The Identification and Role of the Adenosine A_3 Receptor in the Recovery of Cardiac Tissue from Ischaemia.

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SUMMARY OF THESIS

The adenosine A_3 receptor is one of the suspected effectors of intrinsic preconditioning. Its intrinsic ligand, adenosine is produced by ischaemic tissue, where it acts to cause a preconditioning effect, rendering the tissue resistant to damage by any subsequent ischaemia.

Adenosine A_3 receptor stimulation has previously been shown mediate the recovery of the heart following ischaemia. It is thought to be involved in alleviating temporary stunning and modulating infarction by the mechanisms by which it evokes preconditioning.

Using a variety of cardiac ischaemia models from rat tissue, we investigated the effect of a selective A_3 receptor agonist on ischaemic cardiac tissues and whether it is possible to evoke a cardioprotective effect by stimulation of adenosine A_3 receptor alone.

The administration of selective A_3 receptor agonist alleviated post-ischaemic contractile dysfunction in the isolated left atria. No effect was found in right ventricle strips, while in the Langendorff heart it was found that there was a pro-infarct effect, with no observed effect of contractility. Attempts to classify the effects as being adenosine A_3 receptor -mediated or another adenosine subtype by use of selective antagonists was difficult for reasons discussed.

Using immunological methods is was possible to identify a protein specific for the adenosine A_3 receptor in membrane fractions obtained from both normoxic and ischaemic rat left atria. This strengthens the claim that the effect reported in this thesis are adenosine A_3 receptor mediated.

In summary it is recommended that A_3 receptor manipulation and its effect on post-ischaemic recovery remains a viable route of further investigation. The work in this field is required to form new strategies for the treatment coronary heart disease and other cardiac conditions.

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Synthesis, Metabolism and Uptake of Adenosine

1.1.1. Adenosine

The purine nucleoside adenosine is endogenous to all cells. Its tri-phosphorylated precursor, adenosine triphosphate (ATP) is the ubiquitous cellular energy source.

Adenosine is comprised of adenine attached to a D-ribose (ribofuranose) moiety via a β-N^9-glycosidic bond (see Figure 1.1).
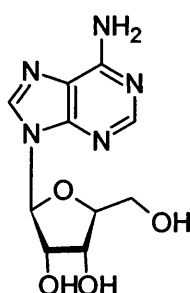
Figure 1.1 The chemical structure of adenosine. Its IUPAC name is (2R,3R,4S,5R)-2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol, has the formula $C_{10}H_{13}N_5O_4$ and a molecular mass of 267.242 g/mol

1.1.1.1. Adenosine and Physiology

The physiological significance of adenosine was first realised in 1929 when its actions on the cardiovascular system were first described by Drury and Szent-Gyorgyi. Since this initial description, the biological effects of adenosine have been extensively studied. Adenosine regulates such a diverse range of physiological functions that almost all tissue and organ systems are affected by it. The responses to adenosine are complex and dependent on the species, tissue type and metabolic state of the tissue, and in what is to be later discussed, the activated receptor subtype involved.

In relation to the heart, adenosine is known to cause slowing of atrioventriclar nodal conduction and so a reduction in the sinus rate of cardiac contraction, the reduction of β-adrenergic responses, a decrease ventricular automaticity and inotropy, inducement of coronary vasodilation and the alteration of cardiac

energy metabolism, especially in ischaemia. The last of theses modes of action was intensively studied in this thesis.

1.1.2. Adenosine Production

An understanding of the conditions where adenosine is evolved is required in order to study adenosine's action. Intracellularly, two pathways can produce adenosine. They are the dephosphorylation of adenosine monophosphate (5'-AMP): the 'ATP pathway', and the hydrolysis of S-adenosylhomocysteine (SAH): the 'transmethylation pathway'. (Both pathways are illustrated in Figure 1.2.)

The importance of each pathway in the production of adenosine is dependent on both the tissue type and the metabolic state of that tissue. The 'ATP pathway' produces adenosine by dephosphorylation of 5'-AMP by the action of the 5'-nucleotidase enzyme. This enzyme is present both in the cytosol and bound to the cell membrane as ecto-5'-nucleotidase, therefore allowing this mechanism of adenosine synthesis to also occur extracellularly. AMP dephosphorylation is termed the ATP pathway as its initial precursor is ATP. In ischaemic cardiomyocytes, the adenosine accumulates as a by-product of energy production. Most of the adenosine in the normal heart is made from AMP (Ingwall, 2002)

Adenosine may also be produced by the 'transmethylation pathway'; where both adenosine and homocysteine are formed by the hydrolysis of SAH by SAH-hydrolase. SAH is formed from the methylation of S-adenosylmethionine (SAM) by SAM-transferase. This mechanism of adenosine synthesis only occurs intracellularly and is an oxygen-independent mechanism. The equilibrium of this reaction normally is towards SAH formation, however, under some physiological conditions the equilibrium changes to the hydrolysis of SAH. Although it is thought that this pathway accounts for approximately one third of cardiac adenosine production under normoxic conditions (Deussen et al., 1989), under hypoxic stress the proportional contribution that SAH hydrolysis makes to adenosine production decreases (Lloyd and Schrader, 1993). Furthermore, adenosine produced by this pathway is thought to be

salvaged by intracellular adenosine kinase, resulting in little extracellular release (Kroll et al., 1993).

This suggests that the main mechanism of adenosine production that allows adenosine to act as an effector in ischaemia is the action of intracellular 5'-nucleotidase on AMP.

1.1.2.1. Adenosine Synthesis

Adenosine itself does not have its own synthetic mechanism in ischaemia and is only formed as a by-product of the ATP breakdown cycle (Schultz et al., 1982). The production of adenosine by *de novo* synthesis is metabolically expensive and so it is thought that this pathway does not make a major contribution to adenosine production during ischaemia, instead its function is to balance the low rate of purine loss (Dow et al., 1985).

1.1.2.2. Adenosine Production in the Ischaemic Heart

When tissue becomes ischaemic or hypoxic, or has an increased oxygen requirement, a imbalance between the oxygen supply and the oxygen demand of the tissue develops (Bardenheuer and Schrader, 1986; Mubagwa et al., 1996; Shryock and Belardinelli, 1997). As in the heart, the high-energy phosphate bond of ATP serves as the main source of energy for myocardial contraction (Katz, 1970), a shortfall in ATP occurs and ATP's metabolites, AMP and adenosine then accumulates.

Intracellular adenosine can then be transported out of the cell by facilitated diffusion which is further discussed in Section 1.1.4.

1.1.3. Adenosine Catabolism

The regeneration of the depleted adenine nucleotide pools are essential in order to maintain the energy levels in the cell. Intracellular adenosine can be regenerated by three routes, which are illustrated in Figure 1.2. The routes are by phosphorylation to AMP by adenosine kinase, deamination to inosine by adenosine deaminase or by washout into the circulation.

Figure 1.2 A diagrammatic representation of the main purine metabolic pathways that influence adenosine concentrations both intra- and extracellularly. The pathways shown do not represent any particular energy state of the cell. Enzymes are shown in *italic* print. Abbreviations used: Mito, Mitochondria
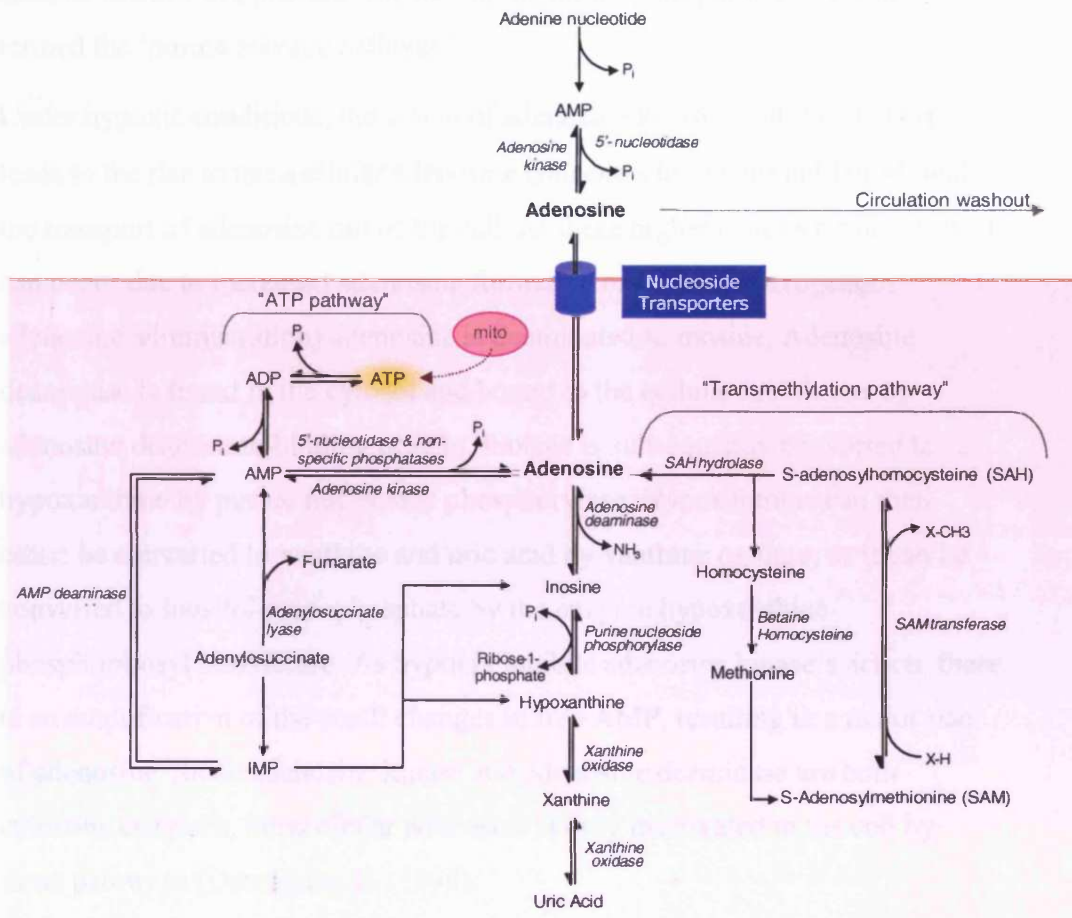
At normal physiological conditions, adenosine is preferentially converted into AMP by adenosine kinase. Basal concentrations of adenosine are kept low by the action of adenosine kinase which phosphorylates adenosine to produce 5'-AMP. It has been estimated that most (90-95%) of the adenosine is salvaged by adenosine kinase's action (Lloyd and Schrader, 1993). Concentrations of intracellular adenosine are kept to approximately 20-300nM. As regeneration of adenine nucleotides prevents the loss of the purine, this pathway of action is termed the 'purine salvage pathway'.

Under hypoxic conditions, the action of adenosine kinase is inhibited. This leads to the rise to intracellular adenosine concentration to around 10μM, and the transport of adenosine out of the cell. At these higher concentrations, (which can occur due to increased adenosine formation or following exogenous adenosine administration) adenosine is deaminated to inosine. Adenosine deaminase is found in the cytosol and bound to the cellular membrane by adenosine deaminase-binding protein. Inosine is subsequently converted to hypoxanthine by purine nucleoside phosphorylase. Hypoxanthine can then either be converted to xanthine and uric acid by xanthine oxidase, or it can be converted to inositol monophosphate by the enzyme hypoxanthine phosphoribosyl transferase. As hypoxia inhibits adenosine kinase's action, there is an amplification of the small changes in free AMP, resulting in a major rise of adenosine. Since adenosine kinase and adenosine deaminase are both cytosolic enzymes, intracellular adenosine is only inactivated in the cell by these pathways (Deussen et al., 1999).

1.1.4. Adenosine Transport

Though there are several fates for the intracellular adenosine produced during ischaemia, in order to have an action at membrane receptors it must be transported into the extracellular space. This can occur by a combination of simple diffusion and nucleoside transportation. Nucleoside transporters mediate the cellular uptake of adenosine. There are two types of transporter from separate nucleoside transporters gene families (Podgorska et al., 2005). They are the equilibrative nucleoside transporter, which consist of facilitated carrier

proteins, and the concentrative nucleoside transporters that are driven by the Na^+ gradient. These Na^+-dependent nucleoside transporters are symporters that couple the outward movement of nucleosides to the inward movement of Na^+. Intracellular Na^+ concentration rises in the heart during ischaemia. Upon reperfusion there is a transient rise followed by a return toward basal levels (Williams et al., 2007).

While there is a potential for the action of intracellular Na^+ to influence adenosine transport in ischaemia, further discussion is beyond the remit required for this thesis.

Unlike adenosine, synthetic adenosine analogues are not substrates for the uptake process by these transporters, which makes them more metabolically stable than adenosine itself. The nucleoside transporter's actions are the reason why administered adenosine has a half life in the order of minutes in the circulation (Blardi et al., 1993).

1.2. Function of Endogenous Adenosine in the Heart

One of the functions of endogenous adenosine is thought to be as a protective "retaliatory" metabolite, preventing tissue damage during traumas such as hypoxia and ischaemia and in other conditions such as hypotension and seizures (Shryock and Belardinelli, 1997). It has the ability to adjust oxygen consumption of the tissue in order to meet its oxygen demands. Adenosine achieves this using several mechanisms. It acts to reduce contractility and the rate of contraction, increases oxygen delivery via coronary vasodilatory actions and reduces sensitivity to stimulation via adrenoceptors, with this anti-adrenergic effect opposing the enhanced sympathetic stimulation within the myocardium (Mubagwa et al., 1996).,

More specifically, there is now accumulating evidence that adenosine exerts a cardioprotective effect (Lasley and Mentzer, 1992; Maddock et al., 2003; Randhawa et al., 1995; Zhao et al., 1993). Endogenous adenosine reduces the infarct size following ischaemia and reperfusion by receptor-mediated mechanisms (Zhao et al., 1993). This cardioprotection is most pronounced during the early phase of reperfusion. The cardioprotective effects can be seen

when adenosine is administered both before the ischaemia and at reperfusion of the tissue (Olafsson et al., 1987; Thornton et al., 1992; Toombs et al., 1992).

Enhancement of the endogenous levels of adenosine by inhibition of the adenosine deaminase enzyme with deoxycoformycin, and the inhibition of adenosine uptake with p-nitrobenzylthioinosine both improve the recovery of the stunned myocardium following coronary artery occlusion (Abd-Elfattah et al., 1993; McClanahan et al., 1995).

Application of exogenous adenosine has been shown to augment post-ischaemic recovery in *in-situ* dog and rabbit hearts (Ogawa et al., 1996; Randhawa et al., 1995). Adenosine also attenuates myocardial stunning in isolated perfused rat hearts (Lasley et al., 1990a). Adenosine and adenosine analogues have been shown in several models to limit the size of infarct induced by ischaemia and subsequent reperfusion (Toombs et al., 1992).

1.2.1. The Clinical Use of Adenosine

At present the only clinical application of adenosine is in the treatment of supraventricular tachycardia, by restoring sinus rate by decreasing AV node conductance. Adenosine's clinical use is limited by its short half-life in the body and the large potential for non-specific actions elsewhere in the body (see Table 1.2 for other non-therapeutic effects). Other potential therapeutic applications which arise from adenosine's mechanisms of action may need to be free of the bradycardic and hypotensive effects which are characteristic of adenosine's pharmacological action in the body.

1.3. Classification and nomenclature of Adenosine Receptors

1.3.1. Original Purinergic Discovery, Classification and Function

The purine receptors were first subdivided into two groups based on their order of potency for ATP, ADP, AMP and adenosine, their sensitivity to antagonists such as the methylxanthines and by the group of secondary messengers evoked upon the receptor's simulation. In this classification, the adenosine receptors fell into the P_1-receptor class.   -

1.3.2. Adenosine Receptors

Further sub classification of the group was based upon the action of adenosine agonists on the receptor (van Calker et al., 1979). The first subdivision made was the separation of A_1 and A_2 subtypes, with A_1 causing inhibition of adenylyl cyclase, and A_2, which stimulated adenylyl cyclase.

The advent of molecular cloning has allowed further division of the receptor classification. To date four distinct adenosine receptors subtypes have been identified using pharmacological interaction and cloning techniques (see Table 1.1). The receptors cloned are the A_1, A_{2A}, A_{2B}, and the most recently identified A_3 receptor (Collis and Hourani, 1993).

1.3.3. Structure and Function

All receptor subtypes have been shown to be G-protein coupled receptors (GPCRs), as all couple to guanine nucleotide binding protein. All adenosine receptors have seven transmembrane domains, fitting the structural model for G protein coupled receptors. Table 1.1 summarises the differences between the subtypes such as that the A_{2A} and A_{2B} subtypes couple to G_s protein, which activates adenylyl cyclase, which increases intracellular cyclic AMP (cAMP) concentrations. The A_1 receptor and A_3 receptor couple to G_i proteins, which inhibits adenylyl cyclase and reduce cAMP formation. The downstream effectors are also shown in table 1.1.

Table 1.1 Differences in structure and function between the four adenosine receptor subtypes. Adapted from Mubagwa and Flameng (2001). Abbreviations used: AC, adenylyl cyclase; $I_{K(Ado)}$, Adenosine activated potassium channel current; NOS, nitric oxide synthase; PLC, phospholipase C; PLD, phospholipase D; PKC, Protein kinase C; $I_{K(ATP)}$, ATP-inhibited potassium channel current.

Receptor subtype	Amino acid length	Effect on adenylyl cyclase	Coupling G-protein	Effector(s)
A_1 receptor	328	Inhibition	Gi/o	AC, $I_{K(Ado)}$
			?	NOS, PLC, PLD,PKC, $I_{K(ATP)}$
A_{2A} receptor	410	Stimulation	Gs	AC
A_{2B} receptor	320	Stimulation	Gs	AC
			Gq	PLC
A_3 receptor	320	Inhibition	Gi/o	AC, $I_{K(Ado)}$
			?	PLC, PLD, PKC, $I_{K(ATP)}$

All four adenosine receptor subtypes have been cloned from a variety of mammalian species including humans. For an in-depth discussion on the homology between the adenosine subtypes please refer to Chapter Seven, section 7.1.2.

A role in energy balance has been attributed to the A_1 and A_{2B} receptors (Shryock and Belardinelli, 1997). The activation of these receptors generally causes a decrease in the rate of ATP catabolism in cells and an increase in the oxygen supply to the tissue thus restoring energy balance (Lasley and Mentzer, 1992; Lasley et al., 1990b). A_1 and A_{2A} receptors are activated by nanomolar concentrations of adenosine, whereas A_{2B} and A_3 subtypes are activated by concentrations in the micromolar range (Jacobson, 1995; Olah and Stiles, 1995).

1.3.3.1. Cardiac and Cardiovascular Effects

The cardiac and cardiovascular effects mediated by the adenosine receptors are shown below in Table 1.2

Table 1.2 The direct physiological effects of adenosine receptor activation on the cardiovascular system. Data taken from Olsson and Pearson (1990), and Lorenzen and Schwabe (2001).

Receptor subtype	Cardiac and Cardiovascular effects attributable to receptor activation
A_1 receptor	Bradycardia (negative chronotropy) Slowing of atrioventricular nodal conduction (negative dromotropy) Reduction of atrial contractility (negative inotropy) Inhibition of the cardiac pacemakers Modification of gycolysis Cardioprotection by ischaemic preconditioning
A_{2A} receptor	Coronary Vasodilation
A_{2B} receptor	Angiogenesis
A_3 receptor	Cardioprotection by ischaemic preconditioning

A_1 and A_3 receptor-mediated cardioprotection is thought to be the single most studied means of cardioprotection, of these the adenosine A_1 receptor is the most highly studied adenosine receptor subtype.

The signal transduction cascade activated by the A_1 receptor is similar to other protective GPCR cascades, but there is ongoing debate on how this cascade evokes protection. At first adenosine's actions via adenosine A_1 receptor activation was thought to induce protection by coronary and collateral vessel vasodilation, which then increased oxygen supply, and decreased myocardial oxygen demand. The negative inotropism, chronotropism, and dromotropism all acted to preserve ATP in ischaemia, thus balancing the oxygen supply and demand (Ely and Berne, 1992). Later, A_1 receptor agonism was linked to protein signalling cascades (in cardiac tissue specially to PKC), with end

effectors converging at the mitochondria. The cardioprotective effects of endogenous adenosine can be mimicked by the addition of A_1 receptor agonists prior to ischaemia (Louttit et al., 1999). A_1 receptor stimulation has other cardioprotective effects. Adenosine's bradycardic effects are produced by the A_1 receptor activation and its vasodilatatory, and so hypotensive effects, are mediated by the A_{2A} receptor.

Ischaemic preconditioning was found not to be the sole preserve of the A_1 receptor as work performed in A_1 receptor knock-out mice (Reichelt et al., 2005) and investigations with A_1 blockers (Auchampach et al., 2004) both indicated that A_1 receptor block or absence did not modify the effect of ischaemic preconditioning.

The A_3 receptor was shown to potentially provide myocardial protection through the use of A_3 receptor agonist drugs, for examples pre-treatment with adenosine A_3 receptor agonists was shown to protect against infarction in rabbit isolated perfused heart (Hill et al., 1998b; Tracey et al., 1997) and against contractile dysfunction in rat isolated perfused heart (Thourani et al., 1999a). An adenosine A_3 receptor agonist has been shown to attenuate stunning and infarction after coronary occlusion in rabbits (Auchampach et al., 1997b). For further in depth discussion please refer to Chapter Four, section 4.1.1.

1.4. The Adenosine A_3 Receptor

1.4.1. Expression

The adenosine A_3 receptor is the most recently identified and so the least characterised of the adenosine receptor subtypes (Auchampach and Gross, 1994; Hammarberg et al., 2004). Following its identification, a number of studies found that A_3 receptor activation had a protective effect against ischaemia, as well as being implicated in other cellular process such as apoptosis, cellular growth, immune function and mast cell degranulation (Abbracchio et al., 1997; Brambilla et al., 2000; Salvatore et al., 2000b; Strickler et al., 1996; Tilley et al., 2000).

Northern blot analysis has shown that the human A_3 receptor gene is expressed mainly in the lungs, liver, kidney and heart, with some expression in brain or skeletal muscle (Ramkumar et al., 1993). Neither radioligand binding nor Northern blot analysis identified the expression of adenosine A_3 receptor in the murine myocardium (Dixon et al., 1996). This issue has led to a divergence in opinion on whether adenosine A_3 receptor activation has a direct action on the myocyte or whether the effects of adenosine A_3 receptor agonism may be indirect. For instance, some protection *in vivo* may derive from the modulation of neutrophil function or vascular-dependent responses (Vinten-Johansen et al., 1999).

The A_3 receptor couples to G_i protein (Fredholm et al., 1996), thus its activation results in the inhibition of adenylyl cyclase, decreasing cAMP concentrations via the $G_{i\alpha}$ protein (illustrated in Figure 1.3.). *In vitro* the $G_{i\beta\gamma}$ of the A_3 subunit also activates phospholipase C leading to an increase in inositol-trisphosphate (IP_3) and diacylglycerol (DAG) and ultimately the release of calcium from intracellular stores (Abbracchio et al., 1995; Englert et al., 2002; Palmer and Stiles, 1995; Ramkumar et al., 1993; Schulte and Fredholm, 2002). The A_3 receptor also activates other intracellular signalling cascades, detailed in Table 1.1.

This signalling following receptor activation shares some aspects with the A_1 receptor signalling such as the involvement of PKC, but the difference in signalling involvement between A_1 and A_3 receptor responses may provide for differing temporal characteristics upon endogenous activation (Lee et al., 2001; Parsons et al., 2000).

1.5. Myocardial Ischaemia

1.5.1. Ischaemic Injury to the heart

Myocardial ischaemia comes about when the oxygen availability to tissue is insufficient to maintain cellular oxidation, which primarily occurs in coronary heart disease, but also other events such as bypass and transplantation surgery to the heart.

1.5.2. Ischaemic Contracture

During a period of ischaemia, the contractile function of the heart declines rapidly. The oxygen supply is insufficient to allow the formation of high-energy phosphates and maintenance of ATP concentration and anaerobic glycolysis becomes the predominant mechanisms of ATP catabolism (Ingwall, 2002). However, this is insufficient to meet the needs of contractile tissue and lactate accumulates as the by-product of this metabolism.

In this state the tissue exhibits a rapid decline in contractile function followed by an arrest of contraction. Soon after contractile failure a sustained period of diastolic contraction occurs, known as the ischaemic contracture. The precise cause of the contracture is under debate. Further discussion occurs in Chapter Three, section 3.1.1.1.

1.5.3. Myocardial Stunning

Depending on the severity of the ischaemic injury, contractility can return to pre-ischaemic levels following reperfusion of the tissue via the coronary circulation. Until it returns fully, the tissue is described as 'stunned'. Myocardial stunning is contractile dysfunction that persists after a period of cardiac ischaemia. It was first described by Braunwald and Kloner in 1982 as a "delayed recovery of regional myocardial contractile function after reperfusion despite the absence of irreversible damage and despite restoration of normal flow". The definition of stunning makes the conditions that the injury must be fully reversible if giving time to recover, that it is sub-lethal and separated from the irreversible injury of infarct and it is in the presence of normal blood flow.

1.5.3.1. Generation of Myocardial Stunning

Despite extensive studies, the mechanism underlying the pathogenesis of myocardial stunning has not been definitively established, but a number of likely mechanisms of stunning have been postulated. The main hypothesis is that it is caused by the depletion of the total adenine nucleotide pool, the generation of oxygen derived free radicals, and calcium overload in the cells (Schulz et al., 1995). The mechanisms thought to produce stunning are varied

and complex, and are reviewed in depth by (Bolli and Marban, 1999). Briefly, the depletion of nucleotides uncouples the excitation-contraction of the muscle due to dysfunction of the sarcoplasmic calcium ATP metabolism. The transient calcium overload may resulting in proteolysis of myofibrils and the time required for resynthesis of damaged proteins would explain in part the delayed recovery of function in stunned myocardium. Also, in ischaemia, there is experimental support that enhanced oxidative stress and generation of oxygen-free radicals occurs and is a critical component in the pathophysiology of stunning.

The range of hypothesis into the cause allows us to think of the stunned myocardium as caused by a "syndrome", rather than a single entity. The oxyradical and calcium hypotheses are not mutually exclusive and are likely to represent different facets of the same pathophysiological cascade.

1.5.3.2. Treatment of Myocardial Stunning

Stunning is thought to occur in several clinical situations, such as delayed recovery from effort angina, unstable angina, early thrombolytic reperfusion, ischaemic cardioplegia, cardiac transplantation, coronary angioplasty, and cardiac arrest (El-Menyar, 2005). A clinical intervention that could alleviate or attenuate stunning would be beneficial as the dysfunction and the accompanying contractile abnormalities caused by myocardial stunning does delay the benefits of reperfusion therapy. In some cases patient recovery slows or the deterioration is even exacerbated by prolonged contractile failure, particularly following cardiac surgery (Hess and Kukreja, 1994)

Some work has found that the use of oxygen derived free radial scavengers, such as superoxide dismutase and catalase improve the recovery of contractile function after ischaemia (Miura et al., 1997; Myers et al., 1985). Other work has found that in experimental preparations calcium antagonists and neutrophil depletion have each been found to be helpful in minimizing stunning (Braunwald, 1991).

Of interest is the observation that A_3 receptor stimulation reverses myocardial stunning in isolated atria and papillary muscles (Gardner et al., 2004) and in

whole heart (Auchampach et al., 1997b; Maddock et al., 2003). This observation is the main basis of investigation of this thesis, which examined the A_3 receptor's involvement in cardioprotection.

1.5.4. Myocardial Infarction

If ischaemia is either longer of more severe, then contractile function may remain depressed after reperfusion, and/or cell death would occur. Cell death results in a irreversible injury that leads to the formation of permanently infarcted or 'dead' tissue. Cell death in the heart can occur by both mechanisms of necrosis and apoptosis, though the predominate mechanism in the damaged heart is necrosis possibly due to the energy demands of the apoptotic pathway.

The mechanical stress placed on the tissue has been a suggested to be a critical determinant of what form of myocyte death occurs during the early phase of reperfusion (Otani et al., 2006), and whether these forms of injury occur during the ischaemic period itself, or upon reperfusion of the tissue. When cell death occurs following coronary reperfusion, it is termed 'lethal reperfusion injury', which is detailed below.

1.5.5. Reperfusion injury, a double edged sword phenomenon.

Reperfusion is essential in order to salvage myocardium cells and maintain cardiac function. However, the restoration of coronary flow and subsequent oxygen supply to the ischaemic myocardium itself causes profound changes in the contractile function of cardiac tissue. Reperfusion itself initiates a cascade of cellular events that then leads to injury in the myocardium (Braunwald, 1990; Moukarbel et al., 2004). One form of reperfusion injury is the transient state of myocardial stunning, which has previously been discussed.

During reperfusion there is generation of reactive oxygen species both in ischaemia and reperfusion leads to lipid peroxidation, protein oxidation and the formation of DNA strand breaks.

It is unclear whether reperfusion injury is a knock-on event in addition to ischaemia or an event inescapably initiated during ischaemia. As reperfusion of

the ischaemic myocardium carries with it a component of injury, reperfusion is termed in the literature as a 'double edged sword' (Kukreja and Janin, 1997).

Though the largest component of cell death during reperfusion is through necrosis there is evidence that apoptosis can contributes to myocyte death during the reperfusion period (Fliss and Gattinger, 1996; Gottlieb et al., 1994), and that the apoptotic programme initiated at the onset of ischaemia is magnified by reperfusion (Scarabelli et al., 1999).

1.5.6. The Impact of Ischaemia in Man

Coronary heart disease (CHD, also know as ischaemic heart disease) arises when coronary vessels become partially occluded by atheromatous plaque deposits. CHD is estimated to be present in at least 75% of the adult population of developed countries but is asymptomatic for the most part. As CHD progresses, it reduces myocardial perfusion to the point where oxygen supply can become insufficient and angina occurs. CHD increases in severity as the extent of occlusion to the coronary vessel becomes greater. The coronary vessel can become completely occluded by a thrombus forming in the atheromatous deposits. When this occurs the blood supply to the segment of myocardium is blocked and the tissue becomes ischaemic. It takes between twenty and forty minutes from the onset of ischaemia before there is irreversible injury to the myocardium and myocardial infarction (MI) occurs.

Sudden death occurs in a quarter of myocardial infarction (MI) cases with 90% of survivors suffering a range of serious acute and chronic complications. Mortality following MI is 35% in the first year, and 10% every year thereafter. Cardiovascular diseases are the main cause of death and one of the main causes of premature death (i.e. before the age of 75) in the UK. The main form of cardiovascular disease is CHD, which accounts for half of all deaths from cardiovascular disease and by itself is the most common cause of premature death in the UK, causing over 40,000 premature deaths in the UK in 2002 (Petersen et al., 2004).

1.5.6.1. Clinical Setting

The aim of existing therapeutic treatment for myocardial infarction is the prevention of irreversible ischaemic damage to the myocardium. The two main clinical interventions used to treat infarction are pharmacotherapy and surgery. Pharmacotherapies include thrombolytics such as streptokinase and tissue Plasminogen Activator (tPA), anti-platelet therapy (aspirin) and anticoagulant drugs such as warfarin. Other drugs are used concomitantly achieve coronary vasodilation which lower blood pressure and ease the symptoms of MI (American Heart Association, 2007).

The occlusion can also be removed using surgical techniques such as the angioplasty procedure and coronary bypass grafts. Spontaneous lysis of the thrombosis is also known to occur.

In addition to the medical or spontaneous lysis of thrombi, another state of reperfusion of the myocardium is brought about following cardioplegic arrest of the heart during some types of cardiac surgery, such as transplantation and bypass surgeries. Cardioplegia is induced by infusion of cold hyperkalemic solutions, which electromechanically arrest the heart's contraction.

1.6. Ischaemic Preconditioning

When the heart is subjected to brief periods of ischaemia and reperfusion, it exhibits an improved ability to recover from further prolonged ischaemic insult (Murry et al., 1986). Ischaemic preconditioning is an endogenous protective mechanism and has been demonstrated experimentally and partly characterised in many species (Alkhulaifi et al., 1993; Asimakis et al., 1992; Downey et al., 1993; Murry et al., 1990). Ischaemic preconditioning also limits post-ischaemic arrhythmias and enhances the contractile function in recovery (Gross, 2003; Raeburn et al., 2002). A greater understanding of this phenomenon could potentially be beneficial therapeutically.

Protection by preconditioning is a receptor-mediated event (de Jong et al., 2000). One type of receptors implicated in this are the adenosine receptors, by the protective action of adenosine (Cohen and Downey, 1996; Goto et al., 1996). Maximal preconditioning by these receptors has been found to require

the activation of both the A_1 and the A_3 adenosine receptor subtype (Lee and Emala, 2000; Wang et al., 1997). In addition, adenosine receptors also have a role in many intracellular signalling pathways, which may act to give anti-ischaemic myocardial protection.

1.6.1. Classical Preconditioning

Two phases of preconditioning have been identified (Bolli, 2000). There is an early, acute phase where cardioprotection is present for up to 1 to 3 hours after ischaemia. The delayed or second phase reappears about 24 hours after ischaemia and may last for up to 72 hours. It has been reported that along with adenosine receptors, opioid peptide and bradykinin receptors, and other G-protein coupled receptors initiate preconditioning, signalling through both tyrosine and calcium dependent protein kinases, specifically PKC (Otani et al., 2003). The downstream mechanisms of cardioprotection induced in preconditioning remain to be elucidated, although several mechanisms have been suggested (Hiraoka, 1997). One suggestion centres on the mitochondria as the source of preconditioning. It is thought that the opening of the mitochondrial K_{ATP} channels by an ischaemic stimulus decreases the mitochondria calcium uptake and thus improves the mitochondrial function during the resulting calcium overload during reperfusion (Crestanello et al., 2002). Opening of K_{ATP} channels may also lead to generation of reactive oxygen species which are triggers for the protective signalling cascade.

Pharmacological opening of the mitochondrial K_{ATP} channels has been demonstrated to be cardioprotective by slowing the mitochondrial energy production during reperfusion (Dos Santos et al., 2002; Garlid et al., 1997). This transient K_{ATP} channel pore opening induces the nuclear translocation of transcriptional regulators thus transforming the cell to a preconditioned state (Raeburn et al., 2002). The pore opening is also thought to prevent or attenuate mitochondrial Ca^{2+} overload (Wong and Wu, 2003). Pain et al. (2000) found that opening of mitochondrial K_{ATP} channels triggers the preconditioned state by activation of protective signalling cascades.

1.6.2. Delayed Preconditioning

Delayed preconditioning is initialised by a similar sequence as acute preconditioning. Reactive oxygen species are again released which induce the transformation of the cardiac myocyte to a 'preconditioned state'. Evidence is accumulating that characteristic cytoprotective effectors found in preconditioned cells include nitric oxide synthase, manganese superoxide dismutase, cyclooxygenase 2 and 12-lipoxygenase (Kodani et al., 2002; Post and Heusch, 2002; Shinmura et al., 2002).

The main component of adenosine-mediated cardioprotection seems to involve protein kinase C (PKC) as when this is inhibited adenosine induced protection effect is lost (de Jong et al., 2000), making any adenosine A_3 receptor mediated effect dependant on PKC. The mechanism by which the protection is achieved remains unclear. There is further intracellular signalling downstream of PKC involving multiple effectors, including the activation of K_{ATP} channels (Liang, 1997), and other kinases (Nakano et al., 2000a; Nakano et al., 2000b). Activated PKC is able to produce a preconditioning effect in the absence of adenosine and in the presence of adenosine antagonists. This indicates PKC activation is downstream of adenosine activation (Iliodromitis et al., 1998; Liang, 1998). As preconditioning also involves effectors downstream of PKC activation including actions such as modification of sarcolemmal and mitochondrial ion transport, changes to cytoskeletal structure and intracellular signalling could be what provides the preconditioning effect.

1.6.3. Physiological relevance of preconditioning

The relevance of the 'preconditioned' tissue is evidenced by the cardioprotection afforded by some cardiac conditions. For example, the occurrence of angina induces a cardioprotective effect which is a mimic of both ischaemic and pharmacological preconditioning (Loubani et al., 2004). Pre-existing angina can both reduce the size of the infarct caused by myocardial infarction (Ottani et al., 1995), and reduces the occurrence of other cardiac events (Kloner et al., 1998). Cardioprotection evoked by an ischaemic insult has

been estimated to have a limited time window of protection of 29-70 hours post initial insult (Loubani et al., 2004).

A limitation to the practicality of using therapeutic ischaemic preconditioning is the ethical issue of initially cause harm with the ischaemic insult which lowers the feasibility of bringing this in a clinical setting. Perhaps the only anticipated situation where the application of deliberate physical preconditioning could occur is in patients undergoing surgery.

1.6.4. Postconditioning

A relative new phenomenon of postconditioning has been described in the literature. The phenomenon was first termed by Zhao et al. (2003), and occurs when reperfusion to the tissues is either graded or intermittent for a period of time before full reperfusion is allowed to occur. It has been found that postconditioning can mimic the effect of ischaemic preconditioning (Vinten-Johansen et al., 2005). Adenosine has been implicated as a mediator of cardioprotection during postconditioning (Kin et al., 2005), along with other mediators such as nitric oxide and guanylyl cyclase, opening of K_{ATP} channels and closing of the mitochondrial permeability transition pore. Postconditioning also modifies intracellular survival pathways, which affect cell death, and attenuates apoptosis (Sun et al., 2006).

There are distinct differences between the pathways involved in preconditioning and postconditioning. Ischaemic preconditioning followed by postconditioning was found not to be synergistically cardioprotective in a canine model of regional ischaemia and reperfusion (Halkos et al., 2004).

1.6.5. Pharmacological Pre- & Post-conditioning

The use of pharmacological agents to bring about preconditioning and postischaemic effects seems to have the great clinical potential, and the mechanism of preconditioning has been especially extensity studied to this effect.

There is evidence that both physical and pharmacological stimuli of preconditioning can have the same magnitude of effect, with none being more

beneficial that the other (Loubani et al., 2004). The two forms of stimuli have been shown to be synergic when used together in a sheep heart model of ischaemia (McCully et al., 1999).

1.6.6. Role of Adenosine A₃ Receptor in Cardioprotection

Since its first characterisation, the adenosine A_3 receptor has consistently been shown to mediate cardioprotection in multiple species and ischaemic models. Selective adenosine A_3 receptor activation causes acute protection and more sustained preconditioning effect (Headrick and Peart, 2005). The mechanisms by which adenosine A_3 receptor activation is thought to evoke preconditioning are illustrated in Figure 1.3.

The level of protection afforded to the cell by A_3 receptors is reported to be a balance, as high A_3 receptor occupancy has also been shown to have detrimental effect (Maddock et al., 2003). There is the suggestion that the A_3 receptors mediate cell survival by attenuating apoptosis, however, the mechanics of how this occurs remains to be found. The precise interactions between the receptor, protein kinase C, mitochondrial K_{ATP} channels, nitric oxide and reactive oxygen species also remains unclear.

In some model the cardioprotection evoked by post-ischaemic adenosine A_3 receptor agonism in rat hearts and myocytes has been shown to be abolished by atractyloside, a mitochondrial permeability pore opener, thereby implicating inhibition of mitochondrial permeability pore formation as a mechanism of protection (Park et al., 2006). Post-ischaemic adenosine A_3 receptor agonism also caused phosphorylation of glycogen synthase kinase-3β and the cell survival kinase Akt (Park et al., 2006), which are co-factors known to cause protection in cardiac tissues (Armstrong, 2004; Gross et al., 2004).

In addition to the adenosine A_3 receptor, other work has collectively implicated the involvement of the other adenosine receptor subtypes in the postconditioning effect (Lu et al., 2006; Philipp et al., 2006; Yang et al., 2005), as well the action as endogenous adenosine release itself (Solenkova et al., 2006).

A precise signalling cascade still to be found, but the evidence provides a strong argument that modifications that take place at reperfusion are indeed capable of altering the post-ischaemic function in the heart, and possibly contributing to a effect that could mimic ischaemic post-conditioning.

The ability of selective adenosine A_3 receptor agonists to trigger potent protective responses in isolated cardiomyocytes and isolated cardiac muscle preparations suggest a direct myocardial response is present and therefore there must be adenosine A_3 receptors expression in myocytes. But due to the lack of very selective ligands for the adenosine A_3 receptor there remains the question of whether the adenosine A_3 receptor is expressed in the normal non-ischaemic myocardium.
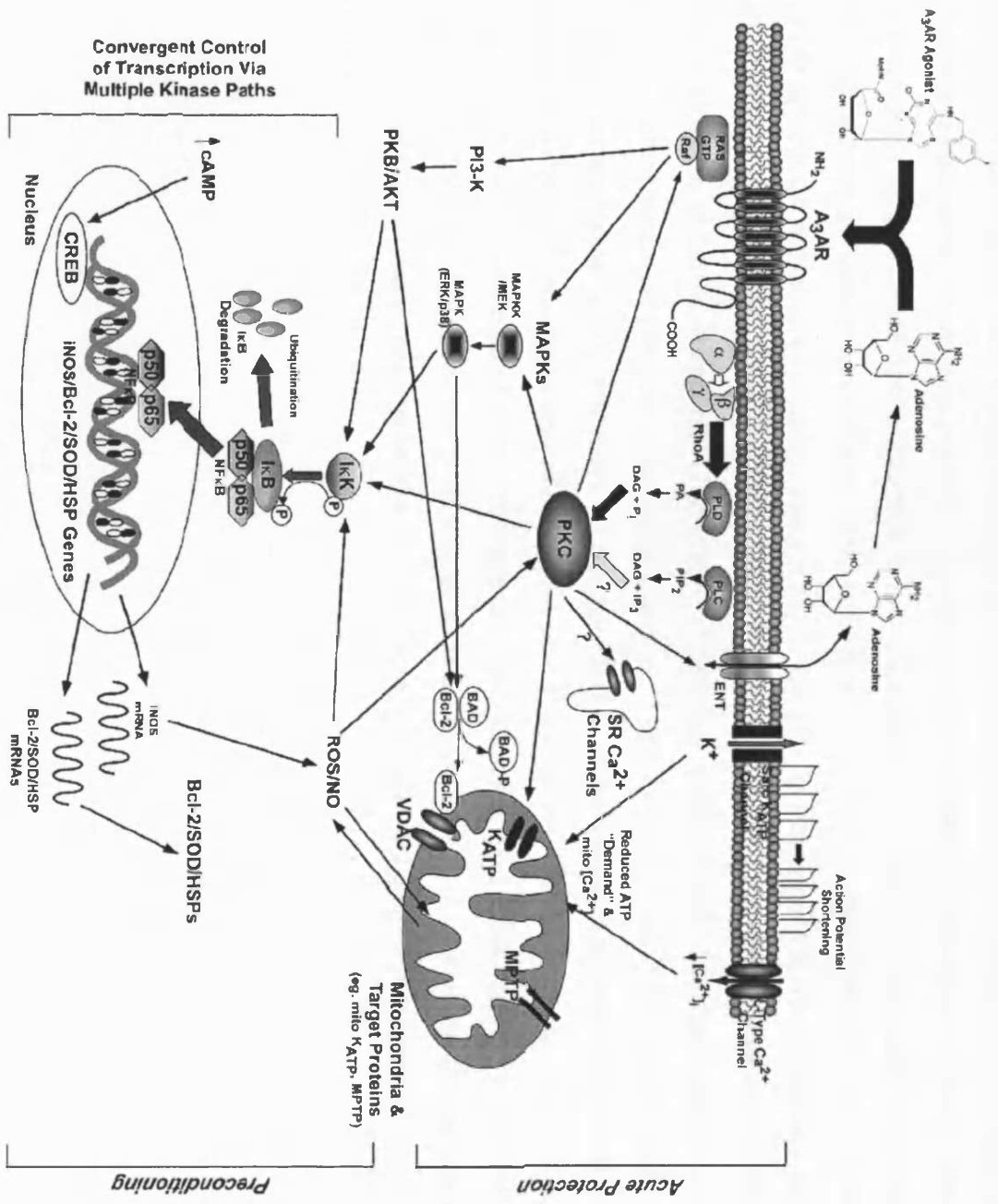
Figure 1.1 Illustration taken from Headrick and Peart (2005). See over page for descriptive text.

Figure 1.3 Illustration taken from Headrick and Peart (2005); the potential mechanisms by which adenosine A_3 receptor agonism evokes cardioprotection. Potential mechanisms of both acute protection and early and delayed preconditioning responses are depicted. Upon stimulation of the adenosine A_3 receptor cAMP concentration falls due to the adenylyl cyclase inhibition by the $Gi\alpha$ subunit (not illustrated), while the $Gi\beta\gamma$ subunit activates phosopholipase. DAG is formed by the action of phospholipase C (PLC), while (phospholipase D) PLD induces the activation of protein kinase C (PKC). The PKC is translocated to the sarcolemma and mitochondrial membranes (Wang and Ashraf, 1999). Precise interactions between PKC, mitochondrial K_{ATP} channels, nitric oxide (NO) and reactive oxygen species (ROS) generation, and sarcoplasmic reticulum (SR) Ca^{2+} handling remain unclear, as is the identity of end-effectors of protection (though mitochondrial targets are strongly implicated (Chan et al., 1999; Kennedy et al., 1999; Schulte and Fredholm, 2000; Stambolic et al., 1999; Yellon and Baxter, 1999). Protective actions include (but are not restricted to) opening of mitochondrial and potentially sarcolemmal K_{ATP} channels (reducing energy consumption and enhancing mitochondrial function), inhibition of Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+} release, modulation of adenosine transport via equilibrative nucleoside transporters (ENTs), transcriptional induction of inducible nitric oxide synthase (iNOS) and protective proteins Bcl-2, superoxide dismutase (SOD) and heat shock proteins (HSPs), phosphorylation of Bcl-2-associated death promoter (BAD) to permit Bcl-2 association with and inhibition of voltage-dependent anion channels (VDAC).

1.7. Objective of this thesis

The objective of this work was to establish if adenosine A_3 receptor activation could evoke cardioprotection in the recovery of post ischaemic cardiac tissue. This would be achieved from the investigation using drugs with act at the adenosine receptors and additionally at the presence of adenosine A_3 receptor in the heart.

The development of a rat model of ischaemia-induced stunning and infarction is first required due to the limitations of the established guinea pig model previously used in this research group. The limitation of the previous model was despite showing the cardioprotective action of adenosine A_3 receptor agonists drug the presence of the actual receptor in the same species could not be established as the there is a lack of commercially available species specific adenosine A_3 receptor antibodies and drugs.

Commercial rat adenosine A_3 receptor antibodies are available and so work was switched to using tissue from this species. The first stage was to establish adenosine A_3 receptor mediated cardioprotection in rat cardiac tissue. There is compelling evidence that adenosine and its receptors have an important role in cardioprotection from ischaemic injury and adenosine may serve as an endogenous mediator of protection from myocardial stunning. Whether A_1 or A_3 receptors or both are involved remains to be established.

The focus of this thesis is on the role that adenosine A_3 receptor activation has in the modification in a model of ischaemic injury. The primary hypothesis is that A_3 receptors are present in the myocardium and under hypoxia and/or ischaemic conditions they exert beneficial cardioprotective effects against myocardial stunning and infarction. Whether selective A_3 receptor agonists can improve contractile recovery or protection from infarction in isolated rat heart tissues following ischaemia will be examined.

In initial investigations we examined the effects of an adenosine A_3 receptor agonist, upon myocardial stunning of rat isolated cardiac tissues by hypoxia and simulated ischaemia followed by reperfusion/reoxygenation. The agonist was added at reperfusion, as this can be regarded as a clinical relevant time point in

this model. It corresponds to where treatment of myocardial infarction would occur in a clinical setting by either surgically or by pharmacotherapy means. Administration of drug at the onset of ischaemia is not as clinically relevant as myocardial infarction is a rarely foreseen condition. This time point for drug intervention was varied as the investigation progressed.

Isolated left atria and right ventricular strips preparations were used at first in order to remove the complication of coronary vasculature involvement. Also detailed is the method for isolated perfused rat hearts to examine infarct size as well as contractile function.

Understanding of adenosine A_3 receptor stimulation in myocardial stunning may permit the introduction of new strategies in the management of the stunned myocardium and the myocardium at risk of infarction.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Animals and Animal Husbandry

Male Wistar rats obtained from Harlan Limited (Bicester, U.K) were used for all work described. Upon delivery rats were housed at an in-house animal facility within Cardiff University, and were left to acclimatise to their surroundings for a period of not less than four days. The rats were housed in cages containing up to a maximum of six, in a room maintained at an ambient temperature of 22°C ± 2°C with a 12 hour light - dark cycle. The rats were fed standard rat diet and had unlimited access to food and water at all times. At the time of their use each rat had a mass of 250 – 350 grams. The conduct of all experiments conformed to the guidelines of the Animals (Scientific Procedures) Act 1986.

2.2. The Isolated Cardiac Tissue Model

2.2.1. Dissection of the Cardiac Tissues

Rats were euthanased by a Home Office approved Schedule 1 method of concussion followed by destruction of the brain. Entrance was gained to the thoracic cavity through an incision through the skin and muscle of the chest wall and removal of the anterior rib cage, exposing the heart. The pericardium and excess fat were removed and the heart was immobilised by attaching a Spencer-Wells forcep to the very tip of the ventricular apex. With the heart remaining in the chest, a needle was used to pass sutures (5.0 black braided silk, non-absorbable surgical suture, Harvard Apparatus, Kent, UK) through the apex of the left atrial appendage and the atrioventricular junction. The left atrium was removed and placed into a petri dish containing oxygenated Krebs solution (composition detailed in 2.2.2). The latter thread was then used to attach the atrium to the electrode tips of a bipolar platinum electrode (Harvard Apparatus). The tissue and attached electrode was transferred to the organ bath.

Similarly when used strips of the right ventricle wall were also isolated by using a needle to pass sutures through two points anterior and posterior of the ventricular wall. A strip of the right ventricular wall was then dissected around the two structures, removing a strip approximately 2-4mm wide by 5-8mm long.

The strip was placed into a petri dish containing oxygenated Krebs solution, The one suture thread was then used to attach the strip to the tips of a bipolar platinum electrode, the tissue and attached electrode was then transferred to the organ bath.

2.2.1.1. Order of Dissection

The left atrium was the first tissue to be isolated from the animal, except in one set of specific experiments where the effect of the order of dissection was investigated. In these experiments the atria remained in the open chest for 3 to 5 minutes longer after the commencement of the dissection in order to allow for the dissection of other tissues first. When this protocol was used it is clearly indicated in the text.

2.2.2. Organ Bath And Data Recording

The atrium was fully immersed in 50ml-volume organ baths containing warmed Krebs-bicarbonate buffer solution. The Krebs-bicarbonate buffer (termed throughout as 'Krebs solution' or simply 'Krebs') was made up in distilled water and had the following composition: NaCl 118 mM, $NaHCO_3$ 25.0 mM, D-glucose 11.7 mM, KCl 4.69 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.25 mM, KH_2PO_4 1.18mM. All reagents were of Analytical™ grade (Fisher Scientific UK). The organ bath's water jacket temperature was maintained at 37°C ± 1°C by a Grant Circulator (Grant Instruments Limited, Cambridge, UK). Krebs bathing solution in both the organ bath and the aspirator reservoir was continuously aerated with 5% CO_2 / 95% O_2 gas (BOC Gases, UK) during normoxia.

The free suture attached to the tissues was attached to an isometric transducer (Dynamometer UF1 isometric transducer, 57 g sensitivity range. Pioden Controls Limited. Canterbury) and a resting diastolic tension of 0.5 g ± 0.1 g was applied. The tissues were then electrically paced at 2 Hz with square waves pulses of 5 milliseconds duration, at a voltage of 50% above threshold. Pacing was delivered by a Harvard 50-72 Stimulator (Harvard Apparatus, Edenbridge, UK). The parameters used for the electrical stimulation of the tissues have been previously shown to drive cardiac contraction without causing significant

autonomic transmitter release when delivered by electrodes in direct contact with the tissue (Koch-Weser and Blinks, 1963). Isometric tension on the transducer was recorded on a Lectromed MT8P multi-trace 8-chart polygraph recorder (Lectromed Limited, Jersey, UK). The tissues were then allowed to equilibrate for 25 to 40 minutes, until a steady baseline of contraction was achieved and the experiment could commence. During this period the bathing solution was changed frequently.

2.2.2.1. Exclusion Criteria

Preparations were excluded if the developed tension of contraction after the equilibration period was less than 0.40g for both tissues.

2.2.3. Experimental Protocols

2.2.3.1. Simulated Ischaemia followed by Reoxygenation

Ischaemia was simulated by switching the bathing Krebs solution to a glucose-free Krebs solution (composition as described in section 2.2.2. but with 7mM choline chloride substituted for glucose. The choline chloride maintains the iso-osmolarity of the Krebs in the absence of D-glucose (Carr et al., 1997). Previous work in this laboratory has shown that the maintenance of iso-osmolarity is 99.7% accurate using this method (Yates, 2004)) and changing the aeration to a 5% CO_2 / 95% N_2 gas. Simulated ischaemia was performed for thirty minutes during which the bathing solution was not changed. After this thirty minute period the tissues were reoxygenated by returning the aeration to a 5% CO_2 / 95% O_2 gas and changing the bathing solution back to normal Krebs solution. Reoxygenation was continued for one hour, with the bathing solution being refreshed at fifteen minutes intervals. All bathing solution were pre-gassed and warmed before filling the tissue bath.

2.2.3.2. Drug Administration Protocols

Various protocols used for the administration of drugs at times both during ischaemia and reoxygenation. These are detailed the methods section in the relevant experimental chapter.

