BETA ADRENERGIC FUNCTION IN ACUTE MYOCARDIAL ISCHAEMIA

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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DECLARATION

The work presented in this thesis was performed solely by the author apart from areas of acknowledged collaboration.

M. HELEN REDDY

HYPOTHESIS

That there are changes in the beta adrenergic post-synaptic responsiveness to catecholamines in acute myocardial ischaemia; these alterations may have a role in arrhythmogenesis.

ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
cAMP	Adenosine 3':5'-cyclic monophosphate
ATP	Adenosine triphosphate
CP	Creatine phosphate
CPK	Creatine phosphokinase
cpm	Counts per minute
DDW	Double distilled water
DHA	Dihydroalprenolol
DTT	Dithiothreitol
GDP	Guanosine diphosphate
Gpp(NH)p	Guanylyimidodiphoshate
GTP	Guanosine triphosphate
ICYP	Iodocyanopindolol
IMP	Inosine monophosphate
(-)-IPin	[^{125}I]-iodopindolol
Isop	Isoprenaline
NA	Noradrenaline
NMR	Nuclear magnetic resonance
PEI	Polyethyleneimine
SD	Standard deviation
TLC	Thin-layer chromatography
Tris	Tris(hydroxymethyl)-aminomethane
UV	Ultra-violet radiation

ABSTRACT

Excess stimulation of the beta adrenergic system has been implicated in the development of serious ventricular arrhythmias during acute myocardial ischaemia, presumably by increasing levels of cAMP. The responsiveness of the myocardium to catecholamines under these conditions has not been fully investigated.

Adenylate cyclase activity and beta adrenoceptors were characterised in membranes prepared from normal and ischaemic rat hearts. Acute myocardial ischaemia increased the number of beta adrenergic receptors with no change in receptor affinity. Prolonged ischaemia led to further increases in the number of receptors. In contrast the activity of adenylate cyclase (basal, sodium fluoride and isoprenaline stimulated), was significantly reduced during both acute and prolonged myocardial ischaemia. Reduction in pH, and adenosine accumulation, as observed in ischaemia, both lower adenylate cyclase activity measured in normal membranes.

During acute myocardial ischaemia the responsiveness of the myocardium to catecholamines is reduced rather than enhanced, despite an increase in the number of beta adrenergic receptors. The degree of reduction may vary according to duration of ischaemia, increase in acidosis and the magnitude of adenosine accumulation. This may lead to a heterogeneous response, likely to contribute to the development of serious ventricular arrhythmias.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Identification of the causes of heart attacks, with a view to prevention, is the focus of much research in the western world. Many deaths from coronary heart disease occur suddenly, as a result of a cardiac arrhythmia. Such a disturbance in cardiac rhythm often occurs during a reduction in blood supply to the myocardium (ischaemia). It has been suggested that catecholamines are released from the heart during acute myocardial ischaemia and are involved in the genesis of lethal arrhythmias. Cyclic AMP has also been implicated in the genesis of arrhythmias.

Investigators have attempted to quantify the release of catecholamines occurring during acute myocardial ischaemia, which despite much work remains controversial. Perhaps a more pressing question is, what is the responsiveness of the myocardium to catecholamines during ischaemia?

This introduction reviews some aspects of beta adrenergic receptors and adenylate cyclase, followed by discussion of myocardial ischaemia and arrhythmias. The final section examines the current literature on the effect of ischaemia on beta adrenergic function.

1.1 THE BETA ADRENERGIC / ADENYLATE CYCLASE SYSTEM IN THE HEART

Noradrenaline (NA), the principal myocardial catecholamine, is stored in vesicles in sympathetic nerve terminals and is released in response to nerve stimulation, whereas adrenaline is released from the adrenal medulla and is carried by the coronary circulation to the heart. Catecholamines bind to specific membrane-bound proteins and initiate postsynaptic responses, such as an increase in heart force. The predominant adrenergic receptor on cardiac myocytes is the beta adrenergic receptor, which when occupied by a beta adrenergic agonist stimulates the production of the intracellular second messenger - cyclic AMP. The beta adrenergic cyclase system consists of three major components - the beta adrenergic receptor (R), a guanine nucleotide regulatory protein (G_s) and (C) the catalytic unit of adenylate cyclase (Fig 1.1). The rise in intracellular cyclic AMP then brings about the intracellular responses to catecholamines.

1.1.1 Adrenergic Receptors

It was first thought that only one type of receptor for catecholamines existed and that specificity was determined solely at the transmitter level (Cannon and Rosenblueth, 1933). This theory was eventually disproved by Ahlquist in 1948, when he subclassified adrenergic receptors into alpha and beta on the basis of relative affinities for sympathomimetic amines. Later, both these receptors were pharmacologically subclassfied into $beta_1$ and

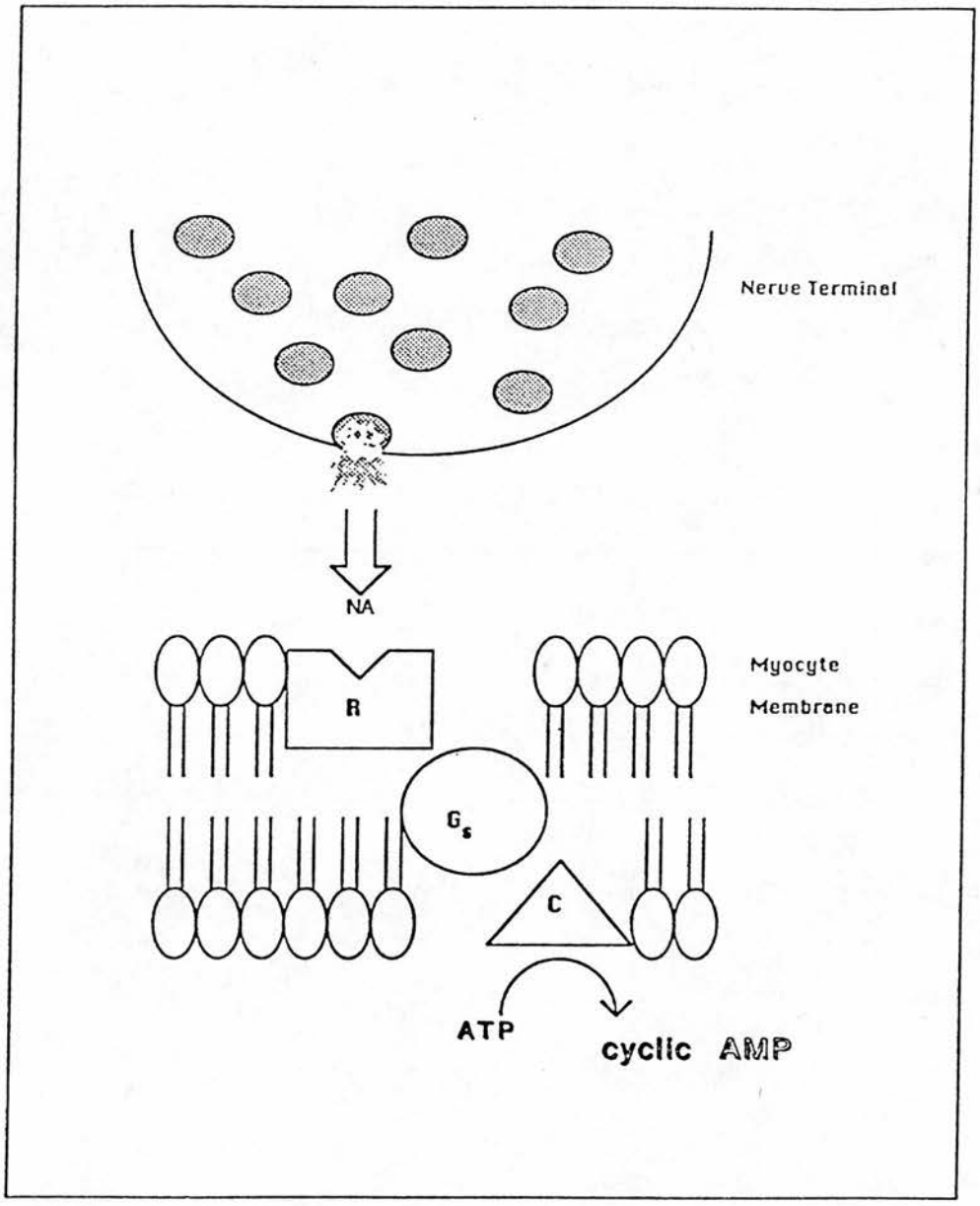

Fig 1.1 **Beta Adrenergic / Adenylate Cylase System**
Schematic diagram of noradrenaline (NA) release, binding to a beta adrenergic receptor (R) and subsequent coupling through a guanine nucleotide regulatory protein (G_s) to adenylate cyclase (C).

$beta_2$ (Lands et al., 1967), $alpha_1$ and $alpha_2$ (Langer, 1974). This classification may not be universal; there is the possibility of additional adrenergic receptor sub-types (McGrath, 1983).

$Beta_1$ adrenergic receptors have been referred to as the cardiac adrenergic receptor and although functionally probably the most important myocardial adrenergic receptor, $beta_2$ and alpha adrenergic receptors are now also known to be present in the mammalian heart. There are regional differences, in the heart, in both the degree of sympathetic innervation and also occurrence of adrenergic receptors. Ariëns and Simonis (1983) have proposed that $beta_1$ adrenergic receptors are innervated but not $beta_2$ adrenergic receptors. The coronary vasculature is also under sympathetic control through alpha adrenergic receptor-mediated vessel constriction and beta-mediated relaxation (Feigl, 1983). In addition to adrenergic receptors on heart muscle and vessels, there are pre-junctional adrenergic receptors which modulate catecholamine release from sympathetic nerve terminals (Fuder, 1985).

As with other receptors, beta adrenergic receptors are proteins which have specific recognition sites, in this case for catecholamines. After binding catecholamine, the receptors are also responsible for initiation of a sequence of events which bring about the physiological response. These separate steps can be monitored in different ways; quantification of the specific binding of a radiolabelled compound, usually an adrenergic receptor antagonist, to a receptor, gives information on the recognition site, but not on subsequent intracellular events. For this, measurement of the intracellular response is required, for example quantification

of adenylate cyclase activity.

Beta$_1$ and beta$_2$ adrenergic receptors both couple in a stimulatory fashion to adenylate cyclase, whereas stimulation of alpha$_2$ adrenergic receptors actually decreases adenylate cyclase activity. In contrast, cyclic AMP is not involved in alpha$_1$ adrenergic stimulation. It is postulated that stimulation of alpha$_1$ adrenergic receptors leads to an increase in intracellular calcium through hydrolysis of phosphatidylinositol-4,5-bisphosphate to myoinositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Fain and Garcia-Sainz, 1980).

Beta adrenergic receptors are intrinsic glycosylated membrane proteins with an apparent molecular weight of 64 kDa (Cubero and Malbon, 1984). Biochemically, most information is available for the beta$_2$ adrenergic receptor, which may exist as a dimer (Homcy et al., 1983). The genes encoding beta adrenergic receptors have been cloned and sequenced from several sources. These studies have revealed a high degree of sequence identity between beta adrenergic receptors and muscarinic cholinergic receptors (Kerlavage et al., 1986). From hydrophobicity plots of the amino acid sequences it appears that the beta$_2$ adrenergic receptor has seven membrane-spanning regions (Kobilka et al., 1987). Biochemical and immunological comparisons suggest that beta$_1$ and beta$_2$ adrenergic receptors are very similar, but they are pharmacologically distinguishable. However, less structural information is available for alpha adrenergic receptors.

Ligand binding studies on myocardial membranes have shown species differences in the ratio of beta$_1$:beta$_2$ adrenergic receptors;

for example, there is a ratio of 86:14 in human left ventricle, whereas the ratio is 93:7 for rabbit left ventricle (Summers et al., 1987). These studies have also indicated the presence of alpha adrenergic binding sites on myocardium (Glossman et al., 1980), however calculation of the relative proportions of alpha to beta adrenergic receptors in the myocardium has been neglected. It should be emphasised that there are difficulties in the exact quantification of adrenergic receptors in the heart. Receptor numbers are often measured on the basis of radioligand binding to 'purified' sarcolemmal preparations. However, Tomlins et al. (1986) discovered that there is a high degree of endothelial cell contamination in some purified sarcolemmal preparations. Furthermore, it has been suggested that the $beta_2$ adrenergic receptors, described as myocardial, are actually present on endothelial cells and not myocytes (Buxton and Brunton, 1985).

The pertinent question is not how many receptors are found by radioligand binding studies, but what functional importance do the receptors have in vivo? The physiological and biochemical consequences of $beta_1$ adrenergic receptor stimulation in the myocardium have been well documented. Catecholamines modulate metabolic events, contractility and heart rate, probably by raising intracellular cyclic AMP levels. Calcium influx into the myocardium is increased by catecholamines (Reuter, 1983) thereby increasing the force of contraction. Chronotropic responses to catecholamines are thought to be mediated by an increase in the hyperpolarization-activated inward current (I_f) (Noble, 1984). The functional importance of the $beta_2$ subtype is not yet clear (Kaumann

and Lemoine, 1987). It is widely accepted that stimulation of myocardial alpha adrenergic receptors is postively inotropic, however, chronotropic responses are controversial; there are reports of both positive and negative chrontropic effects on alpha adrenergic receptor stimulation (Flavanan and McGrath, 1982; Dukes and Vaughan Williams, 1984). Although the physiological roles of $beta_2$ and alpha adrenergic receptors in the myocardium are not well understood, these receptors may be important under pathological conditions.

The mechanisms controlling receptor regulation are being elucidated. There appear to be two ways in which the beta adrenergic / adenylate cyclase system may undergo desensitization to an agonist. These have been described as homologous - reduced sensitivity to a particular class of agonists, and heterologous desensitization - general unresponsiveness of adenylate cyclase. Uncoupling of beta adrenergic receptors from adenylate cyclase and a fall in beta adrenergic receptor binding sites are found after intense beta adrenergic stimulation - this is described as 'down regulation' (Limas and Limas, 1985). Conversely, chronic catecholamine depletion increases beta adrenergic binding sites and potentiates beta-mediated responses - described as 'up regulation' (Chess-Williams et al., 1985). In addition, the number of beta adrenergic binding sites in cardiac membranes has been shown to be altered by ageing and by some disease states such as hyperthyroidism (Lefkowitz et al., 1984). A 'down regulation' of myocardial beta adrenergic receptors has been found to occur in congestive heart failure (Bristow, 1984). Measurement of

changes in the density of receptor binding sites under pathological conditions has become popular. The information derived from such studies is limited unless authors establish that any alteration in binding sites translates to a corresponding alteration in function.

1.1.2 Guanine Nucleotide Regulatory Proteins

The second component in the beta adrenergic / cyclase system is a guanine nucleotide regulatory protein, interchangeably known as G_s, N_s, N or G/F. The importance of this protein to catecholamine stimulation of adenylate cyclase has been well documented. A mutant of the S49 lymphoma cell line (-cyc) has both beta adrenergic receptors and adenylate cyclase but no functional G_s protein, these cells are unresponsive to catecholamines (Northup et al., 1983). G_s is a heterotrimer composed of alpha (45 kDa), beta (35 kDa) and gamma (8 kDa) subunits. The alpha subunit has GTPase activity and recognition sites for both the beta adrenergic receptor and adenylate cyclase. In addition, the alpha subunit is necessary for stimulation of adenylate cyclase.

Information on the coupling of beta adrenergic receptors to G_s has come from radioligand binding studies. Guanine nucleotides such as GTP and the non-hydrolysable analogue Gpp(NH)p have been shown to cause a shift in displacement curves of a specific radioligand in the presence of a competing agonist (Fig 1.2). This shift is not observed if an antagonist is employed to displace the radioligand. These findings have implicated the involvement of two affinity states for agonist binding to beta adrenergic receptors (Fig1.3).

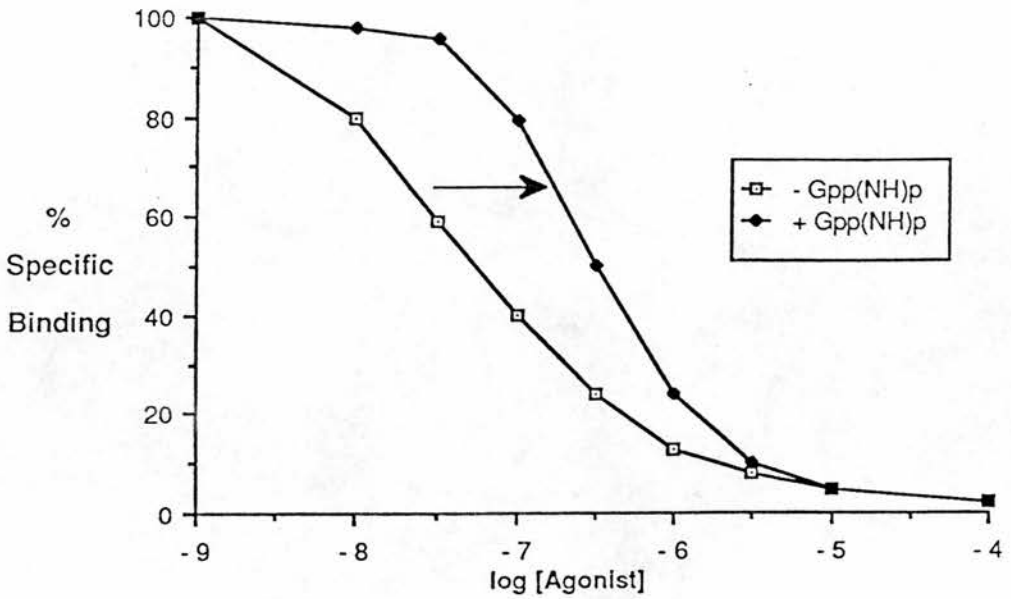

Fig 1.2 Beta adrenergic receptor agonist binding shift in the presence of guanine nucleotides. In the presence of Gpp(NH)p the curve of agonist displacement of ligand is shifted to the right.

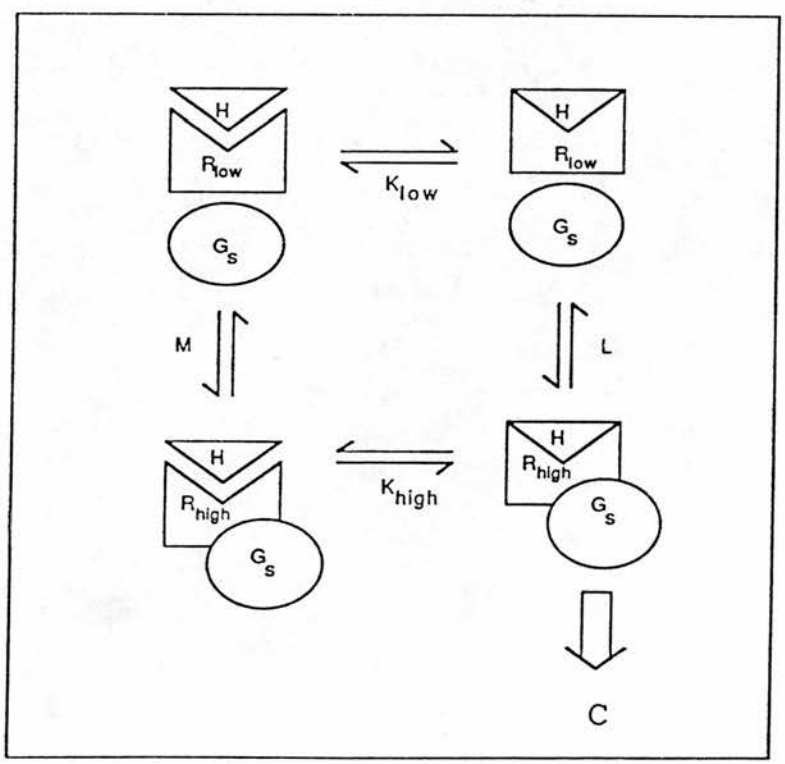

Fig 1.3 Model of ternary complex formation. Agonist (H) binding to beta adrenergic receptor (R) promotes formation of the ternary complex ($H\text{-}R_{high}\text{-}G_s$), which activates adenylate cyclase (C).

De Lean et al. (1980) have postulated that GTP induces a shift in affinity, converting all the receptors to the low affinity form. Agonist binds with higher affinity to the receptor-G_s complex than to receptor alone - the amount of receptor which is precoupled to G_s being dependent on a tissue specific factor (M). Antagonist binds to either form with equal affinity. The active species which transduces the signal to influence adenylate cyclase is a ternary complex H-R-G_s, the formation of which is dependent on the intrinsic activity of the agonist (L). From displacement curves the proportions of receptors in high and low affinity states may be calculated. On a cautionary note, it has not yet been conclusively demonstrated that the shift in these curves represents coupling of beta adrenergic receptors to G_s.

Other receptors, such as those for prostaglandin E_1 and glucagon, are also coupled via G_s proteins, in a stimulatory fashion, to adenylate cyclase. Conversely, occupation of receptors, such as $alpha_2$ adrenergic receptors, leads to inhibition of adenylate cyclase. This inhibitory process also involves a G-protein, known as G_i. The beta and gamma subunits of G_i and G_s are very similar, but these proteins have different alpha subunits. The $alpha_s$ subunit is a substrate for ADP-ribosylation catalysed by cholera toxin, leading to a persistent activation of adenylate cyclase by GTP. In contrast, the alpha subunit of G_i is the substrate for pertussis toxin. The mechanism of inhibition of adenylate cyclase, directly or indirectly, involves the beta and gamma subunits of G_i (Gilman, 1984).

It is becoming apparent that there is a family of G-proteins, each with distinctive alpha subunits, involved in different

transduction systems. Recent exciting findings have shown that some G-proteins have the ability to couple directly to membrane ion channels, by-passing the traditional requirement for an intracellular second messenger. Thus stimulation of the cardiac muscarinic receptor has been shown to promote coupling of G-proteins to potassium channels (Pfaffinger et al., 1985). Moreover, G-proteins can also directly regulate Ca^{2+} channels in guinea-pig cardiac sarcolemma (Yatani et al., 1987). It is conceivable that catecholamines binding to beta adrenergic receptors modulate important events without involvement of cyclic AMP. Indeed, catecholamines promote the phosphorylation of the beta adrenergic receptor via a mechanism which does not involve increases in cyclic AMP or the presence of G_s (Strasser et al., 1986).

1.1.3. Adenylate Cyclase

The enzyme adenylate cyclase (EC 4.6.1.1., ATP pyrophosphate-lyase cyclizing) is an integral membrane protein, the substrate for which is Mg.ATP. The catalytic unit of cardiac adenylate cyclase from rabbit ventricular muscle has been characterised and separated from G_s (Drummond, 1985). Structural information about adenylate cyclase is limited due to difficulty in purifying and raising antibodies to adenylate cyclase (Mollner and Pfeuffer, 1988).

In addition to receptor-mediated stimulation or inhibition of adenylate cyclase there are also receptor-independent modulators of adenylate cyclase activity. As previously mentioned, the presence of GTP or guanine nucleotide analogues can stimulate adenylate cyclase acting at the level of G_s. It has been known for some time

that sodium fluoride stimulates adenylate cyclase (Rall et al., 1957). More recently, it has been demonstrated that an aluminium fluoride complex is the active species (Sternweis and Gilman, 1982). Forskolin, a diterpene, stimulates cyclase via an as yet unknown mechanism, which does appear to be at the level of the catalytic unit, however G_s may also be involved (Gilman, 1984).

As discussed earlier, formation of a high affinity ternary complex of agonist-receptor-G_s is thought to be important in stimulating adenylate cyclase. Current thinking on the interaction of the beta adrenergic receptor, G_s and adenylate cyclase is as follows: catecholamine binding to the receptor causes a conformational change in the receptor. This allows the association of the hormone-receptor with the G_s protein. Under conditions where G_s is unassociated with receptor or adenylate cyclase, GDP is bound to the alpha subunit of G_s. After association of the receptor and G_s there is a conformational change in the alpha subunit, this allows the displacement of GDP by GTP and the dissociation of the alpha subunit of G_s from the beta/gamma subunits. The activated alpha subunit binds to adenylate cyclase thereby stimulating the production of cyclic AMP. The alpha unit's inherent GTPase activity causes the hydrolysis of the bound GTP to GDP. Subsequently, the alpha subunit dissociates from the catalytic unit inactivating adenylate cyclase. The bound catecholamine dissociates from the receptor and the system is back in the unstimulated state.

1.1.4 Cyclic AMP And The Heart

Measurement of absolute levels of cyclic AMP in the heart, and relating these levels to subsequent events, has proved controversial. Although large increases in cyclic AMP levels are observed in adipocytes in the presence of forskolin, the expected activation of lipolysis, as is obtained with beta adrenergic agonists, is poor (Litosch et al., 1982). In the heart similar levels of cyclic AMP are generated by prostaglandin E_1 and a beta adrenergic receptor agonist, isoprenaline, but only cyclic AMP produced by isoprenaline stimulates glycogen phosphorylase. These observations have led to the suggestion that there may be compartmentalization of cyclic AMP and/or cyclic AMP-dependent protein kinase in the myocyte (Buxton and Brunton, 1983). Technically, measurement of cyclic AMP levels in tissues is problematic, in that samples need to be frozen rapidly. Several different assays for the determination of cyclic AMP in tissue samples have not proved reliable (van Belle, 1985).

It has been proposed that cyclic AMP generated by adenylate cyclase binds to the regulatory subunits of cyclic AMP-dependent protein kinase, leading to dissociation of the catalytic subunits of this enzyme (Corbin et al., 1977). The catalytic subunits are then free to phosphorylate intracellular proteins. Cyclic AMP-dependent phosphorylation of proteins is probably the major route by which occupation of beta adrenergic receptors modulates intracellular events.

