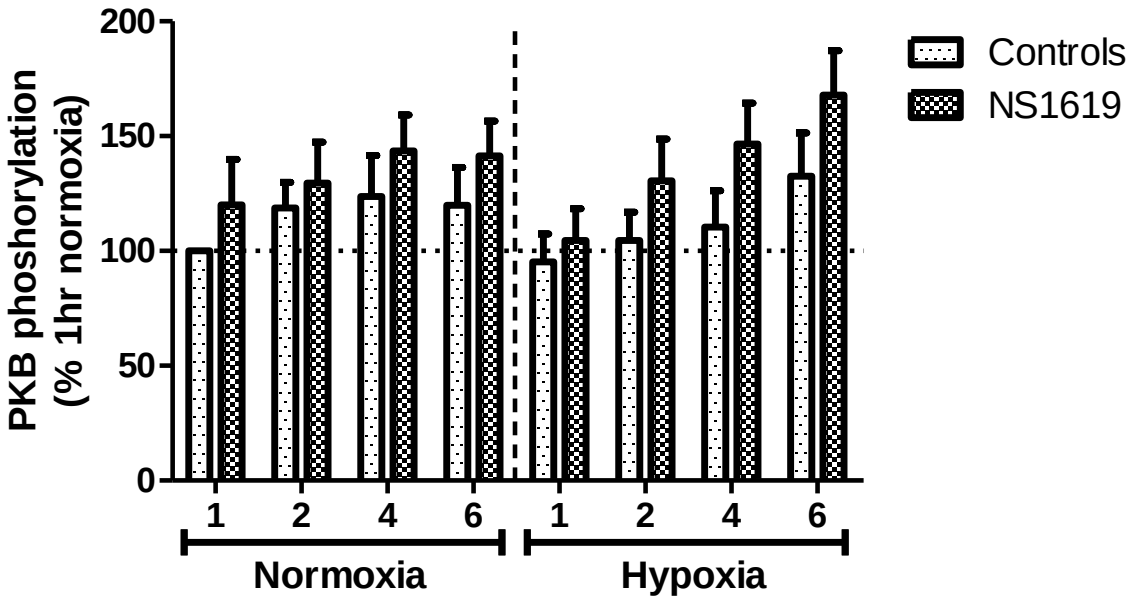
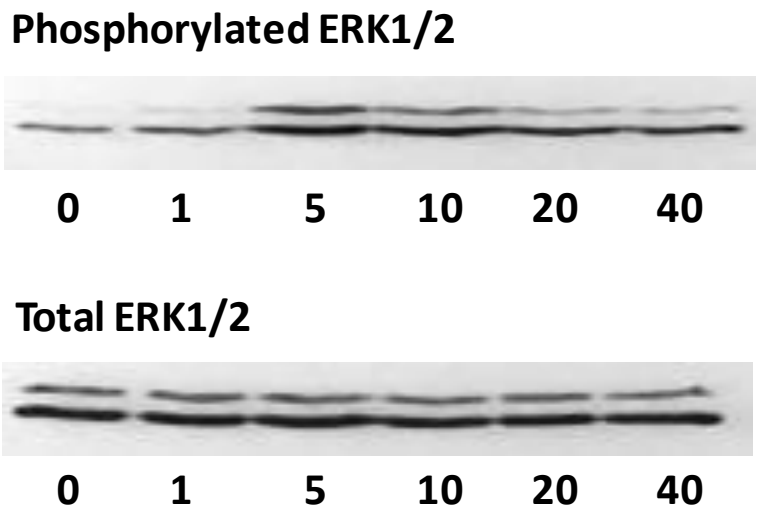


Figure 5.6: The effect of NS1619 on PKB phosphorylation during hypoxia. H9c2 cells were treated with 10 μM NS1619 (or serum-free DMEM for controls) for 30 minutes prior to hypoxic/normoxic incubation for the indicated amount of time (hours; see graph). Cell lysates were then collected and probed for phosphorylated PKB (Ser 473) and total PKB. (a) Representative blot for phosphorylated PKB (b) Representative blot for total PKB (c) Data expressed as percentage of 1 hour untreated normoxic control, total PKB not shown. Each bar represents mean ± S.E.M. of 6 separate experiments.

(a)



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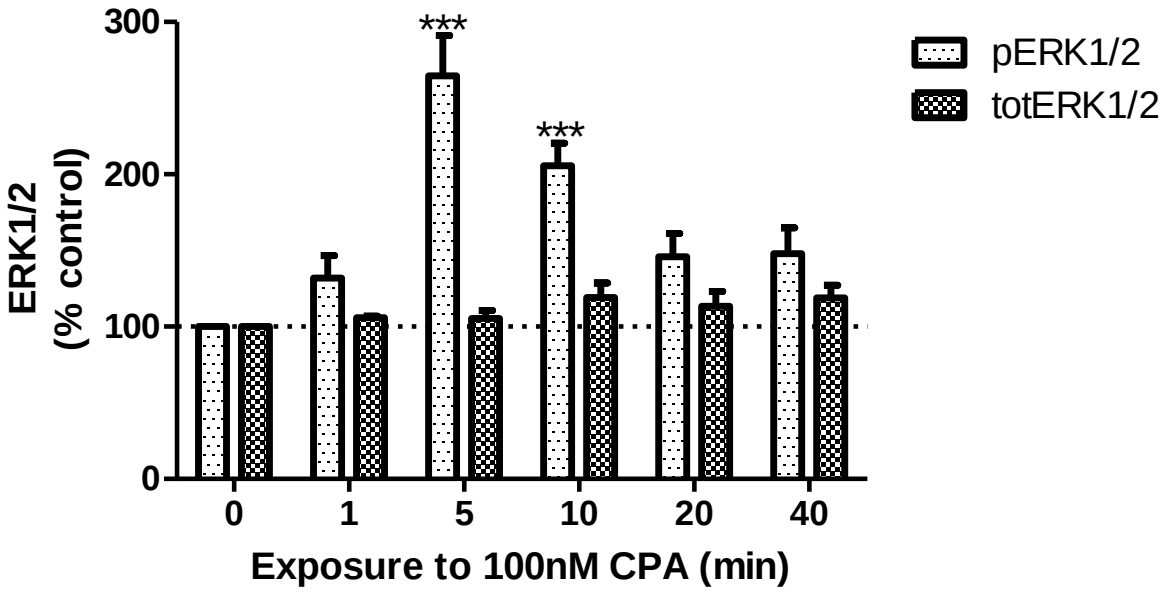
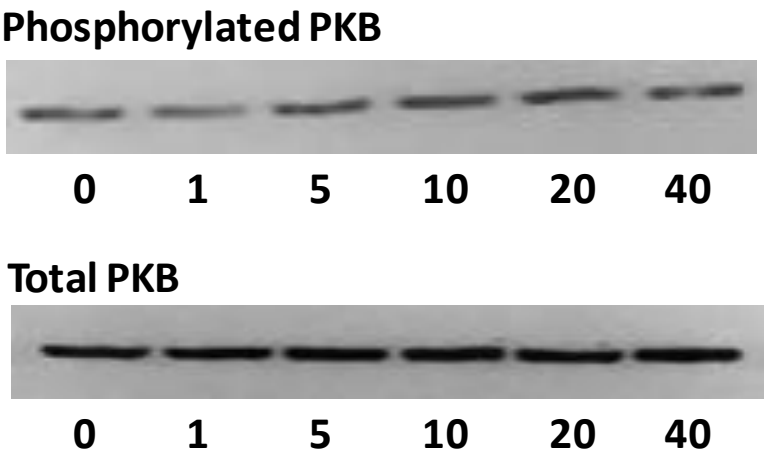


Figure 5.7: CPA-induced phosphorylation of ERK1/2 in H9c2 cells. Cells were treated with 100 nM CPA for the indicated amounts of time then cell lysates were collected and probed for phosphorylated ERK1/2 and total ERK1/2. (a) Representative blot with exposure time (in minutes) underneath. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 4 separate experiments. *** $p<0.001$ vs. untreated pERK1/2 control.

(a)



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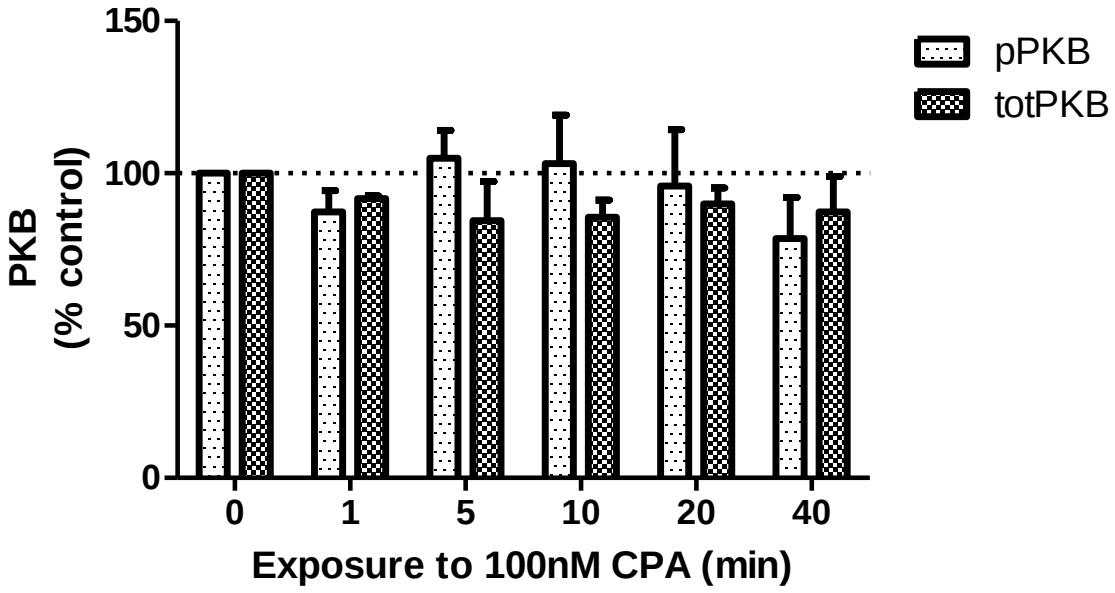
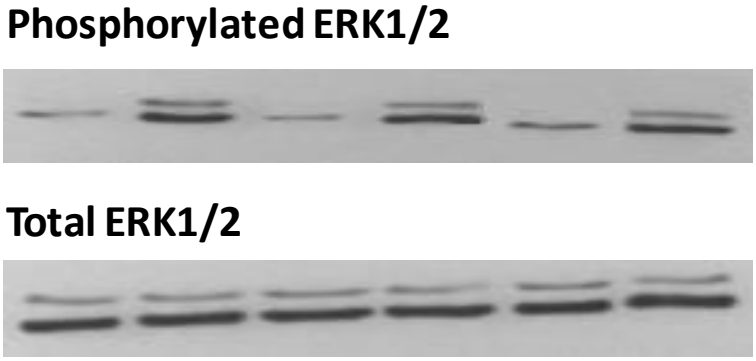


Figure 5.8: The effect of CPA on PKB phosphorylation in H9c2 cells. Cells were treated with 100 nM CPA for the indicated amounts of time then cell lysates were collected and probed for phosphorylated PKB and total PKB. (a) Representative blot with exposure time (in minutes) underneath. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 4 separate experiments.