2.2.3.3. Materials

Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride hexahydrate ($CaCl_2.6H_2O$), calcium chloride dihydrate ($CaCl_2.2H_2O$), magnesium sulphate ($MgSO_4.7H_2O$), sodium hydrogen carbonate ($NaHCO_3$), potassium dihydrogen orthophosphate ($KH_2PO_4.2H_2O$) and D-glucose were obtained from Fisher Scientific, Loughbourgh, UK. Choline chloride was obtained from Sigma Aldrich Company Limited, Poole, UK.

5% CO_2 / 95% O_2 and 5% CO_2 / 95% N_2 gases were obtained from BOC Gases, Gilford, UK. Dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG) were obtained from Fisher Scientific, Loughbourgh, UK.

2.2.4. Data Analysis

The data obtained was expressed in various ways. These methods and any statistical test used are detailed the methods section in the relevant experimental chapter.

2.3. Langendorff Perfused Heart

2.3.1. Setup of the Langendorff Perfused Heart

2.3.1.1. Langendorff Apparatus

The apparatus used was a complete Langendorff System (Catalogue number ML870B2) purchased from ADInstruments Limited, Chalgrove, UK. The system was operated in constant pressure mode, which is controlled by the supplied Powerlab STH Pump Controller. The STH Pump Controller receives the coronary perfusion pressure signal from a pressure transducer and then uses a negative feedback control circuit to alter the flow rate of the peristaltic pump that controls the in-flow of the perfusate. Therefore a constant pressure of perfusate can be maintained into the apparatus. The perfusate used was a modified Krebs-Henseleit solution, composition of which was NaCl 118mM, D-glucose 11mM, KCl 4.7mM, $CaCl_2$ 2.5mM, KH_2PO_4 1.2mM and $MgSO_4$ 1.2mM, with the addition of insulin (100mU/l), which had been previously filtered by passing through Glass Mircofibre paper filter (Whatman, UK). The perfusate reservoir was aerated with a 5% CO_2 / 95% O_2 gas throughout. The

perfusate was prewarmed before entering the heart and was not re-circulated. The water jacket's temperature was maintained at $37 \pm 1°C$ by a Grant Circulator (Grant Instruments Limited, Cambridge).
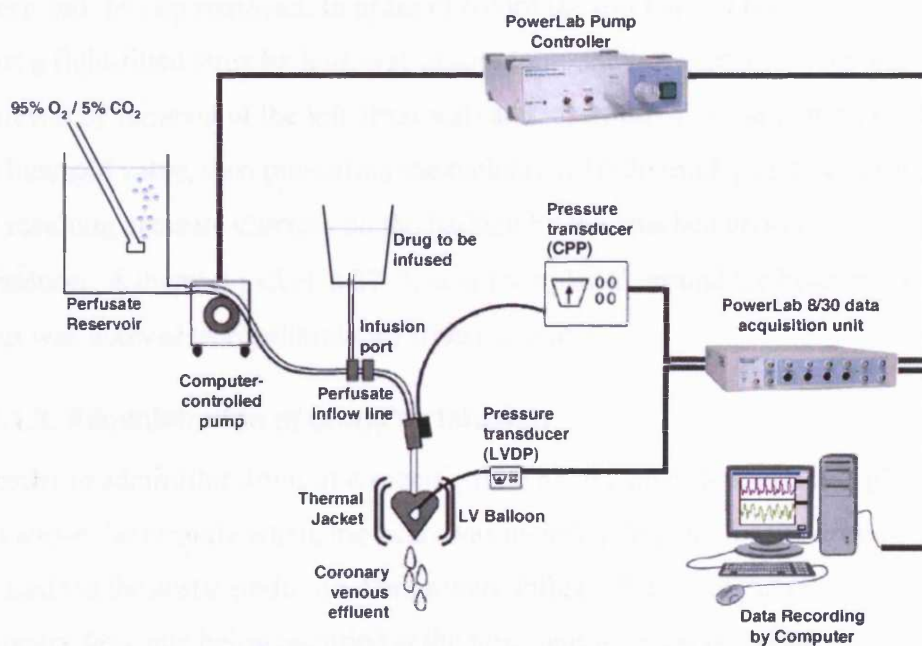

Figure 2.1 Schematic diagram of the Langendorff apparatus and Powerlab™ system used. Warming apparatus is not shown.

2.3.1.2. Anaesthesia and Excision of the Heart

Excision of the heart was performed after general anaesthesia. Male Wistar rats were anaesthetised by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) administered with heparin (100 U/kg). Surgery commenced when the response to a painful stimuli was lost. The animal was placed supine and a transabdominal incision was made to remove the skin of the chest, followed by removal of the sternum and necessary ribs to order to expose the thoracic cavity. The heart and lungs were excised as one by cutting through the aorta and trachea above the heart. The removed organs were then placed in a beaker of ice cold Krebs-Henseleit buffer. The heart was then mounted onto the apparatus by cannulation of the aorta, and temporarily held in place by a surgical clip. Perfusion to the heart (in a physiological retrograde mode) was begun immediately once the aorta was fastened to the cannula in order to re-establish coronary vascular flow. Perfusion pressure was held at 60mmHg $\pm$ 1mmHg for

this study. The superfluous pericardial and lung tissue was removed, and a small incision made in the pulmonary artery to allow adequate drainage of the coronary perfusate. The aorta was securely fastened to the cannula using cotton suture and the clip removed. In order to record the mechanical function of the heart a fluid-filled latex balloon was inserted into the left ventricle. This was achieved by removal of the left atrial wall and insertion of the balloon through the bicuspid valve, then pressuring the balloon to 10-20 mmHg and capturing the resulting pressure changes on the balloon by the attached pressure transducer. A thermal jacket at 37 °C was then placed around the heart and the heart was allowed to equilibrate for fifteen minutes.

2.3.1.3. Administration of drugs by infusion

In order to administer drugs at a specific time points, an inflow line was placed just above the cannula where the heart was mounted in order for the drug to be infused via the aortic perfusate. Drugs were infused at 10% of the rate of the coronary flow rate being recorded at the time, and which was constantly adjusted. The infusion pump was a Watson-Marlow perislotic pump (Smith & Nephew Watson-Marlow, Cornwall, UK).

2.3.1.4. Equilibration period

Before any intervention, the heart was allowed to equilibrate for 15 minutes to determine baseline values for the haemodynamic parameters.

2.3.1.5. Data Capture and Recording

All transducer signals were captured by a PowerLab 8/30 data acquisition unit, and then recorded by a computer (AppleMac) running charting software (PowerLab Chart 5, ADInstruments, Chalgrove, UK) The pressure applied on the latex balloon inserted into the left ventricle was recorded as left ventricular developed pressure (LVDP) via a pressure transducer connected to the balloon. Heart rate (HR) was calculated from LVDP by the charting software. Coronary perfusion pressure (CPP) was recorded by pressure transducer connected just above the cannula holding the heart. After an equilibrative period the CPP was held at 60mmHg. Perfusate flow was measured by the charting software

following calibration of the perfusate pump. Perfusate flow was considered to be coronary flow (CF) once the pump controller had been set to apply a constant perfusate pressure of 60mmHg. The perfusate pump and pressure transducers were calibrated weekly.

2.3.2. Experimental Protocols

2.3.2.1. Global Ischaemia and Reperfusion

Global no-flow ischaemia was produced by stopping the perfusate pump thereby attenuating perfusion into the aorta. Ischaemia was maintained for 30 minutes. Reperfusion commenced by starting the perfusion pump. If a drug was to be infused the infusion pump was also started. Reperfusion continued for one hour.

2.3.2.2. Drug Administration Protocols

The various protocols were used for the administration of drugs are detailed in the methods section of the relevant experimental chapter.

2.3.3. Triphenyltetrazolium chloride staining

At the end of reperfusion, the heart was detached from the Langendorff apparatus and frozen at -20 °C for 30 minutes. Freezing enabled easier sectioning and enhances the staining achieved. Each heart was sliced into six or seven approximately 3-5mm thick transverse sections using a scalpel. The sections were stained in 1% w/v triphenyltetrazolium chloride (TTC) in Krebs buffer at room temperature for 10-15 minutes. The stained sections were fixed in 10% formalin (phosphate buffered formaldehyde solution, pH 7.3) at 4°C overnight. This fixed the stain allowing contrast between viable tissue (termed Area At Risk or ARR) which the TTC stains red, and non-viable tissue, which does not stain and so appears pale, indicating infarction. The slices were flattened between two glass plates that had a 2.5mm separation. The total area of each section plus all areas that showed an absence of red stain (infarct area) were traced onto an acetate sheet which was then computer scanned to a JPEG file. Using computerised planimetry software (SigmaScan Pro 5, Systat Software, Hounslow, Middlesex, U.K) the total area (in pixels) for each heart

section and the total area of infarction was measured. This allowed the infarct size of each heart to be expressed as a percentage of the total area of the heart.
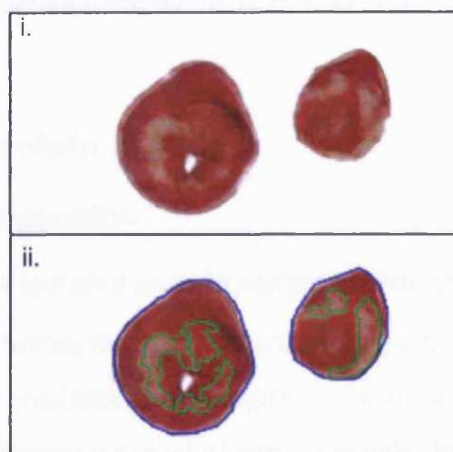

Figure 2.2. Triphenyltetrazolium chloride (TTC) staining. (i) shows the appearance of the heart sections after triphenyltetrazolium chloride staining and flattened between glass places. (ii.) shows the tracing that was performed, the blue line shows the total area of the section and the green line encompasses the infarcted area of the heart, which show up as pale after TTC stain. All non-infarcted tissue stains red.

2.3.4. Materials

Material for the modified Krebs-Henseleit solution and gases were obtained from the same sources detailed in section 2.2.3.3. Triphenyltetrazolium chloride and sodium pentobarbital were obtained from Sigma Aldrich Company Limited, Poole, Poole UK. biphasic isophane porcine Insulin (Hypurin®) and heparin sodium were obtained from AAH Hospital Services, Bristol, UK. Glass Microfibre filter paper GF/C grade was obtained from Whatman International Limited, Maidstone, UK.

2.3.5. Data Analysis

The data obtained was expressed in various ways. These methods and any statistical test used are detailed the methods section in the relevant experimental chapter.

2.4. Membrane Protein Samples

2.4.1. Membrane Preparation

2.4.1.1. Preparation of isolated atria to obtain membrane fractions

In order to obtain membranes that were relevant to the other studies preformed two types of atria were obtained. The first group of membranes were prepared from atria that had undergone a period of normoxia only. For this the atria were first removed from the animal and placed in a tissue bath according to the methods described in section 2.2.1. and paced as detailed in section 2.2.2. Normoxic atria were left in the organ bath for 30 minutes during which time the Krebs bicarbonate bathing solution was changed frequently. The second group of membranes were prepared from atria that had undergone a 30 minute period of simulated ischemia. Simulated ischaemia was induced by switching the bathing solution to a glucose-free Krebs solution, and gassing the bathing solution to 5% CO_2 in 95% N_2. (See section 2.2.3.1. for details) The bathing solution was not changed during the ischemic period.

At the end of either the normoxic or ischaemic periods the atria were removed from the tissue bath and the electrode, and then immediately frozen in liquid nitrogen. The isolated atria in each experimental group (typically six atria were prepared for each) were pooled together in order to provide an adequate amount for experimentation following preparation.

Kidney, spleen, testis, lung and ileum tissue were removed from the rat and prepared for western blotting in order to be used as a comparison in the subsequent stages. This tissue was removed from the rat after the dissection of the cardiac tissues and then immediately frozen in liquid nitrogen, without undergoing any tissue bath experimentation.

All tissues samples were then stored at -80°C prior to the preparation of membrane fractions.

The cell membranes each tissue were isolated by a process of centrifugations. Tissues were homogenised in ice cold Tris buffer (50mM Tris methylamine, 150mM NaCl and 1mM EDTA, pH 7.4) that had been supplemented with the peptidase inhibitors AEBSF hydrochloride (1mM) and bacitracin (0.1mg/ml) in order to prevent breakdown by endogenous peptidase. Homogenising was performed in three 10 second bursts using an Ultra-Turrax Polytron Homogeniser (Janke and Kunkel gmbH and Co. Staufen, Germany) with the homogenate being kept on ice in-between use. The homogenate was then centrifuged at 500g for 10 minutes at 4°C using a Harrier 18/80 centrifuge (Sanyo-Gallenkamp Plc., Loughbough, UK). The resulting supernatant was retained for later use and the pellet resuspended in Tris buffer, homologised and centrifuged again at 500g as above. The two resulting supernatants were combined and centrifuged at 48,000g for 15 minutes at 4°C using a Sorvall Ultracentrifuge (Dupont, Stevenage, UK). Following that, the supernatant was discarded and the pellet resuspended in Tris buffer and centrifuged again at 48,000g. This resulting pellet contains the membrane fraction. It was resuspended in 50mM Tris buffer that contained no peptidase inhibitors and then stored at -80 °C until future use. The protein concentration of the resulting membrane fractions were determined by BCA assay.

2.4.1.2. Measurement of the protein concentration of membrane fractions by BCA assay

The protein concentration of each membrane fraction was assayed using the bicinchoninic acid (BCA) assay. A standard curve for a range of bovine serum albumin concentrations from 0 to 1 mg/ml was also obtained. BCA solution was prepared by adding one part copper sulphate solution to 50 parts bicinchoninic acid (BCA) and added to each sample. Each sample was assayed in duplicate, on a 96-well plate. The plate was incubated at 37°C for 30 minutes, then cooled to room temperature and the absorbance of each sample at 562nm was recorded by a MRXII plate reader (Dynex Technologies, Worthing, UK). An absorbance

of the known concentrations of bovine serum albumin concentrations underwent linear regression on computer in order to construct a standard curve (Prism 4, GraphPad Software Inc. San Diego CA, USA), thus allowing the protein concentration of the membrane fraction to be determined.

2.4.1. Materials

Tris methylamine (Tris base), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA) were obtained from Fisher Scientific Limited, Loughbourgh, UK. Pierce Bicinchoninic acid reagent A and B and Pierce Bovine Albumin standard were obtained from Perbio Science UK Limited, Cramlington, UK. AEBSF hydrochloride and bacitracin were both obtained from Calbiochem, Nottingham, UK.

Liquid nitrogen, 5% CO_2 / 95% O_2 and 5% CO_2 / 95% N_2 gases were obtained from BOC Gases, Gilford, UK.

2.5. Electrophoresis and Western Blotting

2.5.1. Electrophoresis

2.5.1.1. Membrane Fraction Preparation for Electrophoresis

The membrane fractions from various tissues were denatured and made monomeric by boiling in the presence of β-mercaptoethanol and sodium dodecyl sulphate (SDS). β-mercaptoethanol is responsible breaking the disulphide bonds, which occur between proteins. SDS completely disrupts protein-protein interactions, denaturing the proteins resulting in the formation of linear molecules. SDS binds to proteins through hydrophobic interactions, conferring a negative charge to the polypeptide in proportion to its length i.e. denatured polypeptides become 'rods' of negative charge.

All membrane protein samples were resuspended in 50mM Tris methylamine (pH 7.2) and sample buffer (62.7mM Tris methylamine, 4% SDS, 10% glycerol, 5% β-mercaptoethanol, 1% bromophenol blue (0.5mg/ml)) with a 1:3 sample buffer: Tris ratio. The samples were vortexed and heated at 95°C for five minutes by placing in a water bath (Grants Instruments Cambridge UK). Where

possible a protein concentration of $1\mu g/\mu l$ was made in order to load comparative amounts of protein on to the gel.

2.5.1.2. SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous gel system. This acts to concentrate the protein samples into a small volume before separation, which increases the resolution power of the gel. Initially the protein sample moves quickly through a 5% polyacrylamide 'stacking gel' (5% acrylamide/bis 37.5:1, 7.5% 0.5M Tris aminomethane hydrochloride, pH 6.8/SDS (0.4% w/v), 0.15% APS (10% w/v) and 0.024% TEMED in H_2O) and forms a tight band. The sample then enters the 10% polyacrylamide 'separating gel' (10% acrylamide/bis 37.5:1, 3.75% 3M Tris aminomethane hydrochloride pH 8.8, 0.3% SDS (10% w/v), 0.15% APS (10% w/v) and 0.015% TEMED in H_2O) which separates proteins dependent on their molecular weight. Electrophoresis was performed using X-Cell Sure Lock™ system (Novex, CA, USA) and electrophoresis tank buffer (25mM Tris methylamine, 190mM glycine, 0.05% SDS pH 8.3). Protein samples were loaded (37.5-50 μl) onto the gel along with the appropriate pre-stained molecular weight marker (Precision Plus Protein™ All Blue Standards) which containing a broad range of proteins of known molecular weight enabling comparison to the unknown weights. Electrophoretic separation was started at 50 volts by a Bio-Rad PowerPac 300 (Bio-Rad, UK) for 30 minutes to ensure the protein samples were all stacked before entering the separating part of the gel. Electrophoresis was then continued at 110-130 volts until the sample's dye front reached the bottom of the gel.

2.5.2. Western Blotting

2.5.2.1. Semi-Dry Electrophoretic Transfer

In order to immobilise them the separated membrane samples were transferred from the gel onto a nitrocellulose membrane using the semi-dry western blotting technique. Transfer is achieved by sandwiching nitrocellulose membrane (Hybond™-ECL Amersham Bioscience, Little Chalfont, UK) and the

polyacrylamide gel together between a stack of filter papers. Two plate electrodes are placed either side of the sandwich in order to create a uniform electric field over a short distance. The protein sample will move on to the nitrocellulose paper as they move towards the positive electrode. A Multiphor II semi dry blotter (Amersham Bioscience) and transfer buffer (42.9 mM Tris methylamine, 38.9 mM glycine, 0.038% SDS, 20% methanol) were used. The filter papers are saturated in transfer buffer in order to prevent the nitrocellulose membrane and gel polyacrylamide from drying and to provide resistance. Electrophoretic transfer to the nitrocellulose membrane was run at $0.8\text{mA} \times \text{cm}^3$ of the membrane for 1 hour.

2.5.2.2. Blocking Non-Specific Binding

Following the transfer, the nitrocellulose membrane was incubated in a concentrated protein solution of 5% 'BLOTTO' consisting of Tris buffered saline (TBS) (20mM Tris methylamine, 0.15M NaCl, pH 7.5), 5% w/v non-fat dry milk and 0.1% v/v Tween® 20). Incubation in this 5% BLOTTO was performed for one hour in order to block the non-specific binding of proteins.

2.5.2.3. Antibody Incubation

The nitrocellulose membrane was then probed using the rabbit anti-rat adenosine A_3 receptor primary antibody (Autogen Bioclear, UK) The nitrocellulose membrane was incubated with the primary antibody in a 1% BLOTTO solution overnight at 4°C in a sealed plastic bag. Various dilutions were investigated ranging from 1: 500 to 1:5000 v/v in 1% or 3% BLOTTO). Following this the membrane was washed with TBST (TBS (for composition see section 2.5.2.2.) and 0.1% v/v Tween® 20), to remove the excess primary antibody. The nitrocellulose membrane was then incubated with a secondary antibody, peroxidase labelled anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) (Dilution used were 1:20,000 and 1:30,000 in 1% BLOTTO), for one hour at room temperature. This was followed by rapid washes in TBST to remove excess secondary antibody.

2.5.2.4. Detection of protein-antibody complex

In order to visualise the protein-antibody complex, the nitrocellulose membrane was incubated with 1ml of enhanced chemiluminescence (ECL) solution (Pierce SuperSignal® West Dura Extended Duration Substrate) for five minutes. The ECL solution contains luminol and hydrogen peroxide which reacts with the conjugated peroxidase enzyme on the secondary antibody, (Figure 2.3). Photons are emitted as a by-product of this reaction, which were then be detected by x-ray film. The nitrocellulose membrane was exposed to x-ray film (HyperFilm™ ECL, Amersham Biosciences) in darkness in order to detect the immunolabelled proteins. The length of exposure was dependent on the strength of the signal achieved on the day.
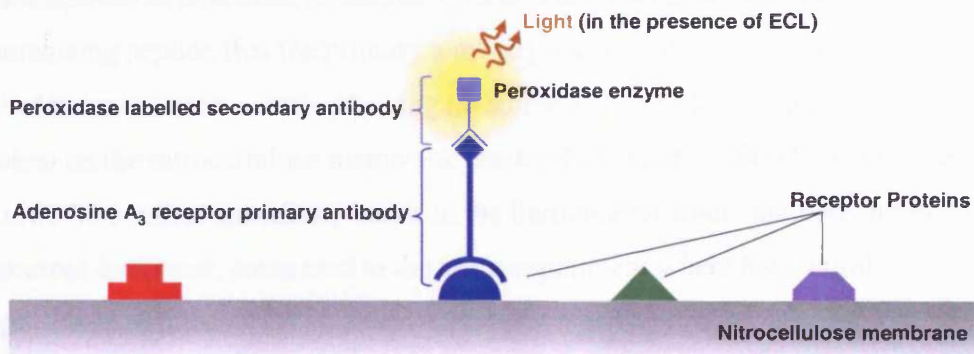

Figure 2.3 The arrangement of the antibody complex at the detection stage. The secondary antibody is conjugated to horse radish peroxidase enzyme which, in the presence of enhanced chemiluminescence (ECL) solution, catalyses a reaction which gives off light (photons) as a by-product. The light was detected by x-ray film which identifies the immunolabelled protein.

Molecular weight determination was made by preparing a standard curve from the molecular weight standards with are included on each gel. The length of migration of each standard was measured and a standard curve constructed using linear regression on a computer running Prism 4 (GraphPad Sofeware Inc. San Diego CA, USA), It was then possible to calculate the molecular weight of the labelled bands from the standard curve.

2.5.2.5. Control Peptide Assay

In order to confirm antibody specificity, a control antibody assay was performed. The adenosine A_3 receptor primary antibody was titrated out of the primary antibody from the solution used in the primary antibody incubation (Section 2.5.2.3.). This was achieved by applying $20\mu g$ of the immunising peptide for rabbit anti-rat adenosine A_3 receptor primary antibody (Autogen Bioclear, UK) on to a small piece of nitrocellulose membrane, and allowing it to dry. This nitrocellulose membrane was then incubated with the primary antibody solution overnight at 4 °C. A separate incubation using a clean piece of nitrocellulose was also performed as a control. The following day the nitrocellulose pieces were removed and the solutions used to the incubation the nitrocellulose as described in section 2.5.2.3. This procedure uses the immunising peptide that the primary antibody was raised to titrate the antibody out of the incubation mixture, leaving no antibody free to bind to the actual protein on the nitrocellulose membrane during the primary antibody incubation. In order to confirm specificity bands in the immunoblot where antibody binding is present disappear, compared to the same experiment where the control peptide was omitted.

2.5.3. Materials

Rabbit anti-rat adenosine A_3 receptor antiserum and its control peptide were obtained from Autogen Bioclear Mile Elm Calne, UK. Peroxide labelled anti-rabbit antibody HRP conjugate was obtained from Vector Laboratories, Burlingame CA, USA. Hybond™-ECL nitrocellulose membrane and HyperFilm ECL x-ray film were obtained from Amersham Biosciences, Little Chalfort, UK. The enhanced chemiluminescence (ECL) solution Pierce SuperSignal® West Dura Extended Duration Substrate was obtained from Perbio Science UK Limited, Cramlington, UK). Precision Plus Protein™ All Blue Standards, acrylamide/bis 37.5:1 and sodium dodecyl sulphate (SDS) were obtained from Bio-rad Laboratories Limited, Hemel Hempstead, UK. Non-fat dried milk (Marvel™) was obtained from Premier International Foods, Spalding, UK. β-mercaptoethanol, bromophenol blue, TEMED

(N,N,N',N'-Tetramethylethylenediamine) and Tween® 20
(polyoxyethylene-sorbitanmonolaurate) were obtained from Sigma Aldrich
Company Limited, Poole UK. APS (Ammonium persulfate), Tris base, (Tris
hydroxymethyl methylamine), Tris.HCl (tris aminomethane hydrochloride),
glycine, methanol and sodium chloride (NaCl) were all obtained from Fisher
Scientific Limited, Loughbourgh, UK.

CHAPTER THREE

THE EFFECT OF SIMULATED ISCHAEMIA & REPERFUSION

ON THE ISOLATED CARDIAC TISSUE

3.1. Introduction

3.1.1. Cardiac Metabolism in Normoxia

Maintaining an adequate amount of ATP is vital to the heart's functions by preserving cell viability and contractility. The oxygen required for cardiac metabolism is delivered to the myocardium by blood carried in the coronary circulation. When the oxygen supply is adequate the cardiac muscle is under aerobic conditions and is able to produce and maintain the ATP amounts adequate for contractile function. There are numerous synthetic pathways which maintain the ATP level in the heart, such as oxidative phosphorylation in the mitochondria, glycolysis and by phosphotransferase reactions (Ingwall, 2002).

3.1.2. Cardiac Metabolism in Ischaemia

Physiological ischaemia is defined as a reduction in the blood supply, usually to an organ or limb. An insufficient blood supply reduces oxygen availability. This insufficient oxygen supply is the definition of hypoxia, a state of having a shortage of oxygen. An imbalance between the oxygen supply and the demand of the heart results in myocardial ischaemia. *In vivo* this is usually brought about by compromised coronary flow.

As venous oxygen saturation is ~70% at rest it allows skeletal muscle to draw out more oxygen from the blood during periods of increased demand. However the oxygen saturation in coronary sinus blood is 25-35% (Van Der Werf, 1980). This low level of oxygen saturation means that the coronary circulation does not possess a reservoir of oxygen for periods of increased demand. If the oxygen demand of the myocardium increases only arteriolar dilation and the subsequent increase in blood flow will provide the supply.

3.1.1.1. The Ischaemia Contracture

Under normoxic conditions, myocyte contraction is brought about by the interaction between actin and myosin. This is triggered by an increase in intracellular calcium. The increase of calcium removes the inhibitory action of the enzyme Tropin I. The myosin heads attach into actin filaments and flex

using energy produced by ATP hydrolysis and the myocyte contracts. ATP then binds to the myosin heads and detaches them from the actin (Solaro, 1999).

During ischaemia the oxygen supply to the mitochondria becomes insufficient to support ATP synthesis by oxidative phosphorylation. Cellular stores of ATP are rapidly consumed. Anaerobic metabolism, which accounts for 1% of ATP production in normoxia, increases and can account for up to 10% of the ATP synthesis in hypoxia. However all ATP synthesis pathways may be insufficient to meet the demand. When this occurs during ischaemia, the interaction between the myosin heads and actin is maintained, due to the unavailability of ATP, and an ischaemic contracture will develop. A moderate ischaemic contracture does not cause major structural damage but it leads to cytoskeletal defects and cardiomyocytes become more fragile and susceptible to mechanical damage. The time of development of contracture seems to coincide with the decrease in ATP availability and corresponds to fully depleted glycogen (Pantos et al., 2006).

3.1.3. Reperfusion

The reperfusion of the myocardium and the cessation of ischaemia is a clinical objective in the treatment of myocardial infarction. Reperfusion restores the oxygen supply to the tissue salvaging any tissue from cell death and to restore the function of the myocytes as much as possible. Metabolism then has the potential to revert to the same as in normoxia.

Following reperfusion, contractile function may be depressed for some time despite an absence of irreversible damage, a condition know as myocardial stunning (Braunwald and Kloner, 1985). In this study the effect of interventions on myocardial stunning in a model of ischaemia was used as detailed below.

3.1.4. Experimental Model

In order to study drug interventions, a model of ischaemia in cardiac tissue is required. Cardiac activity can be studied in a variety of models each with its own advantages and disadvantages. For this study, functional data relating to contractility was required. In order for functional data to have accuracy all

measurements need to be taken from a steady baseline. Baselines are easier to achieve in *ex vivo* tissues as it removes the innumerable extraneous physiological influences such as the autonomic nervous system that are present *in vivo*. The isolation also allows the experimental conditions to be both rigidly controlled and easily altered. During interpretation of any results one must bear in mind that the action of isolating the tissue from the body unavoidable alters its metabolism and function.

3.1.4.1. Isolated atria and isolated right ventricular strips

The left atria were used for a large part of this thesis. The left atria can be isolated whole, thus allowing a speedy set up of the experiment. This is imperative to ensure tissue viability and the reduction of any ischaemic preconditioning. The atrium has thin walls allowing the rapid diffusion of gases through the tissue.

In addition to the atria, strips of the right ventricle wall were used in the model of ischaemia. Strips of the right ventricle were taken while the heart was *in situ*, mainly after the removal of the atria (except on one occasion, which is indicated in the text). The strips were approximately 2-4mm wide by 5-8mm long.

Both tissues do not contain pacemaker node cells so they do not beat autonomously. In order to bring about rhythmic beating, the tissue receives electrical stimulation. This allows the rate of contraction to be fixed by an artificial pacemaker, which delivers the electrical simulation through an attached electrode. Although normal heart rate of the rat is between 320 - 480 beats per minute the stimulator provided stimulation at 2 Hz, equivalent to 120 beats per minute. This slower rate is commonly used in research papers in this area. It provides a reasonable work rate that produces a consistent response and maintains the viability for the time required to complete the experiment. *In vivo*, both tissues receives blood, which provides the preload stretch that dictates the force with which it will contract during the next beat, in accordance with the Frank-Starling law. In this model the preload stretch is fixed to a tension of 0.5 grams, which ensures that a constant tension was applied to the atria and the ventricular strip throughout the experiment.

3.1.4.2. Simulated Ischaemia

The coronary circulation delivers the oxygen necessary for cardiac metabolism in vivo. The oxygen demand of the heart reduces the oxygen saturation in the coronary blood from 98-100% to 35-25%, so it is imperative that the oxygen saturation is as high as possible through normoxia and as low as possible for simulating ischaemia. This was achieved by gassing with 95% O_2 in normoxia and 95% N_2 in simulated ischaemia. The 95% proportion was required to maintain the buffering capacity of the bathing Krebs solution, another facet to ensuring a steady baseline. In simulated ischaemia the 95% N_2 gas lowers the partial pressure of oxygen in the bathing solution. The amount of oxygen available to the tissue becomes insufficient for its normal function. Removing glucose from the Krebs removes the substrate for glucose uptake and glycolysis in the cell thereby halting ATP synthesis by these pathways. These two changes simulate ischaemia in the model. An advantage of this model is that the conditions can quickly be changed, and the time required for transition between the two conditions is around thirty seconds, which aids in the precision of the model. Another advantage is that the absence of coronary circulation allows us to eliminate this as a source of any changes in contractile function.

3.1.5. Vehicle Solvents

Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent used throughout this thesis as a vehicle for water-insoluble drugs. It was important to establish whether DMSO in the concentrations used would alone have any effect. A series of control experiments were performed using DMSO to establish whether that was the case. In initial studies polyethylene glycol (PEG) was also considered for use as a vehicle but its use was abandoned for reasons detailed in this chapter.

3.1.6. Aims of this Chapter

The aims of the chapter were to:

- Establish simulated ischaemia of the isolated cardiac tissues and to examine the effect of change the conditions to which the tissues are subjected to e.g. the time of dissection.

- Investigate the effect of varying the conditions that the tissue was subjected to on the ischaemic contracture developed.

- Establish if the vehicle solvent DMSO has an effect during ischaemia and what affect that would have on the planned future work.

3.2. Methods

Isolated left atria and right ventricular strips from male Wister rats were set up as described in Chapter Two, section 2.2. Simulated ischaemia was induced by switching the bathing solution to a glucose-free Krebs solution and gassing the bathing solution with 5% CO_2 in 95% N_2 for a period of thirty minutes. Gassing with 95% N_2 has been previously shown in this apparatus to reduce the partial pressure of oxygen (pO_2) to 46-54 mmHg compared to pO_2 of 560-620 mmHg which is present in the Krebs during normoxic gassing (Gardner, 1997). No wash of the organ bath occurred during this time. The left atrium was the first tissue to be isolated from the animal, except in one set of experiments where the effect of the order of dissection was investigated. In these experiments the atria remained in the open chest for 3 to 5 minutes longer after the commencement of the dissection in order to allow for the dissection of the ventricular strip first.

In vehicle control experiments, vehicle was administered to the organ bath at the onset of simulated ischaemia. This gave an organ bath concentration of 0.2% v/v DMSO. This remained in the bath until the end of the simulated ischaemia, with no wash out. The tissues were reoxygenated by returning the aeration to a 5% CO_2 in 95% O_2 gas and changing the bathing solution back to normal Krebs solution. Reoxygenation was continued for one hour, with the bathing solution being refreshed at fifteen minutes intervals.

3.2.1. Data Analysis

The diastolic tension was measured every minute during simulated ischaemia. The baseline diastolic tension prior to ischaemia was then subtracted giving a change in diastolic tension, which was used in analysis. The mean diastolic tension at each time point was used in the graphs ± the standard error of the mean (S.E.M). The systolic tension and diastolic tension were measured every 0.5 minutes during the first five minutes of reoxygenation, then every minute until 15 minutes post reoxygenation, then every 5 minutes until 60 minutes post reoxygenation. The developed contractile tension was calculated as the systolic tension minus the diastolic tension. This developed tension was expressed as a percentage of the baseline developed tension before onset of simulated

ischaemia at each time point. The mean developed contractile tension at each time point was used in the graphs ± S.E.M. All values quoted in the text are mean ± S.E.M.

Statistical analysis was performed to establish if any difference in a response between experimental groups was significant if the P value ≤ 0.05. Two-way analysis of variance (ANOVA) was performed to establish if a difference existed. If a difference was observed then analysis of each time point was performed to establish if there was a difference in response at that time-point. If there were two experimental groups a two-tailed t-test was performed or if there were more than two experimental groups a one way analysis of variance (ANOVA) was performed followed by Bonferroni's post-hoc test to identify the differing groups.

3.3. Results

3.3.1. Response to simulated ischaemia

Following the onset of simulated ischaemia there was a rapid loss of developed tension (systolic tension – diastolic tension) resulting in contractile failure. Figure 3.1 shows a typical experimental trace. A gradual increase in developed diastolic tension indicative of the development of ischaemic contracture was then seen.

Contracture in the left atria increased the diastolic tension by 0.90 grams ± 0.06, this peak contracture occurring after 15.5 ± 0.8 minutes (n=30) of ischaemia. In the right ventricular strips contracture increased the diastolic tension by 0.69 ± 0.07 grams, occurring after 13.6 ± 0.8 minutes (n=21) of ischaemia. Thirty minutes of simulated ischaemia abolished the developed tension in all preparations.

3.3.2. Effect of the reduction in time required to setup the tissue

As the work progressed, the time required to set-up the tissues into the experimental apparatus shortened. The frequency of the preparations that were excluded due to having an initial developed tension of 0.4g or less was also reduced.

The effect that the progression of time had on the response of the isolated atria during simulated ischaemia is shown in Figure 3.1. The polygraph trace obtained from right ventricular strips has similar features as the atrial trace. At the start of experimentation, atria experienced the most prolonged set up, during simulated ischaemia they did exhibit a contracture, which remained at a plateau once it was achieved. After six months the time required to isolate the atria was considerably less and the contracture actually peaks then fades in strength. At nine months the time required for setup was considered as short as possible. At this point atria exhibited a greater peak ischaemic contracture, but the time to peak contracture was considerably shortened.
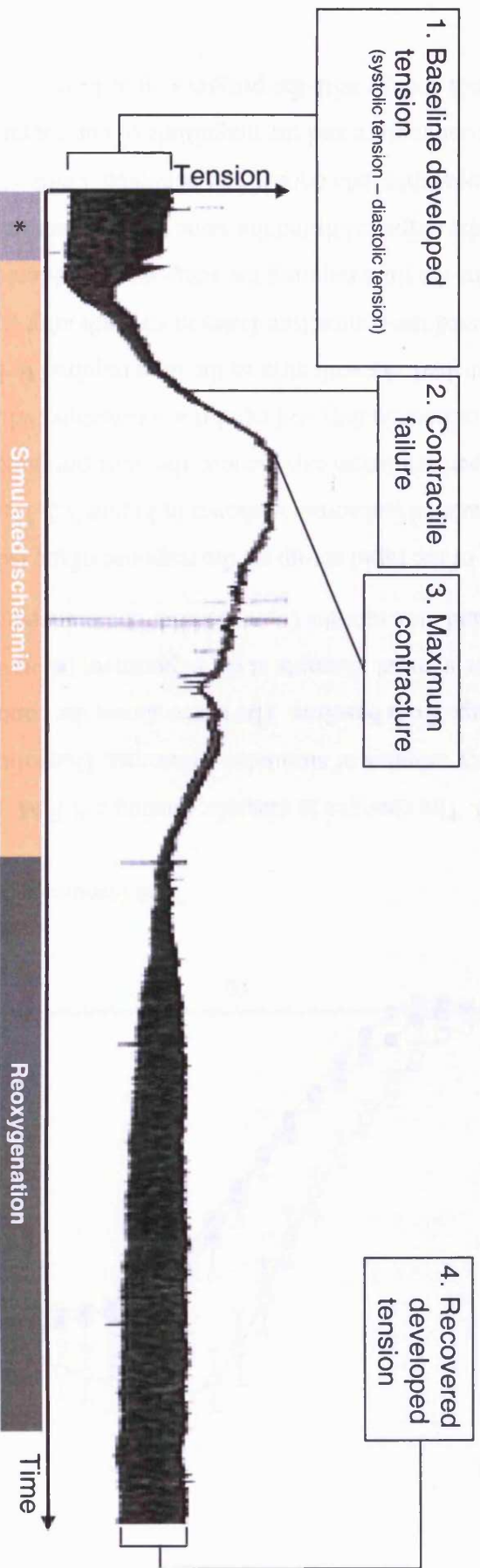
Figure 3.1. a typical polygraph trace from an atrial preparation. The trace beings on the left hand side with the developed tension of the left atria during the initial equilibrative period (*). This is followed by a period of simulated ischaemia and by subsequent reoxygenation.
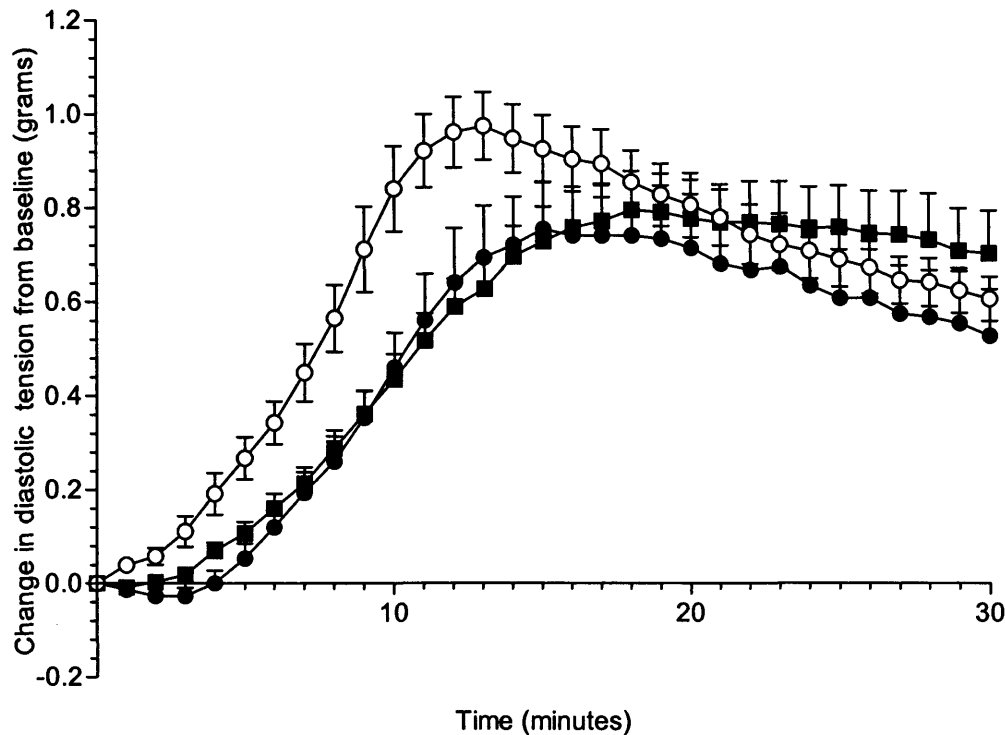
Figure 3.2. The changes in diastolic tension ± S.E.M. of isolated paced left atria during thirty minutes of simulated ischaemia. Diastolic tension was expressed as the change from baseline. The curve shows the contracture seen during the investigator's initial attempts at the experiment (■, $n=15$) and at six months (●, $n=6$), and nine months (○, $n=9$) after commencement of experimentation.

The effect of the rapid set-up on the response of the isolated ventricular strips during simulated ischaemia is shown in Figure 3.3. Here the strips set up at the start of experimentation experienced the most prolonged set up and during simulated ischaemia they did exhibit a contracture, which remained plateau once established. As with atria as the time required to isolate the ventricular strips lessened the contracture fades in strength after it reaches its maximum. At nine months the time required for setup was considered as short as possible. At this point the strips exhibited the same peak ischaemic contracture, but there was a considerable fade once it was achieved. Unlike the atria the time to the maximum contracture and the magnitude of contracture with the ventricular strips did not change with the progression of time.
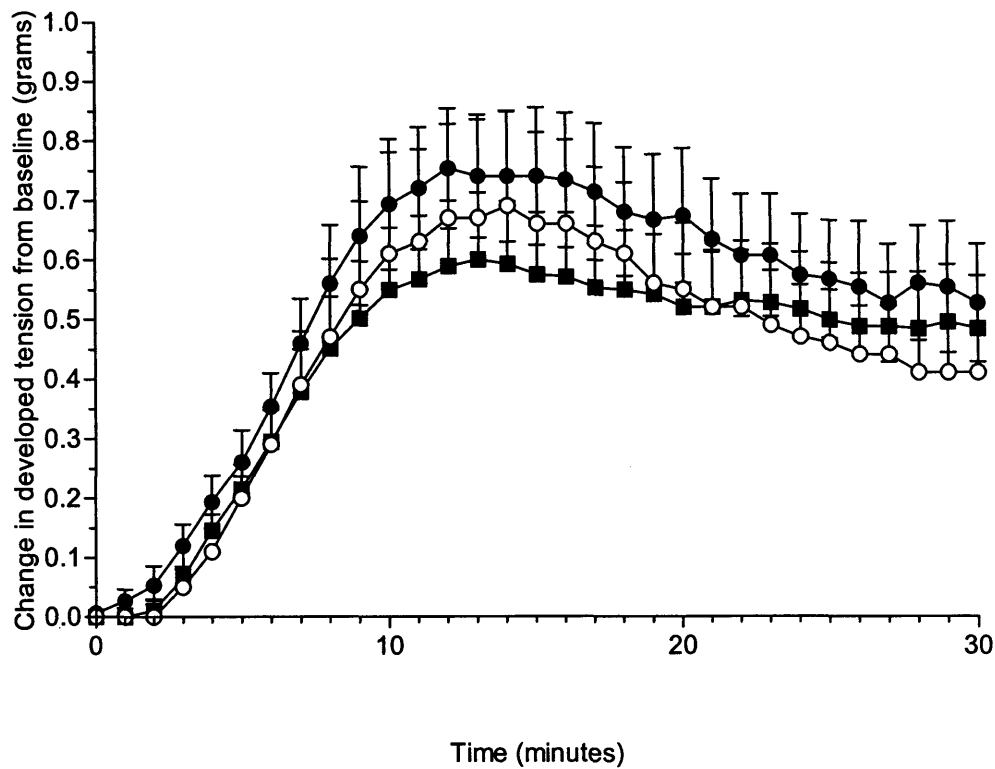
Figure 3.3 The changes in diastolic tension ± S.E.M. of isolated paced right ventricular strips during thirty minutes of simulated ischaemia. Diastolic tension was expressed as the change from baseline. The curve shows the contracture seen during the investigator's initial attempts at the experiment (■, $n=12$) and at six months (●, $n=6$), and nine months (○, $n=4$) after commencement of experimentation.

Table 3.1 Table detailing the response of isolated atria and ventricular strips during thirty minutes of simulated ischaemia. Values are shown ± the S.E.M, and are derived from the data illustrated in Figure 3.2 and Figure 3.3.

	Isolated Right Ventricular strips			Isolated Left Atria		
	Start of experimentation	6 months after commencement of experimentation	9 months after commencement of experimentation	Start of experimentation	6 months after commencement of experimentation	9 months after commencement of experimentation
	$n=11$	$n=4$	$n=6$	$n=15$	$n=6$	$n=9$
Maximum increase in diastolic tension	0.57 ± 0.10 g	0.75 ± 0.10 g	0.690 ± 0.16 g	0.79 ± 0.08 g	0.75 ± 0.10 g	0.97 ± 0.07g
Time to Maximum increase in diastolic tension	11.1 ± 0.62 minutes	12.5 ± 0.76 minutes	12.5 ± 1.44 minutes	17.53 ± 0.94 minutes[†]	15.00 ± 0.97 minutes	12.00 ± 0.66 minutes*
Mean Area Under the Curve	13.09 ± 2.38 g min	14.33 ± 2.22 g min	13.38 ± 2.71 g min	15.85 ± 1.59 g mins	14.43 ± 2.24 g mins	19.11 ± 1.44 g mins

*($P<0.01$, 1-way ANOVA, followed by Bonferroni's comparison to the value obtained at the start of experimentation) considered significant.

[†]($P<0.01$, unpaired t-test comparison to the equivalent parameter between tissue types) considered very significant.

3.3.3. Effect of delayed isolation of isolated cardiac tissues on contracture during simulated ischaemia

During the initial benchmarking phase, the effect of the order of dissection was investigated. For one set of experiments the isolation of the atria was delayed in order to remove other tissues first. During this time the atria remained in the open chest for three to five minutes longer after the commencement of dissection. It was subsequently isolated and set-up in the organ bath. The effect of simulated ischaemia on the tissue was then compared to the atria that were isolated immediately after death. It was found that the time needed for the atria to reach peak contracture increased significantly ($P= 0.0016$) from 12.44 ± 0.84

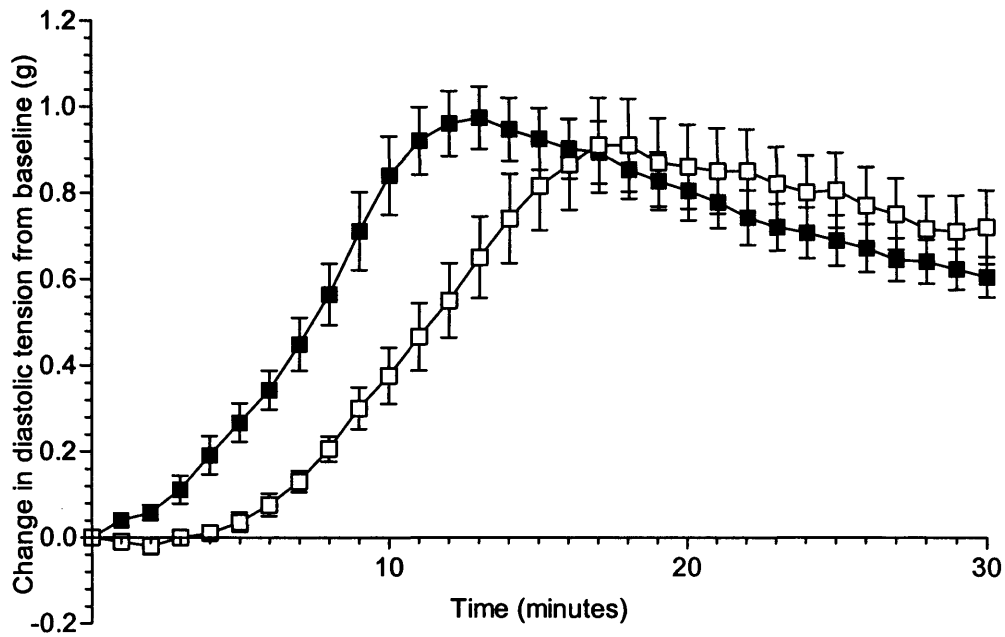minutes to 17.56 ± 1.06 minutes after the onset of simulated ischaemia when the delay occurred (Figure 3.4).

Figure 3.4 The effect of a delay of three to five minutes in the isolation on the ischaemic contracture of the left atria during thirty minutes of simulated ischaemia (□, n=9). The contracture of the left atria when it was isolated immediately also shown (■, n=9).

Table 3.2 Table detailing the response of isolated atria during thirty minutes of simulated ischaemia when immediately set-up and when set-up was delayed. Values are shown ± the S.E.M., and are derived from the data illustrated Figure 3.4.

	Immediately Set-up atria *n*=9	Atria set-up after Delay *n*=9
Maximum increase in diastolic tension	1.01 ± 0.08 g	0.93 ± 0.11 g
Time to Maximum increase in diastolic tension	12.44 ± 0.84 minutes	17.6 ± 1.06 minutes*
Mean Area Under the Curve	19.10 ± 1.44 g min	16.38 ± 1.84 g min

*($P<0.01$, unpaired t-test comparison to the value obtained from Immediately set up atria) considered very significant

For one set of experiments the ventricular strips were isolated immediately after death and its function compared to the ventricular strips remained in the body for 3-5 minutes longer during dissection. It was found that the delay in dissection made the time to reach peak contracture for the ventricle significantly increased from 11.2 ± 0.99 minutes to 16.7 ± 1.34 minutes after the onset of simulated ischaemia (Figure 3.5).
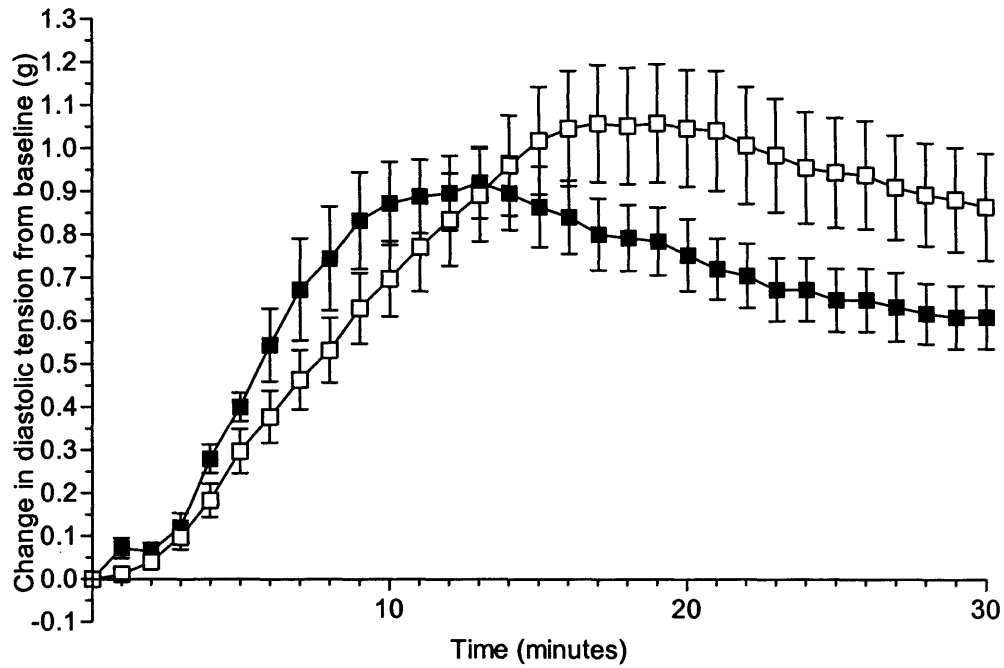
Figure 3.5 The effect of a delay of three to five minutes in the isolation on the ischaemic contracture of the right ventricular strips during thirty minutes of simulated ischaemia (□, *n*=9). The contracture of the right ventricular strips when it was isolated immediately also shown (■, *n*=5).

Table 3.3 Table detailing the response of isolated ventricular strips during thirty minutes of simulated ischaemia when immediately set-up and when set-up was delayed. Values are shown ± the S.E.M., and are derived from the data illustrated in Figure 3.5.