Our understanding of the mechanism by which protein phosphorylation mediates the effects of beta adrenergic receptor stimulation is poor. An increase in calcium transport across the

sarcolemmal membrane has been postulated to be of central importance to catecholamine stimulation of heart cells. The biochemistry of calcium channels is at present a focus of much research. Interestingly, a cardiac calcium channel has recently been identified which is responsive to cyclic AMP-dependent protein phosphorylation (Hofmann et al., 1987). Catecholamine regulation of cardiac calcium channels in vivo, by cyclic AMP-dependent or independent mechanisms, requires further investigation. Although in vitro cyclic AMP-dependent phosphorylation of many sarcolemmal proteins has been demonstrated, the function and physiological significance of this remains uncertain. Several proteins involved in the myocardial contractile apparatus are phosphorylated in response to catecholamines, including troponin I and C-protein (England, 1983). Phospholamban, which is also a substrate for cyclic AMP-dependent protein kinase, is found predominantly in sarcoplasmic reticulum, and has been implicated as having a role in sequestration of intracellular calcium during relaxation of cardiac muscle (Tada and Katz, 1982).

In addition to changing heart rate and force, a rise in cyclic AMP also has metabolic effects. For example, the activation of glycogen phosphorylase b by phosphorylation leads to an increased rate of glycogenolysis; this enzyme has recently provided the first demonstration of a conformational change in protein structure as a result of phosphorylation (Sprang et al., 1988).

The enzyme phosphodiesterase is responsible for the breakdown of cyclic AMP to 5'AMP. However, little is known about the regulation of cyclic AMP levels by this enzyme in vivo, probably due to the presence

of several myocardial isoenzymes of phosphodiesterase, each with different substrate specificities (Weishaar et al., 1987).

In summary, it is apparent that there has been much interest in the beta adrenergic / adenylate cyclase system but there are still gaps in our knowledge. Certainly the degree of sympathetic activity and disease states can regulate the responsiveness of the myocardium to catecholamines. As discussed earlier, myocardial catecholamines have been implicated in the development of serious arrhythmias occurring in acute myocardial ischaemia. It follows therefore, that investigation of the responsiveness of the beta adrenergic system to catecholamines under ischaemic conditions may be important.

1.2 MYOCARDIAL ISCHAEMIA

The myocardium is highly dependent on oxygen delivered by the arterial coronary circulation. Oxygen is necessary for the production of high energy phosphates which are required to meet the energy demands of the continuously contracting heart. In the presence of an arterial obstruction there is a reduction in blood flow to an area of myocardium. The resultant oxygen delivery may then be below the tissue oxygen requirement. This situation has been termed myocardial ischaemia. In addition to a reduction in blood supply, there is a concomitant reduction in removal of waste products, for example CO_2 and lactate. When ischaemia is localised to a region of the heart it is termed regional ischaemia, if the whole heart is rendered ischaemic this is termed global ischaemia.

The resultant changes that occur within the myocardium during myocardial ischaemia are complex. Within seconds of the onset of ischaemia myocardial contractility is depressed (Harden et al., 1979). There is an increase in anaerobic metabolism of glycolytic substrates, as evidenced by early lactate accumulation in the ischaemic area (Khuri et al., 1975). However aerobic metabolism is still the major source of ATP production even under severely ischaemic conditions (Neely et al., 1975). Ischaemia also results in mobilization of glycogen stores as an energy source (Rovetto et al., 1975). Beta-oxidation of fatty acids is inhibited (Whitmer et al., 1978), resulting in a build up of triglycerides and acyl CoA. However, it is not until about 60 minutes of ischaemia that the levels of free fatty acids start to increase (van der Vusse et al., 1982).

Creatine phosphate levels decline rapidly in ischaemia (Braasch et al., 1968), followed later (15-20 min), by a fall in the levels of ATP (Neely et al., 1973). Associated with this turnover of high energy phosphates is a build-up in adenosine and associated metabolities (Jennings et al., 1981) and accumulation of inorganic phosphate (Lavanchy et al., 1984).

The ionic gradients in the normal cell are also disturbed during ischaemia. Intracellular pH is lowered early in the ischaemic myocardium (Cobbe and Poole-Wilson, 1980), with a progressive increase in extracellular potassium and hydrogen ion concentrations (Hirche et al., 1982). However, the measurement of changes in other intracellular ion concentrations during ischaemia has proved methodologically difficult. Although there is a marked increase in intracellular levels of Ca^{2+} on reperfusion of ischaemic myocardium (Poole-Wilson et al., 1984), information on changes in Ca^{2+} levels during ischaemia has been lacking. Recent fluorine NMR measurements have demonstrated an early increase in free intracellular Ca^{2+} in ischaemic rat hearts (Steenbergen et al., 1987).

In early ischaemia there is a marked coronary vasodilation, probably due to multiple factors such as accumulation of adenosine, H^{+} ions and inorganic phosphate (Berne and Rubio, 1974). Pre-existing coronary collaterals may aid perfusion of an ischaemic area; however, the development of collaterals is species dependent. Pig heart has very few collaterals, whilst in canine and human myocardium there are variable degrees of collateral development (Meesman, 1982).

Initially, ischaemic damage is reversible but after a prolonged ischaemic period cells become irreversibly damaged and ultimately

die. The reason for the transition from reversible to irreversible ischaemia is unknown. There are changes in cell ultrastructure with prolonged ischaemia; for example, mitochondrial swelling and disruption of myocyte plasmalemma (Jennings et al., 1978). An increase in tissue osmolarity has also been shown (Steenbergen et al., 1985).

The exact time-course for the above changes cannot be stated with certainty, as the above information has been collated from different models of myocardial ischaemia in different species. It is clear though, that within the first 30 minutes of ischaemia a number of dramatic changes occur. These changes may be very important in the genesis of serious ventricular arrhythmias.

1.3 ARRHYTHMIAS RESULTING FROM MYOCARDIAL ISCHAEMIA

Clinically, arrhythmias occurring in myocardial ischaemia may be divided into two temporal groups; early arrhythmias occurring within minutes of the onset of ischaemia, and late arrhythmias occurring hours after the onset of ischaemia. Early arrhythmias are associated with a high incidence of the lethal arrhythmia, ventricular fibrillation. The high frequency of ectopic beats during late ischaemia is generally considered benign (Bigger et al., 1977).

Experimental evidence also indicates the presence of distinct early and late phases for development of arrhythmias occurring in ischaemia (Harris, 1950). The early malignant arrhythmias have been sub-divided into 1a and 1b phases (Kaplinsky et al., 1979). In addition to arrhythmias during ischaemia, paradoxically, reperfusion of the ischaemic myocardium is also associated with ventricular fibrillation. The significance of reperfusion arrhythmias to sudden death in man requires further clarification. Understanding the development of the early arrhythmias occurring in acute myocardial ischaemia may give an important insight into the causes of sudden death in man.

Early lethal arrhythmias occur against a backdrop of rapid, complex metabolic and ionic alterations; consequently it has proved difficult to identify the trigger for these arrhythmias. Current literature implicates several candidates as having a role in the genesis of the early ischaemic arrhythmias. In particular the arrhythmogenic roles of cyclic AMP and myocardial catecholamines during ischaemia will be discussed.

1.3.1 The Cyclic AMP Hypothesis

Initial observations showed an increase in cyclic AMP levels 5 seconds after onset of ischaemia which was maintained for at least 10 minutes (Wollenberger et al., 1969). Subsequently, other authors have described a later increase in cyclic AMP levels, 10-20 minutes after the onset of myocardial ischaemia. These authors have related this increase to the occurrence of ventricular arrhyhmias on a temporal basis (Corr et al., 1978; Lubbe et al., 1978; Opie et al., 1979; Podzuweit et al., 1982). This has lead to the 'cyclic AMP hypothesis' that an increase in cyclic AMP in the ischaemic area may precede and account for ventricular fibrillation (Podzuweit et al., 1980). Agents which increase intracellular levels of cyclic AMP, such as inhibitors of phosphodiesterase, do indeed increase the severity of ischaemic arrhythmias (Kane et al., 1985).

The mechanism of the arrhythmogenic effect of cyclic AMP is uncertain. Cyclic AMP may enhance the calcium dependent slow inward current, thereby reducing the rate of action potential propagation which may then predispose the myocardium to re-entry arrhythmias (McDonald, 1982).

An unanswered question in this area is, how is cyclic AMP elevated in ischaemia? Catecholamines, as discussed in the following section, may be released in the ischaemic area leading to the stimulation of adenylate cyclase. Alternatively, the metabolic and ionic changes which accompany acute myocardial ischaemia may have direct effects on the activities of adenylate cyclase and phosphodiesterase. In addition, as discussed earlier, there is

controversy over the relevance of tissue levels of cyclic AMP, in normal myocardium, to the resultant physiological responses; therefore the importance of tissue levels of cyclic AMP under ischaemic conditions must be questioned.

Doubt as to the arrhythmogenicity of cyclic AMP has been raised by several subsequent studies. The occurrence of coronary occlusion induced arrhythmias in the anaesthetised rat were not accompanied by temporally related increases in cyclic AMP levels, although an unsustained increase in cyclic AMP in early ischaemia was observed (Kane et al., 1985). Manning et al. (1985) have shown that in the in vitro coronary artery ligated rat heart, administration of isoprenaline increases both cyclic AMP levels and the incidence of ventricular fibrillation in ischaemic myocardium. Forskolin, in contrast, also increases the level of cyclic AMP in the ischaemic area but was anti-arrhythmic.

It appears that many, though not all, agents which increase cyclic AMP exacerbate arrhythmias when administered during myocardial ischaemia. A direct link between cyclic AMP and arrhythmias, in vivo, has yet to be established. Demonstration of a temporal relationship is not sufficient to ascribe a causative role to cyclic AMP.

1.3.2 Catecholamine Involvement

Clinically, raised plasma levels of catecholamines have been shown in patients after myocardial infarction and related to the occurrence of arrhythmias (Strange et al., 1974; Nadeau and De Champlain, 1979). However, these clinical studies do not reveal the

source of the catecholamines, that is, whether myocardial catecholamines are released during ischaemia.

Early experimental studies did report an increase in catecholamine release from the myocardium in early ischaemia (Wollenberger and Shahab, 1965). Subsequent investigations have not confirmed this finding (Riemersma and Forfar, 1982; Marshall and Parratt, 1980; McGrath et al., 1981). In vitro studies, with isolated perfused rat hearts, have not shown an increased overflow of catecholamines in early ischaemia, unless the myocardium is previously depleted of glycolytic substrate (Dart et al., 1987). There is however, a marked overflow of catecholamines on reperfusion of ischaemic myocardium (Dart and Riemersma, 1988). One study has demonstrated increased overflow of [^{3}H]-noradrenaline from prelabelled perfused rat myocardium during early ischaemia (Abrahamsson et al., 1985), but it is not known how the radioactive neurotransmitter distributes within the nerve terminal. Fluorescence studies of nerve terminals have shown decreases in fluorescence early in ischaemia, however simultaneous measurements of tissue catecholamine content have not shown corresponding decreases (Holmgren et al., 1981) suggesting redistribution of catecholamines. Measurement of catecholamine release in myocardial ischaemia is confounded by several problems; inherent in myocardial ischaemia is a reduction in blood flow, therefore in particular areas of the myocardium there may not be sufficent flow to wash out catecholamines. There is a further complication in that there may be enhanced neuronal reuptake of catecholamines in ischaemia.

Several possible mechanisms for an early release of catecholamines have been put forward. There is evidence of increased efferent sympathetic nerve activity in acute ischaemia (Malliani et al., 1969). Catecholamine release has also been demonstrated which is independent of nerve stimulation, perhaps due to the local ischaemic environment; for example, the build up of extracellular K^+ or local acidosis. It has been postulated that there is ischaemic release of catecholamines by a carrier mediated outward transport of noradrenaline from the nerve terminal and that this release is independent of nerve stimulation (Schömig et al., 1985). These studies have been hampered by the limited knowledge of the effects of ischaemia on the nerve terminal. In prolonged ischaemia when cell necrosis is occurring there is leakage of catecholamines probably due to membrane damage. Eventually the ischaemic area becomes depleted of catecholamines (Mathes and Gudbjarnsson, 1971).

Experimentally, it has not been possible to measure catecholamine release at the synapse and relate it to the development of serious arrhythmias. Therefore, indirect methods of assessing catecholamine involvement in arrhythmogenesis have been undertaken. Studies looking at the effect of depleting myocardial catecholamines, by either chronic administration of 6-hydroxydopamine or reserpine, demonstrated an anti-arrhythmic effect (Sethi et al., 1973; Sheridan et al., 1980). Chronic, but not acute, surgical autonomic denervation has also proved protective against the arrhythmias occurring during myocardial ischaemia (Ebert, 1970; Thomas et al., 1981). Stimulation of the left stellate ganglion during ischaemia increased the incidence of ventricular fibrillation (Schwartz and

Vanoli, 1981).

The anti-arrhythmic effectiveness of beta adrenergic antagonists (beta-blockers) is disputed. Many authors have observed an in vivo protective effect of beta-blockade following coronary artery ligation in several different species (Campbell et al., 1984; Coker and Parratt, 1984; Patterson et al., 1984). In contrast, others have not observed protection with beta-blockade (Botting et al., 1983; Daugherty et al., 1986). Some authors have proposed a significant role for alpha adrenergic antagonism during ischaemia (Sheridan et al., 1980). Again this is controversial with Bolli et al. (1984) unable to demonstrate a beneficial effect of phentolamine in ischaemic canine myocardium.

The effectiveness of beta-blockade against sudden death in man is difficult to quantitate. However, beta-blockade has been demonstrated to be significantly anti-arrhythmic when administered in the acute phase of myocardial infarction (Sleight, 1988). Clinically, beta-blockers are used as a preventitive measure against secondary myocardial infarction; pooled data suggests a 33% decrease in sudden death in this group after chronic beta-blockade (Frishmann et al., 1984). However, definitions of 'sudden' are variable (1-24 hours), and this population has already survived acute myocardial infarction and is therefore not a group in which to study early arrhythmogenesis. The benefical effects from beta-blockade may not necessarily be due to actions at the level of the beta adrenergic receptors. Beta-blockers are not a homogeneous group of drugs and some have other properties such as intrinsic sympathomimetic activity and membrane stabilising ability.

Administration of catecholamines during acute myocardial ischaemia has been shown to exacerbate arrhythmias (Manning et al., 1985). An important consideration is how catecholamines exert their arrhythmogenic effect. Catecholamines may exert an arrhythmic effect via occupancy of beta adrenergic receptors thereby increasing intracellular cyclic AMP levels as previously discussed. There is the additional possibility that catecholamines may be arrhythmogenic by a cyclic AMP independent mechanism. It has been postulated that under ischaemic conditions catecholamines form highly reactive species, adrenochromes, which lead to tissue damage (Dhalla et al., 1978). Speculatively, occupation of beta adrenergic receptors may allow coupling of G_s to sarcolemmal proteins other than adenylate cyclase. As previously mentioned, alpha adrenergic receptor blockade has proved protective against some arrhythmias, suggesting that catecholamines may also be arrhythmogenic through stimulation of these receptors during myocardial ischaemia (Sheridan et al., 1980).

From the literature discussed in this section it is clear that to date conclusive evidence demonstrating a release of myocardial catecholamines at an early time after the onset of ischaemia is not available. However, indirect evidence, such as chronic depletion of myocardial catecholamines, implicates catecholamines in the development of arrhythmias in myocardial ischaemia.

1.3.3 Other Suspects

Other arrhythmogenic factors have been proposed; lysophosphoglycerides have been shown to accumulate in ischaemic myocardium, and cause in vitro electrophysiological abnormalities

similar to those found in ischaemia (Corr et al., 1979). Synder et al. (1981) have shown that long chain acyl-carnitine accumulates in ischaemic myocardium, furthermore when applied externally acyl-carnitine has electrophysiological effects similar to those found with lysophosoglycerides (Heathers et al., 1987).

Arachidonic acid metabolities have also been implicated in the genesis of arrhythmias; within 1-2 minutes of myocardial ischaemia in the anaesthetised greyhound there is release of prostacyclin and thromboxane from the ischaemic myocardium followed later by PGE_2 and $PGF_{2\alpha}$ (Coker et al., 1981). It is thought that the balance of generation and release of thromboxane and prostacyclin may be important in early arrhythmogenesis (Parratt and Coker, 1985). An indication that prostanoids may be important in arrhythmias comes from the observation that aspirin is effective in reducing mortality of patients with unstable angina (Lewis et al., 1983).

Free radicals have been implicated in the development of reperfusion arrhythmias (Hearse and Tosaki, 1987). It is unlikely that these reactive species have a role in the genesis of ischaemic arrhythmias due to the reduced oxygen availability during ischaemia.

This is not a comprehensive review of all factors proposed to be arrhythmogenic, however it demonstrates that at present there are several schools of thought over which is thethefactor. At present there is very little information on the relative importance of any of the above factors, or on the interplay between different factors.

1.4 EFFECT OF ISCHAEMIA ON BETA ADRENERGIC FUNCTION

There are different levels at which myocardial ischaemia may modify beta adrenergic function; by alterations in beta adrenergic receptors, G_s or in the activity of adenylate cyclase. In addition, events subsequent to cyclic AMP formation such as phosphodiesterase activity and cyclic AMP dependent protein phosphorylation may be subject to alterations during myocardial ischaemia.

1.4.1 Ischaemia And Beta Adrenergic Receptors

The results from several authors investigating the effect of ischaemia on the number of beta adrenergic binding sites, as measured by radioligand binding, are summarised in Table 1.1. Animals were anaesthetised and hearts rendered ischaemic by ligation, in vivo, of the left anterior descending coronary artery which produces an ischaemic region in the myocardium. Vatner et al. (1988) are the exception to this protocol; these authors occluded the left circumflex coronary artery in conscious dogs.

After the indicated ischaemic period, hearts were excised and membranes prepared from control and ischaemic tissue. Membranes were then incubated, to determine specific binding, in the presence of a radiolabelled antagonist ([^{125}I]-iodocyanopindolol (ICYP) or [^{3}H] dihydroalprenolol (DHA)).

TABLE 1.1

EFFECT OF ISCHAEMIA ON MYOCARDIAL BETA ADRENERGIC RECEPTORS

Period of Ischaemia (min.)	Species	Change in Bmax# (% Control)	Reference
15	Guinea-pig	0	Maisel et al. 1985
15	Canine	+ 18%	Murkherjee et al. 1979
30	Guinea-pig	+ 45%	Maisel et al. 1985
30	Cat	0	Corr et al. 1981
60	Guinea-pig	+ 67%*	Maisel et al. 1985
60	Canine	+ 56%*	Vatner et al. 1988
60	Canine	+ 90%**	Mukherjee et al. 1979
90	Canine	0	Freissmuth et al. 1987
180	Canine	+ 107%***	Mukherjee et al. 1979
300	Canine	+ 35%**	Devos et al. 1985

Estimates from original papers.

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Before one hour of myocardial ischaemia there is no significant increase in the number of beta adrenergic binding sites (B_{max}) in guinea-pig, canine and cat myocardium (Maisel et al., 1985; Mukherjee et al., 1979; Corr et al., 1981). The number of beta adrenergic binding sites increased at one hour of ischaemia in canine and guinea-pig myocardium (Vatner et al., 1988; Mukherjee et al., 1979; Maisel et al., 1985). At later times of ischaemia results are contradictory. Freissmuth et al. (1987) found no alteration in the number of beta adrenergic binding sites after 90 minutes of myocardial ischaemia. In addition, only a 35% increase in beta adrenergic binding sites was observed by Devos et al. (1985) after five hours of ischaemia. At present, it is unclear whether increasing periods of ischaemia, of more than one hour, are associated with corresponding increases in the number of binding sites. No statistically significant alteration in receptor affinity was noted at any time of ischaemia.

The ligands [^{3}H] DHA and [^{125}I] ICYP are non-selective beta adrenergic receptor antagonists, therefore it is not possible to predict whether the increase in binding sites are of the $beta_1$ or the $beta_2$ adrenergic receptor subtype. Autoradiography has demonstrated that the increase in beta adrenergic binding sites appears to be at the level of the myocyte (Muntz et al., 1984). The observation of an increase in the number of beta adrenergic receptors in prolonged iscahemia was unexpected. As previously discussed, ischaemia has been associated with increased levels of circulating catecholamines and therefore 'down-regulation' of myocardial beta adrenergic receptors would be expected.

1.4.2 Ischaemia And G_s

As previously described, an indication of the ability of receptors to couple to G_s has been inferred from agonist displacement curves (Fig 1.2). Very few authors have investigated the effect of ischaemia on agonist displacement curves. It is known that both 1.5 and 5 hours of myocardial ischaemia, in canine hearts, is associated with a reduction in the portion of receptors in a high affinity state (Freissmuth et al., 1987; Devos et al., 1985). These results suggest that in prolonged ischaemia despite an increase in the number of beta adrenergic binding sites, there may be reduced coupling of beta adrenergic receptors to G_s.

1.4.3 Ischaemia And Adenylate cyclase

Several authors have documented the effect of ischaemia on adenylate cyclase (Table 1.2). Hearts were rendered ischaemic under in vivo or in vitro conditions and after a period of ischaemia, membranes were prepared from control and ischaemic ventricles. Most authors have quantitated both basal and stimulated adenylate cyclase activity. Both early and late periods of ischaemia have been shown to be associated with a decrease in adenylate cyclase activity with the exception of Mori (1976) and Maisel et al. (1985) who found an increase in adenylate cyclase activity.

TABLE 1.2

EFFECT OF ISCHAEMIA ON MYOCARDIAL

ADENYLATE CYCLASE ACTIVITY

Period of Ischaemia (min.)	Species	Adenylate Cyclase Activity[#]	Reference
10	Rat	-50%	Will-Shahab et al. 1985
15	Dog	+20%	Mori 1976
20	Dog	-20%	Drummond and Sordahl 1981
30	Rabbit	-44%	Bersohn et al. 1982
60	Dog	-61%	Mori 1976
60	Dog	-54%	Drummond and Sordahl 1981
60	Guinea-pig	+27%	Maisel et al. 1985
60	Dog	-38%	Vatner et al. 1988
90	Dog	-20%	Freissmuth et al. 1987
300	Dog	-65%	Devos et al. 1985

Estimates from original papers.

The molecular level at which these alterations in activity occur is unclear. In general authors have not published sufficient information to enable accurate determination of the level of alteration. No author looked at the effect of acute myocardial ischaemia (ie. less than 60 minutes of myocardial ischaemia) on both beta adrenergic binding sites and adenylate cyclase activity in the same model of ischaemia and the same species.

1.4.4 Ischaemia And Cyclic AMP

As previously described, several authors have shown increases in cyclic AMP levels in ischaemia and have related the increases to occurrence of ventricular fibrillation. Krause and England (1982), found a progressive decline in the ability of catecholamines to increase cyclic AMP levels when adminstered in early ischaemia. By 5 minutes of ischaemia there was no detectable change in cyclic AMP levels in response to isoprenaline.

In light of the data available on adenylate cyclase activity, it is difficult to reconcile alterations in adenylate cyclase with the increase in cyclic AMP levels. In the presence of a high concentration of catecholamines there may still be increases in the level of cyclic AMP, although total adenylate cyclase activity is reduced. It is also possible that there are alterations in the activity of phosphodiesterase in ischaemia. There is very little information on the effects of ischaemia on the activity of

phosphodiesterase, however Mori et al. (1976) found no change in phosphodiesterase activity after 15 or 60 minutes of myocardial ischaemia.

Alterations in protein phosphorylation in myocardial ischaemia have been investigated. Krause et al. (1984) found no change in the phosphorylation state of troponin I, myosin P-light chain, an 11 kD protein (probably phospholamban) or the proportion of phosphorylase a in the first 5 minutes of in vitro ischaemia in the rat heart. Challenge with isoprenaline during ischaemia resulted in a progressive decline in the ability of isoprenaline to stimulate phosphorylation of these proteins with increasing periods of ischaemia. These results suggest that the responsiveness of the myocardium to catecholamines falls early in myocardial ischaemia. Long-term ischaemia (1-4 hours) is associated with a reduction in the phosphorylation of phospholamban (Lamers et al., 1986).

1.5 PURPOSE OF THESIS

The hypothesis that there is altered responsiveness of the myocardium to catecholamines during ischaemia is a reasonable one in view of the preceeding literature. This thesis sets out to clarify the effects of, in particular, acute myocardial ischaemia on beta adrenergic function. The effect of both acute, and prolonged ischaemia, on adenylate cyclase activity and beta adrenergic binding sites, in the same model of myocardial ischaemia and in the same species are investigated. It is hoped that these results, in combination with an understanding of the effects of the local ischaemic environment on beta adrenergic function, may provide an increased basic knowledge of the responsiveness of the acutely ischaemic myocardium to catecholamines at the time of onset of ventricular arrhythmias.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Biochemicals and other materials were purchased from the following:-

Sigma Chemical Company Ltd. (Poole, Dorset, England): Adenosine deaminase EC 3.5.4.4 (A 9876), cyclic AMP (A 6885), ATP (A 2383), alumina Type WN-3:Neutral (A 9003), bovine serum albumin (A6003), creatine phosphate (P 6502), creatine phosphokinase EC 2.7.3.2 (C 3755), dithiothreitol (D 0632), GTP (G 8502), imidazole (I 0125), (-)-isoprenaline (I 6504), DL- propranolol (P 0884) and sodium fluoride (S 1504).

Amersham International plc (Buckinghamshire): [2,8-^{3}H]cyclic AMP (TRK.498), [alpha-^{32}P]ATP (PB.171), [^{125}I]sodium iodide.

BDH Ltd (Poole): Cocktail T scintillation fluid.

