

An Investigation into the Antiadrenergic Effect of
Adenosine on the Ventricular Myocardium of
Guinea-pig and Rabbit.

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Abstract.

The effect of adenosine and its analogues on the developed tension of various cardiac preparations, was investigated.

10 μ M adenosine slightly decreased basal force and completely abolished the isoprenaline (0.1-1nM) stimulated tension in Langendorff-perfused guinea-pig hearts. With papillary muscles adenosine decreased basal force slightly and decreased the isoprenaline (1-10nM) stimulated force by approximately 40%. When 15 μ M 2-Chloroadenosine was used in conjunction with adenosine deaminase, a 100% inhibition of the isoprenaline stimulated tension was seen. This greater effect of 2-chloroadenosine with adenosine deaminase shows that the presence of endogenous adenosine may mask the effect of added adenosine. 2-chloroadenosine decreased basal force by 35%, this was not observed with reserpinised animals suggesting that 2-chloroadenosine decreases basal tension primarily by reducing the stimulation due to endogenous catecholamines. The antiadrenergic effect of 2-chloroadenosine was blocked by 8-phenyl-theophylline, showing the effect to be mediated by extracellular A-type receptors. Rabbit papillary

muscles were clearly different from guinea-pig in that there was little effect of 2-chloroadenosine (plus deaminase) on isoprenaline stimulated tension. The antiadrenergic effect of 2-chloroadenosine was reduced by pretreatment of guinea-pigs with pertussis toxin, showing the involvement of N_i in this action of 2-chloroadenosine.

Adenylate cyclase activity was measured in sarcolemmal membranes isolated from guinea-pig and rabbit ventricle. There was never more than a 25% inhibition mediated by A-type receptors using concentrations of Mg^{++} , Na^+ and GTP reported as optimal for receptor expression. The concentrations of these components were varied but no consistent improvement was seen. The presence of isoprenaline increased adenylate cyclase activity but did not affect inhibition mediated by A-type receptors. An almost total block of cyclase activity could be seen with P-site specific analogues. However, the intracellular P-site is not thought to be of physiological significance.

It is concluded that the antiadrenergic effect of adenosine is mediated by A-type receptors and is seen in guinea-pig but not rabbit. The effect may be masked by endogenous adenosine and catecholamines. The inhibitory guanine nucleotide binding

protein Ni is involved in this antiadrenergic effect. There is no conclusive evidence for A-type receptors coupled to adenylate cyclase in isolated membranes.

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Contents.

Abstract	2
Acknowledgements	5
Contents	6
Figures and Tables	9
Publications arising	13

<u>General Introduction</u>	14
-----------------------------	----

<u>Chapter 1 Methods</u>	53
--------------------------	----

Section A. Experimental procedures for isolated heart muscle preparations	54
--	----

1. Perfusion of an isolated non- working whole heart.	54
--	----

Freeze-clamping of hearts and measurement of tissue cAMP.	58
--	----

2. Superfusion of rabbit and guinea-pig papillary muscle	62
---	----

3. preparation of isolated guinea- pig atria and papillary muscles in organ baths for simultaneous measurement of contractile force.	67
---	----

4. Guinea-pig injection protocol	71
----------------------------------	----

Section B. Measurement of adenylate	
-------------------------------------	--

cyclase activity in membranes isolated from the ventricular myocardium of guinea-pig and rabbit.	72
1. Isolation of membranes	72
2. Assay of cAMP	77
3. Setting up of adenosine-free assay systems	94
4. Non-methylxanthine phosphodiesterase inhibitors	111

<u>Chapter 2</u> The effect of adenosine on basal and isoprenaline stimulated tension in isolated langendorff perfused guinea-pig hearts and papillary muscles.	116
--	-----

<u>Chapter 3</u> The effect of 2-chloroadenosine on the positive inotropic response of guinea-pig papillary muscle to isoprenaline.	136
---	-----

<u>Chapter 4</u> The effect of adenosine and 2-chloroadenosine on tension in rabbit papillary muscle.	153
---	-----

<u>Chapter 5</u> The effect of adenosine and its analogues on the adenylate cyclase activity of rabbit and guinea-pig ventricular myocardium.	163
---	-----

<u>Chapter 6</u> The effect of 2'-deoxyadenosine-3'-monophosphate (a P-site agonist) on the activity of rabbit and guinea-pig ventricular adenylate cyclase.	192
<u>Chapter 7</u> Inhibition of rabbit cardiac adenylate cyclase by theophylline.	214
<u>Chapter 8</u> The effect of adenosine and 2-chloroadenosine on the developed tension of isolated guinea-pig atria.	228
<u>Chapter 9</u> The effect of pertussis toxin on the direct and indirect actions of adenosine and 2-chloroadenosine on guinea-pig heart.	246
Concluding Remarks	259
Appendix: Materials used	271
References	277

List of figures and tables.

	Page
Introduction	
Figure 1	17
Figure 2	21
Methods	
Figure 1.1	55
Figure 1.2	63
Figure 1.3	68
Figure 1.4	79
Figure 1.5	85
Figure 1.6	90
Figure 1.7	96
Figure 1.8	102
Figure 1.9	104
Figure 1.9a	105
Figure 1.10	108
Figure 1.11	109
Figure 1.12	112
Figure 1.13	114
Table 1.1	86
Table 1.2	93
Table 1.3	98
Table 1.4	99
Table 1.5	106

Chapter 2

Figure 2.1	124
Figure 2.2	127
Figure 2.3	129

Chapter 3

Figure 3.1	140
Figure 3.2	141
Figure 3.3	142
Figure 3.4	144
Figure 3.5	145

Chapter 4

Figure 4.1	157
Figure 4.2	158
Figure 4.3	159
Figure 4.4	161

Chapter 5

Figure 5.1	172
Figure 5.2	183
Table 5.1	174
Table 5.2	175
Table 5.3	177
Table 5.4	178
Table 5.5	180
Table 5.6	181
Table 5.7	184
Table 5.8	185

Chapter 6

Figure 6.1	199
Figure 6.2	201
Figure 6.3	204
Figure 6.4	205
Figure 6.5	206
Figure 6.6	209
Table 6.1	200
Table 6.2	202
Table 6.3	208

Chapter 7

Figure 7.1	219
Figure 7.2	220
Figure 7.3	224
Figure 7.4	225
Table 7.1	217
Table 7.2	221
Table 7.3	222

Chapter 8

Figure 8.1	233
Figure 8.2	234
Figure 8.3	236
Figure 8.4	237
Figure 8.5	239
Figure 8.6a	241
Figure 8.6b	242

Figure 8.6c	243
Chapter 9	
Figure 9.1	250
Figure 9.2	251
Figure 9.3	253
Table 9.1	255

Publications Arising

1. Ridgeway A.M., Harding S.E. and Harris P. (1983)
Use of deoxy-ATP as substrate for cardiac adenylate cyclase: measurement of cdAMP by radioimmunoassay.
J Mol Cellular Cardiol 15 suppl 1 p88
2. Ridgeway A.M., Harding S.E. and Harris P. (1983)
DeoxyATP as a substrate for cardiac adenylate cyclase: a radioimmunoassay for cyclic deoxyAMP.
J Pharm Pharmacol 35 p692
3. Hopwood A.M., Harding S.E. and Harris P. (1984)
Theophylline Inhibition of rabbit cardiac adenylate cyclase
J Mol Cellular Cardiol 16 suppl 2 p2.
4. Hopwood A.M., Harding S.E. and Harris P (1985).
Inhibition of rabbit cardiac adenylate cyclase by theophylline.
J Pharm Pharmacol 37 p924-926.
5. Hopwood A.M., Harding S.E. and Harris P. (1985)
The antiadrenergic effect of adenosine on the ventricular myocardium of guinea-pig and rabbit.
J Mol Cellular Cardiol 17 Suppl 3 p160.

General Introduction

A. General Introduction.

Drury and Szent-Gyorgi in 1929 demonstrated that adenosine administration caused widespread effects such as hypotension, arrest of intestinal movement, sedation, inhibition of respiration and renal vasoconstriction. Concentrating their attention on the heart they noted that adenosine could dilate the coronary arteries, inhibit impulse generation and conduction in sinus and atrio-ventricular nodes as well as depressing the contractile force in atrium. The indirect effects which adenosine has on ventricular myocardium went unnoticed at this time.

Adenosine may be released from the heart in response to hypoxia [Schrader et al 1975a] or to sympathetic stimulation [Schrader et al 1977]. If the end result of all the actions of adenosine on the heart are examined, it can be seen that the nucleoside is having a cardioprotective effect against such stimuli. The dilation of the coronary arteries means that the oxygen supply to the tissues is increased. By reducing the rate of firing of pacemaker cells and lowering the force of contraction of atrial and ventricular tissues the myocardial tissues are relieved of excess stress.

Thus, the different actions of adenosine on the heart are complementary and tend to be functionally antagonistic to β -stimulation.

B. The β -receptor adenylate cyclase system.

Over the years much evidence has accumulated which strongly suggests a cause and effect relationship between β -stimulation of adenylate cyclase and the observed gross physiological effects of β -agonists on the myocardium [Drummond and Severson 1979]. A simplified diagram of the β -receptor-adenylate cyclase system is shown in figure 1. The catalytic subunit, C, faces the inside of the cell and catalyses the formation of cAMP from Mg.ATP. Coupled to this is the 'N' protein or guanine nucleotide binding protein, which also possesses GTPase activity. The N protein is considerably more complex than was originally thought and is discussed more fully in section B1. On the outside of the cell are found a number of hormone receptors. These bind the relevant hormone leading to changes in the N protein which cause activation (in this case) of the catalytic sub-unit. Guanine nucleotides modulate the binding of β -agonists [Davies et al 1981].

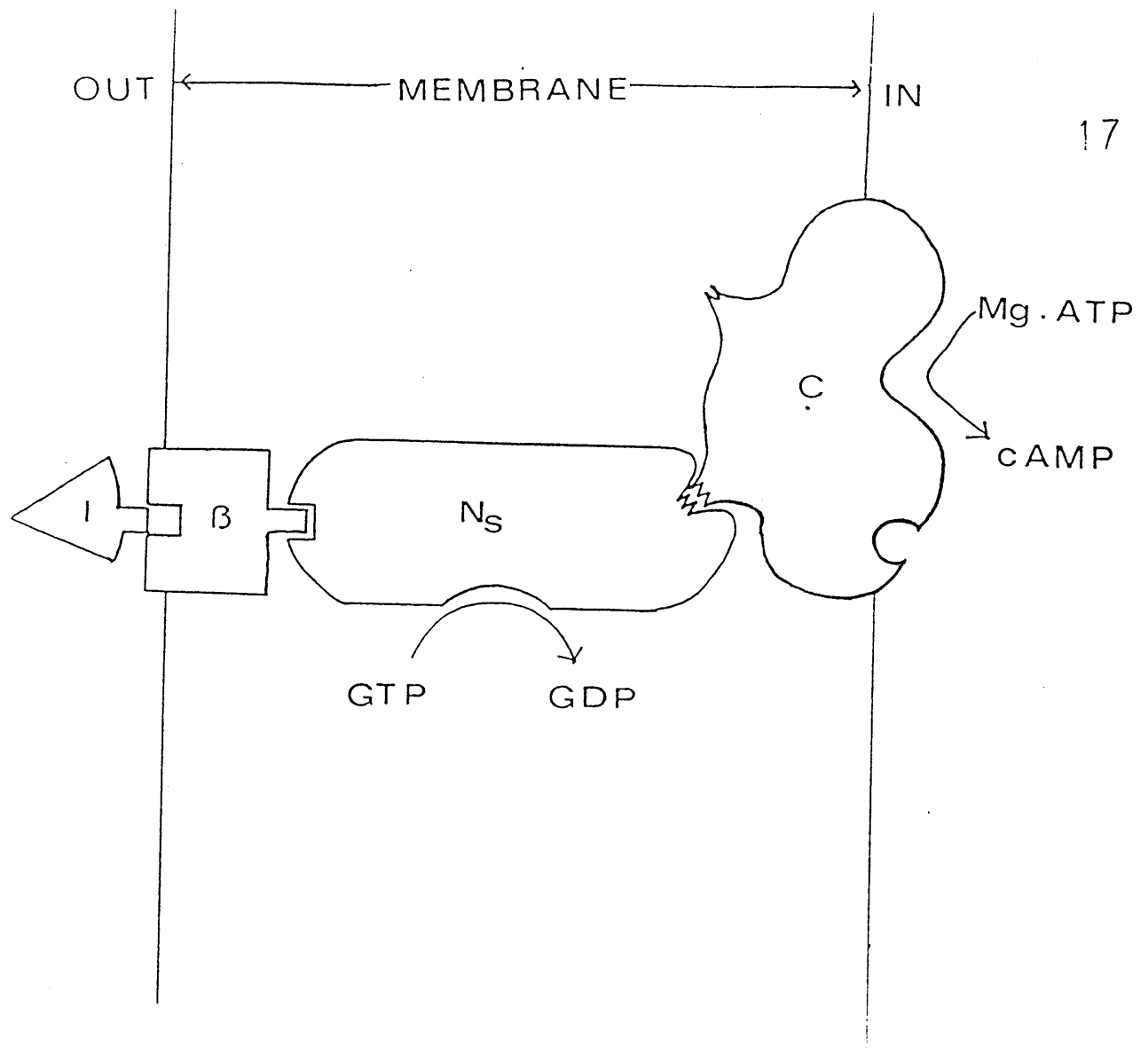


Figure 1. The B-receptor-N protein-adenylate cyclase system.