	Immediately set-up Ventricular strips *n*=5	Ventricular strips set-up after delay *n*=9
Maximum increase in diastolic tension	0.968 ± 0.08 g	1.13 ± 0.125 g
Time to Maximum increase in diastolic tension	11.2 ± 0.99 minutes	16.86 ± 1.34 minutes*
Mean Area Under the Curve	19.2 ± 1.85 g min	22.05 ± 2.57g min

* (*P*<0.05, unpaired t-test comparison to the value obtained from Immediately set up ventricular strips) considered significant

3.3.4. Effect of the addition of DMSO during simulated ischaemia

Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent used as a vehicle for drug administration. In order to establish whether it has a pharmacological effect by itself an investigation was performed into the effect of the vehicle DMSO administered at the appropriate concentrations and relevant time points. The presence of 0.2% DMSO in the bathing Krebs significantly diminished the contracture during simulated ischaemia in the isolated atria (Figure 3.6) but not in the ventricular strips (Figure 3.7). In the atria DMSO delayed the time to peak contracture and the size of the developed tension, and overall time the contracture was smaller, shown by the Area Under the curve measure.
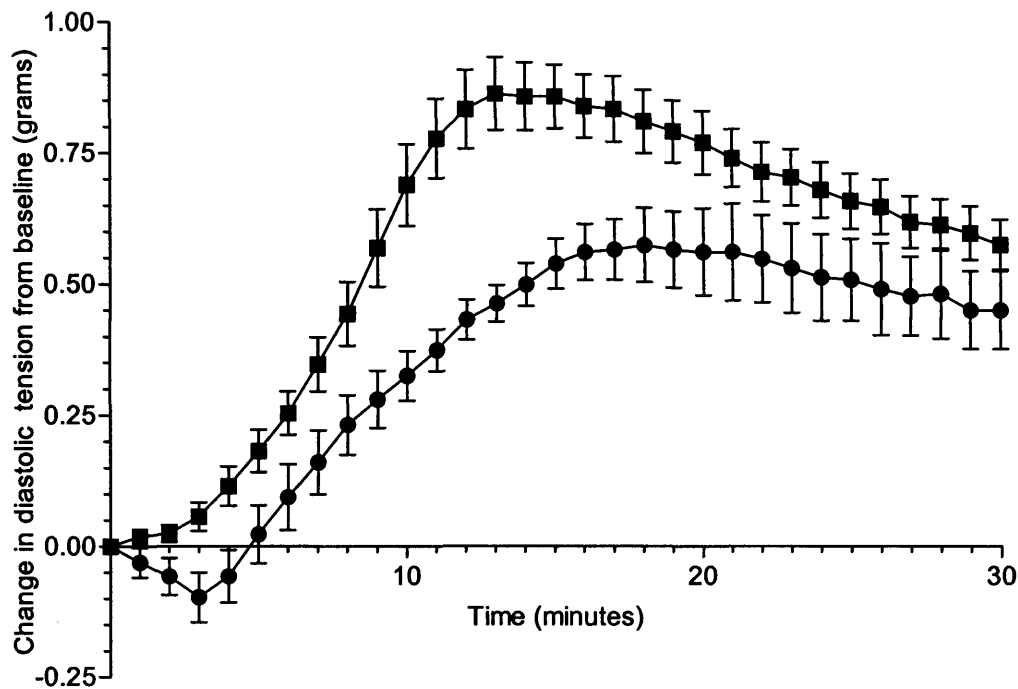
Figure 3.6 The contracture of the isolated left atria during thirty minutes of simulated ischaemia when bathing in unadulterated Krebs solution (■, $n=15$) and Krebs that contained the vehicle DMSO at a 0.2% v/v concentration (●, $n=9$).

Table 3.4 Table detailing the response of isolated left atria during thirty minutes of simulated ischaemia when bathed in unadulterated Krebs and when the Krebs contained 0.2% DMSO v/v. Values are shown ± the S.E.M., and are derived from the data illustrated in Figure 3.6

	Atria bathed in unadulterated Krebs $n=15$	Atria bathed in Krebs containing 0.2% DMSO v/v $n=9$
Maximum increase in diastolic tension	0.93 ± 0.07 g	0.63 ± 0.08 g*
Time to Maximum increase in diastolic tension	13 ± 0.67 minutes	16.0 ± 1.15 minutes*
Mean Area Under the Curve	17.24 ± 1.34 g min	11.61 ± 1.34 g min*

* ($P<0.05$, unpaired t-test comparison to the value obtained from atria bathed in unadulterated Krebs) considered significant
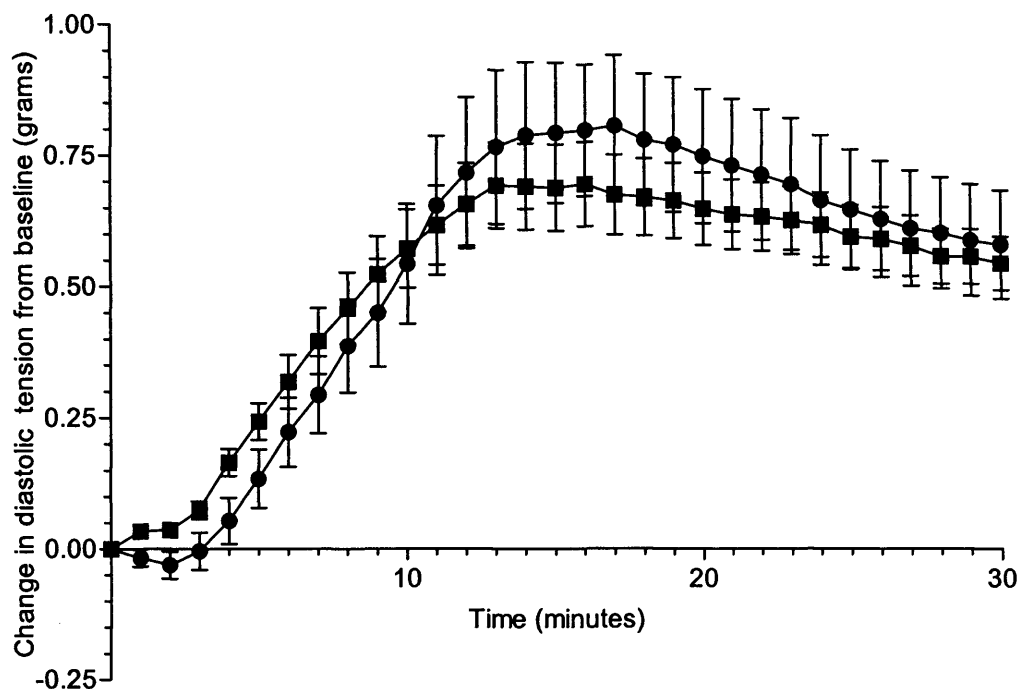
Figure 3.7 The contracture of the isolated right ventricular strips during thirty minutes of simulated ischaemia when bathing in unadulterated Krebs solution (■, $n=18$) and Krebs that contained the vehicle DMSO at a 0.2% v/v concentration (●, $n=9$).

Table 3.5 Table detailing the response of isolated right ventricular strips during thirty minutes of simulated ischaemia when bathed in unadulterated Krebs and when the Krebs contained 0.2% DMSO v/v. Values are shown ± the S.E.M., and are derived from the data illustrated in Figure 3.7.

	Strips bathed in unadulterated Krebs $n=18$	Strips bathed in Krebs containing 0.2% DMSO v/v $n=9$
Maximum increase in diastolic tension	0.77 ± 0.08 g	0.876 ± 0.14 g
Time to Maximum increase in diastolic tension	14.9 ± 1.34 minutes	14.9 ± 0.72 minutes
Mean Area Under the Curve	15.14 ± 1.59 g min	15.92 ± 2.8 g min

3.3.5. Recovery of developed tension in cardiac tissue upon reoxygenation following simulated ischaemia

The left atria were reoxygenated following thirty minutes of simulated ischaemia. During this the contractile developed tension recovered to 45.3 ± 6.8% and 61.0 ± 10.6% of the pre-ischaemic value at 15 and 60 post minutes reoxygenation, respectively (Figure 3.8). When the vehicle control PEG 0.01% v/v was added to the tissue bath for the first 15 minutes of reoxygenation the contractile developed tension was 43.2 ± 5.39% and 61.3 ± 6.55 % of the pre-ischaemic value at 15 and 60 minutes, respectively. When the other vehicle control, DMSO, was added to the tissue bath at 0.02% v/v for the first 15 minutes of reoxygenation, the contractile developed tension was 47.4 ± 7.44% and 66.6 ± 6.96% of the pre-ischaemic value at 15 and 60 minutes respectively. No significant difference was found between the recoveries of each experimental group using two-way ANOVA.
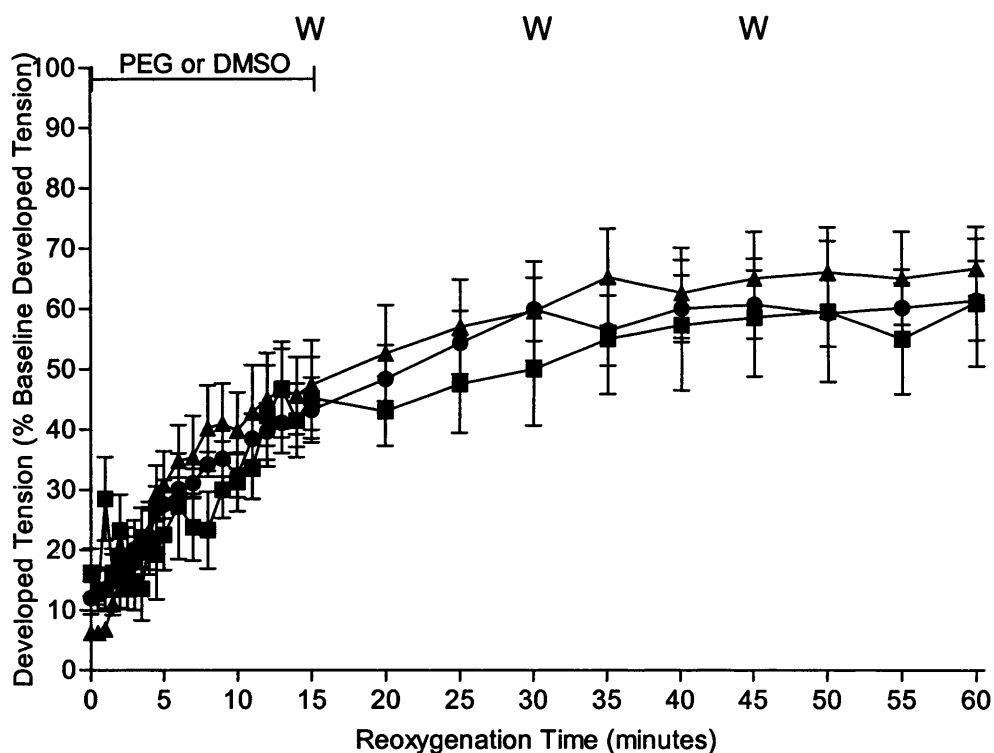
Figure 3.8 The recovery of developed tension in the left atria following thirty minutes of simulated ischaemia (■, $n=4$), and when in the presence of various vehicles. 0.01% v/v PEG (●, $n=9$) and 0.02% v/v DMSO (▲, $n=5$) was administered for the first fifteen minutes of reoxygenation then washed out. 'W' indicates the time-point the tissue bath was washed out.

In right ventricular strips, following reoxygenation after thirty minutes of simulated ischaemia the contractile developed tension recovered to $34.6 \pm 5.26\%$ ($n=7$) and $47.0 \pm 5.41\%$ of the pre-ischaemic value at 15 and 60 post minutes reoxygenation, respectively (Figure 3.9). When the vehicle control PEG 0.01% v/v was added to the tissue bath for the first 15 minutes of reoxygenation the contractile developed tension was $33.3 \pm 8.45\%$ ($n=4$) and $53.8 \pm 8.97\%$ of the pre-ischaemic value at 15 and 60 minutes, respectively. When the other vehicle control, DMSO, was added to the tissue bath at 0.02% v/v for the first 15 minutes of reoxygenation, the contractile developed tension was $27.6 \pm 2.75\%$ and $53.2 \pm 8.55\%$ ($n=5$) of the pre-ischaemic value at 15 and

60 minutes respectively. No significant difference was found between the recoveries of each experimental group using two-way ANOVA.
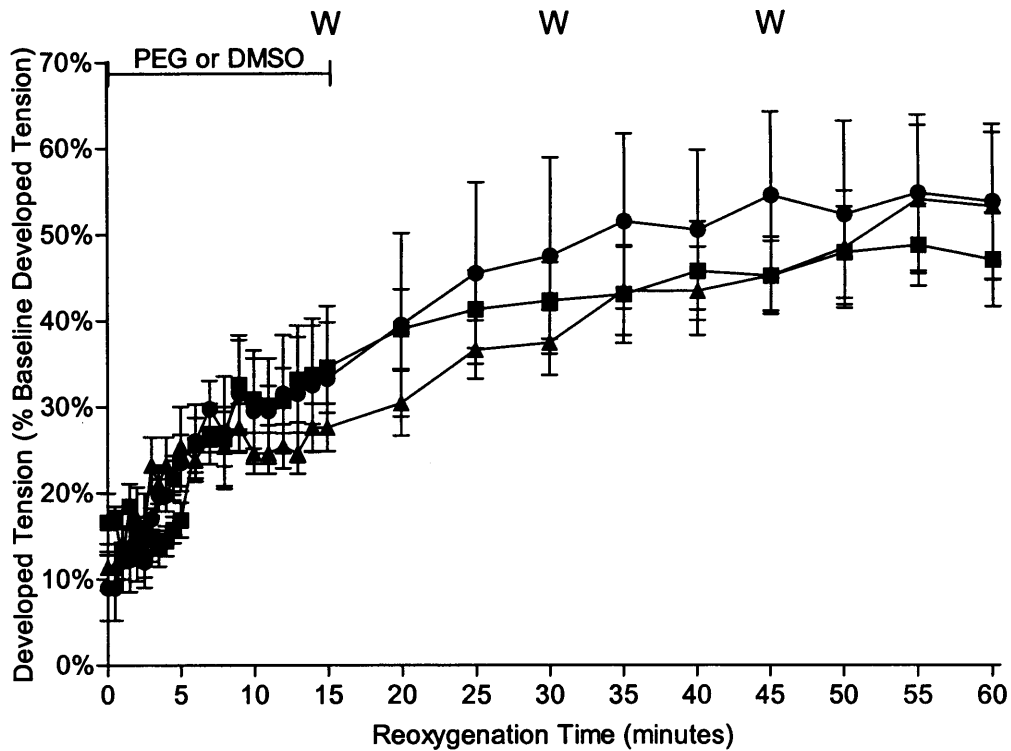

Figure 3.9 The recovery of developed tension in the right ventricle following thirty minutes of simulated ischaemia (■, n=7), and when in the presence of various vehicles. 0.01% v/v PEG (●, n=4) and 0.02% v/v DMSO (▲, n=5) was administered for the first fifteen minutes of reoxygenation then washed out. 'W' indicates the time-point the tissue bath was washed out.

3.3.6. Effect of delaying the isolation of the cardiac tissue on the recovery upon reoxygenation

Two-way ANOVA showed a significant difference (P=0.02) between the recovery from simulated ischaemia of atria immediately isolated after death and atria that remained in the body for 3-5 minutes longer. Atria that were dissected after remaining in the animal for 3 to 5 minutes longer during dissection showed a significantly quicker recovery of developed tension for the first fifteen minutes post reoxygenation (46.6 ± 3.0% recovery of baseline developed

tension at fifteen minutes) compared to 35.4 ± 3.6% recovery in atria immediately set up in the apparatus (Figure 3.10). The rate of recovery then slows so that at 30 and 60 minutes post reoxygenation there is no difference in the recovery of developed tension. The recovery at 60 minutes in atria delayed in being set up was 64.8 ± 4.18 % (n=9) of the pre-ischaemic levels, and atria immediately set up had recovered to 58.4 ± 6.78% (n=5).
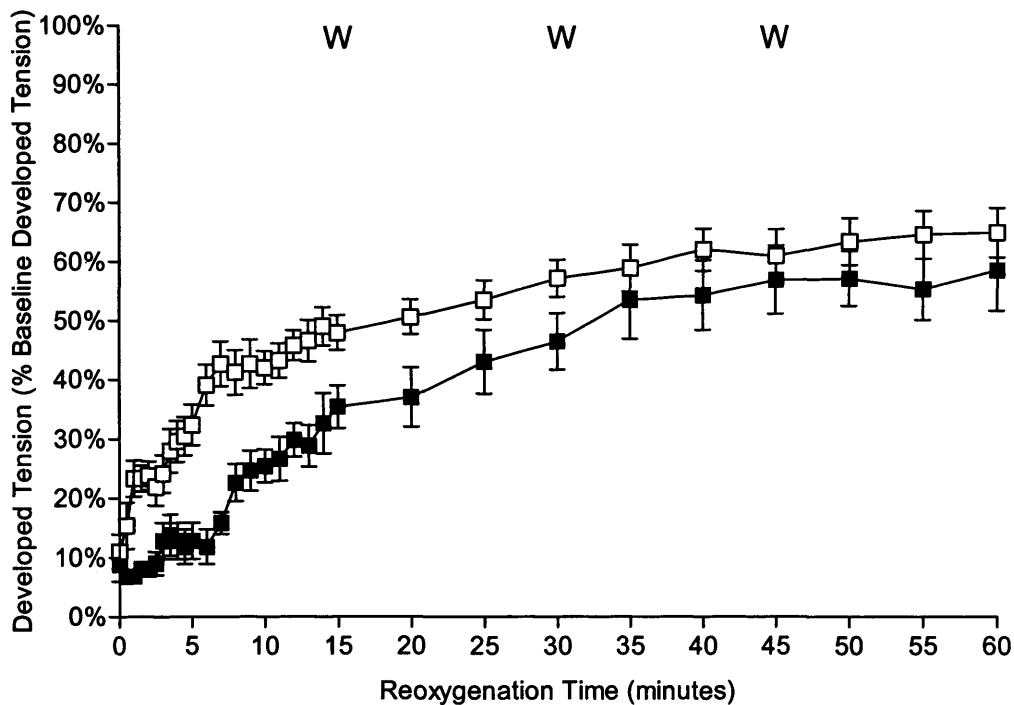

Figure 3.10 The recovery of the left atria developed tension from thirty minutes of simulated ischaemia from atria immediately isolated (■, n=5) and when the removal of the atria was delayed by 3 to 5 minutes (□, n=9). Each point represented the mean developed tension ± S.E.M. expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the time-point the tissue bath was washed out.

Two-way ANOVA showed that there was no significant difference between the recovery from simulated ischaemia of ventricular strips that were immediately isolated after death and those that were isolated 3-5 minutes following death. At fifteen minutes post reoxygenation ventricular strips that were isolated immediately had recovered to 34.5 ± 5.26% of their original developed tension and ones that were delayed to 36.7 ± 4.9% (Figure 3.11). At sixty minutes post

reoxygenation the ventricular strips that were immediately isolated recovered to 47.0 ± 5.41% and the delayed to 59.9 ± 6.28%.

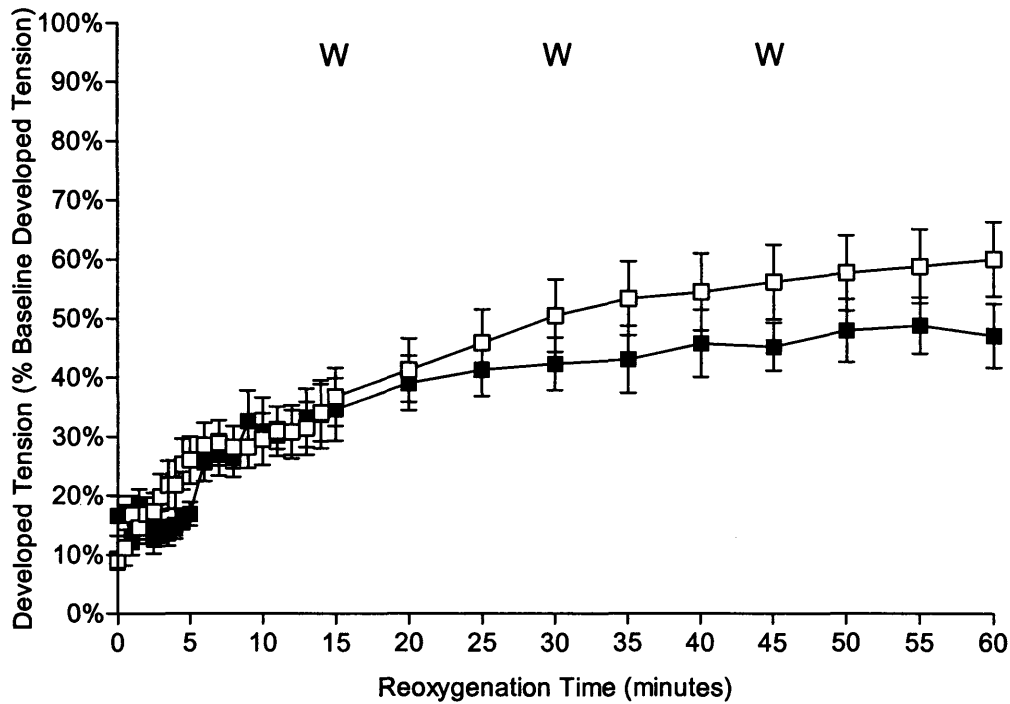**Figure 3.11** The recovery of the right ventricular strips developed tension from thirty minutes of simulated ischaemia from atria immediately isolated (■, $n=7$) and when the removal of the atria was delayed by 3 to 5 minutes (□, $n=9$). Each point represented the mean developed tension ± S.E.M. expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the time-point the tissue bath was washed out.

Table 3.6 Table detailing the recovery of developed tension in paced isolated atria and ventricular strips following thirty minutes of simulated ischemia. Values shown are the recovery of the developed tension ± the S.E.M. expressed as a % of the preischaemic baseline. Derived from the data illustrated in Figure 3.10 and Figure 3.11.

	Atria Immediately set-up $n=5$	Atria 3-5 minutes delay in setup $n=9$	Ventricular strips Immediately set-up $n=7$	Ventricular strips 3-5 minutes delay in setup $n=10$
15 minutes post reoxygenation	35.4 ± 3.6 %*	46.6 ± 3.0 %*	34.6 ± 5.3 %	36.7 ± 4.9 %
30 minutes post reoxygenation	46.4 ± 4.8 %	57.1 ± 3.1 %	42.3 ± 4.4 %	50.4 ± 6.2 %
60 minutes post reoxygenation	58.4 ± 6.8 %	64.8 ± 4.2 %	47.0 ± 5.4 %	59.9 ± 6.3 %

*(P<0.01 One way ANOVA, followed by Bonferroni's comparison to the value at in immediately set up atria) considered significant.

As Table 3.6 illustrated there is no significant difference between the tissue types at the time points measured.

3.4. Discussion

3.4.1. Loss of contractile tension

Upon the onset of simulated ischaemia there was a rapid decline in developed tension almost immediately which halts cardiac contraction despite continued electrical stimulation. This phenomenon closely mirrors the effect of global ischaemia in the whole heart. When a beating heart is made globally ischaemic there is a rapid decline in the developed intraventricular pressure, which becomes apparent within one minute and is generally complete within 5-10 minutes. It is only after this occurs that the gradual rise in diastolic pressure and the ischaemic contracture becomes apparent (Lee and Allen, 1991). If what occurs in the whole heart can be extrapolated to the left atria and the ventricular strips, then the implication is that the onset of simulated ischaemia immediately disrupts the mechanism of normal cardiac contraction. This was seen in all instances of simulated ischaemia with diminished contractility immediately apparent upon onset and with contractile failure occurring with 5 minutes after the onset of ischaemia. This is shown on the typical trace provided in Figure 3.1.

Work in isolated ferret papillary muscle and the measurement of Ca^{2+} has demonstrated that the early fall in contractile force is not due to a failure of the Ca^{2+} transient current, which in this case would be induced by the electrical impulse delivered by the electrode and not an action potential. Instead it was found to be the effect of intracellular metabolite accumulation on the Ca^{2+} sensitivity and maximum Ca^{2+} activated force of the contractile proteins (Blinks and Endoh, 1986; Fabiato and Fabiato, 1978; Lee and Allen, 1990). In the first instance, the increase in intracellular inorganic phosphate due to the phosphocreatine breakdown has a major inhibitory force and a smaller contribution being made by a decrease in intracellular pH.

The contractile failure of the tissues occurs by a separate mechanism to that of ischaemic contracture.

3.4.2. Development of ischaemic contracture

A dissociation between ischaemic contracture and cardioprotection has been observed (Kolocassides et al., 1996). The profile of ischaemic contracture is not necessarily related to the post ischaemic recovery of function. In the rat heart, ATP production needs to be maintained at >3µmol/g per minutes for ischaemic contracture not to occur. Therefore as our preparation demonstrated a contracture we can say that ATP production was falling below 3µmol/g per minute. It has been calculated that only 2.5 µmol/g per minute of ATP can be produced by anaerobic metabolism.

The development of ischaemic contracture coincides with the decrease in ATP availability (Owen et al., 1990) and corresponds to full depletion of glycogen (Pantos et al., 2006). Ischaemic contracture occurs earlier in hearts that have been depleted of glycogen, for example hearts that have already undergone brief ischaemia, such as a preconditioning stimulus. The development of contracture is delayed when myocardial glycogen content is increased.

The point at which the availability of ATP falls below 3µmol/g per minute so that it becomes limited and contracture is initiated will depend on the initial tissue content of ATP and any glycolytic ATP production. Because anaerobic glycolysis increases after as little as 10 seconds of ischaemia (Neely et al., 1973) and is principally involved in the consumption of glycogen, it is likely to be rapidly stimulated during each episode of ischaemic preconditioning, and the endogenous glycogen is likely to be reduced (Volovsek et al., 1992).

3.4.3. Improvement in technique with time

The results show that in regard to the atria there was an effect caused by the improvement of the investigator's skill over time. This demonstrates the need to be vigilant when using such an experimental set-up. It was found that the atria's performance was very sensitive to its setup, and generally when the process of removing and attaching the tissue was slow, the subsequent developed tension was smaller, and if it was less than 0.4 grams it was excluded from the results (as dictated by the exclusion criteria in section 2.2.2.1). This demonstrated the need for control groups to be built into batches of experiments as the skill of the

investigator is a definite variable, and as such has to be controlled. From this point the experiments were conducted generally in batches, over as short a time period as feasible. These results indicate that comparisons between a group conducted early in the study and those performed later are invalid.

Interestingly, ventricular strips did not exhibit such an effect but the time to being set up being shorter the contracture did peak rather that plateau. This was seen in the atria also.

3.4.4. Effect of the delaying the tissue isolation

Delaying the isolation of both the left atria and the right ventricular strips brings about a physiological change in the tissue's behaviour during ischaemia. Because of this there is a requirement to set-up the tissues as quickly as possible, as even a small delay will affect any results. Only left atria immediately isolated were used in all the following work.

With hindsight a practical control measure could be included in the experimental set-up in order to account for this effect. A time limit, say from a designated start point e.g. the euthanasia, to a designated end point, e.g. when the atria was placed in the organ bath, could be included. This control measure may then allow any atria with a long set-up time to be excluded, and ensuring that experiments are only performed on atria that were rapidly set-up. This measure could easily be included in future work, but it may increase the number of experiments that would have to be performed.

As discussed earlier, it was shown that decreasing the time to set-up the atria had the effect of shortening the time to its peak contracture when in ischaemia. Assuming that the atria was ischaemic from the point of concussion to when is was placed in the organ bath then it can be said that the shorter this period is then the time to peak contracture is shorter.

Ischaemic contracture has been shown to occur earlier in isolated hearts that have been preconditioned by a short period of ischaemia. Preconditioning also afforded protection of post ischaemic contractile function and improved recovery (Kolocassides et al., 1996). However we found the opposite in this

study in that the longer the set-up time, the more prolonged time to peak contracture is. This supports Kolocassides other conclusion that the utility of ischaemic contracture as a predictor of the protective efficacy of anti-ischaemic interventions is questionable. The 3-5 minute delay causes the onset of the ischaemic contracture to be delayed, without altering its magnitude. The 3-5 minute delay maybe evoking some sort of protective mechanism, presumably ischaemic preconditioning, and that is delaying the contracture and in the atria, alleviates stunning. Also atria that had a delayed set-up in the apparatus showed a better recovery, which is evidence that these tissues underwent cardioprotective preconditioning. The atria that were delayed in being set-up showed a quicker recovery in the first 15 minutes of reoxygenation, but after an hour of reoxygenation the overall recovery was the same as the immediately isolated atria. It seems the delaying set up, which may precondition, causes the tissue to show a reduction in the stunning caused by simulated ischaemia.

Conversely, immediately set-up tissue may have minimal preconditioning and that was evidenced by the significant difference in the contracture between the two groups. This shows that the model is capable of showing cardioprotective effects if the time to set-up the tissue is as minimal as possible. Otherwise if the tissue had undergone significant preconditioning it would make the study of drug interventions difficult, as preconditioning will already protect the tissue. If this occurred the model may not be able to show preconditioning effects of the drug.

In the ventricular strips the effect of the delay in setting up was less marked. This suggested that there maybe preconditioning already even in the most rapidly set up tissues. There seems to be the balancing factor to what the experimental model can achieve. All tissues will undergo a period of ischaemia before use in the model, but if this period is significantly longer this causes a significant effect of damage, but in turn more preconditioning and so more protection occurs. The action of one seems to offset the other. It also shows that if a tissue that have been preconditioned may not react to drug intervention in the same way as non- preconditioned tissue, and that preconditioning may mask the effect of drugs. From this I was assumed that the time to set up tissue

needed to be the quickest possible, so that any the effect of any intervention seen is the result of its action and not of preconditioning.

For example, the effect of DMSO on the recovery during reoxygenation is present in the atria, which was the first tissue removed during dissection, but is not evident in the ventricular strips, which were the second tissue removed during the dissection. It may not be that DMSO has no effect on the ventricle, but just by the fact that it was dissected second and so received a greater amount of preconditioning, and that the preconditioning may be masking any effect of DMSO.

This conclusion means that in order to study drugs in an ischaemia-reperfusion model, it is essential that the tissue received as little ischaemic preconditioning as possible. Any preconditioning that a tissues received in a matter of 3-5 minutes is capable of interfering with the tissue response during subsequent ischaemia and reperfusion.

3.4.5. Effect of the vehicle solvent

The effect of solvents DMSO and PEG during ischaemia-reperfusion were examined in order to determine if either had an effect. Both DMSO and PEG showed no effect on the tissue during reperfusion. However in the atria DMSO has a significant inhibitory effect on the contracture during simulated ischaemia, but in reoxygenation it did not affect the recovery. DMSO may not have this effect in the ventricular strips as they can be regarded as undergone preconditioning. PEG did not affect the contracture during ischaemia or reoxygenation (data not shown). Therefore PEG would be the favoured solvent for use in the study in this model due to its lack of biological effect in both ischaemia and reperfusion. While the solvent DMSO could also be used, its effects during ischaemia must be borne in mind, and the appropriate vehicle control measures must be employed.

CHAPTER FOUR

THE EFFECT OF IB-MECA,

THE ADENOSINE A$_3$ RECEPTOR AGONIST, ON THE RECOVERY OF

THE ISOLATED LEFT ATRIA & RIGHT VENTRICULAR STRIPS

FOLLOWING A PERIOD OF SIMULATED ISCHAEMIA.

4.1. Introduction

4.1.1. Cardioprotection and the adenosine A_3 receptor

Adenosine exerts its numerous pharmacological effects, through stimulation of a family of adenosine receptors, whose role and function were described in Chapter One. The adenosine A_3 receptor is the most recently identified adenosine receptor subtype. It was first cloned in the early 1990s, first in rat and then in other species. Messenger RNA for the rat adenosine A_3 receptor has been reported in a number of tissues using the sensitive detection technique of reverse transcription PCR (Sajjadi and Firestein, 1993; Zhou et al., 1992), as well as other tissues including the spleen, testis, lung, liver, and brain. Although the adenosine A_3 receptor has a wide distribution in the body, little is still known about the endogenous function of the adenosine A_3 receptor. 'Moderate amounts' of adenosine A_3 receptor transcript have been found in the heart (Salvatore et al., 1993), and in Chapter Seven we describe locating the adenosine A_3 receptor in the left atria using western blotting. The location of the adenosine A_3 receptor in the heart suggests that there is a role of the adenosine A_3 receptor in this tissue that must then relate to myocardial function. Liu et al. (1994) used antagonists of the other adenosine receptor subtypes to suggest that the effect of preconditioning is not exclusively mediated by the adenosine A_1 receptor in rabbit heart and could involve the adenosine A_3 receptor. Armstrong and Ganote (1994) in the same year suggested that preconditioning in isolated rabbit myocytes requires the adenosine A_3 receptor.

Although selective agonists for the adenosine A_1 and A_{2A} receptor have been available for several years, highly selective adenosine A_3 receptor agonists have been synthesised only in the last 12 years. IB-MECA and it 2-chloro derivative 2-Cl-IB-MECA (Figure 4.2) were the first selective agonists made available (Kim et al., 1994). Through the combination of their use and the use of antagonists of the other adenosine receptors a picture is emerging of the role adenosine A_3 receptor activation plays in cardioprotection. A summary of IB-MECA and 2-Cl-IB-MECA cardioprotective effectiveness in myocardial ischaemic models is shown in Table 4.1.

Table 4.1 Summary of published literature demonstrating the cardioprotective effect of adenosine A₃ receptor activation on cardioprotection.

	The action of adenosine A₃ receptor activation	Model of ischaemia used	Animal	Adenosine A₃ receptor agonist used	Time point of administration	Reference
1	Protection against myocardial stunning	Coronary occlusion	Rabbit (conscious)	IB-MECA	Pre-treatment	(Auchampach et al., 1997b)
2	Reduction in infarct size	Left anterior descending coronary artery occlusion in dog	Dog	IB-MECA	Pre-treatment and on reperfusion	(Auchampach et al., 2003)
3	Protection against simulated ischaemia	Isolated human atrial trabeculae	Human	IB-MECA	Pre-treatment	(Carr et al., 1997)
4	Protection of functional responses (energy metabolism and cytosolic pH) against ischaemia	Isolated ischaemic -reperfused heart	Mouse (including adenosine A₃ receptor gene knock out mice)	2-Cl-IB-MECA	Pre-treatment	(Harrison et al., 2002)
5	Decreases polymorphonuclear neutrophil-endothelial cell interaction	Isolated heart	Rabbit	2-Cl-IB-MECA	Pre-treatment	(Jordan et al., 1997)
6	Attenuates neutrophil function and neutrophil mediated reperfusion injury	Isolated artery	Dog	2-Cl-IB-MECA	On reperfusion	(Jordan et al., 1999)

	The action of adenosine A$_3$ receptor activation	Model of ischaemia used	Animal	Adenosine A$_3$ receptor agonist used	Time point of administration	Reference
7	Protection against myocardial stunning	Coronary occlusion	Rabbit (conscious)	IB-MECA	Pre-treatment	(Kodani et al., 2001)
8	Limits myocardial injury in the isolated rat heart and improves survival in isolated cardiomyocytes,	Isolated ischaemic -reperfused heart and isolated cardiomyocytes	Rat	2-Cl-IB-MECA	On reperfusion	(Maddock et al., 2002b)
9	Protects against myocardial stunning	Isolated ischaemic -reperfused working hearts	Guinea pig	IB-MECA	On reperfusion	(Maddock et al., 2003)
10	Direct preconditioning	Cultured chick ventricular myocytes	Chicken	2-Cl-IB-MECA	Pre-treatment	(Strickler et al., 1996)
11	Late preconditioning against infarction	Coronary occlusion	Conscious rabbits	IB-MECA	Pre-treatment	(Takano et al., 2001)
12	Attenuates post ischaemic dysfunction	Isolated ischaemic -reperfused heart	Rat	2-Cl-IB-MECA	Pre-treatment	(Thourani et al., 1999a)
13	Attenuates post ischaemic cardiac dysfunction	Isolated cardioplegic arrest reperfused heart		2-Cl-IB-MECA	Pre-treatment	(Thourani et al., 1999b)
14	Cardioprotection (reduction in infarct size)	Langendorff ischaemic -reperfused heart	Rabbit	IB-MECA	Pre-treatment	(Tracey et al., 1997)

With effects that could augment cardioprotection, the activation of the adenosine A_3 receptor has been proposed to enhance the release of inflammatory mediators from mast cells (Fozard et al., 1996; Hannon et al., 1995; Van Schaick et al., 1996), lower blood pressure (Carruthers and Fozard, 1993; Fozard and Carruthers, 1993), and both induce (Jacobson et al., 1993) and reduce apoptosis (Yao et al., 1997).

IB-MECA has been found to be one of the more potent agonist for the adenosine A_3 receptor, with an affinity of 1.1nM, 50 fold selectivity over A_1 receptor and A_{2A} receptor. However the data regarding the measured affinity of IB-MECA and other adenosine A_3 receptor agonists is varied. Although for many receptors the measured affinity and the measured EC50 of functional assay varies, there is a wide range of these values quoted in the literature, and they span four orders of magnitude, which is unusual for a single species of receptor (Jacobson, 1998).

IB-MECA	2-Cl-IB-MECA
(1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-*β*-D-ribofuranuronamide	2-Chloro-N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide

	IB-MECA	2-Cl-IB-MECA
Adenosine A_1 receptor	54nM	820nM
Adenosine A_{2A} receptor	56nM	470nM
Adenosine A_3 receptor	1.1nM	0.33nM

Figure 4.1. Structures of IB-MECA and its 2-chrolo derivative 2-Cl-IB-MECA, both adenosine A_3 receptor agonists. The receptor binding affinities (K_i) at the rat adenosine A_1, A_2 and A_3 receptors are shown. Taken from Jacobson (1998).

4.1.2. Time point of intervention with A_3 receptor agonist

From Table 4.1 the majority of understanding of the cardioprotective effects of adenosine A_3 receptor activation has been obtained from protocols that administer the agonist at the onset of ischaemia. While this aids understanding, in order for adenosine A_3 receptor activation to be utilised in a clinical setting, the action of activation must be able to be seen when the receptor is activated

after the onset of ischaemia. This is because acute periods of myocardial ischaemia are often unexpected.

For this study, the time point of initial IB-MECA administration was upon reoxygenation, though some investigation of the effect of introducing the drug during simulated ischaemia was also performed. This is a clinically relevant time-point when it comes to extrapolating the results of the study in a clinical situation; all drug interventions for the treatment of acute myocardial ischaemia will have to occur after the ischaemia takes place. An intervention such as drug administration or graded low-level ischaemia given after the major ischaemic insult is now sometimes referred to as post-conditioning (Yellon and Opie, 2006).

It is known that the adenosine receptor agonist R-PIA induced desensitization of recombinant human A_3 receptors within twenty minutes (Palmer et al., 1996), so a drug contact time of fifteen minutes was deemed appropriate.

4.1.3. Myocardial stunning and its clinical relevance

Myocardial stunning is defined as transient contractile dysfunction that appears after reperfusion despite the absence of irreversible damage and restoration of normal or near normal coronary flow (Braunwald and Kloner, 1982). In rat models, stunning has been induced by global ischaemia in isolated heart preparations. In rabbit models, multiple, completely reversible episodes of regional ischaemia result in stunning. In large animals single or multiple completely reversible episodes of regional ischaemia or prolonged coronary stenosis were shown to induce myocardial stunning (reviewed in (Kim et al., 2003)). Although the pathogenesis of myocardial stunning has not been definitively established, the major hypotheses are that it is caused by the generation of oxygen derived free radicals and by calcium overload in reperfusion. These two hypotheses are not mutually exclusive and are likely to represent different facets of the same pathophysiological cascade.

Protective strategies to reduce myocardial ischaemia and reperfusion injury can potentially lead to better recovery of the post ischaemic heart, decrease post operative complication and lower patient mortality and morbidity (Thourani et

al., 1999a). Post ischaemic myocardial injury is mediated during both the ischaemia phase and the myocardial reperfusion. Adenosine has been shown to attenuate myocardial stunning (Lasley and Mentzer, 1992; Randhawa et al., 1995; Zhou et al., 1992). The cardioprotective effects of adenosine are exerted both during ischaemia and at reperfusion.

4.1.4. Aims of this chapter

The aims of the chapter were:

- to investigate what effect the administration of IB-MECA, an adenosine A_3 receptor agonist, had on the contractile dysfunction immediately following a period of simulated ischaemia.

- to determine what role adenosine A_3 receptor activation may have on myocardial stunning in rat cardiac tissues.

4.2. Methods

Isolated left atria and right ventricular strips from male Wister rats were used as described in detail in Chapter Two, section 2.2. The left atrium was the first tissue to be isolated from the animal, with the ventricular strips being isolated, approximately 3 to 5 minutes later after the commencement of dissection.

Simulated ischaemia was induced by switching the bathing solution to a glucose-free Krebs solution and gassing the bathing solution with 5% CO_2 in 95% N_2 for a period of thirty minutes. No wash of the organ bath occurred during this time. After this the tissues were reoxygenated by returning to gassing with 5% CO_2 in 95% oxygen and changing the bathing solution back to normal Krebs. Reoxygenation continued for one hour, with a wash of the tissue bath every fifteen minutes. The protocol is diagrammatically represented in Figure 4.2, Protocol i.

4.2.1. The vehicle solvents

In the previous chapter the effects of DMSO (0.2% v/v) and PEG (0.01% v/v) on the tissue's response were examined as both solvents have the potential to be used as a vehicle for the drugs employed in this thesis. Previously, PEG was seen as the ideal candidate as it had no effect on the response of the tissue's ischaemic response during the simulated ischaemia – reperfusion protocol. However there was a significant difficulty in replicating known effects of drugs when both solvents were used together, with PEG used as the vehicle for the agonist and DMSO as the vehicle for the antagonist (data not shown). It was theorised that the presence of two solvents caused the drug molecules to behave as if a complex partition co-efficient system existed in the bathing medium affecting the equilibrative distribution of a drug and effectively being able to alter its pharmacological capabilities. It was therefore decided to limit the solvent used as vehicles to one. It was agreed that although DMSO showed a significant inhibitory effect on the contracture of the atria during simulated ischaemia, its use in the remaining work was favoured over PEG as it was the more versatile solvent to use with the prospective drugs that were going to be used in this study. As two way ANOVA showed no significant difference

between the recovery of contractility on reoxygenation when different vehicle controls were used (see Chapter Three), DMSO could be used as long as the appropriate controls were performed for each experimental group. Control experiments were performed where the tissues underwent the experimental protocols detailed in Figure 4.2 but with vehicle instead of IB-MECA. When necessary the appropriate volume and composition of the vehicle was added to the organ bath.

4.2.1.1. Drug administration protocols

The adenosine A_3 receptor agonist IB-MECA was administered from a stock solution to the organ bath to give final bath concentrations of 3nM, 10nM, 30nM, 100nM, 300nM or 1µM when needed. Doses were added to the tissue bath at the point of reoxygenation and remained there for 15 minutes until the first wash of the organ bath at fifteen minutes post reoxygenation, as represented by Figure 4.2, Protocol ii.

An alternative time point of IB-MECA administration was investigated by adding IB-MECA at an organ bath concentration of 300nM fifteen minutes prior to reoxygenation and again upon reoxygenation, where is remained until the first wash of the organ bath at fifteen minutes post reoxygenation. The total contact time with the tissue was thirty minutes. This protocol is represented in Figure 4.2, Protocol iii.

Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific, Loughbourgh, UK. N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) was obtained from Tocris Limited, Bristol UK.

4.2.2. Data analysis

From the polygraph trace obtained from each atria or ventricular strip, the diastolic tension was measured every minute during simulated ischaemia. The baseline diastolic tension prior to ischaemia was then subtracted from each measurement giving a change in diastolic tension. The mean diastolic tension ± the standard error of the mean (S.E.M) at each time point was used in analysis and graphed. The systolic tension and diastolic tension were measured every 0.5

minutes during the first five minutes of reoxygenation, then every minute until 15 minutes post reoxygenation, then every 5 minutes until 60 minutes post reoxygenation. The developed contractile tension was calculated as the systolic tension minus the diastolic tension. The developed tension was expressed as a percentage of the baseline developed tension at each time point before onset of simulated ischaemia. The mean of each time point in each experimental group was used in graphing the data, with error bars describing the standard error of the mean.

Statistical analysis of the curves was performed by a two-way analysis of variance (ANOVA). Statistical analysis at a single time point was performed using either a two tailed t-test or, for more than two groups, by a one way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A P-value of less than 0.05 was considered statistically significant.

Figure 4.2 A diagrammatic representation of the experimental protocols used for both isolated left atria and the right ventricular strips in this chapter. All protocols were preceded by at least 25 minutes stabilisation until a steady baseline of contraction was achieved. "W" indicated when wash-out of the organ bath occurred, and the replenishment with fresh oxygenated Krebs.

4.3. Results

4.3.1. Effect of IB-MECA on the left atria during the recovery from simulated ischaemia

4.3.1.1. Recovery of developed contractile tension in isolated atria

As previously described in Chapter Three, the left atrial developed tension gradually recovers upon reoxygenation following simulated ischaemia. The atrium begins to beat again and a developed contractile tension returns. At 60 minutes post reoxygenation, and with no drug intervention, the developed tension of the atria was $50.8 \pm 7.7\%$ of its baseline pre-ischaemic developed tension.

For the initial detailed study, the adenosine A_3 receptor agonist IB-MECA was administered at concentrations of 300nM and $1\,\mu M$ upon reperfusion. It remained in contact with the tissue until the first wash of the organ bath at fifteen minutes post reoxygenation. When 300nM IB-MECA was administered, the developed tension at sixty minutes post reoxygenation was $76.5 \pm 5.8\%$ of its pre-ischaemic value, significantly increased from the control, in fact administration of 300nM IB-MECA significantly increases the recovery of developed tension from 25 minutes post reoxygenation. The time course of recovery is shown in detail in Figure 4.3. However when the concentration of IB-MECA administered was increased to $1\,\mu M$, the recovery seen at sixty minutes was not significantly different from vehicle controls, its recovery being $64.3 \pm 5.7\%$, at 60 minutes post reoxygenation. However in the first seven minutes of recovery the administration of $1\,\mu M$ IB-MECA also significantly improves the recovery from ischaemia. But that effect was lost past seven minutes post reoxygenation time.
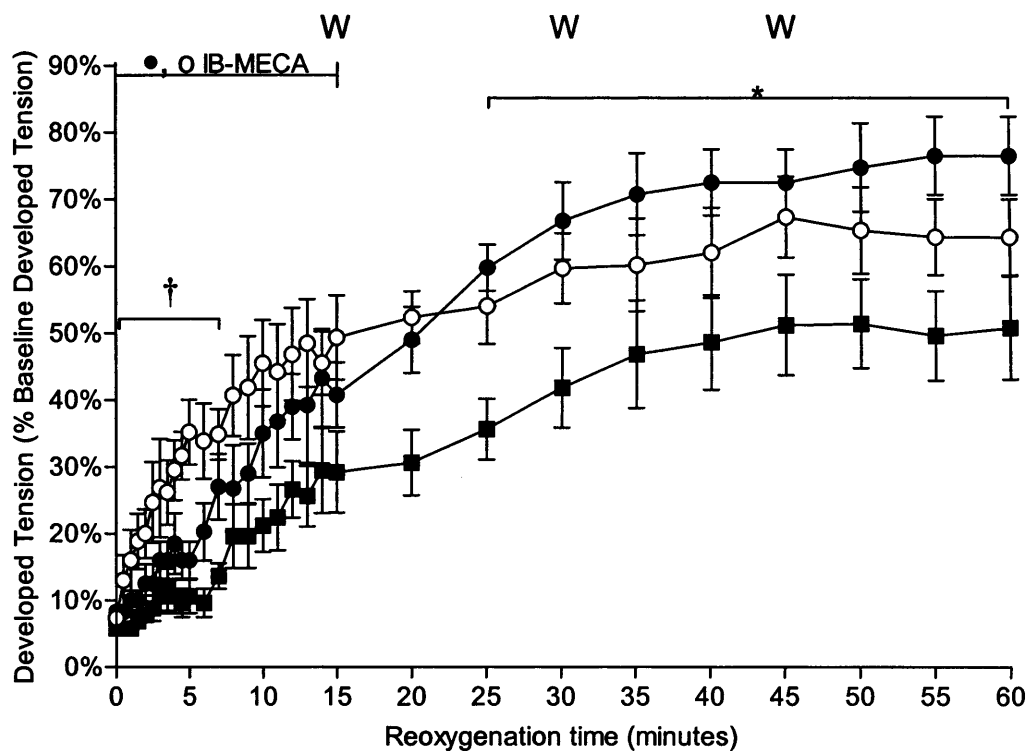
Figure 4.3a The effect of administration of IB-MECA on the recovery of developed tension of the left atria following thirty minutes of simulated ischaemia. IB-MECA 300nM (●, *n*=6) and 1μM (○, *n*=6) were administered for the first fifteen minutes of reoxygenation. A vehicle control is also shown (■, *n*=5). Each point represented the mean (± S.E.M). 'W' indicates the point of wash-out of the organ baths. * indicates a significant difference between the recoveries in the presence of 300nM IB-MECA compared to controls.

† indicates a significant difference between the recoveries in the presence of 1μM IB-MECA compared to controls.

However, looking that the early stages of reperfusion, the recovery by 1 μM
IB-MECA at five minutes post reperfusion was 35.17 ± 4.86% (*n*=6), which
was significantly greater than the recovery of the control at five minutes, which
was 10.6 ± 2.50% (*n*=5, (*P*<0.01)). This significance lasted until seven minutes
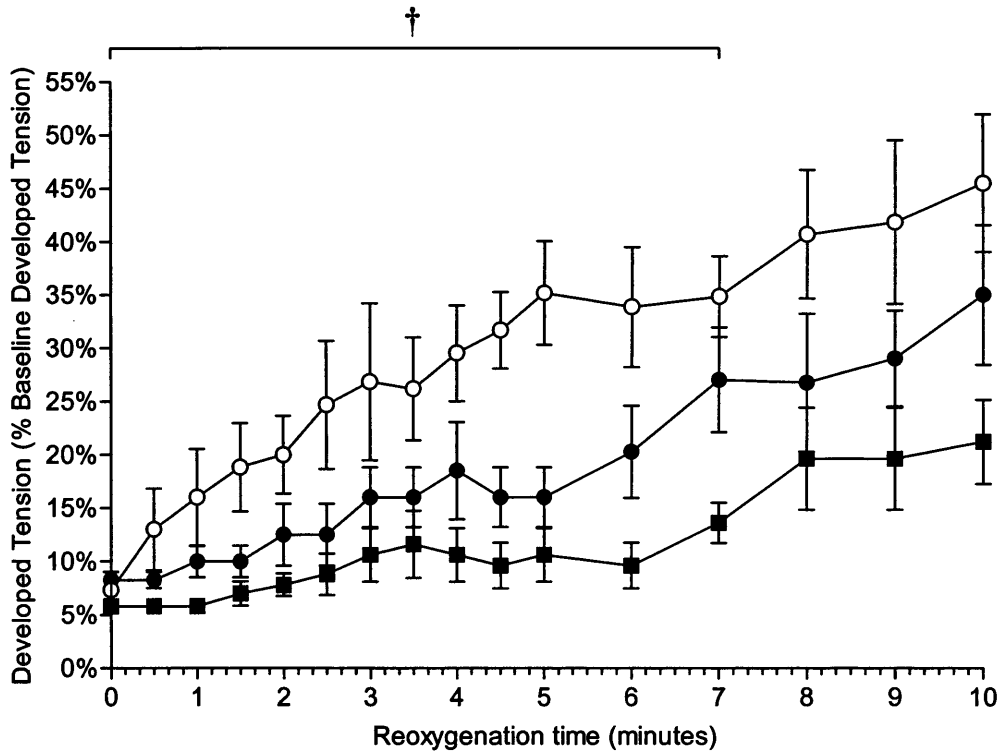of reperfusion (Figure 4.3b).

Figure 4.3b detail The effect of administration of IB-MECA on the first ten
minutes of recovery of the developed tension following thirty minutes of
simulated ischaemia in the left atria. IB-MECA 300nM (●, *n*=6) and 1 μM (○,
n=6) were administered upon reoxygenation. A vehicle control is also shown
(■, *n*=5). Each point represented the mean (± S.E.M). [†]indicates a significant
difference between the recoveries in the presence of 1 μM IB-MECA compared
to controls.

An analysis of the rate of recovery in the five minute periods was performed
and detailed in Table 4.2.

Table 4.2 The analysis of the rate of recovery of developed tension during reperfusion of the isolated atria following thirty minutes of simulated ischaemia. Values shown are the % increase in the developed tension (as a % or the baseline developed tension) per minute ± S.E.M. Statistical comparison was made by ANOVA vs. the appropriate time control.