Biorad Laboratories Ltd. (Watford, Hertfordshire): Dowex AG 50W-X4 Resin (200-400 mesh size).

Flow Laboratories Ltd. (Hertfordshire): 'Micronic' tubes and multi-channel pipettors for adenylate cyclase assay.

Merck (Darmstadt, W. Germany): TLC plastic sheets polyethyleneimine (PEI)-cellulose pre-coated, with a UV fluorescent background (20 x 20 cm).

Pierce & Warriner (U.K.) Ltd. (Chester): Polystyrene columns -29920 (8mm I.D. x 102 mm) for adenylate cyclase assays.

Sarstedt Ltd. (Leicester): Polypropylene tubes for ligand binding studies (Sarstedt No. 55 476).

Whatman International Ltd. (Maidstone, England): Glass fibre filters (GF/C) 2.5 cm, for ligand binding studies.

(-)-Pindolol was a gift from Dr Engel, Sandoz Ltd. (Basle): All other reagents were of the highest purity available and were purchased from Sigma Chemical Co. Ltd.

2.2 PREPARATION OF MYOCARDIAL MEMBRANES

Male Sprague/Dawley rats (300 g) were sacrificed by stunning followed by cervical dislocation, the hearts quickly removed and placed in ice-cold 50 mM Tris-HCl 0.25 M sucrose, pH 7.4 at 4°C. The atria and large vessels were trimmed from the hearts and the ventricles minced throughly and homogenised in 20 volumes of the above buffer in a Potter-Elvehjem homogeniser. The homogenate was filtered through two layers of gauze and the filtrate centrifuged at 3,600 rpm (1600 g_{av}) for 10 min in a Beckman rotor (JA20). The resultant substantial pellet, containing red blood cells, cell debris and nuclei, was discarded. The supernatant was centrifuged at 25,000 rpm (42,000 g_{av}) for 25 min in a Beckman rotor (type 45Ti). Crude membranes were prepared by gentle homogenisation of the pellet in an all glass, loose, homogeniser in 50 mM Tris-HCl 10 mM $MgCl_2$, pH 7.5. All steps were carried out at 4°C. A Beckman J2.21 centrifuge and a Beckman L8 ultracentrifuge were used for centrifugal procedures.

Preparation of myocardial membranes by the above method is rapid (approximately 90 min), allowing all experiments to be carried out on freshly prepared membranes.

2.3 PROTEIN DETERMINATION

Two methods of determination of the protein content in myocardial membranes in 50 mM Tris-HCl 10mM $MgCl_2$ were compared; that of Lowry et al. (1951) and a dye binding method (Bradford, 1976). Tris positively interferes with protein determination by the Lowry method. Removal of Tris from membranes was achieved by precipitation of the protein in perchloric acid, however triplicate determinations revealed that this step introduced inaccuracies. The Lowry method for protein determination was also found to be sensitive to temperature fluctuations. The Bradford method was adopted for estimation of protein content in myocardial membranes.

The Bradford dye binding assay was linear over the range 2-8 µg protein. Bovine serum albumin (BSA) was used as a protein standard; the absorbance at 280 nm was used to calculate the concentration of BSA. A [x5] dye reagent stock solution was prepared containing 25 mg serva blue G dissolved in 25 mls of 96% ethanol, 50 mls orthophosphoric acid and 25 mls double distilled water. This solution was stored at $4^{o}C$ for four months with no alteration in the reproducibility of the standard curve.

2.4 ADENYLATE CYCLASE ASSAY

The assay of adenylate cyclase employed was basically that of Salomon (1979) with modifications, which follows the conversion of [alpha-^{32}P]ATP to [^{32}P]cyclic AMP. Separation of [^{32}P]cyclic AMP formed from the assay substrate was achieved by chromatography on alumina (White and Zenser, 1971).

2.4.1 Assay

Membranes (~100 µg protein) were incubated at 37°C for 5 minutes (unless otherwise stated) in a final volume of 100 µl containing 22 kBq [alpha-^{32}P]ATP, 0.4 mM ATP, 25 mM Tris-HCl pH 7.5 (37°C), 5 mM $MgCl_2$, 20 mM creatine phosphate, 50 units/ml creatine phosphokinase, 2.5 mM cyclic AMP, ~10,000 c.p.m. [^{3}H]cyclic AMP, 1 mM dithiothreitol, 10 µM GTP and 0.02% (w/v) ascorbic acid. The reaction was terminated by the addition of 25 µl of 0.5 N HCl. Each sample was assayed in quadruplicate. Basal adenylate cyclase was measured under the conditions described above. Stimulation of adenylate cyclase by (-)-isoprenaline and sodium fluoride was measured at drug concentrations described in the text.

2.4.2 Separation of [^{32}P]cyclic AMP from [^{32}P]ATP

Separation of the assay product, [^{32}P] cyclic AMP, from the substrate was achieved by anion exchange chromatography. Multivalent nucleotides, such as ATP, are adsorbed to the alumina while monovalent

nucleotides, such as cyclic AMP, are eluted at neutral pH. Assay tubes were placed in a boiling bath for 2 minutes and the contents cooled and then neutralised with 25 µl 1 M imidazole. This boiling step reduced the blank values obtained by 50% without altering the recovery of [^{3}H]cyclic AMP. Boiling also denatures the membrane protein thereby speeding up the subsequent chromatography. Samples were applied sequentially to pre-washed alumina columns with 1 ml 0.1 M imidazole pH 7.3 (Column Buffer). A further 1 ml of Column Buffer was applied but the eluent discarded, subsequent application of 2 mls of Column Buffer eluted (65-80%) of [^{3}H]cyclic AMP, the eluent was collected into scintillation vials and 15 mls of Cocktail T scintillant added to each vial. [^{3}H]Cyclic AMP was present as a marker of the recovery of [^{32}P]ATP during the assay.

2.4.3 Standards and assay blanks

Standards were prepared containing [^{3}H]cyclic AMP and [^{32}P]ATP and counted under the same conditions as assay samples. Assay blanks were measured by incubation of assay components without membrane ('no membrane blank') or with denatured membranes under assay conditions ('membrane blank'). Neither of these methods yeilded significantly different blank levels. The level of the blank in the assay was affected by different batches of [^{32}P]ATP and alumina. The value of the blank was between 0.002% and 0.02% of the amount of [^{32}P]ATP in the assay.

2.4.4 Features of the assay

During development of the assay a number of important features emerged; such as the importance of an ATP regenerating system, adequate protection of cyclic AMP formed in the assay and the lability of cardiac adenylate cyclase.

The membrane preparation used is crude and contains ATPases which cause hydrolysis of the reaction substrate (Johnson, 1980). To maintain ATP levels during the assay an ATP regenerating system was included, consisting of 20 mM creatine phosphate (CP) and 50 units/ml creatine phosphokinase. In order to establish that these regenerating conditions were sufficient, ATP levels and CP levels were measured in samples, under adenylate cyclase assay conditions but without [^{32}P]ATP or [^{3}H]cyclic AMP, in the presence of the ATP regenerating system. Both basal and (-) isoprenaline (0.1 mM) stimulated adenylate cyclase activity were investigated. After indicated times samples were deproteinised with perchloric acid. Measurements of ATP and CP in the deproteinised assay extracts were carried out spectrophotometrically in a Cobas Bio centrifugal analyser, employing a linked enzyme assay (Lamprecht et al., 1974).

Under basal adenylate cyclase assay conditions there is a rapid decline in CP levels as shown in Fig 2.1; after 5 mins only 50% of initial CP remains. In contrast, ATP levels are still approximately 0.4 mM at 5 minutes, however by 30 minutes both ATP and creatine phosphate levels are unmeasurable. In the presence of (-)-isoprenaline an identical profile was obtained to that shown in Fig 2.1. These results suggest that under the assay conditions described the regenerating system is sufficient.

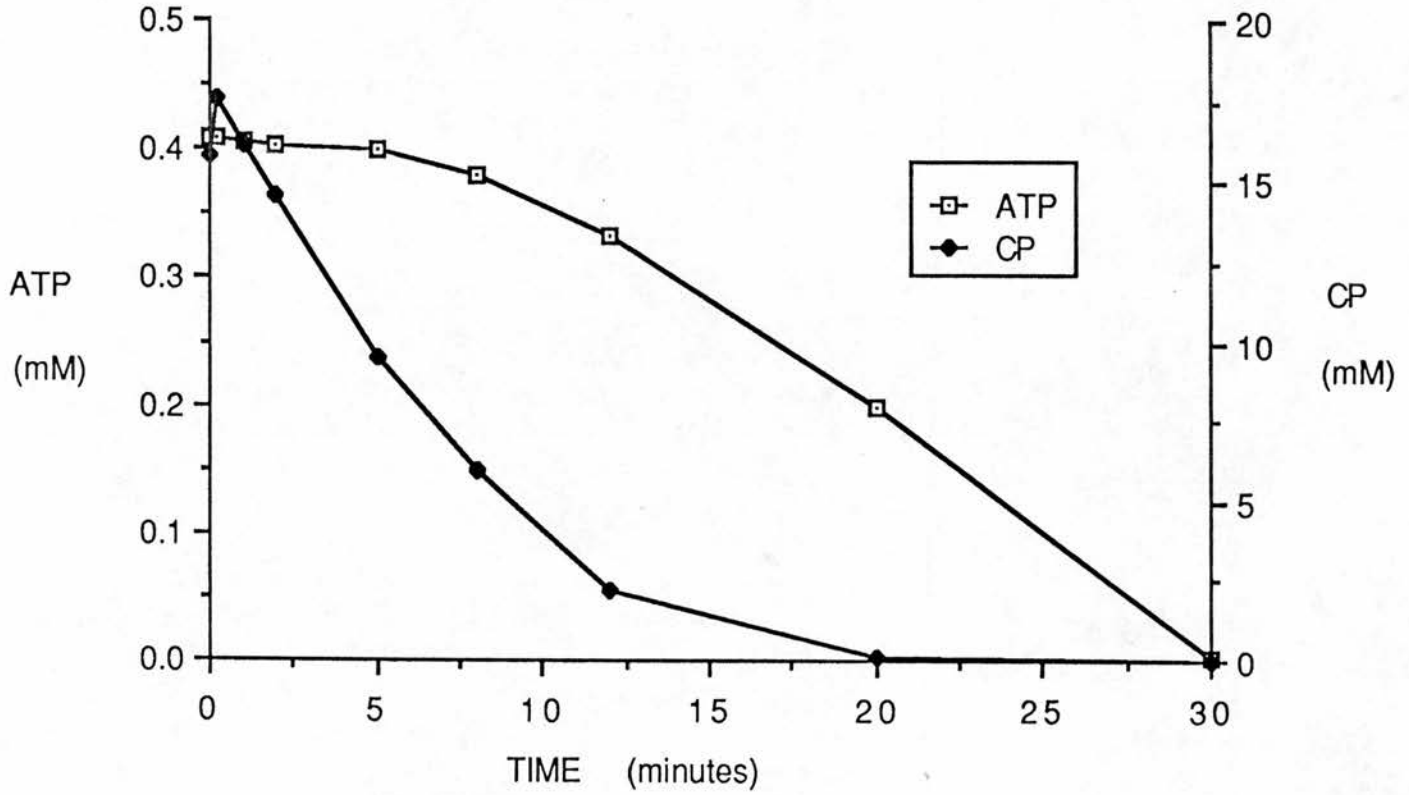

Fig 2.1 Levels of ATP and creatine phosphate (CP) in rat myocardial membranes under adenylate cyclase assay conditions. Duplicate determinations.

Cyclic AMP formed during the adenylate cyclase assay may be degraded by phosphodiesterases also present in the membrane preparation. Breakdown of the the product from the assay may be minimised by the addition of unlabelled cyclic AMP. The concentration of unlabelled cyclic AMP required to minimise the effect of myocardial phosphodiesterase was determined by incubating [^{3}H]cyclic AMP with myocardial membranes (1 mg/ml) for 30 minutes under assay conditions in the presence of a range of unlabelled cyclic AMP concentrations. Fig 2.2 shows that the inclusion of 2.5 mM unlabelled cyclic AMP reduces the effect of phosphodiesterase to a negligible level.

The reducing agent dithiothreitol (DTT) is included in the assay due to its ability to stabilise adenylate cyclase activity. DTT reduces S-S groups and has also been shown to inhibit nucleotide pyrophosphatase and therefore protect GTP levels (Johnson 1980). Adenylate cyclase in membranes is labile, the enzyme is particularly labile in cardiac membranes incubated at 37^{o}C (Synder and Drummond, 1978). Cardiac membranes were therefore not pre-incubated at 37^{o}C. Assays were carried out quickly, two sets of quadruplicates were assayed simultaneously with the use of 'micronic' test tubes and multichannel pipettors.

2.4.5 Preparation of alumina columns

Columns were prepared by scooping 1 g alumina into a tube containing 2 mls Column Buffer, mixing and applying the contents to a column. The columns were then washed with 15 mls Column Buffer and stored at room temperature in Column Buffer until required. Before applying assay samples, the columns were washed with a further 5-

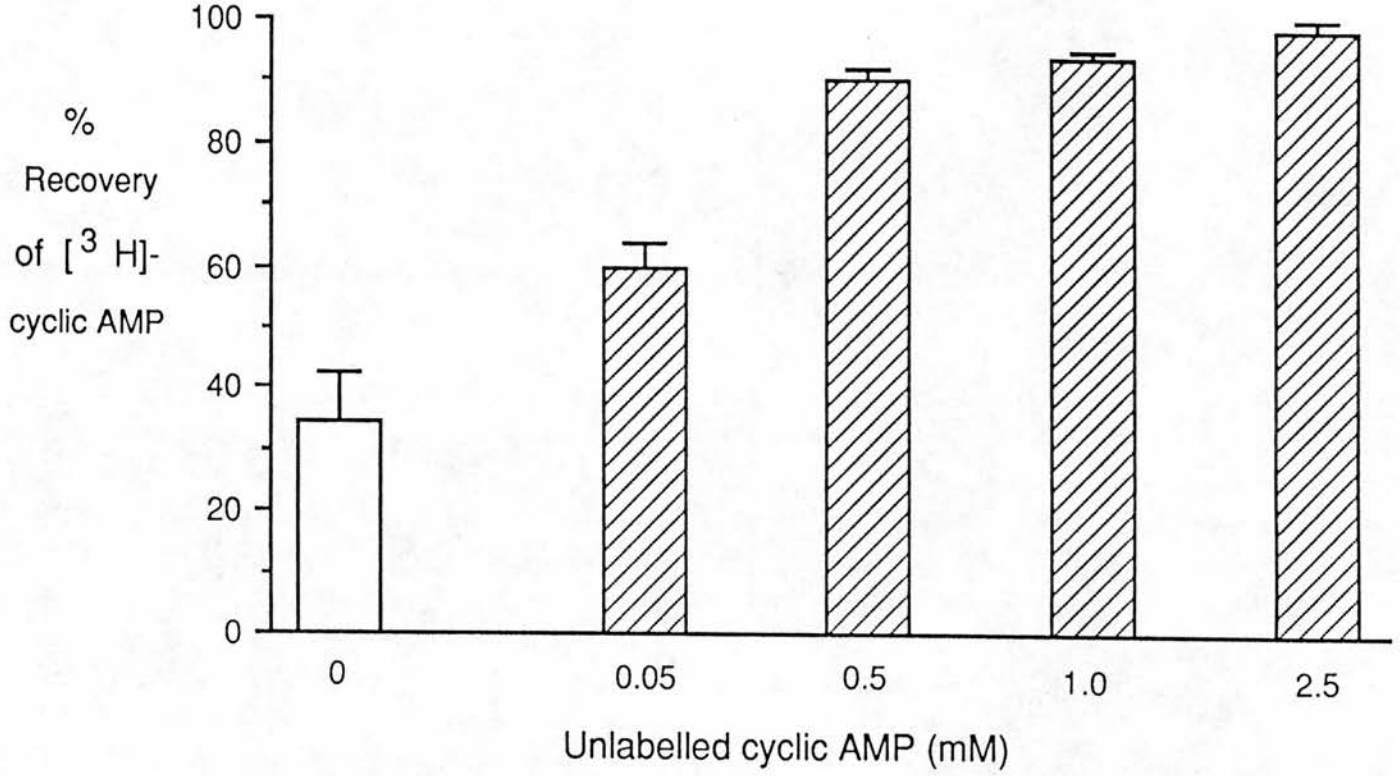

Fig 2.2 Effect of unlabelled cyclic AMP concentration on the recovery of [^{3}H]cyclic AMP from adenylate cyclase assay of myocardial membranes. 100 % recovery is that obtained in the absence of myocardial membranes.

10 mls of Column Buffer. A reduction in the magnitude of the blank from the cyclase assay was obtained when alumina was oven dried overnight and subsequently stored in a dessicator before use as suggested by (Thompson et al., 1973). Alumina columns were not reused, this elminated uncertainity about contamination from previous assays (approximately 99% of applied ^{32}P remains bound to alumina after [^{32}P]cyclic AMP elution).

2.4.6 Validation of assay product

Most authors publishing data on adenylate cyclase in myocardial tissue have not attempted to ensure that the product from their assay is actually cyclic AMP. In order to identify the product from the described assay, the end products from adenylate cyclase assays were chromatographed on PEI phosphocellulose TLC plates and the nucleotide formed was identified. Basal and stimulated (sodium fluoride (10 mM), (-)-isoprenaline (0.1 mM)) adenylate cyclase were investigated and in addition the identity of the 'no membrane' and 'membrane' blanks obtained in the assay.

Membranes were incubated under the previously described conditions for measuring adenylate cyclase activity, but without [^{3}H]cyclic AMP. The eluent from alumina columns was applied directly to columns containing Dowex AG 50W-X4 (chloride form). This step ensures the removal of imidazole buffer from samples which would, when concentrated, interfere with the subsequent nucleotide separation. Washing columns with 20 mls DDW removed imidazole as verified by measurement of the optical density at 300 nm. The ^{32}P-product remained tightly bound to the dowex as shown by liquid scintillation counting of the washes. A 10 ml wash of 0.1 N HCl

eluted greater than 90% of ^{32}P applied to the columns. This wash was subsequently freeze-dried and reconstituted in DDW and applied to PEI-phosphocellulose TLC plates with a mixture of reference unlabelled nucleotides (20 nmoles of each). The plates were developed first in butanol:methanol:DDW (1:1:8, by vol.) for 5 cm of the plate length and then DDW for 3 cm and finally 0.55 M LiCl to the top of the TLC plates, witout drying the plates between solvents. This method for separation of nucleotides is based on that described by Reibel and Rovetto (1978) and optimises conditions for the separation of cyclic AMP from other nucleotides (Table 2.1).

TABLE 2.1

PEI-Phosphocellulose Chromatography of nucleotides

Nucleotide	R_F
cAMP	0.36
IMP	0.30
AMP	0.25
ADP	0.05
ATP	0.01

Nucleotides were visualised under ultraviolet light (254 nm), before drying, and the plates cut into bands of length approximately 1 cm. Radioactivity was eluted from the TLC bands by shaking with 1 ml of 2 M LiCl overnight. In addition autoradiographs

of duplicate TLC plates were used to visualise ^{32}P bands.

Figure 2.3 shows the product obtained from an adenylate cyclase assay in the absence of stimulation. The peak of ^{32}P coincided with the position of cyclic AMP on the plate. This demonstrates that basal adenylate cyclase activity is the formation of cyclic AMP. A small shoulder of radioactivity which runs ahead of cyclic AMP was observed. This profile was also found with stimulated samples. The small peak of ^{32}P was also observed in 'membrane' and 'no membrane' blanks but no cyclic AMP peak was found. An autoradiograph of a TLC strip of sodium fluoride (10 mM) stimulated adenylate cyclase is shown in Fig 2.4. This shows a dense band with an identical R_F value to cyclic AMP and a faint discrete band running ahead, confirming the above. It has been assumed that the blank obtained in this assay is non-enzymatically formed cyclic AMP, however this did not appear to be the case. These results suggest that the product of this adenylate cyclase assay is indeed cyclic AMP but that the blank in the assay is not cyclic AMP.

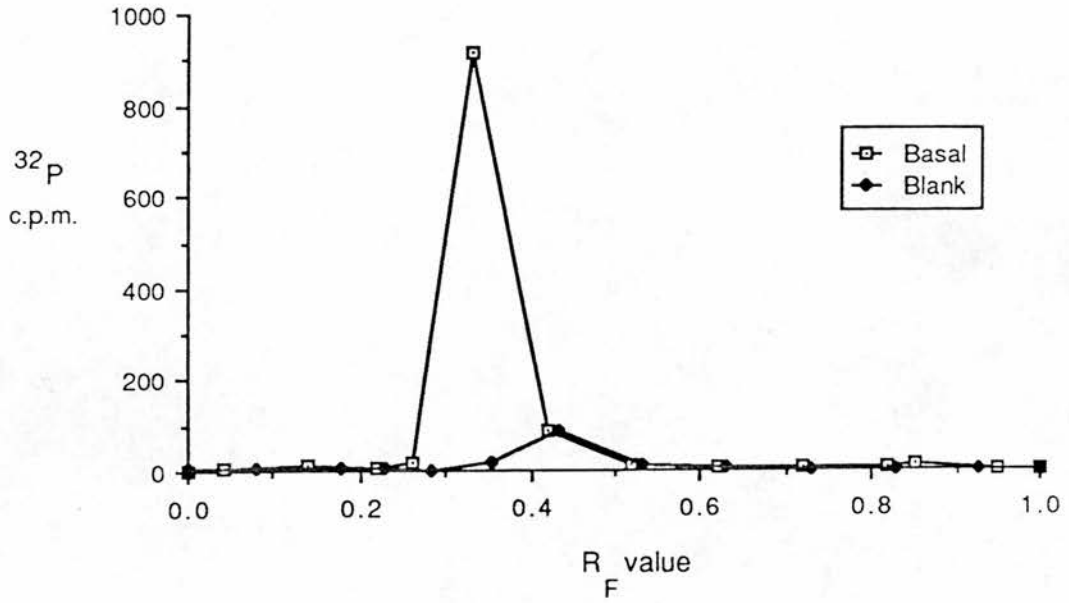

Fig 2.3 Profile of ^{32}P counts obtained following thin-layer PEI-phosphocellulose chromatography of the product from an adenylate cyclase assay.

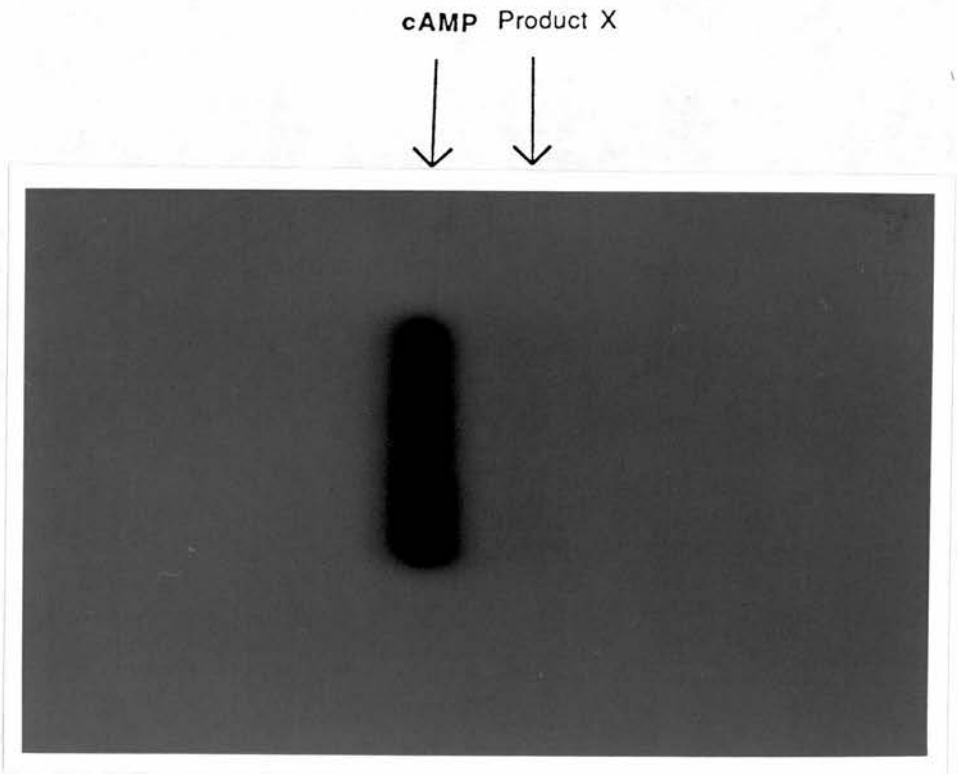

Fig 2.4 Photograph of an autoradiograph of the ^{32}P product from sodium fluoride (10 mM) stimulated adenylate cyclase after thin-layer PEI-phosphocellulose chromatography.

2.4.7 Linearity of assay

The formation of cyclic AMP was proportional to time over a period of 10 minutes for both basal and isoprenaline stimulated adenylate cyclase (Fig 2.5). However sodium fluoride stimulation of adenylate cyclase was associated with a lag phase. This has been reported in other tissues (Manganiello and Vaughan, 1976), however the reason for this lag phase is not clear. The production of cyclic AMP is linear with respect to protein over a range of concentrations (55-350 µg) Fig 2.6.

2.4.8 K_m determination of cardiac adenylate cyclase

The apparent K_m of adenylate cyclase, in rat cardiac membranes, was determined by incubation of membranes in the presence of a range of ATP concentrations (0.2 mM - 0.8 mM) along with the previously described assay components. A Hanes plot of the data obtained is shown in Fig 2.7. Kinetic parameters were determined by linear regression analysis, the observed K_m and V_{max} values are shown in Table 2.2.