Key: C = catalytic subunit.

N_s = Stimulatory guanine nucleotide binding protein.

B = B-adrenergic receptor.

I = Isoprenaline.

B1. The N proteins.

It has long been suspected that the stimulatory and inhibitory responses of adenylate cyclase to hormones are mediated by distinct N proteins. These are defined as Ns (stimulatory) and Ni (inhibitory). Evidence for this comes from the observations of Aktories et al [1982] who found that limited proteolysis with trypsin or treatment with N-ethylmaleimide attenuated inhibitory effects without affecting stimulation. The S49 cyc- mutants of the lymphoma cell line have no stimulatory N protein, but nevertheless respond to inhibitory hormones [Hildebrandt and Birnbaumer 1983]. This provides further evidence for discrete Ns and Ni. More recently the use of bacterial toxins has helped to distinguish between stimulatory and inhibitory hormone effects. Cholera toxin catalyses the ADP ribosylation of Ns leading to an inhibition of the GTPase activity. This results in persistent maximal activation of adenylate cyclase, [Finklestein 1978], effectively preventing the action of stimulatory hormones. Jakobs et al in 1983 demonstrated that cholera toxin treatment of platelets abolished stimulatory hormone action while the inhibitory effects were retained. The discovery of Islet Activating Protein (IAP,

Pertussis Toxin) by Yajima et al in 1978 provided a complementary tool for investigating Ni action. IAP catalyses the ADP ribosylation of Ni leading to, as above, inhibition of the GTPase, in this case resulting in permanent inhibition of Ni. Such inhibition of Ni prevents the action of inhibitory hormones.

The current view of N protein action has recently been reviewed by Gilman [1984]. Both Ns and Ni have an α, β, γ subunit structure. The β subunits (Ni, Mwt= 41,000 , Ns Mwt= 45,000) are the sites of ADP ribosylation by the bacterial toxins. Ni and Ns share a common α subunit (Mwt, 35,000). The function of the γ subunits is at present unknown.

Activation of Ns or Ni by GTP or non hydrolysable analogs is associated with a ligand promoted dissociation of α and β subunits resulting in free β and α -GNT (GNT = guanine nucleotide). The α -GNT complex causes stimulation of the catalytic subunit. Stimulatory effects are turned off by reassociation of α and β subunits. The inhibitory action of Ni is proposed to be due to the release of β -subunit, which binds to and inactivates any α s which is free. This requires there to be a stimulatory 'tone' maintained by free α s. However,

the observation that hormones are able to inhibit adenylate cyclase in cyc- cells [Hildebrandt and Birnbaumer 1983], which are reported to be devoid of α_i weakens the argument. One explanation for this is that α_i has a weak direct effect on the catalytic subunit which is usually masked by the rather larger indirect β effect.

C. Modes of action of adenosine on various tissues.

Two independent groups of workers in 1970 [Sattin and Rall, Shmitzu and Daly] found that administration of adenosine caused an increase in cAMP levels in brain slices. Since then much evidence has accumulated to support the role of adenosine as a local hormone. Adenosine can stimulate or inhibit adenylate cyclase depending on tissue type. Stimulation is seen with brain striatum and hypothalamus, lung, platelets, bone, lymphocytes, skin, adrenals, leydig tumors, and possibly coronary arteries. Adipocytes and brain hippocampus, striatum, frontal and parietal cortex, and cerebellum show inhibition [reviewed by Fain and Malbon 1979].

C1. The sites, A1, A2 and P, nomenclature.

Figure 2 shows a stylised diagram of the way

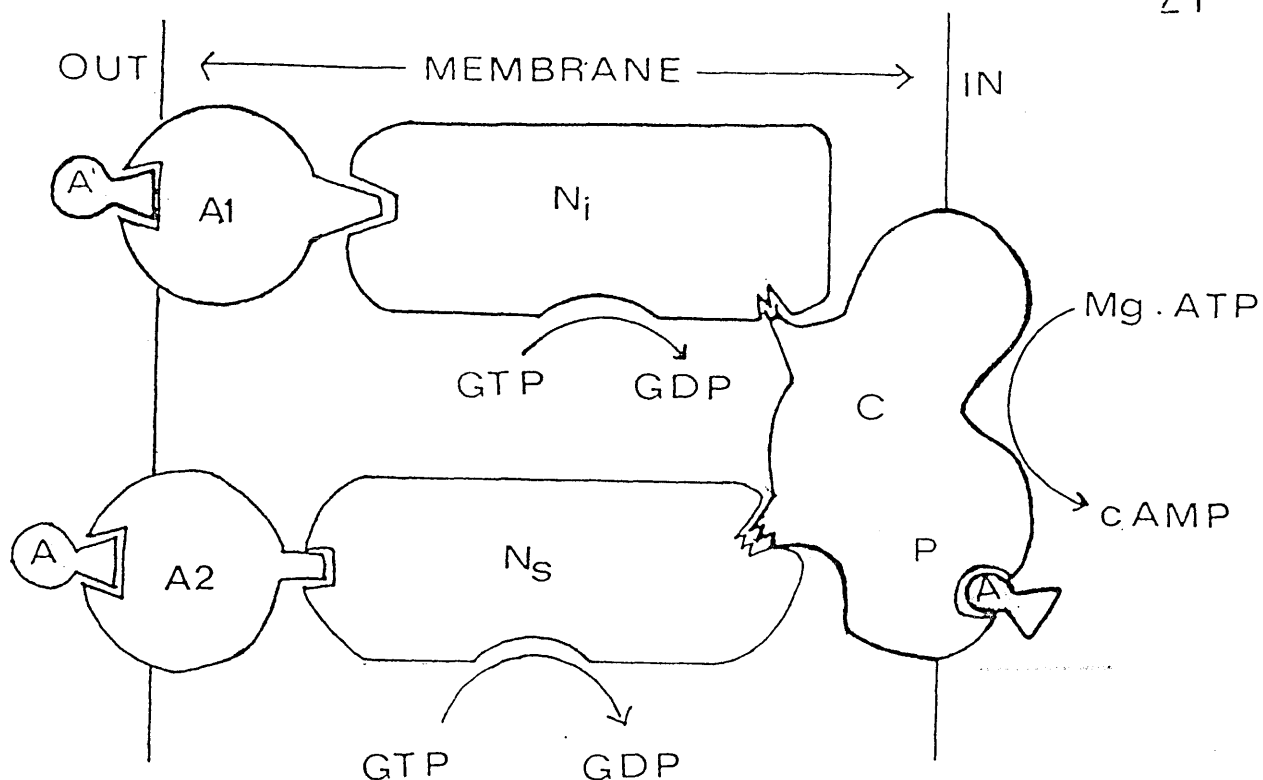


Figure 2. The adenosine receptor-N-protein-adenylate cyclase system.

Key: C = catalytic subunit.

N_s = Stimulatory guanine nucleotide binding protein.

N_i = Inhibitory " .

A1 = Inhibitory adenosine receptor.

A2 = Stimulatory " .

A = Adenosine.

P = Psite.

that adenosine interacts with the adenylate cyclase system. If adenosine binds to an A2 type of receptor then N_s is activated and cAMP production is increased. If an A1 receptor is present, then binding of adenosine results in activation of N_i and an attenuation of adenylate cyclase activity. A1 and A2 receptors are never present together on any given cell. In addition to these conventional hormone receptors, adenosine can also inhibit adenylate cyclase by binding at a further site, the P-site, which is found on the inside of the cell.

A1 and A2 receptors show differences in the way that they are activated by adenosine analogues. Such differences form the basis of the nomenclature. Where N6-phenylisopropyladenosine (PIA) is more potent than 5'-N-ethylcarboxamideadenosine (NECA) inhibition is usually seen (but see below) and the receptors are designated A1 [Van Calcar et al 1978] or R_i [Londos et al 1981]. If the effect of NECA is greater than that of PIA then stimulation of adenylate cyclase is generally seen and the receptors are known as A2 or R_a . These relative potencies are of some use in determining whether a given gross physiological response is mediated by A1 or A2 receptors. However the finding that a particular response is mediated, by say, an A1 receptor does not necessarily mean that

inhibition of adenylate cyclase is responsible for the effect. This was demonstrated by Dunwiddie and co-workers in 1984, who found that in the hippocampus, the receptors mediating the electrophysiological effect are of the A1 subtype whereas the adenylate cyclase coupled receptors are A2.

C2a. Characteristics of stimulatory (A2) adenosine receptors.

The stimulatory effects of adenosine are seen in the low micromolar range. They are competitively antagonised by methylxanthines at the binding site [Sattin and Rall 1970]. The receptors can be shown to be on the outside of the cell by the use of high molecular weight derivatives, which cannot penetrate the cell but nevertheless produce a similar response to adenosine [Olsson et al 1976]. Adenosine uptake blockers tend to potentiate the effects seen, providing further evidence that the receptors face the outside [Haslam and Rosson 1975]. Activation of adenylate cyclase proceeds by a transduction mechanism similar to that used by other stimulatory hormones, and the same general features are seen: a multiplicative rather than additive effect between adenosine and guanine nucleotides to activate adenylate cyclase and a

shortening of the lag of activation by GppNHp. Where adenosine shares a common catalytic subunit with another stimulatory hormone, the stimulation elicited by adenosine is much smaller. The existence of adenosine-sensitive and adenosine-insensitive adenylate cyclase has been suggested to account for this phenomenon [Johnson et al 1979]. It has also been proposed that adenosine receptors are permanently coupled to adenylate cyclase, whereas B-receptors require collision before coupling [Tolkovski and Levitski 1978, Braun and Levitski 1979, Rimon et al 1980]. Adenosine receptors may also be less efficient at stimulating the dissociation of α and β subunits of N_s . Adenosine does not cause a reduction in the maximum stimulation elicited by the other hormone [Lad et al 1980].

C2b. Characteristics of inhibitory (A1) adenosine receptors.

As with stimulatory adenosine receptors, these can be shown to be on the outside of cells. Their effects are blocked by methylxanthines. Here the similarity ends. Rather lower concentrations of adenosine (nM) are generally required to elicit inhibitory effects. Although addition of guanine nucleotides is necessary for the stimulatory

actions, with the inhibition of adenylate cyclase, the actual concentration of GTP is rather more important. In some systems GTP has a biphasic effect on adenylate cyclase activity, at concentrations above 1 μ M inhibition may be seen [Cryer et al 1969]. This inhibitory phase can be suppressed by the addition of Na⁺ [Cooper et al 1983, Londos et al 1981], thus allowing inhibitory hormones to exert their action. The requirement for Na⁺ has recently been questioned by the finding that it could be replaced by an isoosmotic amount of sucrose [Garcia-Sainz et al 1981].