	Control $n=5$	IB-MECA 300nM $n=6$	IB-MECA 1µM $n=6$
From 0-5 minutes	1.12 ± 0.22 %/min	2.06 ± 0.34 %/min[t]	5.82 ± 0.57 %/min[t]
From 5-10 minutes	2.20 ± 0.45 %/min	3.74 ± 0.43 %/min[t]	1.69 ± 0.38 %/min
From 10-15 minutes	1.78 ± 0.25 %/min	1.53 ± 0.27 %/min	0.54 ± 0.26 %/min[*]
From 15-20 minutes	0.28 ± 0.62 %/min	1.65 ± 0.10 %/min[t]	0.60 ± 0.66 %/min
From 20-25 minutes	1.00 ± 0.23 %/min	2.15 ± 0.37 %/min[t]	0.33 ± 0.47 %/min[*]
From 25-30 minutes	1.24 ± 0.54 %/min	1.40 ± 0.57 %/min	1.13 ± 0.18 %/min

[*] (P<0.05, unpaired t-test comparison to controls) considered significant

[t] (P<0.01, unpaired t-test comparison to controls) considered very significant

4.3.1.2. Recovery of diastolic tension of isolated atria

During simulated ischaemia, the diastolic tension increases indicative of contracture, as described in section 3.3.1. Upon reoxygenation, the diastolic tension quickly falls as the tissue recovers from ischaemic contracture. It does not return to pre-ischaemic levels within the 60 minutes recording time following reoxygenation. The change in diastolic tension of the atria is shown in Figure 4.4. In the presence of IB-MECA the diastolic tension fell more rapidly

initially to reach the same levels as the control tissue by 10 minutes of reoxygenation.
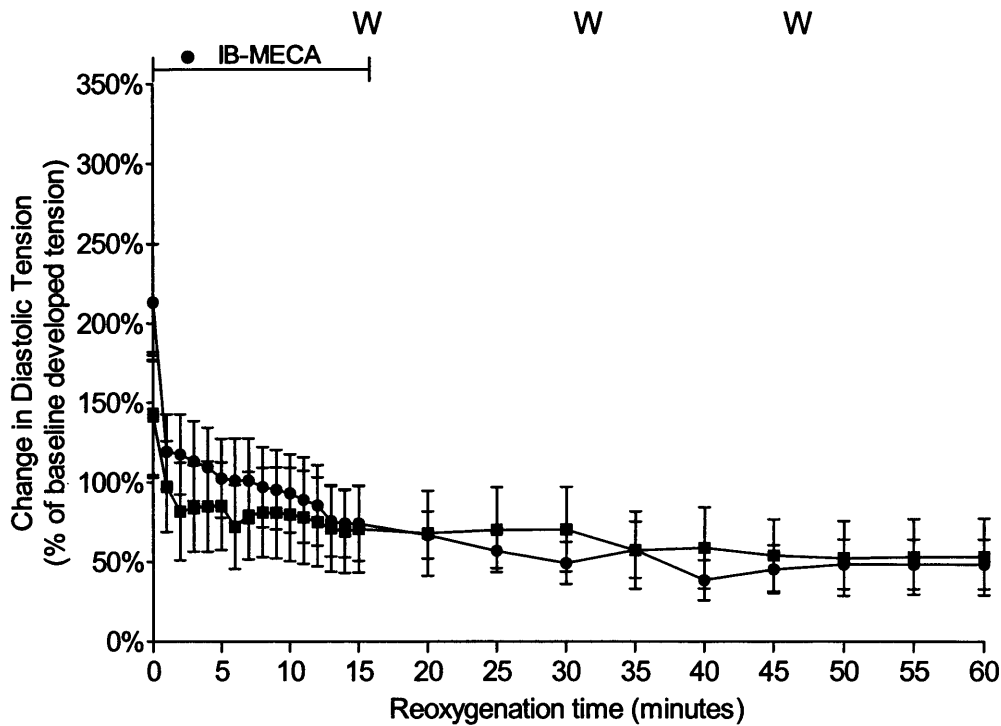

Figure 4.4 The recovery of diastolic tension following thirty minutes of simulated ischaemia in paced isolated left atria (■, $n=9$) and when IB-MECA 300nM (●, $n=6$) was added to the bath at reoxygenation, until the first wash at fifteen minutes post reoxygenation. The mean diastolic tension is expressed as a percentage of the baseline value, with error bars ± S.E.M. 'W' indicates the point of wash of the organ baths.

4.3.2. Effect of IB-MECA on right ventricular strips during the recovery from simulated ischaemia

4.3.2.1. Recovery of developed contractile tension in isolated ventricular strips

Following reoxygenation after thirty minutes of simulated ischaemia, the ventricular strips recover and contractile tension returns. At sixty minutes post reperfusion the contractile developed tension of isolated right ventricular strips recovered to 53.2 ± 8.6% ($n=5$) of the pre-ischaemic value. IB-MECA was

administered at concentrations of 300nM and 1μM upon reperfusion. It remained in contact with the tissue until the first wash of the organ bath at fifteen minutes post reoxygenation. When 300nM IB-MECA was administered, the developed tension at sixty minutes post reoxygenation was $51.9 \pm 7.7\%$ of its pre-ischaemic value. When 1μM of IB-MECA was administered at the same time-point, the recovery at sixty minutes post reoxygenation was $47.0 \pm 8.1\%$. No significant difference was found between the recoveries of each experimental group in comparison to the control recovery and each other, using two-way ANOVA.
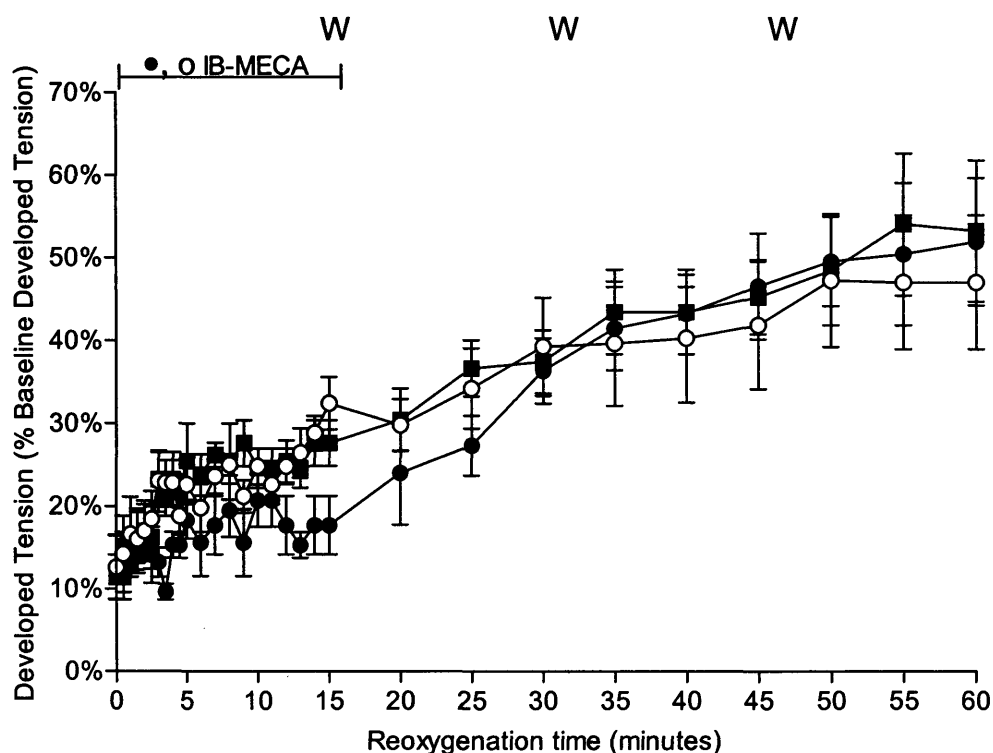

Figure 4.5 The effect of administration of IB-MECA on the recovery of developed tension of right ventricular strips following thirty minutes of simulated ischaemia. IB-MECA 300nM (●, $n=4$) and 1μM (○, $n=6$) was administered for the first fifteen minutes of reoxygenation. A vehicle control is also shown (■, $n=5$). Each point represented the mean (± S.E.M). 'W' indicates the point of wash-out of the organ baths.

Following the discovery that IB-MECA does not exert an effect, a set of experiments were performed when the right ventricular strip was the first tissue to be isolated from the animal following death (as opposed to the previous experimentation when the strip was removed after the dissection of the atria, resulting in a 3-5 minute delay in setting up the strip following the death of the animal). This would allow us to establish if removing this delay in dissection changes the response to IB-MECA during reoxygenation. The resulting recoveries seen during this experiment are shown in Figure 4.6.
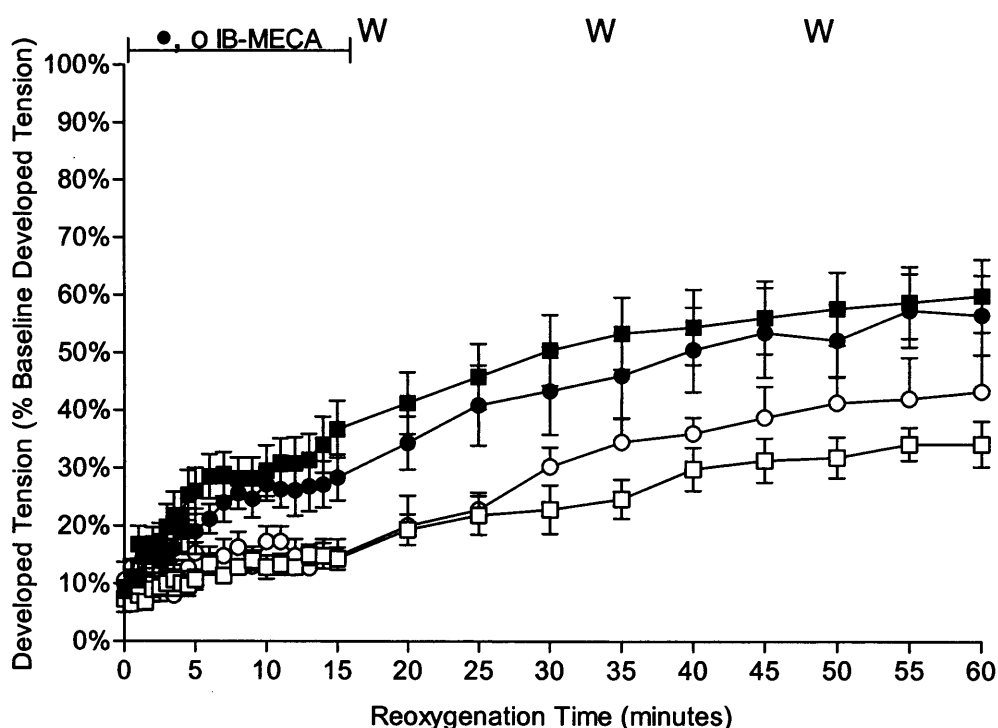

Figure 4.6 The recovery of the right ventricle following simulated ischaemia when the isolation of the ventricle was delayed ($\square$, $n=6$) and the recovery seen when IB-MECA 300nM was administered for the first fifteen minutes of reperfusion ($\circ$, $n=4$). The recovery of the ventricular strips when their isolation was immediately after death is shown ($\blacksquare$, $n=6$) together with the recovery seen when IB-MECA 300nM was administered to the tissue upon reoxygenation ($\bullet$, $n=6$). Each point represented the mean ($\pm$ S.E.M). 'W' indicates the point of wash-out of the organ baths.

Two way ANOVA analysis revealed there is a significant difference between the two experimental groups, that is the order of dissection causes a significant

difference in the recovery following ischaemia. However the administration of IB-MECA did not cause any difference to the recovery in comparison to the respective control recovery.

4.3.2.2. Recovery of diastolic tension of isolated ventricular strips

During simulated ischaemia, the diastolic tension increases, indicative of contracture. Upon reoxygenation, the diastolic tension quickly falls as the tissue recovers from ischaemic contracture. It does not return to pre-ischaemic levels within the 60 minutes recording time following reoxygenation. The change in diastolic tension of the ventricular strips is shown in Figure 4.7. The presence of IB-MECA was found not to statistically change the recovery from ischaemia of the diastolic tension
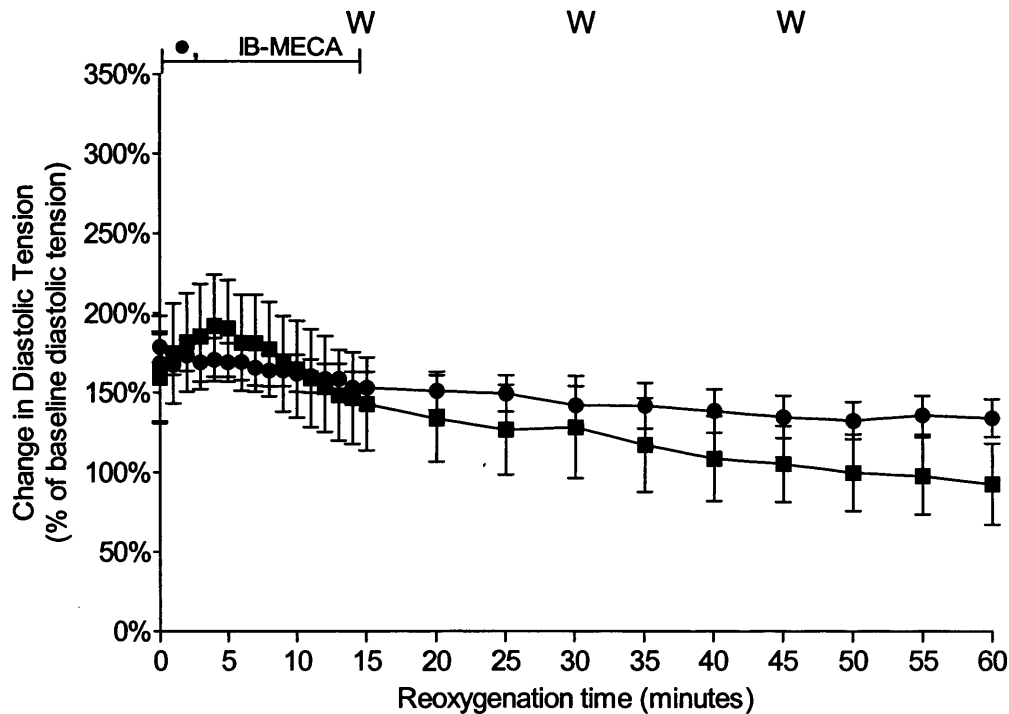
Figure 4.7 The recovery of diastolic tension following thirty minutes of simulated ischaemia in paced isolated right ventricular strips (■, $n=5$) and when IB-MECA 300nM (●, $n=4$) was added to the bath at reoxygenation, until the first wash at fifteen minutes post reoxygenation. The mean diastolic tension is expressed as a percentage of the baseline value, with error bars ± S.E.M. 'W' indicates the point of wash of the organ baths.

4.3.3. The dose response relationship for IB-MECA's effect on isolated atria during recovery from simulated ischaemia

As previously described in Chapter Three the atria recover during reoxygenation and regain contractile developed tension. The adenosine A_3 receptor agonist IB-MECA at 3nM, 10nM, 30nM, 100nM, 300nM and 1μM was administered to the tissue bath at the point of reoxygenation as detailed in Figure 4.2, Protocol ii.. The recovery of developed tension of all doses at 5, 15, 30 and 60 minutes post reoxygenation is detailed in Table 4.3. The dose response curve representing this table is shown in Figure 4.8.

Table 4.3 The effect of IB-MECA (3nM – 1μM) on the recovery of the developed tension of paced isolated atria following thirty minutes of simulated ischaemia. Values shown are the recovery of the developed tension ± S.E.M. error of the mean, expressed as a % of the pre-ischaemia baseline. Statistical comparison was made by two-tailed t-test vs. the appropriate time control.

		IB-MECA					
	Control $n=5$	**3nM** $n=8$	**10nM** $n=5$	**30nM** $n=3$	**100nM** $n=7$	**300nM** $n=6$	**1μM** $n=6$
5 minutes post reoxygenation	10.6 ± 2.50%	39.04 ± 1.58%[t]	40.20 ± 9.63%*	33.40 ± 7.86%*	37.09 ± 12.7%	16.0 ± 2.80%	35.1 ± 4.86%[t]
15 minutes post reoxygenation	29.2 ± 6.09%	54.8 ± 2.64%[t]	57.0 ± 3.56%[t]	54.0 ± 14.4%	54.3 ± 5.24%*	40.8 ± 4.87%	49.3 ± 6.29%
30 minutes post reoxygenation	41.8 ± 5.96%	61.4 ± 3.57%*	75.8 ± 4.12%[t]	63.0 ± 16.7%	62.7 ± 5.78%	66.8 ± 5.81%*	59.7 ± 5.29%
60 minutes post reoxygenation	50.8 ± 7.70%	74.2 ± 3.81%*	80.0 ± 4.43%*	68.0 ± 21.1%	71.3 ± 5.61%*	76.5 ± 5.80%*	64.3 ± 5.70%

* ($P<0.05$, unpaired t-test comparison to controls) considered significant

[t] ($P<0.01$, unpaired t-test comparison to controls) considered very significant

The administration of IB-MECA at 3nM and 10nM at reoxygenation caused a significant increase in the recovery of the developed tension at 5, 15, 30 and 60 minutes post reoxygenation. Other concentrations of IB-MECA used also caused an increase in recovery. Administration of IB-MECA 100nM caused a significant increase in the recovery of the contractile developed tension at 15 and 60 minutes post reoxygenation, with IB-MECA 300nM also increasing the recovery at 30 and 60 minutes post reoxygenation.
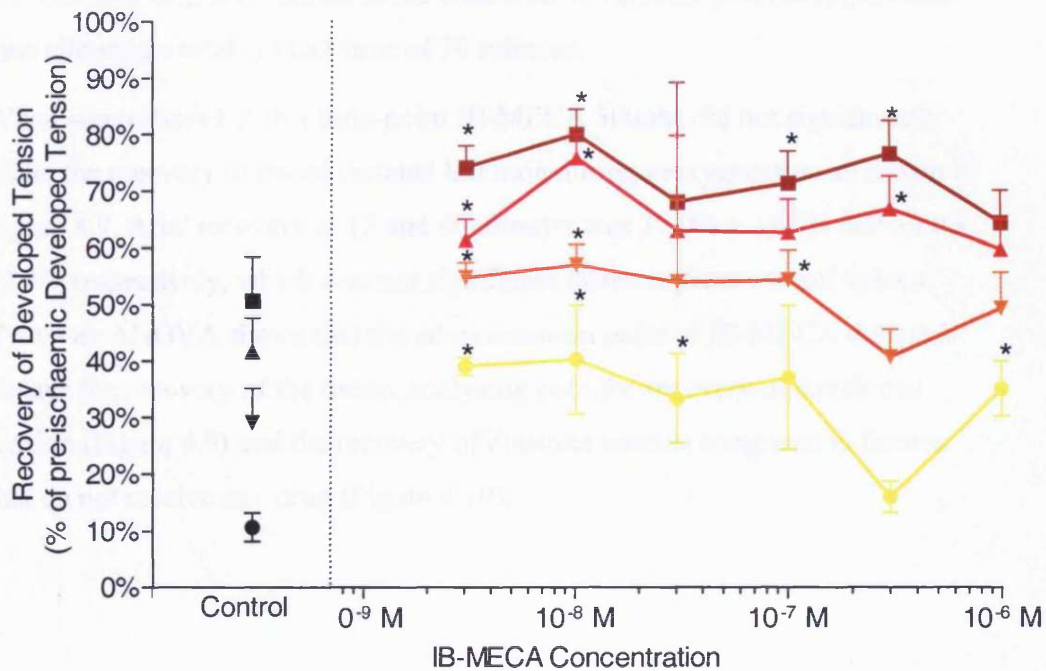
Figure 4.8 The effect of administration of various concentrations IB-MECA and its vehicle control on the recovery of the left atria at 5 minutes (●), 15 minutes (▼), 30 minutes (▲) and 60 minutes (■) post-reoxygenation. Error bars show ± S.E.M. * indicates a significant difference in the recovery exhibited compared to the appropriate time control.

4.3.3.1. Non-linear regression of the dose-response relationship

Attempts were made to use the graphing software Prism to use non-linear regression in order to fit a dose-response curve to the data points shown in Figure 4.8. This would enable greater description of the effects of IB-MECA. However the attempts were unsuccessful due to the data obtained not resembling a sigmoid curve and the program was unable to perform curve fitting.

4.3.4. Effect of IB-MECA when administered prior to reoxygenation on recovery of isolated left atria from simulated ischaemia

IB-MECA 300nM was added to the tissue bath 15 minutes into simulated ischaemia and therefore 15 minutes prior to reoxygenation (detailed in Figure

4.2, Protocol iii.). It remained in the bath until 15 minutes post reoxygenation, thus allowing a total contact time of 30 minutes.

When administered at this time-point IB-MECA 300nM did not significantly affect the recovery of paced isolated left atria during reoxygenation as shown in Figure 4.9. Arial recovery at 15 and 60 minutes was 37.0% ± 11.5% and 53.0 ± 12.3% respectively, which was not significant different from control values. Two way ANOVA shows that the administration point of IB-MECA does not change the recovery of the tissue, analysing both the recovery of developed tension (Figure 4.9) and the recovery of diastolic tension compared to tissues that do not receive any drug (Figure 4.10).
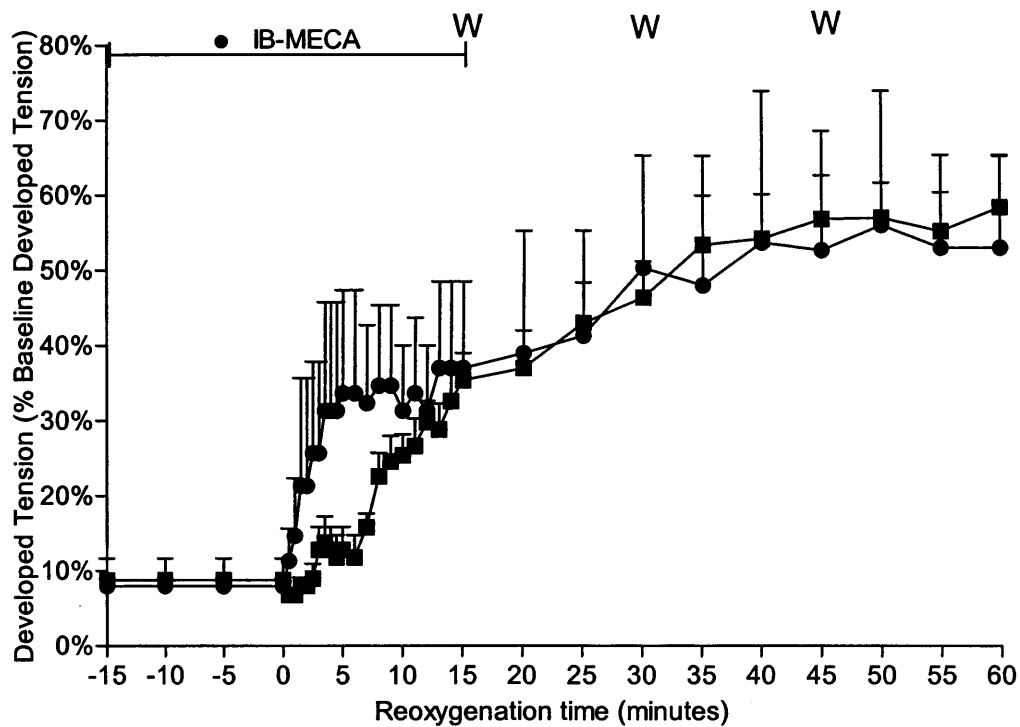
Figure 4.9 The effect of IB-MECA 300nM (●, *n*=3) administered to the organ bath fifteen minutes prior to reoxygenation on the recovery of developed tension of left atria from thirty minutes of simulated ischaemia. IB-MECA was administered fifteen minutes into simulated ischaemia and remained in the bath until fifteen minutes into reoxygenation. A control that received no drug was also performed (■, *n*=5). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the time-point the tissue bath was washed out.
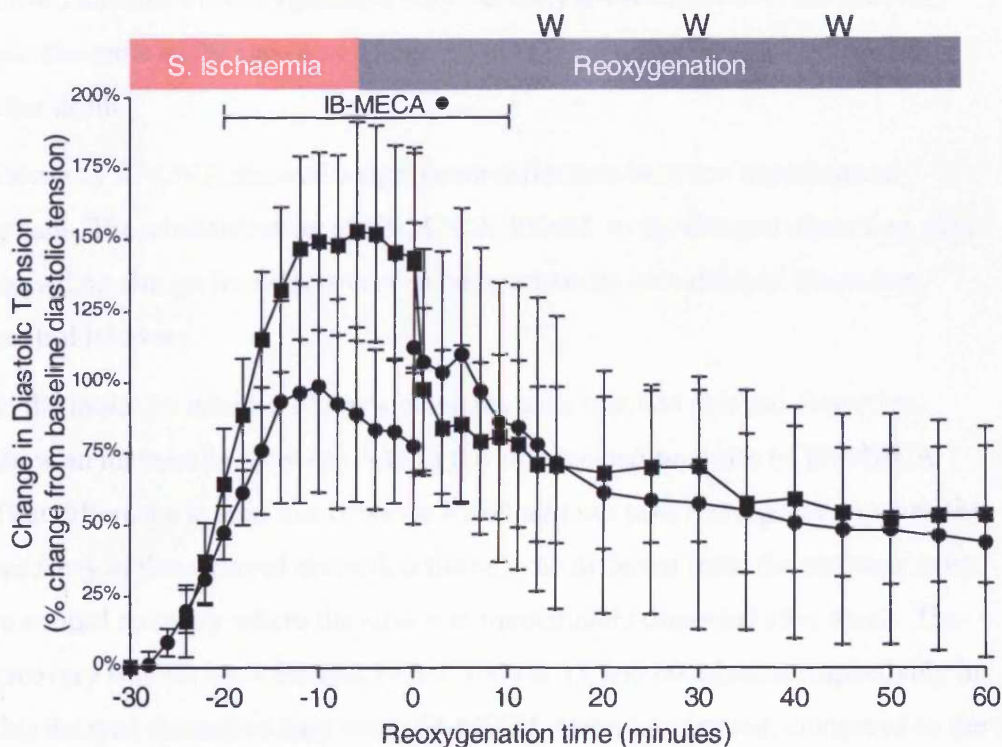
Figure 4.10 The effect of IB-MECA 300nM (●, *n*=3) on the recovery of atrial diastolic tension during and after thirty minutes of simulated ischaemia. IB-MECA was administered to the organ bath fifteen minutes prior to reoxygenation and remained in the bathing perfusate until fifteen minutes after reoxygenation. A control that received no drug was also performed (■, *n*=5). Each point represented the mean diastolic tension (± S.E.M.) expressed as a percentage of the pre-ischaemic diastolic tension. 'W' indicates the time-point the tissue bath was washed out and the ischaemia-reperfusion protocol used is indicated

4.3.5. Effect of delaying dissection on IB-MECA's effect in isolated atria

In one set of experiments, the atria remained in the animal for 3-5 minutes longer after death (see Chapter Two, section 2.2.1.1). As detailed in section 3.3.3., there was a significant difference between the recoveries of the atria from simulated ischaemia. When the atria remained in the body for 3-5 minutes longer after death, a significantly quicker recovery is observed for the first

fifteen minutes of reoxygenation only. At sixty minutes however the recovery was the same as the recovery of the left atria which were isolated immediately after death.

Two-way ANOVA showed a significant difference between experimental groups. The administration of IB-MECA 300nM to the delayed dissection atria caused no change in the recovery in comparison its own delayed dissection control recovery.

In the initial 15 minutes of reperfusion the atria that had delayed dissection show an increase in recovery both in the absence and presence of IB-MECA. That difference is then lost however by 60 minutes post reoxygenation when the recovery of this delayed dissection tissue is no different from the recovery seen in control recovery where the atria was immediately dissected after death. The recovery was $50.3 \pm 4.5\%$ and $59.3 \pm 3.4\%$ at 15 and 60 minutes respectively in this delayed dissection atria when IB-MECA was administered, compared to the control recovery of $47.9 \pm 2.9\%$ and $64.8 \pm 4.2\%$.
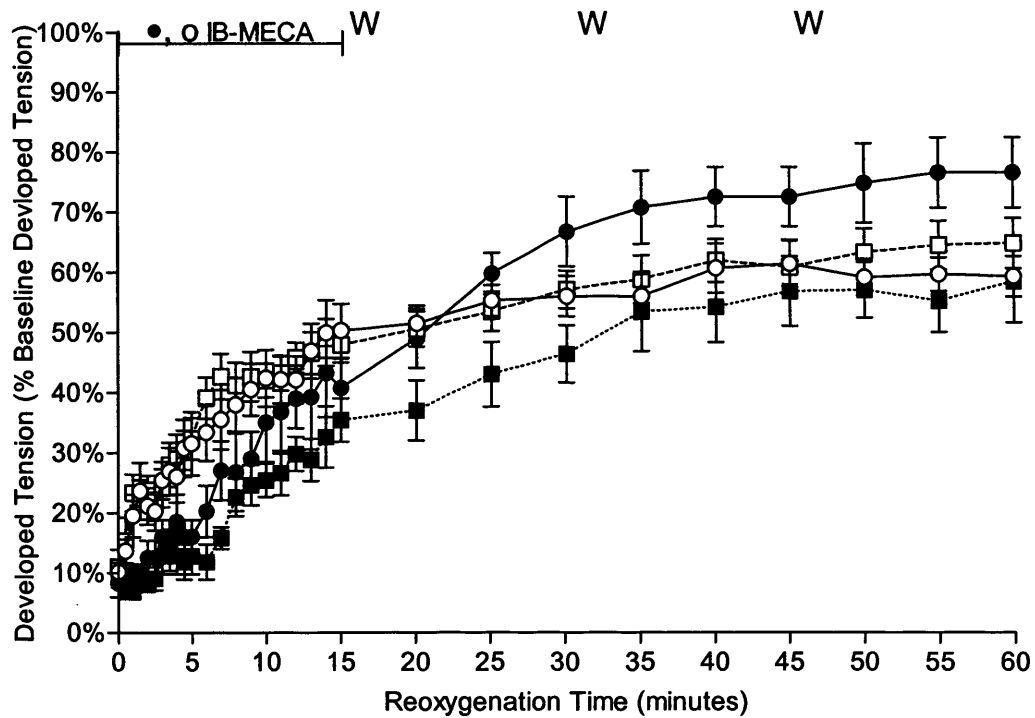
Figure 4.11 The recovery of the left atria following simulated ischaemia when the isolation of the atria was delayed (□, *n*=9) and the recovery seen when IB-MECA 300nm was administered for the first fifteen minutes of reperfusion (○, *n*=8). The recovery of atria when isolation was immediately after death is also shown (■, *n*=6) and when IB-MECA 300nM was administered to atria isolated immediately (●, *n*=5).

4.4. Discussion

4.4.1. The effect of IB-MECA during recovery from simulated ischaemia

The impaired contractility and metabolic dysfunction in the initial stages of reperfusion following a period of ischaemia has been termed myocardial stunning (Braunwald and Kloner, 1982). Myocardial stunning was seen in rat isolated atria and ventricular strips exposed to the simulated ischaemia, as a loss of contractility during ischaemia, which partially returns during reoxygenation to around 60% of the initial resting level, which is indicative of a stunned myocardial tissue.

In this chapter we examined the effect of IB-MECA, an adenosine A_3 receptor agonist, on the recovery of isolated atria and ventricular strips from ischaemia-reperfusion. As previously discussed in the introduction of this chapter there is a lot of support that IB-MECA through its activation of adenosine A_3 receptors is cardioprotective in a number of ischaemia-reperfusion models. If there proved to be an aid to recovery then a possible link to the alleviation of myocardial stunning could be made.

In the isolated left atria we have shown that the adenosine A_3 receptor agonist IB-MECA augments the post ischaemic recovery, when administered at reperfusion. This demonstrates that the activation of adenosine A_3 receptors by IB-MECA can exert a cardioprotective effect against ischaemia-induced myocardial stunning in isolated atria when measured from tension development. This effect occurred when the agonist was added at the onset of reoxygenation. This conclusion is in agreement with the findings of Maddock et al. (2003) who showed that the adenosine A_3 receptor agonist IB-MECA when administered at reperfusion improved the recovery of contractibility in isolated guinea pig atria and papillary muscles to pre-hypoxia levels.

In the isolated ventricular strips IB-MECA did not affect the recovery from ischaemia at both doses used 300nM and 1μM. As noted in Chapter Three the ventricular strips did not have a marked change in the recovery when the isolation was delayed. The lack of IB-MECA's action in the ventricular strips

may be as because the ventricle tissue is not affected by IB-MECA or that the tissue becomes preconditioned in isolated and so is unable for a drug to affect it function. Further investigation would be required to further explain this effect.

In the atria, the effect of a range of concentrations (3nM to 1μM) of IB-MECA was examined. There were indications that the effects of IB-MECA are dose-dependent. IB-MECA has a beneficial effect at all time points measured at the lower concentrations tested (i.e. 3nM and 10nM) but only protective at one or two of the time points measured at the higher doses of 30nM to 1μM. One possible critique of this conclusion is the small number of replicates used for 30nM IB-MECA. The data indicates that the protective effect is maximal at the 10nM dose. This maximum and the observation of dose dependency is in agreement with work of Maddock et al. (2002b) who used the IB-MECA analogue 2-Cl-IB-MECA in isolated rat heart. They showed a dose dependency of cardioprotective action against infarct size and the protection occurred with the range of doses of 0.1-10nM. In hindsight lower doses than 3nM could have been used to better show the dose-response relationship.

4.4.2. Possibility of other effectors involvement

IB-MECA is only relatively selective for A_3 receptors at low doses, but is typically used at higher doses for intact tissue. Subtype-specific doses for IB-MECA have been found to vary between species. For example, doses of IB-MECA (30 nM) used in myocardial ischaemia studies in the rabbit bind to cloned rabbit adenosine A_1 receptors (Hill et al., 1998a). The concentration of 300nM IB-MECA has been shown to have no A_{2A} or A_1 receptor mediated action in isolated normoxic guinea pig heart, but do have action at the human A_{2A} receptors and A_{2A} receptors in the isolated rat heart (Lasley et al., 1999).

In order to confirm if IB-MECA was stimulating adenosine A_1 receptors a further examination of the rate at which tension recovered was performed. If IB-MECA was acting at A_1 receptors, it may be possible to observe a negative inotropic effect of the contractile tension when IB-MECA was present and then observe the relinquishing of this suppression when the drug was washed out. The rates of recovery of contractility were calculated for five minute segments

of the first half hour of reperfusion, and then the rate of recovery seen in the presence of IB-MECA compared to the rate that would otherwise be seen in its absence. The results showed that the rate of recovery of contractile tension is significantly faster between 5-10 minutes of reoxygenation compared to the rates observed both at the first five minutes of reoxygenation and from 15 minutes post reoxygenation. 300nM IB-MECA seems to produce a better recovery after the washout at fifteen minutes post reoxygenation. This suggests that the wash out removes a negatively inotropic effect. Further work would be required to establish the mediator of this inotropic effect.

The action of IB-MECA is to augment the rate of recovery. 300nM increases the rate of recovery both during the initial ten minutes of reoxygenation and then at the later stage of between 15-25 post reoxygenation minutes. What is interesting is that the beneficial effect of IB-MECA seems to be missing in the final five minutes before the wash of the tissue bath and the replenishment of the Krebs solution. This may be due to the accumulation of metabolites in the bath that are either acting to block or diminish the effects of IB-MECA. It is difficult to say whether the suppression of recovery rate is due to negative inotropy as no suppression of contractility compared to controls was seen. The loss in beneficial action of IB-MECA at the 10-15 minute post reoxygenation was probably not due to negative inotropy because the IB-MECA was present for the entire initial 15 minutes and did not hinder it for the first 10 minutes. However, the beneficial action of IB-MECA is seen after its removal from the baths (both it action to the rate of increase recovery of contractility and its overall effect to increase recovery). It has therefore triggered something beneficial, and the fact that it is a short contact time with the tissue is an aid to it actions. In order to prove this theory, further work with IB-MECA remaining in the contact with the tissue throughout reperfusion would have to be performed. Further investigation using A_1 receptor antagonists would be needed in order to confirm if IB-MECA is able to exert an A_1 mediated effect, and to confirm that IB-MECA's cardioprotective effect is A_3 receptor-mediated.

This picture of IB-MECA however is not the same when using the 1 μM dose. Although at this dose IB-MECA increased the initial 5 minutes of recovery, any

effect that it had on the further recovery seen with the lower dose, is lost. The A_1 receptor mediated negative inotropy may be greater at this dose and offset the improved recovery from A_3 receptor stimulation. This would explain why a bell shape dose dependency may occur with this drug's actions on atrial recovery.

It is difficult to say if IB-MECA is able to relieve stunning. IB-MECA cannot protect from any damage that occurred during simulated ischaemia as it was administered from the point of reperfusion. It may have protected the atria from any damage that occurs immediately post reperfusion, such as the generation of stunning. However there is a limitation of the model. The level of recovery in the absence of any drug intervention is around 40% its preischaemic level (Figure 4.3, ■). As the definition is that myocardial stunning is a reversibly injury, then in this model if any stunning has occurred then either that stunning has not been totally reversed at 60 minutes reoxygenation, or some other kind of irreversible damage has also occurred to the tissue in order to cause a depression in contractile function. As we cannot say what type of damage may be occurring it is difficult to draw inferences as to what the action of IB-MECA are. However IB-MECA increased the contractile recovery and so must provide some sort of protection against post-ischaemia dysfunction.

As 1 μM of IB-MECA is four orders of magnitude larger in concentration than the quoted K_i value (Figure 4.1), the loss of beneficial effect at this concentration may be due to non-specific effects, which were interfering with the action of IB-MECA or itself having a physiological effect on the atria. IB-MECA is known to be non-selective at relatively higher concentrations. Other work has been published that indicated that IB-MECA has a dose dependent effect (Maddock et al., 2002b). A point of interest is that in the first fifteen minutes of reperfusion this concentration of IB-MECA provided the greatest amount of recovery, the rate of which was quickest in the initial five minutes. But then after washout the recovery level lost ground to the lower concentration so that at 60 minutes the 300nM provided a significant aid to recovery.

4.4.3. Effect of IB-MECA on the ischaemic contracture

The diastolic tension increased during simulated ischaemia indicating contracture of the atria and ventricular strips. Upon reoxygenation there was a gradual return towards baseline but by 60 minutes the diastolic tension had failed to be restored to the pre-ischaemic level. This may suggest together with the discussion above that simulated ischaemia may be causing some non-reversible change.

The support of this is that IB-MECA does not affect the diastolic tension change and failed to restore diastolic tension to pre ischaemic level during reoxygenation. If an agent were protective it would be expected to have an effect on diastolic tension. IB-MECA did assist in the initial recovery of the contracture but it did not reduce this level at 60 minutes post reoxygenation. It is possible that a cardioprotective agent such as IB-MECA would inhibit or delay the onset of ischaemia induced contracture when administered prior to or during ischaemia. However whether such an agent could reverse or hasten recovery once contracture has been established is another matter. It appears that whatever the mechanism of protection from stunning by IB-MECA, the same pathway does not exert a reversal of the contracture.

4.4.4. Effect of IB-MECA on atrial recovery when dissection was delayed.

The beneficial action of IB-MECA at 300nM was only seen when the atria had been dissected immediately after sacrifice. In tissues removed after a 3-5 minute delay, cardioprotection by IB-MECA was lost. It was assumed that during the delay the tissue undergoes a longer length of ischaemia, and this is capable of exerting a larger preconditioning effect. Preconditioning of the heart can exert a cardioprotective effect against contractile dysfunction and myocardial infarction. Ischaemic preconditioning is the phenomenon whereby brief ischaemic episodes render muscle resistant to subsequent ischaemic damage (Murry et al., 1986). Of the two windows of protection are attributed to preconditioning, it is the "early phase" or classical preconditioning which occurs immediately after the initial ischaemia that appears to be effective here

(Gross and Gross, 2006). So this could be what is causing the tissue to recover differently during the ischaemic-reperfusion protocol. It would appear therefore that if there is already cardioprotection by a preconditioning stimulus (ischaemia) the cardioprotective drug such as IB-MECA fails to exert any additional effect. This is supported by the recovery of the ventricular strips shown in Figure 4.5. In this tissue, IB-MECA administration did not exert a cardioprotective effect at the same dose which had established protection in the isolated atria. Further examination revealed that even when the right ventricular strips were the first tissue isolated, IB-MECA still had no effect on the recovery of the right ventricle (Figure 4.6).

Here it may be concluded that the effect of IB-MECA may be dependent on the preconditioning state of the tissue. We know that delaying dissection of the tissue causes IB-MECA to have no effect in the atria. As ventricular tissue is much thicker than atria then the oxygen diffusion to the ventricle is much poorer during the dissection period. The lack of oxygen may be causing either more ischaemic damage or even establishing intrinsic preconditioning. Either way it is established that IB-MECA only exerts an observational cardioprotective effect in the left atria which has been immediately isolated. This contrasts with previous observations in guinea pig papillary muscle where IB-MECA did cause protection from stunning (Gardner et al., 2004). This may have been due to the fact that ventricular strips used here are relatively thicker than papillary muscles and therefore more likely to become ischaemic. The difference could also be due to a species difference between the two tissue types. This finding caused us to continue using only left atria in this ischaemia reperfusion model in the further work.

4.4.5. Conclusion and Future Steps

In this model of simulated ischaemia-reperfusion, administration of IB-MECA upon reoxygenation caused a greater recovery of contractile function following ischaemia in left atria provided it is isolated immediately. IB-MECA does not have an effect when the dissection of the tissue is delayed, or in ventricular

strips. The action of IB-MECA does support the role of the adenosine A_3 receptor in protection from ischaemia in the atria, but not in the right ventricle.

It is difficult to drawn solid conclusions using solely IB-MECA as the work has indicated that other effectors may be implicated in IB-MECA's role in recovery. In order to therefore tease out the role of the adenosine A_3 receptor in recovery, further investigation in the next chapter was performed using a variety of receptor antagonists, which may allow a greater understanding of the role of adenosine A_3 receptors in ischaemia.

CHAPTER FIVE

INVESTIGATION INTO THE MECHANISM OF THE

CARDIOPROTECTIVE EFFECT OF IB-MECA

5.1. Introduction

5.1.1. Objective

In Chapter Four the cardioprotective action of IB-MECA in an atrial model of recovery from ischaemia was established. Although the action of IB-MECA was established, the effectors by which this cardioprotection was induced needs to be determined.

In this chapter we take a logical approach with the pharmacological tools available to establish what receptors are being activated, what other possible mechanisms may be involved and what the effect of the contact time of IB-MECA has. Since IB-MECA failed to protect against contractile dysfunction in the ventricular strips, further analysis of these tissues was not undertaken in this chapter.

5.1.2. The adenosine receptor family

As previously discussed in Chapter Four, a number of studies have repeatedly demonstrated that activation of adenosine A_3 receptors is cardioprotective, by limiting injury and strongly supporting that A_3 receptors mediate classic ischaemic preconditioning (Headrick and Peart, 2005).

In order to establish that IB-MECA's actions are indeed A_3 receptor mediated, the specific A_3 receptor antagonist MRS1191 was used.

There also came a need to examine the contribution of A_1 receptors in IB-MECA's effect. DPCPX, the A_1 receptor antagonist was used. The adenosine A_3 receptor cascade has many mediators in common with those activated by A_1 receptor stimulation (Thourani et al., 1999a; Tracey et al., 1998), though some differing protective actions have been revealed by work in cultured myocytes; such as that A_1 receptor is linked via G_i to phospholipase C to produce cardioprotective responses while the A_3 receptor is coupled via RhoA to activate phospholipase D to produce cardioprotective responses (Lee et al., 2001; Parsons et al., 2000).

CGS15943 was used in its capacity as an antagonist at all the adenosine receptor subtypes (Kim et al., 1998). NECA was used in it capacity of being an agonist at all receptor subtypes.
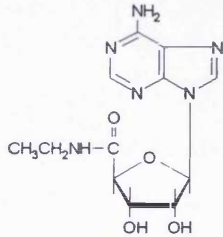
	CGS15943	NECA	Adenosine Deaminase
	9-chloro-2-(-2-funanyl)-[1,2,4]triazolo [1,5-c]quinazoline-5-amine	5'-N – Ethylcarboxamidoadenosine	
Adenosine A_1 receptor	20 nM (rat)[a]	0.59 nM (rat)[b] 14 nM (human)[c]	
Adenosine A_{2A} receptor	3nM (rat)[a]	460 nM (rat)[b] 20 nM (human)[c]	
Adenosine A_{2B} receptor		2400 nM (human)[c]	
Adenosine A_3 receptor	14nM (human)[d]	6.2 nM (human)[c]	

Figure 5.1. The chemical structures and selectivities of CGS15943, NECA and adenosine deaminase. CGS15943 is a potent and selective antagonist at all adenosine receptor subtypes. NECA is a potent adenosine receptor agonist. Adenosine deaminase is an enzyme that irreversibly deaminates adenosine, converting it to the related nucleoside inosine by the removal of an amine group. Receptor binding affinities (Ki) at the each adenosine subtype are shown. Taken from [a]Williams et al. (1987), [b]Bruns et al. (1986), [c]Klotz et al. (1998) and [d]Kim et al. (1996). For the structures and properties of MRS1191 and DPCPX please refer to Figure 6.1, and for IB-MECA refer to Figure 4.1.

5.1.3. Aims of this Chapter

The aims of the chapter were:

- Through the use of a variety of interventions we will attempt to establish the mechanisms by which IB-MECA has a cardioprotective effect.

- Establish if IB-MECA's effect is truly mediated through the adenosine A_3 receptor.

- Establish whether endogenous adenosine interferes with the actions of IB-MECA.

5.2. Methods

5.2.1. Protocols

The isolated paced left atria model was used as described in Chapter Three and Four. A period of 30 minutes simulated ischaemia was performed followed by one hour of reoxygenation. This model has been established as the best model to show the effects of interventions provided that minimum preconditioning takes place during the set up of the tissues.

For further details on the set up of the isolated atria see Chapter Two.

5.2.1.1. Simulated Ischaemia

Simulated ischaemia was induced by switching the bathing solution to a glucose-free Krebs solution and gassing the bathing solution with 5% CO_2 in 95% N_2 for a period of thirty minutes. After this the tissues were reoxygenated by returning to gassing with 5% CO_2 in 95% oxygen and changing the bathing solution back to normal Krebs. Reoxygenation continued for one hour, with a wash of the tissue bath every fifteen minutes. The protocol is diagrammatically represented in Figure 4.2, Protocol i.

5.2.1.2. Drug Administration Protocols

The drug interventions administered and their corresponding concentrations are detailed in Figure 5.2. The control group received the appropriate concentration of the vehicle dimethyl sulfoxide (DMSO). All concentrations quoted are the final concentrations in the tissue bath.

We have previously shown that in this model of ischaemia the administration of the adenosine A_3 receptor agonist IB-MECA at the point of reperfusion brings about a cardioprotective effect at concentrations ranging from 3nM to 300nM in the isolated atria (see section 4.3.3.) For continued use in this chapter the concentration of 300nM IB-MECA was used. 300nM is the dose that have been employed in previous in previous studies in this laboratory. Gardner et al. (2004) used 300nM IB-MECA in a study of myocardial stunning in guinea pig atria and Maddock et al. (2003) also used the same dose in the study of stunning in whole hearts.

The adenosine A_3 receptor agonist IB-MECA was used at a 300nM concentration in a vehicle of a final bath concentration of 0.02% v/v DMSO. The antagonists MRS1191 and CGS15943 were used in a vehicle of 0.2% v/v DMSO. DPCPX was used in a vehicle of 0.02% v/v DMSO. The adenosine receptor agonist NECA was used at a 1μM concentration in a vehicle of 0.1% v/v DMSO. Adenosine deaminase was used at a 1U/ml organ bath concentration and dissolved in distilled water. The manner by which all drug were employed throughout the experiment is detailed in Figure 5.2.
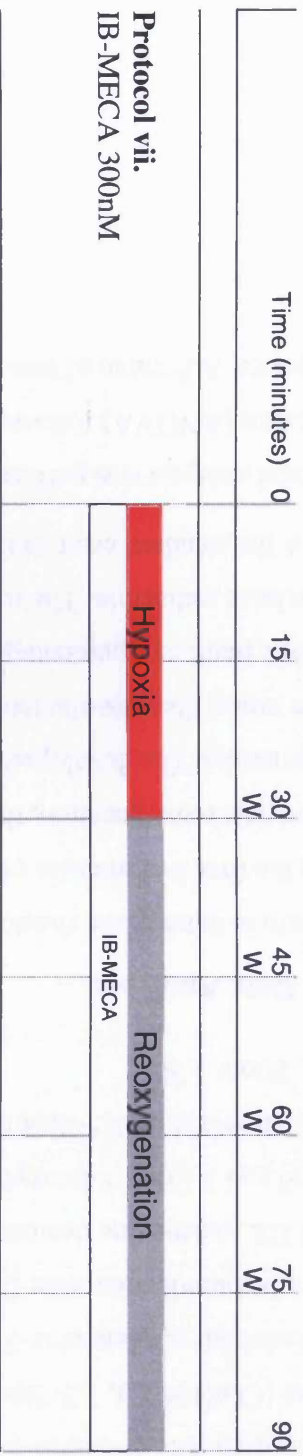
Protocol vii.
IB-MECA 300nM

| Time (minutes) | 0 | 15 | 30 W | 45 W | 60 W | 75 W | 90 |

Hypoxia

IB-MECA

Reoxygenation

Figure 5.2. Experimental protocols of simulated ischaemia for all drug interventions employed in this chapter. All protocols were preceded by at least 25 minutes stabilisation until a steady baseline of contraction was achieved. "W" indicated the point of wash-out of the tissue bath.

Protocol i.
Vehicle only

Protocol ii.
MRS1191 1µM & vehicle
MRS1191 1µM &
IB-MECA 300nM

Protocol iii.
DPCPX 200nM & vehicle
DPCPX 200nM &
IB-MECA 300nM

Time (minutes) 0 15 30 W 45 W 60 W 75 W 90

Hypoxia — Antagonist Vehicle Control — Reoxygenation

Hypoxia — MRS1191 — Reoxygenation — IB-MECA or Vehicle

Hypoxia — DPCPX — Reoxygenation — IB-MECA or Vehicle

Time (minutes): 0 | 15 | 30 W | 45 W | 60 W | 75 W | 90

Protocol iv.
CGS15943 1µM & vehicle
CGS15943 1µM &
IB-MECA 300nM

Hypoxia — Reoxygenation
CGS15943
IB-MECA or Vehicle

Protocol v.
Adenosine Deaminase 1U/ml &
vehicle
Adenosine Deaminase 1U/ml &
IB-MECA 300nM

Hypoxia — Reoxygenation
Adenosine Deaminase
IB-MECA

Protocol vi.
Antagonist vehicle and vehicle.
NECA 1µM & antagonist vehicle
NECA 1µM &
DPCPX 200nM

Hypoxia — Reoxygenation
DPCPX or Antagonist Vehicle
NECA or Vehicle

5.2.2. Drugs

Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific, Loughbourgh, UK. 9-chloro-2-(-2-funanyl)-[1,2,4]triazolo[1,5-c]quinazoline-5-amine (CGS15943), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) and 5'-N – Ethylcarboxamidoadenosine (NECA) were obtained from Tocris Limited, Bristol UK. Adenosine deaminase (Adenosine Deaminase from calf intestinal mucosa) and 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) were obtained from Sigma Alrich, Poole, UK.

5.2.3. Data Analysis

The systolic tension and diastolic tension were measured every 0.5 minutes during the first five minutes of reoxygenation, then every minute until 15 minutes post reoxygenation, then every 5 minutes until 60 minutes post reoxygenation. The developed contractile tension was calculated as the systolic tension minus the diastolic tension. This developed tension was expressed at each time point as a percentage of the baseline developed tension before onset of simulated ischaemia. The mean of each time point in each experimental group ± the standard error of the mean (S.E.M.) was used in graphs and tables.

Statistical analysis was performed using either a paired t-test or paired analysis of variance (ANOVA) followed by Bonferroni's post hoc test, when appropriate. A P value of less than 0.05 was considered statistically significant.

5.3. Results

5.3.1. Use of the antagonist vehicle control

Because in this chapter antagonists were employed throughout, a control group of experiments that used the antagonist's vehicle was required to allow comparison. The highest concentration of vehicle used was 0.2% v/v DMSO / water which was present in the Krebs throughout the protocol. This protocol is illustrated in Figure 5.2, protocol i. The recovery of the left atria from simulated ischaemia is shown in Figure 5.3.

Isolated left atria, when in the presence of 0.2%v/v DMSO, and when IB-MECA (300nM) was administered at reoxygenation had a recovery of 72.3 ± 2.1% of the baseline developed tension ($n=6$), which was significantly greater than the control group with no vehicle (50.8 ± 7.7%, $n=5$) or the control exposed to 0.2% v/v DMSO throughout (47.4 ± 4.8%, $n=5$). The resulting recovery is shown in Figure 5.2.

Statistical analysis showed that 0.2% DMSO v/v the vehicle for the antagonists used in this chapter, did not affect the recovery following simulated ischaemia (■ versus ●). The recovery of the atria following administration of IB-MECA upon reperfusion in the presence of 0.2% DMSO v/v throughout (□ versus ○), was also not significantly different (72.3 ± 2.1% $n=6$) than the IB-MECA affected recovery when DMSO was not present (76.5 ± 5.8%, $n=6$)
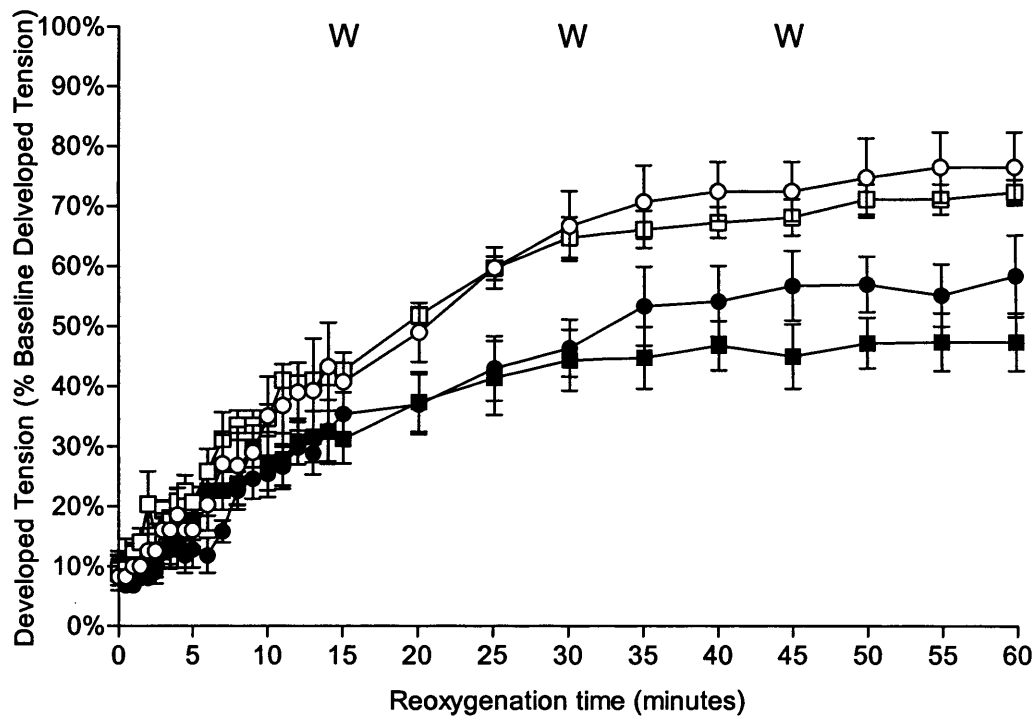
Figure 5.3. The control post ischaemic recovery. Left atrial recovery from thirty minutes of simulated ischaemia when the vehicle control (0.02% v/v DMSO) was present (■, n=5). IB-MECA (300nM) administered upon reoxygenation (300nM, □, n=6), when 0.2% v/v DMSO is present in the Krebs throughout simulated ischaemia and reoxygenation is also shown. For comparison the recovery of left atria in unadulterated Krebs is shown when the agonist vehicle was administered upon reoxygenation (0.02% v/v DMSO, ●, n=5) or IB-MECA was administered upon reoxygenation (300nM, ○, n=6). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.