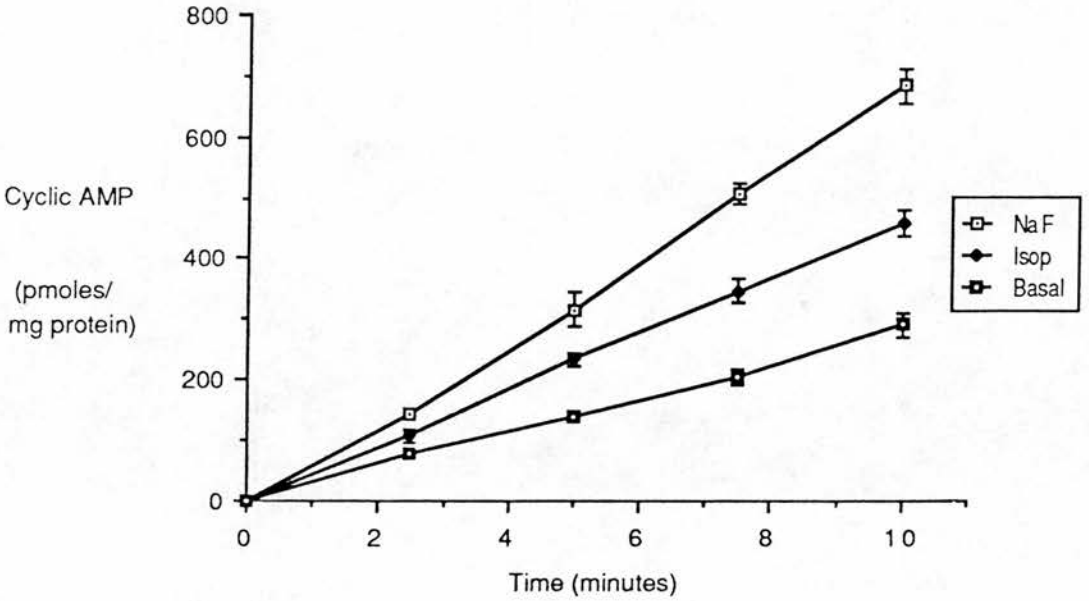

Fig 2.5 Effect of incubation time at 37°C on basal and stimulated (Na F 10 mM, isoprenaline 10^{-4}M) adenylate cyclase activity. Each determination in quadruplicate.

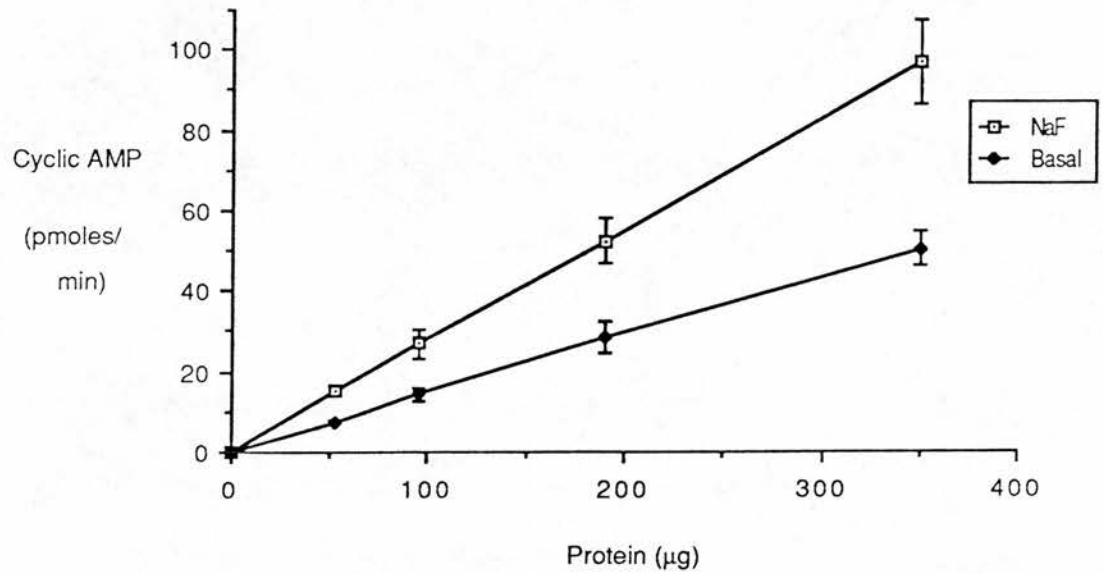

Fig 2.6 Relationship of the rate of cyclic AMP formation to protein concentration in rat heart membranes, in the presence and absence of Na F 10mM. Each determination in quadruplicate.

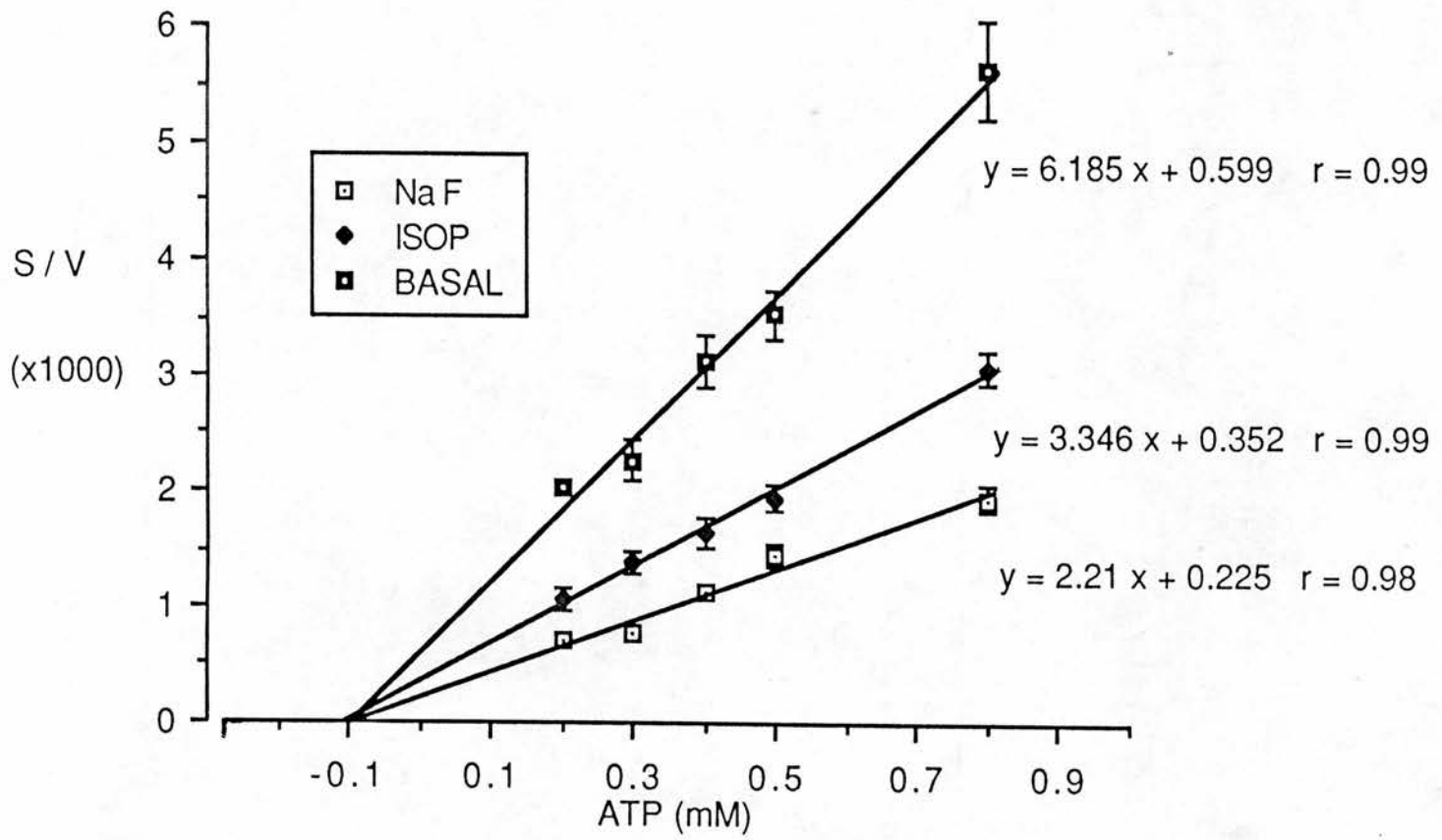

Fig 2.7 K_m determination of cardiac adenylate cyclase. The effect of ATP concentration on unstimulated adenylate cyclase activity (basal) and that in the presence of sodium fluoride (10 mM) and (-)-isoprenaline (10^{-4}M).

TABLE 2.2 Determination of the Km and Vmax values of adenylate cyclase in rat myocardial membranes.

	K_m (mM)	V_{max} (pmoles/min/ mg protein)
Basal	0.097 ± 0.004	162 ± 7.3
Isoprenaline 0.1 mM	0.105 ± 0.004	299 ± 12.4
Sodium Fluoride 10 mM	0.102 ± 0.01	452 ± 45

Cardiac adenylate cyclase obeyed Michaelis-Menten kinetics over the range of ATP concentrations investigated. The data presented in Table 2.2 show that a K_m value of approximately 0.1 mM is observed for adenylate cyclase in rat myocardial membranes. Stimulation of the enzyme with sodium fluoride or (-)-isoprenaline did not alter the apparent K_m but did increase V_{max}. The dervived K_m for rat adenylate cyclase agrees well with that found for human adenylate cyclase (Golf et al., 1984).

2.5 RADIOLIGAND BINDING TO BETA ADRENERGIC RECEPTORS

As previously discussed, ligand binding studies give information on the binding characteristics of receptors and ligands. There are several ligands which are specific to beta adrenergic receptors; most are hydrophobic, for example [^{125}I]-iodopindolol. A hydrophillic ligand is also available ^{3}H - CGP-12177; this is useful for labelling surface receptors as it cannot cross membranes. The iodinated beta adrenergic ligands have several advantages; they have high affinity for beta adrenergic receptors and may be prepared inexpensively with high specific activity. Ligand binding studies were carried out with [^{125}I]iodopindolol synthesized as described below.

2.5.1 Synthesis of (-)[^{125}I]iodopindolol

(-)-Pindolol was iodinated by a modification of the method of Barovsky and Brooker (1980). Briefly, 20 µl 1-2 mCi of [^{125}I]sodium iodide in 0.3 M potassium phosphate buffer pH 7.6 was combined with 10 ul 2 mg/ml (-)-pindolol in HCl (13.5 mM). The iodination was started by the addition of 20 µl fresh chloramine T (0.34 mg/ml) and the reaction was allowed to proceed for 5 minutes at room temperature. Addition of 300 µl of sodium metabisulphite 1 mg/ml stopped the reaction. The contents of the tube were then made alkaline by the addition of 10 µl (1 M) sodium hydroxide and iodinated products extracted into water saturated ethyl acetate containing 0.01% phenol (3-4 washes combined). (-)-[^{125}I]iodopindolol ((-)-IPin) was separated from pindolol and free I^{125} by descending paper chromatography in 0.1 M ammonium formate pH 8.5 / 0.01% phenol at 4^{o}C for 3-4 hours. (-)-IPin was eluted into methanol and quantified by gamma counting.

Figure 2.8 shows a typical chromatographic profile obtained, the R_F value for free pindolol in this system is 0.69. The structure of (-)-IPin is shown in Figure 2.9. The fractions containing iodopindolol were concentrated by a stream of nitrogen and stored at -20°C.

2.5.2 Stability of (-)-IPin

The radiochemical purity of (-)-IPin was asssesed after synthesis by chromatography of an aliquot as described above. Routinely ~93% of ^{125}I coincided with the (-)-IPin peak, ~3% with the free ^{125}I peak, there was no alteration in the radiochemical purity on storage of the ligand. The stability of (-)-IPin in aqueous buffer was monitored (binding studies were carried out under these conditions) and the percentage purity calculated by subsequent chromatography of (-)-IPin in buffer. A reduction in purity of (-)-IPin from 93% to 75% was observed when (-)-IPin was diluted in 25 mM Tris-HCl 5 mM $MgCl_2$ pH 7.5. A concomitant increase in the percentage of free ^{125}I was observed. Pre-spotting either phenol or pindolol on the chromatography paper before (-)-IPin in buffer stopped the reduction in purity. This suggests that decomposition of the ligand occurred during chromatography and not before.

2.5.3 (-)-IPin binding studies

Binding studies were routinely carried out, unless otherwise stated, by incubation of membranes with (-)-IPin, 25 mM Tris-HCl 5 mM $MgCl_2$ pH7.5 and 0.02% ascorbic acid (w/v) in a total volume of 250 µl. Incubations were carried out for 30 min at 37°C in

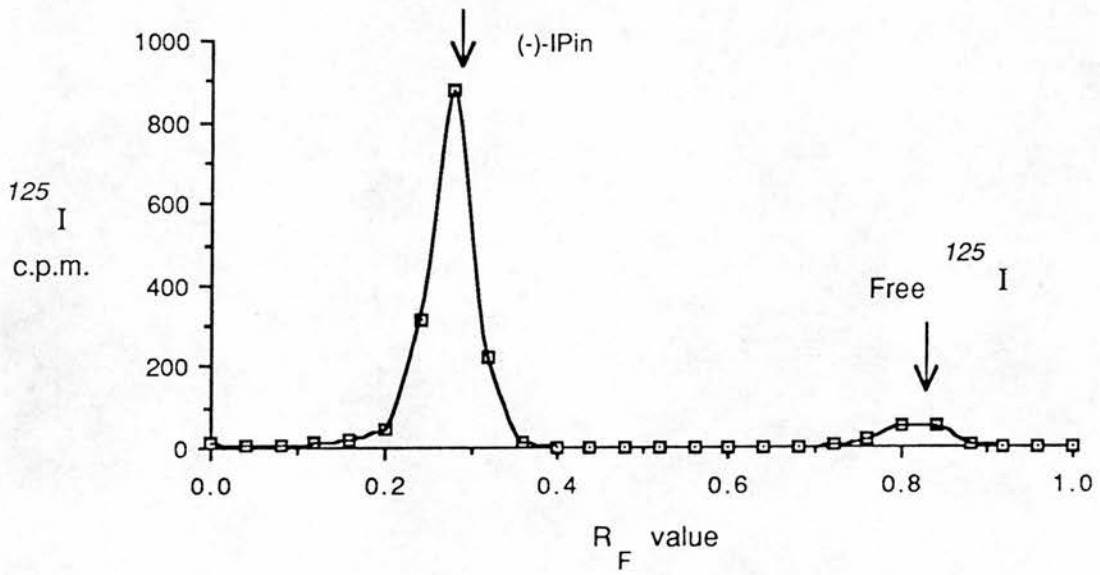

Fig 2.8 Chromatographic profile of the ^{125}I product after preparation of (-)-IPin by iodination of pindolol. The R_F value of pindolol in this system is ~0.7.

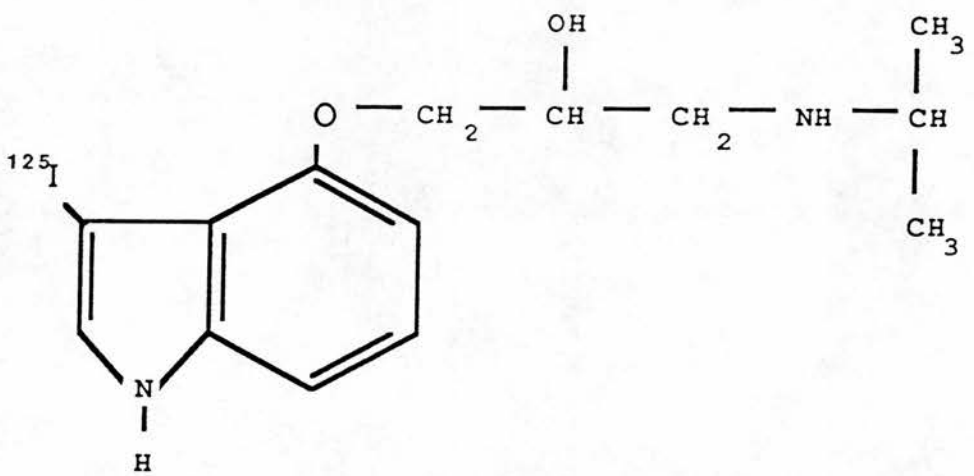

Fig 2.9 Structure of [^{125}I] (-)iodopindolol.

polypropylene tubes (Sarstedt). The reaction was terminated by addition of 1 ml 25 mM Tris-HCl 5mM $MgCl_2$ pH 7.5 and rapid filtration through Whatman glass fibre filters on a Millipore filtration unit. Each filter was washed with a further 15 mls of buffer. Bound ligand was determined by counting filters in an LKB Gamma counter (efficiency for ^{125}I of 83%). Non-specific binding was defined as ligand bound in the presence of $10^{-5}M$ (-) isoprenaline. Specific binding was calculated by subtraction of non-specific binding from total binding.

For equilibrium binding studies membranes were incubated in the presence of at least ten concentrations of (-)-IPin (20-1500 pM) and 0.02% ascorbic acid as described above. Non-specific binding of the ligand was determined at each concentration of (-)-IPin used. Scatchard plots of the resultant data were used to calculate B_{max} and the dissociation constant K_D, the best fit line was determined by the least squares method (Scatchard, 1949).

Competition experiments were carried out to obtain information on agonist and antagonist binding to beta adrenergic receptors. Membranes were incubated with increasing concentrations of (-)-isoprenaline at a constant (-)-IPin concentration.

The kinetics of (-)-IPin binding were quantified by incubating ligand in the presence of membranes at $37^{o}C$ for time points as indicated. From the specific binding at specified times an association rate constant (k_1) may be calculated. After 30 minutes (-)-isoprenaline ($10^{-4}M$) was added to displace (-)-IPin from the membranes. From displacement of ligand with time the dissociation rate constant (k_{-1}) may be calculated.

Calculation of k_1 $\quad R + L \longrightarrow RL$

The association of (-)-IPin (L) with the membrane receptors (R) was estimated graphically from the equation below as described by Hoyer et al. (1982).

$$k_1 = \frac{2.303}{t([L]-[R])} \left(\log \frac{R([L]-[x])}{L([R]-[x])} \right)$$

Where k_1 is the association rate constant

[L] - initial (-)-IPin concentration

[R] - initial receptor concentration

[x] - concentration of occupied receptors at time t

Calculation of k_{-1} $\quad RL \longrightarrow R + L$

This may be calculated from the following equation:-

$$k_{-1}\, t = \ln \frac{[B]}{[B_o]}$$

$[B_o]$ - ligand bound before any dissociation of ligand

[B] - ligand bound after time t

The dissociation constant K_D can then also be calculated:-

$$K_D = \frac{k_{-1}}{k_1}$$

2.6 STATISTICAL ANALYSIS

Unless otherwise stated all analysis of results were paired t-tests. If significance is not shown then the test was not significant at $p < 0.05$. Analysis and data handling was facilitated by the use of the Minitab statistics package on a BBC microcomputer with a second processor.

2.7 QUANTIFICATION OF RADIOACTIVITY

^{32}P and ^{3}H were quantified by liquid scintillation counting in a LKB Rack Beta scintillation counter in the presence of the scintillant, Cocktail T, corrections for spillover of counts from the ^{32}P channel into ^{3}H channel were made. ^{125}I was quantified by counting in an LKB gamma counter, with efficiency of 83%.

CHAPTER 3

CHARACTERISATION OF CARDIAC ADENYLATE CYCLASE AND BETA ADRENERGIC RECEPTORS

CHAPTER 3

Before attempting to understand the function of the myocardial beta adrenergic / adenylate cyclase system under ischaemic conditions, it was important to characterise this system under normal physiological conditions. Therefore both adenylate cyclase activity, and binding to beta adrenergic binding sites by the ligand (-)-IPin, were measured in rat cardiac membranes prepared as described in Chapter 2. The possibility of measuring binding sites and adenylate cyclase activity under the same conditions was also investigated.

3.1 ADENYLATE CYCLASE

The activity of adenylate cyclase in rat myocardial membranes was studied. This enzyme holds a key position in the control of cardiac contractility and metabolism and in determining the responsiveness of the myocardium to catecholamines. As previously discussed, the activity of adenylate cyclase may also be altered by receptor-independent mechanisms, thereby allowing determination of whether any alteration observed is specific to the beta adrenergic / adenylate cyclase system or to a more general alteration.

3.1.1 Basal and stimulated adenylate cyclase

The general profile of adenylate cyclase activity in rat heart membranes is shown in Fig 3.1. Each determination was carried out in quadruplicate. In the presence of (-) isoprenaline (0.1 mM) or sodium

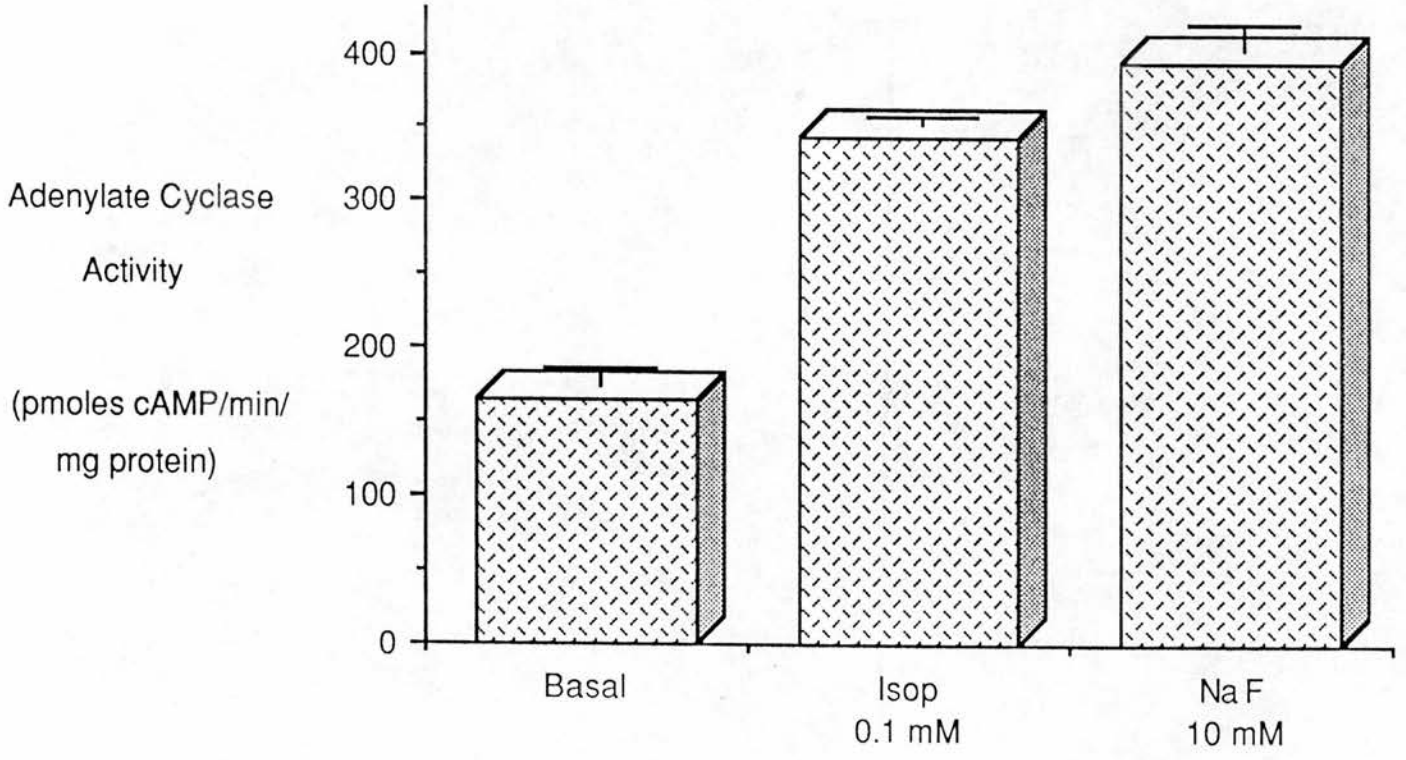

Fig 3.1 Adenylate cyclase activity in rat myocardial membranes. Basal activity and that in the presence of Na F (10 mM) or isoprenaline (0.1 mM) is shown. Quadruplicate determinations.

fluoride (10 mM) stimulation of adenylate cyclase was observed. The mean adenylate cyclase results from 18 separate rat cardiac membrane preparations are presented in Table 3.1. Membranes were assayed immediately after preparation.

TABLE 3.1 Adenylate cyclase activity in rat cardiac membranes. Activity expressed as both pmoles cyclic AMP/min/mg protein and percentage stimulation above basal activity. Means ± SD from 18 separate membrane preparations are shown.

	ADENYLATE CYCLASE ACTIVITY	
	pmoles cAMP/min /mg protein	% Stimulation
Basal	223 ± 94	-
Isoprenaline (10^{-4}M)	450 ± 217	101 ± 16
Sodium Fluoride	572 ± 260	160 ± 24

These results show that there was high variability in the absolute levels of cyclic AMP produced when comparing different membrane preparations. The coefficient of variation (CV) within an experiment was routinely less than 5%, any sample with variable results (CV > 15%) was rejected. Also presented in Table 3.1 are the results were also expressed as percentage stimulation above basal levels. This reduced the variability between different membrane preparations. (-) Isoprenaline (0.1 mM) and sodium fluoride (10 mM) stimulated adenylate cyclase activity by 101% and 160% respectively.

Retrospective analysis of the results in Table 3.1 allowed the different membrane preparations to be divided into three temporal groups as shown in Table 3.2. The Sprague/Dawley rats, from which the cardiac membranes were prepared, were supplied by Bantin and Kingman in batches of twenty. There was a significant increase in the basal adenylate cyclase levels obtained in batches of rats received from May 1986 to July 1986, subsequently the basal adenylate cyclase activities were found to decrease to a value which was not significantly different from the original. Both (-) isoprenaline and sodium fluoride stimulation of adenylate cyclase were affected to the same extent as basal cyclase activity, as evidenced by no alteration in the percentage stimulation above basal levels. Variation in adenylate cyclase activity was not related to heart weight nor to differences in protein recovery from hearts.