More recent evidence suggests that Mg⁺⁺ may also have a role to play in the modulation of inhibitory hormone action, since Bockaert et al [1984] found that hormonal inhibition is suppressed by high Mg⁺⁺ concentrations. Agonist binding to the inhibitory receptors is also modulated by GTP, Na⁺, and Mg⁺⁺ [Schwabe et al 1982, Lohse et al 1984, Berrie et al 1979].

The reduction of cAMP production seen with inhibitory hormones is never complete; at best a 60% decrease may be observed. It is possible that this is due to the presence of two pools of adenylate cyclase, inhibitory hormone sensitive and insensitive, similar to those proposed for

stimulatory adenosine receptors. Differences in receptor-cyclase coupling could also account for this incomplete inhibition.

Earlier studies revealed that non-hydrolysable GTP analogues could not support receptor-mediated inhibition [Jakobs et al 1979, Cooper and Londos 1979]. Indeed in many tissues, stimulation of a GTPase seems to be correlated with the receptor-mediated inhibition of adenylate cyclase [Aktories et al 1982, Onali^{et al} 1983, Koski et al 1982]. This suggests a pivotal role for GTP hydrolysis in the mechanism of action of inhibitory hormones.

The P-Site.

Adenosine can also inhibit adenylate cyclase at a further locus, the P-site. Present in all tissues, this site always mediates inhibition of adenylate cyclase in a GTP-independent manner. It can be shown to be facing the inside of the cell and is thought to function as an allosteric regulator of adenylate cyclase as P-site agonists do not compete with Mg.ATP for the active site [Welton and Simko 1980, Braun and Levitski 1979, Johnson et al 1979, and Haslam et al 1978].

The presence of the N-protein seems to be essential for the expression of P-site mediated

inhibition in some systems [Londos et al 1981], though Neer and Salter [1981] have shown P-site action on an isolated catalytic site from bovine brain. The observation by Sadler and Maller [1983] that 2'5' dideoxyadenosine increases the lag time for stimulation by GppNHp and slows the exchange rate of guanine nucleotides provides a possible reason for the necessity of the presence of the N-protein for P-site action.

There is not yet any firm evidence for a physiological role of this site which means that investigators have to take care to eliminate P-site effects which might otherwise mask those of the A-site. The best method for discriminating between A-site and P-site effects is the use of adenosine analogues. Generally, modifications in the ribose moiety enhance the P-site effect and abolish the A-type receptor activity of the molecule, whereas modifications to the purine leave the molecule with no P-site activity and enhance the receptor mediated effect.

In only one case, [Thomas et al 1984], has inhibition of adenylate cyclase by adenosine through the P-site been correlated with an observed physiological effect. Collis and Brown in 1983 have been able to postulate that an intracellular site

might be involved in the adenosine mediated relaxation of the aorta.

The main interest in this site stems from the fact that the actions of P-site agents are altered by metal ions and other modulators of adenylate cyclase. Substitution of Mn for Mg or raising the Mg concentration to 15 or 20 mM is reported by many to increase the potency of P-site agonists [Cochaux et al 1982, Wolff et al 1978, Bregman et al 1980, Welton and Simko 1980 and de Vente and Zaagsma 1981, Premont et al 1979a, Londos and Wolff 1977, Johnson et al 1979, Peck et al 1976 and Webster and Olsson 1981].

C4.Effects on the phosphodiesterase.

Adenosine may also change cAMP levels by stimulating or inhibiting the cAMP phosphodiesterase. In 1984[a & b], de Mazancourt and Guidicelli reported that adenosine can stimulate the membrane bound cAMP phosphodiesterase in fat cell and brain in a GTP-dependent manner. This resulted in a lower level of cAMP and was found to be mediated by A1 type receptors. Teo et al [1981] observed opposing effects in whole fat cells and isolated membranes. With intact adipocytes,

stimulation of the phosphodiesterase was seen, but with isolated membranes, PIA inhibited this enzyme. The implications of these results are that contradictory observations might be made between whole cells and membranes. This is discussed more fully in sections E and F.

C5.Methodology involved in the study of adenosine receptors coupled to adenylate cyclase.

The progress in understanding the mechanism of action of adenosine in relation to adenylate cyclase has been slow due to the difficulties encountered in the methodology. Assay systems for adenylate cyclase tend to have a high level of endogenous adenosine generated by the metabolism of the substrate ATP by membrane phosphohydrolases and also from the breakdown of cAMP, often added to 'trap' radioactive cAMP. Addition of adenosine deaminase to the assay mixture will remove endogenous adenosine (by converting it to inosine, which has no activity at adenosine receptors). The action of adenosine deaminase resistant analogues can then be determined. However, even this method is felt to be unsatisfactory since the action of adenosine itself cannot be determined. As well as this the possibility remains that the analogues could inhibit adenosine deaminase thus raising the

level of adenosine. A rather better method of reducing the amount of endogenous adenosine is to use deoxyATP as the cyclase substrate, since the 2'-deoxyadenosine which may be generated has no action on the receptors [Londos et al 1981]. The cyclic deoxyAMP (dcAMP) formed may be measured using a modified Salomon assay [Cooper et al 1979] or by a radioimmunoassay method developed by myself [Ridgeway et al 1983] (see methods section B3b).

The other membrane-bound enzyme which causes problems is the cAMP phosphodiesterase which must be blocked to ensure accurate measurement of cAMP generated during the assay. Methylxanthines, which are commonly used cAMP phosphodiesterase inhibitors in other systems, cannot be used here since they antagonise the binding of adenosine to its cell surface receptors [Sattin and Rall 1970]. Compounds such as RO 201724, ICI 63 197 and papaverine (non methylxanthine phosphodiesterase inhibitors) must be used instead. (See appendix for the full names and structures of these compounds.)

D. Other possible mechanisms of action of adenosine.

Some of the gross physiological effects of adenosine can be attributed to its uptake into cells where it is converted to ATP which may

influence cellular responses. Adenosine may also combine with cysteine to form S-adenosyl homocysteine which inhibits methylation in cells, often having toxic effects [Fox 1981].

The presynaptic action of adenosine on neurotransmitter release is likely to be due to modulation of calcium transport [Jackish et al 1984, Jonzon and Fredholm 1984]. In smooth muscle, there is a certain amount of controversy over whether or not cAMP is involved in relaxation. The possible alternative is that adenosine might decrease the density of operative calcium channels. The direct negative inotropic effect of adenosine on atria is independent of cAMP. Instead, increased K⁺ flux is felt to be the primary response [Belardinelli and Isenberg 1983a].

The subunit of Ni appears to be involved in some of the functions regulated by inhibitory receptors, for example Ca⁺⁺ gating and phosphatidylinositol turnover. Indeed, Molski and co-workers [1984] have produced evidence for the involvement of Ni in Ca⁺⁺ gating by chemotactic factors in neutrophils. At the time of writing no firm evidence exists for the hypothesis that adenosine receptors and Ni might be coupled to phosphatidyl inositol turnover in heart.

E. Effects of adenosine on the myocardium-gross effects and relation to tissue cAMP.

E1. Coronary Vasodilation.

That there is a connection between coronary blood flow and myocardial metabolic activity has been known for some years [Berne 1980] Adenosine meets most of the criteria for the required factor; it is a potent vasodilator, readily crosses cell membranes, is effectively metabolised and can be supplied by intracellular ATP. An external receptor for adenosine can be shown by the use of high molecular weight derivatives [Olsson et al 1976, Schrader et al 1981] . However, there are a number of points which ought to be taken into account before adenosine is unequivocally assigned the role of coronary vasodilator. The first is that methylxanthines only partially block this action of adenosine, suggesting either uncharacteristic adenosine receptors, another mechanism of action of adenosine not involving cell surface receptors, or the involvement of another substance such as ATP or inosine. Burnstock [1980] suggests that ATP rather than adenosine is the true regulator of vasodilation in hypoxia since ATP and ADP are four

times more potent in dilating coronary blood vessels than are adenosine derivatives. This ATP is proposed to come from purinergic nerve endings. Aviado [1983] recently reviewed the evidence for the involvement of inosine in vasodilation. Inosine, usually thought to be inactive, interacts with specific Inosine-Benzodiazepine receptors to bring about many of its actions. Adenosine is released from the heart during ischaemia. Aviado reasoned that this is likely to be largely converted to inosine during efflux, since it must come into contact with membrane bound adenosine deaminase. This endogenously released inosine has been shown to mediate vasodilation [Faucon et al 1966]. Alternatively Downey et al [1982], as a result of their observation that coronary vasodilation can occur in the absence of increased tissue adenosine during non-ischaemic hypoxia, suggested that myocardial oxygen tension could have a direct effect on arteriolar vascular smooth muscle.

There is rather better evidence for adenosine being the mediator of vasodilation caused by increased cardiac mechanical activity. In dogs and rats constriction of the aorta, leading to increased cardiac work without hypoxia, increases adenosine levels [Foley et al 1978, McKenzie et al

1980] and there is an inverse relationship between coronary vascular resistance and the myocardial adenosine content [Berne 1980]. Similar results were obtained with exercised dogs [Watkinson et al 1979] and with stellate ganglion stimulation of cellular metabolic activity [Miller et al 1979]. Adenosine and its breakdown products are also released during systole as opposed to diastole in the slowed heart [Thompson et al 1980]. This shows that adenosine formation and release are tightly coupled to the cardiac cycle and can serve to produce very rapid adjustments in coronary blood flow.

The mechanism of action of adenosine on coronary vasodilation is still under dispute. Anand-Strivastava et al [1982], Kukovetz et al [1978], Goldman et al [1983], have evidence for an A₂ receptor coupled to adenylate cyclase on the smooth muscle of blood vessels. Hamet^{etal} [1978] and Ollinger and Kukovetz [1983] could find no evidence for cAMP involvement. The other possible mediator of the action of adenosine is Ca⁺⁺. Adenosine can reduce Ca⁺⁺ uptake by reducing the channel density (see atria below), but there is no firm evidence for this as yet [Berne 1980].

E2.Effects on Atrial Tissue.

Adenosine has direct and indirect actions on atrial tissue. The direct effect takes the form of a depression of contraction of the atrial myocardium. The indirect action, commonly known as the antiadrenergic effect, is an antagonism of the β -stimulation of developed tension. This is seen at lower concentrations of adenosine than are required for the direct reduction of tension. The direct effects have been known for some time but it is only recently that the mechanisms involved have been elucidated. Schrader et al [1975b] and Belardinelli et al [1979] studying the action of adenosine on guinea pig and rat atrial muscle found that the effects of adenosine were antagonised by Ca^{++} , and suggested that the reduction in tension by adenosine was mediated by a depression in the permeability of the membrane to Ca^{++} . No alteration of cAMP levels were seen. More recently [1983a], Belardinelli and Isenberg found that K^+ channels were activated by adenosine in a cAMP independent manner in atrial myocytes. The depression of the electrical conductance of the atrio-ventricular node cells by adenosine seems to be mediated by a similar mechanism [Belardinelli et

al 1980]. The receptor mediating these events has been said to be of the A1 subtype as determined by Evans et al [1982] and Collis [1983].

Rockoff and Dobson in 1980 showed that adenosine at concentrations too low to elicit a direct effect in rat atria could antagonise the positive inotropic effect of isoprenaline. Their experiments suggested the site of action to be at or proximal to cAMP formation. Adenosine was found to be released from atria stimulated by catecholamines [Dobson 1983b & c] and was responsible for the steady decline in elevated contractile responses observed with continuous catecholamine administration. Methylxanthines antagonised all of the above effects of adenosine. Hughes and Stone [1983] examined the order of potency of adenosine analogues in eliciting the indirect effect, and found that the patterns obtained conformed to neither the A1 nor A2 subtype. Dipyridamole, which inhibits the uptake of adenosine, had little effect but theophylline antagonised the responses. On the basis of this data they suggested that the indirect effect of adenosine is mediated by an external receptor distinct from the A1/A2 type.

Endoh and coworkers [1983] studying both the

direct and indirect effects of adenosine on guinea pig atrium found them both to be attenuated by prior treatment of the animals with pertussis toxin. As a change in cAMP is found only with the indirect effect, these results raised the possibility that N_i could couple to other proteins beside adenylate cyclase.

E3.Ventricular Myocardium.