5.3.2. MRS1191

In order to confirm whether the cardioprotection brought about by IB-MECA's administration is mediated by the adenosine A_3 receptor, the use of an adenosine A_3 receptor antagonist is necessary. If protection is indeed A_3 receptor-mediated then an A_3 receptor antagonist should competitively block the action of IB-MECA at that receptor.

The antagonist used was the rat A_3 receptor specific antagonist MRS1191. The use of MRS1191 as oppose to other adenosine A_3 receptor antagonists was to ensure species specificity. This is important as previous investigations in other species have been hampered due to species difference in the receptor structure and specificity of the drugs available. MRS1191 is one of the two commercially available antagonists at the adenosine A_3 receptor (Alexander et al., 2006). MRS1191 was present throughout as it was added to the tissue bath during simulated ischaemia and reoxygenation. This ensured that binding to the receptor was at equilibrium throughout and blocked the receptor before IB-MECA was added.

The K_i of MRS1191 at the human adenosine A_3 receptor has been determined as 31 nM (Jacobson, 1998). The concentration previously in other studies employing MRS1191 has been 1μM (Maddock et al., 2002b), and this was thought to be adequate to ensure receptor block. This concentration was used initially, and then subsequently increased to 3μM. Generally, adenosine A_3 receptor antagonists are "thought to have little effect on ischaemic outcomes" (Headrick and Peart, 2005)

MRS1191 was administered as illustrated in Figure 5.2 protocol ii. The recovery of left atria from simulated ischaemia when administrating MRS1191 alone and with IB-MECA is shown in Table 5.1, and detailed in Figure 5.4 and Figure 5.5.

Table 5.1 Table detailing the recovery of the left atria following ischaemia at various time points following reoxygenation following the interventions with IB-MECA and MRS1191. Values are the mean recovery of the atrial developed tension ± the S.E.M., expressed as a % of the pre-ischaemic developed tension. The values are also graphically displayed in Figure 5.4 and Figure 5.5

	Control $n=5$	IB-MECA 300nM $n=6$		MRS1191 1µM $n=5$	MRS1191 3µM $n=3$		IB-MECA 300nM & MRS1191 1 µM $n=6$	IB-MECA 300nM & MRS1191 3µM $n=5$
15 minutes post reoxygenation	31.2 ± 4.07%	42.7 ± 0.49%*		29.4 ± 8.94%	45.0 ± 3.21%		50.7 ± 5.98%*	47.4 ± 7.01%
30 minutes post reoxygenation	44.4 ± 5.09%	64.8 ± 3.38%†		43.4 ± 11.2%	60.0 ± 4.58%		69.7 ± 5.98%*	55.2 ± 7.51%
60 minutes post reoxygenation	47.4 ± 4.82%	72.3 ± 2.09%†		48.6 ± 9.64%	64.0 ± 6.81%		77.8 ± 5.41%$^{†/‡}$	61.6 ± 5.77%

* (P<0.05, unpaired t-test comparison to controls) considered significant

†(P<0.01, unpaired t-test comparison to controls) considered very significant

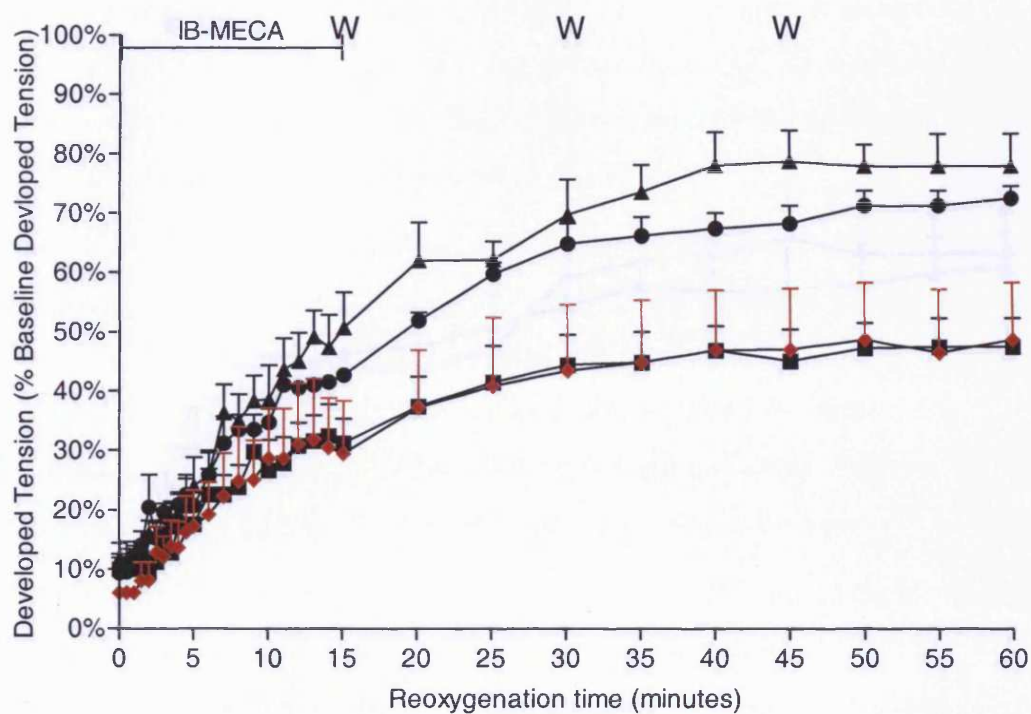‡(P<0.05, unpaired t-test comparison to MRS1191 1µM group) considered significant

Figure 5.4. The Effect of MRS1191 (1μM) on post-ischaemic recovery. The effect of MRS1191 (1μM) on the recovery of the left atrial developed tension following thirty minutes of simulated ischaemia when it is administered alone (◆, $n=5$) and when IB-MECA is additional administered at reoxygenation (300nM ▲, $n=6$). Also shown is the recovery of the left atria from simulated ischaemia when the appropriate vehicle was administered (■, $n=5$), and when IB-MECA alone was administered upon reoxygenation (300nM ●, $n=5$). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.

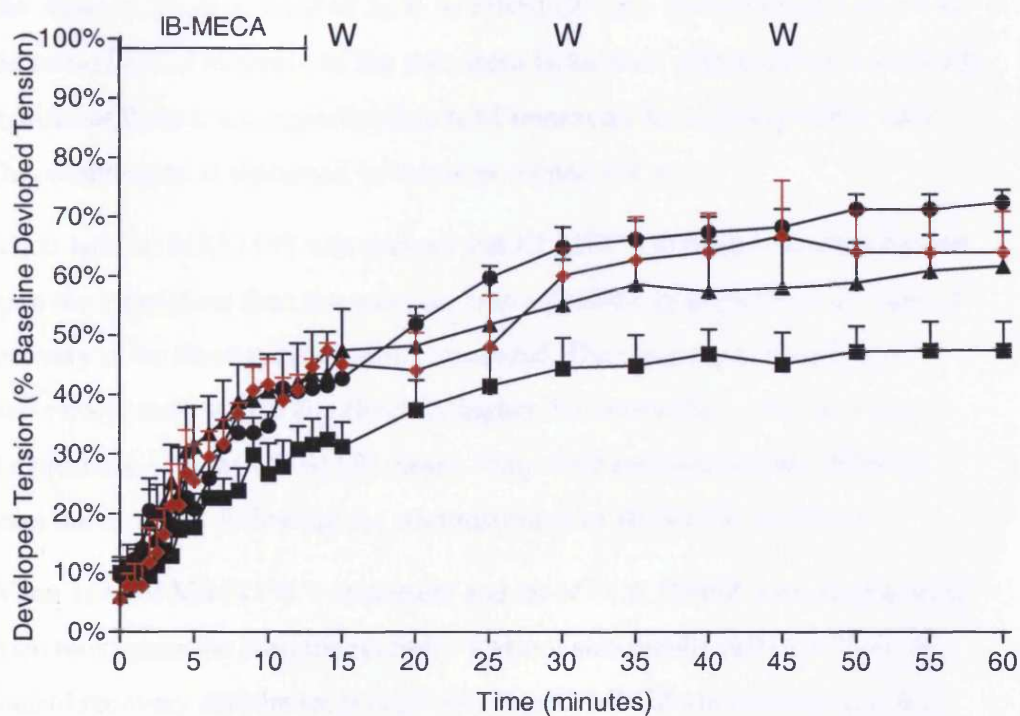**Figure 5.5. The Effect of MRS1191 (3µM) on post-ischaemic recovery.** The effect of MRS1191 (3µM) on the recovery of the left atrial developed tension following thirty minutes of simulated ischaemia when it is administered alone (♦, n=5) and when IB-MECA is additional administered at reoxygenation (300nM ▲, n=6). Also shown is the recovery of the left atria from simulated ischaemia when the appropriate vehicle was administered (■, n=5), and when IB-MECA alone was administered upon reoxygenation (300nM, ●, n=5). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.

The administration of $1\,\mu M$ or $3\,\mu M$ of MRS1191 also upon onset of ischaemia did not affect the recovery of the atria from ischaemia. Although not statistically significant there is a suggestion that $3\,\mu M$ improved the recovery of the atria. This observation is discussed in below in section 5.4.3.

When $1\,\mu M$ of MRS1191 was present and IB-MECA 300nM was administered upon reoxygenation then the recovery was significantly higher that the control recovery at the three minute points measured. The recovery at 60 minutes post-reoxygenation also significantly higher that when the tissue recovered in the presence of $1\,\mu M$ MRS1191 alone. They were not significantly different from the recovery following the administration of IB-MECA on it own.

When $3\,\mu M$ of MRS1191 was present and IB-MECA 300nM was administered upon reoxygenation then the recovery was not statistically different from the control recovery and the recovery following IB-MECA administration alone.

The level of recovery after IB-MECA was administered in the presence of MRS1191 ($3\,\mu M$) was similar following IB-MECA administration alone (Figure 5.5).

5.3.3. DPCPX

IB-MECA's cardioprotective actions could not therefore be fully attributed to the A_3 receptor. The possibility of A_1 receptor activation being involved was next investigated. DPCPX being a specific A_1 receptor antagonist would block IB-MECA's effect if it is A_1 receptor mediated.

DPCPX 200nM was administered alone and together with IB-MECA as illustrated in Figure 5.2 protocol iii. The resulting recovery of developed tension following ischaemia is detailed in Table 5.2, and shown in Figure 5.6.

The presence of 200nM DPCPX caused a significant improvement to the post-ischaemic recovery at 30 and 60 minutes post reoxygenation. When IB-MECA was also administered at reoxygenation the post ischaemic recovery was not significantly different from that of control.

Table 5.2 Table detailing the recovery of the left atria following ischaemia at various time points following the interventions with DPCPX. Values are the mean recovery of the atrial developed tension ± S.E.M., expressed as a % of the pre-ischaemic developed tension.

	Control $n=5$	IB-MECA 300nM $n=6$	DPCPX 200nM $n=7$	DPCPX 200nM & IB-MECA 300nM $n=7$
15 minutes post reoxygenation	31.2 ± 4.07%	42.7 ± 0.49%*	44.3 ± 5.60%	41.3 ± 5.62%
30 minutes post reoxygenation	44.4 ± 5.09%	64.8 ± 3.38%†	62.6 ± 3.32%*	55.63 ± 6.46%
60 minutes post reoxygenation	47.4 ± 4.82%	72.3 ± 2.09%†	69.4 ± 3.42%†	61.63 ± 6.88%

* ($P<0.05$, unpaired t-test comparison to controls) considered significant

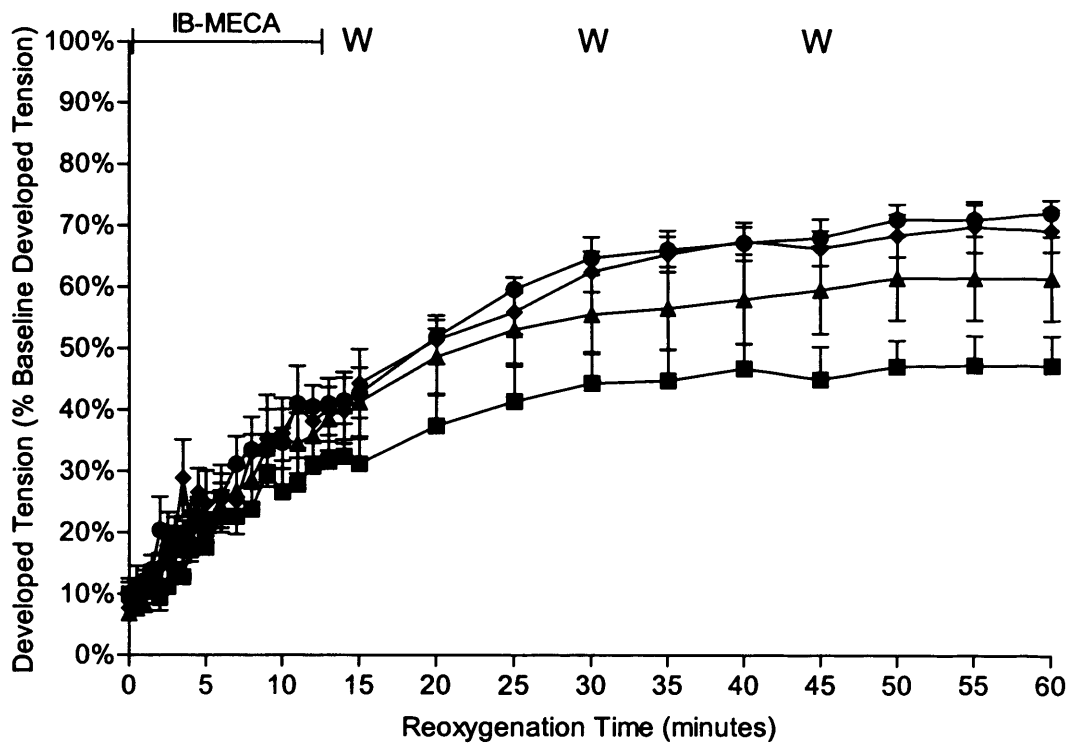† ($P<0.01$, unpaired t-test comparison to controls) considered very significant

Figure 5.6. The effect of DPCPX on post-ischaemic recovery. The effect of DCPCX (200nM) on the recovery of left atrial developed tension following thirty minutes of simulated ischaemia is shown (◆, *n*=5). Also shown is the effect of IB-MECA (300nM) when administered upon reperfusion in the presence of DPCPX (▲, *n*=6). For comparison the recovery from ischaemia in the presence of the appropriate vehicle is shown (■, *n*=5), and when IB-MECA was administered upon reperfusion alone (300nM ●, *n*=5). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.

It was found that DPCPX itself is cardioprotective in this model. It increased recovery after ischemia compared to control at 30 and 60 minutes post reoxygenation.

5.3.4. CGS15943

So far a complex picture of IB-MECA's actions has emerged. The next stage was to eliminate other members of the adenosine receptor family using the adenosine receptor antagonist CGS15943

To eliminate adenosine receptors as a source which evokes cardioprotection, an antagonist that acts to block activation at all four adenosine subtypes was used. It was hoped that using this alone and in combination with IB-MECA would show if IB-MECA mediates cardioprotection by an adenosine receptor-mediated event.

CGS15943 (200nM) was administered as detailed in Figure 5.2, protocol vi., both alone and together with IB-MECA. The resulting recovery of developed tension is shown in Figure 5.7. and detailed in Table 5.3.

The administration of CGS15943 alone throughout the protocol caused a significant increase of the post ischaemic recovery. When IB-MECA was also administered during the first fifteen minutes of reoxygenation while in the presence of CGS15943, an increased recovery was observed. For the first fifteen minutes of reoxygenation the two drug together brought about a higher recovery than either IB-MECA or CGS15943 did when used alone.
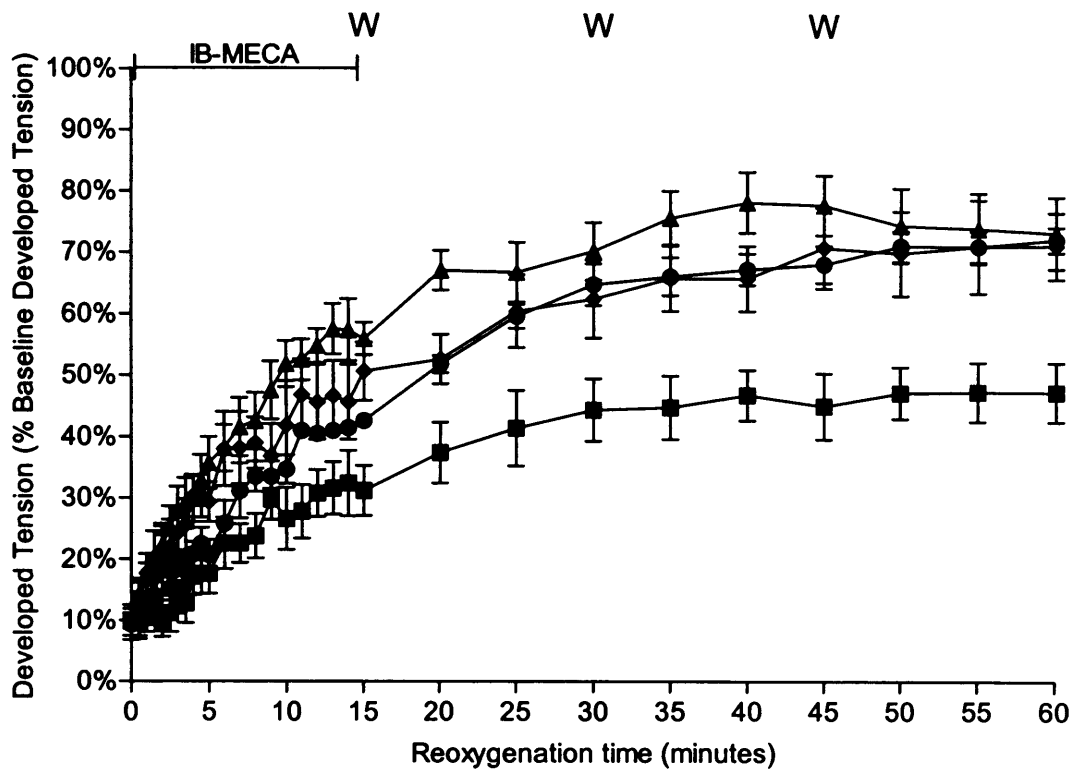
Figure 5.7. Effect of CGS15943 on post ischaemic recovery. The effect of CGS15943 (200nM) on the recovery of the left atria's developed tension following thirty minutes of simulated ischaemia is shown (◆, *n*=5) and when IB-MECA was administered upon reperfusion also (300nM ▲, *n*=6).). For comparison the recovery of the atria following the administration of IB-MECA (300nM) alone upon reoxygenation is also shown (●, *n*=6) and in the presence of the appropriate vehicles (■, *n*=5). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.

Table 5.3 Table detailing the recovery of the left atria following ischaemia at various time points following the interventions with CGS15943, as detailed in Figure 5.2, protocol vi.. Values are the mean recovery of the atrial developed tension ± S.E.M., expressed as a % of the pre-ischaemic resting developed tension.

	Control $n=5$	IB-MECA 300nM $n=6$	CGS15943 200nM $n=6$	CGS15943 200nM & IB-MECA 300nM $n=7$
15 minutes post reoxygenation	31.2 ± 4.07%	42.7 ± 0.49%*	50.7 ± 4.73%*	56.0 ± 2.68%[†]/[‡]
30 minutes post reoxygenation	44.4 ± 5.09%	64.8 ± 3.38%[†]	62.5 ± 5.34%*	70.3 ± 4.71%[†]
60 minutes post reoxygenation	47.4 ± 4.82%	72.3 ± 2.09%[†]	71.3 ± 5.42%*	73.4 ± 5.87%[†]

* ($P<0.05$, unpaired t-test comparison to controls) considered significant

[†] ($P<0.01$, unpaired t-test comparison to controls) considered very significant

[‡] ($P<0.05$, unpaired t-test comparison to IB-MECA 300nM group) considered significant

5.3.5. Adenosine deaminase

It was considered that endogenous adenosine (which is evolved during ischaemia by the catabolism of ATP) may modify the tissue's response to the drug interventions.

In order to say if endogenous adenosine is influencing the tissue's response it must be removed from the bathing medium as soon as it is evolved, preventing any action it may have. To achieve this the enzyme adenosine deaminase was added to the bathing Krebs. Adenosine deaminase irreversibly deaminates adenosine to inosine. The concentration used (1U/ml of Krebs) has been shown in a similar model to abolish the maximum negative inotropic effect of adenosine in guinea pig atria (Gardner and Broadley, 1999). Adenosine deaminase was used as described in Figure 5.2, protocol v., both alone and together with IB-MECA. The resulting recovery is shown in Figure 5.a and b.
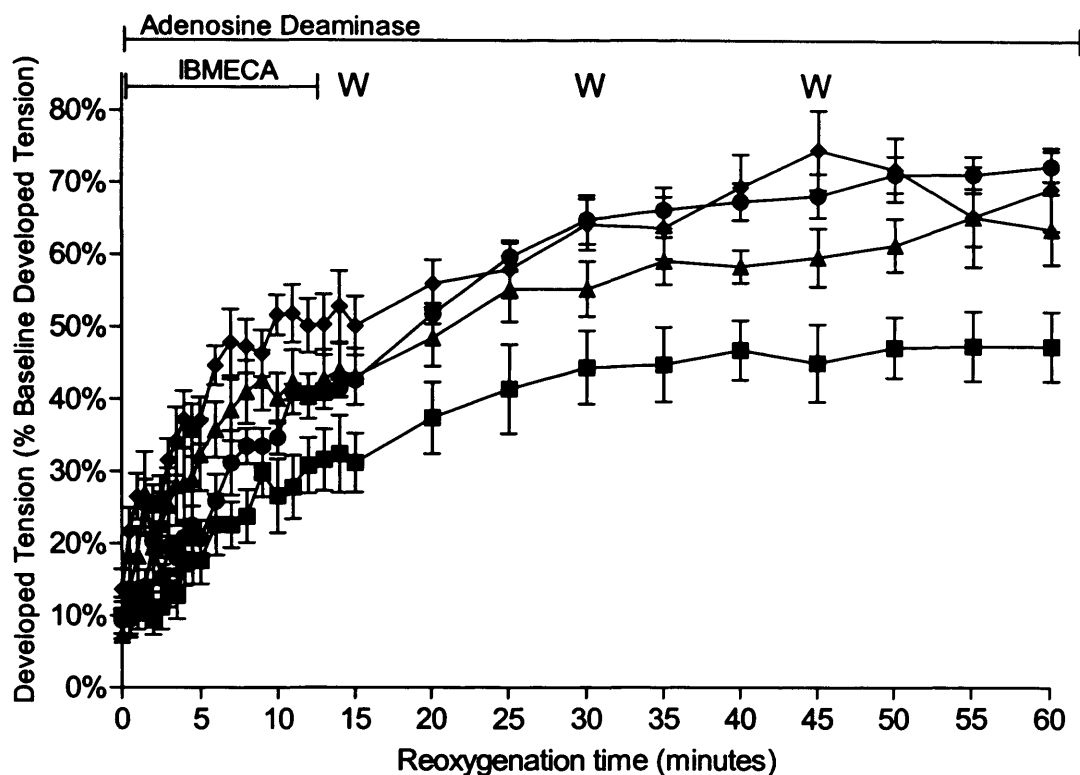
Figure 5.8a. Effect of adenosine deaminase on post ischaemic recovery. The effect of adenosine deaminase (1U/ml) on the recovery of the left atrial developed tension following thirty minutes of simulated ischaemia when present alone (◆, n=6) and when IB-MECA was additionally administered upon reperfusion (300nM ▲, n=7). For comparison the recovery of the atria following the administration of IB-MECA alone upon reoxygenation is also shown (300nM ●, n=6) and in the presence of the appropriate vehicles (■, n=5). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.
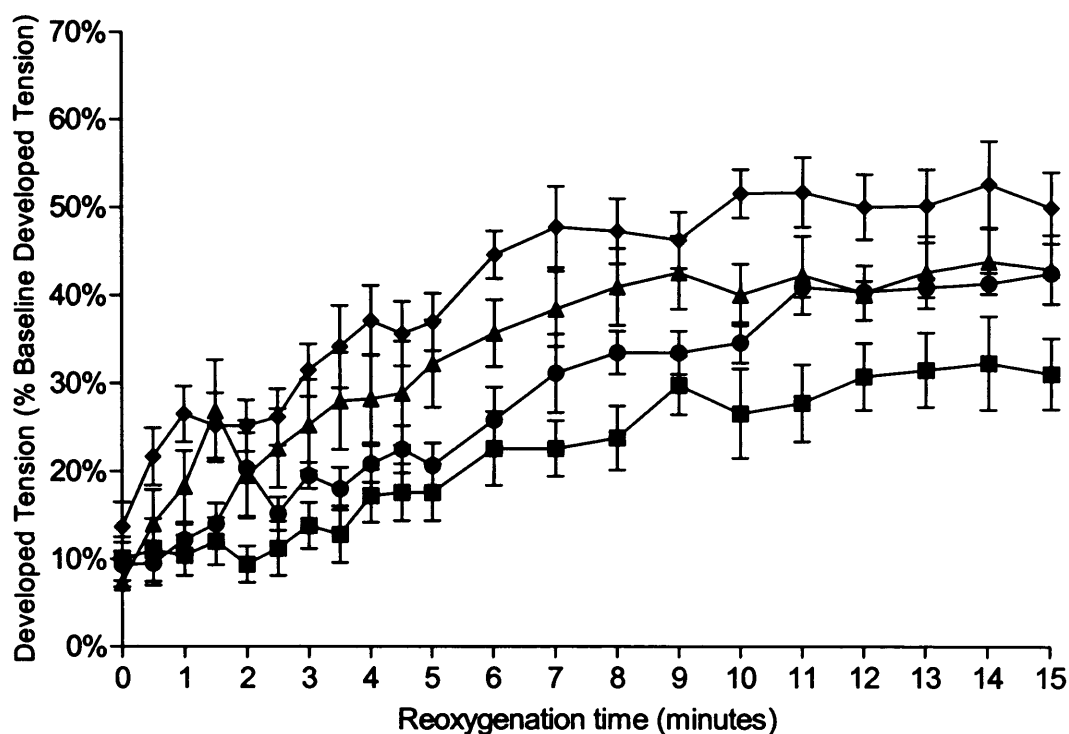
Figure 5.8b. Detail of the initial fifteen minutes of the recovery in the presence of adenosine deaminase. The effect of adenosine deaminase (1U/ml) on the recovery of the left atrial developed tension following thirty minutes of simulated ischaemia when present alone (◆, $n=6$) and when IB-MECA was additionally administered upon reperfusion (300nM ▲, $n=7$). For comparison the recovery of the atria following the administration of IB-MECA alone upon reoxygenation is also shown (300nM ●, $n=6$) and in the presence of the appropriate vehicles (■, $n=5$). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension.

Table 5.4 Table detailing the recovery following simulated ischaemia of the left atria in the presence of adenosine deaminase. Values are the mean recovery of the atrial developed tension ± S.E.M., expressed as a % of the pre-ischaemic resting developed tension.

	Control n=5	Adenosine Deaminase (1U/ml) n=6	IB-MECA 300nM n=6	Adenosine Deaminase (1U/ml) & IB-MECA (300nM) n=8
15 minutes post reoxygenation	31.2 ± 4.07%	50.2 ± 4.08%†	42.7 ± 0.49%*	43.1 ± 3.93%
30 minutes post reoxygenation	44.4 ± 5.09%	64.2 ± 3.52%†	64.8 ± 3.38%†	55.3 ± 3.81%
60 minutes post reoxygenation	47.4 ± 4.82%	69.2 ± 5.77%*	72.3 ± 2.09%†	63.6 ± 4.90%

* (P<0.05, unpaired t-test comparison to controls) considered significant

† (P<0.01, unpaired t-test comparison to controls) considered very significant

Statistical analysis showed that adenosine deaminase increased recovery from ischaemia in comparison to controls, to a level comparable to that achieved when IB-MECA was administered upon reperfusion.

The presence of adenosine deaminase changes the tissues response to IB-MECA so that although recovery was not significantly different from when IB-MECA was administered alone, the recovery was now not significantly different from the control recovery.

5.3.6. IB-MECA present throughout recovery

We investigated if IB-MECA brought about an additional benefit if it was present throughout reoxygenation. While a straightforward conclusion might be drawn if it does, e.g. IB-MECA brings about cardioprotection, it may also allow us to pinpoint when in the reperfusion cascade IB-MECA may act. If administration of IB-MECA throughout reoxygenation caused protection to be less, the conclusion could be that IB-MECA evokes A_1 receptor-mediated

negative iontropy, which less suppresses the tissue's contractility throughout reperfusion.

To investigate this IB-MECA (300nM) was administered throughout ischaemia and reoxygenation, as illustrated in Figure 5.2, protocol vii.
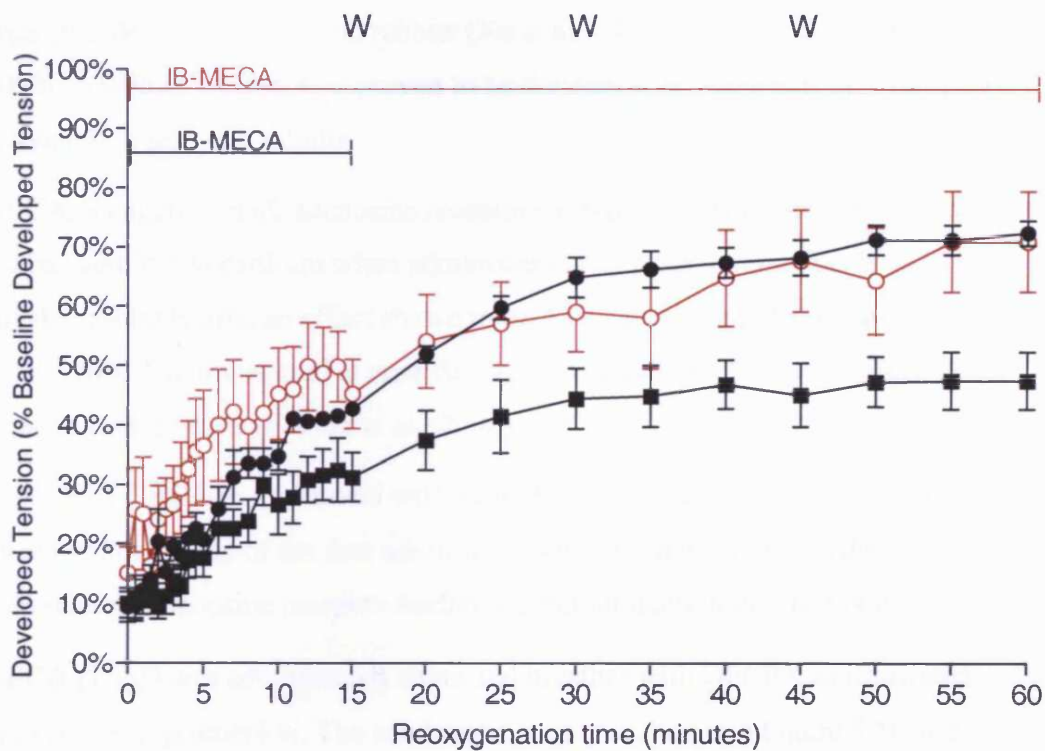

Figure 5.9. The effect of IB-MECA (300nM) administered at reperfusion, when it was administrated for the first fifteen minutes of reperfusion only (●, n=6) or present throughout reoxygenation (○, n=8). The recovery in presence of the appropriate vehicle is also shown (■, n=5). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.

It was found that the presence of IB-MECA throughout reperfusion caused no statistical difference in the recovery of the tissue than if IB-MECA was present for the first fifteen minutes of the reperfusion.

5.3.7. NECA and other interventions

In the literature the use of an adenosine agonist with nearly equivalent affinities for A_1 and A_2 receptors is shown to cause cardioprotection when administered at the time of reperfusion. The adenosine agonist AMP579 when infused at or shortly before reperfusion decreased infarct size in pigs (Smits et al., 1998), dogs (Budde et al., 2000), and rabbits (Xu et al., 2001; Xu et al., 2002; Xu et al., 2000). AMP579's effect was proven to be dependent on adenosine A_{2A} receptor activation in pigs and rabbits.

NECA, an agonist at all adenosine receptor subtypes, has been shown to salvage the ischaemic myocardium when administered just prior to reperfusion in isolated rabbit hearts, an effect shown to be A_2 receptor mediated (Yang et al., 2004). NECA administered at reperfusion has been shown to limit infarct size in isolated rabbit hearts (Forster et al., 2006).

The use of NECA in this model will show if cardioprotection is brought about by activation of any of the four adenosine receptors, and if it is possible to demonstrate adenosine receptor-mediated cardioprotection in this model.

NECA ($1\,\mu M$) was administered alone and together with DPCPX as illustrated in Figure 5.2, protocol vi. The resulting recovery is shown in Figure 5.10 and 5.11

Administering NECA ($1\,\mu M$) increased the recovery of contractile function during reoxygenation indicative of causing cardioprotection. NECA was able to bring about increased recovery to that caused by IB-MECA administration in the initial fifteen minutes of reoxygenation.

As observed earlier DPCPX alone improved post ischaemic recovery. When NECA was administered in the presence of DPCPX, the recovery was equivalent to that seen when DPCPX was used alone (Figure 5.11), and is less than the recovery achieved when NECA alone was used.

Table 5.5 Table detailing the recovery of the left atria following ischaemia at various time points following the interventions with NECA and DPCPX, as detailed in Figure 5.2, protocol vi. Values are mean recovery of tension expressed as a % of the pre-ischaemic developed tension ± S.E.M.

	Control *n=5*	IB-MECA 300nM *n=6*		DPCPX 200nM *n=7*		NECA 1μM *n=5*		DPCPX 200nM & NECA 1μM *n=4*
15 minutes post reoxygenation	31.2 ± 4.07%	42.7 ± 0.49%*		44.3 ± 5.60%		57.2 ± 3.34%$^{t/\ddagger}$		45.5 ± 7.92%
30 minutes post reoxygenation	44.4 ± 5.09%	64.8 ± 3.38%^{t}		62.6 ± 3.32%*		73.2 ± 4.91%^{t}		55.0 ± 11.09%
60 minutes post reoxygenation	47.4 ± 4.82%	72.3 ± 2.09%^{t}		69.4 ± 3.42%^{t}		82.8 ± 3.53%$^{t/\ddagger}$		72.0 ± 9.35% *

* ($P<0.05$, unpaired t-test comparison to controls) considered significant

^{t} ($P<0.01$, unpaired t-test comparison to controls) considered very significant

‡ ($P<0.05$, unpaired t-test comparison to IB-MECA 300nM group) considered significant
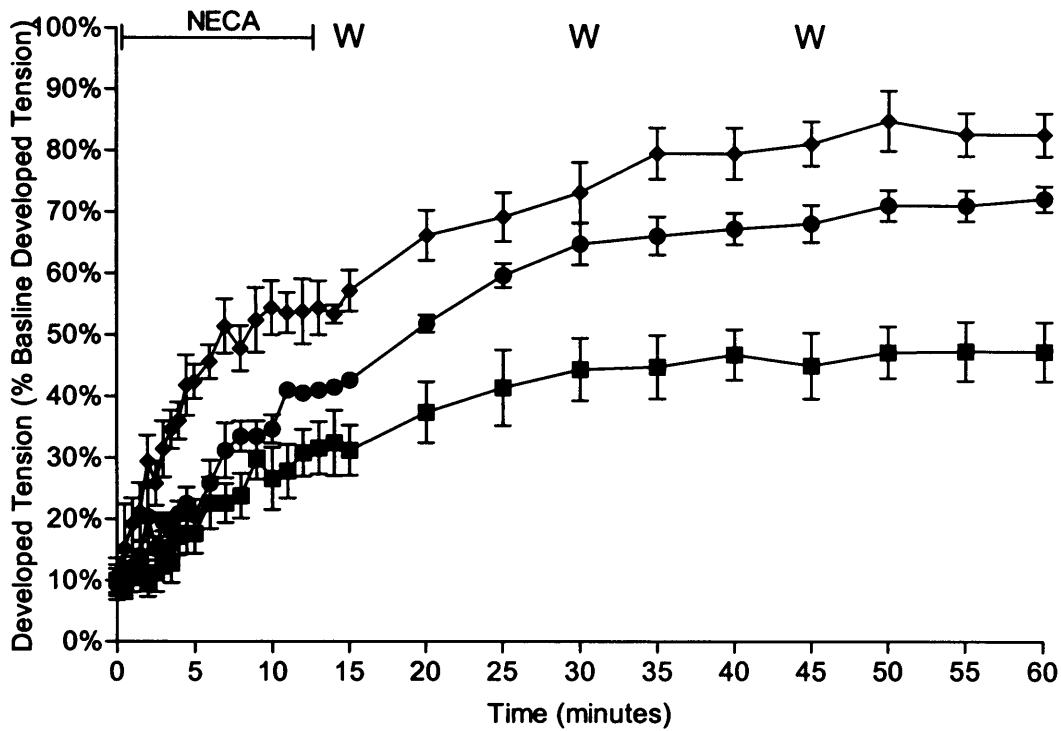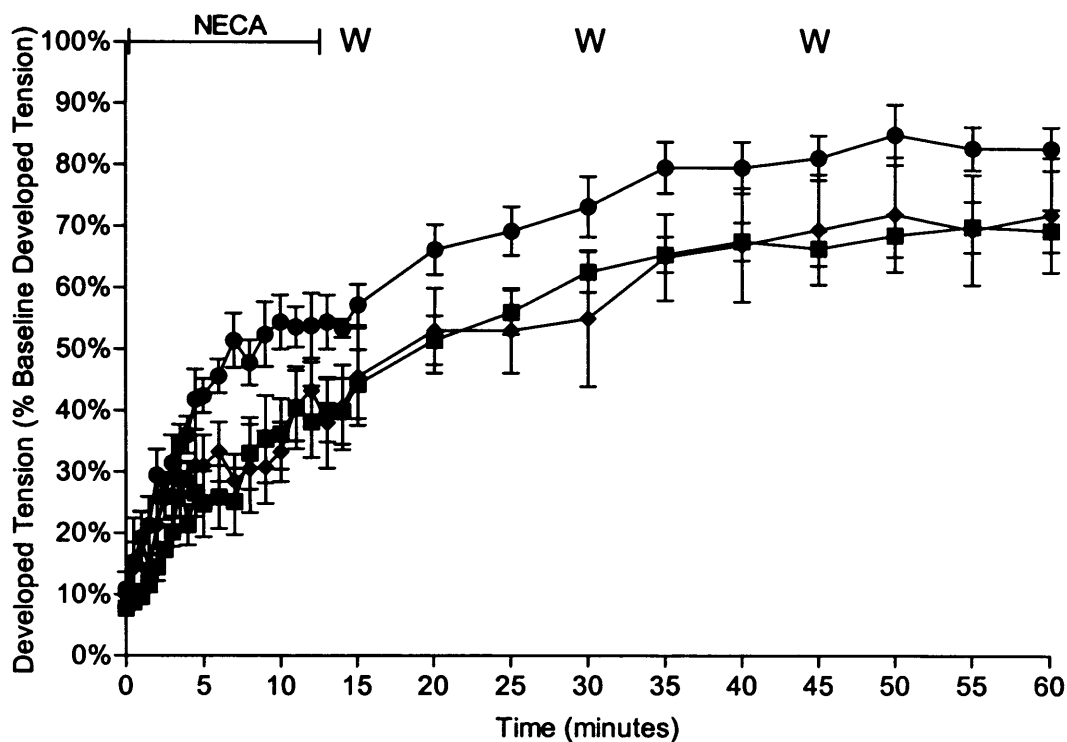
Figure 5.10. Effect of NECA. The effect of NECA (1µM) on the recovery of
the left atrial developed tension following thirty minutes of simulated ischaemia
(◆, *n*=5). For comparison the recovery of the atria following the administration
of IB-MECA (300nM) alone upon reoxygenation is also shown (●, *n*=6) and in
the presence of the appropriate vehicles (■, *n*=5). Each point represents the
mean developed tension (± S.E.M.) expressed as a percentage of the
pre-ischaemic developed tension. 'W' indicates the point of wash-out of the
organ baths.

Figure 5.11. Effect of NECA and DPCPX on recovery. The effect of NECA (1 μM) on the recovery of the left atrial developed tension following thirty minutes of simulated ischaemia when administered alone (●, *n*=5) and together with DPCPX 200nM (◆, *n*=4). Also shown is the recovery when DPCPX was administered alone (200nM ■, *n*=7). Each point represents the mean developed tension (± S.E.M.) expressed as a percentage of the pre-ischaemic developed tension. 'W' indicates the point of wash-out of the organ baths.

5.3.8. Summary

Table 5.5 summarises the observations made in this chapter.

Table 5.5 Summary of the observations and the statistical analysis of this chapter. The effect of the administration of various adenosine receptor agents in isolated left atria ischaemic model are described.

Drugs Used (Concentration)	Adenosine Receptors "stimulated"	Adenosine Receptors "blocked"	General observations
1 **IB-MECA** (300nM)	A₃ receptor		Cardioprotection observed throughout reoxygenation. Increased recovery of pre-ischaemic function from ~50% to ~70% at 60 minutes post-reoxygenation.
2 **MRS1191** (1μM & 3μM)		A₃ receptor	No effect on the recovery. Suggestion that 3μM may act to improve recovery. *Considered a statistically underpowered experiment (see discussion).*
3 **IB-MECA** (300nM) & **MRS1191** (1 μM)	A₃ receptor	A₃ receptor	Cardioprotection observed. Increased recovery of pre-ischaemic function from ~50% to ~80% at 60 minutes post reoxygenation
4 **IB-MECA** (300nM) & **MRS1191** (3μM)	A₃ receptor	A₃ receptor	There is no effect on the recovery. *Considered a statistically underpowered experiment (see discussion).*
5 **DPCPX** (200nM)		A₁ receptor	Cardioprotection observed, in the last 30 minutes of the hour's reperfusion. Increased recovery of pre-ischaemic function from ~50% to ~70% at 60 minutes post reoxygenation
6 **IB-MECA** (300nM) & **DPCPX** (200nM)	A₃ receptor	A₁ receptor	There is no change in the recovery throughout reoxygenation compared to control
7 **CGS15943** (1 μM)		All adenosine receptors	Cardioprotection observed. Increased recovery of pre-ischaemic function from ~50% to ~70% at 60 minutes post reoxygenation

Drugs Used (Concentration)	Adenosine Receptors "stimulated"	Adenosine Receptors "blocked"	General observations
8 IB-MECA (300nM) & CGS15943 (1 μM)	A_3 receptor	All adenosine receptors	Cardioprotection observed. Increased recovery of pre-ischaemic function from ~50% to ~70% at 60 minutes post reoxygenation. At 15 minutes post reoxygenation cardioprotection is higher that that seen when IB-MECA was administered alone.
9 NECA (1 μM)	A_{2A}, A_{2B} and A_1 receptor		Cardioprotection observed. Increased recovery of pre-ischaemic function from ~50% to ~80% at 60 minutes post reoxygenation. At 15 and 60 minutes post reoxygenation cardioprotection is higher than that observed when IB-MECA was administered alone.
10 NECA (1 μM) & DPCPX (200μM)	A_{2A}, A_{2B} and A_1 receptor	A_1 receptor	Cardioprotection observed, Same as the level achieved by DPCPX when administration alone.

5.4. Discussion

5.4.1. Ligand selection

Compounds that exhibit high affinity for only one adenosine receptor subtype are rare. Klotz (2000) concluded in a comparative pharmacological characterization of the four human adenosine receptor subtypes that because of unexpected potencies at the human receptors that the prediction of affinities for drugs using K_i data in another other species may not be applicable for it use in another. It was remarked that DPCPX is one such compound. It is characterised as being A_1 selective agonist, but in fact has marked affinity at the A_{2B} receptor.

5.4.2. Effect of the antagonist vehicle control

The vehicle for the antagonist (0.2% DMSO v/v) does not affect either the recovery without the presence of any drug, nor change the increased recovery caused by the administration of IB-MECA. This observation allowed the progression of work to the use of antagonists without any revision to the experimental protocol.

5.4.3. Effect of MRS1191 on recovery

The administration of $1 \mu M$ MRS1191 before ischaemia did not affect the recovery of the atria from simulated ischaemia in comparison to the appropriate control. This finding is in agreement with work conducted in the Langendorff model of ischaemia, which is described in Chapter Six.

While adenosine A_3 receptor stimulation evokes a cardioprotective effect, this work may suggest that blocking A_3 receptor from activation by its endogenous ligand adenosine does not produce a detrimental effect to the contractile function in this model. It would be expected that endogenous adenosine would be evolved during ischaemia and stimulate all adenosine receptor subtypes and possibly exert a protective effect.

This observation is a possible indicator of the 'modality' of the adenosine A_3 receptor. While activation causes a protective effect only, it may not be possible

to evoke detrimental effects or damage via the A_3 receptor subtype when it is blocked.

This use of $3\mu M$ MRS1191 caused a complex picture of cardioprotection in terms of the A_3 receptor. It must be highlighted that this, being a grouping of only $n=3$, was underpowered for statistical comparison. However for the purpose of discussion the $3\mu M$ group was included in the analysis. Although $3\mu M$ MRS1191 had a recovery that was not significantly different from the control recovery, it was also not different from the effect of 300nM IB-MECA. This suggested that it would be possible to show an improvement of recovery by MRS1191 alone had the experimental numbers been greater.

We were unable to demonstrate antagonism of the cardioprotective effect of IB-MECA using $3\mu M$ MRS1191 because its own cardioprotective action masked any antagonism, making it not possible to conclude whether the cardioprotective effect of IB-MECA was due to adenosine A_3 receptor activation. The lower dose of $1\mu M$ also failed to block the effect of IB-MECA.

How MRS1191 could evoke cardioprotection would have to be further investigated. This action could be due to blockade of A_3 receptors that are activated by endogenous adenosine, which assumes that endogenous adenosine has a deleterious effect. This is not suggested by the literature, where adenosine has a cardioprotective effect in it own right (Przyklenk and Whittaker, 2005).

If A_3 receptor stimulation were cardioprotective (as suggested by the literature) then in this study, blockade of these receptors by MRS1191 would be expected to worsen the recovery, not improve it. It can therefore be concluded that the improved recovery by MRS1191 is unlikely to be due to blockade of the A_3 receptor.

One possible explanation of MRS1911's effect is that it is an unrelated property of the drug such as free radical scavenging, since agents with this property have been shown to be cardioprotective (Myers et al., 1985).

If A_3 receptors could not be shown to be involved, it was decided to proceed to examine whether A_1 receptors were being activated by IB-MECA.

5.4.4. Effect of DPCPX on the beneficial effect of IB-MECA following simulated ischaemia

Having established doubt that IB-MECA's action may not be entirely A_3 receptor-mediated we investigated if IB-MECA was acting at the adenosine A_1 receptor.

Here, DPCPX (200nM) caused a cardioprotective effect on its own. This goes against the considerable literature where A_1 receptor antagonism generally impairs the post ischaemic function (Morrison et al., 2000; Murphy et al., 1993). In the closest methodology to this work, Peart and Headrick (2000), found that DPCPX significantly depressed the post ischaemic recovery in a Langendorff model of ischaemia. This begs the question whether the effect's of DPCPX observed in this model are attributable to the A_1 receptor.

DPCPX blocks A_1 receptors, which are normally stimulated by the endogenous adenosine released in ischaemia. The A_1 receptor exerts a negative inotropic effect when stimulated in the atria. If DPCPX blocks this negative inotropy in isolated atria only then improved contractile function could occur, which is what was observed.

Because of this improvement of recovery of atrial contractility by DPCPX, there was no opportunity for it to reverse the effects of IB-MECA. The recoveries with DPCPX alone and with IB-MECA were not significantly different. Therefore use of DPCPX fails to prove whether A_1 receptors are involved in the cardioprotective action of IB-MECA.

With A_3 and A_1 activation still not categorically ruled out as the effectors when IB-MECA is administered, it was decided to progress with an evaluation of the other adenosine receptors.

5.4.5. Effect of CGS15943 on the beneficial effect of IB-MECA following simulated ischaemia

CGS15943 is an antagonist at all adenosine receptor sub-types. Its administration alone showed cardioprotection, which immediately obscured the aim of identifying the means of IB-MECA's action. The use of IB-MECA and

CGS15943 together also showed cardioprotection but it is impossible to say whether this is due to either one of the drug's actions.

CGS15943 and IB-MECA did not act in a synergistic or additive way to further increase protection. This may be due to the model reaching the limit of cardioprotection via adenosine receptor activation, or that the tissue is at the limit of its recovery.

Since tissues underwent one hour of post-reoxygenation recovery, it was not possible to say whether further time-dependent recovery could have occurred. Since IB-MECA caused recovery of contractile function it can be assumed that the dysfunction is reversible at least up to 75% in this model. It may therefore be classed as stunning up to this level of recovery (Hess and Kukreja, 1994). However, it is possible that recovery above 80% is not possible because this represents non-reversible contractile dysfunction due to ischaemic damage.

CGS15943 could have exerted a cardioprotective effect by the two possible explanations already proposed here. Firstly it behaves in a non-adenosine receptor related manner, as suggested for MRS1191. For example it may have free radical scavenging properties. Secondly, the explanation giving for the apparent cardioprotective by DPCPX may apply. CGS15943, by blocking A_1 receptors may block the negative inotropic actions of endogenous adenosine, thereby increasing contractility at reoxygenation.

5.4.6. Effect of adenosine deaminase following simulated ischaemia

The next question to be asked was whether stimulation of A_1 or A_3 adenosine receptors by the endogenous adenosine released by ischaemia could have masked the antagonistic activities of MRS1191, DPCPX and CGS13943 against IB-MECA's effects. This may have affected all the past protocols used in this thesis, making any interventions post reperfusion difficult to interpret as adenosine could modify or possibly override any of the non-physiological interventions used. Endogenous adenosine was removed by incubating throughout with adenosine deaminase. In the atria incubated throughout with adenosine deaminase, vehicle added at reoxygenation had an improved recovery in comparison to the control recovery.

The presence of adenosine deaminase did not affect the tissue's response to IB-MECA when recovering from simulated ischaemia. Therefore IB-MECA's own effect is not modified by or dependent on the endogenous adenosine generated during ischaemia, therefore adenosine does not need to be present for cardioprotection by IB-MECA to be seen.

Although adenosine was the first preconditioningmimetic agent to be identified, the use of it as a protective agent has yielded mixed results. Some reports claimed cardioprotection occurs when adenosine was administered only at reperfusion (Olafsson et al., 1987; Pitarys et al., 1991; Velasco et al., 1991). Other studies did not produce protection when adenosine or adenosine receptor agonists were administered at the time of coronary reflow (Baxter et al., 2000; Smits et al., 1998; Thornton et al., 1992). With such inconsistency with the use of adenosine itself as a cardioprotective agent then perhaps it is difficult to draw any solid conclusions from this data.

Gardner et al. (2004) also drew the mixed conclusion that endogenous adenosine does not protect guinea pig atria from myocardial stunning, but does protect in other cardiac tissues. They also concluded that IB-MECA's action of increasing recovery rate in the atria was not affected by the presence of adenosine deaminase.

5.4.7. Effects of IB-MECA present throughout simulated ischaemia and subsequent recovery

This experiment was conducted to find if other mechanisms of protection could be evoked later in reoxygenation, apart from the initial effectors activated by IB-MECA in the initial 15 minutes of reoxygenation. There was no difference in the level of recovery of contractile function when IB-MECA remained in the tissue bath throughout the reoxygenation period. As IB-MECA evokes cardioprotection in the initial fifteen minutes the possible effectors being modified by IB-MECA must be present during the initial stage of the reperfusion cascade, and acts to limit contractile dysfunction throughout the later stages of reoxygenation.

5.4.8. Effects of NECA on the isolated left atria following simulated ischaemia.

NECA exerted a cardioprotective action during the recovery in this model of ischaemia. The degree of recovery from simulated ischaemia was in fact greater than that observed with the most effective concentration of IB-MECA. Administration of DPCPX did block the action of NECA to some degree, to the level of protection seen with IB-MECA or DPCPX alone. The residual protection may be due to A_3 receptor activation and therefore A_1 or A_{2A} stimulation can also be suspected of being able to bring about a degree of functional cardioprotection. This observation allows the suspicion that IB-MECA may be acting at A_1 or A_2 receptors to evoke cardioprotection when MRS1191 is used. A way to test this hypothesis would be to see if using MRS1191 and DPCPX together blocks the action of IB-MECA

Liang and Jacobson (1998) found that MRS1191 caused a biphasic inhibition of the protective effect of a brief period of ischaemia (ischaemic preconditioning), and when MRS1191 was incubated with cultured chicken ventricular myocytes. When they used DPCPX along with MRS1191, the combined presence of both antagonists abolished the protective effect induced by the brief ischaemic preconditioning.