(a)



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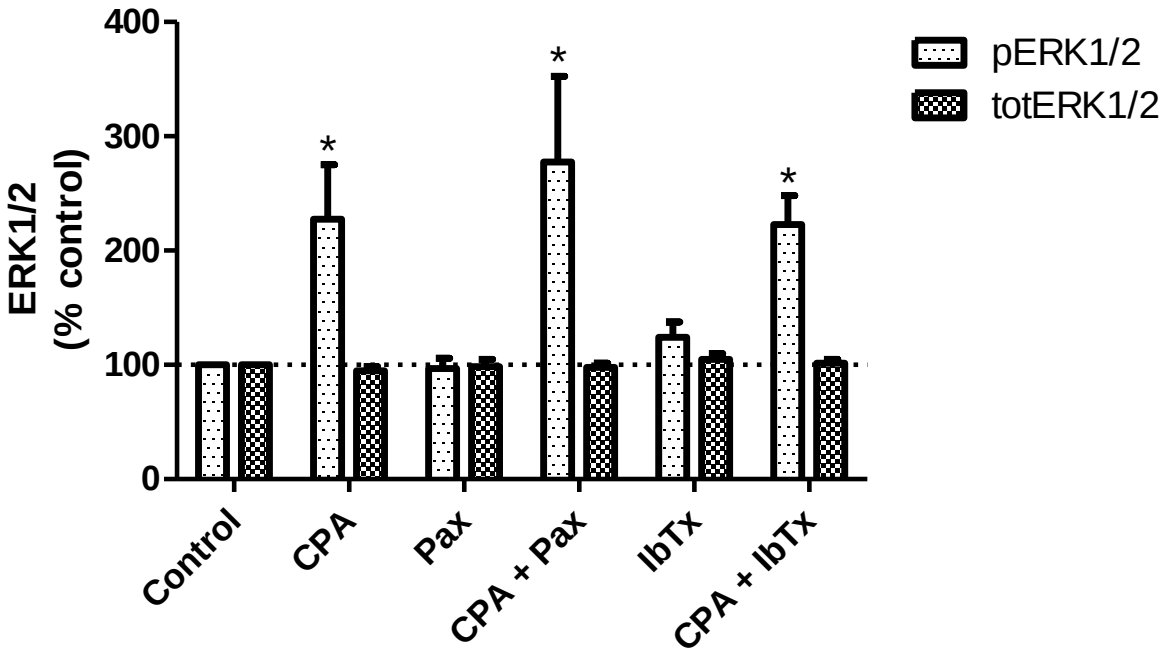
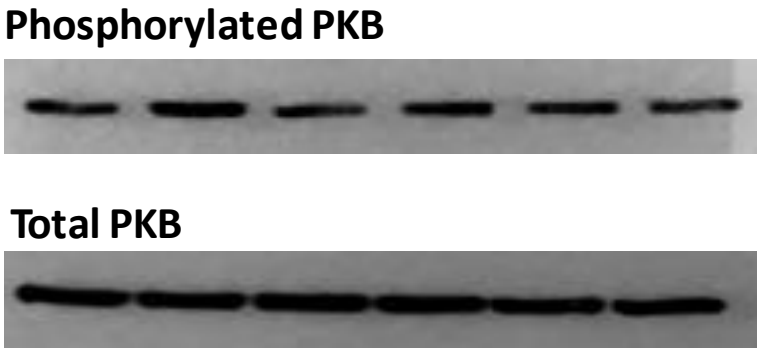


Figure 5.9: The effect of blocking the BKCa channel on CPA-induced ERK1/2 phosphorylation in H9c2 cells. Cells were exposed to paxilline (1 µM) or iberiotoxin (10 nM) for 45 minutes, with the addition of CPA (100 nM) for the final 30 minutes. Cell lysates were then collected and probed for phosphorylated ERK1/2 and total ERK1/2. (a) Representative blot. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 4 separate experiments. * $p<0.05$ vs. untreated pERK1/2 control.

(a)



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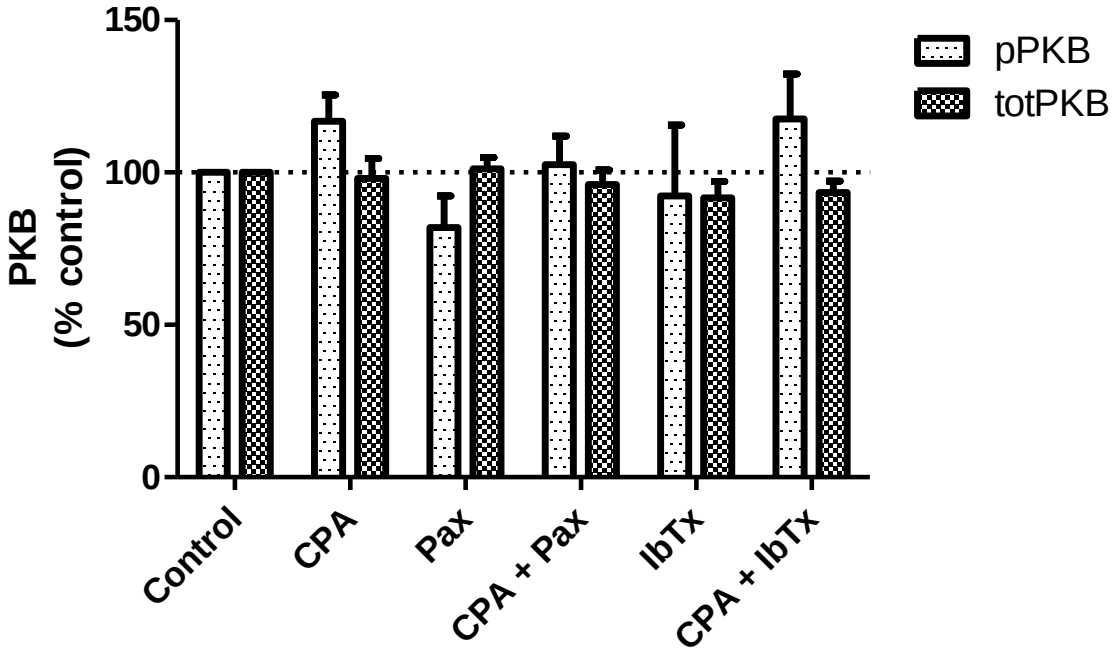


Figure 5.10: The effect of blocking the BK_{Ca} channel on CPA-induced PKB phosphorylation in H9c2 cells. Cells were exposed to paxilline (1 µM) or iberiotoxin (10 nM) for 45 minutes, with the addition of CPA (100 nM) for the final 30 minutes. Cell lysates were then collected and probed for phosphorylated PKB and total PKB. (a) Representative blot. (b) Data expressed as percentage of untreated controls. Each bar represents mean ± S.E.M. of 5 separate experiments.

6. The role of the BK_{Ca} channel in adenosine receptor-mediated protection against hypoxia-reoxygenation

6.1. A model for hypoxia-reoxygenation in H9c2 cells

Models using hypoxia-reoxygenation allow us to gain a more realistic insight into the damage caused by ischaemia/reperfusion and discovery of potential therapeutic agents. This study utilised a model for hypoxia-reoxygenation in H9c2 cells consisting of 6 hours hypoxia as used for previous experiments, followed by 18 hours of reoxygenation – a time point which had previously been used (Germack & Dickenson, 2005). Preliminary data showed that this model was sufficient for initiating cell death in H9c2 cells as measured by LDH release (363 ± 70% compared to normoxic control = 100%, n=6, $p<0.06$, see fig.6.1a), but caspase-3 activity was not increased suggesting that apoptosis had not occurred (100.5 ± 11% compared to normoxic control = 100%, n=6, see fig.6.1b).

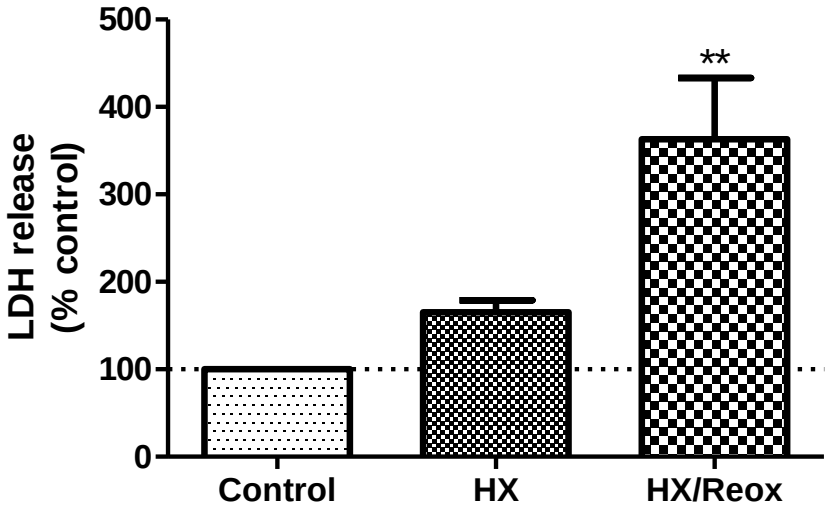
For preconditioning, H9c2 cells were treated with pharmacological agents for 30 minutes before the onset of the hypoxic incubation. Activation of the adenosine A_1 receptor with CPA (100 nM) did not provide any protection against hypoxia-reoxygenation induced cell death as measured by LDH release (100 ± 13% compared to untreated control = 100%, n=7, see fig.6.2). Unexpectedly, treatment with the BK_{Ca} channel opener NS1619 (10 µM) raised LDH release (148 ± 8% of untreated control = 100%, n=11, $p<0.01$ vs. control, see fig.6.2). This augmentation was reversed by co-treatment with paxilline (1 µM, 110 ± 6% of untreated control = 100%, n=5, $p<0.05$ vs. NS1619 alone, see fig.6.3) and iberiotoxin (10 nM, 116 ± 7%

of untreated control = 100%, n=5, $p<0.05$ vs. NS1619 alone, see fig.6.3), confirming the involvement of the BK_{Ca} channel.

To assess the postconditioning potential of pharmacological agents, treatment occurred after hypoxia at the start of reoxygenation. Postconditioning is a more valid method for protection against myocardial infarction in a clinical setting as the stimulus is applied following a bout of ischaemia – with preconditioning it would be difficult to predict when treatment should occur. NS1619- and CPA-mediated postconditioning both proved to be beneficial in the H9c2 model specified earlier (NS1619: 10 µM, 64 ± 4% of untreated control = 100%, n=7, $p<0.001$; CPA: 100 nM, 59 ± 6% of untreated control = 100%, n=7, $p<0.001$, see fig. 6.4). The protection afforded by NS1619 was reversed by paxilline (1 µM, 101 ± 11% of untreated control = 100%, n=5, $p<0.05$ vs. NS1619) and iberiotoxin (10 nM, 103 ± 8% of untreated control = 100%, n=5, $p<0.05$ vs. NS1619, see fig. 6.5), confirming that it is mediated by the BK_{Ca} channel.

CPA-mediated protection was reversed by the adenosine A_1 receptor antagonist DPCPX (10 µM, 92 ± 7% of untreated control = 100%, n=6, $p<0.01$ vs. CPA, see fig. 6.6). Interestingly, the BK_{Ca} channel blockers paxilline and iberiotoxin also significantly attenuated the CPA-mediated response suggesting a role for the BK_{Ca} channel in adenosine A_1 receptor-mediated postconditioning in H9c2 cells (paxilline: 1 µM, 83 ± 5% of untreated control = 100%, n=6, $p<0.05$ vs. CPA; iberiotoxin: 10 nM, 84 ± 4% of untreated control = 100%, n=6, $p<0.05$ vs. CPA).