TABLE 3.2 Temporal alterations in basal adenylate cyclase activity. Groups 1-3 correspond to different delivery batches of Sprague-Dawley rats. The number of different membrane preparations in which adenylate cyclase activity was assayed in each group is shown as the number of experiments.

Batch	Month	No. Experiments	Basal adenylate cyclase activity pmoles/min/mg protein
Group 1	March/April 1986	4	128 ± 25
Group 2	May/June/July 1986	9	286 ± 61**
Group 3	February/March 1987	5	134 ± 21

** $p < 0.001$ vs Group 1 and Group 3 (analysis of variance).

The dose / response relationship of (-)isoprenaline stimulation of adenylate cyclase was investigated (Fig 3.2). Maximal stimulation of adenylate cyclase was achieved with a concentration of 0.1 mM (-) isoprenaline. Graphical extrapolation revealed an EC_{50} for (-) isoprenaline stimulation of adenylate cyclase of 1 µM. Propanolol competitively inhibited isoprenaline induced stimulation of adenylate cyclase with a pA_2 of 8.2.

The effect of increasing concentrations of sodium fluoride on adenylate cyclase activity was investigated. From Fig 3.3 it can be seen that concentrations of sodium fluoride up to 10 mM were associated with a dose-dependent increase in adenylate cyclase activity. Higher concentrations of sodium fluoride decreased adenylate cyclase activity. This unusual profile of activation of adenylate cyclase has been observed by other authors in cardiac membranes (Leclerc et al., 1984). The aluminium-fluoride complex responsible for this stimulation is thought to be stimulatory by causing dissociation of the components of G_s.

The ability of (-) isoprenaline to stimulate adenylate cyclase in guinea-pig cardiac membranes, prepared as described in Chapter 2, was investigated. 0.1 mM (-) Isoprenaline stimulated adenylate cyclase activity by 74% ± 9 (n=3).

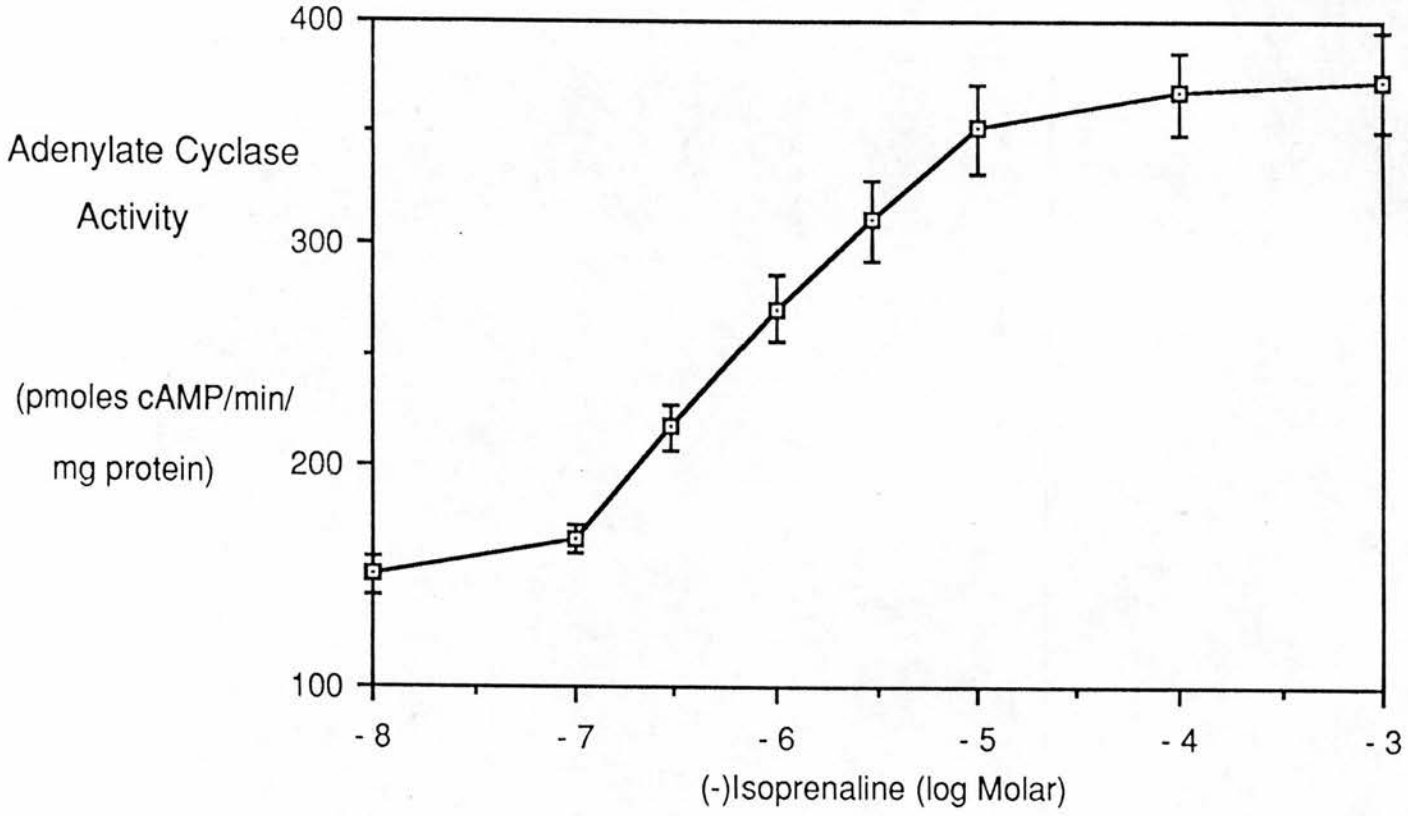

Fig 3.2 Dose-dependent (-) isoprenaline stimulation of adenylate cyclase in rat cardiac membranes. Half maximum stimulation was observed at ~1 μM and maximum stimulation was obtained with 0.1 mM (-) isoprenaline.

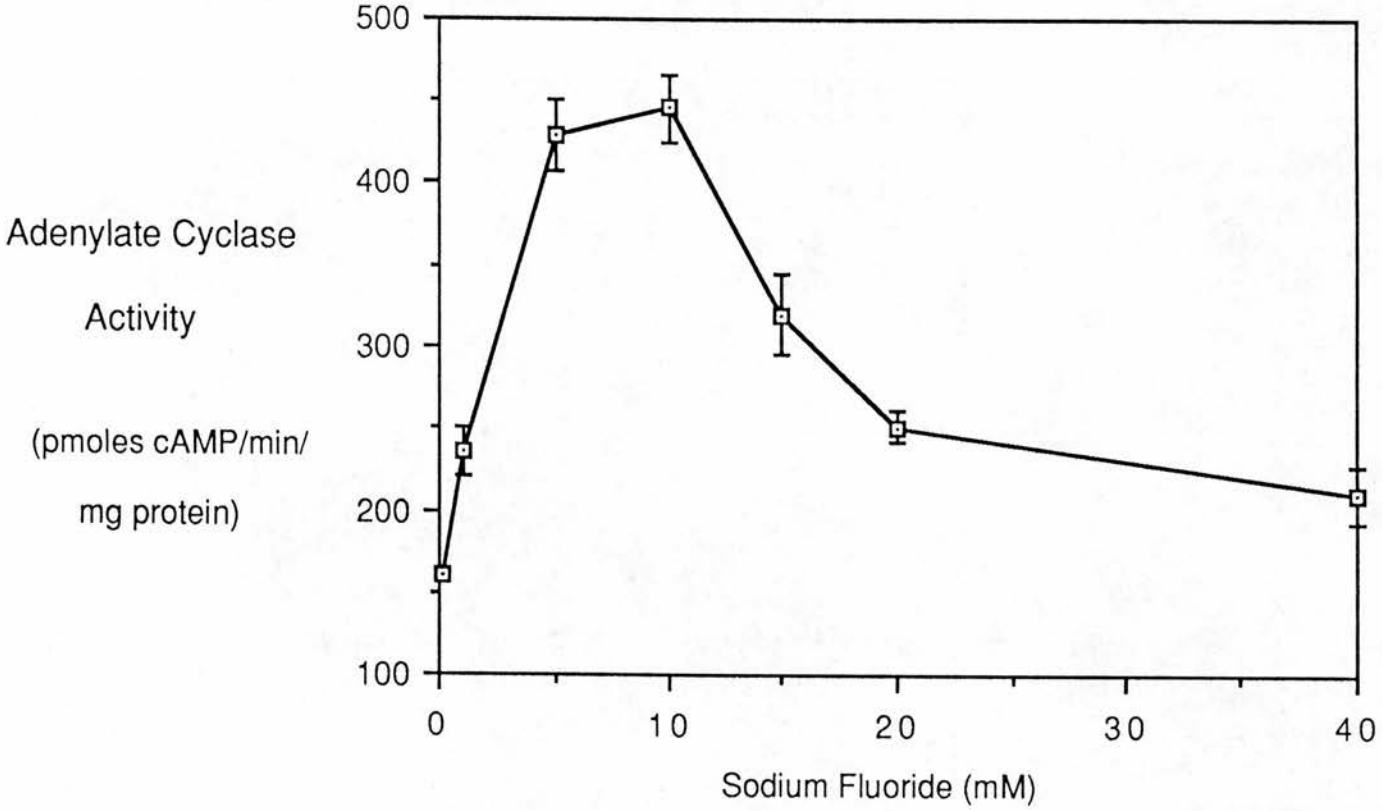

Fig 3.3 Stimulation of rat cardiac adenylate cyclase activity by sodium fluoride. Maximum stimulation of adenylate cyclase was obtained with 10 mM Na F.

3.1.2 Endogenous Catecholamines

The possibility of endogenous catecholamines from the membrane preparation causing artifically high basal adenylate cyclase activity in rat cardiac membranes was investigated. In the presence of propranolol (10^{-5}M) an insignificant reduction in basal adenylate cyclase activity was observed from 223 ± 94 to 216 ± 95 pmoles cyclic AMP/min/mg protein (data from 18 membrane preparations). Nevertheless, basal activity was routinely assayed in membranes in the presence of propranolol (10^{-5}M) to monitor effects of endogenous catecholamines in different membrane preparations.

3.2 LIGAND BINDING TO BETA ADRENERGIC BINDING SITES

In order to characterise beta adrenergic binding sites in rat cardiac membranes, binding of (-)-IPin to membranes was investigated.

3.2.1 Kinetics of (-)-IPin binding to rat heart membranes

The kinetics of (-)-IPin binding to rat cardiac membranes were measured as described in Chapter 2. Fig 3.4 shows specific binding of (-)-IPin to rat heart membranes and displacement of bound (-)-IPin with (-) isoprenaline (10 μM). Maximum specific binding was achieved by 15 minutes incubation with (-)-IPin. The level of non-specific binding obtained with this ligand was routinely less than 10% of the total binding.

The graphical determinations of the association rate constant (k_1) and the dissociation rate constant (k_{-1}) of (-)-IPin binding to rat cardiac membranes are shown in Fig 3.5. The mean determinations of k_1 and k_{-1} from 4 separate membrane preparations are summarised in Table 3.3. The association rate constant is limited mainly by simple diffusion of the ligand, whereas the dissociation rate constant reflects the affinity of the binding site for the ligand.

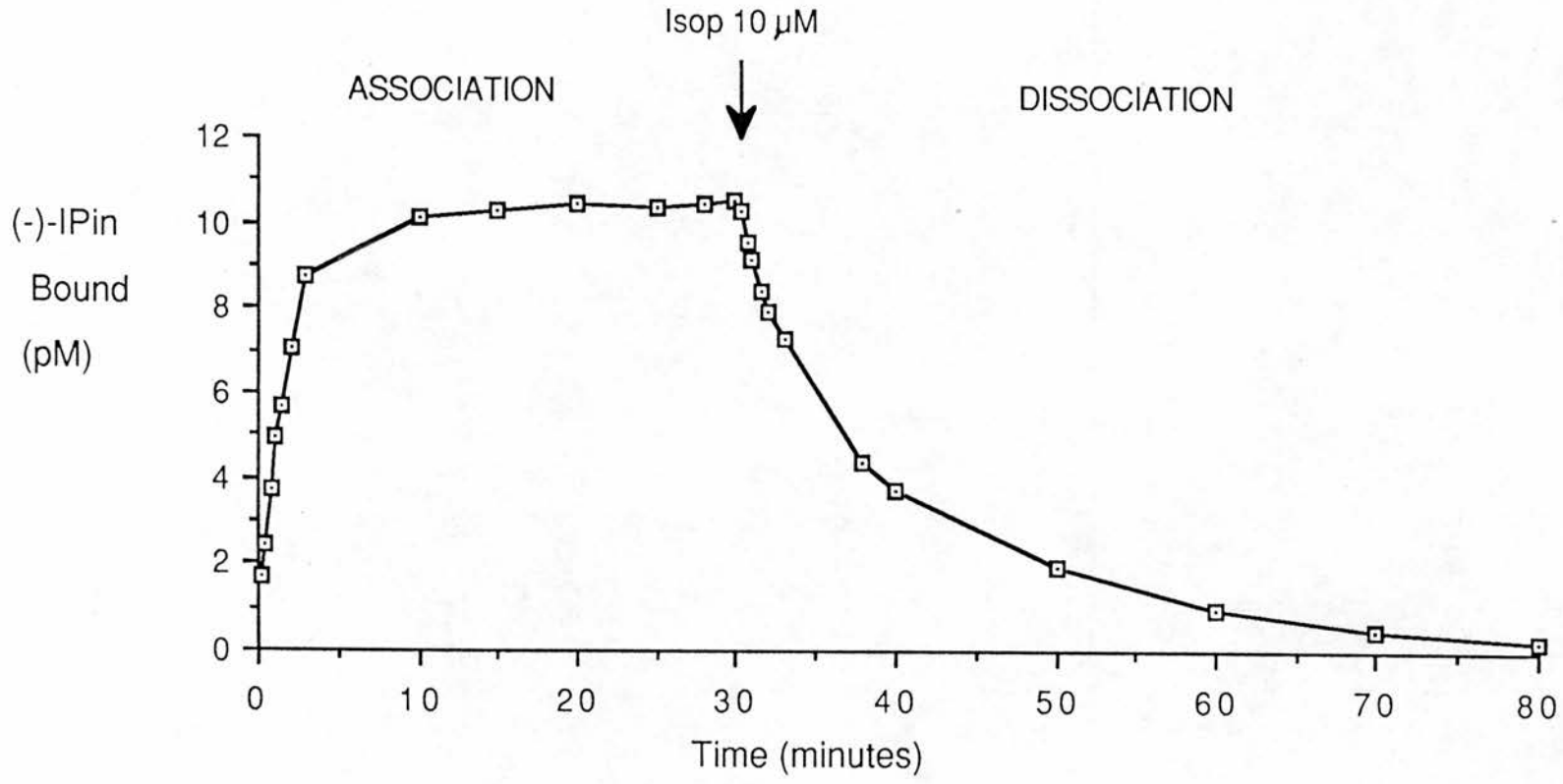

Fig 3.4 The kinetics of (-)-IPin binding to rat cardiac membranes. Specific binding of (-)-IPin (220 pM) to membranes (37°C) with time and the subsequent displacement of ligand in the presence of 10 μM (-) isoprenaline. Non-specifc binding was determined by a simultaneous separate incubation of ligand and membranes with (-) isoprenaline (10 μM). Duplicate determinations.

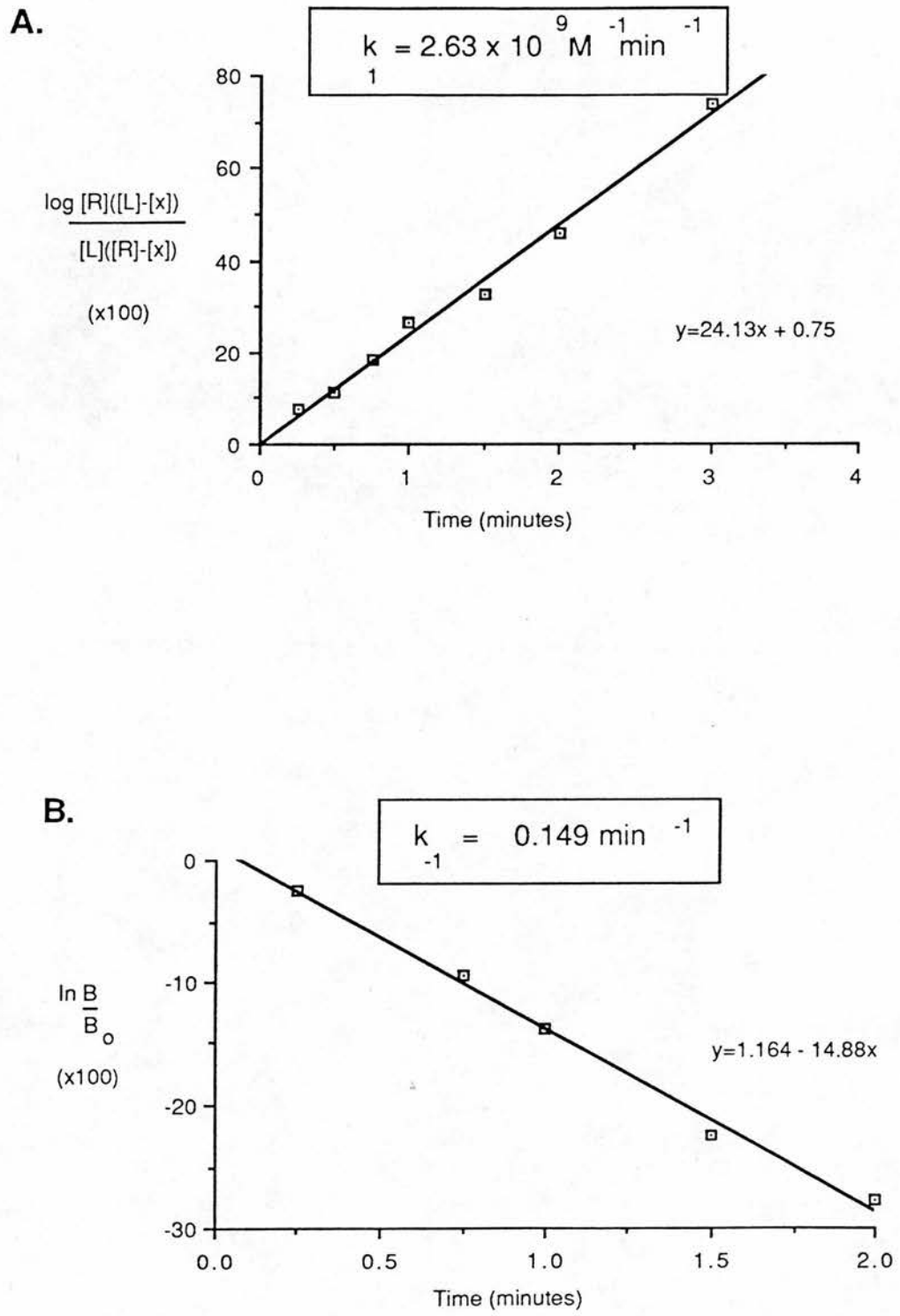

Fig 3.5 Calculation of kinetic parameters of the binding of (-)-IPin to rat cardiac membranes. A. Determination of the association rate constant (k_1) for data shown in Fig 3.4. B. Determination of the dissociation rate constant (k_{-1}).

TABLE 3.3 Kinetics of (-)-IPin binding to rat cardiac membranes. The calculated value of K_D is also shown.

k_1	$2.4 \pm 0.18 \times 10^9$ $M^{-1}min^{-1}$
k_{-1}	0.156 ± 0.011 min^{-1}
Calculated K_D	66 ± 4.6 pM

n=4 (each curve in duplicate).

3.2.2 Equilibrium binding studies

Equilibrium binding of (-)-IPin to rat cardiac membranes was also measured. Fig 3.6 shows total, specific and non-specific binding of (-)-IPin to rat cardiac membranes, from a representitive experiment. The specific binding of (-)-IPin to rat cardiac membranes was found to be saturable. Scatchard plots of the relationship between specifically bound (-)-IPin and the concentration of (-)-IPin were used to determine B_{max}, the density of binding sites in the membranes and K_D, the affinity of the binding sites for (-)-IPin (Fig 3.6). The mean B_{max} and K_D values determined from four separate membrane preparations are shown in Table 3.4.

TABLE 3.4 Equilibrium binding parameters for binding of (-)-IPin to rat cardiac membranes. Means $\pm$ SD from 4 separate membrane preparations.

K_D	102 ± 20 pM
B_{max}	18 ± 2.4 fmoles/mg protein

n=4

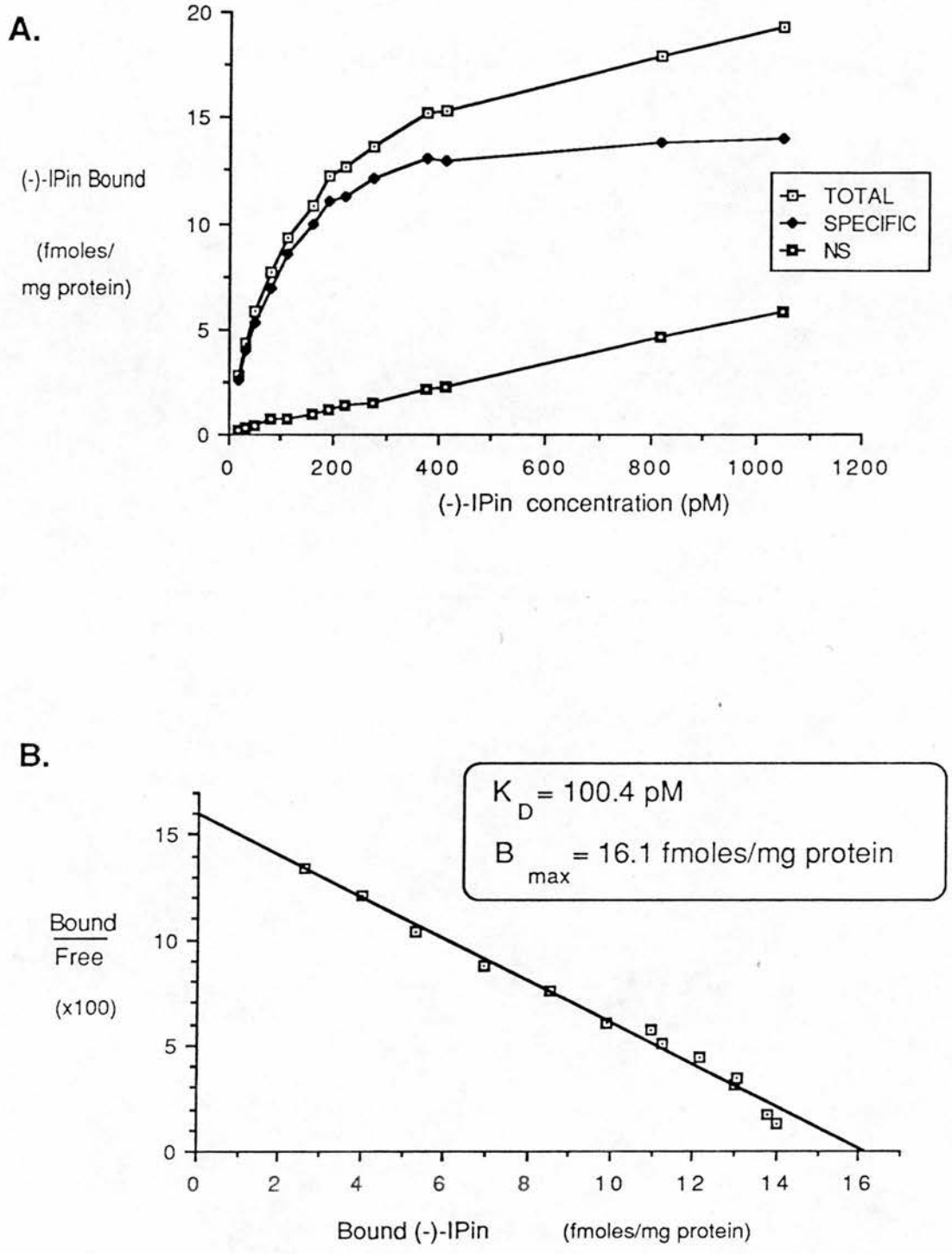

Fig 3.6 Binding of (-)-IPin to rat cardiac membranes. A. Shows the total, specific and non-specific binding of (-)-IPin to rat cardiac membranes. Thirteen different concentrations of (-)-IPin were used (19 -1050 pM). Non-specific binding was determined in the presence of (-) isoprenaline (10 μM). Specific binding was calculated as the difference between total and non-specific binding. All determinations were based on triplicate measurements. B. Scatchard plot of the above data, showing (-)-IPin specifically bound/free plotted against concentration of (-)-IPin bound. From the intercept on the abscissa Bmax was determined and from the negative reciprocal of the slope the value of K_D.

3.3 COUPLING OF RECEPTORS TO G_s

As previously mentioned an indication of the ability of beta adrenergic receptors to stimulate adenylate cyclase has been extrapolated from the ability of agonists to displace specifically bound ligand from receptors in the presence of GTP. Therefore the displacement of specific binding of (-)-IPin to cardiac membranes in the presence and absence of GTP was characterised. From Fig 3.7 it is apparent that no shift in the displacement curve was observed with crude cardiac membranes. In addition, no shift in the displacement curve was observed in the presence of the non-hydrolysable GTP analogue 5'-guanylylimidodiphosphate. In the presence of GTP (10^{-4}M) a 16% increase in B_{max} was observed (n=6, $p < 0.05$) but no shift in affinity.