Before 1975 a range of contradictory results were reported concerning the effect of adenosine on contractile force development in ventricular myocardium [see Schrader 1981 for a review]. When Burger and co-workers [1975] examined the effects of adenosine under rather better defined conditions than had been used previously they found that adenosine at a concentration of 15 μ M had no effect on basal contractile force but greatly attenuated the inotropic effect of isoprenaline. Schrader et al [1977] found that, in addition to affecting the increase in developed tension, adenosine also inhibited the initial elevation in myocardial levels of cAMP raised by isoprenaline. Theophylline antagonised all these effects of adenosine. When the myocardium was stimulated by catecholamines, adenosine was released in sufficiently high

quantities to exert an antiadrenergic effect [Schrader et al 1977].

Endoh and Yamashita in 1980 showed that adenosine could only inhibit B-adrenergic and not α -adrenergic effects in rabbit papillary muscle. However, in addition to depressing B-adrenergic effects, adenosine has also been reported to inhibit the positive inotropic actions of histamine and dopamine [Bauman et al 1981] .

E3a. Species Differences

There may be some species differences in the antiadrenergic effect since the inhibition seen on rabbit papillary muscle was only 40% of the isoprenaline-induced rise in tension [Endoh and Yamashita 1980] compared with a maximum of 90% inhibition seen with the guinea pig Langendorff preparation [Schrader et al 1977]. In fact Burnstock and Megh^ji [1983] were unable to see any action at all on rat ventricular strips but this may be a reflection on the preparation used, since Dobson [1983] using a Langendorff perfused rat heart was able to confirm Schrader's original observations with respect to the effect of adenosine on tension and cAMP levels.

E3b. Electrophysiological studies

Studies on the electrophysiological effects of adenosine have lent support to the impression that only an indirect, antiadrenergic effect is seen on ventricular myocardium. Belardinelli and Isenberg [1983b] working with bovine and guinea pig myocytes were able to demonstrate an antiadrenergic effect of adenosine on the shortening of the action potential and shortening of sarcomeres. The adenosine concentrations required to produce these effects were in the range of the amounts of adenosine released from the cell when the oxygen is limited (ie. in these experiments 1-100 uM). Rardon and Bailey [1984] demonstrated a similar antiadrenergic effect of adenosine on various electrophysiological parameters of isolated Purkinje fibres. An A1 receptor was implicated.

E3c. Lack of Correlation between Tension and cAMP.

Correlation between the observed effects on tension and on cAMP is still lacking. Neither Schrader et al [1977], Bauman et al [1981] nor Dobson [1983a] were able to observe a good correlation between the effects of adenosine on tension and its inhibition of adenylate cyclase activity in a crude membrane preparation. Indeed

their assay conditions for adenylate cyclase (high Mg^{++} , no Na^{+} , no steps taken to reduce endogenous adenosine) would tend to favour the expression of the P-site. Many workers report that hormonal stimulation of adenylate cyclase increased the potency of P-site agents [Cochaux et al 1982, Wolff et al 1978, Glynn and Cooper 1978, Bregman et al 1980, McKenzie and Bar 1973, Rodbell and Londos 1976 and Johnson and Welden 1977]. This observation could explain the antiadrenergic effect obtained by the above groups in their in vitro-assay.

In a recent paper by Bohm and co-workers [1984] evidence was presented against cAMP being the mediator of the effects of adenosine on the heart. Although they could see the usual antiadrenergic effect of adenosine on tension in guinea pig atrial and papillary muscles, the nucleoside failed to attenuate the isoprenaline-stimulated cAMP levels. In agreement with other workers in this field, they found adenosine able to inhibit isoprenaline-stimulated adenylate cyclase, but in a manner which seems more typical of P-site activity. They concluded that in atria the adenosine-induced increase in K^{+} current is likely to be more important, and that the mechanism of action of adenosine in the ventricle must be distal to cAMP, perhaps at the level of the protein

kinases.

In an attempt to determine whether or not the P-site could play a physiological role in the observed antiadrenergic effects of adenosine, Dobson and Schrader collaborated in 1984 on a series of experiments designed to determine if intracellular or extracellular adenosine was more important in attenuating the enhancement of cardiac contractile state and glycogenolysis induced by catecholamines. Nitrobenzylthioinosine (NBMPR) was used to prevent adenosine transport out of the cell. Hypoxia caused adenosine to be released from the myocardium and reduced the isoprenaline-induced rise in myocardial cAMP, cAMP dependent protein kinase activity and contraction. NBMPR completely prevented the reduction of the isoprenaline-induced increases in cAMP and protein kinase but only partially prevented the attenuation of the contractile response. They concluded that an increase in extracellular adenosine is more influential than elevation of intracellular adenosine in attenuating B-adrenoreceptor elicited increases in myocardial cAMP content, cAMP protein kinase activity and to some extent contractile state.

F.Effects of adenosine on myocardial adenylate cyclase.

What the studies described in the previous section have established is that adenosine exerts an indirect, antiadrenergic effect on tension and a similar indirect effect on adenylate cyclase in a particulate preparation. However, a good correlation between the two effects has not yet been shown. This is probably due to the fact that the assay conditions used by Schrader's group and by Dobson would tend to favour the expression of the P-site which would swamp any A-type receptor mediated effects. These problems have prompted many research groups to use more strictly controlled conditions in their experiments to determine whether or not A-type receptors are present in ventricular tissue and are coupled to adenylate cyclase.

F1.Effects of Adenosine on cAMP in Tissue Slices and Myocytes.

Huang and Drummond, [1977,1978] reported that adenosine stimulated cAMP accumulation in atrial and ventricular slice preparations of guinea pig.

This was antagonised by methylxanthines.

Stimulation by adenosine is a surprising finding considering the opposing effects of adenosine and B-agonists on contraction, especially as there is very good evidence for a cause and effect relationship between catecholamine stimulation of cAMP levels and increase in contraction [reviewed by Drummond and Severson 1979]. Adenosine did not interfere with the B-stimulation of adenylate cyclase, the two stimulations being in fact, additive. Dobson [1978] using a similar ventricular preparation from rat heart found that adenosine had an antiadrenergic effect on cAMP levels but no effect on basal cAMP. Differences in experimental conditions may explain this discrepancy. The incubation medium used by Huang and Drummond could favour the expression of stimulatory receptors on coronary arteries, whereas the slightly different conditions used by Dobson could enhance the expression of inhibitory receptors on coronary myocytes. It might be thought that experiments on myocytes would help to clarify the issue since there will be no interference from cAMP production by the other cells of the heart, but some contradictions still remain. Hazeki and Ui [1981] found that IAP treatment prevented adenosine- or muscarinic-receptor-mediated inhibition of

adenylate cyclase while potentiating the stimulation by B-agonists in adult rat myocytes. On the other hand, Anand-Strivastava and Cantin [1983] using cultured cardiocytes from atria and ventricles of neonatal rats found stimulation by adenosine and its analogues in both atrium and ventricle. This stimulation was GTP-dependent and was antagonised by methylxanthines. As observed by Huang and Drummond, stimulation by adenosine and catecholamines was additive, at least with atrial cardiocytes. However, cultured cells from neonatal animals are thought to behave differently from adult cells and receptor-activator mechanisms may change during development, [Kuisak and Pitha 1981].

F2.Effects of Adenosine on Adenylate Cyclase in Isolated Sarcolemmal Membranes.

Early investigations of the effects of adenosine on isolated membranes failed to find any A₁/A₂ mediated actions on the cyclase, only the usual P-site events [Bar and McKenzie 1973, Avdonin et al 1982, Schutz and Tuisl 1981]. Schutz and Tuisl suspected that steps in the preparation of their membranes may have led to loss of receptors. If the data presented in their paper is examined, it will be noted that there is no stimulation by GTP which leads one to suspect some modification of N-protein

function. Webster and Olsson [1981] reported that adenosine stimulated the adenylate cyclase of dog heart, the characteristics being typical of A2 adenosine receptors. It should be noted that their assay mixture contained no Na^+ , and GppNHp was used as the added guanine nucleotide. GppNHp, as mentioned earlier, is reported to prevent the action of inhibitory agonists. In brain striatum, both A1 and A2 receptors are present and adenosine analogues will inhibit or stimulate adenylate cyclase activity depending on the assay conditions [Yeung and Green 1984]. Since, in a heart homogenate there are likely to be inhibitory receptors from cardiac myocytes and stimulatory receptors from coronary smooth muscle cells, modification of the assay conditions could favour the expression of one or the other. The assay conditions used by Webster and Olsson would favour expression of stimulatory receptors.

In 1983 Leung and co-workers reported that adenosine could inhibit adenylate cyclase in homogenates of guinea pig ventricle in a GTP and Na^+ dependent manner. The inhibition exhibited a rather sharp dependency on Na^+ concentration, the optimum being 50 mM. No appreciable inhibition was seen at 100 mM, the concentration usually reported to be optimal in other inhibitory systems [see Londos et

al 1981]. The addition of isoprenaline was not necessary for the expression of this inhibition. This is in contrast with the findings of Hosey et al [1984] who found that PIA could antagonise the effect of isoprenaline on cAMP accumulation and phospholamban phosphorylation in chick heart sarcolemma but had no effect in the absence of B-stimulation. Binding studies indicated the presence of A1 receptors. Lohse and coworkers [1984] have been able to provide evidence for A1 receptors by radioligand binding in isolated membranes from bovine heart but were unable to see any alteration in adenylate cyclase activity. Schutz and coworkers [1984] also report that there are no adenosine receptors coupled to adenylate cyclase in the heart, although using the same assay conditions they were able to demonstrate their existence in cerebral cortical membranes.

G.Scope of This Investigation.

Briefly, this study is concerned with an examination of the effects of adenosine on the atrial and ventricular myocardium of guinea pig and rabbit in relation to changes in tension and adenylate cyclase activity. The main points under

investigation are discussed in more detail below.

G1. Possible Reasons For Discrepancies in The Reported Antiadrenergic Effects of Adenosine

The disparity between the results of Schrader et al [1977] and Endoh and Yamashita [1980] concerning the extent of the antiadrenergic effect of adenosine led me to consider that there might be a species difference between guinea pig and rabbit in the way that the myocardium reacts to adenosine. Rabbit hearts have been reported to have poor autoregulation of coronary vasodilation by adenosine [Day and Johnson 1959] and it may be that adenosine is of limited importance in negative feedback in response to sympathetic overstimulation of the heart. Other agents such as prostaglandins may be more important here [Moretti and Abraham 1978]. If the disparity can be shown to be due to species differences rather than experimental artefact, then this would be an important observation.

However, such a difference in inhibitory effect could have arisen in a number of ways. Pressure recordings in the Langendorff preparation are susceptible to artefact due to the effects of coronary vasodilatation combined with the erectile

properties of the vascular system. Papillary muscles, on the other hand, may be in a condition of hypoxia in which high concentrations of endogenous adenosine may mask the effects of adenosine added to the superfusate. Also the concentration of isoprenaline used to elicit the positive inotropic effect may be important, if the effect of adenosine is confined to the lower doses of this positive inotrope.

The experiments described⁵ in chapters 2 and 3 were designed to determine if such artefacts were sufficient to account for the observed discrepancy. Chapter 4 explores the possibility of a species difference, taking the findings of the earlier chapters into account.

62.Determination of The Involvement of the Known Components of Adenylate cyclase, N-protein, adenosine receptor system.

To find if the extracellular A-type receptors are involved in the response of the myocardium to adenosine, the influence of methylxanthines was studied. Since methylxanthines, in addition to being potent antagonists of adenosine receptors, are amongst the most well-used phosphodiesterase inhibitors, parallel experiments were performed

using papaverine, a phosphodiesterase inhibitor with no action at adenosine receptors. The use of methylxanthines will also provide indirect evidence as to whether or not the P-site is involved in the response, since the action of ribose-modified analogues at the P-site is not antagonised by methylxanthines, although theophylline may directly inhibit adenylate cyclase through this site, (see later, Chapter 7).

The involvement of N_1 can be determined using pertussis toxin. If a given response is abolished in toxin treated animals, then it can be stated that the effect is mediated via the inhibitory N -protein. The response of guinea pig papillary muscles and atria to 2-chloroadenosine was examined using hearts from control and pertussis treated animals to find if N_1 is involved in the indirect effect of adenosine on ventricle and/or the direct effect of adenosine on atria.