(a)



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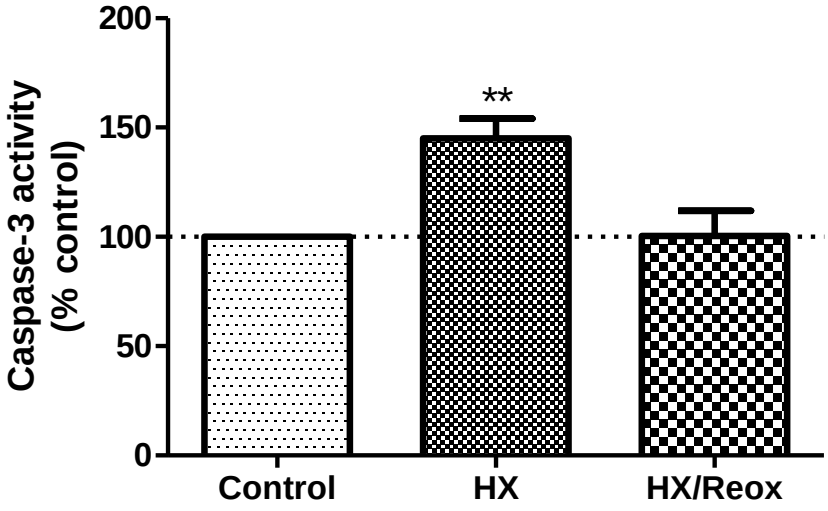

Figure 6.1: Cell death induced by hypoxia-reoxygenation in H9ce cells. Cells were exposed to 6 hours normoxia (control), 6 hours hypoxia (HX) or 6 hours hypoxia followed by 18 hours reoxygenation (HX/Reox) then cell death was assessed by (a) LDH release or (b) caspase-3 activity. Data expressed as percentage of control. Each bar represents mean ± S.E.M from 6 separate experiments; for each experiment the mean was taken from 2 (caspase-3) or 6 (LDH) replicates. ** $p<0.01$ vs. control.

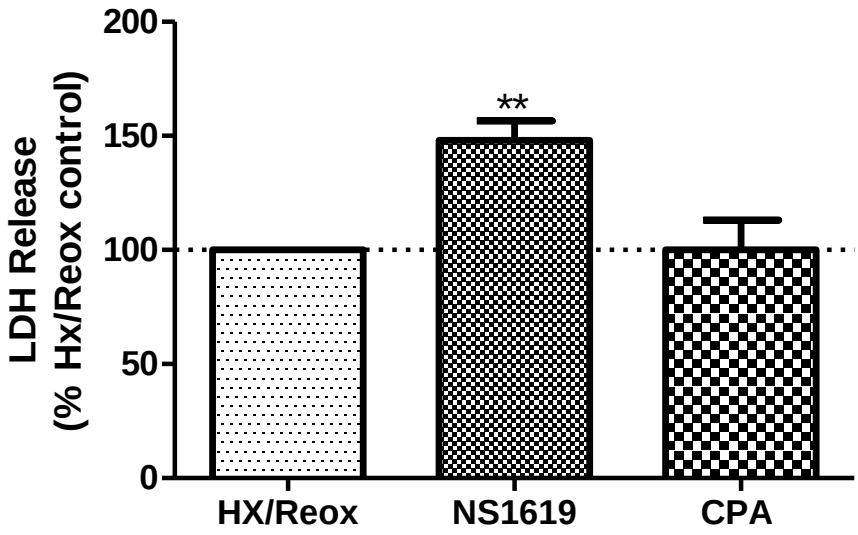

Figure 6.2: Preliminary data investigating the potential cardioprotective effects of preconditioning with CPA and NS1619. H9c2 cells were exposed to NS1619 (10 μM) or CPA (100 nM) for 30 minutes prior to 6 hours of hypoxa followed by 18 hours of reoxygenation. Data expressed as percentage of untreated controls exposed to hypoxia/reoxygenation (HX/Reox = 100%). Each bar represents mean ± S.E.M. of 7-11 separate experiments, for each experiment the mean was taken from 6 replicates. ** $p<0.01$ vs. HX/Reox.

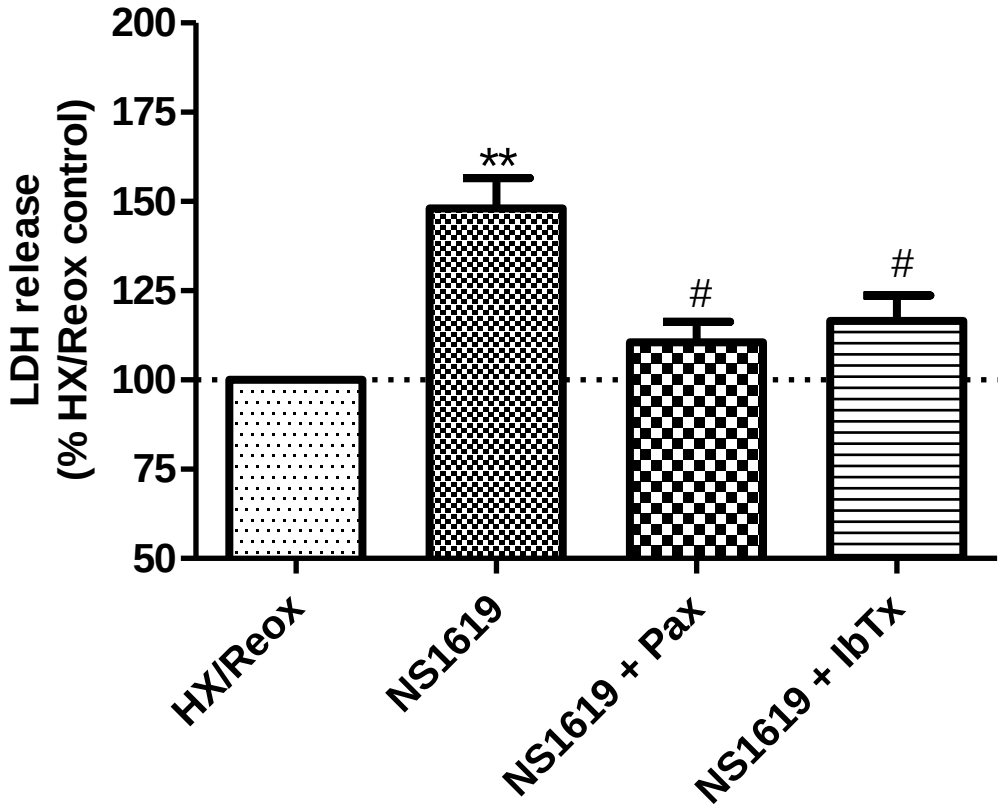

Figure 6.3: The role of the BKCa channel in NS1619-mediated preconditioning in H9c2 cells. Cells were treated with paxilline (Pax, 1 μM) or iberiotoxin (IbTx, 10 nM) for 45 minutes, with the addition of NS1619 (10 μM) for the final 30 minutes. This preceded 6 hours hypoxia followed by 18 hours reoxygenation. Data expressed as percentage of untreated controls exposed to hypoxia/reoxygenation (HX/Reox = 100%). Each bar represents mean ± S.E.M. of 5-11 separate experiments, for each experiment the mean was taken from 6 replicates. ** $p<0.01$ vs. HX/Reox; # $p<0.05$ vs. NS1619.

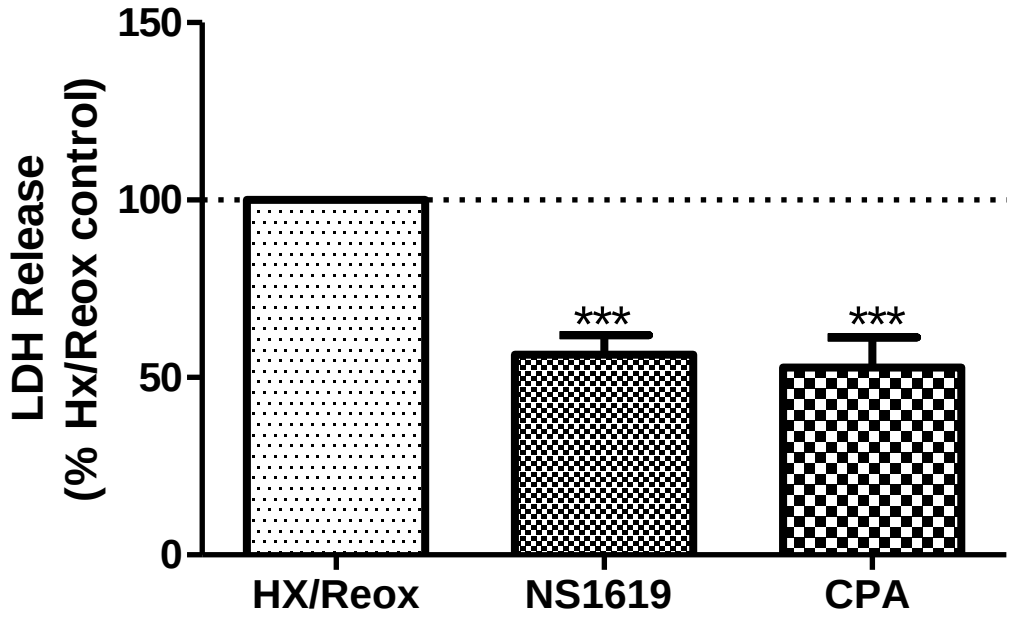

Figure 6.4: Preliminary data investigating the potential cardioprotective effects of postconditioning with CPA and NS1619. H9c2 cells were exposed to 6 hours of hypoxia followed by 18 hours of reoxygenation. For the first 30 minutes of reoxygenation, cells were exposed to NS1619 (10 μM) or CPA (100 nM) which was then removed for the remainder of reoxygenation. Data expressed as percentage of untreated controls exposed to hypoxia/reoxygenation (HX/Reox = 100%). Each bar represents mean ± S.E.M. of 7 separate experiments, for each experiment the mean was taken from 6 replicates. *** $p<0.001$ vs. HX/Reox.