Administration of DPCPX did not completely block NECA's action. Therefore cardioprotection by NECA has been proven not to be entirely A_1 receptor mediated but other receptor mechanism appears to be involved. The fact that DPCPX was able to block the action of NECA in the first 30 minutes suggests that adenosine receptor mediated cardioprotection is an event that is brought about in the initial stages of reperfusion or at least is strongest in the initial stages. An investigation into the time point of intervention would be of further interest.

NECA brought about an increase in cardioprotection above that of maximally effective dose of IB-MECA (300nM), so a dual activation of receptors may be acting with this agonist. As DPCPX is only blocking the A_1 receptor-mediated component involved in the first 30 minutes of reoxygenation, a second non-A_1 aspect is revealed that gives protection to the 60 minute post reoxygenation time-point.

5.4.9. Conclusion

In conclusion, IB-MECA has been shown to improve cardiac contractile recovery following thirty minutes of simulated ischaemia in rat isolated left atria. Since IB-MECA is regarded as a relatively selective adenosine A_3 receptor agonist, it was assumed that the protective effect of introducing it at reoxygenation was due to adenosine A_3 receptor stimulation. Furthermore, since IB-MECA has been shown to exert similar cardioprotective effects through its action at the adenosine A_3 receptor in guinea pig isolated atria and papillary muscle (Gardner and Broadley, 1999), and in rabbit isolated perfused hearts (Tracey et al., 1997) and guinea-pig isolated perfused hearts (Maddock et al., 2003), it suggested that the effect in this study could have been mediated via A_3 receptors. However, the effect was not antagonised by the rat A_3 receptor selective antagonist MRS1191 at a concentration of $1\mu M$. At $3\mu M$ there was also no antagonism but this was due to the antagonist itself exerting a protective effect, thereby masking any antagonism. The protective response was also not antagonised by the A_1 receptor selective antagonist DPCPX or the non-selective antagonist CGS15943. One possible explanation is that the dose of IB-MECA (300nM) used in these studies was too high for the $1\mu M$ concentration to overcome. As MRS1191 is a competitive antagonist then a higher concentrations than $1\mu M$ might be required to compete with 300nM of IB-MECA. Unfortunately $3\mu M$ of MRS1191 exerted effects on its own and this could not reveal the desired antagonism. In hindsight a lower concentration of IB-MECA should have been employed for the antagonism studies. The results from Chapter Four showed that 300nM was clearly a supramaximal concentration for cardioprotection. Even a concentration 100-fold less (3nM)

would have exerted near maximal protection and would have been more suitable for testing the antagonists.

CHAPTER SIX

THE EFFECT OF IB-MECA,

THE ADENOSINE A$_3$ RECEPTOR AGONIST, ON FUNCTIONAL

RECOVERY AND INFARCT SIZE FOLLOWING GLOBAL ISCHAEMIA

IN ISOLATED HEARTS

6.1 Introduction

6.1.1. IB-MECA and Cardioprotection

Having established that the administration of IB-MECA causes a cardioprotective effect in the rat isolated atria, further studies were undertaken to investigate this effect in another experimental model of ischaemia to see if protection could be established in the whole heart organ. The model used in this investigation was the whole Langendorff heart. While this model and the isolated atria both ascertain the acute effects of ischaemia, the Langendorff model does have a different set of characteristics. It allows the direct measurement of ischaemic damage to be made following the ischaemic protocol, by allowing the measurement of infarct size. This is the measure of actual physical damage occurring and so allows the investigation of interventions that may limit or exacerbate ischaemic damage. The Langendorff heart also allows measurement of functional characteristics. Contractility can be measured from an intraventriclar balloon, as well as additional measures such as determination of rate of contraction and the coronary perfusion flow, which is an index of coronary vascular resistance.

6.1.2. The Langendorff Isolated Perfused Heart Model

The mammalian isolated perfused heart preparation was first described and named after Oscar Langendorff (Langendorff, 1895). It is a widely used and characterised experimental model. A number of physiological measurements can now be obtained with its use, with high accuracy and reproducibility, making this method a very useful tool in modern cardiovascular and pharmacological research (Skrzypiec-Spring et al., 2006). There are recognised limitations of the Langendorff setup such as an absence of innervation and circulating hormonal factors. However the absence of these also provides an advantage of excluding the peripheral influences thus giving uncomplicated information of the heart's function.

Ischaemia can be rapidly induced and different modes can be employed. Regional ischaemia, is induced using a snare ligation of the coronary artery,

stopping perfusion to a part of the myocardium. The method of ischaemia employed in this work was a global ischaemia to the whole heart. Global ischaemia was employed so that the model parameters where as similar to the isolated tissue model as possible, thus enabling comparisons between both models easier to make. For this reason the ischaemia time was set to thirty minutes.

The delivery of drugs to the heart can also be rapidly made via the perfusate line and can be precisely controlled. Infusion of drugs was started from the point of reperfusion onwards. This both kept conformity between the previously used model and to maintain the clinical relevance of being administered after a period of myocardial ischaemia.

Two modes of perfusate delivery can be applied in the Langendorff depending on the requirements of the experiment; either delivery at a constant hydrostatic pressure or at a constant rate of flow. In either mode the Langendorff model allows the measurement of perfusion pressure in order to assess vascular smooth muscle function. As direct measurement of contractility of the coronary vessels is difficult, the resistance to constant flow, which is directly proportional to coronary perfusion pressure and inversely proportional to coronary flow, can be determined.

Perfusion delivery at a constant flow overrides the autoregulatory mechanisms of the heart, so perfusate delivery cannot be altered in response to changes in heart rate or contraction, or as a result of pathological conditions such as ischaemia. Perfusion at a constant pressure, which was employed here, is therefore the preferential mode to be used, in particular for studies of ischaemia (Sutherland and Hearse, 2000). The systolic *in-situ* perfusion pressure for the rat heart ranges from 70 to 90 mmHg. This range is not recommended for the isolated heart on account of an approximately 50% lowering of pressure required for perfusion, due to tissue oedema because of the saline based perfusate solution and the potential of aortic valve incompetence (Doring and Dehnert, 1988). Therefore the perfusion pressure used was 60mmHg.

6.1.3. The Advantages of using the Langendorff model

The advantages of the Langendorff heart are that it can yield data on number of different parameters, including infarct size, contractility and coronary vascular resistance.

Infarct size is determined by triphenyltetrazolium staining. The technique relies on the ability of dehydrogenase enzymes and cofactors in viable tissue to react with the tetrazolium salts to form formazan pigment (Nachlas and Shnitka, 1963). When pigment forms in viable tissue it stains a red colour, while infarcted tissue is unable to form pigment so the tissue stays its pale tan colour. Infarct size itself was determined by measuring the area of the heart sections and the visible infarct area within each section. Computerised planimetry is then used to calculate the total infarct area.

When global ischaemia is used the entire heart is considered to be at risk of ischaemia so the infarct size is expressed as a percentage of the region at risk. This is the typical unit used in the literature to express infarct size in rat hearts (Fishbein et al., 1981).

The measurement of the left ventricular developed pressure assesses the contractility of the heart by determining the rate and force of cardiac contraction. This will reveal any chronotropic and inotropic response of the interventions performed. The measurement of coronary flow assesses the effects of drugs on the coronary vasculature and mechanical function of the smooth muscle of the coronary vessels.

As discussed in the Chapter Four, section 4.1.1., there is a wealth of literature supporting the cardioprotective effect of adenosine A_3 receptor activation in the heart and specifically in the Langendorff heart model. The effect of adenosine A_3 receptor agonists on haemodynamic parameters differs among species. IB-MECA has been observed by others to have no haemodynamic effects in rabbits and dogs (Auchampach et al., 2003) and it is thought that in rodents any haemodynamic effect would be the result of the release of vasoactive mediators from mast cells.

6.1.4. Using DPCPX, an adenosine A₁ receptor antagonist and MRS1191, an adenosine A₃ receptor antagonist

The use of DPCPX, a potent, selective adenosine A_1 receptor antagonist was deemed necessary in order to rule out the possibility of a non-specific activation of the adenosine A_1 receptor by endogenous adenosine which is generated during ischaemia. Adenosine A_1 receptor stimulation would influence the outcome of the experiment by exerting a negative inotropic effect. The knock-on effect of this would be a reduction in rate which may reduce the work load on the heart, possible effecting the parameters being measured and thus masking the effect of other drug interventions.

The initial suggestion that adenosine A_3 receptor activation may cause cardioprotection in the whole heart came from the observation that the protective action of adenosine A_1 receptor agonists, which also cause protective effect against infarction in the rabbit heart can be blocked by unselective adenosine antagonists but, paradoxically, DPCPX could not. This indicated that protection by adenosine receptor stimulation was not exclusively mediated by the adenosine A_1 receptors and could involve the A_3 receptor (Lasley et al., 1990b). Additional studies in isolated rat hearts have shown that DPCPX was unable to block ischaemic and hypoxic preconditioning (Lasley et al., 1993). The numerous reports of cardioprotection with the adenosine A_3 receptor agonists IB-MECA and Cl-IB-MECA further bolstered this hypothesis. These observations are all consistent with the hypothesis that adenosine A_3 receptor play a role in adenosine-related cardioprotection.

There is controversy about the mechanism by which adenosine A_3 receptor agonists exert their cardioprotective effects. One sticking point is the lack of proof of a cardiac adenosine A_3 receptor (Tracey et al., 1997) and the immunological characterisation performed as described in Chapter Seven supports the notion that it is indeed present. Some recent speculation is that IB-MECA in fact mediates its protective effect via the activation of adenosine A_1 receptor . Despite the insensitivity of rat and rabbit A_3 receptor to DPCPX and the use of this antagonist in ischaemic preconditioning studies, Kilpatrick et al. (2001) argued that they had evidence that the cardioprotection of

Cl-IB-MECA and IB-MECA in the ventricular myocardium are abrogated with DPCPX.

With this controversy in mind, DPCPX was used as an additional control. It was added to the circulating perfusate solution at a 200nM, a concentration previously used in similar studies as suitable for the specific inhibition of binding to the adenosine A_1 receptor. The structure and K_i values are detailed in Figure 6.1.

The first stages of work in the Langendorff heart involved establishing baseline values with and without DPCPX present in the circulating perfusate. Some conclusions were drawn from these results alone but the bulk of the remaining investigation examined adenosine A_3 receptor activation performed with DPCPX included in the circulating perfusate in order to exclude adenosine A_1 receptor interaction.
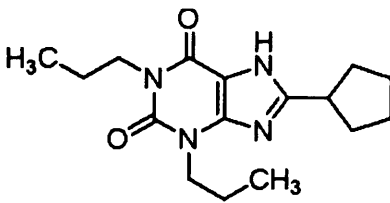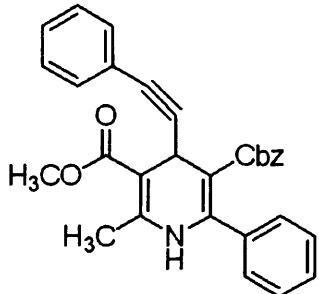
DPCPX	MRS1191
1,3-dipropyl-8-cyclopentylxanthine	3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate

	DPCPX	MRS1191
Adenosine A_1 receptor	3.9nM (human)[a] 0.9nM (rat)[b]	40,000 nM (rat)[c]
Adenosine A_{2A} receptor	130nM (human)[a] 470nM (rat)[b]	> 100,000 nM (rat)[c]
Adenosine A_{2B} receptor	50nM (human)[a]	
Adenosine A_3 receptor	4000 nM (human)[a] 5300 nM (rat)[b]	31 nM (human)[c]

Figure 6.1 The structure of adenosine receptor antagonists DPCPX (a A_1 receptor antagonist) and MRS1191 (a A_3 receptor antagonist). Receptor binding affinities (K_i) at the each adenosine subtype are shown. Taken from [a]Klotz et al. (1998) and [b]Jacobson and Suzuki (1996) and [c]Jacobson (1998).

In order to establish the functional role of adenosine A_3 receptor activation IB-MECA was administered both alone and in the presence of MRS1191. The use of MRS1191 allows the further definition of the role of adenosine A_3 receptor activation in the recovery from ischaemia and determines the possibility that non-specific activation of other adenosine receptors by IB-MECA, and what this may contribute to the overall observed effect.

Previously MRS1191 has been shown to block the IB-MECA-induced production of nitric oxide, a result of adenosine A_3 receptor stimulation in ischaemic/reperfused Langendorff hearts (Zhao and Kukreja, 2002).

6.1.5. Aims of this Chapter

The aims of the chapter were:

- Establish whether the adenosine A_1 receptor agonist DPCPX has an effect on the heart and what affect that would have on the planned future work.

- Investigate the effect of IB-MECA on the ischaemic damage and cardiac contractile function immediately following a period of ischaemia.

- Establish the action of adenosine A_3 receptor activation in ischaemia, using the adenosine A_3 receptor antagonist MRS1191 alone and with IB-MECA.

6.2 Methods

Isolated hearts from male Wistar rats (350g-350g) were mounted on a Langendorff perfusion set-up using the methods described in Chapter Two, section 2.3.1.2. Hearts underwent global ischaemia for thirty minutes followed by one hour of reperfusion under a constant perfusion pressure of 60 mmHg. Measurements of coronary flow, heart rate and the left ventricular developed pressure were recorded throughout. Following this the heart was cut into 3mm sections and stained using 1% TTC to identify infarcted areas (as previously described in Chapter Two: Methods). This infarct size was then expressed as a percentage of the whole myocardium sections.

6.2.1. Protocols

6.2.1.1. Global Ischaemia of the Whole Heart

Global no-flow ischaemia was produced by attenuating perfusion to the aorta by switching off the perfusion pump. Ischaemia was maintained for 30 minutes. Reperfusion commenced by restarting the perfusion pump. Reperfusion continued for one hour. The control group of tissues underwent the same

protocol with the administration of the appropriate vehicles at the appropriate time-point in order to act as an experimental control (Figure 6.2, protocol i.)

6.2.1.2. Drug Administration Protocols

The adenosine A_1 receptor antagonist DPCPX was used at a 200nM concentration in a vehicle of 0.02% v/v dimethyl sulfoxide (DMSO). It was included in the circulating Krebs perfusate throughout (Figure 6.2, Protocol ii.). This concentration was used as affinity data shown that it will selectively block A_1 receptors in the rat leaving the other adenosine receptor subtypes unblocked (see Figure 6.1 for K_i values).

Adenosine A_3 receptor agonists were infused at the onset of reperfusion by means of an infusion pump, the infusion added to the aortic perfusate just above the aortic cannula. The solution to be infused was ×10 concentrated than the effective concentration required and so was infused at a rate of 10% of the coronary flow rate being recorded at the time. The infusion rate was constantly adjusted and infusion continued for the hour of reperfusion.

The agonists were administered are detailed in Figure 6.2, Protocol iii. The adenosine A_3 receptor agonist IB-MECA was infused so there was an effective concentration of 1nM in the heart, in a vehicle of 0.001% v/v DMSO. This concentration was shown by Maddock et al. (2002b) to give the greatest reduction in infarct size in a markedly similar model when using an analogue 2-Cl-IB-MECA. Previously we have shown that 3nM afforded a greater recovery from ischaemia in the isolated atria than control. The adenosine A_3 receptor antagonist MRS1191 was infused so there was an effective concentration of 1μM in the heart, in a vehicle of 0.1% v/v DMSO. Again 1μM was used as this is estimated concentration to selective block the A_3 receptor subtype. When agonist was not used the appropriate vehicle control concentration of DMSO was infused.

Time (minutes)	-15	0	15	30	45	60	75	90

Protocol i
No drug administered

| Equilibrative | Ischaemia | Reoxygenation |

Protocol ii
DPCPX 200nM

| Equilibrative | Ischaemia | DPCPX | Reoxygenation |

Protocol iii
DPCPX 200nM, IB-MECA
1nM & vehicle
DPCPX 200nM, MRS1191
1µM & vehicle
DPCPX 200nM & IB-MECA
1nM & MRS1191 1µM

| Equilibrative | Ischaemia | DPCPX | Reoxygenation | IB-MECA and vehicle or MRS1191 and vehicle or IB-MECA and MRS1191 |

Figure 6.2 Experimental protocols used in the Langendorff heart studies. All protocols were preceded by at least 15 minutes equilibrative period as shown to achieve a steady baseline of contraction was achieved.

6.2.2. Drugs

Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific,

Loughbourgh, UK. 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and

N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) was obtained

from Tocris Limited, Bristol UK.

3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-

3,5-dicarboxylate (MRS1191) was obtained from Sigma Alrich, Poole, UK.

6.2.3. Data Analysis

Coronary flow (CF), left ventricular pressure (LVDP) and heart rate (HR) were

recorded as described in section 2.3.1.5. Measurements of each were taken at 10

minutes intervals throughout the protocol, and were then expressed as a

percentage of the baseline value before the onset of ischaemia. Each time point

was expressed as the mean obtained from hearts in that experimental group. The

means of each experimental group were expressed in the text and in the graph.

Each mean is accompanied by ± the standard error of the mean (S.E.M.).

Statistical analysis was performed to establish a significance of differences

between either infarct sizes or the functional measurement at each time point.

Analysis was performed using one-way analysis of variance (ANOVA)

followed by either an unpaired t-test or Dunnet's post hoc test if a significant

difference was found. A P value ≤ 0.05 was considered statistically significant.

6.3 Results

6.3.1. Infarct Size

For an illustration of the result of tetrazolium staining, which indicates infarct size, see Section 2.3.3. in Chapter Two: Methods. In hearts that underwent thirty minutes of ischaemia followed by one hour of reperfusion the mean infarcted area was 20.9 ± 4.7% (n=12) of the myocardium (see Figure 6.3). When the A_1 receptor antagonist DPCPX (200nM) was present throughout the experiment, the infarcted area was 28.5 ± 2.4% (n=13). No significant difference was found between the two treatments.
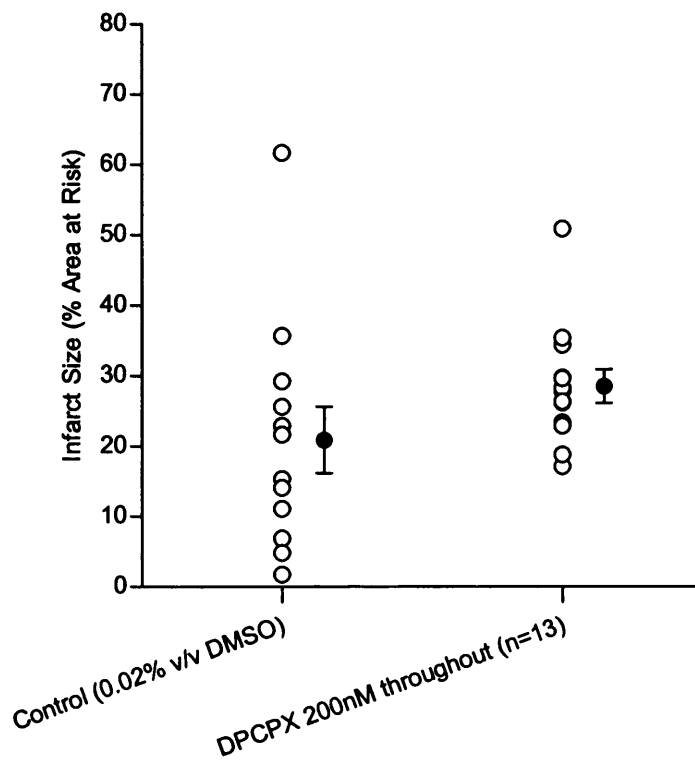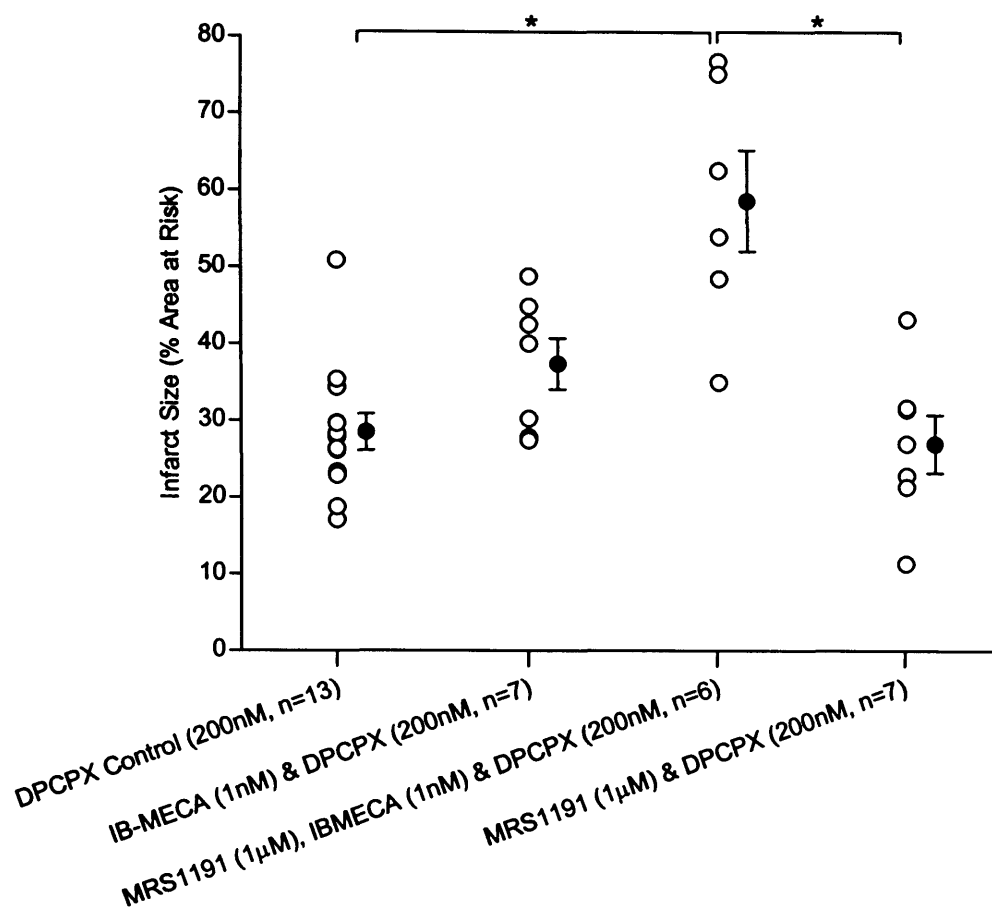
Figure 6.3 Infarct size after 30 minutes of global no-flow ischaemia followed by 1 hour of reperfusion. Infarct size was determined by tetrazolium staining and normalised as a percentage of the region at risk of infarction. When used, DPCPX (200nM) was present in the circulating Krebs throughout. Open symbols (O) represent individual hearts, whereas solid symbols (●) represent the mean (± S.E.M.).

From this point on DPCPX (200nM) was included in the circulating Krebs at all times (see Figure 6.2, protocol iii. for a diagrammatic), and the infarct size following reperfusion was considered as the control or baseline for comparison.

The adenosine A_3 receptor agonist IB-MECA (effective concentration of 1nM in the heart) was infused upon reperfusion of the heart in addition to the DPCPX included in the perfusate. After one hour of reperfusion the mean infarct size was 37.4 ± 3.3% (see Figure 6.4) which was not significantly different from the DPCPX control.

The adenosine A_3 receptor antagonist MRS1191 (effective concentration of 1μM in the heart) was infused upon reperfusion of the heart n addition to the DPCPX included in the perfusate. The mean infarct size following reperfusion was 27.3 ± 3.8% (n=6) (see Figure 6.4), again not significant from the DPCPX control.

IB-MECA (effective concentration of 1nM) and MRS1191 (effective concentration of 1μM) were infused together throughout reperfusion in addition to the DPCPX included in the perfusate. The mean infarct size following reperfusion was 58.7 ± 6.6% (n=7), which was significantly larger infarct size that the infarct in the control hearts which were infused with DPCPX only and hearts that were infused with MRS1191 (see Figure 6.4).

Figure 6.4 Infarct size after 30 minutes of global no-flow ischaemia followed by one hour reperfusion. Infarct size was determined by tetrazolium staining and normalised as a percentage of the region at risk of infarction. All experiments were in the presence of DPCPX (200nM), with other drug administered for the one hour of reperfusion. Open symbols (O) represent an individual heart, whereas solid symbols (●) represent the means (± S.E.M.) of each group. * indicates $P<0.05$ between indicated groups.

6.3.2. Effect on Coronary Flow

Following thirty minutes of ischaemia, upon reperfusion the coronary flow to the heart initially returned, to rate of 108.6 ± 9.2% ($n=11$) of the pre-ischaemic coronary flow at 10 minutes following reperfusion. From reperfusion on, the

coronary flow steadily fell so that at the end of the hour's reperfusion the coronary flow was at 56.8 ± 12.5% ($n=11$) of the pre-ischaemic flow rate.

DPCPX (200nM) in the circulating Krebs significantly lowered the coronary flow at 10, 20 and 30 minutes post reperfusion. At 10 minutes post reperfusion the coronary flow was reduced to 69.3 ± 6.7% ($n=15$) of the pre-ischaemic coronary flow, significantly lower that the heart were subjected to ischaemia and reperfusion only (see Figure 6.5). At 20 and 30 minutes post-ischaemia the coronary flow remain lower than control. At 20 minutes post reperfusion coronary flow was 61.0 ± 5.9% compared to 92.5 ± 11.7% and at 30 minutes is was 53.6 ± 5.3% compared to a control of 83.3 ± 13.3%.
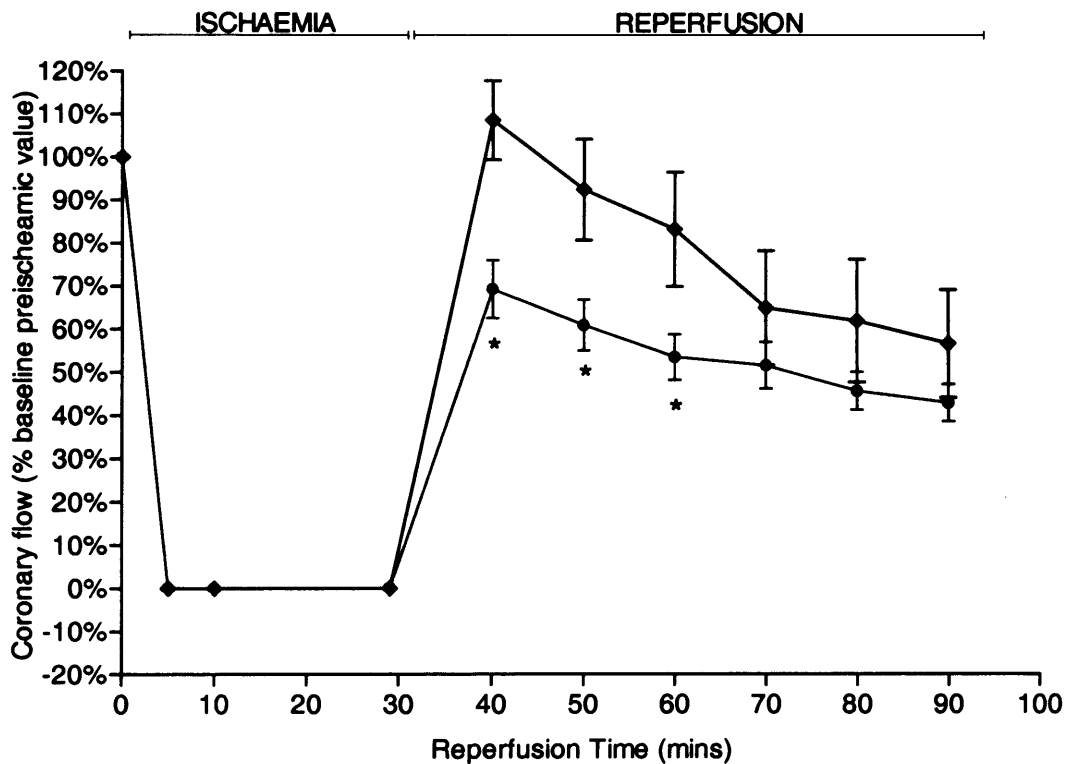
Figure 6.5 Effect of DPCPX on coronary flow in the isolated heart. Data is presented as percentage of the mean baseline value ± S.E.M. * indicates a significant difference between the 'ischaemia only' hearts (◆, $n=11$) and hearts that underwent ischaemia in the presence of DPCPX (200nM, ●, $n=15$) at that time point, as measured by a unpaired t-test.

When IB-MECA (1nM) was administered upon reperfusion in the presence of DPCPX, it caused no statistically significant change to the coronary flow during reperfusion in comparison to the appropriate control experiments performed with DPCPX. MRS1191 (1μM) administered had no statistically significant effect on coronary flow during reperfusion. When administered together MRS1191 (1μM) and IB-MECA (1nM) has no statistically significant effect to the coronary flow compared to both the DPCPX control recovery of coronary flow, and the coronary flow seen when either one was administered alone. Therefore no drug intervention had any significant effect of coronary flow when in the presence of DPCPX (200nM), as shown in Figure 6.6.
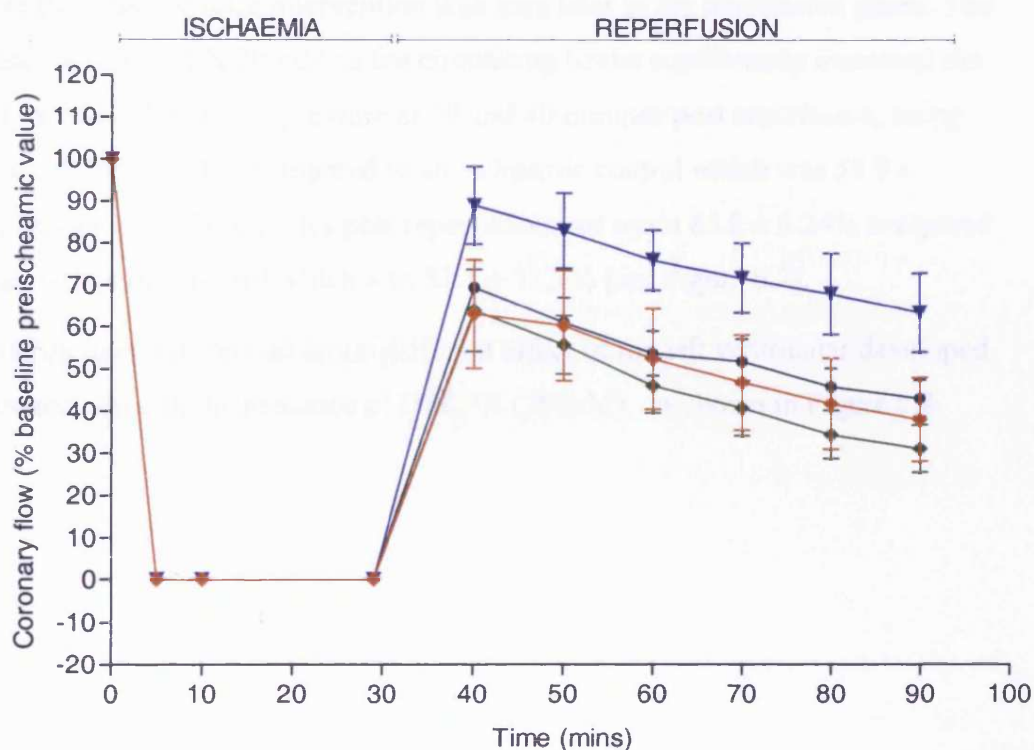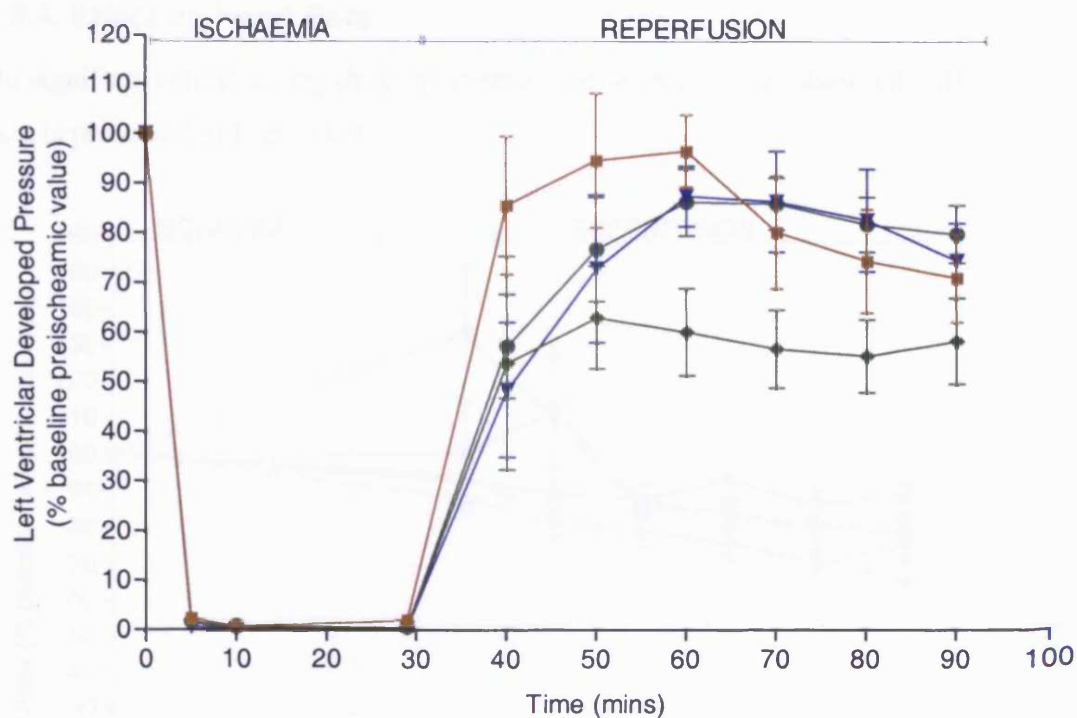

Figure 6.6 The effect of IB-MECA (1nM, ▼, $n=6$) and MRS1191 (1μM, ■, $n=6$), and when both administered together (◆, $n=6$) on the recovery of the coronary flow in isolated heart. The appropriate control is also shown (the effect of in the presence of DPCPX 200nM (●, $n=15$)). Data is presented as percentage of the mean baseline value ± S.E.M.

6.3.3. Effect on Left Ventricle Developed Pressure

Following thirty minutes of ischaemia, upon reperfusion the left ventricular developed pressure returned, to $77.7 \pm 16.2\%$ ($n=11$) of the pre-ischaemic coronary flow at 10 minutes following reperfusion. From reperfusion on the pressure steadily fell up until 40 minutes post reperfusion, at that point the left ventricular developed pressure was $52.1 \pm 11.9\%$ ($n=10$). Then until the end of the experiment at 60 minutes post reperfusion the left ventricular developed pressure then rose. At 60 minutes post reperfusion the left ventricular developed pressure was $62.0 \pm 10.4\%$ ($n=9$) of the pre-ischaemic left ventricular developed pressure.

Here the effect of drug intervention was seen later in the reperfusion phase. The presence of DPCPX 200nM in the circulating Krebs significantly increased the left ventricle developed pressure at 30 and 40 minutes post reperfusion, being $86.1 \pm 6.79\%$ ($n=15$) compared to an ischaemic control which was $58.9 \pm 11.9\%$ ($n=11$) at 30 minutes post reperfusion and again $85.8 \pm 5.24\%$ compared to an ischaemic control which was $52.1 \pm 11.9\%$ (see Figure 6.7).

No drug intervention had any significant effect of the left ventricular developed pressure when in the presence of DPCPX (200nM), as shown in Figure 6.8.
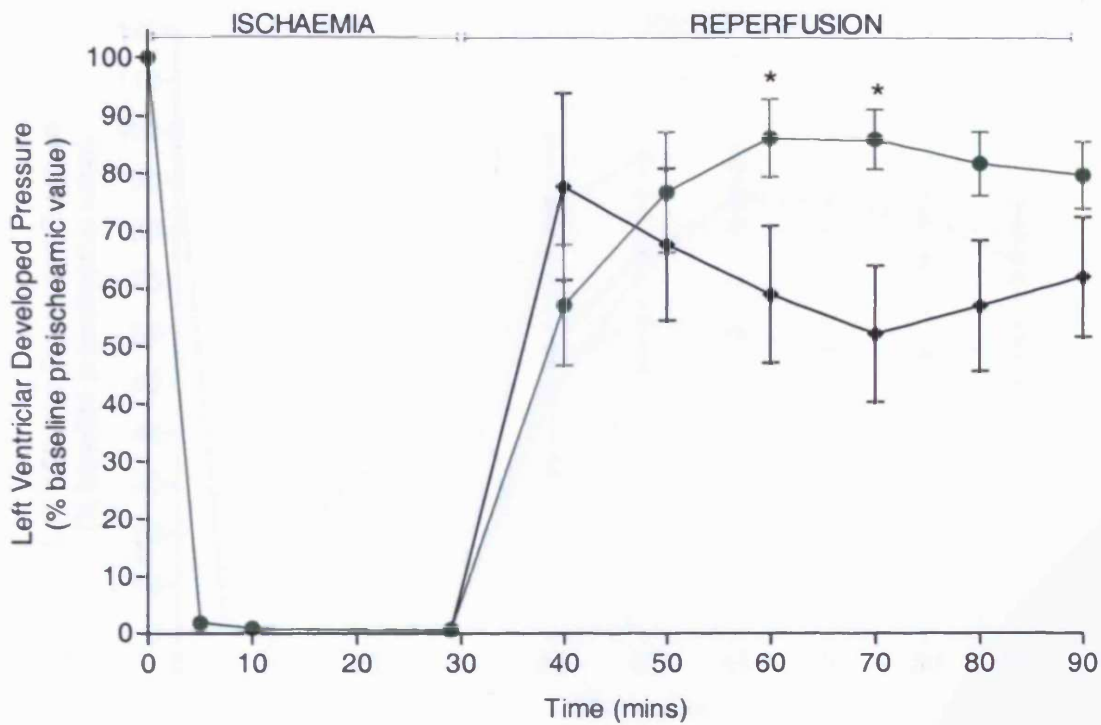
Figure 6.7 The effect of DPCPX (200nM) on the recovery of the left ventricular developed pressure in the isolated heart (●, $n=15$) compared to hearts that acted as a control (◆, $n=11$) . Data is presented as the mean percentage of baseline left ventricular pressure ± S.E.M. * indicates a significant difference between the "ischaemia only" heats and the hearts that underwent ischaemia in the presence of DPCPX 200nM at that time point, as measured by a unpaired t-test.

Figure 6.8 The effect of IB-MECA (1nM, ▼, *n*=6) and MRS1191
(1μM, ■, *n*=6), and when both administered together (◆, *n*=6) on the recovery
of the left ventricular developed pressure in the isolated heart. The appropriate
control is also shown (the effect of in the presence of DPCPX 200nM
(●, *n*=15)). Data is presented as percentage of the mean baseline value ±
S.E.M.

6.3.4. Effect on Heart Rate

No significant effect of any drug intervention on heart rate was observed. All data is presented in Figure 6.9.
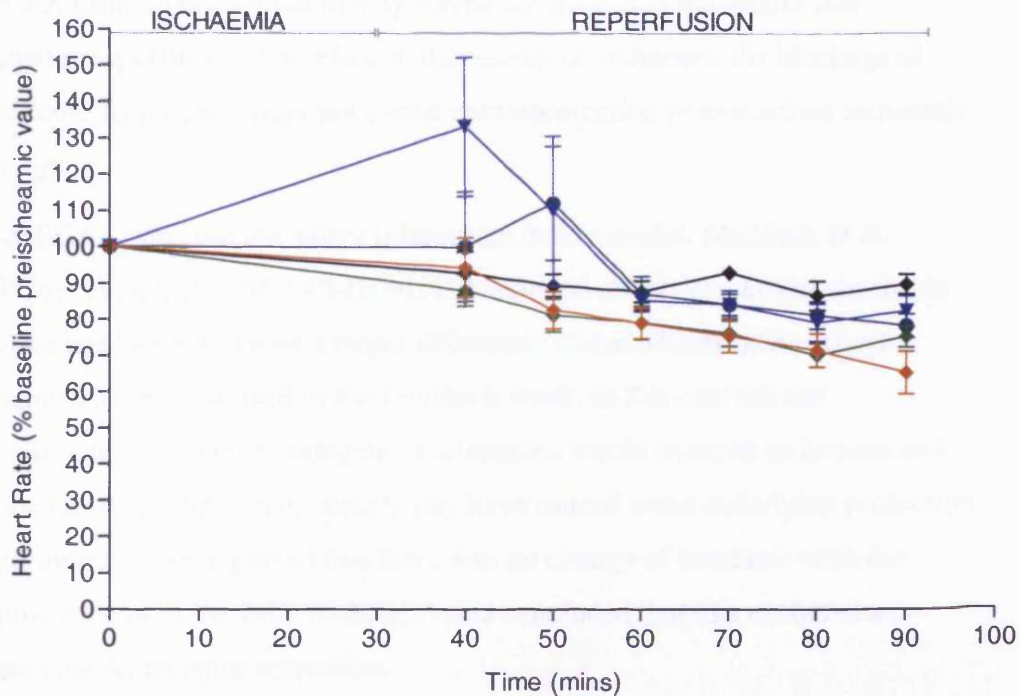
Figure 6.9 The effect of various drug interventions on the recovery of heart rate following ischaemia. The recovery of control hearts with received the appropriate control (◆, *n*=7) and those which received DPCPX (200nM, ●, *n*=15) upon reperfusion are shown, together with the recovery when IB-MECA (1nM, ▼, *n*=7) and MRS1191 (1μM, ◆, *n*=6) and MRS1191 and IB-MECA together (◆, *n*=7) in the presence of 200nM DPCPX is also shown. Data is presented as percentage of the mean baseline value ± S.E.M.

6.4 Discussion

6.4.1. The effect of adenosine A_3 receptor and the adenosine A_1 receptor on infarct size

DPCPX (200nM) in the circulating Krebs did not affect the infarct size following reperfusion. Therefore in this model of ischaemia the blockage of adenosine A_1 receptor does not evoke cardioprotection or exacerbate ischaemic damage.

IB-MECA (1nM) did not affect infarct size in this model. Maddock et al. (2002b) found that 1nM 2-Cl-IB-MECA reduced infarct in a model similar to the one used here however a major difference is that adenosine A_1 receptor antagonists were not used in the Maddock work. In this case normal physiological release of endogenous adenosine would occur in ischaemia and act on the A_1 receptor also, which may have caused some underlying protection from infarct. They reported that there was no change of heart rate with the administration of the 2-Cl-IB-MECA and concluded that this excluded any adenosine A_1 receptor activation.

Controversy remains on the role that adenosine A_3 receptor plays in cardioprotection. While administration of adenosine A_3 receptor agonists have proved to be cardioprotective in certain models (see Table 4.1) the mechanism on how this effect is brought about has still to be found. Though Maddock et al. (2002b) found cardioprotection upon adenosine A_3 receptor agonist administration, they did not block and so exclude adenosine A_1 receptors. This observation is in agreement with Kilpatrick et al. (2001) who showed that DPCPX blocked the anti infarct effect of IB-MECA administration in globally ischaemic rat and rabbit hearts. Could it be that DPCPX blocks the protective actions of IB-MECA which is cardioprotective through the adenosine A_1 receptor? The conclusions of Kilpatrick et al. (2001) were commented on by Headrick (2002) who observed that the publication did not reveal the results of the appropriate control to the drugs that were used. In order to identify the effect of exogenous agonism in which background endogenous responses exist, the control is treatment with antagonist alone.

This forces us to agree with Headrick (2002) that the effects of agonists are not dependant on the targeted receptor and/or competitive antagonism fails to effectively counter the responses to the applied agonists. The apparent lack of protection during co-treatment with A_3 agonist and A_1 antagonist may reflect a balance between beneficial effects of adenosine A_3 receptor agonism and injury arising from antagonism of the protective effects of endogenous adenosine via the adenosine A_1 receptors. IB-MECA's binding affinities at the adenosine receptor subtypes is only separated by one order of magnitude. The use of antagonists is essential in order to elude specific receptor actions by an agonist. Maddock discounted adenosine A_1 receptor activation by IB-MECA on account that no effect on heart rate was observed, with any change being a consequence of adenosine A_1 receptor activation. Though this is a suggestion it does not thoroughly discount that IB-MECA does not activated adenosine A_1 receptor at all. Changes in heart rate could be small enough not to be detected as doses of IB-MECA were below the K_i at the A_1 receptor, but the activation effect could be far more prevalent.

It was observed that the infarct sizes in the control were visibly more variable that the other experimental groups. This is a consequence of them being the first experimental group to be performed. In the future possible randomisation of the order of experiments performed could be conducted, together with a statistical test to see it the order the experiments are performed has a effect on infarct size.

Auchampach et al. (2004) showed that adenosine A_1 receptor antagonism alone reduced infarct size. We did not observe this effect, but at the same time concluded that adenosine A_1 receptor antagonism did not exacerbate the infarct sustained by the heart that underwent the ischaemic protocol. Zhao et al. (1994) conclude that adenosine itself exerts its predominate modulation of infarct size during reperfusion and the cardioprotection mediated by adenosine A_1 receptor mechanism is modest and exerted principally at the ischaemic time period. Therefore IB-MECA may not add to a small threshold of A_1 receptor-mediated cardioprotection, as it was administered during reperfusion.

MRS1191 had no effect on altering the infarct size following reperfusion. Blocking the adenosine A_3 receptor alone does not appear to induce cardioprotection in this model. This could be due to the MRS1191 being administered at the onset of reperfusion, and so being unable to influence the damage brought about during ischaemia. This time point of administration in the same as the one employed by Maddock et al. (2002b), but they found that the action of 2-Cl-IB-MECA can be blocked by an adenosine A_{2A} receptor antagonist (8-3 chlorostyryl caffeine), and not be evoked by A_{2A} receptor agonists. Therefore in this model of ischaemia the blockage of both A_3 and A_1 adenosine receptors does not evoke either cardioprotection or exacerbation of ischaemic damage.

When IB-MECA was administered in the presence of MRS1191 a rise in infarct size occurred. This further suggests that IB-MECA may have non adenosine A_3 receptor mediated pro-infarct effects because the presence of the adenosine A_3 receptor antagonist MRS1191 would prevent adenosine A_3 receptor-mediated responses.

The results suggest that IB-MECA has a duality of action in the model. IB-MECA has a destructive effect when the adenosine A_3 receptor is blocked. Therefore could its potential beneficial cardioprotective effect (evidenced by the raft of literature supporting the notion that adenosine A_3 receptor activation is cardioprotective) which was lacking in this model, because it is being offset by some other action of IB-MECA. The notion that this could be occurring was investigated in Chapter Five with the use of other adenosine receptor ligands.

6.4.2. The effect of adenosine A_3 receptor and the adenosine A_1 receptor on coronary flow during reperfusion

Our work revealed that the presence of DPCPX throughout ischaemia and reperfusion lowered the coronary flow the first half hour of reperfusion. A lowering of coronary flow in a constant pressure Langendorff system implies that the presence of DPCPX causes constriction of the coronary vessels. The adenosine A_1 receptor block caused by the DPCPX is either blocking the

normal vasodilatory response post ischaemia or activating an effort that is somehow counteracting this response.

This is surprising as the adenosine A_{2A} receptor and not A_1 receptors, is shown to cause vasodilation of the coronary arties. Possible blocking the endogenous action of adenosine acting on the adenosine A_{2A} receptor may explain this effect of DPCPX. The K_i of DPCPX at A_1 is 0.9nM and at the A_{2A} it is 470nM. At a 200nM effective concentration there is only a small likelihood of this occurring. To prove it was occurring further work would have to be performed using A_{2A} receptor antagonists.

There was a suggestion from the results that IB-MECA has a vasodilatory effect when administered at reperfusion, which is not present when MRS1191 was administered, although we were unable to prove significance of this. The suggestion that IB-MECA may be acting at either the A_{2A} or the A_{2B} can be discounted for two reasons; DPCPX is present throughout, and from the examination of the Ki of DPCPX a 200nM concentration will block both receptors. Secondly, MRS1191 seems to be blocking the effect of IB-MECA. This suggests that an adenosine A_3 receptor mechanism has an effect of coronary flow. Further work would be needed to substantiate these claims, especially as previous literature has suggested that the cardioprotection of adenosine A_3 receptor agonists occurs by a mechanism independent of coronary flow (Auchampach et al., 2003; Maddock et al., 2002a).

6.4.3. The effect of adenosine A_3 receptor and the adenosine A_1 receptor on developed pressure during reperfusion

Adenosine A_1 receptor antagonism has been shown to impair post ischaemic function (Thourani et al., 1999a) which goes against our findings that the administration of DPCPX improved the left developed ventricular pressure at 30 and 40 minutes post reperfusion.

This could be caused by an A_1 receptor-mediated negative inotropic effect being blocked. Or conversely the actions of endogenous effectors which occur during ischaemia and reperfusion act on function and it is their actions that DPCPX blocks which leads to improvement in the left ventricular developed pressure.

6.4.4. The Post-Reperfusion Effects

It should be said that the interaction of DPCPX during reperfusion is interesting and alludes to the function of the heart during reperfusion, but at 60 minutes post reperfusion all functional characteristics of the heart are not significantly different from each other and infarct size is not significantly different either. This may because the adenosine A_1 receptor plays a role in myocardial stunning, which is the temporary dysfunction following reperfusion and not in the permanent damage resulting from the ischaemia.

It remains difficult to tease out the function of adenosine A_3 receptors on myocardial contractile recovery and infarct size by just concentrating on using drugs directed at it. It has become clear that the role the adenosine A_3 receptor plays is complicated and made further complicated by the possibility of the other adenosine receptor subtypes and other classes of receptor both being physiologically involved or being implicated because of the non-specific actions of the drug we used. Either way further investigation using a raft of adenine receptor drugs is needed in order to elucidate the role of adenosine A_3 receptors in myocardial ischaemia.

CHAPTER SEVEN

IMMUNOLOGICAL CHARACTERISATION OF THE
ADENOSINE A₃ RECEPTOR IN RAT CARDIAC TISSUE

7.1. Introduction

7.1.1. The adenosine receptor family

To date adenosine receptors are found to exist as at least four different subtypes: A_1, A_{2A}, A_{AB} and A_3 receptor (Olah and Stiles, 1995), all of which have been cloned. All adenosine receptors are seven transmembrane domain G protein-coupled receptors. Although initially the adenosine receptors were classified by their pharmacological effect on adenylyl cyclase, they were later found to have differing amino acid sequences and molecular weights, tissue distribution, ligand affinity and signal transduction mechanisms. Also subtle interspecies differences exist in the primary structure of each receptor subtype (Linden, 1994).

7.1.2. Identification of the adenosine A_3 receptor

The adenosine A_3 receptor was first cloned by the use of the polymerase chain reaction that used degenerate oligonucleotides primers directed against conserved regions of the G-protein coupled receptors. The receptor we now know was first reported as a novel receptor clone from rat testis (Meyerhof et al., 1991). Zhou et al. (1992) identified a clone to be the adenosine A_3 receptor. The adenosine A_3 receptor cDNA fragment from rat brain was isolated, expressed and its amino acid sequence deduced. The cDNA open reading frame encodes a protein of 320 amino acids, with a predicted molecular weight of 36,664 Da (based on the sequence of Rattus norvegicus adenosine receptor accessed though the National Library of Medicine's NCBI Entrez-Protein database, accession number AAA40680). Hydrophobicity analysis of the amino acid sequence found that it contains seven hydrophobic domains of 21-26 amino acids that are possible transmembrane helices. It also contained putative N-glycosylation sites in the second extracellular loop and on two sites of the N-terminus; three potential phosphorylation sites are also present. Since this work, adenosine A_3 receptors have been cloned from sheep, human, canine, rabbit and chick tissues (Auchampach et al., 1997a; Durand and Green, 2001; Hill et al., 1997; Linden et al., 1993; Salvatore et al., 1993).

Evidence of specific binding at the cell surface of adenosine A_3-receptor selective ligands has been found in a small number of studies (Gessi et al., 2002; Salvatore et al., 1993; Varani et al., 2000), together with previous work from this laboratory which identified inhibition of binding of the selective ligand for the adenosine A_3 receptor, ^{125}I-AB-MECA, in a Chinese hamster ovary cell line expressing the human adenosine A_3 receptor (Yates et al., 2006). In man, adenosine A_3 receptor mRNA transcripts have been found in the lungs, liver, kidney, heart and aorta, with a lower density being found in the brain and testis (Sajjadi and Firestein, 1993; Salvatore et al., 1993). To date, detection of adenosine A_3 receptor protein from native tissue has been reported once. Di Tullio et al. (2004) reported identification of adenosine A_3 receptor protein through immunoblotting. They identified a band of 52kDa from the membranes of the rat pial (cerebrovascular) artery. No other literature was found which cites this work (ScienceDirect search, performed 18 December 2006). There are no reports in the literature of detection of the adenosine A_3 receptor protein in peripheral cardiovascular tissues.