6.3. Relationship Between The Observed Changes in Tension and Effects on cAMP levels, Adenylate Cyclase Activity and cAMP Breakdown.

Those workers who have been able to demonstrate an antiadrenergic effect of adenosine on tension have also been able to show a similar

effect on cAMP levels in whole muscle [Schrader et al 1977, Dobson 1983]. However, in biochemical studies involving measurements of cyclase activity in ventricular membrane preparations the picture is not so clear, with the effects reported ranging from stimulation through no effect to inhibition of adenylate cyclase (see above, section F).

Some of these discrepancies can be attributed to species differences, but most of them are likely to be due to differences in experimental design. As discussed earlier, if both A1 and A2 receptors are present in a given homogenate the manipulation of the assay constituents will tend to favour the expression of one or other of the receptors.

In chapter 2 I describe experiments performed to determine the effect of adenosine on cAMP levels in guinea-pig heart. The extraction procedure used was the acid/alcohol one recommended by Bates et al in 1978 to ensure inactivation of all enzymes involved with adenine nucleotide metabolism.

In chapter 5 I have attempted to find the optimal conditions for expression of A1 receptors in membranes from guinea pig and rabbit heart. Other factors which might influence receptor expression were also explored. Finally, the effect of PIA on breakdown of cAMP was studied. The

experiments on adenylate cyclase were performed in the presence and absence of isoprenaline to determine if the antiadrenergic effect is reflected in changes in adenylate cyclase activity. Rabbit and guinea pig were studied together to determine whether differences noted for the effects of adenosine on contraction were also seen with the adenylate cyclase activity of isolated membranes.

G4. Species Differences With Respect to P-site Inhibition of Adenylate cyclase.

Schrader et al [1977], Dobson [1983], and Bohm et al [1984] also performed experiments on a crude homogenate of myocardium. They all saw inhibition of adenylate cyclase when stimulated by isoprenaline, but in a manner more typical of P-site activity. This antiadrenergic effect at the P-site is interesting, and could be explained if we assume that B-stimulation renders this site more sensitive to inhibition by P-site agents (see earlier, section E). The P-site of the heart has never been studied extensively, so in chapter 6 I have characterised the inhibition of adenylate cyclase by a P-site agent, 2'-deoxyadenosine 3'-monophosphate, before determining if this site could be of importance physiologically. I compared the responses of rabbit and guinea pig membranes to

the P-site agonist to see if there was any species difference.

This then, is a summary of the problem under investigation and the intended approach to it; a more detailed explanation of the rationale behind each group of experiments will be found in the introductions to the separate chapters.

Methods

SECTION A. EXPERIMENTAL PROCEDURES FOR ISOLATED
HEART MUSCLE PREPARATIONS.

1. Perfusion of an Isolated Non-Working Whole
Heart.

The basic method for the perfusion of an isolated non-working heart remains similar to that used by Langendorff in 1885. A physiological salt solution is delivered to the heart via a cannula tied into the aorta so that its tip is above the aortic valve and the coronary ostia. The position of the cannula allows perfusion of the coronary circulation, but prevents fluid from entering the chambers of the heart directly. The perfusion fluid enters the right and left coronary arteries and after traversing the capillary bed drains into the right atrium via the coronary sinus, or into the left ventricle via the thebesian veins. The rate of perfusion is controlled by a roller pump.

a. Animals. Dunkin Hartley guinea pigs weighing from 200 to 500 g were used in these experiments. They were given 2,500 units of Heparin intraperitoneally before being killed by cervical dislocation.

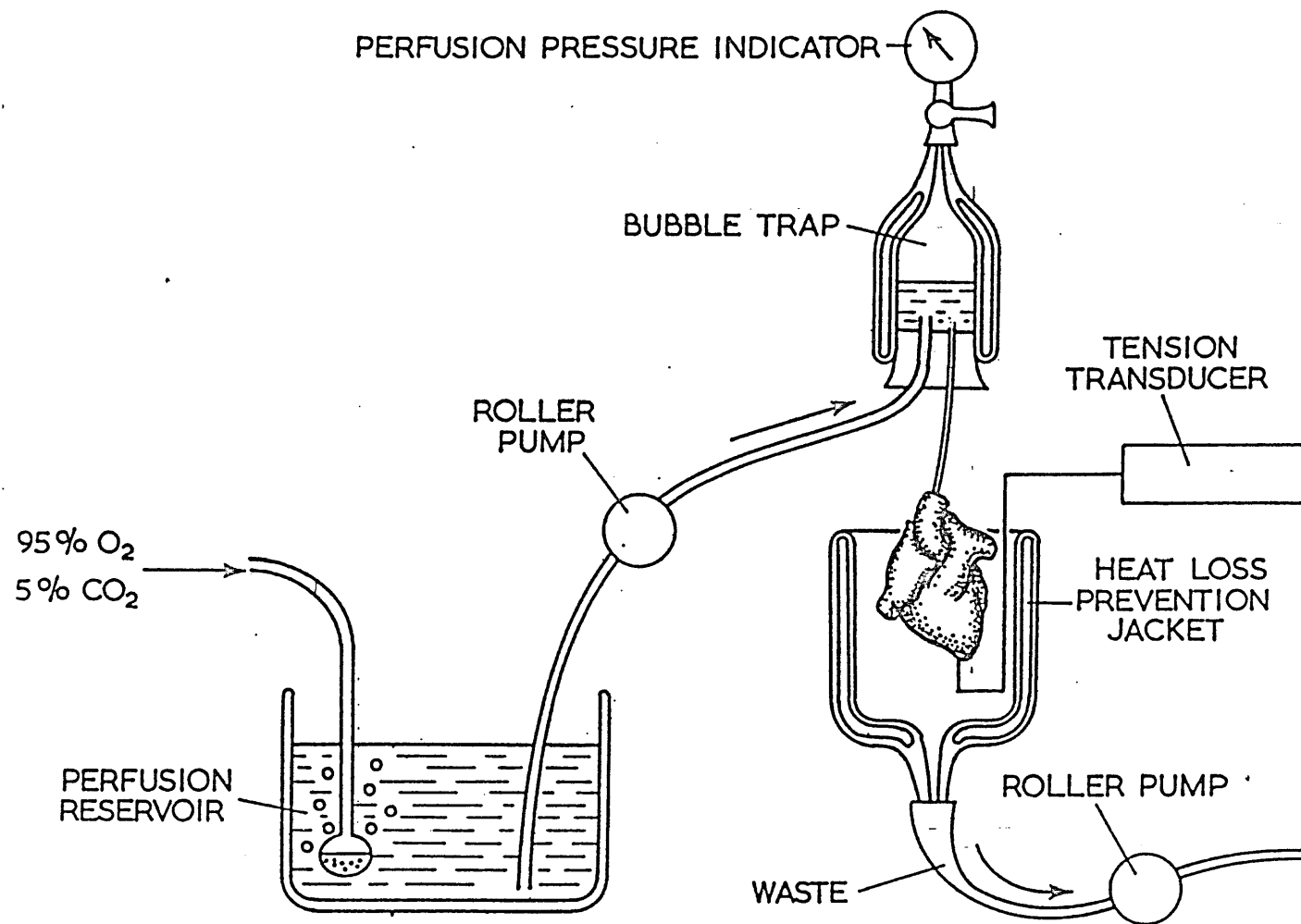


Figure 1.1 Diagrammatic representation of the arrangement of the equipment used for the measurements of tension with the langendorff perfused heart.

b. Perfusion of hearts. The hearts were rapidly removed from the animal and immersed in ice-cold perfusate (the composition of which was (mM): NaCl 119, KCl 4.7, MgSO₄.7H₂O 0.94, KH₂PO₄ 1.2, NaHCO₃ 25, Glucose 11.5, Ascorbate 0.1 and CaCl₂ 2.5). After the extraneous fat and connective tissue had been removed, the aorta was exposed and the heart suspended from an aluminium cannula (1.0 mm in diameter). The hearts were perfused with a physiological salt solution. The solution was gassed in the perfusion reservoir with 95% O₂, 5% CO₂. After cannulation, flow was gradually increased until the systolic perfusion pressure reached about 60 mm Hg. The wall of the left ventricle was pierced so that the ventricular cavity could drain. Contraction was recorded with a Devices recorder. A stabilisation period of 20 minutes was allowed after cannulation before any experimental intervention was made.

c. Control of Heart Rate. Since administration of adenosine at micromolar concentrations causes atrio-ventricular block [Schrader et al 1977], the hearts were paced at 4Hz using supramaximal rectangular pulses of 0.2mS duration delivered from a model 6032 stimulator (Scientific and research instruments, Croydon, England). The stimulus was

delivered by a pair of platinum electrodes inserted into the left ventricular wall. The stimulus voltage was usually less than 10 v.

d. Control of Temperature. Perfusate was kept at 37°C . Heat loss from the perfusion system was minimised by placing the perfusate in a water-bath maintained at 37°C, and by surrounding the adjustable height reservoir and bubble trap by a heated water jacket in which the circulating water was maintained at 37°C

e. Method of Perfusion. A roller pump (model MHRE 200, Watson Marlowe, Falmouth, England) pumped perfusate to the bubble trap. Flow rate was changed by altering the speed of the pump.

f. Measurement of Resting and Developed Force. Contraction was monitored with a force displacement transducer (model UF1, Devices, Welwyn Garden City, England), the output from which was linear for loads up to 30g . Enclosing the heart within an isothermic chamber prevented direct connection between the transducer and the heart. A steel rod, 1mm in diameter was bolted to the tension transducer and inserted into the chamber beside the bubble trap. The steel rod was bent underneath the

heart and attached to the apex with a ligature (Figure 1.1). The force of myocardial contraction was relayed through the rigid rod to the transducer.

g. Freeze-clamping of hearts and measurement of tissue cAMP.

- i. Hearts were removed, weighed, and set up as described above (sections a-e).
- ii. After the 20 minute equilibration period, inotropic interventions were made and the hearts were freeze clamped using aluminium freeze clamp tongs precooled in liquid nitrogen. Preliminary experiments showed that cAMP formation peaked 1 minute after the isoprenaline solution had reached the bubble trap, and isoprenaline treated hearts were clamped after this interval.
- iii. After clamping the heart was broken up into slivers and placed in a test tube containing liquid nitrogen where it could be stored until all the hearts to be clamped that day had been collected.
- iv. When all the day's hearts had been collected, each heart in turn was tipped into a mortar and ground up with a pestle under liquid nitrogen, and the slurry tipped into a marked test-tube at -10°C . 2mls of 10% trichloroacetic acid containing 25 % methanol was added to this. All samples were

homogenised using a Polytron homogeniser (setting 5 for 2x 3 secs). The homogenate of each heart was tipped into a marked centrifuge tube and the blade of the Polytron washed in a further 2mls TCA/MeOH. The Polytron blade was also rinsed in distilled water between samples.

v. When all the hearts had been homogenised, a 1.5 ml sample of the homogenate was removed for the determination of the recovery of cAMP. 10 μ l of a radioactive cAMP solution (containing 250 mCi in 250 μ l) was added to each of the recovery controls. All the radioactive tubes were vortexed and all heart samples centrifuged at 9k x g for 20 minutes at 2°C.

vi. The supernatant was taken and the methanol extracted from this twice with an equal volume of ether. The pH was determined and each aqueous extract neutralised with Tris crystals. The extracts were freeze dried overnight. 1 ml of the Amersham kit Tris EDTA buffer (see section B2, this chapter) was added to the freeze dried extract. More Amersham buffer and cAMP standard was made up containing the same amount of extra Tris as was added to the heart extracts. This 'extra-Tris' buffer was used for the cAMP standard curve and for further dilutions of the extract. Preliminary studies showed that other substances in the extract

interfered with the cAMP assay , and the extract had to be diluted 1 to 20 to avoid this. Samples were taken and assayed with the Amersham International cAMP assay kit as described in section B2b of this chapter.

vii. The pellet was washed with ether and centrifuged at $9k \times g$ for 20 minutes. The ether was decanted off and the washing/centrifugation step repeated. The ether was decanted off and the pellet dried under a stream of nitrogen. 0.9 ml of water per 100 mg wet weight (approx) was added to each tube and the appropriate amount of 3M NaOH added to this to give a solution containing 0.3M NaOH . Glass beads were added to the tubes, and the tubes stoppered and mixed. The protein solutions were usually left to dissolve overnight before the protein was assayed by the Biuret method [Gornall et al 1949].

viii. Biuret reagent was made up as follows; 1.5 g $CuSO_4 \cdot 5H_2O$ was dissolved in 5 ml water, 6g of NaK tartrate and 30 g NaOH was added to this and the solution was diluted to 900 ml with water before 1 g of KI and 15 g deoxycholate were added, the solution was warmed to dissolve the latter two components before making up to 1 L with water. A volume of 0.5 ml of either sample or bovine serum albumen standard was placed in plastic test tubes

in duplicate. 3ml of Biuret reagent was added to this, the tubes covered and boiled for 2-6 minutes. After cooling, the absorbance at 540 nm was read.

ix. Recovery of cAMP was determined as follows. 2 x 200 ul of the reconstituted freeze dried radioactive extracts were placed in 10 mls ACS scintillation fluid. Values for the maximum counts obtainable were found by placing 10 ul of the radioactive cAMP in 1ml 'extra-Tris' buffer (see above), and taking 2 x 200 μ l samples for counting. The counts obtained were converted to disintegrations per minute. The dpm for the samples were expressed as a percentage of the maximum. The average recovery of this method proved to be 75.4% . Since there seemed to be no systematic variation in recovery between differently treated hearts, the average recovery for each day's hearts was used to correct for the actual cAMP obtained.