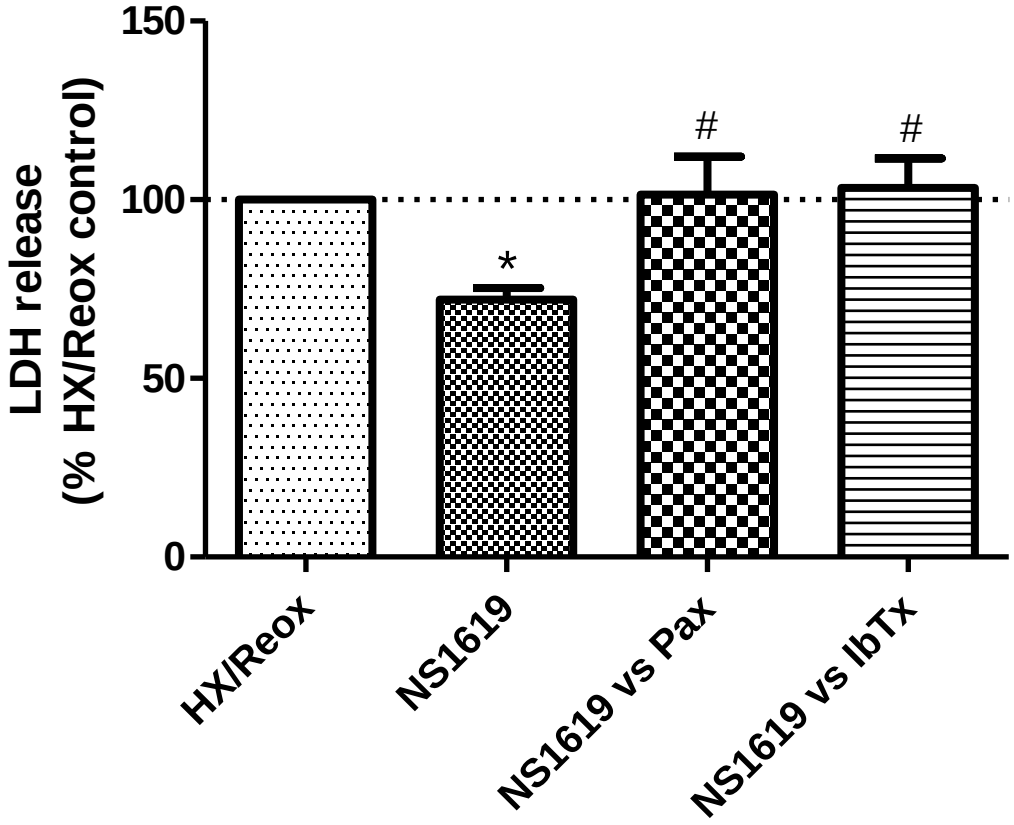

Figure 6.5: The role of the BK_{Ca} channel in NS1619-mediated postconditioning in H9c2 cells. Cells were exposed to 6 hours of hypoxia followed by 18 hours of reoxygenation. For the first 45 minutes of reoxygenation the cells were treated with paxilline (Pax, 1 μM) or iberiotoxin (IbTx, 10 nM) with the addition of NS1619 (NS, 10 μM) for the final 30 minutes of treatment. Data expressed as percentage of untreated controls exposed to hypoxia/reoxygenation (HX/Reox = 100%). Each bar represents mean ± S.E.M. of 5 separate experiments, for each experiment the mean was taken from 6 replicates. * $p<0.05$ vs. HX/Reox; # $p<0.05$ vs. NS1619.

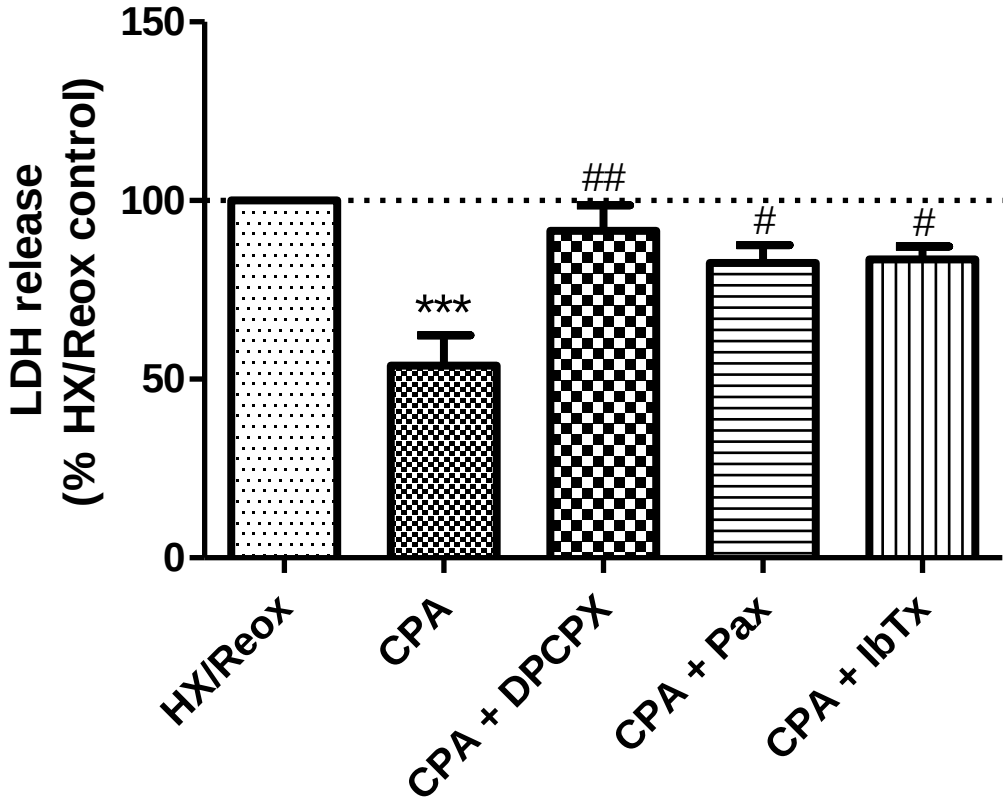

Figure 6.6: The role of the BKCa channel in CPA-mediated postconditioning in H9c2 cells. Cells were exposed to 6 hours of hypoxia followed by 18 hours of reoxygenation. For the first 45 minutes of reoxygenation the cells were treated with DPCPX (10 μM), paxilline (Pax, 1 μM) or iberiotoxin (IbTx, 10 nM) with the addition of CPA (100 nM) for the final 30 minutes of treatment. Data expressed as percentage of untreated control (=100%). Each bar represents mean ± S.E.M. of 5 separate experiments, for each experiment the mean was taken from 6 replicates. *** $p<0.001$ vs. control; ## $p<0.01$ vs. NS1619; # $p<0.05$ vs. NS1619.

6.2. A model for hypoxia-reoxygenation in rat ventricle strips

The previous experiments assessed the cytoprotective effects of activation of the adenosine A_1 receptor and the BK_{Ca} channel in H9c2 cells. To test the hypothesis that the BK_{Ca} channel is involved in A_1 receptor-mediated signal transduction in a more physiologically relevant model the contractile function of rat ventricle strips following hypoxia with/without pharmacological preconditioning was assessed. One strip per heart was taken from the right ventricle wall and set up in an organ bath. Control strips were exposed to 40 minutes of oxygenation to allow for equilibration, a further 30 minutes oxygenation, 30 minutes hypoxia and then 30 minutes reoxygenation – this lead to a 5 ± 2% recovery of function during reoxygenation, compared to the loss of function during hypoxia. The loss of contractile function during hypoxia and reoxygenation was measured, and the recovery of function during reoxygenation expressed as a percentage of original function compared to the loss observed during hypoxia. For pharmacological preconditioning the agents were added for the final 2 minutes of oxygenation then washed out before the onset of hypoxia. Where appropriate, blockers were added for the whole 30 minutes of oxygenation. For hypoxic preconditioning strips were exposed to 40 minutes of oxygenation to allow for equilibration, 10 minutes preconditioning hypoxia, 20 minutes oxygenation, 30 minutes hypoxia and then 30 minutes reoxygenation. When appropriate, strips were exposed to iberiotoxin for 30 minutes prior to the preconditioning period of hypoxia.

Firstly, it was verified that activation of the BK_{Ca} channel has a beneficial effect in this model. Preconditioning with NS1619 significantly increased the return of contractile function of the ventricle strips during the reperfusion stage (10 μM, 36 ±

4% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.01$ vs. control, see fig.6.7). To ensure that the response observed was due to the BK_{Ca} channel ventricle strips were then co-treated with NS1619 and the channel blockers paxilline and iberiotoxin. Preliminary data with 1 μM paxilline did not reveal any effect upon the NS1619-mediated return of contractile function (38 ± 4% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.001$ vs. control, see fig.6.7), 10 μM paxilline slightly attenuated the return of response (29 ± 6% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.01$ vs. control, see fig.6.7); it is possible that the concentrations of paxilline tested were insufficient in this model, however, to reduce the number of animals used it was decided to continue using solely iberiotoxin to block the BK_{Ca} channel. Iberiotoxin considerably decreased the NS1619-mediated effect (50 nM, 14 ± 6% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.05$ vs. NS1619, see fig.6.7), confirming that it was due to activation of the BK_{Ca} channel.

A brief period of hypoxia has been shown to be beneficial against a subsequent, prolonged period of hypoxia (hypoxia preconditioning) in many clinical setting and models – including the rat ventricle strip (Button *et al*, 2005). This study also observed a large return of contractile function when strips were preconditioned in this manner (35 ± 4% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.001$ vs. control, see fig.6.8), and found that this could be significantly attenuated by treatment with iberiotoxin (50 nM, 18 ± 6% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.05$ vs. hypoxic preconditioning, see fig.6.8). These data indicate that hypoxic preconditioning in rat ventricle strips involves activation of the BK_{Ca} channel.

Using specific agonists for the adenosine A_1, A_{2A} and A_3 receptors (CPA, CGS 21680 and 2-CI-IB-MECA respectively) it was found that activation of the A_1 and A_{2A} receptors lead to significant return of contractile function (see next paragraphs for details), however, a large response with activation of the A_3 receptor was not observed (1 μM, 13 ± 4% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p>0.05$ vs. control, data not shown).

Activation of the A_1 receptor with CPA lead to a robust return of function (1 μM, 39 ± 6% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.001$ vs. control, see fig.6.9) and this was considerably decreased by co-treatment with DPCPX (10 μM, 14 ± 5% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.01$ vs. CPA alone, see fig.6.9) – confirming involvement of the A_1 receptor.

Activation of the A_{2A} receptor with CGS 21680 also increased return of function (1 μM, 32 ± 3% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.001$ vs. control, see fig.6.10) which was attenuated by co-treatment with the A_{2A} antagonist ZM 241385 (12 ± 3% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.01$ vs. CGS 21680 alone, see fig.6.10). Furthermore, return of function mediated by both CPA and CGS 21680 was significantly inhibited by co-treatment with 50 nM iberiotoxin (CPA: 14 ± 2% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.01$ vs. CPA alone, see fig.6.9; CGS 21680: 15 ± 3% contractile recovery compared to 5 ± 2% observed in control strips, n=4, $p<0.01$ vs. CGS 21680 alone, see fig.6.10).

These data, for the first time, demonstrate a role for the BK_{Ca} channel in adenosine A_1 and A_{2A} receptor-mediated preconditioning – with evidence regarding the A_1

receptor collected from both *in vitro* cell viability work and experiments using an *ex-vivo* model measuring mechanical function.