There is therefore a paradox that despite mounting evidence of cardioprotection being mediated by the adenosine A_3 receptor, there has been no identification of expression of the adenosine A_3 receptor in heart tissue. Controversy remains as to whether adenosine A_3 receptor signalling is directly acting on the myocyte or whether the cardioprotective actions are due to activation of adenosine A_3 receptors on other cell types. Adenosine A_3 receptor-mediated effects have been found to mediate the vasoconstriction response to adenosine stimulation of mast cells (Shepherd et al., 1996) and alteration of the glycocalyx at the cell surface (Platts and Duling, 2004).

Mubagwa and Flameng (2001) proposed that due to the multiplicity of receptor subtypes in a given cell or tissue, the species-related structural properties of each subtype and the divergence in the coupling of each receptor subtype to its various effectors, make it difficult to determine the subtype underlying a particular response. The adenosine A_3 receptor has been pharmacologically identified as being responsible for the cardioprotective action in isolated tissues in this thesis and by others (see Chapter Four for summary).

It was therefore desirable to ascertain the presence of adenosine A_3 receptors in cardiac tissue, namely the rat atria tissue, the most relevant tissue to use because of the improvement of functional recovery induced by IB-MECA in this thesis. A direct immunological approach was used with antibodies to the adenosine A_3 receptor. Previous work performed in this laboratory attempted to identify the adenosine A_3 receptor protein in guinea pig cardiac tissue using a human anti-adenosine A_3 receptor antibody, hoping that the epitope of the antibody would be similar to a conserved region in the guinea pig adenosine A_3 receptor protein and be able to bind specifically. This approach was unsuccessful and blamed on the species-dependent lack of homology between the human and guinea pig receptor.

This study used rat tissues that enabled me to take advantage of a commercially available anti-rat adenosine A_3 receptor antibody. Hopefully, this approach would eliminate the problem of lack of species homology. The adenosine A_3 receptor in the rat has unusually high divergence in sequence homology from the other species from which the receptor has been cloned, as demonstrated in Table 7.1.

Table 7.1 The percentage sequence homology that exists between adenosine receptor subtypes in differing species. The adenosine A_3 receptor in the rat contains a lower sequence homology to the A_3 receptor from other species (Table 7.1a) compared to the other adenosine receptor subtypes that have been cloned from rat tissues (Table 7.1b). Adapted from (Linden, 1994).

a	Sheep A_3 receptor	Human A_3 receptor
Rat A_3 receptor	73.8%	73.9%
Sheep A_3 receptor		85.2 %

b	Human A_1 receptor	Human A_{2A} receptor	Human A_{2B} receptor
Rat A_1 receptor	94.8 %		
Rat A_{2A} receptor		84.3 %	
Rat A_{2B} receptor			86.1 %

7.1.3. Aims of this chapter

The aims of the chapter were:

● To optimise the western blotting conditions for the anti-rat adenosine A_3 receptor antibody to allow subsequent detection of binding.

● To determine the presence of the adenosine A_3 receptor by western blotting in rat cardiac tissues.

● To establish if there is a detectable difference in the density of adenosine A_3 receptor protein in membranes prepared from both ischaemic and normoxic atria.

7.2. Methods

Membrane fractions were prepared from rat kidney, lung, spleen and testis in order to provide ample membrane samples to optimise the western blotting stage. The optimisation was performed using the membrane fractions from rat kidney and spleen. This is because a large amount of membrane protein was obtained from these tissues. A total of three atria underwent each experimental protocol in order to obtain normoxic and ischaemic tissue samples. The three atria were pooled together in order to prepare the membrane fraction but even so atrial tissues yielded ten-fold smaller amounts of protein, which limited the number of blots possible.

Membranes from the ischaemic and normoxic left atria were prepared using the method described in detail in Chapter Two, section 2.4. The gassing protocol used to obtain normoxic and hypoxic tissue is detailed in Figure 7.1.
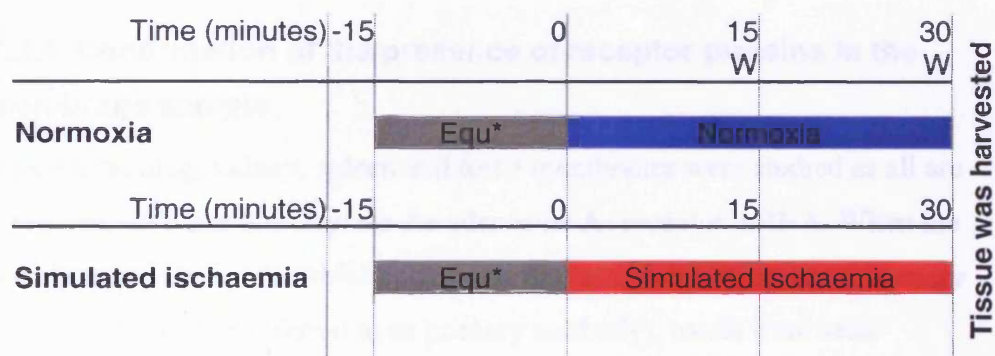
Figure 7.1. A diagrammatic representation of the experimental protocols used to obtain both normoxic and ischaemic tissue prior to membrane preparation. Both protocols were preceded by at least a 15-minute stabilisation equilibrative period. 'W' indicated when wash out of the organ bath was performed. 'Equ*' indicates the equilibrative period before the commencement of the protocol.

The membrane fractions underwent electrophoretic separation in polyacrylamide gels and electrophoretic transfer to nitrocellulose membranes (as described in section 2.5 of Chapter Two). The separated proteins from the membrane sample were probed using an anti-adenosine A_3 receptor antibody.

The initial optimisation stages established the conditions for the western blot to resolve the maximum signal relative to background. A series of logical alterations to the v/v concentration of antibodies and BLOTTO solution were performed in order to establish the best detection results.

Once optimisation was achieved, the control peptide assay was performed as described in section 2.5.2.5. of Chapter Two. It was used in order to confirm the specificity of primary antibody binding to the adenosine A_3 receptor protein in the atria.

The developed films of the probed blots were scanned to JPEG image files using an HP ScanJet 4370 optical scanner (Hewlett Packard, Bracknell, UK). Image manipulation was performed by Paint Shop Pro 8 (Jasc Software USA). Any image enhancement was applied equally across an entire image, no selection of parts of an image was made.

7.3. Results

7.3.1. Confirmation of the presence of receptor proteins in the membrane sample

Initially rat lung, kidney, spleen and testis membranes were studied as all are known tissue types that express the adenosine A_3 receptor mRNA. When the membranes were immunoblotted with anti-rat adenosine A_3 receptor primary antibody (here after referred to as primary antibody), bands were seen confirming binding to a protein present in the membrane sample, making it suitable for further investigation.

This first attempt used a primary antibody dilution concentration of 1:500 v/v with a secondary anti-rabbit HRP conjugated antibody (here after referred to as secondary antibody) at a concentration of 1:20,000 v/v. The resulting western blot detection using the enhanced chemiluminescence is shown in Figure 7.2. A band, indicating the positive detection with the conjugate antibody was seen in the kidney sample. Its size was compared to the known size standards included on the blot and found to be approximately 40 kDa.

This initial blot (see Figure 7.2) showed that the antibody system of binding and detection was working and the next stage required in order to get clearer pictures was optimisation of the antibody conditions.
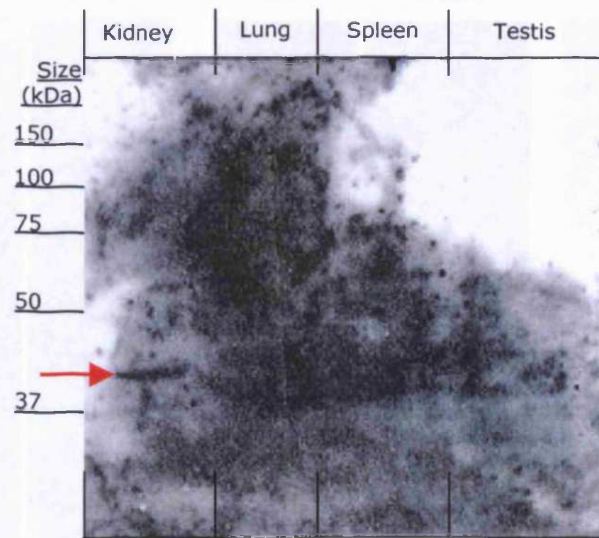
Figure 7.2 Western blot of 50μg of each membrane samples (the membrane type is shown above the respective lane) probed with the anti-adenosine A_3 receptor (primary) antibody. The red arrow indicates a band of the expected molecular weight, suggesting that this antibody is effective in immunoblotting for the adenosine A_3 receptor.

7.3.2. Optimisation of the antibody conditions

To enable optimisation of the immunoblotting conditions, the protocol was repeated with sequential dilutions of the primary antibody concentration keeping the secondary antibody concentration constant. The higher concentrations of primary antibody caused dark areas of background making detection of specific bands difficult. This is due to the primary antibody binding non-specifically to proteins in the membrane sample. Optimization allows the balance between specificity and detection to be achieved allowing distinct bands to be detected.

A concentration of 1:5000 v/v of primary antibody was found to be optimum (Figure 7.3).

Antibody dilution (v/v)	Primary 1:1500 Secondary 1:20,000		Primary 1:2500 Secondary 1:20,000		Primary 1:5000 Secondary 1:20,000	
Membrane Sample Size (kDa)	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen
150						
100						
75						
50						
37						
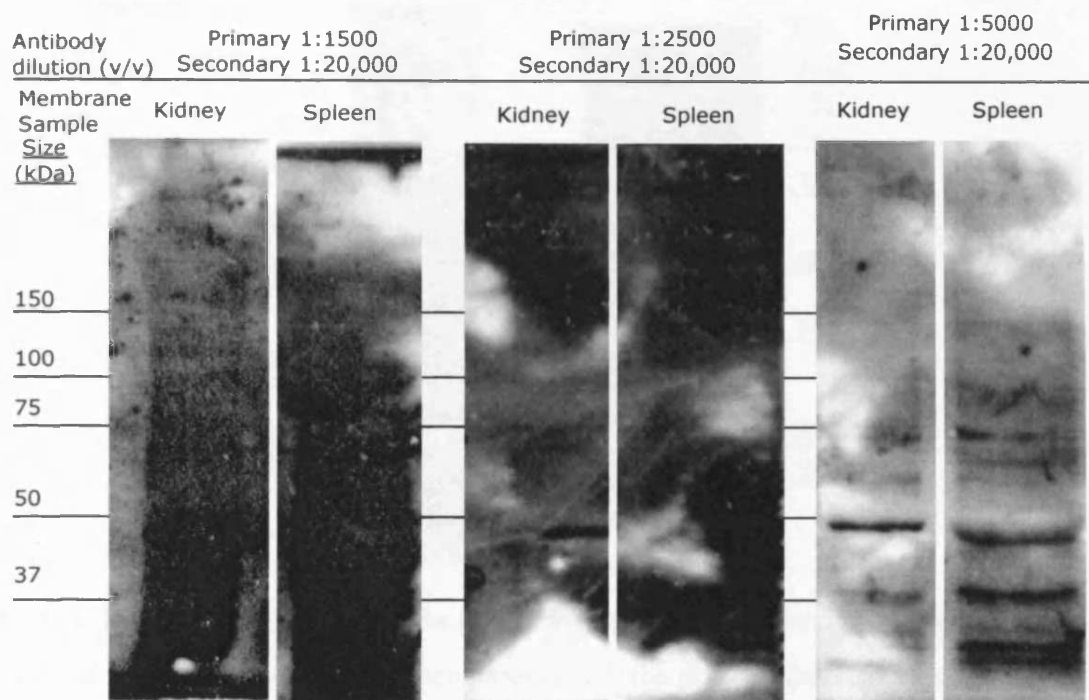
Figure 7.3 The optimisation of the primary antibody concentration. Western blots of 50µg kidney and spleen membranes probed with the primary (anti-adenosine A_3 receptor) antibody. Differing dilutions of the primary antibody were used (shown above the respective lane) and the resulting blot with the most prominent banding indicated the optimum primary antibody concentration to be used, in this case 1:5,000 v/v.

An adjustment of the secondary antibody concentration to 1:30,000 v/v was found to be favourable by increasing the contrast between the bands and the rest of the blot where no binding occurred. Therefore the concentration of secondary antibody used was changed to 1:30,000 v/v (Figure 7.4).
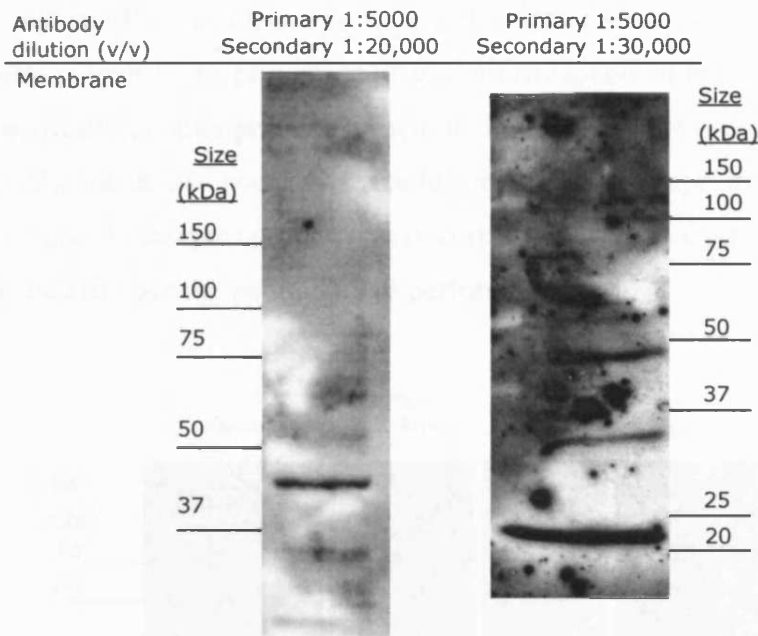
Figure 7.4 The optimisation of the secondary antibody concentration. Western blots of 50μg of kidney membranes probed with the primary (anti-adenosine A_3 receptor) antibody. Differing dilutions of the secondary (anti-rabbit HRP conjugated) antibody were used (shown above the respective lane) and the resulting blot with the most prominent banding indicates the optimum secondary antibody concentration.

In all of the above work the antibodies were incubated with the membrane in 1% v/v BLOTTO. An adjustment to 3% BLOTTO for both the primary and secondary antibody solutions was found not to be favourable, so the BLOTTO concentration was kept at 1% v/v (data not shown).

The optimised conditions for adenosine A_3 receptor detection were found to be a primary antibody concentration of 1:5000 v/v and a secondary antibody concentration of 1:30,000 v/v, both in 1% BLOTTO.

7.3.3. Anti-adenosine A_3 receptor antibody binding

Using the optimised conditions the membrane samples prepared from ischaemic and normoxic hearts were probed using the anti-adenosine A_3 receptor (primary) antibody. The result is shown in Figure 7.5. Bands were seen around the 30-50 kDa range, which is the size expected for a G-protein coupled receptor protein. Therefore the primary antibody was binding to a protein that

potentially could be the adenosine A_3 receptor. However, as there are other antibodies present in the primary antibody serum capable of binding non-specifically to other proteins present in the atrial membranes, other non-specific bands also occur. In order to confirm whether specific binding to the adenosine A_3 receptor protein was occurring, an assay with the control peptide used to raise the antibody was performed.
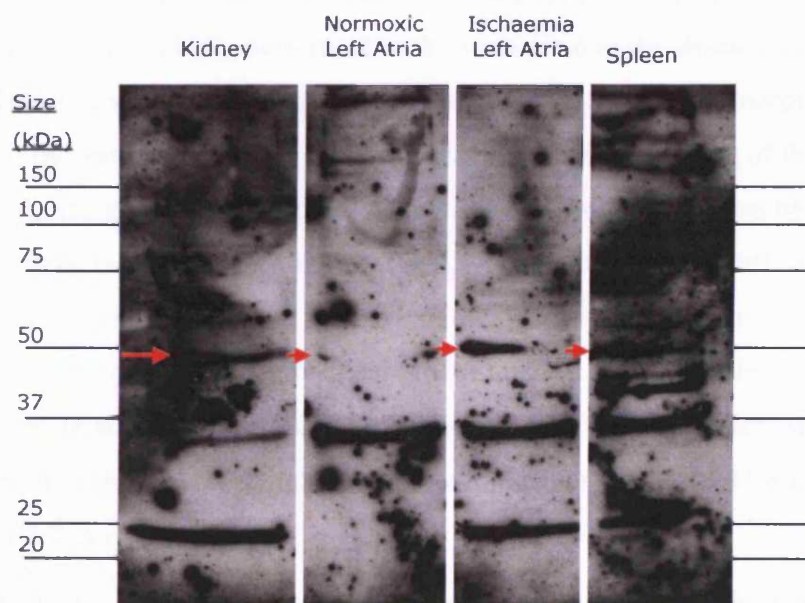
Figure 7.5 Suspected adenosine A_3 receptor protein. Western blot of 37.5 µg of ischaemic and normoxic left atria membranes, together with kidney and spleen membrane samples which acted as controls. Following probing with the anti-adenosine A_3 receptor antibody, the presence of banding of the expected size (with in the red arrow indicating) shows the adenosine A_3 receptor protein is potentially in both cardiac membrane samples.

7.3.4. Control peptide assay to confirm specificity

To confirm that the primary antibody is binding specifically to the adenosine A_3 receptor, a control peptide assay was performed. This was repeated three times in order to confirm that the protein from the adenosine A_3 receptor was present in the membrane samples obtained from the atria. This assay is able to reveal the site of specific anti-adenosine A_3 receptor antibody binding by absorption of the specific antibody from the primary antibody sample by the peptide.

Therefore two blots were performed using identical antibody samples, one of which had the specific antibodies removed by incubation with the control peptide and therefore should not produce any specific bands.

The primary antibody was pre-incubated with 20 μg of the immunising control peptide as described in Chapter Two, section 2.5.2.5. The resulting western blots showed that the band of the suspected size of the adenosine A_3 receptor was reduced in size and intensity (Figure 7.6) compared to the band obtained from identical western blots performed at the same time in the absence of peptide. These bands are therefore assumed to be the adenosine A_3 receptor protein and by comparison of the autoradiographs, the identification of the adenosine A_3 receptor protein is possible on the corresponding control blot. By measurement of the known molecular weight markers included on each blot a standard curve were constructed. The size of the band suspected to be the adenosine A_3 receptor was estimated using the standard curve, and the resulting estimates are detailed in Table 7.2. The bands suspected to be the adenosine A_3 receptor protein showed an estimated average molecular weight of 41.8 kDa (S.E.M. of 2.7, n=6).

Table 7.2 The size measurements of adenosine A_3 receptor protein in western blots calculated as unknowns from a standard curve constructed from the migration of known molecular weight markers.

	Estimated Size (kDa)
Blot 1	35.3
Blot 1 + Control Peptide	42.9
Blot 2	39.9
Blot 2 + Control Peptide	53.4
Blot 3	36.3
Blot 3 + Control Peptide	43.1
Average (± S.E.M.)	**41.8 (± 2.7)**
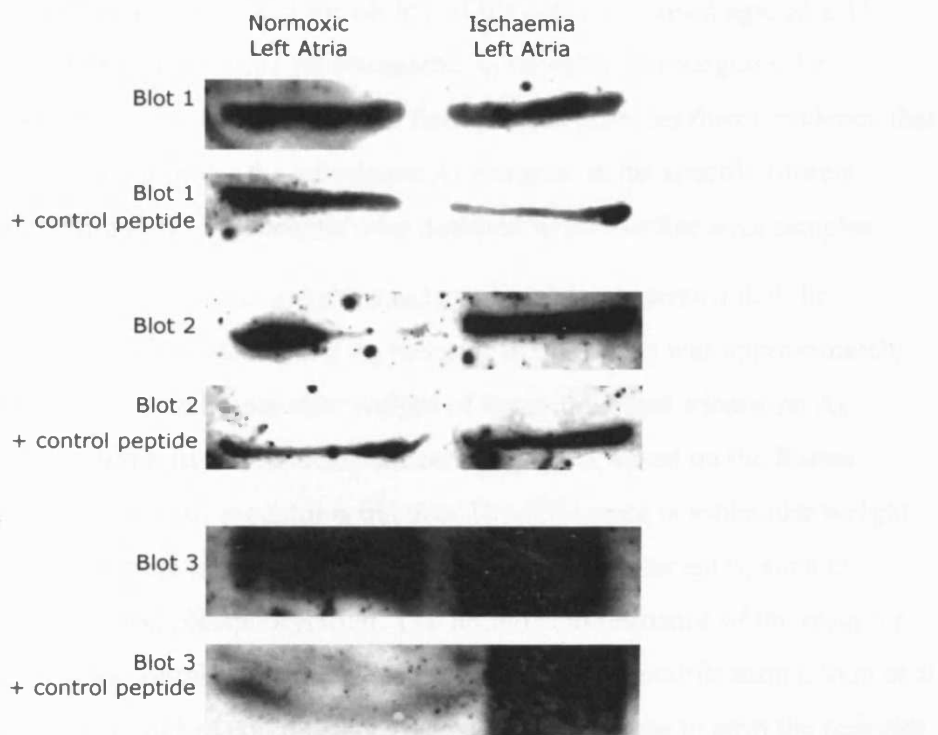
Figure 7.6 Western blot of ischaemic and normoxic left atria membrane samples (37.5μg) probed with the anti-adenosine A_3 receptor antibody. Antibody pre-incubated with 20μg of immunising control peptide before use is shown underneath the normal blot. The bands in these samples were reduced in size and intensity compared to the western blot of the same membrane samples in the absence of peptide.

7.4. Discussion

This study has demonstrated the ability of the antibody raised against a 15 amino acid sequence of the rat adenosine A_3 receptor, to recognise the adenosine A_3 receptor from left atria tissues. This provides direct evidence that rat atrial tissues express the adenosine A_3 receptor as the specific protein corresponding to the A_3 receptor was detected in the cardiac atria samples.

Comparison to molecular weight standards have demonstrated that the molecular size of the adenosine A_3 receptor in this tissue was approximately 42kDa. The expected molecular weight of the unmodified adenosine A_3 receptor based on its amino acid sequence is 37kDa, based on the Rattus norvegicus adenosine receptor sequence. The difference in molecular weight may be due to post-transcriptional modifications of the receptor, such as glycosylation and phosphorylation. The amino acid sequence of the receptor reveals it does contain potential sites for both types of modification (Zhou et al., 1992). Further studies could use deglycosylation enzymes to strip the receptor of its modifications to see if the molecular weight reduced to the expected size.

No detectable differences in the size or intensity of the band were detected for normoxic and ischaemia samples. This may indicate that 30 minutes of simulated hypoxic gassing has no effect on either post-transcriptional modifications or the degradation of the adenosine A_3 receptor protein on the plasma membrane of the atrial cells, compared to atria cells frozen immediately after death. Further work, however, would be required in order confirm this.

Although not performed here, it would be possible to further characterise the differences between adenosine A_3 receptor bands in normoxic and hypoxic atria by using densitometry measurements of the banded areas and normalizing the data to a house-keeping gene, such as β-actin.

There is a raft of support for cardioprotective actions via adenosine A_3 receptor activation, which is summarised in Chapter Four. It is not clear whether the cardioprotection provided by adenosine A_3 receptor agonists used in these studies was the result of their actions at A_3 receptors expressed in

cardiomyocytes or at A_3 receptors expressed in other cell types within the heart (Auchampach and Bolli, 1999).

However, this work concluded that the adenosine A_3 receptor protein is indeed expressed by myocytes in the left atrium, and is present at the plasma membrane of the cell. This supports the argument that adenosine A_3 receptor-mediated actions could be due to a direct effect on the adenosine A_3 receptor and downstream modifications caused by adenosine A_3 receptor activation.

The fact that these receptors are expressed in the normoxic tissue suggests that ischaemia or hypoxia is not required to reveal the receptors in order for them to exert their cardioprotective role. This raises the question why in earlier studies from this laboratory, IB-MECA was found to exert cardioprotection in guinea-pig isolated atria only when added at reperfusion and not when added before ischaemia (Gardner et al., 2004; Maddock et al., 2003). Although there is the possibility that species differences may account for the effect, the present results indicate that it may not related to an increased expression of the adenosine A_3 receptor in ischaemia. It may be due to adenosine A_3 receptors become desensitised with prolonged agonist exposure. It is known that the A_3 agonist R-PIA induces desensitisation of recombinant human A_3 receptors within twenty minutes (Palmer et al., 1996) and that exposure to the A_3 receptor agonist Cl-IB-MECA (100 nM) caused rapid receptor desensitisation and receptor internalization within 30 minutes of exposure in human astrocytoma cells (Trincavelli et al., 2002). Therefore the longer exposure when IB-MECA is added before ischaemia may result in loss of activity.

7.4.1. Conclusion

The presence of adenosine A_3 receptor in atria cells was confirmed using western blotting using rat tissue. The estimated size of the adenosine A_3 receptor protein obtained from the membrane was 42kDa. No detectable difference between the bands obtained from normoxic and ischaemic tissue was visually detected.

CHAPTER EIGHT

GENERAL DISCUSSION

8.1. Introduction

The experimental data obtained throughout this thesis has been extensively discussed in the relevant chapters. The purpose of this chapter is to summarise the relevant findings of the work, provide a critique of it and discuss how these findings fit into the field of knowledge for adenosine receptors and myocardial ischaemia and cardioprotection. The potential advancements that this field of knowledge could provide to medical science are also discussed.

8.2. Development of the appropriate model

8.2.1. Species

The work contained in this thesis was performed on only one species of animal; rats. This was a move away from the use of guinea pig tissue, the species that was previously used in this laboratory. The decision to move to the Wistar rats appears to be justified because of the successful identification of a protein specific for the adenosine A_3 receptor in membrane fractions prepared from left atria tissue (Chapter Seven).

In that respect we have completed one objective of the thesis that adenosine A_3 receptors are present in the myocardium. It is hoped that this work will be published as the first immunological characterisation of the adenosine A_3 receptor in cardiac tissues.

Obviously this finding supports further advancement of research into the adenosine A_3 receptors role in recovery from ischaemia in this animal species. Other support for the rat to be a suitable species for adenosine A_3 receptor targeted investigations is the finding that in the left atria, IB-MECA had a greater efficacy than at first anticipated based on previous studies on the guinea-pig (discussed in section 8.4.).

8.2.2. Type of model

A variety of models of cardiac ischaemia were used. In this thesis, isolated cardiac tissues were the main models used, in which the measured endpoints

were the recovery of baseline developed tension upon each contraction, which then was used as a assessment of the contractile function of the tissue.

In contrast, investigations using the isolated heart measured contractile function, coronary vascular function and infarct size as endpoints. However, when comparing the similar investigation of administration of 300nM IB-MECA on reperfusion/reoxygenation of the tissue the models all gave conflicting outcomes in their response to post ischaemic IB-MECA administration. The general outcomes from each model are summarised in Table 8.1, below.

Table 8.1 Summary of the main findings following the post ischaemic administration of IB-MECA (300nM) in the three cardiac ischaemia models used in this thesis.

	IB-MECA vs. Control	IB-MECA vs. Control in the presence of MRS1191
Isolated left atria	IB-MECA exhibited a cardioprotective effect	Cardioprotection by IB-MECA not prevented
Isolated right ventricular strips	No significant effect	Not performed
Isolated heart	No significant effect on infarct No significant effect on contractility*	IB-MECA exhibited a pro-infarct effect No significant effect on contractility*

*also in the presence of DPCPX an A_1 receptor antagonist.

IB-MECA in the isolated left atria yields a cardioprotective effect on contractile function, but in the isolated heart it has no effect on contractility yet yields a damaging pro-infarct effect. Because of this discrepancy, and the lack of effect in the ventricular strips it is not possible to state the general effect IB-MECA has in the heart. Improvement of the contractile recovery by IB-MECA was only exhibited in the isolated left atria. IB-MECA can therefore be shown to provide some sort of protection against post-ischaemia dysfunction with recovery of contractile function occurring in only one cardiac model of ischaemia.

Why it was not seen in the ventricular strips or the isolated heart may warrant further investigation. The possible reason for the lack of response to IB-MECA

in the isolated ventricle strips is discussed in section 8.4. Why it would have no effect in the isolated heart could be to it being a more complex model with mediators involved in the heart regulation involved which may counter-act the possible effect of IB-MECA.

The improvement of contractility effect of IB-MECA may be limited to the atria only, and if so IB-MECA may have little impact on the function of the whole heart.

Whereas some aspects of contractile dysfunction may be taken to be a temporary injury, infarction is a permanent injury to the tissue. A direct comparison of IB-MECA effects on contractile function and infarct from the models are not valid as there is too great a difference between the two modes of injury.

A modification when using isolated tissues would be to measure infarct size in isolated tissue, using the TTC method previously described in Chapter Two, section 2.3.3. Other published literature has achieved this with isolated left ventricle slices (Veeravalli and Akula, 2004). Having this data would make the isolated tissues a more clinically relevant model for investigation.

8.3. Use of IB-MECA

The selective adenosine A_3 agonist IB-MECA has been the principal drug tool used throughout this thesis (See Chapter Four, table 4.1 for examples). Of the experiments conducted in isolated tissues IB-MECA administration has exhibited a cardioprotective effect when used in concentrations ranging between 10nM (Tracey et al., 1997) and 300nM (Maddock et al., 2003). Tracey et al. (1997) demonstrated that in rabbit tissue 10nM and 50nM, but not 1nM of IB-MECA exhibited a cardioprotective effect in isolated hearts. The Ki of IB-MECA at the rabbit adenosine A_3 receptor is 2nM (Hill et al., 1997).

Of these and other experiments exhibiting IB-MECA's cardioprotective effect in isolated tissues at lower dose, none were performed in the rat. We concluded that as explained in Chapter Five the dose of 300nM was supra-maximal, and cardioprotection can be exhibited by a dose of 3nM.

As IB-MECA's Ki value at the rat adenosine A_3 receptor is 1.1nM, (Jacobson, 1998) using a 10 fold higher dose (~10nM) seems a feasible lower concentration that could have been used to demonstrate the satisfactory efficacy of IB-MECA. This finding identifies the need for further investigation into the action of IB-MECA as lower doses, which are discussed in section 8.4.1.

However as discussed in section 4.4.1. the failure of IB-MECA to evoke a response in the ventricular strips when used at a concentration 333-fold higher than the lowest concentration that evoked a response in the isolated atria, makes it unlikely that the concentration was too low in isolated ventricular strips. It maybe that there is a bell-shaped dose response curve for the cardioprotective effect of IB-MECA and that the 300nM concentration is not only supra-maximal but also less effective thank lower doses.

Other possible reasons may be due to the extent of injury that occurs to the strip during removal, such as a preconditioning effect or permanent damage that renders the strips immune to cardioprotection by drugs. This point also merits further investigations.

8.3.1. Possible further study

The major limitation to the findings of this thesis is that we failed to block the cardioprotection by IB-MECA using MRS1191, a specific rat adenosine A_3 receptor antagonist and failure to attribute IB-MECA's action to another adenosine receptor subtype. As such a strong body of work supports the action of adenosine A_3 receptor stimulation as cardioprotective, and also that IB-MECA administration evokes cardioprotection, is it difficult to conclude that IB-MECA's cardioprotective actions here are a result of exclusively evoking a non-specific effector, especially as we have proven the presence of the adenosine A_3 receptor in the left atria. Thus a alternative explanation for this result can be sought.

It was concluded that IB-MECA was used at a supra-maximal concentration which prevented MRS1191 from blocking its action. This finding was an unforeseen occurrence, but with hindsight possibly could have been anticipated from the published Ki data for IB-MECA at the rat (Jacobson, 1998) and rabbit

(Hill et al., 1997) adenosine A_3 receptor. Possible reasons why this concentration may have had a higher efficacy in this model may therefore be due to the animal species used.

Following the dose-response investigation (Section 4.3.2) it was found that lower concentrations all gave a comparable level of cardioprotection, up to and including 3nM, which was the lowest concentration used. This suggests that there is a justification in repeating the investigation using the antagonist MRS1191, with a lower concentration of IB-MECA. If MRS1191 then blocked the cardioprotective effect of this lower concentration of IB-MECA then that would prove that it stimulation of adenosine A_3 receptors by IB-MECA that is evoking cardioprotection in this model. If it was found that MRS1191 did not block IB-MECA's cardioprotective actions then that would suggest that the cardioprotection evoked by IB-MECA is due to non-specific mechanisms.

8.4. Characterisation of adenosine A_3 receptor

As discussed above one of the aims of this thesis, to confirm the presence of adenosine A_3 receptors in the myocardium, was achieved. We have proven that adenosine A_3 receptor protein is present in membrane fractions from left atria. Extension of this study would use the same immunological methods to show if adenosine A_3 receptors are present in the other regions of the heart.

If it was proven that adenosine A_3 receptors are indeed absent from the right ventricle fraction then this would provide one possible explanation why IB-MECA did not affect the recovery of the ventricle strips when used (discussed in Chapter Four, section 4.4.1.). Further characterisation using immunological techniques will provide some clarification on whether this is a reasonable explanation.

Clarification on the cell types within the fraction that express the adenosine A_3 receptors may yield further information such as whether the receptor is expressed on the mast cells within the cardiac fraction. As mast cells degranulation is regulated by A_3 receptor stimulation (Reeves et al., 1997), finding these cells in the fraction will implicate this cell type in the tissue's response to A_3 receptor stimulation.

8.4.1. Limitations of the study

A review of the work performed in the thesis has identified a number of points that could have been improved upon. Throughout the work the control groups used where not included with the experimental groups. This reliance on historical controls is not ideal as illustrated in section 3.3.2. there the results obtained using the same protocol change with time. Ideally controls should have been performed concurrently with the experiments

The numbers of each experimental group were frequently below the numbers required for a successful statistical comparison. At the commencement of work a sample size of 6 per group was regarded as being sufficient. A later power analysis of the typical percentage values and the standard deviation of the actual experimental groups revealed that sample size of around 12-15 is the minimum needed for acceptable statistical comparison. Therefore comparison between groups with a sample size of less than 12 has to be considered to be underpowered.

8.4.2. Possible further study

The possibility of non-specific effects occurring with the use of any drug is an aspect of any work performed in native tissue. The possible interference that this may have has to be considered from any of the results drawn from these experiments. To better characterise the specific effects of a drug other fields of study need to be considered. I have discussed below other ways, which could be employed to obtain further characterisation of the adenosine A_3 receptor.

Cultured cells expressing a single receptor type could be used. Surrogate indicators of ischaemic damage or cardioprotection could then be used to assess cardioprotection of pharmacological agents. While it would not be possible to fully evaluate the role of adenosine A_3 receptor with this approach, the work may indicate the best possible potential avenues for further investigation. Some aspects of my thesis could be refined and repeated. I have discussed below other ways, which could be employed to obtain further characterisation of the adenosine A_3 receptor.

One area of research that ties in with the models used here is the use of genetically modified 'knock-out' mice that have the receptor gene deleted. Previous studies have produced adenosine A_3 receptor 'knock out' mice strains (Salvatore et al., 2000a). Though removed from the normal physiology, work in this field has recently brought some strong conclusions.

Ge et al. (2006) used A_3 receptor-knock out mice to "Provide the first .. definitive evidence that adenosine A_3 receptor agonists provide protection against myocardial ischaemia and reperfusion injury by activating adenosine A_3 receptor". Earlier studies by Guo et al. (2001) where A_3 receptor-knock out mice exhibited a cardioprotective phenotype were rejected because of what they deemed use of the inappropriate strains that were used to gather control measures.

As discussed in Chapter Five, involvement of the adenosine A_3 receptor could not be proven definitively due to being unable to selectivity block the action of a selective agonist using a selective antagonist. Using A_3 receptor-knock out animals may have the potential to allow clearer conclusions to be drawn. While any work conducted in gene knockout studies of this type will add to our knowledge of ischaemia, it is not a road to go down regarding finding a clinical 'treatment' for ischaemia as the model is a less natural model of ischaemia. However it would be a useful tool, as the present pharmaceutical agents available now simply do not have the selectivity index that would allow them to be used clinically.

8.4.2. Use of radiolabelled ligands

Following on from the immunological characterisation radioligand binding using the iodinated form of IB-MECA, [^{125}I]AB-MECA, with membrane fractions from the cardiac tissue sample will be able to evaluate if IB-MECA has a receptor binding site in the tissue. To assess whether there are possible non-specific binding site for IB-MECA, these experiments could be performed on tissue obtained from adenosine A_3 receptor knockout animals. Also ischaemic and normoxic tissues could be used to assess if ischaemia brings about a different phenotype to the cell.

8.5. The Context of this thesis

In the clinical context, adenosine A_3 receptor signalling manipulation in order to provide a clinically use is by nature a far more complex issue. Multiple intercellular signalling cascades provide regulatory control at a cellular level right through to the whole body responses of the nervous system on the heart. Also A_3 receptors are thought to be widely distributed in the body therefore increasing the scope for systemic side effects.

However, therapeutic strategies targeting the A_3 receptor may have greater applicability in the clinical setting than the other adenosine receptor subtypes. Auchampach and Bolli (1999) suggested that adenosinergic therapy has to take advantage of receptor reserve, and that adenosine agonists will elicit more potent responses in tissues where the receptors are in excess. Work where A_{2A} receptor agonist increased coronary conductance without change to systemic hemodynamic suggests this hypothesis is feasible (Shryock et al., 1998).

With that in mind the use of A_3 receptor manipulation is discussed below.

8.5.1. Other forms of ligand mediated cardioprotection

Extrinsic cardioprotection is not exclusively adenosine-mediated and many receptor ligands have also been found to be cardioprotective. Bradykinin, opioids, Erythropoietin and adrenergic compounds been identified as cardioprotective ligands in animal models (Gross and Gross, 2006).

Analogous with adenosine, bradykinin is also elevated in ischaemia. Two bradykinin receptors are found in cardiomyoctyes. However the expression of the bradykinin receptors is stress-induced. Bradykinin administration mimics ischaemic preconditioning, which has been found to be mediated by a bradykinin receptor. Opioids peptides are also released as a result of myocardial ischemia. Of the three opidod receptor subtypes identified, two have been found in cardiomyocytes. Morphine administration has been found to be cardioprotective. Erythropoietin has been found to be cardioprotective in animal models of ischaemia, and its receptor are found in myocytes. Adrenergic receptor blockade at reperfusion has been shown to reduce infarct size. Opiods, like adenosine has been shown to have a dose dependant effect.

8.5.2. Adenosine A_3 receptor manipulation therapy

Envisioning the use of IB-MECA solely as an agent to activate adenosine A_3 receptors is made difficult for the reasons of potential non-selectivity at the different adenosine receptor subtypes, but its high potency at the A_3 receptors could overcome this and allow low dose administration. For example the $100\mu g/kg$ intravenous dose has a post-ischaemic protective effect in dog (Auchampach et al., 2003) and rabbit (Takano et al., 2001) translates to a reasonable 8.5mg dose for a male of average weight (190 pounds, US Department of Health and Human Services, 2006). This low dose may limit clinical side effects.

However with further investigation and discovery of more truly selective A_3 receptor agonists, A_3 receptor manipulation could have a greater potential for use as a clinical strategy for treating myocardial ischaemia and its subsequent after effects in the heart. Poulsen and Quinn (1998) remarked that specific tissue localisation will overcome the barrier to developing adenosine based therapeutics, a step we have taken in this thesis, but stressed the importance that any human therapy has to be based on work performed on the human receptors due to the difference in receptor structure between the species.

A_3 receptor stimulation has been shown to cause mast cell degranulation in rat and mice, but not rabbits (Hannon et al., 1995). Whether this would occur in man remained to be seen and if not then A_3 receptor manipulation has greater potential if it can be administered without causing any hemodynamic changes.

Another theoretically step would be to modify the A_3 receptor expression in the therapeutic-target tissue in order to intrinsically increase it responsiveness to A_3 receptor targeted therapy. This approach was effective when used to over-express A_1 receptors in a model of ischaemic tolerance (Matherne et al., 1997). In humans this approach would be a branch of gene therapy, a technology described at present as being in it infancy.

The present work concentrates on the function of post-ischaemic tissue for a relatively short period of time after an acute ischaemic insult. This makes it difficult to say that adenosine A_3 manipulation therapy would be a viable

therapeutic idea based on this work, which is far removed from the clinical situation. However on the basis that A_3 receptor activation has the potential to augment recovery after insult, this offers great potential for the treatment of myocardial ischaemia conditions such as myocardial infarction and angina.

There is a question whether a therapeutic time window for adenosine A_3 receptor therapy exists since here the intervention occurs at the onset of reperfusion or before. It may be possible to dictate the administration time point in clinical situations where myocardial ischaemia occurs (e.g. after cardioplegia, angioplasty and transplant surgery). But in cases of myocardial ischaemia associated with myocardial infarction there is an inevitable delay from onset of symptoms to presenting into hospital, during which reperfusion may spontaneously occur. Reperfusion may not be possible to achieve immediately and with a delay the therapeutic window may close in this time. Further investigation would be needed to assess this issue of whether a therapeutic window exists. To assess this in an experimental model, the time point for intervention may have to be moved to a later point during reoxygenation/reperfusion.

8.5.3. Clinical Trials

At the present time the author is not aware of any clinical trial being conducted in which the A_3 receptor is a therapeutic target. Pharmalicensing Ltd, a licencing agengy in the UK, reports that currently a variety of adenosinergic compounds advancing toward clinical trials (Pharmalicensing Ltd, 2007).

A global search of patents through the European Patent Office online database found that a number of patents pertaining to targeting the adenosine A_3 receptor have been filed by global pharmaceutical companies (Sanofi-aventis, Novartis and Merck & Co.), as well as a number of smaller companies, academic intuitions and the US government (European Patent Office, 2007).

A search of the National Library of Medicine's ClinicalTrial.gov website performed 25th June 2007 revealed there is one ongoing studies assessing the benefit of adenosine administration in effecting endpoints of infarction (ClinicalTrial.gov identifier NCT00284323) and a French Cardiology Society

trial assessing whether ischaemic post conditioning can reduce coronary endothelial dysfunction and infarct size in humans (ClinicalTrial.gov identifier NCT00333320). Two large clinical trials have been performed to assess whether adenosine was a beneficial adjunct to reperfusion therapy in anterior myocardial infarction. The first study AMISTAD (Acute Myocardial Infarction Study of Adenosine) found that infarct size was reduced by intravenoius adenosine administration in certain pathphysological ischaemic conditions (Mahaffey et al., 1999). The second trial, AMISTAD-II, was performed to assess the actual clinical outcome of this reduction in infact size. It found that the clinical outcomes of myocardial infaction were not imporoved, although infact size and adverse clinical events were reduced (Ross et al., 2005). Its findings recommend further investigations with a higher dose of adenosine than the one used.

Post hoc analysis of the AMISTAD-II study revealed that in certain conditions if the time to therapy was less than 3.2 hours and the manner of reperfusion was either by thrombolytics or percutaneous coronary intervention then various mortality end points were reduced (Kloner et al., 2006). This was a post-hoc analysis, the results therefore should be considered hypothesis-generating only but it does seem that systemic adenosine infusion is capable of reducing mortality though its own receptors and intrinsic signalling pathways.

Clinical trials can be used to assess likelihood of an individual experiencing myocardial ischaemia. The studies of the risk factors for CHD are extensive, some well know examples are the INTERHEART study (Yusuf et al., 2004), and the Framingham Heart Study which was published in 1951 (Dawber et al., 1951). These studies identified a consistent set of criteria for individuals that will have a greater risk of coronary heart disease (CHD). Identifying these risk factors in individuals using their medical examination and history (e.g. smoking status, history of hypertension or diabetes, etc.) it is possible to identify individuals with a greater risk of CHD. This therefore provides a situation where a therapeutic agent may be administered as a prophylactic measure in order to create a "cardioprotective" state in normal tissue which is then protected against subsequent ischaemia in the event that the individual

subsequently goes on to suffer myocardial ischaemia (angina or myocardial infarction). Support for this approach came from preclinical animal studies where an adenosine A_3 receptor agonist has been administered as a pre-treatment before ischaemia which was shown to be cardioprotective in a number of models (see table 4.1). Not all literature has reported this effect (Maddock et al., 2003).

This clinical situation will relate more to the literature where the pharmacological agents were administered before the onset of ischaemia. This course of action has potential problems, such as desensitisation of the receptors affecting the outcome of other therapies. I have not come across any demonstration of an adenosine A_3 receptor functional response of the heart that is exclusive to normoxic situations. The immunological characterisation performed in Chapter Seven has shown that adenosine A_3 receptor may be present in the normoxic atria. But whether it is possible to demonstrate adenosine A_3 receptor-mediated responses in the heart under normal normoxic conditions is a further valid path of investigation. It may be that in normoxia the receptors do not couple to any functional response.

8.6. Conclusion

In conclusion, this work demonstrates that after 20 years from the first identification of adenosine as a cardioprotective agent (Olafsson et al., 1987), there are still gaps in our knowledge of the mechanisms that evoke the protection. Although the limitation of the tools available for research have prevented a fuller explanation of events, there are still a number of investigations possible that have the potential to further the medical treatment of myocardial ischaemia based upon adenosine receptor pharmacology and in particular adenosine A_3 receptors. Because of this potential further work in this area would be justified.

BIBLIOGRAPHY

ABBRACCHIO, M. P., BRAMBILLA, R., CERUTI, S., KIM, H. O., VON LUBITZ, D. K., JACOBSON, K. A. & CATTABENI, F. (1995). G protein-dependent activation of phospholipase C by adenosine A_3 receptors in rat brain. *Mol Pharmacol* **48(6)**, 1038-1045.

ABBRACCHIO, M. P., CERUTI, S., BRAMBILLA, R., FRANCESCHI, C., MALORNI, W., JACOBSON, K. A., VON LUBITZ, D. K. & CATTABENI, F. (1997). Modulation of apoptosis by adenosine in the central nervous system: a possible role for the A_3 receptor. Pathophysiological significance and therapeutic implications for neurodegenerative disorders. *Ann N Y Acad Sci* **825**, 11-22.

ABD-ELFATTAH, A. S., DING, M., DYKE, C. M. & WECHSLER, A. S. (1993). Protection of the stunned myocardium. Selective nucleoside transport blocker administered after twenty minutes of ischemia augment recovery of ventricular function. *Circulation* **88**, 11336-11343.

ALEXANDER, S. P. H., MATHIE, E. & PETERS, J. A. (2006). Guide to receptors and channels, 2nd edition. *Br J Pharmacol* **147(Suppl. 3)**, S1-S180.

ALKHULAIFI, A. M., PUGSLEY, W. B. & YELLON, D. M. (1993). The influence of the time period between preconditioning ischemia and prolonged ischemia on myocardial protection. *Cardioscience* **4(3)**, 163-169.

ARMSTRONG, S. & GANOTE, C. E. (1994). Adenosine receptor specificity in preconditioning of isolated rabbit cardiomyocytes: evidence of A_3 receptor involvement. *Cardiovasc Res* **28(7)**, 1049-1056.

ARMSTRONG, S. C. (2004). Protein kinase activation and myocardial ischemia/reperfusion injury. *Cardiovasc Res* **61(3)**, 427-436.

ASIMAKIS, G. K., INNERS-MCBRIDE, K., MEDELLIN, G. & CONTI, V. R. (1992). Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart. *Am J Physiol* **263(3 Pt 2)**, H887-894.

AMERICAN HEART ASSOCIATION. (2007). *Cardiac Medications At-A-Glance*. Available at: <URL: http://americanheart.org/presenter.jhtml?identifier=3038846> [Accessed: 10 July 2007].

AUCHAMPACH, J. A. & BOLLI, R. (1999). Adenosine receptor subtypes in the heart: therapeutic opportunities and challenges. *Am J Physiol* **276(3 Pt 2)**, H1113-1116.

AUCHAMPACH, J. A., GE, Z.-D., WAN, T. C., MOORE, J. & GROSS, G. J. (2003). A$_3$ adenosine receptor agonist IB-MECA reduces myocardial ischemia-reperfusion injury in dogs. *Am J Physiol Heart Circ Physiol* **285(2)**, H607-613.

AUCHAMPACH, J. A. & GROSS, G. J. (1994). Reduction in myocardial infarct size by the new potassium channel opener bimakalim. *J Cardiovasc Pharmacol* **23(4)**, 554-561.

AUCHAMPACH, J. A., JIN, X., MOORE, J., WAN, T. C., KRECKLER, L. M., GE, Z. D., NARAYANAN, J., WHALLEY, E., KIESMAN, W., TICHO, B., SMITS, G. & GROSS, G. J. (2004). Comparison of three different A$_1$ adenosine receptor antagonists on infarct size and multiple cycle ischemic preconditioning in anesthetized dogs. *J Pharmacol Exp Ther* **308(3)**, 846-856.

AUCHAMPACH, J. A., JIN, X., WAN, T. C., CAUGHEY, G. H. & LINDEN, J. (1997a). Canine mast cell adenosine receptors: cloning and expression of the A$_3$ receptor and evidence that degranulation is mediated by the A$_{2B}$ receptor. *Mol Pharmacol* **52(5)**, 846-860.

AUCHAMPACH, J. A., RIZVI, A., QIU, Y., TANG, X. L., MALDONADO, C., TESCHNER, S. & BOLLI, R. (1997b). Selective activation of A$_3$ adenosine receptors with N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide protects against myocardial stunning and infarction without hemodynamic changes in conscious rabbits. *Circ Res* **80(6)**, 800-809.

BARDENHEUER, H. & SCHRADER, J. (1986). Supply-to-demand ratio for oxygen determines formation of adenosine by the heart. *Am J Physiol* **250(2 Pt 2)**, H173-180.

BAXTER, G. F., HALE, S. L., MIKI, T., KLONER, R. A., COHEN, M. V., DOWNEY, J. M. & YELLON, D. M. (2000). Adenosine A$_1$ agonist at reperfusion trial (AART): results of a three-center, blinded, randomized, controlled experimental infarct study. *Cardiovasc Drugs Ther* **14(6)**, 607-614.

BLARDI, P., LAGHI PASINI, F., URSO, R., FRIGERIO, C., VOLPI, L., DE GIORGI, L. & DI PERRI, T. (1993). Pharmacokinetics of exogenous adenosine in man after infusion. *Eur J Clin Pharmacol* **44(5)**, 505-507.

BLINKS, J. R. & ENDOH, M. (1986). Modification of myofibrillar responsiveness to Ca^{++} as an inotropic mechanism. *Circulation* **73(3 Pt 2)**, III85-98.

BOLLI, R. (2000). The late phase of preconditioning. *Circulation Research* **87(11)**, 972-983.

BOLLI, R. & MARBAN, E. (1999). Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev* **79(2)**, 609-634.

BRAMBILLA, R., CATTABENI, F., CERUTI, S., BARBIERI, D., FRANCESCHI, C., KIM, Y. C., JACOBSON, K. A., KLOTZ, K. N., LOHSE, M. J. & ABBRACCHIO, M. P. (2000). Activation of the A_3 adenosine receptor affects cell cycle progression and cell growth. *Naunyn Schmiedebergs Arch Pharmacol* **361(3)**, 225-234.

BRAUNWALD, E. (1990). The stunned myocardium: newer insights into mechanisms and clinical implications. *J Thorac Cardiovasc Surg* **100(2)**, 310-311.

BRAUNWALD, E. (1991). Stunning of the myocardium: an update. *Cardiovasc Drugs Ther* **5(5)**, 849-851.

BRAUNWALD, E. & KLONER, R. A. (1982). The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation* **66(6)**, 1146-1149.

BRAUNWALD, E. & KLONER, R. A. (1985). Myocardial reperfusion: a double edged sword? *J Clin Invest* **76**, 1713-1719.

BRUNS, R. F., LU, G. H. & PUGSLEY, T. A. (1986). Characterization of the A_2 adenosine receptor labelled by ^{3}H-NECA in rat striatal membranes. *Mol Pharmacol* **29(4)**, 331-346.

BUDDE, J. M., VELEZ, D. A., ZHAO, Z., CLARK, K. L., MORRIS, C. D., MURAKI, S., GUYTON, R. A. & VINTEN-JOHANSEN, J. (2000). Comparative study of AMP579 and adenosine in inhibition of neutrophil-mediated vascular and myocardial injury during 24h of reperfusion. *Cardiovasc Res* **47(2)**, 294-305.

CARR, C. S., HILL, R. J., MASAMUNE, H., KENNEDY, S. P., KNIGHT, D. R., TRACEY, W. R. & YELLON, D. M. (1997). Evidence for a role for both the adenosine A_1 and A_3 receptors in protection of isolated human atrial muscle against simulated ischaemia. *Cardiovasc Res* **36(1)**, 52-59.

CARRUTHERS, A. M. & FOZARD, J. R. (1993). Effect of pertussis toxin treatment on the putative adenosine A_3 receptor-mediated hypotensive response in the rat. *Eur J Pharmacol* **250(1)**, 185-188.

CHAN, T. O., RITTENHOUSE, S. E. & TSICHLIS, P. N. (1999). AKT/PKB and other D_3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* **68**, 965-1014.

COHEN, M. V. & DOWNEY, J. M. (1996). Myocardial preconditioning promises to be a novel approach to the treatment of ischemic heart disease. *Annu Rev Med* **47**, 21-29.

COLLIS, M. G. & HOURANI, S. M. (1993). Adenosine receptor subtypes. *Trends Pharmacol Sci* **14(10)**, 360-366.

CRESTANELLO, J. A., DOLIBA, N. M., BABSKY, A. M., DOLIBA, N. M., NIIBORI, K., WHITMAN, G. J. R. & OSBAKKEN, M. D. (2002). Ischemic Preconditioning Improves Mitochondrial Tolerance to Experimental Calcium Overload. *Journal of Surgical Research* **103(2)**, 243-251.