2. Superfusion of Rabbit and Guinea Pig Papillary Muscles.

In this laboratory a method for superfusing small mammalian papillary muscles has been developed. This has been used by myself during these studies and is described in detail below.

a. Animals. Male Dunkin-Hartley guinea pigs, 200-500g and male New Zealand white rabbits, 2.0-2.5 Kg were used. The rabbits were killed by a sharp blow to the head, and the guinea pigs were heparinised and killed as described in the previous section.

b. Superfusion of Muscles.

i. Rabbit hearts were rapidly removed and immersed in ice-cold perfusate. After the extraneous fat, connective tissue and atria had been removed, the ventricles were pinned onto a dissecting dish. The wall of the right ventricle was dissected open close to the intraventricular septum and pinned back. A papillary muscle was selected, clipped with the removable clip from the apparatus (figure 1.2) at the muscle end and dissected free of the heart. The clip with the muscle was then replaced in the

Equipment for superfusion of guinea-pig and rabbit papillary muscles

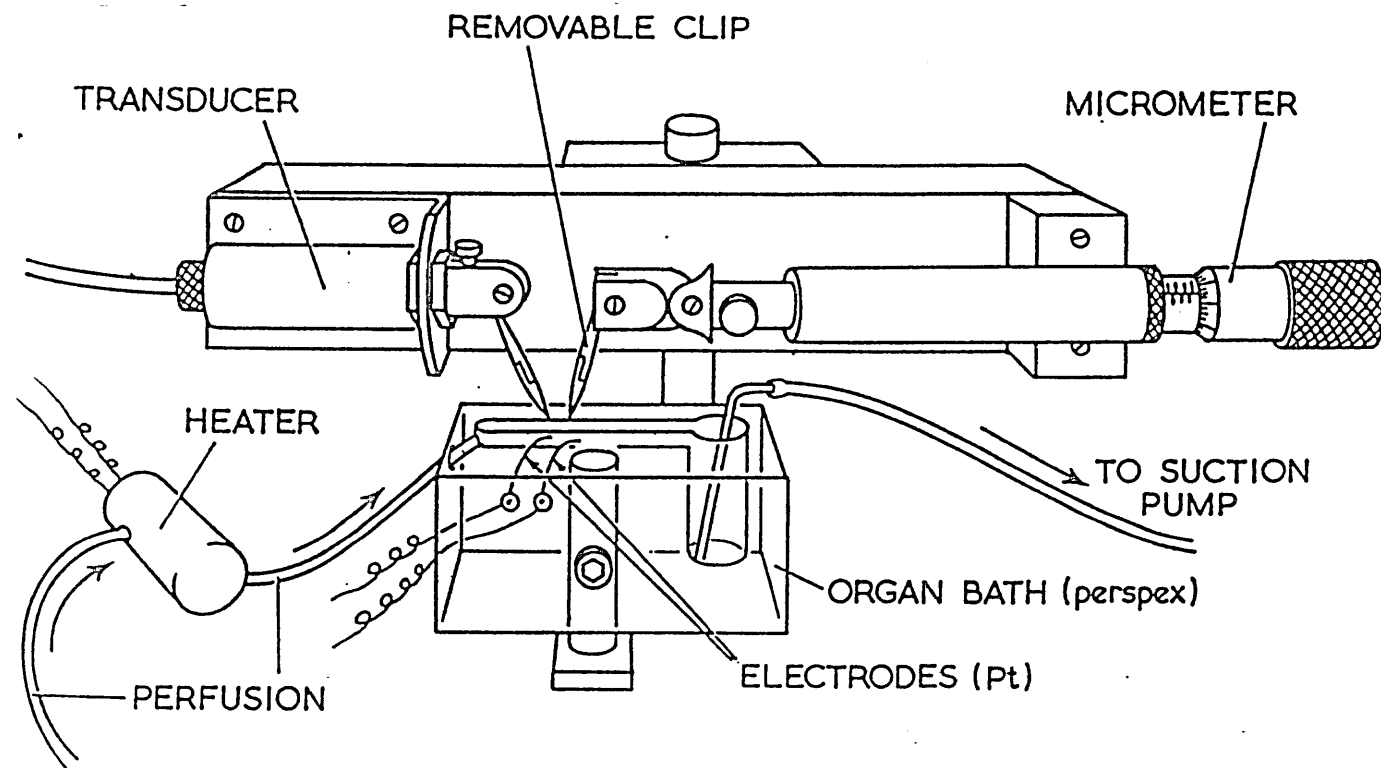


Figure 1.2 The arrangement of the equipment with rabbit or guinea pig papillary muscle.

end of the micrometer, and the bath raised so that the muscle entered the superfusing fluid. Using the microscope for viewing the muscle, the micrometer was adjusted until the tendon of the muscle entered the opened clip attached to the tension transducer. Physiological salt solution was kept flowing through the muscle bath while the muscle was being set up. (See section A1b for composition of salt solution). The solution was gassed in the distribution bottle or perfusion reservoir with 95% O₂, 5% CO₂. The muscle was stretched to close L_{max} (the length which gives the maximum contraction) by moving the micrometer in small stages during the equilibration period of 1 hr.

ii. Guinea pig papillary muscles. The hearts were removed and dissected open in the same way as described for rabbit. Guinea pig papillary muscles tend to be very small or part of a branched system. A thin layer of connective tissue often covers the muscles making it difficult to remove them easily and insulating them electrically against the contraction stimulus. After the heart was dissected open, the dissecting dish was transferred to a platform beneath the dissecting microscope and the rest of the dissection carried out there. Using small straight forceps, a piece of braided suture

silk was passed beneath the muscle at one end and tied. This was repeated at the other end of the muscle. The papillary muscle was then cut free of the surrounding tissue and the braided suture silk put into the clips. The position of the clips was moved and the suture silk pulled through them until the muscle itself or the knots were in the grip of the clips. The muscle was now stretched and stimulated as described above for rabbit papillary muscle.

c. Control of Contraction Rate. The muscle was stimulated by field stimulation, (using the two electrodes fixed permanently in the bath) or by point and field, the point stimulation being through the clip attached to the micrometer. The muscles were usually stimulated at 1 Hz using suprathreshold rectangular pulses of 0.2mS duration delivered from a model 6032 stimulator. The stimulus voltage was usually less than 10 v.

d. Control of Temperature. The superfusate was maintained at 30°C or 37°C by a heating coil immediately before the organ bath, and monitored using a thermistor in the bath. The thermistor reading was linearly related to temperature in the range 20-40°C. The thermistor reading was displayed

on a purpose-built digital readout meter.

e. Method of Superperfusion. The flow rate through the system was maintained at 6 ml min⁻¹ either by adjusting the height of the distribution bottle containing the superfusate or by altering the speed of the roller pump which was used in the later experiments. When the distribution bottle system was in use, small volumes of drug, etc. were delivered from 60 ml syringes which fitted into the tap system. It was found that there was a drop in flow rate as the syringe emptied and this method was eventually abandoned in favour of the roller pump method.

f. Measurement of Resting and Developed Tension. Contraction was measured using a force displacement transducer (Statham, USA). This was calibrated in the range of tensions likely to be elicited by rabbit or guinea pig muscles before any series was started and at intervals of one month thereafter. The resting and developed tension was recorded on a Devices pen recorder, as was the differentiated signal. Both signals were displayed on a Telequipment oscilloscope (model DM64). The force of contraction was relayed to the transducer via the clip holding the tendons.

3. Preparation of isolated guinea pig atria and papillary muscles in organ baths for simultaneous measurement of contractile force.

Animals were killed and the hearts removed as described previously (section A1a).

a. Atria. After removal of extraneous fat and tissue, the left atrium was selected, removed and placed in Krebs-Henseleit solution at room temperature. The ventricles were placed in Krebs-Henseleit solution which was oxygenated with 95% O₂ 5% CO₂. The atrium was returned to the dissecting dish and a length of braided suture silk was passed through the atrium, using a curved needle, at the point where it would have been attached to the ventricles. This silk was tied in such a way as to leave a loop from which the atrium could be suspended. The atrium was hung from the small hook attached to the tension transducer. (see figure 1.3). The free end of the atrium was clamped into the small bulldog clip attached to the stimulation rod. The whole unit was then lowered into the organ bath. The muscle was stretched by

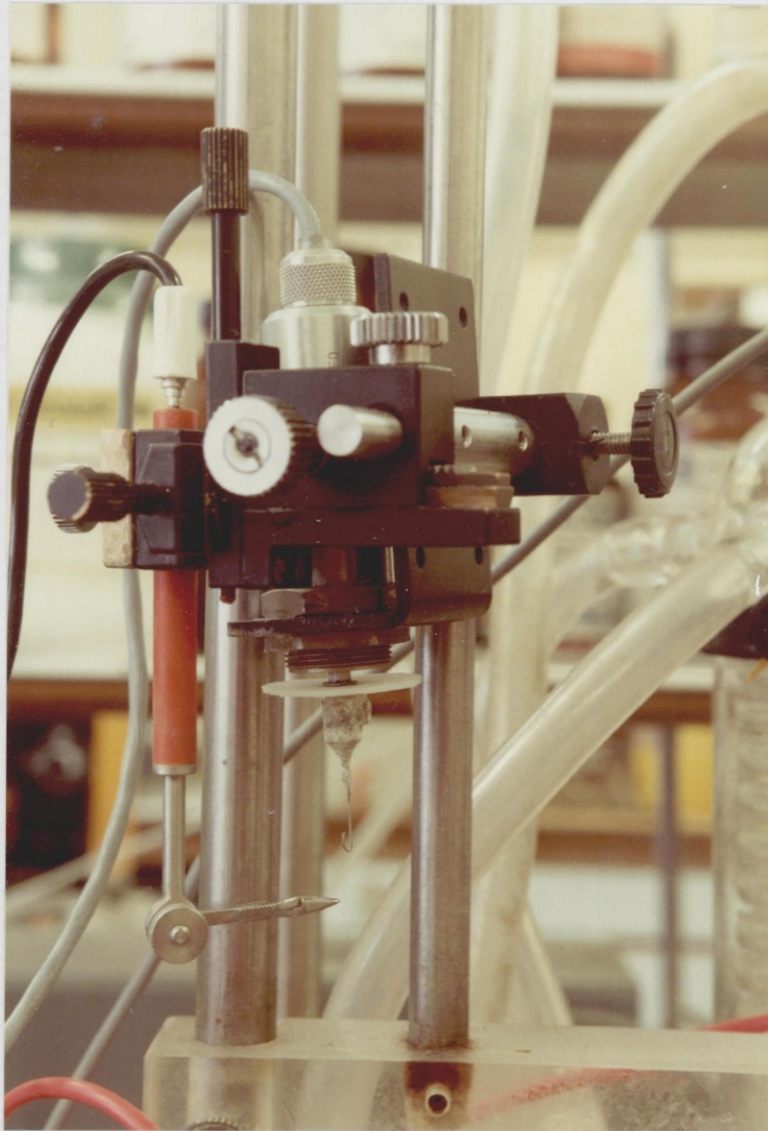


Figure 1.3 A Photograph of the equipment used for the measurement of tension of either guinea-pig atria or papillary muscles.

during the 1 hour equilibration period.

b. Papillary muscles. Guinea pig papillary muscles were removed as described previously (methods A2b), except that a double ended hook was tied onto one end of the muscle before removal of the papillary muscle from the heart. The muscle was then set up on the apparatus in a similar manner to that described for atria.

c. Control of contraction rate. Both atria and papillary muscles were stimulated by point and field electrical stimulation. The point stimulation was delivered through the clip which held the muscle at the lower end while the field stimulation was provided by a platinum electrode attached to the side of the bath. Both preparations were usually stimulated at 1 Hz using suprathreshold rectangular pulses of 0.2 ms duration delivered from separate model 6032 stimulators. The stimulation voltage was usually less than 10 v.

d. Control of temperature. The bathing solution was maintained at 30°C by means of circulating water (at 32°C) which passed through the water jacket surrounding the bath. Fresh perfusate entering the bath was pre-heated to 30°C by passing it through a

surrounding the bath. Fresh perfusate entering the bath was pre-heated to 30°C by passing it through a similar water-jacketed column.

e. Filling and emptying of organ bath. The bath was filled from the reservoir by means of the taps located on the front of the apparatus.