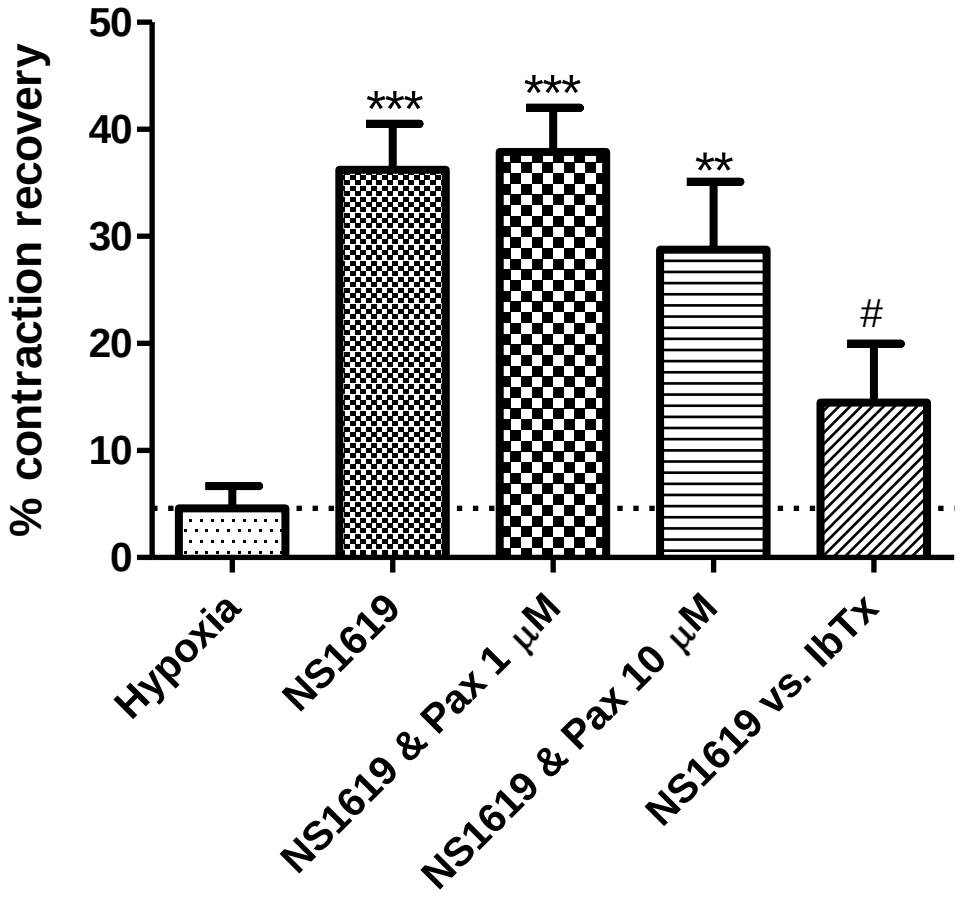

Figure 6.7: The role of the BK_{Ca} channel in NS1619-mediated pharmacological preconditioning in rat ventricle strips. Rat ventricle strips were pretreated with paxiline (Pax; 10 μM) or iberiotoxin (IbTx, 50 nM) for 30 minutes prior to cardioprotection induced by 10 μM NS1619. Data expressed as a percentage of contractile recovery compared to the level of recovery observed in control strips exposed to 30 minutes hypoxia alone. Each bar represents mean ± S.E.M. of 4 experiments. *** $p<0.001$ vs. hypoxia control; ** $p<0.01$ vs. hypoxia control; # $p<0.05$ vs. NS1619 alone.

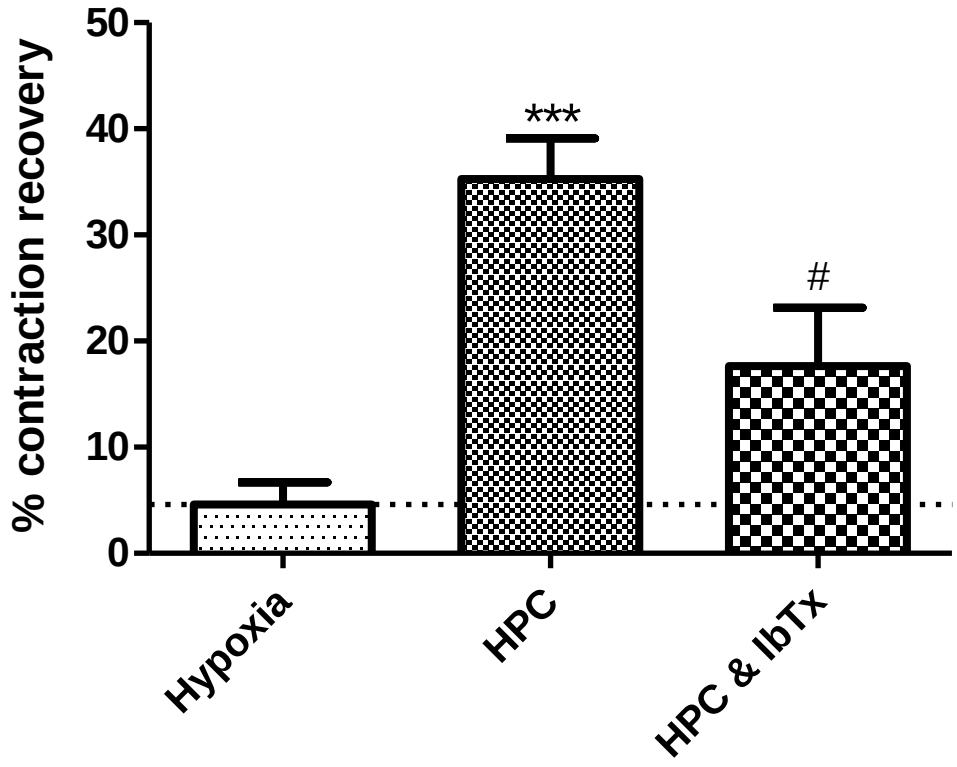

Figure 6.8: The role of the BK_{Ca} channel in hypoxic preconditioning in rat ventricle strips. Rat ventricle strips were pretreated with iberiotoxin (IbTx, 50 nM) for 30 minutes prior to cardioprotection induced by hypoxic preconditioning (HPC). Data expressed as a percentage of contractile recovery compared to the level of recovery observed in control strips exposed to 30 minutes hypoxia alone. Each bar represents mean ± S.E.M. of 4 experiments. *** $p<0.001$ vs. hypoxia control; # $p<0.05$ vs. HPC alone.

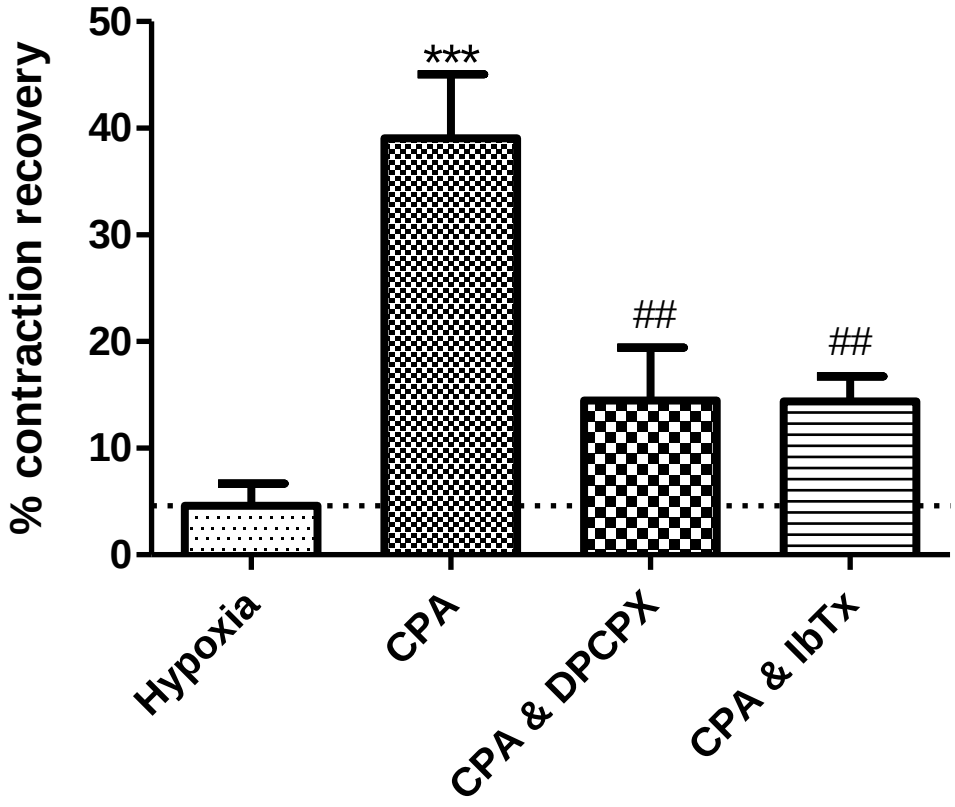

Figure 6.9: The role of the BK_{Ca} channel in pharmacological preconditioning mediated by the adenosine A_1 receptor in rat ventricle strips. Rat ventricle strips were pretreated with iberiotoxin (IbTx; 50 nM) or DPCPX (A_1 antagonist, 10 µM) for 30 minutes prior to cardioprotection induced by 1 µM CPA. Data expressed as a percentage of contractile recovery compared to the level of recovery observed in control strips exposed to 30 minutes hypoxia alone. Each bar represents mean ± S.E.M. of 4 experiments. *** $p<0.001$ vs. hypoxia control; ## $p<0.01$ vs. CPA alone.

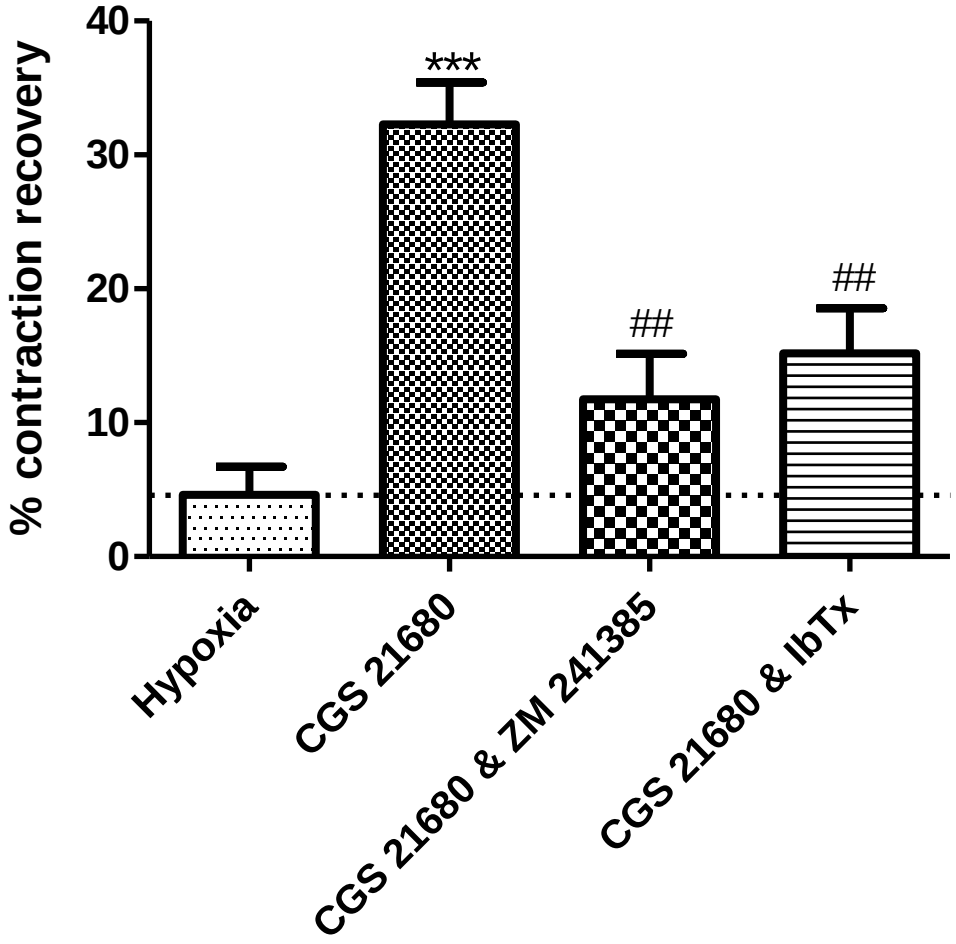

Figure 6.10: The role of the BKCa channel in pharmacological preconditioning mediated by the adenosine A_{2A} receptor in rat ventricle strips. Rat ventricle strips were pretreated with iberiotoxin (IbTx; 50 nM) or ZM 241385 (A_{2A} antagonist, 1 μM) for 30 minutes prior to cardioprotection induced by 1 μM CGS 21680. Data expressed as a percentage of contractile recovery compared to the level of recovery observed in control strips exposed to 30 minutes hypoxia alone. Each bar represents mean ± S.E.M. of 4 experiments. *** $p<0.001$ vs. hypoxia control; ## $p<0.01$ vs. CGS 21680 alone.