3.4 LIGAND BINDING UNDER ADENYLATE CYCLASE ASSAY CONDITIONS

The possibility of looking at binding of (-)-IPin under the same conditions was investigated. From Chapter 2 it is apparent that a number of biochemicals, such as dithiothreitol (DTT), are required to measure adenylate cyclase activity. Initially, therefore, the effect of the adenylate cyclase assay components on the specific binding of (-)-IPin to rat cardiac membranes was investigated. Kinetic binding studies to rat cardiac membranes with (-)-IPin were carried out in the presence of the components required for the adenylate cyclase assay, excluding [^{32}P]-ATP and [^{3}H]-cyclic AMP. Specific binding was reduced to 30% of control specific binding under these conditions. Incubation of cardiac membranes in the presence of 1 mM DTT alone decreased specific binding of (-)-IPin by 50%.

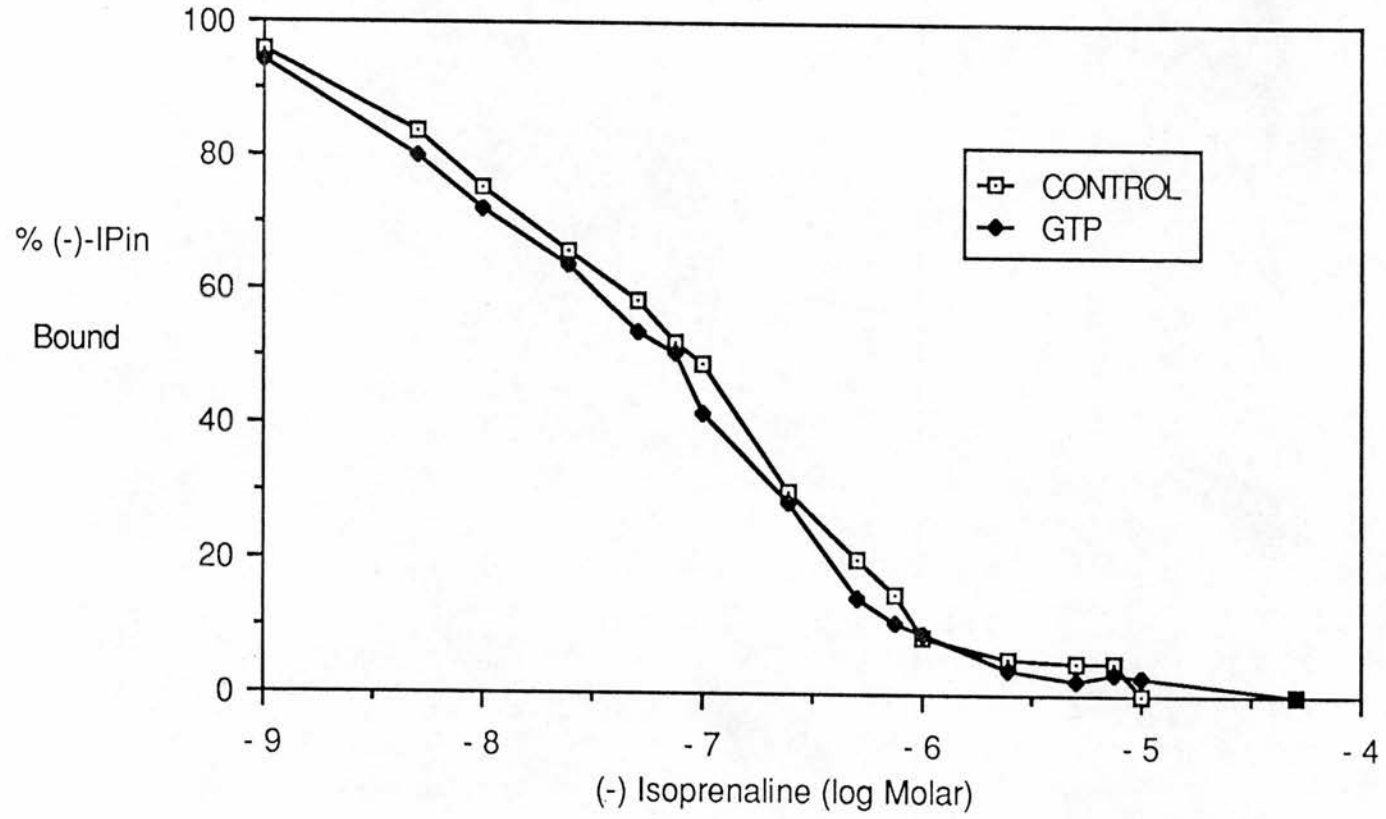

Fig 3.7 Displacement of specifically bound (-)-IPin from rat cardiac membranes by (-) isoprenaline in the presence and absence of 0.1 mM GTP.

3.5 DISCUSSION

The intial section of this chapter was concerned with investigating adenylate cyclase activity in rat cardiac membranes. It is clear that although the coefficient of variation for the measurement of basal, isoprenaline and sodium fluoride stimulated adenylate cyclase activity in any one preparation was reasonable, there was considerable variability between different membrane preparations. This was also observed by Golf et al. (1985) in human myocardial membranes. Similarily, expression of their results as a percentage of stimulation above basal activity reduced the variability. These authors could not discern any reason for the variability in the levels of cyclic AMP formed between different membrane preparations. The data presented here showed a temporal relationship with the level of adenylate cyclase activity which was related to different batches of rats. Adenylate cyclase activity is known to be altered by diet (Alam et al., 1987). Rats were purchased, therefore breeding and subsequent weanling rat diets could not be controlled. It would therefore be desirable to breed rats on the premises under ridgedly controlled conditions, although this was not possible for this project. There is the additional possibility that there is seasonal variation in adenylate cyclase activity; this requires further investigation. Due to the variation in adenylate cyclase activity between membrane preparations, results are expressed as a percentage of the maximum control response to sodium fluoride (10 mM) in all experiments comparing activity in different preparations. In addition control adenylate cyclase activity was characterised in all membrane preparations in order to carry out

paired comparisons.

In order to determine whether guniea-pig hearts would have been more suitable for investigating the beta adrenergic/adenylate cyclase system, isoprenaline stimulated adenylate cyclase in this species was measured. The presence of 0.1 mM (-) isoprenaline stimulated guinea-pig adenylate cyclase activity by 74% above basal levels, while rat cyclase was stimulated by 101%. These results suggest that rat myocardium is a better choice in which to study beta adrenergic function.

Propranolol did not significantly alter basal adenylate cyclase activity, suggesting that endogenous catecholamines have little effect on adenylate cyclase in these experiments. The value of measuring total catecholamine levels in myocardial membrane preparations is limited as has been shown by other workers in this field - there are disparate effects between measured catecholamines and their actual effects, this may be due to tightly bound endogenous catecholamines (Forfar and Riemersma, 1982; Nerme et al., 1985).

Binding of (-)-IPin to rat cardiac membranes was saturable, the K_D determined by kinetic analysis was lower than that dervived from equilibrium binding. There was no apparent effect of different batches of rats on ligand binding of (-)-IPin to rat cardiac membranes. A lower kinetic derived dissociation constant has been observed for adrenergic receptors by several authors (Forfar and Riemersma, 1982; Greenberg et al., 1978).

As previously described, the shift in agonist displacement curves of radioligand by GTP has been used as an indication of coupling between receptors and G_s. The data presented here show no effect of

GTP on agonist displacement curves although an increase in B_{max} was observed. It has been postulated that rat cardiac membranes bind endogenous catecholamines tightly at high affinity sites and that GTP destabilises these complexes allowing ligand to gain access to these sites, thereby increasing B_{max} (Nerme et al., 1985). The results presented here confirm the earlier finding of an increase in B_{max} in the presence of GTP and further suggest that tightly bound endogenous catecholamines may also interfere with the GTP shift of agonist displacement curves.

Ideally adenylate cyclase activity and the character of beta adrenergic binding sites should be characterised under identical conditions. Fressimuth et al. (1987) investigated the effect of ischaemia on both beta adrenergic receptors and adenylate cyclase in canine hearts. However, these authors looked at the receptors in a different membrane preparation to that used for adenylate cyclase activity. In these studies an attempt was made to characterise both adenylate cyclase and beta adrenergic receptors in the same membrane preparation and under identical conditions. This was not possible as it was found that the assay conditions dramatically reduced the specific binding of (-)-IPin to cardiac membranes. Sulphydrl reagents, such as N-ethylmaleimide (NEM) and 1,4-dithiothreitol (DTT) have been shown to effect binding of agonists and antagonists to several different receptors (Carman-Krzan, 1983). DTT (1 mM), as present in the cyclase assay, decreased the ability of (-)-IPin to bind to rat myocardial membranes. In addition, the other components of the assay (excluding DTT) still caused a reduction in specific binding of ~20%. It was therefore decided that binding studies should

be carried out as described in Chapter 2 because components of the adenylate cyclase assay interfered with specific binding of (-)-IPin.

The fact that DTT reduced specific binding of (-)-IPin to rat cardiac membranes has mechanistic implications for beta adrenergic receptors. Comparison of the deduced amino acid sequences of several beta adrenergic receptors, from different sources, has shown that there are ten conserved cysteine residues (Kerlavage et al., 1986). It seems that disulphide bridges and thiol groups may be important in the 'activation ' of beta adrenergic receptors; it has been proposed that the conserved cysteine residues may have a role in signal transduction to G_s (Malbon et al., 1987). These results suggest that an intact disulphide bridge is important in the binding of a beta adrenergic antagonist to cardiac beta adrenergic receptors. In contrast to the results presented here, no alteration in [^{3}H]DHA binding to $beta_1$ adrenergic receptors to turkey erythrocyte membranes, was observed in the presence of DTT (Vauquelin et al., 1980).

This preliminary characterisation of adenylate cyclase and beta adrenergic binding sites allowed subsequent investigation of the beta adrenergic/adenylate cyclase system in ischaemic rat myocardium.

CHAPTER 4

EFFECT OF ISCHAEMIA ON ADENYLATE CYCLASE ACTIVITY AND BETA ADRENERGIC BINDING

CHAPTER 4

Alterations in beta adrenergic function in myocardial ischaemia have become a focus of attention for several groups of researchers. From the introduction there seem to be contradictory observations, in that, most authors show increased numbers of beta adrenergic receptors for example Mukherjee et al. (1979), while both increases and decreases in adenylate cyclase activity have been observed, Maisel et al. (1985) and Will-Shahab et al. (1985) respectively. Actual levels of cyclic AMP in the ischaemic myocardium have been shown to increase (Wollenberger, 1969) but the the ability of (-) isoprenaline to promote protein phosphorylation declines rapidly in ischaemia (Krause and England, 1982). A problem with these studies is that in most cases adenylate cyclase activity and beta adrenergic receptors were not measured at the same time point of myocardial ischaemia or in the same tissue and very little attention has been given to acute as opposed to prolonged myocardial ischaemia. Therefore, it is still not clear how the ischaemic beta adrenergic/adenylate cyclase system in the myocardium responds to catecholamines, particularly in early ischaemia. In order to help clarify this area ligand binding and adenylate cyclase studies were carried out on membranes prepared from both acute and prolonged ischaemic rat myocardium.

4.1 MODEL OF MYOCARDIAL ISCHAEMIA

Most previous studies on the responsiveness of the beta adrenergic/adenylate cyclase system were carried out using in vivo models of myocardial ischaemia, such as coronary artery ligation which produces regional ischaemic areas. These studies may result in heterogeneous ischaemic regions in which there are areas of well perfused myocardium (Becker et al., 1973). This heterogenity may be due to collateral flow leading to variability in the degree of ischaemia from area to area and also between individual animals. This series of experiments sets out first to compare a more simple in vitro model of myocardial ischaemia with the previous in vivo findings. By investigating beta adrenergic function in an in vitro model of myocardial ischaemia the inherent variability of in vivo coronary artery occlusion is avoided. In the isolated heart uniform, total ischaemia can be investigated. The simplest model of in vitro ischaemia is adopted here, that of stop-flow global ischaemia.

Hearts were excised from male Sprague Dawley rats and placed in ice cold Krebs-Hensliet (glucose-free) solution containing heparin 10 U/ml. Ischaemia was induced by incubating hearts in a small volume of Krebs-Hensliet (glucose-free) solution, gassed with N_2/CO_2 (95%/5%), at $37^{\circ}C$ for the described time periods. Hearts were then removed, cooled and membranes immediately prepared as described in Chapter 2. Control hearts were kept on ice for the duration of the ischaemic period. Membranes were prepared simultaneously from ischaemic and control hearts. Adenylate cyclase activity and beta adrenergic binding sites were then investigated in these membranes.

4.2 EFFECT OF PROLONGED ISCHAEMIA ON BETA ADRENERGIC FUNCTION

At about 60 minutes myocardial ischaemia the myocardium becomes irreversibly damaged (Jennings et al., 1960). This is therefore an important stage of myocardial ischaemia to study with respect to beta adrenergic function. The effect of a prolonged period of myocardial ischaemia (60 minutes) on both adenylate cyclase activity and beta adrenergic binding sites was investigated in membranes prepared from control and globally ischaemic rat myocardium.

4.2.1 Adenylate cyclase activity in prolonged ischaemia

Prolonged ischaemia, of 60 minutes, produced a significant reduction in adenylate cyclase activity in rat myocardial membranes (Fig 4.1). The decrease in adenylate cyclase activity was found in both stimulated and basal adenylate cyclase. Table 4.1 expresses numerically the decline in adenylate cyclase activity in myocardial membranes following 60 minutes of myocardial ischaemia. A period of myocardial ischaemia of 60 minutes has a marked effect on adenylate cyclase activity - reducing all activities by approximately 76%.

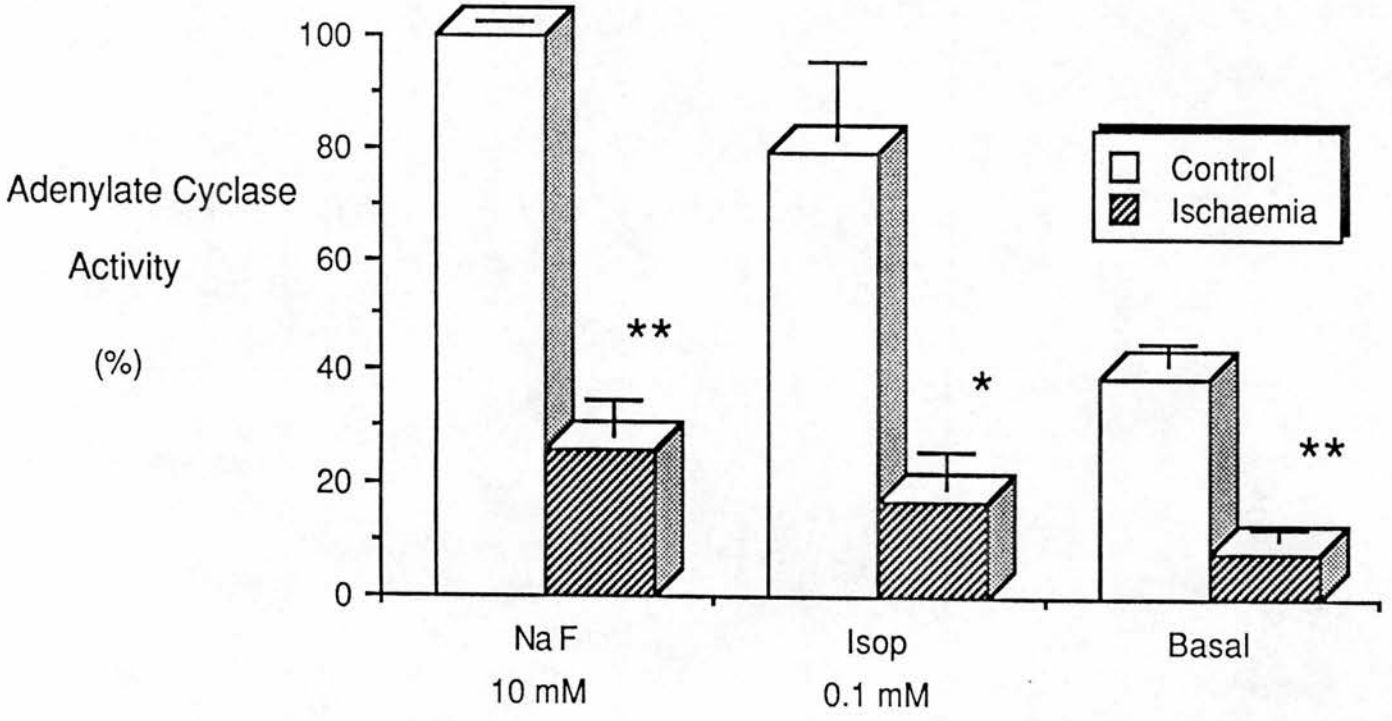

Fig 4.1 Effect of prolonged ischaemia (60 minutes) on basal and stimulated adenylate cyclase activity in rat cardiac membranes. Results expressed as a percentage of control sodium fluoride stimulation. (n=4).

** $p < 0.001$, * $p < 0.005$, vs the appropriate control.

TABLE 4.1 Effect of 60 minutes of ischaemia on adenylate cyclase activity. Results expressed as a percentage of control sodium fluoride (10 mM) activity. Basal and (-) isoprenaline (0.1 mM) stimulated adenylate cyclase activities are also shown.

	ADENYLATE CYCLASE ACTIVITY %		
	Na F	Isop	Basal
Control	100	79.2 ± 13.9	39.2 ± 3.4
Ischaemia	25.6 ± 6.0	17.2 ± 5	8.3 ± 1.6
% Decline in activity	74.4**	78.3*	78.8**

n=4 ** $P < 0.001$ vs appropiate control.
* $P < 0.005$

The effect of 60 minutes myocardial ischaemia on the dose/response of (-)isoprenaline stimulation of adenylate cyclase activity was investigated. A representitive experiment is shown in Fig 4.2, a decline in the maximum response to (-) isoprenaline from 331 to 58 pmoles/min/mg protein was observed. The EC_{50} was not altered by prolonged myocardial ischaemia.

4.2.2 Beta adrenergic receptors and prolonged ischaemia

The density and affinity of beta adrenergic binding sites under ischaemic conditions were measured as described in Chapter 3. Figure 4.3 shows a representitive Scatchard plot obtained after 60 minutes global myocardial ischaemia compared with the relevant control Scatchard plot. Table 4.2 summarises the mean results from three experiments.

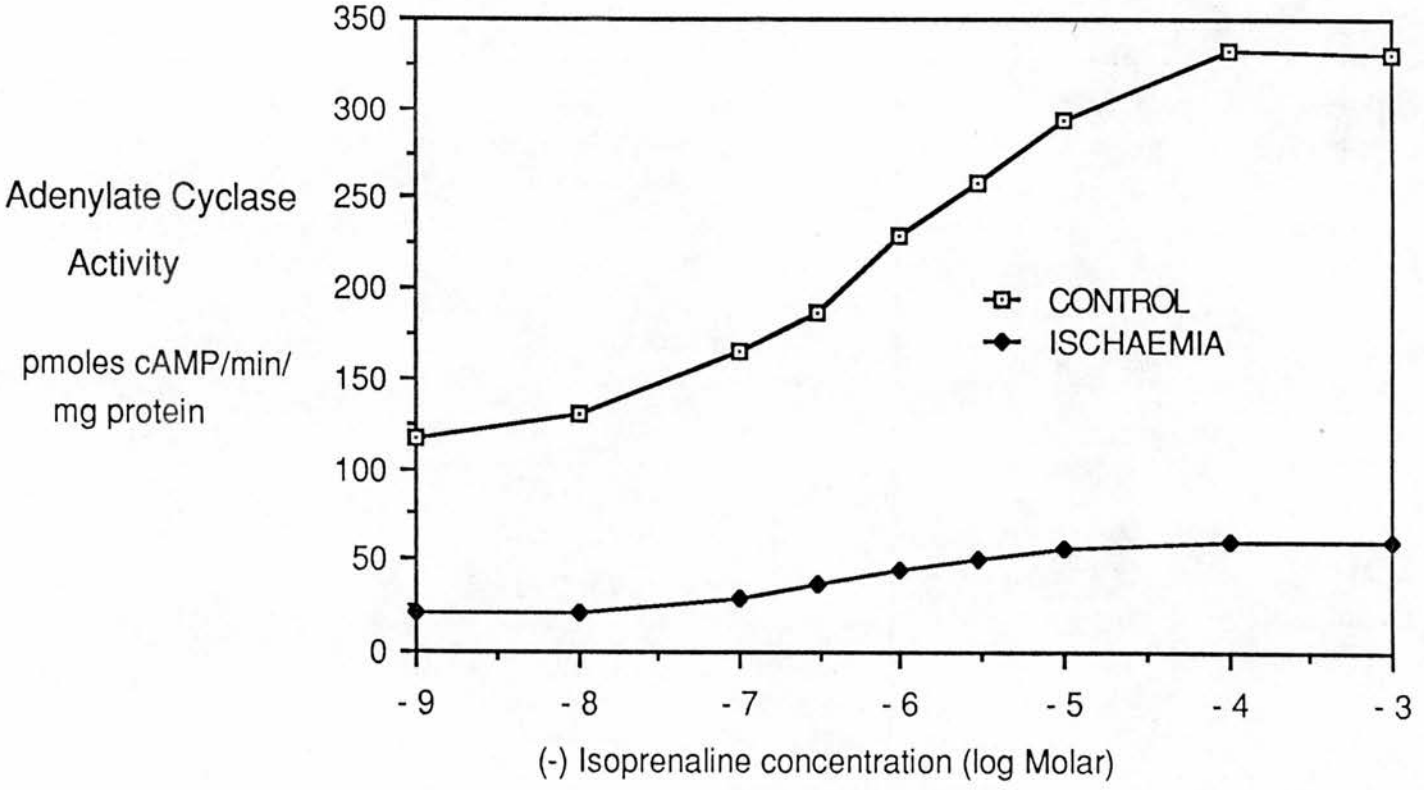

Fig 4.2 Dose-response of (-) isoprenaline stimulation of adenylate cyclase in control and ischaemic (60 minutes)rat cardiac membranes. Results are means of quadruplicate determinations.

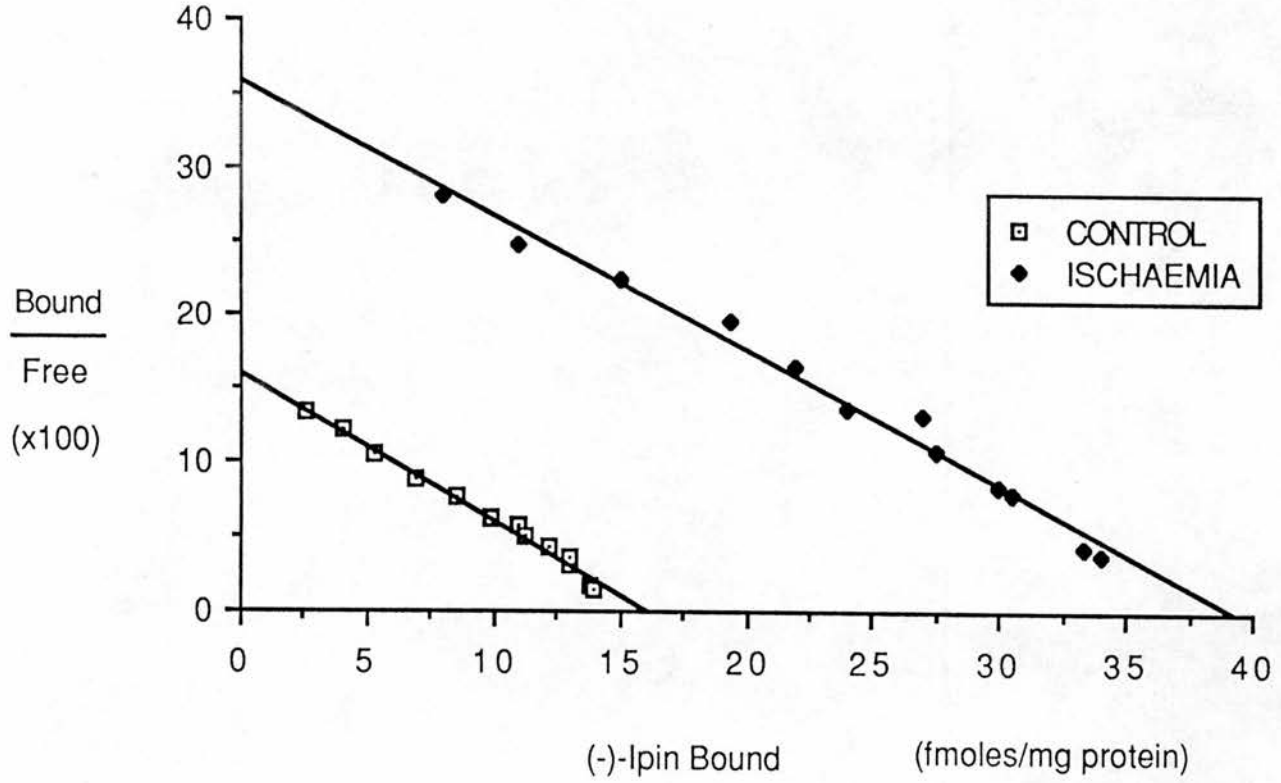

Fig 4.3 Scatchard plots of (-)-IPin binding to control and ischaemic (60 minutes) rat cardiac membranes. The calculated values of B_{max} in control and ischaemic tissue were 16.1 and 39.2 fmoles/mg protein, the K_D values were 101 and 109 pM respectively.