Krebs-Henseleit solution was allowed to enter the bath until it started to escape through the overflow. To change the bathing solution, the bath was completely emptied and refilled twice, since attempts to change the bathing solution using the overflow proved unsatisfactory. Muscles were oxygenated through the sintered glass at the bottom of the bath.

f. Measurement of resting and developed tension. Contraction was measured using a force displacement transducer (Statham, USA). This was calibrated by hanging small weights on the hook attached to the transducer before the start of each day's experiments. The resting and developed tension was recorded on a Devices pen recorder or an Elcomatic E740 pen recorder, Elcomatic, Kirktonfield road, Neilston, Glasgow.

4. Guinea Pig Injection Protocol.

a. Reserpinisation. When reserpinised hearts were required, guinea pigs were treated with reserpine 24 hours before being killed. Reserpine was dissolved in the minimum amount of Tween 80 and diluted with saline to give a final concentration of 10 mg ml⁻¹ reserpine. Guinea pigs were given 10 mg Kg⁻¹ body weight, delivered intraperitoneally.

b. Treatment with Pertussis Toxin. Pertussis toxin was obtained frozen on dry ice. It was dissolved in 50 mM Tris, 1M NaCl; pH 8.0. The sample was thawed and aliquoted so that there was enough to dose an animal weighing up to 400g in each tube. An equal volume of glycerol was added to this. The tubes were labelled and stored at -80 until required. After thawing, the sample was diluted with saline to give a convenient dose in ug/ml injection volume. (eg 10 ug per 100 ul). Control animals were injected with 50 mM Tris, 1M NaCl pH 8.0 / glycerol (50:50) diluted with saline in the same ratio as for the test animals. The animals were left for 3-4 days until required.

SECTION B MEASUREMENT OF ADENYLATE CYCLASE ACTIVITY
IN MEMBRANES ISOLATED FROM THE VENTRICULAR
MYOCARDIUM OF GUINEA PIG AND RABBIT.

1. Isolation of Membranes.

a. Isolation of Sarcolemma Enriched Fractions from
Cardiac Muscle.

This method is a modification of the one described from this laboratory by Mas-Oliva et al [1979], which was adapted from that described by St Louis and Sulakhe [1976]. The process involves homogenisation followed by solubilisation of the contractile proteins, differential centrifugation and density equilibration in discontinuous gradients. The fractions to be taken were characterised as having sarcolemmal properties in this laboratory by Mas-Oliva et al [1979] with respect to (Na⁺, K⁺) Mg⁺⁺ ATPase, ouabain sensitivity, (Ca⁺⁺, Mg⁺⁺) ATPase, NaN₃ sensitivity, Cytochrome C oxidase and adenylate cyclase. Structural differences with sarcoplasmic reticulum and mitochondrial preparations were determined by Mas-Oliva et al using electron microscopy.

Procedure. i. Removal of the Hearts and Homogenisation.

Rabbits were killed by a sharp blow to the head. Three or four hearts were quickly removed from the rabbits and transferred to 10mM Tris-Acetate buffer at pH 7.5 containing 2mM Dithiothreitol (TD buffer) at 4°C . The ventricles were trimmed of fat, atria and great vessels before being cut into small pieces. The tissue was homogenised in 10 volumes (v/w) of TD buffer with an Ultra-Turrax homogeniser on setting 9 or with a Polytron homogeniser on setting 6 for two three second bursts separated by a period of cooling on ice.

ii. Solubilisation of Contractile Proteins.

The resulting homogenate was filtered on gauze (50 u), its volume measured and 4M KCl in TD buffer added to give a final concentration of KCl of 1.25 M. The homogenate was kept on ice and stirred gently for 10 minutes. After stirring, it was centrifuged for 10 minutes at 9K x g ($K=1,000$) (in a Sorval RC-5B or MSE 18 or 21). The resulting pellet was suspended in 10 volumes of 1.25 M KCl in TD, stood on ice and given 10 minutes' gentle stirring.

The homogenate was centrifuged for 10 minutes at 4K x g . The resulting pellet was suspended in 10 volumes of TD buffer and centrifuged at 3K x g. The last step was repeated.

iii. Sucrose Density Centrifugation.

The pellet was resuspended in 10% sucrose in TD and layered on a sucrose gradient of 1.83, 1.60, 1.39 and 1.17 M (2,5,5,5 ml). This sucrose was made up in TD buffer at pH 8.2. The membranes were centrifuged on the gradient for one hour at 40K x g. The fraction banding between 1.39 and 1.60 was taken, washed in TD buffer and centrifuged for 20 minutes at 11K x g. The pellet was resuspended in TD and placed on a sucrose gradient of 1.83, 1.60, 1.53 and 1.39 M (2,5,5,5). This was centrifuged for one hour as before. The fraction between 1.39 and 1.53 as well as the fraction between 1.53 and 1.60 were collected (since both of these fractions were found to have high adenylate cyclase activity), diluted x3 with TD buffer and centrifuged for 20 minutes at 11K x g . The resulting pellets were homogenised in an appropriate amount of TD buffer and 0.5 ml aliquots dispensed into small plastic containers which were sealed, labelled and stored at -80 °C until required. Previous work in this laboratory had shown that this preparation could be

kept at -80°C for up to 8 weeks without loss of cyclase activity [Leclerc et al 1984].

b. Preparation of a Washed Homogenate from Guinea Pig Ventricles.

The above preparation was repeated with guinea pig hearts, but it was found that the increase in cyclase activity due to the purification of the membranes did not compensate for the loss of activity during the prolonged purification procedure.

The average basal cyclase activity of a washed crude homogenate was 58.7 ± 14.0 pmoles cAMP min/mg/protein ($n=3$), whereas the activity of the sarcolemma enriched fraction was only 69.3 pmoles cAMP min/mg/protein. As very little product is obtained by this method, it was felt that it would be better to use a washed homogenate. This has the added advantage of not having a potentially damaging 'high salt' step.

Procedure

A heart was quickly removed and transferred to TD buffer at pH 7.5 and 4°C . The ventricles were trimmed of fat and blood vessels before being cut

into small pieces. The tissue was homogenised in 10 volumes (v/w) TD buffer with the Polytron on setting 6 for two 3 second bursts separated by cooling on ice. The resulting homogenate was filtered on gauze (50 u) and centrifuged for 10 minutes at 11K x g in the Sorvall or MSE centrifuges. The pellet was resuspended and recentrifuged. After the second centrifugation, the pellet was suspended in the appropriate amount of TD buffer. Aliquots of 1ml were dispensed into small containers, sealed, labelled and stored at -80°C until required. *The preparation* was usually stable up to four weeks with regard to cyclase activity when prepared by this method.

2. Assay of cAMP.

A procedure for determining the cAMP produced by isolated cardiac membranes has been developed and characterised by Drs Leclerc, Harding and Harris in this laboratory [Leclerc et al 1984]. They determined that cAMP production under the conditions described below was linear with a protein concentration of up to 0.2 mg/ml and an incubation period of up to 30 minutes.

a. General Experimental Procedure.

i. Preparation of Membranes and Assay of Protein.

Rabbit or guinea pig ^{ventricular} cardiac membranes which had been isolated as described in the previous section were thawed and diluted with TD buffer so that the protein concentration was in the range 0.2 - 1 mg/ml. Protein concentration was determined using Coomassie blue [Bradford 1976]. The Coomassie blue reagent was made up as follows; 10mg of Coomassie blue G250 was dissolved in 5ml 95% ethanol, to which was added 10 ml of conc. phosphoric acid and the solution made up to 100 ml with distilled water. Bovine serum albumin at 1mg/ml was used as

standard. 3ml of the protein reagent was added to 100 μ l of a range of protein standards and the unknowns in duplicate. The tubes were vortex mixed, left for three minutes for the colour to develop and the absorbance read at 595 nm. A sample standard curve is shown in figure 1.4. The protein concentration was adjusted to between 0.25 and 0.75 mg/ml since the curve is not linear above 0.75.

ii. Preparation of Assay Solutions and Experimental Procedure. The phosphodiesterase inhibitor (see later, section B4) and creatine phosphate at 5mM (final concentration in assay) were dissolved in adenylate cyclase assay buffer (25 mM Tris-acetate at pH 7.5 containing 0.4 mM dithiothreitol, 0.4 mM EDTA, 0.1 mM Ascorbate and MgAcetate at 0, 3, 3.53, 4 or 12 mM as stated). This was then adjusted back to pH 7.5 . All test compounds were dissolved and diluted in this buffer. 50 μ l of membranes were added to each tube and a total volume of 150 μ l test solutions added to this. The pipette tip was changed after each addition to avoid contamination. Experiments were performed in duplicate or triplicate. The tubes were placed in a water bath at 30°C to equilibrate for 5-10 minutes. A solution of ATP was made up, its pH checked and adjusted to pH 7.5 before creatine kinase at 2mg/ml (final

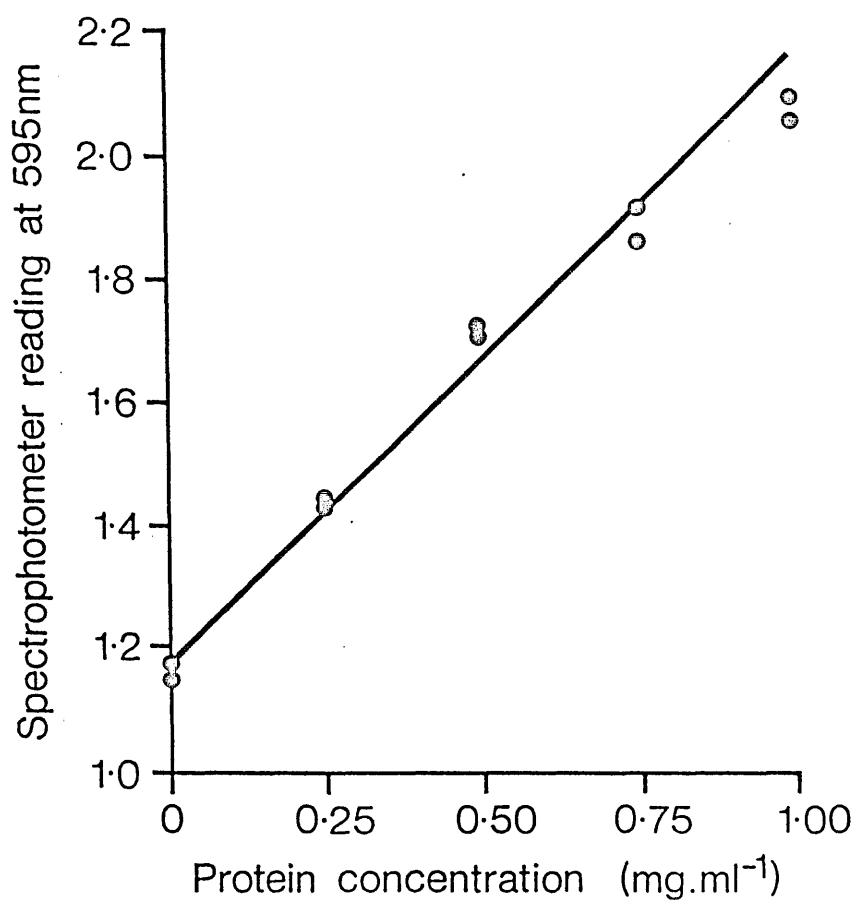


Figure 1.4 A sample standard curve for the Coomassie blue protein assay, showing the relationship of absorbance at 595 nm to protein concentration.