DAWBER, T. R., MEADORS, G. F. & MOORE, F. E., JR. (1951). Epidemiological approaches to heart disease: the Framingham Study. *Am J Public Health Nations Health* **41(3)**, 279-281.

DE JONG, J. W., DE JONGE, R., KEIJZER, E. & BRADAMANTE, S. (2000). The role of adenosine in preconditioning. *Pharmacol Ther* **87(2-3)**, 141-149.

DEUSSEN, A., LLOYD, H. G. & SCHRADER, J. (1989). Contribution of S-adenosylhomocysteine to cardiac adenosine formation. *J Mol Cell Cardiol* **21(8)**, 773-782.

DEUSSEN, A., STAPPERT, M., SCHAFER, S. & KELM, M. (1999). Quantification of extracellular and intracellular adenosine production: understanding the transmembranous concentration gradient. *Circulation* **99(15)**, 2041-2047.

DI TULLIO, M. A., TAYEBATI, S. K. & AMENTA, F. (2004). Identification of adenosine A_1 and A_3 receptor subtypes in rat pial and intracerebral arteries. *Neurosci Lett* **366(1)**, 48-52.

DIXON, A. K., GUBITZ, A. K., SIRINATHSINGHJI, D. J., RICHARDSON, P. J. & FREEMAN, T. C. (1996). Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol* **118(6)**, 1461-1468.

DORING, H. J. & DEHNERT, H. (1988). Methods for the measurement of cardiovascular parameters. *The Isolated Perfused Heart According to Langendorff.* 1st ed. Biomesstechnik-Verlag March.

DOS SANTOS, P., KOWALTOWSKI, A. J., LACLAU, M. N., SEETHARAMAN, S., PAUCEK, P., BOUDINA, S., THAMBO, J. B., TARIOSSE, L. & GARLID, K. D. (2002). Mechanisms by which opening the mitochondrial ATP- sensitive K^+ channel protects the ischemic heart. *Am J Physiol Heart Circ Physiol* **283(1)**, H284-295.

DOW, J. W., NIGDIKAR, S. & BOWDITCH, J. (1985). Adenine nucleotide synthesis de novo in mature rat cardiac myocytes. *Biochim Biophys Acta* **847(2)**, 223-227.

DOWNEY, J. M., LIU, G. S. & THORNTON, J. D. (1993). Adenosine and the anti-infarct effects of preconditioning. *Cardiovasc Res* **27(1)**, 3-8.

DRURY, A. N. & SZENT-GYORGYI, S. (1929). *Journal of Physiology* **68**, 213.

DURAND, I. H. & GREEN, R. D. (2001). Cloning of a chick A_3 adenosine receptor: characterization of ligand binding and receptor-effector coupling of chick A_1 and A_3 adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol* **363(1)**, 81-86.

EL-MENYAR, A. A. (2005). The resuscitation outcome: revisit the story of the stony heart. *Chest* **128(4)**, 2835-2846.

ELY, S. W. & BERNE, R. M. (1992). Protective effects of adenosine in myocardial ischemia. *Circulation* **85(3)**, 893-904.

ENGLERT, M., QUITTERER, U. & KLOTZ, K. N. (2002). Effector coupling of stably transfected human A_3 adenosine receptors in CHO cells. *Biochem Pharmacol* **64(1)**, 61-65.

EUROPEAN PATENT OFFICE. (2007). *esp@cenet*. Available at: <URL: ep.espacenet.com> [Accessed: 10 July 2007].

FABIATO, A. & FABIATO, F. (1978). Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscles. *J Physiol* **276**, 233-255.

FISHBEIN, M. C., MEERBAUM, S., RIT, J., LANDO, U., KANMATSUSE, K., MERCIER, J. C., CORDAY, E. & GANZ, W. (1981). Early phase acute myocardial infarct size quantification: validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. *Am Heart J* **101(5)**, 593-600.

FLISS, H. & GATTINGER, D. (1996). Apoptosis in Ischemic and Reperfused Rat Myocardium. *Circ Res* **79(5)**, 949-956.

FORSTER, K., PAUL, I., SOLENKOVA, N., STAUDT, A., COHEN, M. V., DOWNEY, J. M., FELIX, S. B. & KRIEG, T. (2006). NECA at reperfusion limits infarction and inhibits formation of the mitochondrial permeability transition pore by activating p70S6 kinase. *Basic Res Cardiol* **101(4)**, 319-326.

FOZARD, J. R. & CARRUTHERS, A. M. (1993). adenosine A3 receptor mediate hypotension in the angiotensin II-supported circulation of the pithed rat. *Br J Pharmacol* **109(1)**, 3-5.

FOZARD, J. R., PFANNKUCHE, H. J. & SCHUURMAN, H. J. (1996). Mast cell degranulation following adenosine A3 receptor activation in rats. *Eur J Pharmacol* **298(3)**, 293-297.

FREDHOLM, B. B., ARSLAN, G., KRULL, B., KONTNY, E. & SVENNINGSSON, P. (1996). Adenosine (P1) Receptor Signalling. *Drug Delivery Research* **39**, 262-268.

GARDNER, N. M. 1997. *Heterogeneity of receptors and signalling in cardiac responses to adenosine.* Ph.D Thesis, Cardiff University.

GARDNER, N. M. & BROADLEY, K. J. (1999). Analysis of the atypical characteristics of adenosine receptors mediating negative inotropic and chronotropic responses of guinea-pig isolated atria and papillary muscles. *Br J Pharmacol* **127(7)**, 1619-1626.

GARDNER, N. M., YATES, L. & BROADLEY, K. J. (2004). Effects of endogenous adenosine and adenosine receptor agonists on hypoxia-induced myocardial stunning in Guinea-pig atria and papillary muscles. *J Cardiovasc Pharmacol* **43(3)**, 358-368.

GARLID, K. D., PAUCEK, P., YAROV-YAROVOY, V., MURRAY, H. N., DARBENZIO, R. B., D'ALONZO, A. J., LODGE, N. J., SMITH, M. A. & GROVER, G. J. (1997). Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. *Circ Res* **81(6)**, 1072-1082.

GE, Z. D., PEART, J. N., KRECKLER, L. M., WAN, T. C., JACOBSON, M. A., GROSS, G. J. & AUCHAMPACH, J. A. (2006). Cl-IB-MECA [2-Chloro-N6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide] Reduces Ischemia/Reperfusion Injury in Mice by Activating the A3 Adenosine Receptor. *J Pharmacol Exp Ther* **319(3)**, 1200-1210.

GESSI, S., VARANI, K., MERIGHI, S., CATTABRIGA, E., IANNOTTA, V., LEUNG, E., BARALDI, P. G. & BOREA, P. A. (2002). A3 adenosine receptors in human neutrophils and promyelocytic HL60 cells: a pharmacological and biochemical study. *Mol Pharmacol* **61(2)**, 415-424.

GOTO, M., COHEN, M. V. & DOWNEY, J. M. (1996). The role of protein kinase C in ischemic preconditioning. *Ann N Y Acad Sci* **793**, 177-190.

GOTTLIEB, R. A., BURLESON, K. O., KLONER, R. A., BABIOR, B. M. & ENGLER, R. L. (1994). Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* **94(4)**, 1621-1628.

GROSS, E. R. & GROSS, G. J. (2006). Ligand triggers of classical preconditioning and postconditioning. *Cardiovasc Res* **70(2)**, 212-221.

GROSS, E. R., HSU, A. K. & GROSS, G. J. (2004). Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts. *Circ Res* **94(7)**, 960-966.

GROSS, G. J. (2003). Role of opioids in acute and delayed preconditioning. *Journal of Molecular and Cellular Cardiology* **35**, 709-718.

GUO, Y., BOLLI, R., BAO, W., WU, W. J., BLACK, R. G., JR., MURPHREE, S. S., SALVATORE, C. A., JACOBSON, M. A. & AUCHAMPACH, J. A. (2001). Targeted deletion of the A₃ adenosine receptor confers resistance to myocardial ischemic injury and does not prevent early preconditioning. *J Mol Cell Cardiol* **33(4)**, 825-830.

HALKOS, M. E., KERENDI, F., CORVERA, J. S., WANG, N. P., KIN, H., PAYNE, C. S., SUN, H. Y., GUYTON, R. A., VINTEN-JOHANSEN, J. & ZHAO, Z. Q. (2004). Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. *Ann Thorac Surg* **78(3)**, 961-969.

HAMMARBERG, C., FREDHOLM, B. B. & SCHULTE, G. (2004). Adenosine A₃ receptor-mediated regulation of p38 and extracellular-regulated kinase ERK1/2 via phosphatidylinositol-3'-kinase. *Biochem Pharmacol* **67(1)**, 129-134.

HANNON, J. P., PFANNKUCHE, H. J. & FOZARD, J. R. (1995). A role for mast cells in adenosine A₃ receptor-mediated hypotension in the rat. *Br J Pharmacol* **115(6)**, 945-952.

HARRISON, G. J., CERNIWAY, R. J., PEART, J., BERR, S. S., ASHTON, K., REGAN, S., PAUL MATHERNE, G. & HEADRICK, J. P. (2002). Effects of A₃ adenosine receptor activation and gene knock-out in ischemic-reperfused mouse heart. *Cardiovascular Research* **53(1)**, 147.

HEADRICK, J. P. (2002). Apparent activation of cardiovascular A₁ adenosine receptors by A₃ agonists. *Am J Physiol Heart Circ Physiol* **282(2)**, H793-796.

HEADRICK, J. P. & PEART, J. (2005). A₃ adenosine receptor-mediated protection of the ischemic heart. *Vascul Pharmacol* **42(5-6)**, 271-279.

HESS, M. L. & KUKREJA, R. C. (1994). Myocardial stunning. *J Card Surg* **9(3 Suppl)**, 382-386.

HILL, R. J., OLEYNEK, J. J., HOTH, C. F., KIRON, M. A., WENG, W., WESTER, R. T., TRACEY, W. R., KNIGHT, D. R., BUCHHOLZ, R. A. & KENNEDY, S. P. (1997). Cloning, expression and pharmacological characterization of rabbit adenosine A_1 and A_3 receptors. *J Pharmacol Exp Ther* **280(1)**, 122-128.

HILL, R. J., OLEYNEK, J. J., MAGEE, W., KNIGHT, D. R. & TRACEY, W. R. (1998). Relative importance of adenosine A_1 and A_3 receptors in mediating physiological or pharmacological protection from ischemic myocardial injury in the rabbit heart. *J Mol Cell Cardiol* **30(3)**, 579-585.

HIRAOKA, M. (1997). Pathophysiological functions of ATP-sensitive K^+ channels in myocardial ischemia. *Japanese Heart Journal* **38(3)**, 297-315.

ILIODROMITIS, E. K., MIKI, T., LIU, G. S., DOWNEY, J. M., COHEN, M. V. & KREMASTINOS, D. T. (1998). The PKC activator PMA preconditions rabbit heart in the presence of adenosine receptor blockade: is 5'-nucleotidase important? *J Mol Cell Cardiol* **30(11)**, 2201-2211.

INGWALL, J. S. (2002). Degradation and synthesis of ATP. In: INGWALL, J.S. ed. *ATP and the Heart*. Boston Dordrecht London: Kluwer Academic Publishers.

JACOBSON, K. A. (1995). A_3 adenosine receptor: design of selective ligands and therapeutic prospects. *Drugs Future* **20**, 689-699.

JACOBSON, K. A. (1998). Adenosine A_3 receptors: novel ligands and paradoxical effects. *Trends Pharmacol Sci* **19(5)**, 184-191.

JACOBSON, K. A., NIKODIJEVIC, O., SHI, D., GALLO-RODRIGUEZ, C., OLAH, M. E., STILES, G. L. & DALY, J. W. (1993). A role for central A_3-adenosine receptors. Mediation of behavioural depressant effects. *FEBS Lett* **336(1)**, 57-60.

JACOBSON, K. A. & SUZUKI, F. (1996). Recent developments in selective agonists and antagonists acting at purine and pyrimidine receptors. *Drug Development Research* **39(3-4)**, 289-300.

JORDAN, J. E., THOURANI, V. H., AUCHAMPACH, J. A., ROBINSON, J. A., WANG, N. P. & VINTEN-JOHANSEN, J. (1999). A_3 adenosine receptor activation attenuates neutrophil function and neutrophil-mediated reperfusion injury. *Am J Physiol Heart Circ Physiol* **277**, H1895-H1905.

JORDAN, J. E., ZHAO, Z.-Q., SAT, H., TAFT, S. & VINTEN-JOHANSEN, J. (1997). Adenosine A_2 receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary

endothelial adherence. *J. Pharmacol. Exp. Ther.* **280**, 301-309.

KATZ, A. M. (1970). Contractile proteins of the heart. *Physiol Rev* **50(1)**, 63-158.

KENNEDY, S. G., KANDEL, E. S., CROSS, T. K. & HAY, N. (1999). Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. *Mol Cell Biol* **19(8)**, 5800-5810.

KILPATRICK, E. L., NARAYAN, P., MENTZER, R. M., JR. & LASLEY, R. D. (2001). Adenosine A_3 agonist cardioprotection in isolated rat and rabbit hearts is blocked by the A_1 antagonist DPCPX. *Am J Physiol Heart Circ Physiol* **281(2)**, H847-853.

KIM, H. O., JI, X. D., SIDDIQI, S. M., OLAH, M. E., STILES, G. L. & JACOBSON, K. A. (1994). 2-Substitution of N6-benzyladenosine-5'-uronamides enhances selectivity for A_3 adenosine receptors. *J Med Chem* **37(21)**, 3614-3621.

KIM, S.-J., DEPRE, C. & VATNER, S. F. (2003). Novel Mechanisms Mediating Stunned Myocardium. *Heart Failure Reviews* **8(2)**, 143-153.

KIM, Y. C., DE ZWART, M., CHANG, L., MORO, S., VON FRIJTAG DRABBE KUNZEL, J. K., MELMAN, N., AP, I. J. & JACOBSON, K. A. (1998). Derivatives of the triazoloquinazoline adenosine antagonist (CGS15943) having high potency at the human A_{2B} and A_3 receptor subtypes. *J Med Chem* **41(15)**, 2835-2845.

KIM, Y. C., JI, X. D. & JACOBSON, K. A. (1996). Derivatives of the triazoloquinazoline adenosine antagonist (CGS15943) are selective for the human A_3 receptor subtype. *J Med Chem* **39(21)**, 4142-4148.

KIN, H., ZATTA, A. J., LOFYE, M. T., AMERSON, B. S., HALKOS, M. E., KERENDI, F., ZHAO, Z. Q., GUYTON, R. A., HEADRICK, J. P. & VINTEN-JOHANSEN, J. (2005). Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. *Cardiovasc Res* **67(1)**, 124-133.

KLONER, R. A., FORMAN, M. B., GIBBONS, R. J., ROSS, A. M., ALEXANDER, R. W. & STONE, G. W. (2006). Impact of time to therapy and reperfusion modality on the efficacy of adenosine in acute myocardial infarction: the AMISTAD-2 trial. *Eur Heart J* **27(20)**, 2400-2405.

KLONER, R. A., SHOOK, T., ANTMAN, E. M., CANNON, C. P., PRZYKLENK, K., YOO, K., MCCABE, C. H. & BRAUNWALD, E. (1998). Prospective temporal analysis of the onset of preinfarction angina versus outcome: an ancillary study in TIMI-9B. *Circulation* **97(11)**, 1042-1045.

KLOTZ, K. N. (2000). Adenosine receptors and their ligands. *Naunyn Schmiedebergs Arch Pharmacol* **362(4-5)**, 382-391.

KLOTZ, K. N., HESSLING, J., HEGLER, J., OWMAN, C., KULL, B., FREDHOLM, B. B. & LOHSE, M. J. (1998). Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol* **357(1)**, 1-9.

KOCH-WESER, J. & BLINKS, J. R. (1963). The Influence Of The Interval Between Beats On Myocardial Contractility. *Pharmacol Rev* **15**, 601-652.

KODANI, E., BOLLI, R., TANG, X. L. & AUCHAMPACH, J. A. (2001). Protection of IB-MECA against myocardial stunning in conscious rabbits is not mediated by the A_1 adenosine receptor. *Basic Res Cardiol* **96(5)**, 487-496.

KODANI, E., XUAN, Y.-T., SHINMURA, K., TAKANO, H., TANG, X.-L. & BOLLI, R. (2002). Delta-opioid receptor-induced late preconditioning is mediated by cyclooxygenase-2 in conscious rabbits. *American Journal Of Physiology. Heart And Circulatory Physiology* **283(5)**, H1943-H1957.

KOLOCASSIDES, K. G., GALINANES, M. & HEARSE, D. J. (1996). Dichotomy of ischemic preconditioning: improved postischemic contractile function despite intensification of ischemic contracture. *Circulation* **93(9)**, 1725-1733.

KROLL, K., DECKING, U. K., DREIKORN, K. & SCHRADER, J. (1993). Rapid turnover of the AMP-adenosine metabolic cycle in the guinea pig heart. *Circ Res* **73(5)**, 846-856.

KUKREJA, R. C. & JANIN, Y. (1997). Reperfusion Injury: Basic Concepts and Protection Strategies. *J Thromb Thrombolysis* **4(1)**, 7-24.

LANGENDORFF, O. (1895). Untersuchungen am uberlebenden Saugetierherzenam (Investigations on the Surviving Mammalian Heart). *Pflugers Archives fur die Gesamte Physiologie des Menschen and der Tiere (Archives for the entire physiology of humans and animals)* **61**, 291-332.

LASLEY, R. D., ANDERSON, G. M. & MENTZER, R. M., JR. (1993). Ischaemic and hypoxic preconditioning enhance postischaemic recovery of function in the rat heart. *Cardiovasc Res* **27(4)**, 565-570.

LASLEY, R. D. & MENTZER, R. M., JR. (1992). Adenosine improves recovery of postischemic myocardial function via an adenosine A_1 receptor mechanism. *Am J Physiol* **263(5 Pt 2)**, H1460-1465.

LASLEY, R. D., NARAYAN, P., JAHANIA, M. S., PARTIN, E. L., KRAFT, K. R. & MENTZER, R. M., JR. (1999). Species-dependent hemodynamic effects of adenosine A_3-receptor agonists IB-MECA and Cl-IB-MECA. *Am J Physiol* **276(6 Pt 2)**, H2076-2084.

LASLEY, R. D., RHEE, J. W., VAN WYLEN, D. G. & MENTZER, R. M., JR. (1990a). A_1 receptor mediated protection of the globally ischemic isolated rat heart. *J Mol Cell Cardiol* **22(1)**, 39-47.

LASLEY, R. D., RHEE, J. W., VAN WYLEN, D. G. & MENTZER, R. M., JR. (1990b). Adenosine A_1 receptor mediated protection of the globally ischemic isolated rat heart. *J Mol Cell Cardiol* **22(1)**, 39-47.

LEE, H. T. & EMALA, C. W. (2000). Protective effects of renal ischemic preconditioning and adenosine pre-treatment: role of A_1 and A_3 receptors. *Am J Physiol Renal Physiol* **278(3)**, F380-387.

LEE, J. A. & ALLEN, D. G. (1990). Calcium sensitisers: a new approach to increasing the strength of the heart. *BMJ* **300(6724)**, 551-552.

LEE, J. A. & ALLEN, D. G. (1991). Mechanisms of acute ischemic contractile failure of the heart. Role of intracellular calcium. *J Clin Invest* **88(2)**, 361-367.

LEE, J. E., BOKOCH, G. & LIANG, B. T. (2001). A novel cardioprotective role of RhoA: new signalling mechanism for adenosine. *FASEB J* **15(11)**, 1886-1894.

LIANG, B. T. (1997). Protein kinase C-mediated preconditioning of cardiac myocytes: role of adenosine receptor and K_{ATP} channel. *Am J Physiol* **273(2 Pt 2)**, H847-853.

LIANG, B. T. (1998). Protein kinase C-dependent activation of K_{ATP} channel enhances adenosine-induced cardioprotection. *Biochem J* **336 (Pt 2)**, 337-343.

LIANG, B. T. & JACOBSON, K. A. (1998). A physiological role of the adenosine A_3 receptor: sustained cardioprotection. *Proc Natl Acad Sci U S A* **95(12)**, 6995-6999.

LINDEN, J. (1994). Cloned adenosine A_3 receptors: pharmacological properties, species differences and receptor functions. *Trends Pharmacol Sci* **15(8)**, 298-306.

LINDEN, J., TAYLOR, H. E., ROBEVA, A. S., TUCKER, A. L., STEHLE, J. H., RIVKEES, S. A., FINK, J. S. & REPPERT, S. M. (1993). Molecular cloning and functional expression of a sheep A$_3$ adenosine receptor with widespread tissue distribution. *Mol Pharmacol* **44(3)**, 524-532.

LIU, G. S., RICHARDS, S. C., OLSSON, R. A., MULLANE, K., WALSH, R. S. & DOWNEY, J. M. (1994). Evidence that the adenosine A$_3$ receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res* **28(7)**, 1057-1061.

LLOYD, H. G. & SCHRADER, J. (1993). Adenosine metabolism in the guinea pig heart: the role of cytosolic S-adenosyl-L-homocysteine hydrolase, 5'-nucleotidase and adenosine kinase. *Eur Heart J* **14 Suppl I**, 27-33.

LORENZEN, A. & SCHWABE, U. (2001). P$_1$ Receptors. In: ABBRACCHIO, M.P. & WILLIAMS, M. eds. *Purinergic and Pyrimidinergic Signalling I.* Vol. I. Berlin: Springer-Verlag and Heidelberg GmbH & Co.

LOUBANI, M., HASSOUNA, A. & GALINANES, M. (2004). Delayed preconditioning of the human myocardium: signal transduction and clinical implications. *Cardiovasc Res* **61(3)**, 600-609.

LOUTTIT, J. B., HUNT, A. A., MAXWELL, M. P. & DREW, G. M. (1999). The time course of cardioprotection induced by GR79236, a selective adenosine A$_1$-receptor agonist, in myocardial ischaemia-reperfusion injury in the pig. *J Cardiovasc Pharmacol* **33(2)**, 285-291.

LU, J., ZANG, W. J., YU, X. J., JIA, B., CHORVATOVA, A. & SUN, L. (2006). Effects of postconditioning of adenosine and acetylcholine on the ischemic isolated rat ventricular myocytes. *Eur J Pharmacol* **549(1-3)**, 133-139.

MADDOCK, H. L., BROADLEY, K. J., BRIL, A. & KHANDOUDI, N. (2002a). Effects of adenosine receptor agonists on guinea-pig isolated working hearts and the role of endothelium and NO. *J Pharm Pharmacol* **54(6)**, 859-867.

MADDOCK, H. L., GARDNER, N. M., KHANDOUDI, N., BRIL, A. & BROADLEY, K. J. (2003). Protection from myocardial stunning by ischaemia and hypoxia with the adenosine A$_3$ receptor agonist, IB-MECA. *Eur J Pharmacol* **477(3)**, 235-245.

MADDOCK, H. L., MOCANU, M. M. & YELLON, D. M. (2002b). Adenosine A$_3$ receptor activation protects the myocardium from reperfusion/reoxygenation injury. *Am J Physiol Heart Circ Physiol* **283(4)**, H1307-1313.

MAHAFFEY, K. W., PUMA, J. A., BARBAGELATA, N. A., DICARLI, M. F., LEESAR, M. A., BROWNE, K. F., EISENBERG, P. R., BOLLI, R., CASAS, A. C., MOLINA-VIAMONTE, V., ORLANDI, C., BLEVINS, R., GIBBONS, R. J., CALIFF, R. M. & GRANGER, C. B. (1999). Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. *J Am Coll Cardiol* **34(6)**, 1711-1720.

MATHERNE, G. P., LINDEN, J., BYFORD, A. M., GAUTHIER, N. S. & HEADRICK, J. P. (1997). Transgenic A_1 adenosine receptor overexpression increases myocardial resistance to ischemia. *Proc Natl Acad Sci U S A* **94(12)**, 6541-6546.

MCCLANAHAN, T. B., IGNASIAK, D. P., MARTIN, B. J., MERTZ, T. E. & GALLAGHER, K. P. (1995). Effect of adenosine deaminase inhibition with pentostatin on myocardial stunning in dogs. *Basic Res Cardiol* **9**, 176-183.

MCCULLY, J. D., UEMATSU, M. & LEVITSKY, S. (1999). Adenosine-enhanced ischemic preconditioning provides myocardial protection equal to that of cold blood cardioplegia. *Ann Thorac Surg* **67(3)**, 699-704.

MEYERHOF, W., MULLER-BRECHLIN, R. & RICHTER, D. (1991). Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Letters* **284(2)**, 155.

MIURA, H., MORGAN, D. A. & GUTTERMAN, D. D. (1997). Oxygen-derived free radicals contribute to neural stunning in the canine heart. *Am J Physiol* **273(3 Pt 2)**, H1569-1575.

MORRISON, R. R., JONES, R., BYFORD, A. M., STELL, A. R., PEART, J., HEADRICK, J. P. & MATHERNE, G. P. (2000). Transgenic overexpression of cardiac A_1 adenosine receptors mimics ischemic preconditioning. *Am J Physiol Heart Circ Physiol* **279(3)**, H1071-1078.

MOUKARBEL, G. V., AYOUB, C. M. & ABCHEE, A. B. (2004). Pharmacological therapy for myocardial reperfusion injury. *Current Opinion in Pharmacology* **4**, 1-7.

MUBAGWA, K. & FLAMENG, W. (2001). Adenosine, adenosine receptors and myocardial protection: an updated overview. *Cardiovasc Res* **52(1)**, 25-39.

MUBAGWA, K., MULLANE, K. & FLAMENG, W. (1996). Role of adenosine in the heart and circulation. *Cardiovasc Res* **32(5)**, 797-813.

MURPHY, E., FRALIX, T. A., LONDON, R. E. & STEENBERGEN, C. (1993). Effects of adenosine antagonists on hexose uptake and preconditioning in perfused rat heart. *Am J Physiol* **265(4 Pt 1)**, C1146-1155.

MURRY, C. E., JENNINGS, R. B. & REIMER, K. A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74(5)**, 1124-1136.

MURRY, C. E., RICHARD, V. J., REIMER, K. A. & JENNINGS, R. B. (1990). Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* **66(4)**, 913-931.

MYERS, M. L., BOLLI, R., LEKICH, R. F., HARTLEY, C. J. & ROBERTS, R. (1985). Enhancement of recovery of myocardial function by oxygen free-radical scavengers after reversible regional ischemia. *Circulation* **72(4)**, 915-921.

NACHLAS, M. M. & SHNITKA, T. K. (1963). Macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity. *Am J Pathol* **42**, 379-405.

NAKANO, A., BAINES, C. P., KIM, S. O., PELECH, S. L., DOWNEY, J. M., COHEN, M. V. & CRITZ, S. D. (2000a). Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. *Circ Res* **86(2)**, 144-151.

NAKANO, A., COHEN, M. V. & DOWNEY, J. M. (2000b). Ischemic preconditioning: from basic mechanisms to clinical applications. *Pharmacol Ther* **86(3)**, 263-275.

NATIONAL CENTER FOR HEALTH STATISTICS. (2006) National Health and Nutrition Examination Survey. U.S. Department of Health and Human Services, Hyattsville, MD, U.S.A.

NEELY, J. R., ROVETTO, M. J., WHITMER, J. T. & MORGAN, H. E. (1973). Effects of ischemia on function and metabolism of the isolated working rat heart. *Am J Physiol* **225(3)**, 651-658.

OGAWA, T., MIURA, T., SHIMAMATO, K. & IIMURA, O. (1996). Activation of adenosine receptors before ischemia enhances tolerance against myocardial stunning in the rabbit heart. *J. Am. Coll. Cardiol.* **27**, 225-233.

OLAFSSON, B., FORMAN, M. B., PUETT, D. W., POU, A., CATES, C. U., FRIESINGER, G. C. & VIRMANI, R. (1987). Reduction of reperfusion injury in the canine preparation by intracoronary adenosine: importance of the endothelium and the no-reflow phenomenon. *Circulation* **76(5)**, 1135-1145.

OLAH, M. E. & STILES, G. L. (1995). Adenosine receptor subtypes: characterization and therapeutic regulation. *Annu Rev Pharmacol Toxicol* **35**, 581-606.

OLSSON, R. A. & PEARSON, J. D. (1990). Cardiovascular purinoceptors. *Physiol Rev* **70(3)**, 761-845.

OTANI, H., MATSUHISA, S., AKITA, Y., KYOI, S., ENOKI, C., TATSUMI, K., FUJIWARA, H., HATTORI, R., IMAMURA, H. & IWASAKA, T. (2006). Role of mechanical stress in the form of cardiomyocyte death during the early phase of reperfusion. *Circ J* **70(10)**, 1344-1355.

OTANI, H., OKADA, T., FUJIWARA, H., UCHIYAMA, T., SUMIDA, T., KIDO, M. & IMAMURA, H. (2003). Combined pharmacological preconditioning with a G-protein-coupled receptor agonist, a mitochondrial K^+_{ATP} channel opener and a nitric oxide donor mimics ischaemic preconditioning. *Clinical And Experimental Pharmacology & Physiology* **30(9)**, 684-693.

OTTANI, F., GALVANI, M., FERRINI, D., SORBELLO, F., LIMONETTI, P., PANTOLI, D. & RUSTICALI, F. (1995). Prodromal angina limits infarct size. A role for ischemic preconditioning. *Circulation* **91(2)**, 291-297.

OWEN, P., DENNIS, S., OPIE, L. H. (1990). Glucose flux rate regulates onset of ischemic contracture in globally underperfused rat hearts. *Circ Res* **66(2)**, 344-354.

PAIN, T., YANG, X. M., CRITZ, S. D., YUE, Y., NAKANO, A., LIU, G. S., HEUSCH, G., COHEN, M. V. & DOWNEY, J. M. (2000). Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. *Circ Res* **87(6)**, 460-466.

PALMER, T. M., BENOVIC, J. L. & STILES, G. L. (1996). Molecular basis for subtype-specific desensitization of inhibitory adenosine receptors. Analysis of a chimeric A_1- A_3 adenosine receptor. *J Biol Chem* **271(25)**, 15272-15278.

PALMER, T. M. & STILES, G. L. (1995). Adenosine receptors. *Neuropharmacology* **34(7)**, 683-694.

PANTOS, C., MOUROUZIS, I. & COKKINOS, D. V. (2006). Myocardial Ischemia. Basic concepts. In: COKKINOS, D.V., PANTOS, C., HEUSCH, G. & TAEGTMEYER, H. eds. *Myocardial Ischemia: From Mechanisms to Therapeutic Potentials*. Springer.

PARK, S. S., ZHAO, H., JANG, Y., MUELLER, R. A. & XU, Z. (2006). N6-(3-Iodobenzyl)-adenosine-5'-N-methylcarboxamide confers cardioprotection at reperfusion by inhibiting mitochondrial permeability transition pore opening via glycogen synthase kinase 3beta. *J Pharmacol Exp Ther* **318(1)**, 124-131.

PARSONS, M., YOUNG, L., LEE, J. E., JACOBSON, K. A. & LIANG, B. T. (2000). Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D. *FASEB J* **14(10)**, 1423-1431.

PEART, J. & HEADRICK, J. P. (2000). Intrinsic A$_1$ adenosine receptor activation during ischemia or reperfusion improves recovery in mouse hearts. *Am J Physiol Heart Circ Physiol* **279(5)**, H2166-2175.

PETERSEN, S., PETO, V. & RAYNER, M. (2004). *Coronary heart disease statistics*. London: British Heart Foundation.

PHARMALICENSING LIMITED. (2007). *Outlicensing A$_3$ Adenosine Receptor Agonists*. Available at: <URL: pharmalicensing.com/licensing/displicopp/3500> [Accessed: 10 July 2007].

PHILIPP, S., YANG, X. M., CUI, L., DAVIS, A. M., DOWNEY, J. M. & COHEN, M. V. (2006). Postconditioning protects rabbit hearts through a protein kinase C-adenosine A$_{2B}$ receptor cascade. *Cardiovasc Res* **70(2)**, 308-314.

PITARYS, C. J., 2ND, VIRMANI, R., VILDIBILL, H. D., JR., JACKSON, E. K. & FORMAN, M. B. (1991). Reduction of myocardial reperfusion injury by intravenous adenosine administered during the early reperfusion period. *Circulation* **83(1)**, 237-247.

PLATTS, S. H. & DULING, B. R. (2004). Adenosine A$_3$ receptor activation modulates the capillary endothelial glycocalyx. *Circ Res* **94(1)**, 77-82.

PODGORSKA, M., KOCBUCH, K. & PAWELCZYK, T. (2005). Recent advances in studies on biochemical and structural properties of equilibrative and concentrative nucleoside transporters. *Acta Biochim Pol* **52(4)**, 749-758.

POST, H. & HEUSCH, G. (2002). Ischemic preconditioning: Experimental facts and clinical perspective. *Minerva Cardioangiologica* **50(6)**, 569-605.

POULSEN, S. A. & QUINN, R. J. (1998). Adenosine receptors: new opportunities for future drugs. *Bioorg Med Chem* **6(6)**, 619-641.

PRZYKLENK, K. & WHITTAKER, P. (2005). Cardioprotection with adenosine: 'a riddle wrapped in a mystery'. *Br J Pharmacol* **146(6)**, 699-700.

RAEBURN, C. D., ZIMMERMAN, M. A., ARYA, J., BARSNESS, K. & HARKEN, A. H. (2002). Ischemic preconditioning: fact or fantasy? *Journal Of Cardiac Surgery* **17(6)**, 536-542.

RAMKUMAR, V., STILES, G. L., BEAVEN, M. A. & ALI, H. (1993). The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J Biol Chem* **268(23)**, 16887-16890.

RANDHAWA, M. P., LASLEY, R. D. & MENTZER, R. M., JR. (1995). Salutary effects of exogenous adenosine administration on in vivo myocardial stunning. *J Thorac Cardiovasc Surg* **110(1)**, 63-74.

REEVES, J. J., JONES, C. A., SHEEHAN, M. J., VARDEY, C. J. & WHELAN, C. J. (1997). Adenosine A_3 receptors promote degranulation of rat mast cells both in vitro and in vivo. *Inflamm Res* **46(5)**, 180-184.

REICHELT, M. E., WILLEMS, L., MOLINA, J. G., SUN, C. X., NOBLE, J. C., ASHTON, K. J., SCHNERMANN, J., BLACKBURN, M. R. & HEADRICK, J. P. (2005). Genetic deletion of the A_1 adenosine receptor limits myocardial ischemic tolerance. *Circ Res* **96(3)**, 363-367.

ROSS, A. M., GIBBONS, R. J., STONE, G. W., KLONER, R. A. & ALEXANDER, R. W. (2005). A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *J Am Coll Cardiol* **45(11)**, 1775-1780.

SAJJADI, F. G. & FIRESTEIN, G. S. (1993). cDNA cloning and sequence analysis of the human A_3 adenosine receptor. *Biochim Biophys Acta* **1179(1)**, 105-107.

SALVATORE, C. A., JACOBSON, M. A., TAYLOR, H. E., LINDEN, J. & JOHNSON, R. G. (1993). Molecular Cloning and Characterization of the Human A_3 Adenosine Receptor. *Proc Natl Acad Sci U S A* **90(21)**, 10365-10369.

SALVATORE, C. A., TILLEY, S. L., LATOUR, A. M., FLETCHER, D. S., KOLLER, B. H. & JACOBSON, M. A. (2000a). Disruption of the A$_3$ adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *J Biol Chem* **275(6)**, 4429-4434.

SALVATORE, C. A., TILLEY, S. L., LATOUR, A. M., FLETCHER, D. S., KOLLER, B. H. & JACOBSON, M. A. (2000b). Disruption of the A$_3$ adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *J Biol Chem* **275(6)**, 4429-4434.

SCARABELLI, T. M., KNIGHT, R. A., RAYMENT, N. B., COOPER, T. J., STEPHANOU, A., BRAR, B. K., LAWRENCE, K. M., SANTILLI, G., LATCHMAN, D. S., BAXTER, G. F. & YELLON, D. M. (1999). Quantitative assessment of cardiac myocyte apoptosis in tissue sections using the fluorescence-based tunel technique enhanced with counterstains. *J Immunol Methods* **228(1-2)**, 23-28.

SCHULTE, G. & FREDHOLM, B. B. (2000). Human adenosine A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$ receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. *Mol Pharmacol* **58(3)**, 477-482.

SCHULTE, G. & FREDHOLM, B. B. (2002). Signalling pathway from the human adenosine A$_3$ receptor expressed in Chinese hamster ovary cells to the extracellular signal-regulated kinase 1/2. *Mol Pharmacol* **62(5)**, 1137-1146.

SCHULTZ, W., SCHRADER, J. & GERLACH, E. (1982). Different Sites of adenosine formation in the heart. *Am J Physiol* **240**, H936-H970.

SCHULZ, R., EHRING, T. & HEUSCH, G. (1995). Stunned myocardium: inotropic reverse and pharmacological attenuation. *Basic Res Cardiol* **90**, 294-296.

SHEPHERD, R. K., LINDEN, J. & DULING, B. R. (1996). Adenosine-induced vasoconstriction in vivo. Role of the mast cell and A$_3$ adenosine receptor. *Circ Res* **78(4)**, 627-634.

SHINMURA, K., NAGAI, M., TAMAKI, K., TANI, M. & BOLLI, R. (2002). COX-2-derived prostacyclin mediates opioid-induced late phase of preconditioning in isolated rat hearts. *American Journal of Physiology - Heart and Circulatory Physiology* **283(6 52-6)**, H2534-H2543.

SHRYOCK, J. C. & BELARDINELLI, L. (1997). Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am J Cardiol* **79(12A)**, 2-10.

SHRYOCK, J. C., SNOWDY, S., BARALDI, P. G., CACCIARI, B., SPALLUTO, G., MONOPOLI, A., ONGINI, E., BAKER, S. P. & BELARDINELLI, L. (1998). A_{2A} adenosine receptor reserve for coronary vasodilation. *Circulation* **98(7)**, 711-718.

SKRZYPIEC-SPRING, M., GROTTHUS, B., SZELAG, A. & SCHULZ, R. (2007). Isolated heart perfusion according to Langendorff-Still viable in the new millennium. *J Pharmacol Toxicol Methods* **55(2)**, 113-126.

SMITS, G. J., MCVEY, M., COX, B. F., PERRONE, M. H. & CLARK, K. L. (1998). Cardioprotective effects of the novel adenosine A_1/ A_2 receptor agonist AMP 579 in a porcine model of myocardial infarction. *J Pharmacol Exp Ther* **286(2)**, 611-618.

SOLARO, R. J. (1999). Integration of myofilament response to Ca^{2+} with cardiac pump regulation and pump dynamics. *Am J Physiol* **277(6 Pt 2)**, S155-163.

SOLENKOVA, N. V., SOLODUSHKO, V., COHEN, M. V. & DOWNEY, J. M. (2006). Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt. *Am J Physiol Heart Circ Physiol* **290(1)**, H441-449.

STAMBOLIC, V., MAK, T. W. & WOODGETT, J. R. (1999). Modulation of cellular apoptotic potential: contributions to oncogenesis. *Oncogene* **18(45)**, 6094-6103.

STRICKLER, J., JACOBSON, K. A. & LIANG, B. T. (1996). Direct preconditioning of cultured chick ventricular myocytes. Novel functions of cardiac adenosine A_{2A} and A_3 receptors. *J Clin Invest* **98(8)**, 1773-1779.

SUN, H. Y., WANG, N. P., HALKOS, M., KERENDI, F., KIN, H., GUYTON, R. A., VINTEN-JOHANSEN, J. & ZHAO, Z. Q. (2006). Postconditioning attenuates cardiomyocyte apoptosis via inhibition of JNK and p38 mitogen-activated protein kinase signalling pathways. *Apoptosis* **11(9)**, 1583-1593.

SUTHERLAND, F. J. & HEARSE, D. J. (2000). The isolated blood and perfusion fluid perfused heart. *Pharmacol Res* **41(6)**, 613-627.

TAKANO, H., BOLLI, R., BLACK, R. G., JR., KODANI, E., TANG, X. L., YANG, Z., BHATTACHARYA, S. & AUCHAMPACH, J. A. (2001). A(1) or A_3 adenosine receptors induce late preconditioning against infarction in conscious rabbits by different mechanisms. *Circ Res* **88(5)**, 520-528.

THORNTON, J. D., LIU, G. S., OLSSON, R. A. & DOWNEY, J. M. (1992). Intravenous pretreatment with A_1-selective adenosine analogues protects the heart against infarction. *Circulation* **85(2)**, 659-665.

THOURANI, V. H., NAKAMURA, M., RONSON, R. S., JORDAN, J. E., ZHAO, Z. Q., LEVY, J. H., SZLAM, F., GUYTON, R. A. & VINTEN-JOHANSEN, J. (1999a). Adenosine A_3 receptor stimulation attenuates postischemic dysfunction through K_{ATP} channels. *Am J Physiol* **277(1 Pt 2)**, H228-235.

THOURANI, V. H., RONSON, R. S., JORDAN, J. E., GUYTON, R. A. & VINTEN-JOHANSEN, J. (1999b). Adenosine A_3 pretreatment before cardioplegic arrest attenuates postischeamic dysfunction. *Ann Thorac Surg* **67**, 1732-1737.

TILLEY, S. L., WAGONER, V. A., SALVATORE, C. A., JACOBSON, M. A. & KOLLER, B. H. (2000). Adenosine and inosine increase cutaneous vasopermeability by activating A_3 receptors on mast cells. *J Clin Invest* **105(3)**, 361-367.

TOOMBS, C. F., MCGEE, S., JOHNSTON, W. E. & VINTEN-JOHANSEN, J. (1992). Myocardial protective effects of adenosine. Infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation* **86(3)**, 986-994.

TRACEY, W. R., MAGEE, W., MASAMUNE, H., KENNEDY, S. P., KNIGHT, D. R., BUCHHOLZ, R. A. & HILL, R. J. (1997). Selective adenosine A_3 receptor stimulation reduces ischemic myocardial injury in the rabbit heart. *Cardiovasc Res* **33**, 410- 415.

TRACEY, W. R., MAGEE, W., MASAMUNE, H., OLEYNEK, J. J. & HILL, R. J. (1998). Selective activation of adenosine A_3 receptors with N6-(3-chlorobenzyl)-5'-N-methylcarboxamidoadenosine (CB-MECA) provides cardioprotection via K_{ATP} channel activation. *Cardiovasc Res* **40(1)**, 138-145.

TRINCAVELLI, M. L., TUSCANO, D., MARRONI, M., FALLENI, A., GREMIGNI, V., CERUTI, S., ABBRACCHIO, M. P., JACOBSON, K. A., CATTABENI, F. & MARTINI, C. (2002). A_3 Adenosine Receptors in Human Astrocytoma Cells: Agonist-Mediated Desensitization, Internalization, and Down-Regulation. *Mol Pharmacol* **62(6)**, 1373-1384.

U.S. NATIONAL INSTITUTES OF HEALTH. (2007). *Clinical Trial: Salvage: Postconditioning With Adenosine for STEMI (NCT00284323)*. Available at: <URL: clinicaltrials.gov/ct/show/NCT00284323?order=4> [Accessed: 10 July 2007].

U.S. NATIONAL INSTITUTES OF HEALTH. (2007). *Clinical Trial: Positive Effect of Ischaemic Postconditioning During Acute Myocardial Infarction (NCT00333320)*. Available at: <URL: clinicaltrials.gov/ct/show/NCT00333320?order=1> [Accessed: 10 July 2007].

VAN CALKER, D., MULLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* **33(5)**, 999-1005.

VAN DER WERF, T. (1980). *Cardiovascular Pathophysiology*. Oxford University Press.

VAN SCHAICK, E. A., JACOBSON, K. A., KIM, H. O., JZERMAN, I. A. P. & DANHOF, M. (1996). Hemodynamic effects and histamine release elicited by the selective adenosine A_3 receptor agonist 2-Cl-IB-MECA in conscious rats. *Eur J Pharmacol* **308(3)**, 311-314.

VARANI, K., MERIGHI, S., GESSI, S., KLOTZ, K. N., LEUNG, E., BARALDI, P. G., CACCIARI, B., ROMAGNOLI, R., SPALLUTO, G. & BOREA, P. A. (2000). ^{3}H-RE3008F20: a novel antagonist radioligand for the pharmacological and biochemical characterization of human A_3 adenosine receptors. *Mol Pharmacol* **57(5)**, 968-975.

VEERAVALLI, K. K. & AKULA, A. (2004). Involvement of nitric oxide and prostaglandin pathways in the cardioprotective actions of bradykinin in rats with experimental myocardial infarction. *Pharmacological Research* **49(1)**, 23.

VELASCO, C. E., TURNER, M., COBB, M. A., VIRMANI, R. & FORMAN, M. B. (1991). Myocardial reperfusion injury in the canine model after 40 minutes of ischemia: effect of intracoronary adenosine. *Am Heart J* **122(6)**, 1561-1570.

VINTEN-JOHANSEN, J., THOURANI, V. H., RONSON, R. S., JORDAN, J. E., ZHAO, Z. Q., NAKAMURA, M., VELEZ, D. & GUYTON, R. A. (1999). Broad-spectrum cardioprotection with adenosine. *Ann Thorac Surg* **68(5)**, 1942-1948.

VINTEN-JOHANSEN, J., ZHAO, Z. Q., ZATTA, A. J., KIN, H., HALKOS, M. E. & KERENDI, F. (2005). Postconditioning--A new link in nature's armour against myocardial ischemia-reperfusion injury. *Basic Res Cardiol* **100(4)**, 295-310.

VOLOVSEK, A., SUBRAMANIAN, R., REBOUSSIN, D., NEELY, J. R., ROVETTO, M. J., WHITMER, J. T. & MORGAN, H. E. (1992). Effects of duration of ischaemia during preconditioning on mechanical function, enzyme release and energy production in the isolated working rat heart. *J Mol Cell Cardiol* **24(9)**, 1011-1019.

WANG, J., DRAKE, L., SAJJADI, F., FIRESTEIN, G. S., MULLANE, K. M. & BULLOUGH, D. A. (1997). Dual activation of adenosine A_1 and A_3 receptors mediates preconditioning of isolated cardiac myocytes. *Eur J Pharmacol* **320(2-3)**, 241-248.

WANG, Y. & ASHRAF, M. (1999). Role of protein kinase C in mitochondrial K_{ATP} channel-mediated protection against Ca^{2+} overload injury in rat myocardium. *Circ Res* **84(10)**, 1156-1165.

WILLIAMS, I. A., XIAO, X. H., JU, Y. K. & ALLEN, D. G. (2007). The rise of Na^+_i during ischemia and reperfusion in the rat heart-underlying mechanisms. *Pflugers Arch.* [E-published ahead of print]

WILLIAMS, M., FRANCIS, J., GHAI, G., BRAUNWALDER, A., PSYCHOYOS, S., STONE, G. A. & CASH, W. D. (1987). Biochemical characterization of the triazoloquinazoline, CGS 15943, a novel, non-xanthine adenosine antagonist. *J Pharmacol Exp Ther* **241(2)**, 415-420.

WONG, T. M. & WU, S. (2003). Roles of kappa opioid receptors in cardioprotection against ischemia - The signalling mechanisms. *Acta Physiologica Sinica* **55(2)**, 115-120.

XU, Z., DOWNEY, J. M. & COHEN, M. V. (2001). AMP579 reduces contracture and limits infarction in rabbit heart by activating adenosine A_2 receptors. *J Cardiovasc Pharmacol* **38(3)**, 474-481.

XU, Z., JIAO, Z., COHEN, M. V. & DOWNEY, J. M. (2002). Protection from AMP579 can be added to that from either cariporide or ischemic preconditioning in ischemic rabbit heart. *J Cardiovasc Pharmacol* **40(4)**, 510-518.

XU, Z., YANG, X. M., COHEN, M. V., NEUMANN, T., HEUSCH, G. & DOWNEY, J. M. (2000). Limitation of infarct size in rabbit hearts by the novel adenosine receptor agonist AMP579 administered at reperfusion. *J Mol Cell Cardiol* **32(12)**, 2339-2347.

YANG, X. M., KRIEG, T., CUI, L., DOWNEY, J. M. & COHEN, M. V. (2004). NECA and bradykinin at reperfusion reduce infarction in rabbit hearts by signalling through PI3K, ERK, and NO. *J Mol Cell Cardiol* **36(3)**, 411-421.

YANG, X. M., PHILIPP, S., DOWNEY, J. M. & COHEN, M. V. (2005). Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. *Basic Res Cardiol* **100(1)**, 57-63.

YAO, Y., SEI, Y., ABBRACCHIO, M. P., JIANG, J. L., KIM, Y. C. & JACOBSON, K. A. (1997). Adenosine A3 receptor agonists protect HL-60 and U-937 cells from apoptosis induced by A3 antagonists. *Biochem Biophys Res Commun* **232(2)**, 317-322.

YATES, L. 2004. *A3 receptor-mediated protection from ischaemia- and hypoxia-induced myocardial stunning.* Ph.D. Thesis, Cardiff University.

YATES, L., CLARK, J. H., MARTIN, T. J., JAMES, S., BROADLEY, K. J. & KIDD, E. J. (2006). Radioligand binding and functional responses of ligands for human recombinant adenosine A3 receptors. *Auton Autacoid Pharmacol* **26(2)**, 191-200.

YELLON, D. M. & BAXTER, G. F. (1999). Reperfusion injury revisited: is there a role for growth factor signalling in limiting lethal reperfusion injury? *Trends Cardiovasc Med* **9(8)**, 245-249.

YELLON, D. M. & OPIE, L. H. (2006). Postconditioning for protection of the infarcting heart. *Lancet* **367(9509)**, 456-458.

YUSUF, S., HAWKEN, S., OUNPUU, S., DANS, T., AVEZUM, A., LANAS, F., MCQUEEN, M., BUDAJ, A., PAIS, P., VARIGOS, J. & LISHENG, L. (2004). Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* **364(9438)**, 937-952.

ZHAO, T. C. & KUKREJA, R. C. (2002). Late preconditioning elicited by activation of adenosine A3 receptor in heart: role of NF- kappa B, iNOS and mitochondrial K_{ATP} channel. *J Mol Cell Cardiol* **34(3)**, 263-277.

ZHAO, Z. Q., CORVERA, J. S., HALKOS, M. E., KERENDI, F., WANG, N. P., GUYTON, R. A. & VINTEN-JOHANSEN, J. (2003). Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* **285(2)**, H579-588.

ZHAO, Z. Q., MCGEE, S., NAKANISHI, K., TOOMBS, C. F., JOHNSTON, W. E., ASHAR, M. S. & VINTEN-JOHANSEN, J. (1993). Receptor-mediated cardioprotective effects of endogenous adenosine are exerted primarily during reperfusion after coronary occlusion in the rabbit. *Circulation* **88(2)**, 709-719.

ZHAO, Z. Q., NAKANISHI, K., MCGEE, D. S., TAN, P. & VINTEN-JOHANSEN, J. (1994). A_1 receptor mediated myocardial infarct size reduction by endogenous adenosine is exerted primarily during ischaemia. *Cardiovasc Res* **28(2)**, 270-279.

ZHOU, Q. Y., LI, C., OLAH, M. E., JOHNSON, R. A., STILES, G. L. & CIVELLI, O. (1992). Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. *Proc Natl Acad Sci U S A* **89(16)**, 7432-7436.

APPENDIX

ABBREVIATIONS

AAR	area at risk
AC	adenylyl cyclase
ADP	adenosine diphosphate
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AMISTAD	Acute Myocardial Infarction Study of Adenosine
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
BAD	Bcl-2-associated death promoter
BCA	bicinchoninic acid
BLOTTO	Bovine Lacto Transfer Technique Optimiser
Ca^{2+}	calcium divalent cation
cAMP	cyclic adenosine monophosphate
CF	coronary Flow
CHD	coronary heart disease
CO_2	carbon dioxide
CPP	coronary perfusion pressure
Da	daltons
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC50	half maximal effective concentration
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ENTs	equilibrative nucleoside transporters
g	g-force
G protein	guanine nucleotide binding protein
G_i	inhibitory G protein
$G_i\beta\gamma$	$\beta\gamma$ subunit of the inhibitory G protein
GPCR	G-protein coupled receptors
G_s	stimulatory G protein

HR	heart rate
HRP	horse radish peroxidase
HSP	heat shock proteins
Hz	hertz
IgG	immunoglobulin G
$IK_{(Ado)}$	adenosine activated potassium channel current
$IK_{(ATP)}$	ATP inhibited potassium channel current
INOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
IUPAR	International Union of Pure and Applied Chemistry
JPEG	Joint Photographic Experts Group
K_{ATP}	ATP-sensitive potassium channel
K_i	dissociation constant of an inhibitor
LVDP	left ventriclar developed pressure
MAP	mitogen-activated protein kinases
MI	myocardial Infarction
Mito	mitochondria
mm	millimetre
N_2	nitrogen (in molecular form)
Na^+	sodium monovalent cation
NO	nitric oxide
NOS	nitric oxide synthase;
O_2	oxygen (in molecular form)
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
ROS	reactive oxygen species
S.E.M.	standard error of the mean
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine

SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
TBS	tris buffered saline
TBST	tris buffered saline plus Tween®
TEMED	tetramethylethylenediamine
TTC	tetrazolium chloride
UK	United Kingdom
v/v	volume by volume
VDAC	voltage-dependent anion channel
w/v	weight by volume

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