7. Discussion

7.1. Summary of main findings

i) H9c2 myocardial cell model

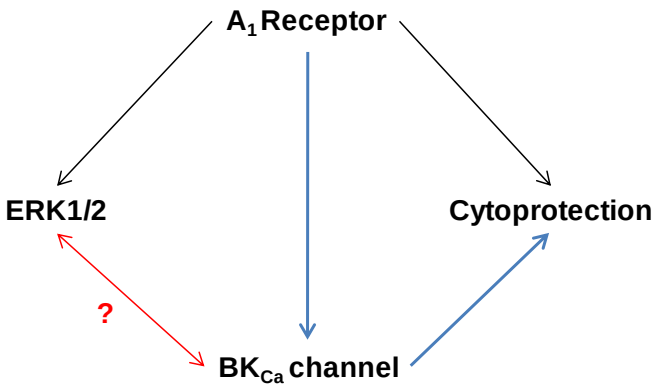

Figure 7.1.i) Cytoprotective signal transduction in H9c2 cells. Activation of the adenosine A_1 receptor leads to protection against hypoxia and hypoxia-reoxygenation-induced cell death, and this involves activation of the BK_{Ca} channel and possibly ERK1/2. Black arrow = well documented event; red arrow = possible link; blue arrow = novel pathway.

ii) Rat ventricle strip model

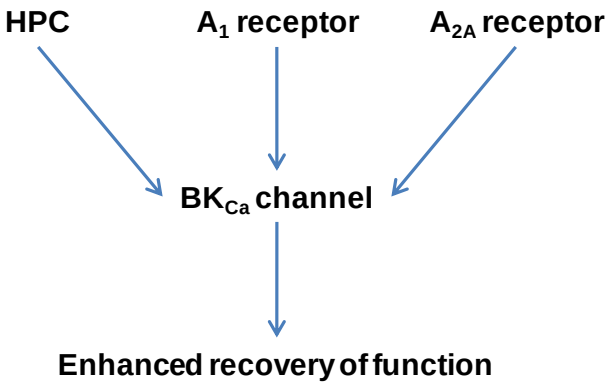

Figure 7.1.ii) The BKCa channel in cardioprotection of rat ventricle strips. For the first time it has been shown that the BK_{Ca} channel mediates cardioprotection triggered by hypoxic preconditioning (HPC) and activation of adenosine A_1 and A_{2A} receptors in rat ventricle strips.

Figure 7.1.i) summarizes the main findings from this current project using H9c2 cells. It was shown that pharmacological activation of the adenosine A_1 receptor and the BK_{Ca} channel both lead to protection against hypoxia and hypoxia-reoxygenation-induced cell death. Regarding hypoxia-reoxygenation-induced damage, the A_1 receptor agonist CPA was most beneficial as a preconditioning agent, whereas the BK_{Ca} channel activator NS1619 was most beneficial as a postconditioning agent. Interestingly, the protection afforded by A_1 receptor activation was abolished by blockade of the BK_{Ca} channel – implying that the cytoprotective signal transduction pathway triggered by this receptor involves activation of the BK_{Ca} channel. However, the role of ERK1/2 in this pathway remains controversial; more work is required to elucidate whether ERK1/2 activation occurs upstream of BK_{Ca} channel activation, or not at all.

Figure 7.1.ii) summarizes the findings from the work with the rat ventricle strip model. Hypoxic preconditioning and pharmacological preconditioning with agonists at the adenosine A_1 receptor (CPA) and the A_{2A} receptor (CGS 21680) all led to significantly enhanced contractile recovery following a hypoxic insult. Blockade of the BK_{Ca} channel prevented such preconditioning, showing that the this channel has an important role in preconditioning of rat ventricle strips mediated by hypoxia, activation of the adenosine A_1 receptor and activation of the adenosine A_{2A} receptor.

For the first time, and in two different models, a role for the BK_{Ca} channel in adenosine receptor-mediated cytoprotective signal transduction has been demonstrated.

7.2. Characterisation of GPCRs functionally expressed on H9c2 cells

i) Adenosine receptors

This report provides evidence for the functional expression of G_i-coupled adenosine A_1 receptors on myocardial H9c2 cells. The specific A_1 receptor agonist CPA provided a robust inhibition of forskolin-stimulated [^{3}H]-cAMP accumulation, which was reversed by the G_i-protein blocker pertussis toxin (PTX) and the specific adenosine A_1 receptor antagonist DPCPX. The adenosine A_3 receptor agonist CI-IB-MECA did not inhibit cAMP accumulation, but at a high concentration actually amplified the forskolin response; interestingly, this was blocked by DPCPX but not the A_3 receptor antagonist MRS 1220. This observation is suggestive of a non-specific action of CI-IB-MECA resulting in A_1 activation, indeed this was reported by Kilpatrick *et al* (2001) who showed that CI-IB-MECA-induced cardioprotection was blocked by DPCPX.

The adenosine A_1 receptor usually couples to G_i protein, producing an inhibitory effect on adenylyl cyclase, but it has been shown that high agonist concentrations can lead an increase in cAMP production (Cordeaux *et al*, 2004). This biphasic adenylyl cyclase response following A_1 receptor activation is due to direct coupling of the A_1 receptor with both G_i and G_s protein; and agonist potency at the G_s-coupled A_1 receptor was in the micromolar range, compared to nanomolar responses at the G_i-coupled receptor (Cordeaux *et al*, 2004; Baker & Hill, 2007). This finding is in keeping with the biphasic cAMP response to CPA observed in this current report, and could explain the increase in cAMP measured following treatment with 10 μM CI-IB-MECA.

A previous report showed that stimulation of the A_1 receptor transiently activates ERK1/2 in cardiomyocytes (Germack & Dickenson, 2004). In this current report a robust CPA-induced ERK1/2 phosphorylation was also observed in the H9c2 cell line, further validating a functional role for the A_1 receptor in these cells.

The data presented in this report indicate that adenosine A_{2A} receptors are not functionally expressed on H9c2 cells. This is in contrast to a recent study by Urmaliya *et al* (2009), which described cooperation between adenosine A_1 and A_{2A} receptors in cardioprotection in H9c2 cells. The latter study exposed H9c2 cells to hypoxia (100% N_2) for 12 hours, and noticed that protection against necrosis mediated by a selective A_1 receptor agonist could be attenuated with a selective A_{2A} receptor antagonist. It was also noticed that co-treatment with the A_1 and A_{2A} agonists potentiated the effect of the A_1 agonist alone, but treatment with only the A_{2A} agonist had no effect – suggesting that the receptors are working together during hypoxia. The presence of the A_{2A} receptor in this H9c2 cell model, but not in the functional study in this current report, could be explained by the experimental conditions. It has been demonstrated that adenosine A_{2A} receptor mRNA and protein levels are increased during hypoxia in PC12 cells (Kobayashi & Millhorn, 1999), with a maximum of ~250% increase in mRNA and ~400% increase in protein occurring at 12 hours of hypoxia. It is therefore likely that the expression of A_{2A} receptors on H9c2 cells is low during normal conditions (and so not detectable by functional assay), but 12 hours of hypoxic incubation stimulates a substantial increase in receptor expression so the effects of A_{2A} receptor signalling reach a detectable level.

ii) Opioid receptors

κ-opioid receptors are the predominant subtype of opioid receptor in the heart; and results suggest that these G_i-coupled receptors are also expressed on H9c2 cells. However, no evidence was uncovered for functional expression of δ-opioid receptors. Research suggests that κ-opioid receptors account for 80% of opioid receptors expressed in immature rat hearts (Zimlichman *et al*, 1996), and expression of δ-opioid receptors increases with postnatal development. As H9c2 cells are derived from embryonic rat heart (Kimes & Brandt, 1976) this could explain the lack of functional δ-opioid receptors.

During these experiments it was found that treatment with pertussis toxin (PTX) actually decreased forskolin-induced cAMP accumulation. This was also observed by Robinson & Caron (1997), who noticed that this effect only occurred with HEK293 cells expressing adenylyl cyclase type V. It is thought that PTX may increase intracellular Ca^{2+}, which would inhibit the calcium-sensitive adenylyl cyclase type V – which is the predominant isoform of adenylyl cyclase expressed in the heart, possibly explaining the observed effect in the heart-derived H9c2 cells (Ebina *et al*, 1997).

iii) β-adrenergic receptors

The data in this report regarding β_2-adrenergic receptor expression implies that these receptors are expressed on H9c2 cells, but the data regarding β_1 receptor expression is less convincing. A study using receptor mRNA levels and radioligand binding assays to investigate β-adrenergic receptor expression on H9c2 cells reported a 29%:71% ratio in favour of the β_2 receptor (Dangel *et al*, 1996), but another study

using antibody imaging reported modest expression of both subtypes (Ianoul *et al*, 2005); it is possible that discrepancies regarding expression of the receptor subtypes on H9c2 cells arise from the different methods utilised. Taking all of these data into account, it can be inferred that β_2-adrenergic receptors are expressed on H9c2 cells.

Evidence has also been presented supporting the functional expression of a UTP-sensitive G_q-coupled P2Y receptor on H9c2 cells. To date, this is the first study looking into the functional expression of GPCRs on H9c2 cells; if time permitted it would be interesting to expand on this body of information by confirming the results using other techniques (e.g. Ca^{2+} mobilization for G_q-coupled receptors) and increasing the selection of receptors tested.