TABLE 4.2 Effect of 60 minutes ischaemia on beta adrenergic binding sites. The calculated equilibrium binding parameters B_{max} and K_D are shown.

	Bmax fmoles/mg protein	Kd pM
Control	19.1 ± 1.8	102 ± 23
Ischaemia	42.5 ± 7.3	114 ± 4

n=3

Prolonged ischaemia caused an increase in the density of beta adrenergic binding sites from 19.1 to 42.5 fmoles/mg protein, this is a 122% increase in the number of binding sites. There was no alteration in the affinity of the binding sites for (-)-IPin.

4.3 EFFECT OF ACUTE ISCHAEMIA ON BETA ADRENERGIC FUNCTION

In investigating the role of catecholamines and beta adrenergic function in the genesis of serious myocardial arrhythmias, it is important to study the acute phase of myocardial ischaemia. Ventricular fibrillation is most likely to occur during the first 15-30 minutes of myocardial ischaemia (Janse, 1982). Therefore, in order to characterise beta adrenergic function at a time when serious ventricular arrhythmias occur a time of 15 minutes of ischaemia was investigated.

4.3.1 Effect of acute ischaemia on adenylate cyclase activity

As in the previous section membranes were prepared from control and ischaemic hearts, but in this case after 15 minutes of myocardial ischaemia. Figure 4.4 shows the effect of 15 minutes global stop-flow ischaemia on adenylate cyclase activity. The percentage decline, some 50%, in adenylate cyclase activity was less than that observed after prolonged ischaemia (Table 4.3). There was no significant difference in the degree of reduced activity between basal and sodium fluoride and isoprenaline stimulated adenylate cyclase.

TABLE 4.3 Effect of 15 minutes of myocardial ischaemia on adenylate cyclase activity. Results are expressed as a percentage of control sodium fluoride (10 mM) activity. Basal and (-) isoprenaline (0.1 mM) stimulated activity are also shown.

	ADENYLATE CYCLASE ACTIVITY %		
	Na F	Isop	Basal
Control	100	75.6 ± 5.0	43.4 ± 4.1
Ischaemia	48.9 ± 6.7	36.2 ± 5.6	20.2 ± 4.7
% Decline in activity	51.1**	52.1**	53.5*

n=4, ** $p < 0.001$ vs the appropriate control.
* $p < 0.005$

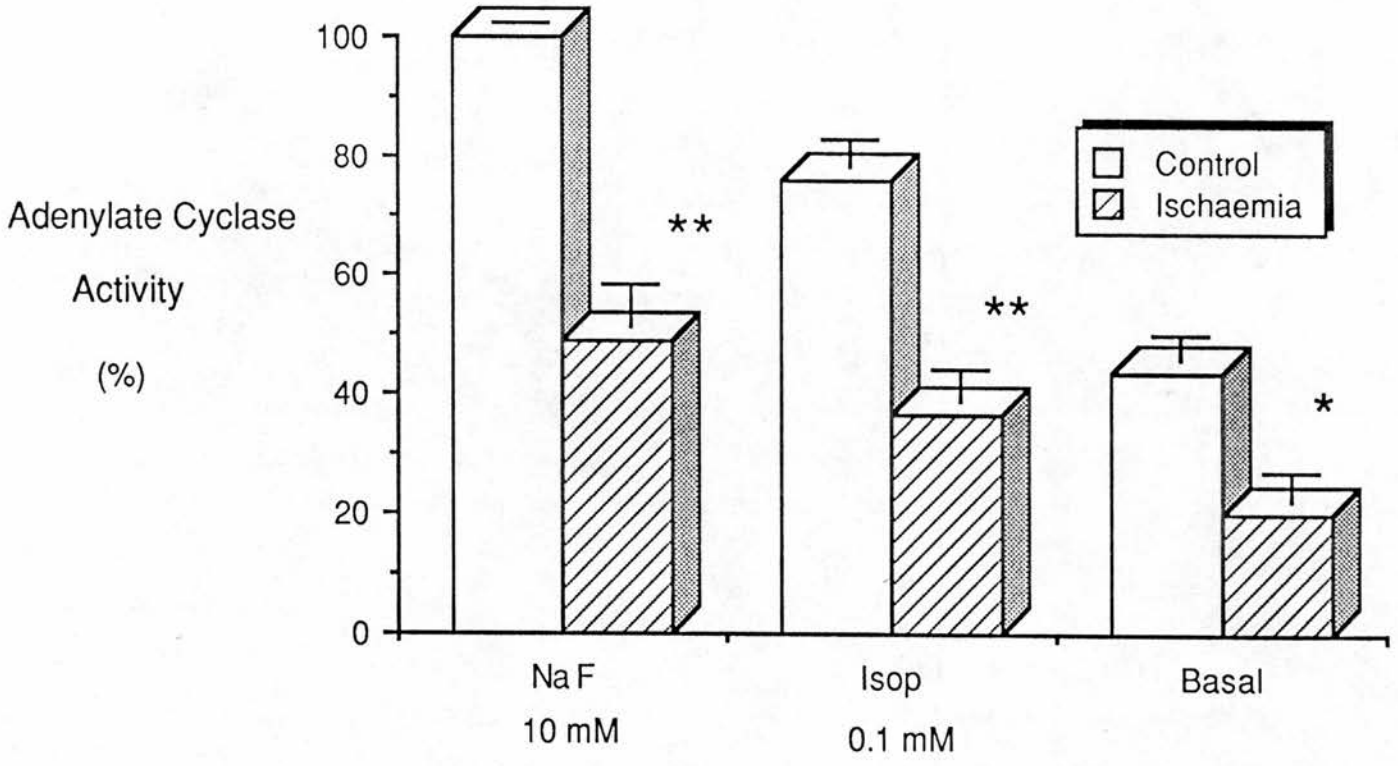

Fig 4.4 Effect of acute myocardial ischaemia (15 minutes) on basal and stimulated adenylate cyclase in rat cardiac membranes. Results are expressed as a percentage of control sodium fluoride stimulation. (n=4)

** $p < 0.001$, * $p < 0.005$, vs the appropriate control.

The maximum adenylate cyclase response of the acutely ischaemic myocardium to (-) isoprenaline was reduced with no alteration in EC_{50} apparent, data not shown.

4.3.2 Effect of acute ischaemia on beta adrenergic binding sites

The effect of 15 minutes of myocardial ischaemia on (-)-IPin binding to myocardial membranes was also investigated. The results are summarised in Table 4.4. An increase from 18.7 to 29.6 fmoles/mg protein in the density of beta adrenergic binding sites was observed, that is, a 47.6% increase after 15 minutes of myocardial ischaemia. There was no alteration in the affinity of the binding sites for (-)-IPin.

TABLE 4.4 Effect of 15 minutes ischaemia on specific binding of (-)-IPin to rat cardiac membranes. The calculated equilibrium binding parameters B_{max} and K_d are shown.

	Bmax fmoles/mg protein	KD pM
Control	18.7 ± 2.5	114.7 ± 5.9
Ischaemia	29.6 ± 1.3*	116.7 ± 11.8

n=3, * $p < 0.05$ vs control.

4.4 DISCUSSION

Prolonged myocardial ischaemia (60 minutes) was associated with a decline in adenylate cyclase activity of about 76%. The density of beta adrenergic binding sites was increased by 122%. The fall in adenylate cyclase activity was not restricted to (-) isoprenaline stimulated adenylate cyclase; both basal and sodium fluoride stimulated adenylate cyclase activity were reduced to a similar degree. These results obtained in the same membranes demonstrate that after prolonged ischaemia the responsiveness of the beta adrenergic/adenylate cyclase system to catecholamines is reduced despite an increase in the number of beta adrenergic binding sites. In addition, the results show that the in vitro global ischaemia model yields results in agreement with the majority of researchers in this controversial field (Table 1.1 and Table 1.2). The results looking at prolonged ischaemia therefore provide a sound basis for the subsequent characterisation of acute myocardial ischaemia. Acute myocardial ischaemia (15 minutes) was associated with a 50% decline in adenylate cyclase activity while the density of beta adrenergic binding sites was increased by 48%. Therefore, at a time of myocardial ischaemia when ventricular fibrillation is occurring there is actually reduced responsiveness of adenylate cyclase to catecholamines, although this reduction is not as severe as observed in prolonged myocardial ischaemia.

The reduction in adenylate cyclase activity was found in all cyclase activities; basal, isoprenaline and sodium fluoride stimulation. This information suggests that a stage subsequent to the

beta adrenergic receptor is altered, that is, probably at the level of adenylate cyclase, however the function of G_s may also be modified by myocardial ischaemia.

There are, however, several authors who have found that adenylate cyclase activity is not increased in myocardial ischaemia (Mori, 1976; Maisel et al., 1985; Strasser 1988, personal communication[#]). Mori (1976) has not published sufficient details to allow full interpretation of his adenylate cyclase results. Adenylate cyclase activity was found to be increased in membranes prepared from in vitro perfused rat hearts, rendered ischaemic (15 minutes) by ligation of the left anterior descending coronary artery (Strasser 1988, personal communication). However this work is as yet unpublished therefore it cannot be fully discussed here.

Maisel et al. (1985) have suggested that their findings of increased numbers of beta adrenergic receptors, at 30 minutes of myocardial ischaemia, and increased adenylate cyclase activity, at 60 minutes of myocardial ischaemia[*], demonstrates an enhanced sensitivity of the ischaemic myocardium to catecholamines (Fig 4.5). They suggest that externalization of beta adrenergic receptors may be important in arrhythmogenesis.

Oral presentation by Dr R Strasser, Adrenergic System and Ventricular Arrhythmias in Myocardial Infarction, International Workshop, Heidelberg 1988.

* Maisel et al. (1985) did not publish the period of ischaemia after which the alterations in adenylate cyclase activity occurred, however subsequently the authors have stated that adenylate cyclase activity was found to be increased after one hour of ischaemia (Insel, Personal communication, 1988).

There are, however, several problems with this work. The model of ischaemia employed was that of coronary ligation in guinea-pig heart, which is, an unsatisfactory model of myocardial ischaemia as coronary artery ligation in this species does not produce a reliably ischaemic area, due to well-developed collateral circulation (Johns and Olson, 1954; Maxwell et al., 1984). Furthermore, as was demonstrated in Chapter 3, this species is not one of choice in which to investigate isoprenaline stimulated adenylate cyclase. In fact, in control hearts these authors observed only 22% stimulation of adenylate cyclase activity, above basal levels, in the presence of isoprenaline (Fig 4.5). In view of the above criticisms these results must be interpreted carefully.

Adenylate cyclase is not the only enzyme activity known to be altered in early ischaemia. The function of other sarcolemmal enzymes is also altered, Na/K ATPase activity and Na-Ca exchange are decreased in acute myocardial ischaemia (Suyatna et al. 1988). The cause for the decline in adenylate cyclase activity in myocardial ischaemia is not clear. As discussed in Chapter 1 there are complex alterations occurring within the ischaemic myocardium; such as a rapid fall in pH, accumulation of metabolities and breakdown of normal ion homeostasis. In prolonged ischaemia it is reasonable to assume that adenylate cyclase may be irreversibily damaged and therefore, that preparation of membranes and assay of the enzyme under conditions of normal pH and high levels of ATP will not reverse the damage caused by ischaemia. However, the same assumption may not necessarily be true for the acutely ischaemic myocardium, in that there could be alterations in the activity of the enzyme in vivo which are reversed

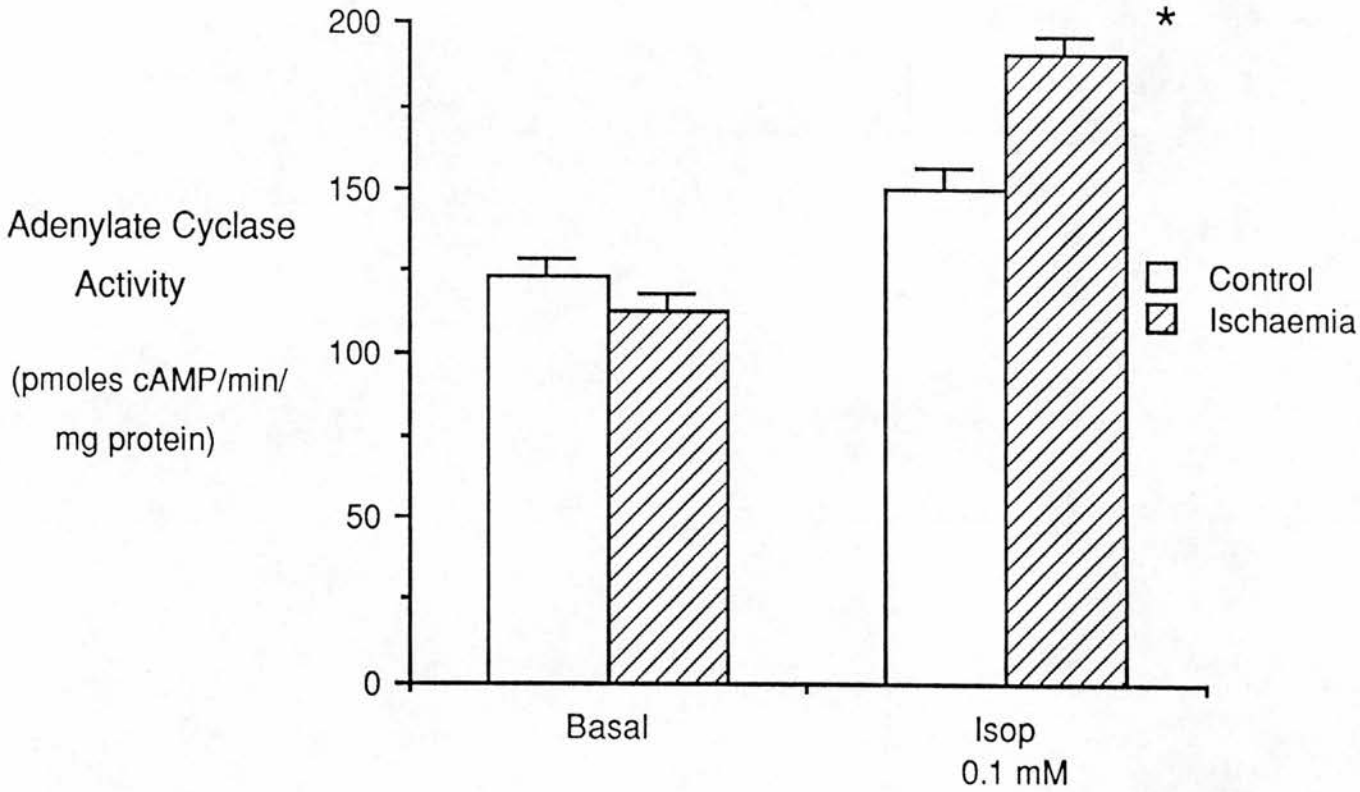

Fig 4.5 Effect of ischaemia (60 minutes) on adenylate cyclase activity in guinea-pig cardiac membranes. Data from Maisel et al. (1985) Science 230: 183-186. (n=3), * $p < 0.003$, vs isoprenaline control.

by washing out the ischaemic environment and assaying the enzyme under normal conditions. The results presented in this chapter do show essentially 'irreversible' damage to adenylate cyclase by prolonged myocardial ischaemia. Acute myocardial ischaemia was also associated with 'irreversible' damage to adenylate cyclase. However, it is possible that much information on the function of adenylate cyclase and therefore its responsiveness to catecholamines in vivo is lost by preparation and assay of ischaemic membranes under normal conditions. This possibility is investigated in Chapter 5.

The results presented here show that there is an increase in the number of beta adrenergic binding sites in both acute and prolonged myocardial ischaemia but as discussed earlier the responsiveness of adenylate cyclase to catecholamines is reduced. These results illustrate the danger in interpretating data solely on the basis of ligand binding studies. Several authors in various species and models of myocardial ischaemia have now shown increases in the number of beta adrenergic binding sites in myocardial ischaemia (Table 1.1).

Whether or not the ischaemic alterations are specific to beta adrenergic receptors has been investigated. Muscarinic receptors in canine myocardium remained unaltered at any time point of ischaemia, while the number of beta adrenergic binding sites were increased (Mukherjee et al., 1979). Alpha adrenergic binding sites are increased in feline myocardium after 30 minutes of global ischaemia (Dillon et al., 1988) and after in vivo occlusion of the left anterior descending coronary artery (Corr et al., 1981). In contrast, alpha adrenergic binding sites were unaltered in globally ischaemic rat myocardium (Dillon et al., 1988).

The mechanism of the increase in number of beta adrenergic binding sites in ischaemia is uncertain. Several possibilities have been proposed. There may be an increased externalization of beta adrenergic receptors in ischaemia (Maisel et al., 1985). It is also conceivable that there is decreased internalization of beta adrenergic receptors, as has been demonstrated to occur during impaired energy metabolism (Buja et al., 1985). Nokin et al., (1983) have shown that propranolol is able to reduce the increase in beta adrenergic binding sites measured in myocardial ischaemia. Ischaemia may also unmask receptors already present in membranes; phospholipid methylation unmasks cryptic beta adrenergic sites in rat reticulocytes (Strittamatter et al., 1979).

It is possible that membranes prepared from ischaemic myocardium have altered protein recoveries. Several authors have investigated this by comparing the activity of membrane markers in ischaemic membranes and control membranes. The activity of 5'-nucleotidase has been found to be unaltered by five hours myocardial ischaemia (Nokin et al., 1983). The total recoveries of protein from ischaemic and non-ischaemic membranes have been reported as similar, but there is still the possiblity that total protein remains unaltered but that there is differential recovery of particular proteins.

In summary, these experiments show that both prolonged and acute myocardial ischaemia are associated with a reduced responsiveness to catecholamines despite an increase in the number of beta adrenergic binding sites.

CHAPTER 5

THE ISCHAEMIC ENVIRONMENT AND BETA ADRENERGIC FUNCTION

CHAPTER 5

In the previous chapter the activity of adenylate cyclase and the character of beta adrenergic binding sites in membranes prepared from ischaemic tissue were investigated. This approach involves the ischaemic membranes being assayed under 'normal' conditions, therefore it is likely that some of the in vivo changes (accumulation of H^+, lactate, adenosine etc.) are normalized during the preparation of the membranes and by assaying the enzyme and binding sites under normal conditions. In order to obtain a clearer picture of beta adrenergic function during acute myocardial ischaemia the effect of several factors which contribute to the early ischaemic environment on normal cardiac membranes were individually investigated.

5.1 pH AND MYOCARDIAL ISCHAEMIA

As discussed in Chapter 1 there is a rapid decline in myocardial pH in acute myocardial ischaemia. The decline in pH may be of importance in considering beta adrenergic function under ischaemic conditions, therefore the effect of lowering the pH of control membranes from pH 7.5 to pH 6.8 on both adenylate cyclase and beta adrenergic receptors was investigated.

5.1.1 pH and adenylate cyclase

The effect of lowering pH from 7.5 to 6.8 was investigated in rat cardiac membranes. Cardiac membranes were prepared as described in

Chapter 2 from the same rat hearts, but the final pellets were resuspended in Tris.HCl at either pH 7.5 or pH 6.8 and subsequently assayed at the appropriate pH.

Reduction of pH from 7.5 to pH 6.8 caused a significant reduction in both basal and stimulated adenylate cyclase (Fig 5.1). Basal rat cardiac adenylate cyclase at pH 6.8 was reduced by 73.5% compared to the activity at pH7.4, while (-)-isoprenaline (0.1 mM) and sodium fluoride (10 mM) were reduced 75.7% and 76.3% respectively, (there was no significant difference between the percentage reductions observed). The dose-response relationship of stimulation of adenylate cyclase by (-) isoprenaline was investigated at pH 7.5 and pH 6.8. The EC_{50} was unaltered but the maximum response to (-) isoprenaline was reduced at pH 6.8.

In order to examine the nature of this pH dependent effect on adenylate cyclase, it was of interest to examine whether the observed reduction in activity was reversible. Three aliquots of rat cardiac membranes were incubated at 37°C for 5 minutes, one at pH 7.5 and two at pH 6.8. After cooling the membranes on ice, the pH of one aliquot was brought up to pH 7.5 with Tris and the membranes immediately assayed for adenylate cyclase activity.

These experiments confirmed the earlier observation: reducing pH to 6.8 reduced adenylate cyclase activity to 25% of the control (Fig 5.2). Reversing the pH from 6.8 to 7.5 leads to a partial recovery of adenylate cyclase activity; all activities are restored to ~ 60% of the corresponding control activity (Table 5.1).

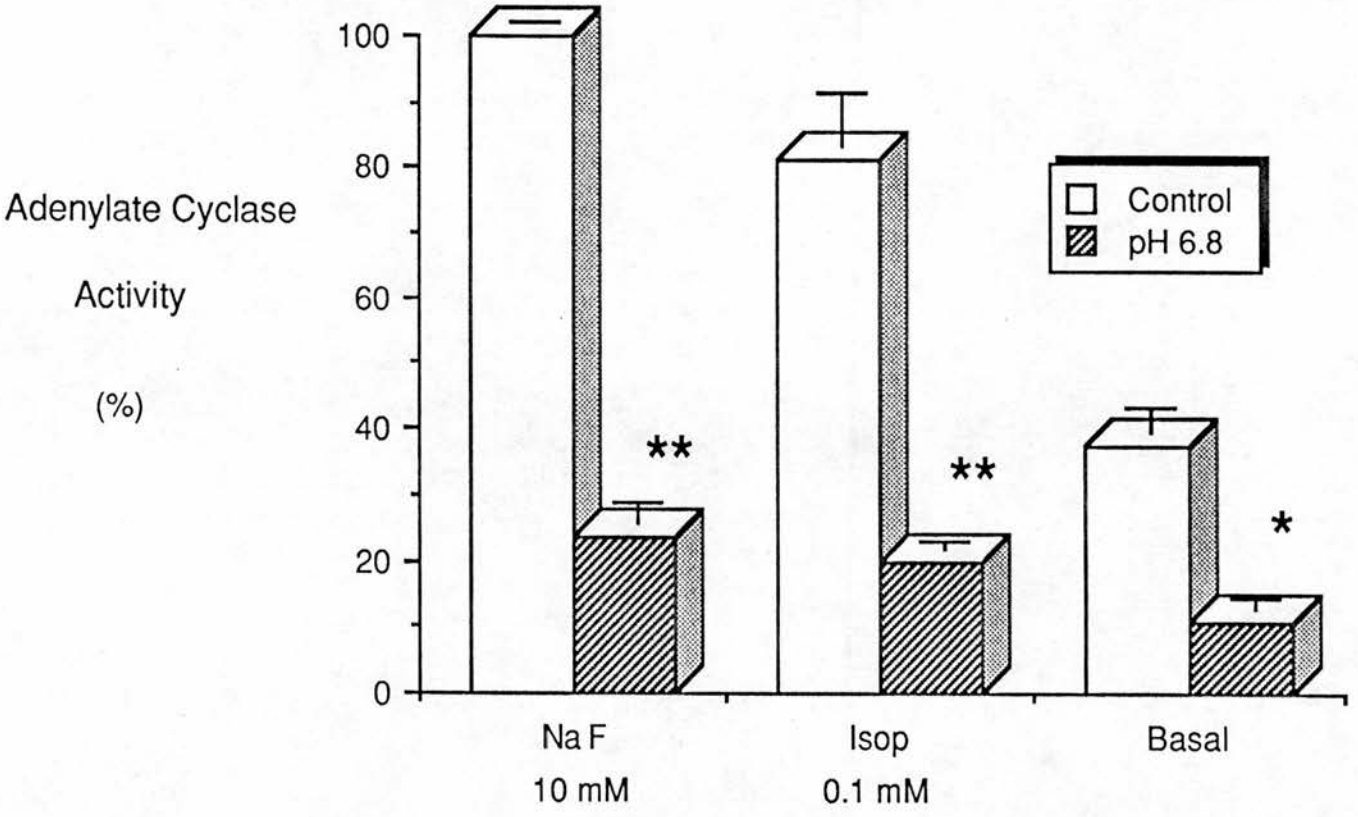

Fig 5.1 Effect of ischaemic pH (6.8) on the activity of adenylate cyclase in rat cardiac membranes. Results are expressed as a percentage of control sodium fluoride stimulation. (n=4).