7.2. The cardioprotective potential of functionally expressed receptors and K^+ channels on H9c2 cells

i) Adenosine A_1 receptor

H9c2 cells are commonly used for studies into cardioprotection, as they display similar biochemical and electrophysiological properties to cardiac tissue (Hescheler *et al*, 1991) but are less demanding to culture (Zordoky & El-Kadi, 2007). Activation of the G_i-coupled adenosine A_1 receptor has a well-documented role in cardioprotection in many models, including primary rat cardiomyocytes (Safran *et al*, 2001; Fei *et al*, 2009), so it was proposed that this would also be the case in H9c2 cells. Stimulation of the adenosine A_1 receptor with the selective agonist CPA, prior to 6 hours hypoxic incubation, significantly increased mitochondrial reduction of MTT, and decreased LDH release and caspase-3 activity. Loss of mitochondrial

function or cell membrane integrity (as measured by MTT metabolism and LDH leakage, respectively) signifies irreversible cell death (i.e. necrosis), but can also occur at the late stages of apoptosis, so caspase-3 activity (which is specific to apoptosis) was also measured to fully distinguish between necrosis and apoptosis. These findings (that CPA increased mitochondrial reduction of MTT, and decreased LDH release and caspase-3 activity) point to a role for the A_1 receptor in regulation of apoptosis and necrosis during hypoxia - confirming the receptors part in cardioprotection. This role for the A_1 receptor in both apoptosis and necrosis has also been documented in murine hearts; in isolated hearts from transgenic mice over-expressing the cardiac A_1 receptor it was reported that, following ischaemia/reperfusion, less apoptosis and less necrosis occurred compared to wild-type controls (Regan *et al*, 2003).

ii) κ-opioid receptor

Activation of the G_i-coupled κ-opioid receptor with (-)-U-50488 conferred a different pattern of protection to the adenosine A_1 receptor. Hypoxia-induced LDH release was attenuated, but neither MTT metabolism or caspase-3 activity were affected. This suggests that activation of the κ-opioid receptor provides some protection against necrotic cell death in H9c2 cells, but does not modulate apoptosis or mitochondrial function. There is great variability regarding the anti-apoptotic nature of the κ-opioid receptor when using different experimental models; one study stated that activation of κ-opioid receptors in a human epithelial tumour cell line increased apoptosis (Diao *et al*, 2000), but more recently activation of this receptor has been reported to attenuate myocardial apoptosis following ischaemia/reperfusion (Rong *et al*, 2009). When measuring different parameters, even the beneficial role of cardiac

κ-opioid receptor activation can be questioned, as activation of such receptors can be arrhythmogenic during ischaemia/reperfusion (Wong *et al*, 1990). The role of the κ-opioid receptor may be well documented in cardioprotection, but it is important to note that inconsistencies do arise between different *in vitro* and *in vivo* models.

iii) β-adrenergic receptors

As mentioned previously, the role of β-adrenergic receptor activation in cardioprotection is unclear and appears to differ between various models and experimental procedures. In this current study it was found that the non-selective β-adrenergic receptor agonist isoprenaline significantly attenuated hypoxia-induced caspase-3 activity in H9c2 cells. The functional receptor assays implied that the β_2 but not β_1 adrenergic receptor was present on these cells; the β_2 receptor has a well documented role in cardioprotection (Tong *et al*, 2005), and the β_1 receptor reportedly has a role in cardioprotection and ischaemia-reperfusion injury (Spear *et al*, 2007). It is thought that the cardioprotective properties of β-adrenergic receptor activation arise from coupling of β_2 receptors with G_i protein, which is dependent on PKA activity (Tong *et al*, 2005) - PKA phosphorylates the receptor, creating a higher affinity for G_i protein over G_s protein, and inhibition of PKA has been shown to block isoprenaline-mediated cardioprotection in mouse hearts (Tong *et al*, 2005). Taking this into account, it could be presumed that the effect observed from isoprenaline is mediated by the β_2 receptor. However, in this case it is interesting to note that the selective β_2 agonist procaterol did not provide any significant protection against hypoxia-induced cell death (as measured my MTT reduction, LDH release and caspase-3 activity) in H9c2 cells, but did increase MTT reduction in control cells exposed to 6 hours normoxia in glucose and serum-free DMEM. It is possible that

this is mediated by a non-β_2 adrenergic receptor effect, otherwise it would be expected that isoprenaline would also induce an increase in MTT reduction.

Overall, from the agonists used in this study it can be concluded that stimulation of the adenosine A_1 receptor provided the greatest cardioprotective effect in H9c2 cells.

iv) K_{ATP} and BK_{Ca} channels

Activation of mitochondrial K_{ATP} and BK_{Ca} channels are reported to protect against ischemic injury (Ardehali, 2005), and this also appears to be the case when H9c2 cells are exposed to a 6 hour hypoxic insult. Both diazoxide (K_{ATP} activator) and NS1619 (BK_{Ca} activator) significantly reduced LDH release, which was blocked by specific channel inhibitors (5-HD, K_{ATP} inhibitor; paxilline and iberiotoxin, BK_{Ca} inhibitors). NS1619 also significantly inhibited hypoxia-induced caspase-3 activity, suggesting an anti-apoptotic role for the BK_{Ca} channel in H9c2 cells.

It has been reported that NS1619-induced delayed neuronal preconditioning occurs independently of the BK_{Ca} channel, and is instead mediated by activation of the PI3K/PKB pathway, generation of reactive oxygen species and inhibition of caspases (Gáspár et al, 2008). Taking this into account two specific BK_{Ca} channel inhibitors (paxilline and iberiotoxin) were used in this present study to confirm the presence of a BK_{Ca} channel in H9c2 cells via NS1619-mediated cytoprotection, however, there is doubt surrounding the specificity of diazoxide for K_{ATP} channels. Kim *et al* (2006) suggest that activation of K_{ATP} channels by diazoxide is indirect, with diazoxide inducing translocation of PKC which then leads to opening of K_{ATP} channels. Alternatively, Dröse *et al* (2006) found that diazoxide induced oxidation of a reactive oxygen species indicator in the absence of potassium or ATP, so questioning the

involvement of K_{ATP} channels. This article also postulated that 5-HD acts as a metabolic substrate rather than an inhibitor of K_{ATP} channels. Despite these reservations regarding the specific actions of diazoxide, it is still widely accepted that it has a role in cardioprotection.

Due to the large body of work and knowledge regarding mitoK_{ATP} channels, it was decided to concentrate on BK_{Ca} channels for the remainder of the project. However, by finding evidence for functional expression of K_{ATP} channels the expression profile of receptors and channels on H9c2 cells has been expanded.

Although mitochondrial, not sarcolemmal, BK_{Ca} channels are reported to be expressed in myocardial cells, the BK_{Ca} channel opener NS1619 does not discriminate between channels expressed at the two locations so further investigation was required to uncover the location of BK_{Ca} channels in H9c2 cells. By probing the mitochondrial and cytoplasmic fractions of these cells with antibodies specific to the α and β4 subunits of the BK_{Ca} channel it was shown that myocardial H9c2 cells express a BK_{Ca} channel in the mitochondria, but not cytoplasm (therefore, not in the sarcolemmal membrane). As mentioned previously, mitochondrial BK_{Ca} channels are reported to be expressed in cardiomyocytes but the presence of a plasma membrane channel is less definite (Ko *et al*, 2009), so this finding is as expected. Interestingly, the BK_{Ca} channel β4 subunit is believed to be the predominant subunit in the brain, with β1 mainly expressed in the heart (Piwonska *et al*, 2008; Jiang *et al*, 1999) – but the use of a β1-specific antibody did not provide evidence for protein expression of this subtype in H9c2 cells (data not shown). However, a study using RT-PCR has identified both the β1 and β4 subunits in rat cardiac tissue (Poulsen *et al*, 2009), demonstrating that the β4 subunit is not purely localised to neural tissue.

BK_{Ca} channels with associated β4 subunits are less sensitive to the channel blockers charybdotoxin and iberiotoxin (Meera *et al*, 2000), so the presence of the β4 subunit in H9c2 cells could explain why iberiotoxin did not block NS1619-mediated cytoprotection as fully as paxilline in this report.

In conclusion, BK_{Ca} channels display cytoprotective properties in H9c2 cells, and the channels in question are located to the mitochondria. These mitochondrial BK_{Ca} channels have been linked with signalling pathways mediated by GPCR agonists such as (-)-U-50488 (Cao *et al*, 2005) and adrenomedullin (Nishida *et al*, 2008), but for the first time this present study discovered a link between mitochondrial BK_{Ca} channels and adenosine A_1 receptor-mediated signal transduction. It was revealed that hypoxia-induced LDH release and caspase-3 activity were attenuated by pre-treatment with CPA, the selective adenosine A_1 receptor agonist, and this effect could be reversed by blockade of the BK_{Ca} channel with paxilline and iberiotoxin. This finding demonstrates that BK_{Ca} channels (presumably mitochondrial BK_{Ca} channels) mediate the cytoprotective and anti-apoptotic effects of the adenosine A_1 receptor against hypoxia-induced cell death in H9c2 cells.

7.3. The role of protein kinases ERK1/2 and PKB in adenosine A_1 receptor and BK_{Ca} channel-mediated cytoprotection

Having shown that stimulation of the adenosine A_1 receptor can lead to activation of the BK_{Ca} channel it was then postulated that cellular protein kinases mediate some of the intermediate signal transduction pathways. Extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase B (PKB) have well documented roles in the

Reperfusion Injury Salvage Kinase (RISK) pathway (Hausenley *et al*, 2007), and have both been implicated in cardioprotection mediated by adenosine receptors (Germack & Dickenson, 2005; Germack *et al*, 2004) and mitochondrial K_{ATP} channels (de Toit *et al*, 2008; Grossini *et al*, 2009), so it was postulated that they may be involved in A_1 receptor/BK_{Ca} channel signalling in H9c2 cells. Initially, cells were treated with 10 µM NS1619 for varying amounts of time, but at no point was an increase in ERK1/2 or PKB phosphorylation observed. It was then decided to use 100 µM NS1619, as one study reported that this activates PKB in neurones (although by a BK_{Ca} channel-independent mechanism; Gáspár *et al*, 2008). However, this finding was not replicated in H9c2 cells – in fact 100 µM NS1619 significantly inhibited phosphorylation of PKB, and ERK1/2. It is likely that due to the high concentration used, the observed effects of NS1619 on dephosphorylation of ERK1/2 and PKB are independent of the BK_{Ca} channel – possibly occurring via activation of a phosphatase. It is interesting to note that Quercetin, a flavonoid which is reported to activate BK_{Ca} channels in coronary arteries (Cogolludo *et al*, 2007), also dephosphorylates ERK1/2 and PKB when used at a high concentration (Spencer *et al*, 2003).

As PKB and ERK1/2 are both cell survival kinases activated during periods of stress, and activation of the BK_{Ca} channel has an anti-apoptotic effect in H9c2 cells, it was proposed that increased phosphorylation of these kinases may be observed during hypoxia following pre-treatment with NS1619 (10 µM); however this was not observed – suggesting that the anti-apoptotic effect of BK_{Ca} channel-mediated signalling occurs independently of ERK1/2 and PKB, or that BK_{Ca} channel activation occurs downstream of ERK1/2 or PKB phosphorylation. It is interesting to note that

activation of both of these protein kinases has been reported following pharmacological activation of the mitochondrial K_{ATP} channel, implying that the anti-apoptotic effects of the mitochondrial K_{ATP} channel and the BK_{Ca} channel are differentially mediated (Ahmad *et al*, 2006; Naitohel *et al*, 2006).

An alternative explanation is that compartmentalisation of ERK1/2 and PKB occurs upon activation. During the resting state of a cell ERK1/2 and PKB are distributed throughout the cytoplasm, but both have been reported to localise elsewhere when activated. Despite the lack of a nuclear localisation signal ERK1/2 has been found to accumulate in the nucleus upon activation (Chuderland *et al*, 2008), where is then able to generate signal responses most effectively. Translocation of PKB to the mitochondria upon activation is reported in rat cardiomyocytes, where it then exerts an anti-apoptotic effect by phosphorylating hexokinase-II which prevents opening of the mPTP (Miyamoto *et al*, 2008). If this was the case, phosphorylation of ERK1/2 or PKB at specific subcellular localities may not be apparent when using whole cell lysates.

Activation of the adenosine A_1 receptor with CPA has been reported to produce an increase in activity of ERK1/2 and PKB in rat cardiomyocytes (Gemack & Dickenson, 2004; Germack et al, 2004). In H9c2 cells CPA produced a significant, transient increase in ERK1/2 phosphorylation, but had no effect on PKB – suggesting that in these cells adenosine A_1 receptor signalling is mediated by ERK1/2 but not PKB.

Using the knowledge that adenosine A_1 receptor stimulation leads to activation of ERK1/2 and the BK_{Ca} channel, it was hypothesised that ERK1/2 forms part of the A_1 receptor/BK_{Ca} channel signal transduction pathway. However, in H9c2 cells

blockade of the BK_{Ca} channel did not affect CPA-mediated ERK1/2 activation – suggesting that these kinases are not phosphorylated downstream of the BK_{Ca} channel.

Overall, these data suggest that activation of the BK_{Ca} channel, and the signalling pathway between adenosine A_1 receptors and the BK_{Ca} channel, are likely to occur independently of ERK1/2 and PKB activation in H9c2 cells.

7.4. The role of the BK_{Ca} channel in adenosine receptor-mediated protection against hypoxia-reoxygenation

i) A model for hypoxia-reoxygenation in H9c2 cells

Studies into protection against hypoxia-induced cell death are important to help elucidate the mechanisms involved in such phenomena, but models using hypoxia-reoxygenation can provide more clinically relevant data, especially when relating to myocardial infarction. By creating a model for hypoxia-reoxygenation in H9c2 cells it was possible to then test the pre- and postconditioning potential of pharmacological activation the adenosine A_1 receptor and the BK_{Ca} channel. It was observed that a protocol of 6 hours hypoxia (0.5% O_2 with glucose/serum-free DMEM to mimic ischaemia) followed by 18 hours reoxygenation (DMEM containing glucose and 1% serum) was sufficient to induce necrotic cell death, as measured by LDH release. No caspase-3 activity was observed though, indicating that apoptosis had not occurred. Nevertheless, it is possible that during the 24 hour long protocol of hypoxia and reoxygenation, any cell death initiated by hypoxia

could have progressed beyond the apoptotic stage – consequentially caspase-3 would not be detected but LDH would be released from the damaged and dying cells.

ii) Pre- and postconditioning in H9c2 cells

Pre-treating H9c2 cells with CPA prior to the period of hypoxia did not have any effect on LDH release, implying that adenosine A_1 receptor-mediated pharmacological preconditioning is not protective in H9c2 cells. Interestingly, preconditioning with NS1619 augmented LDH release, and this was reversed with paxilline and iberiotoxin – indicating that pharmacological preconditioning via activation of the BK_{Ca} channel amplifies cell death in this model. This finding contrasts with the majority of reports regarding BK_{Ca} channels in preconditioning, so more work would be required to uncover the mechanisms involved. It is possible that the observed increase in LDH release could be a cell specific effect, or due to the experimental protocol used. It has been shown that the time of agonist/antagonist treatment can greatly affect the experimental outcome. For example, Schröter *et al* (2005) found that excitotoxic NMDA receptor-evoked rises in neuronal intracellular calcium could be attenuated by pre-treatment with nitric oxide, but nitric oxide treatment at the time of NMDA receptor stimulation actually potentiated the toxicity. Therefore it can be expected that the treatment protocol used would affect the end result.

Conversely, activation of the BK_{Ca} channel during the first few minutes of reoxygenation (i.e. postconditioning) significantly reduced hypoxia-reoxygenation-induced LDH release. This was also attenuated by co-treatment with paxilline and iberiotoxin, suggesting that the effect was mediated by the BK_{Ca} channel. Currently,

this is the first report of postconditioning triggered by direct activation of the BK_{Ca} channel.

Despite the lack of efficacy of CPA as a preconditioning agent, activation of the adenosine A_1 receptor created a postconditioning effect in H9c2 cells as LDH release was significantly reduced. Not only was this reversed by antagonism of the A_1 receptor, but also by blockade of the BK_{Ca} channel – providing the first evidence of a role for the BK_{Ca} channel in adenosine A_1 receptor-mediated postconditioning.

iii) Preconditioning in rat ventricle strips

To validate the use of H9c2 cells as a model for investigating adenosine A_1 receptor and BK_{Ca} channel mediated signalling, it was decided to verify the observed results in a more physiologically relevant model. Strips of rat ventricle are often used as a method for measuring mechanical contractile function of the tissue following various treatment protocols (for examples see: Button *et al*, 2005; Bravo *et al*, 2007; Vassallo *et al*, 2008;). In this case, strips from the right ventricle were used as the right ventricle wall is thinner than the left, allowing for greater penetration of drugs. Following a period of hypoxia (glucose/oxygen-free KREBS solution to mimic ischaemia) contractile function of the ventricle strips returned to approximately 5% of that observed prior to hypoxia, i.e. the control phase. This was important as it conveyed that the tissue was still viable – if there had been no return of function it would be possible that the tissue had died, therefore the beneficial effects of preconditioning would be masked.

Preconditioning with NS1619 significantly enhanced the return of contractile function following hypoxia, and this was blocked by iberiotoxin. This is the first

time that activation of the BK_{Ca} channel has been investigated, and indeed shown to be beneficial, in this model. Hypoxic preconditioning has been well documented in many different models, including rat ventricle strips (Button *et al*, 2005). However, in this model the role of the BK_{Ca} channel had not been examined. This present study demonstrates that blockade of the BK_{Ca} channel is sufficient to significantly inhibit the effect of hypoxic preconditioning in rat ventricle strips.

Preconditioning with selective agonists of adenosine A_1, A_{2A} and A_3 receptors has previously been reported to increase the return of contractile function in this rat ventricle strip model (Button *et al*, 2005). However, in this current study it was observed that the A_1 and A_{2A} receptors were capable of inducing preconditioning, but not the A_3 receptor. There is no obvious explanation for this discrepancy, as the same drug was used at the same concentration in both studies (2-CI-IB-MECA, 1 μM) – more work would need to be done to clarify the effects of adenosine A_3 receptor activation.

The roles of the A_1 and A_{2A} receptors in preconditioning of rat ventricle strips were confirmed with the observation that the beneficial effect of pre-treating with an agonist at either receptor was blocked by co-treatment with a receptor-appropriate antagonist. Following on from earlier work with H9c2 cells, it was postulated that the BK_{Ca} channel – direct stimulation of which has been proven to be beneficial in both the H9c2 cell viability model and the ventricle tissue mechanical function model - was also involved in A_1 receptor-mediated preconditioning in rat ventricle strips. Indeed this was the case, as co-treatment with the A_1 receptor agonist CPA and the BK_{Ca} channel blocker iberiotoxin significantly attenuated the protective effect afforded by treatment with CPA alone.

This study also revealed that blockade of the BK_{Ca} channel could inhibit preconditioning mediated by the G_s-coupled adenosine A_{2A} receptor in rat ventricle strips. Although the adenosine A_1 and A_{2A} receptors couple to different G proteins, they are both reported to activate ERK1/2 (Germack & Dickenson, 2004). It is possible, therefore, that in rat ventricle strips this common ground of ERK1/2 activation provides an intermediate step between adenosine receptor stimulation and BK_{Ca} channel activation, or is a consequence of BK_{Ca} channel signalling. It would be interesting to further research the signal transduction pathways involving adenosine receptors and the BK_{Ca} channel, as this current report demonstrates a beneficial effect of such pathway activation in two separate models.

7.5. Conclusions and further work

i) General conclusions

Overall, this body of work has provided novel data supporting a role for the BK_{Ca} channel in adenosine A_1 receptor-mediated protection against hypoxia and hypoxia-reoxygenation-induced cell death in a H9c2 myocardial cell model. This data was then validated using a model utilising isolated rat ventricle strips, and it was discovered that in this more physiologically relevant model the BK_{Ca} channel has a pivotal role in hypoxic preconditioning and in pharmacological preconditioning mediated by the adenosine A_1 and adenosine A_{2A} receptors.

ii) Further work

If this body of work were to be extended, it would be important to elucidate the signal transduction pathway that occurs between the adenosine A_1 and A_{2A} receptors and the BK_{Ca} channel. Regarding the H9c2 cell model, by using subcellular fractionation it would be possible to probe distinct localities of the cells for protein kinase activity. The possible role of ERK1/2 and PKB could then be uncovered, and PKA, PKC and PKG would also make good targets for future work as they have all been linked with adenosine A_1 receptor-mediated signalling and/or BK_{Ca} channel-mediated signalling. Inhibitors of PKA, PKC and PKG are also commercially available, therefore increasing the scope for examining the signalling pathways and determining the triggers, modulators and effectors.

Although there is not a commercially available direct inhibitor of ERK1/2, inhibitors of MEK1/2 are available (e.g. PD 98059) and are often used to study ERK1/2-mediated responses. The role of ERK1/2 as an upstream second messenger in adenosine A_1 receptor-mediated signalling is confirmed, but it is not known if ERK1/2 is part of the BK_{Ca} channel signalling pathway. By preventing ERK1/2 activity via inhibition of MEK1/2 it would be possible to elucidate whether ERK1/2 phosphorylation occurs upstream of the BK_{Ca} channel, as if this was the case inhibition of ERK1/2 would prevent NS1619-mediated protection.

Due to the anti-apoptotic nature of adenosine A_1 receptors and BK_{Ca} channel activation, it is likely that the signal pathway between these two proteins focuses on the mitochondria. Activation of the mitochondrial BK_{Ca} channel has been reported to reduce Ca^{2+} overload (Kang *et al*, 2007), which would create an environment favouring closure of the mPTP – indeed it has already been suggested that the mPTP is a downstream modulator of BK_{Ca} channel signalling (Cao *et al*, 2005 & 2005a;

Gao *et al*, 2005), and that adenosine A_1 receptor activation leads to inhibition of mPTP (Fei *et al*, 2009), so it appears quite likely that the anti-apoptotic effects of the adenosine A_1 receptor/BK_{Ca} channel-mediated signal transduction pathway arises from inhibition of the mPTP. As yet though, this has not been looked into.

Another area of interest would be to determine the BK_{Ca} channel subtype/s expressed and responsible for A_1 receptor/BK_{Ca} channel-mediated cardioprotection in the rat heart. Western blotting with antibodies to the specific subtypes would reveal which are expressed, and then by using knock-out animals lacking the individual subtypes it would be possible to 1) ascertain the general effects of knocking out each BK_{Ca} channel subunit (e.g. β1 or β4), and 2) establish if cardioprotection triggered by the adenosine A_1 receptor can still take place.

A further avenue to explore would be whether the expressions of BK_{Ca} channel subunits are upregulated following treatment. It has been shown that the K_{ATP} channel – which has a well documented role in preconditioning – contains a hypoxia-inducible Kir6.1 subunit (Melamed-Frank *et al*, 2001); is this also the case for the BK_{Ca} channel and if so is this effect modulated by adenosine receptor activation?

iii) Concluding remarks

In conclusion, this study has shown that activation of the BK_{Ca} channel can provide protection against hypoxia and hypoxia-reoxygenation-induced cell death and loss of function, and that these effects are mediated by adenosine receptor signalling. Alongside the potential cardioprotective properties, opening of the BK_{Ca} channel has been proposed to be beneficial in ischaemic stroke. *In vivo* experiments using the BK_{Ca} channel opener BMS-204352 have shown very promising data, but clinical

trials were disappointing (Jenson, 2002), highlighting the need for more research into the mechanisms involved in BK_{Ca} channel-mediated signalling. The data in this current report will contribute to the increasing body of knowledge regarding BK_{Ca} channel-mediated signal transduction, and hopefully lead to further investigation and potential drug discovery.

8. References

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