** $p < 0.005$, * $p < 0.01$, vs the appropriate control.

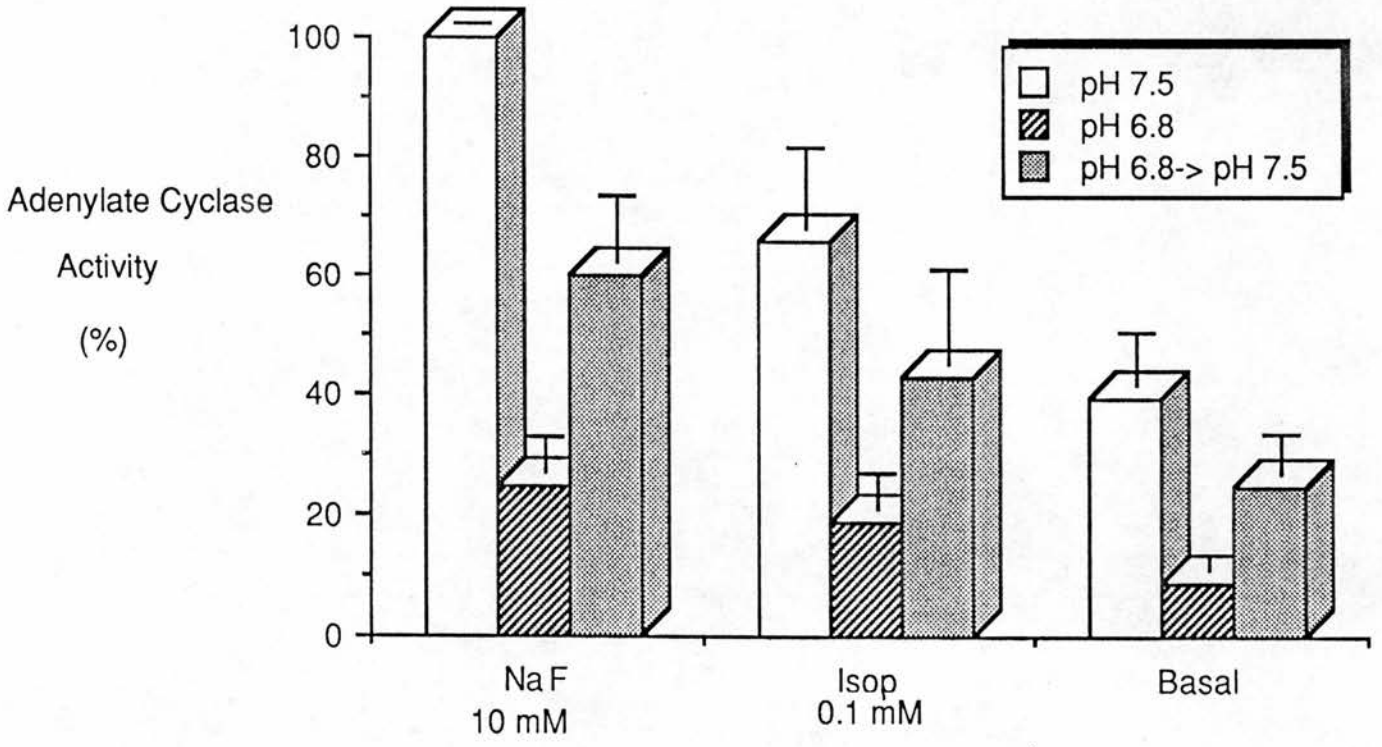

Fig 5.2 Reversibility of the effect of pH 6.8 on adenylate cyclase activity in rat cardiac membranes. Results are expressed as a percentage of control sodium fluoride stimulation. (n=3).

TABLE 5.1 Reversal of the pH effect on adenylate cyclase activity. Results are expressed as a percentage of control sodium fluoride (10 mM) activity at pH 7.5. The effect on basal and (-) isoprenaline (0.1 mM) stimulated activities are also shown.

	% ADENYLATE CYCLASE ACTIVITY		
	Na F	Isop	Basal
Control (pH 7.5)	100	66.0 ± 13.3	39.3 ± 9
pH 6.8	24.9 ± 5.8	19.1 ± 5.4	9.0 ± 1.9
pH 6.8 —> pH 7.5	60.0 ± 11.0	43.2 ± 15.6	24.9 ± 6.5
% Reversal	60.0%	65.0%	63.3%

(n=3)

5.1.2 pH and beta adrenergic binding sites

The kinetics and equilibrium binding of membranes under pH 7.4 and pH 6.8 were investigated. A decrease in the affinity of rat cardiac membranes at pH 6.8 for the ligand (-)-IPin was observed, K_D increased from 119 ± 14 pM to 241 ± 12 pM at pH 6.8. Ischaemic pH decreased B_{max} by 17% (n=3). The observed effects of (-)-IPin binding to rat cardiac membranes at pH 6.8 were independent of the anion used to adjust the pH to 6.8; hydrochloric, nitric and lactic acids produced effects of the same magnitude. These results suggest that the increase in hydrogen ion concentration caused a decrease in B_{max} and an increase in the value of K_D of (-)-IPin binding to rat cardiac membranes.

5.2 ADENOSINE AND MYOCARDIAL ISCHAEMIA

Within the first 2 minutes of myocardial ischaemia the levels of adenosine have been shown to increase in rat myocardium (Meghji et al., 1988). There is also increasing evidence that adenosine exerts anti-adrenergic effects in the myocardium through binding to A_1 adenosine receptors which lead to inhibition of adenylate cyclase (Henrich et al., 1987). Adenosine is formed during the time-course of adenylate cyclase assays from the breakdown of ATP, this therefore complicates investigation of the effect of adenosine on adenylate cyclase activity. In order to show the inhibitory effect of adenosine, investigators have included the enzyme adenosine deaminase to convert adenosine to inosine, thereby unmasking the inhibitory effect of adenosine (Hopwood et al., 1985).

The effect of inclusion of adenosine deaminase in the adenylate cyclase assay on the activity of control rat cardiac membranes was investigated. Adenylate cyclase activity was increased in the presence of 1U/ml adenosine deaminase (Fig 5.3); basal activity was increased by 23.6%, while (-) isoprenaline (0.1 mM) and sodium fluoride (10 mM) stimulated activities were increased by 22.4% and 28.9% respectively. Inclusion of the adenosine analogue (-)-N6 (R-phenyl-isopropyl) adenosine (R-PIA) (0.1 mM) was found to abolish the increase in adenylate cyclase activity found in the presence of adenosine deaminase (1U/ml).

Ischaemic membranes (60 minutes) prepared as described in Chapter 4 were investigated in the presence and absence of adenosine deaminase (1U/ml). From Fig 5.4 it is apparent that adenylate cyclase in ischaemic rat membranes is increased in the presence of

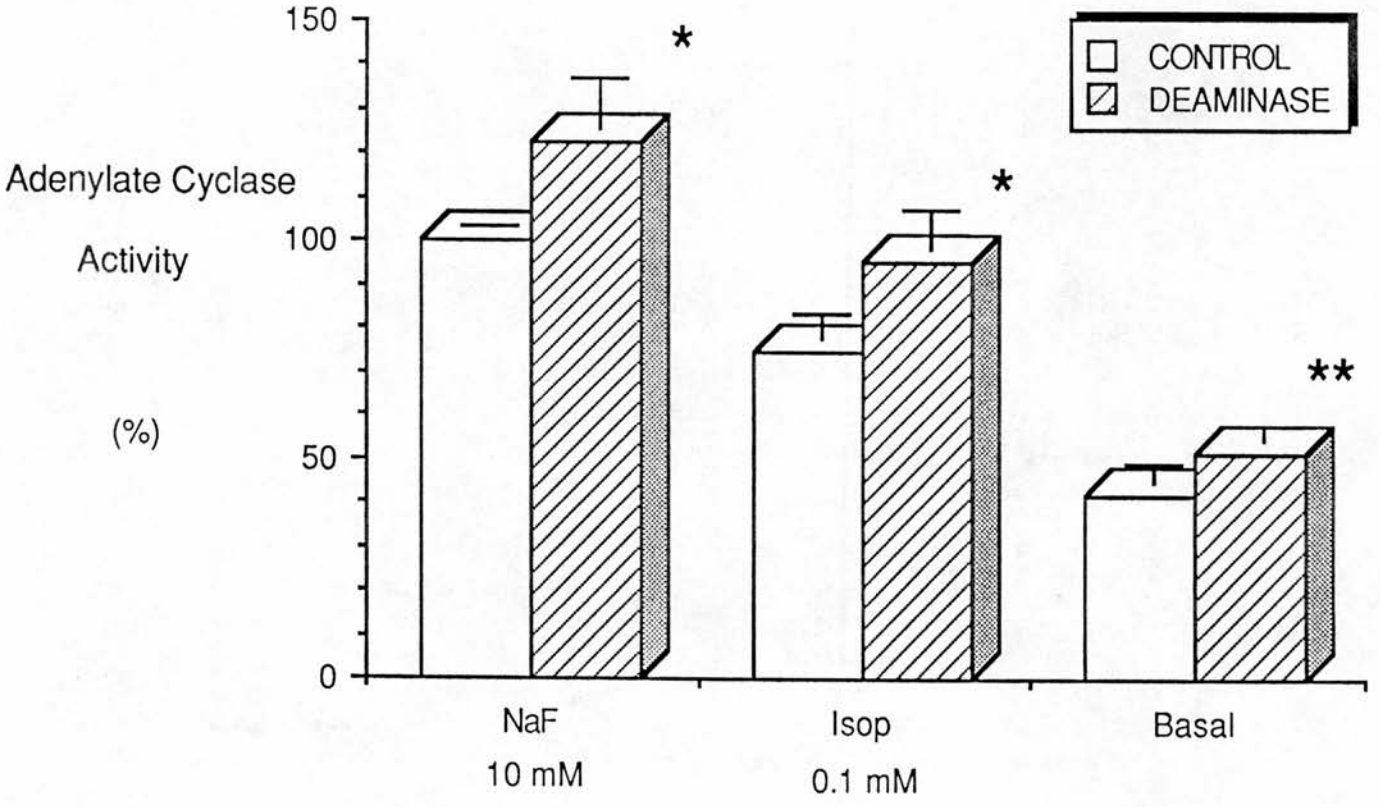

Fig 5.3 Effect of adenosine deaminase (1U/ml) on adenylate cyclase activity in control rat cardiac membranes. Results are expressed as a percentage of control sodium fluoride stimulation. (n=5).

** $p < 0.01$, * $p < 0.05$, vs the appropriate control.

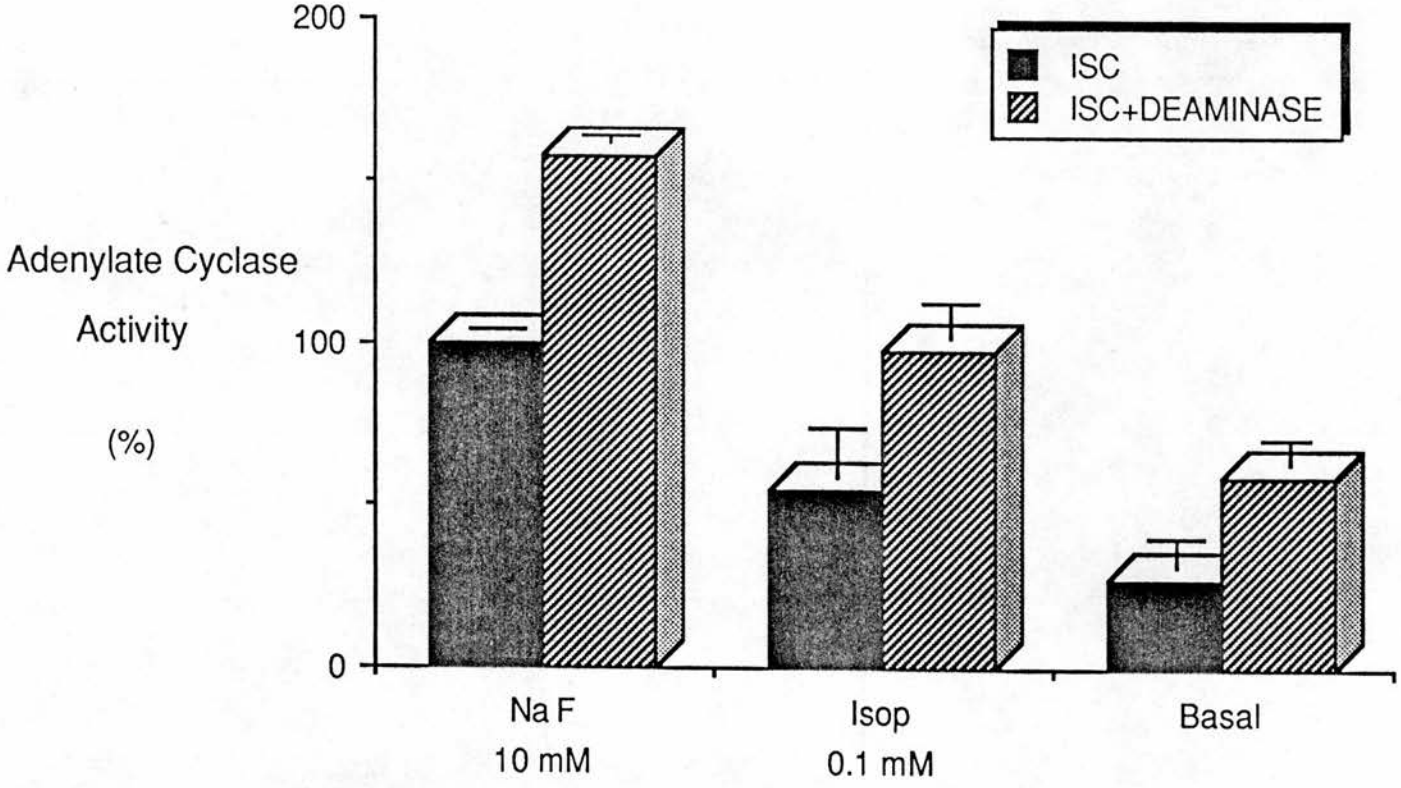

Fig 5.4 Effect of prolonged myocardial ischaemia (60 minutes) on the response of adenylate cyclase in the presence of adenosine deaminase (1U/ml). Results are expressed as a percentage of ischaemic sodium fluoride stimulation. (n=3).

adenosine deaminase; basal activity was increased by 105%, while (-)-isoprenaline (0.1 mM) and sodium fluoride (10 mM) stimulated adenylate cyclase activities were increased by 77% and 55% respectively.

5.3 DISCUSSION

A metabolic acidosis is one of the earliest changes in myocardial ischaemia (Ichihara et al., 1984). By 15 minutes of left anterior descending coronary artery ligation in canine hearts a decline in intracellular pH from ~ 7.5 to ~ 6.8 was observed (Ichihara et al., 1984). The results presented here show that lowering pH from 7.5 to 6.8 caused a reduction in all adenylate cyclase activities by approximately 75%. This suggests that under conditions of low pH, cardiac adenylate cyclase sensitivity to catecholamines is markedly reduced and that this effect is most probably at the level of the enzyme and not at the level of the beta adrenergic receptor. The results presented here show that the reduction in activity found at pH 6.8 was partially reversed by assaying the membranes at pH 7.5. It would be predicted on the basis of enzymology that the the pH effect would be fully reversible. The rat cardiac membrane preparation is likely to be vesicular in nature therefore simply altering the pH to 7.5 may not change the intra-vesicular pH which may remain at pH 6.8. It is also possible that incubation of membranes at 37°C at pH 6.8 does cause some degree of irreversible inhibition of adenylate cyclase.

Lowering pH from 7.5 to 6.8 also had dramatic effects on the binding of (-)-IPin to rat cardiac membranes. A decrease in the affinity of beta adrenergic binding sites for (-)-IPin and a decrease in B_{max} was observed at pH 6.8. A fall in the affinity of (-)-[^{3}H] dihydroalprenolol for $beta_2$ adrenergic receptors has been shown when the pH is lowered from 7.5 to 6.7; over this pH range there is minimal alteration in the ionization of (-)-[^{3}H] dihydroalprenolol or (-)-IPin (Ijzerman et al., 1984). These results show that reduction of pH from 7.5 to 6.8, as occurs during myocardial ischaemia, causes an reduction in affinity probably due to an alteration at the level of the receptor. Nakanishi et al. (1987) have also observed a decrease in beta adrenergic binding sites in membranes, from newborn rabbit hearts, on lowering the binding assay pH from 7.4 to 6.0, using the ligand iodohydroxybenzylpindolol. The decrease in B_{max} observed under conditions of low pH may be due to pH-mediated conformational alterations in beta adrenergic binding sites. Extrapolating these results to acute myocardial ischaemia, the affinity of beta adrenergic receptors for catecholamines is likely to be reduced.

Evidence has been accumulating that the function of the beta adrenergic/adenylate cyclase system is reduced under acidotic conditions. Reduced inotropic and chronotropic responses to isoprenaline were found on lowering pH from 7.4 to 6.8 in perfused newborn rabbit heart and isolated rat atria (Nakanishi et al., 1987; Camilion de Hurtado et al., 1981). Interestingly acidosis has also been described as desensitizing the chronotropic action of histamine in rabbit atria (Hughes and Coret, 1976). Adenylate cyclase in rat fat cells has also been shown to be inhibited by low pH, these authors

also found inhibition of phosphodiesterase at low pH (Fredholm and Hjemdahl, 1976). However, effects of ischaemic pH on cardiac phosphodiesterase have not yet been throughly investigated.

The results presented here show evidence of the presence of an inhibitory effect of adenosine on adenylate cyclase activity in rat cardiac membranes, this inhibitory effect has proved difficult to see in many 'purified' membrane preparations (Schutz et al., 1986). These results provide support for the theory that adenosine has anti-adrenergic effects on the myocardium through inhibition of adenylate cyclase. As discussed previously adenosine levels increase early in myocardial ischaemia. Accumulation of adenosine during acute myocardial ischaemia may alter the responsiveness of the myocardium to endogenous catecholamines. To investigate this, the effect of adenosine deaminase on ischaemic membranes was investigated. Adenosine deaminase caused an increase in adenylate cyclase activity in ischaemic membranes. This suggests that even after 60 minutes of global ischaemia the inhibitory effect of adenosine on adenylate cyclase activity is still functional. The magnitude of the increase in adenylate cyclase activity in response to adenosine deaminase in ischaemic membranes is larger than in normal membranes. This may be due to higher levels of endogenous adenosine trapped in membranes prepared from ischaemic myocardium but without measuring adenosine levels this is speculative. Alternatively there may be enhanced inhibitory effects of adenosine during myocardial ischaemia.

The effects of adenosine and pH on beta adrenergic / adenylate cyclase function have been investigated in this chapter, however there many are other alterations in early ischaemia which may change

the responsiveness of the myocardium to catecholamines: free cytosolic calcium levels have been shown to increase as early as 10 minutes in the ischaemic rat heart (Steenbergen et al., 1987). Cardiac adenylate cyclase is inhibited by calcium through a calmodulin independent process (Cros et al., 1984). Will-Shahab et al. (1985) have shown that a degree of protection of adenylate cyclase from myocardial ischaemia may be afforded by the presence of calcium channel blockers.

In summary, these in vitro experiments suggest that the responsiveness of the myocardium to catecholamines is reduced under the conditions of acute myocardial ischaemia.

CHAPTER 6

CONCLUSION

CHAPTER 6

The aim of the project was to clarify the responsiveness of the myocardium to catecholamines during myocardial ischaemia. Initial experiments were geared to characterisation of the beta adrenergic adenylate cyclase system in control membranes prepared from rat myocardium. The results of which demonstrated that both beta adrenergic receptor stimulated adenylate cyclase activity and receptor independent activity could be quantified. Binding of the ligand (-)-IPin to beta adrenergic binding sites was both saturable and specific allowing the determination of kinetic and equilibrium parameters.

The effect of in vitro stop flow myocardial ischaemia on adenylate cyclase activity and the density of beta adrenergic binding sites was investigated. Acute myocardial ischaemia was associated with a 50% decline in adenylate cyclase activity, in both basal and stimulated activities, despite an increase in the number of beta adrenergic binding sites found in myocardial ischemia. The fact that these alterations were found in vitro demonstrates that these effects are not reflex alterations to ischaemia but that they are direct effects of ischaemia on the myocardium. Prolonged ischaemia was associated with a further decline in adenylate cyclase activity and increases in the number of beta adrenergic binding sites.

In addition to the traditional approach of quantitating adenylate cyclase activity in ischaemic membranes, the effect of the local ischaemic environment on the responsiveness of the myocardium to

catecholamines was investigated. Two candidates were examined in detail - pH and adenosine. The experiments presented showed that cardiac adenylate cyclase is reversibly inhibited at pH 6.8. There are also effects of pH directly on the beta adrenergic receptor causing reduced affinity at pH 6.8. In addition, adenosine continues to exert anti-adrenergic effects in myocardial ischaemia. The results of these experiments are particularily pertinent to the acutely ischaemic myocardium, where local effects on beta adrenergic function are lost by assaying membranes under 'normal' conditions. Some effects of local ischaemic environment on beta adrenergic function are summarised in Fig 6.1.

These results show that neither approach, separately, can give sufficient information on the effects of myocardial ischaemia on beta adrenergic function. However the combination of the stop-flow global ischaemia model and the approach of examining individual factors (pH, adenosine) may give a clearer picture of the events occurring in vivo.

The results presented here are further supported by the observation of Krause and England (1982) that the phosphorylation response to a bolus injection of isoprenaline in rat hearts was completly abolished by 5 minutes of myocardial ischaemia. These authors related this inhibition to accumulation of lactate in ischaemia, however it is likely to be due to pH inhibition of the responsiveness of the myocardium to isoprenaline.

The observation of reduced responsiveness to catecholamines during myocardial ischaemia has implications with regard to the arrhythmogenesis of catecholamines. The question therefore remains

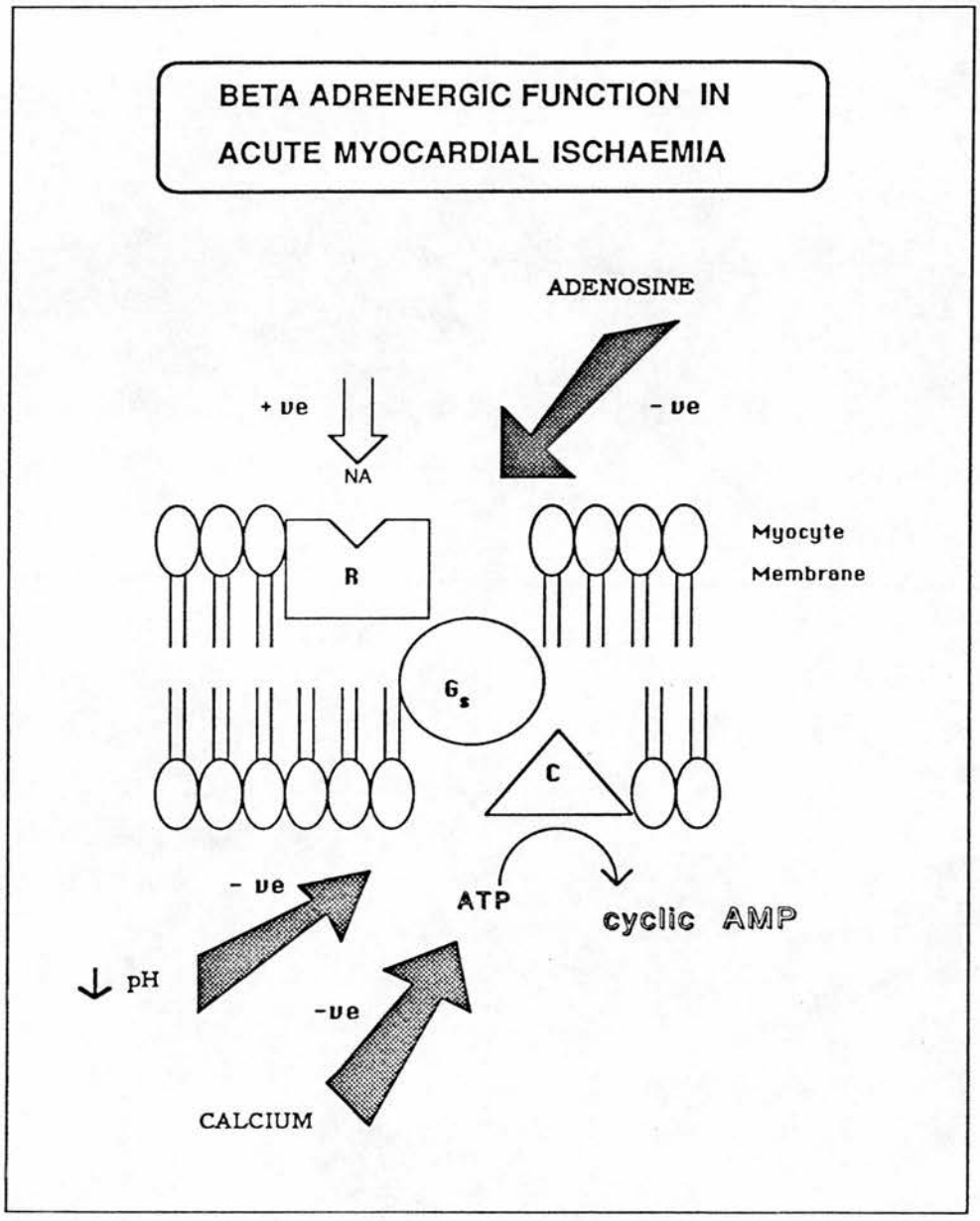

Fig 6.1 Beta adrenergic function in acute myocardial ischaemia. Summary of the environmental effects of acute myocardial ischaemia on the function of adenylate cyclase, and therefore the responsiveness of the enzyme to catecholamines. pH falling in myocardial ischaemia reduces the activity of adenylate cyclase, as does the accumulation of calcium. The build up of extra cellular adenosine will also contribute to an overall reduction in the responsiveness of adenylate cyclase to catecholamines in acute myocardial ischaemia.

how are catecholamines arrhythmogenic ?

A. Catecholamines may be arrhythmogenic by a mechanism which by-passes adenylate cyclase. Speculatively, by the coupling of beta adrenergic receptors to G_s and then directly to another membrane protein eg. an ion channel for calcium entry.

B. Catecholamines may be arrhythmogenic through an alpha adrenergic receptor mechanism.

C. Through a receptor independent mechanism such as formation of peroxides.

D. The reduction in beta adrenergic function in global stop-flow myocardium represents areas of the myocardium in vivo receiving no flow, however the in vivo ischaemic area is more heterogeneous with respect to flow and, therefore, severity of ischaemia. From the results presented it is likely that there will be a heterogeneous response of the myocardium to catecholamines. This may precipitate the development of serious arrhythmias.

> "...redistribution of endogenous catecholamines in the acutely ischemic myocardium, perhaps coupled with increased adrenergic sensitivity, because of larger numbers of exposed receptors, could increase calcium influx to the myocyte. The latter could contribute to both the progression of ischemic cell injury and the increased propensity for cardiac arrhythmias."
> - Riemer and Jennings, 1986.

This thesis challenges the above hypothesis for genesis of arrhythmias during myocardial ischaemia. The evidence presented here shows that the acutely ischaemic myocardium has actually reduced sensitivity to catecholamines.

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