concentration in assay) was added and dissolved . The creatine kinase and creatine phosphate act as a regenerating system for ATP. The creatine kinase/ATP solution was also put to equilibrate. The reaction was started by the addition of ATP to each tube in turn, each tube being vortex-mixed after the addition. After 20 minutes' incubation the reaction was stopped by taking a 50 μ l aliquot from each vortex mixed tube and putting this into 500 μ l of the appropriate stopping buffer (see section B2b & c). A zero time control was prepared by putting the appropriate amounts of ATP, membrane etc. into stopping buffer, the tube being vortex mixed between each addition. The buffer was then neutralised with 3M Tris (Amersham kit) or adjusted to pH 6.2 with 1 M Tris (N.E.N. kit) before aliquots were taken for assay with the appropriate assay kit (see later for description of procedure for use of kits).

iii. Variations of Main Assay Procedure.

Assay of cAMP When The Cyclase is Stimulated by Guanine Nucleotides. The procedure was as above except that all the assay constituents (except for ATP/creatine kinase) were incubated together for 20-30 minutes before the reaction was started in

the usual way.

Determination of cAMP Breakdown in the Absence of Phosphodiesterase Inhibitor . In this case cAMP (or cdAMP) was added to start the reaction. Both the phosphodiesterase inhibitor and the regenerating system were omitted^t from the buffer (the latter since there was no high energy triphosphate to be conserved). A zero was made by putting the appropriate amounts of cAMP, membrane etc into stopping buffer. In this case the zero was made up in triplicate as it represents the maximum amount of cAMP measureable if none is broken down. This is not an assay of phosphodiesterase activity, but merely an estimation of how much cAMP is broken down during the assay (although the phosphodiesterase is recognised as being responsible for most of this reduction, see section B4 for fuller explanation).

b. Assay of cAMP Using The Amersham International Assay Kit.

cAMP was usually determined using the Amersham International assay kit (code TRK 432). This assay is based on the competition between unlabelled cAMP and a fixed quantity of tritium-labelled compound

for binding to a protein which has a high specificity and affinity for cAMP. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP in the sample.

Separation of the protein-bound cAMP from the unbound nucleotide is achieved by adsorption of free nucleotide onto coated charcoal followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

Procedure i. Reconstitution of Reagents and Preparation of Standards. The freeze-dried reagents provided were reconstituted and kept on ice. Serial dilutions of the standard cAMP solutions were prepared to give 5 levels of cAMP solution, 50 μ l of which will give 16,8,4,2, and 1 pMol per assay tube.

ii. Addition of Radioactive compound and Binding Protein. 14 microcentrifuge tubes with caps (plus additional tubes for unknowns) were placed in a rack in an ice tray. 150 μ l of the buffer was pipetted into tubes 1 and 2 for the determination of blank counts per minute. 50 μ l of buffer was

pipetted into tubes 3 and 4 for the determination of binding in the absence of unlabelled cAMP. Starting with the lowest level of standard cAMP, 50 μ l of each dilution was added to each successive pair of tubes. 50 μ l of each unknown (in duplicate) was added to the additional assay tubes. In this case the unknowns were in stopping buffer of 0.1 M NaAcetate and 0.1 M Acetic acid containing 1mM EDTA, originally at pH 4.0, but adjusted to pH 7.0 with 3M Tris. 50 μ l of the labelled cAMP was added to all assay tubes before 100 μ l of the binding protein was added to all tubes except for tubes 1 and 2. An Eppendorf multipipette dispenser was used for the addition of radioactive cAMP and binding protein, since this speeded up the additions and aided accuracy. The tubes were capped and vortex mixed for 5 seconds before being stored in a cold room or fridge at 2-4°C for 1.5-18 hours.

iii. Separation of Bound and Unbound cAMP. 15

minutes before the end of the incubation time, 20 ml of distilled water was added to the charcoal reagent and the container stood on ice and stirred continuously. The ice bath was removed from the fridge and 100 μ l charcoal suspension added to the first 24 tubes in turn, each tube being capped and vortex mixed after the addition. The first 12 were

centrifuged for 2 minutes in an Eppendorf centrifuge 5412. While they were spinning, charcoal was added to the next 12 tubes. This process was repeated until all tubes had been treated with charcoal, left for 1-6 minutes and centrifuged for 2. Without disturbing the sediment, a 200 μ l sample was removed from each tube and placed in a scintillation vial (already containing 10 mls ACS scintillant) for counting.

iv. A Programme for Calculating the Results has been written by Dr S Harding in this laboratory and was used in these studies. A sample standard curve is shown in figure 1.5.

v. Cross Reactivity of Adenosine Analogs. As adenosine and its analogues resemble (to some extent) the structure of cAMP it is possible that they might interfere with binding. The various compounds were dissolved in the Amersham Tris-EDTA buffer, PIA and NECA at 0.1mM ($\times$ 50/570 -to allow for the dilution by the stopping buffer) and all others at 1 mM ($\times$ 50/570) since these are the maximum concentrations that would be expected to come into contact with the binding protein. The results are shown in table 1.1. As can be seen, none of the compounds displaced the radioactive

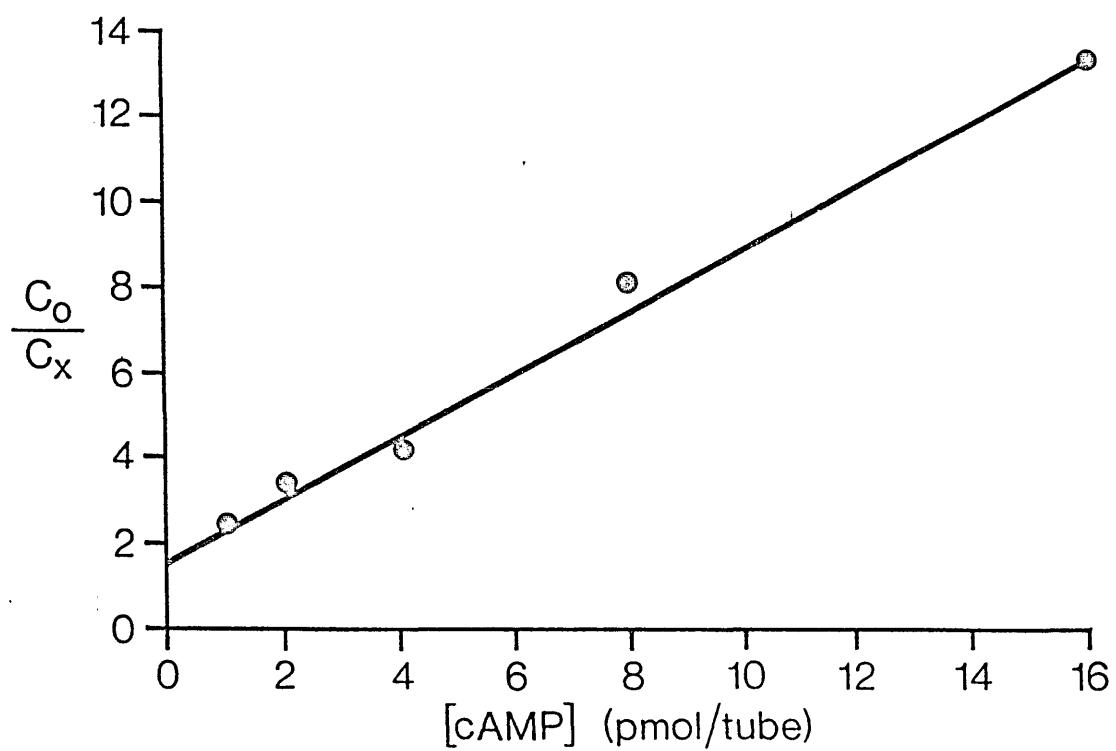


Figure 1.5 A sample standard curve, showing the relationship between the ratio C_o/C_x and picomoles cAMP using the Amersham kit.

Table 1.1 Cross-reactivity of The Amersham Binding Protein With Adenosine and its Analogues.

Analogue	% Displacement
Adenosine	2.9
2-Cl Adenosine	1.6
2'deoxyadenosine-	+4.0
3'monophosphate	
2'5'dideoxyadenosine	+7.4
2'deoxyadenosine	0.3
Eritadenine	1.5
PIA	+4.7
NECA	+0.2

% displacement refers to the displacement of the radioactive compound from its binding sites on the binding protein. A '+' indicates that there is greater radioactivity bound in this case compared to the zero additions control.

antigen to an appreciable extent.

c. Assay of cAMP using the New England Nuclear 125I RIA kit.

Principle of the Method. The technique of radioimmunoassay depends on the competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. If increasing amounts of nonradioactive antigen and a fixed amount of radioactive antigen are allowed to react with a constant amount of antibody, an increasing amount of non-radioactive antigen is bound to the antibody. This relationship can be expressed as a standard curve, and the amount of unlabelled antigen in a sample determined from interpolation from this curve. Free antigen must be separated from bound antigen

In the case of the NEN RIA kit, the labelled antigen is a succinyl tyrosine-[125I]-methyl ester derivative of cAMP. Separation of bound and free antigen is achieved by the use of a pre-reacted primary and secondary antibody complex. The primary antibody was prepared in rabbits against a succinyl-cAMP albumin conjugate while the second antibody was prepared in sheep against rabbit

γ -globulin. After the single overnight incubation at 2-8 °C, buffer is added, the tubes are centrifuged, the supernatant is discarded and the precipitate counted in a gamma counter.

Procedure i. Preparation of Standard curve. This was prepared by serial dilutions of the standard cAMP solution. The concentration range of the curve was between 0.1 and 25 pMoles/100 μ l sample. A blank was set up by substituting buffer for both radioactive antigen and antiserum complex. In the '0' standard, 100 μ l buffer was added in place of a standard solution of cAMP. The samples were in a stopping buffer containing 0.05 Na Acetate and 0.05 N acetic acid originally at pH 4.0, adjusted to pH 6.2 with 1 M Tris.

ii. Treatment of Samples and Standards. Radioactive antigen was added to all tubes. Antiserum complex was added to all except the blank and total counts tubes. The Eppendorf multipette dispenser was used for both these operations. After mixing, the tubes were covered with aluminium foil and incubated in the refrigerator overnight. Ice-cold sodium acetate buffer was added to all tubes except the total counts tubes which were set aside. The tubes were vortex mixed and centrifuged at 2K xg for 15

minutes. The supernatant was aspirated, taking care not to disturb the antibody complex at the bottom of the tube. All tubes were counted in a gamma counter.

iii. Summary of Calculations. The average background counts was subtracted from each of the averaged counts for the standards and samples. The average net counts for the '0' standard represents the counts bound to the antibody in the absence of added cAMP standard (B_0). The average net counts per minute of each cAMP standard or sample (B) may be expressed as a percentage of the average net counts per minute of the '0' standard, = normalised % bound.

Using semilog graph paper, the % B/B_0 for each standard was plotted against the corresponding concentration of cAMP in pMoles added/tube. The concentration of cAMP in each sample tube was determined by interpolation from this standard curve. A sample standard curve is shown in figure 1.6.

It was noticed that the middle portion of the standard curve was reasonably linear (ie. excluding the 0.1 and 25 pMoles points). This made it possible to write and use a computer programme

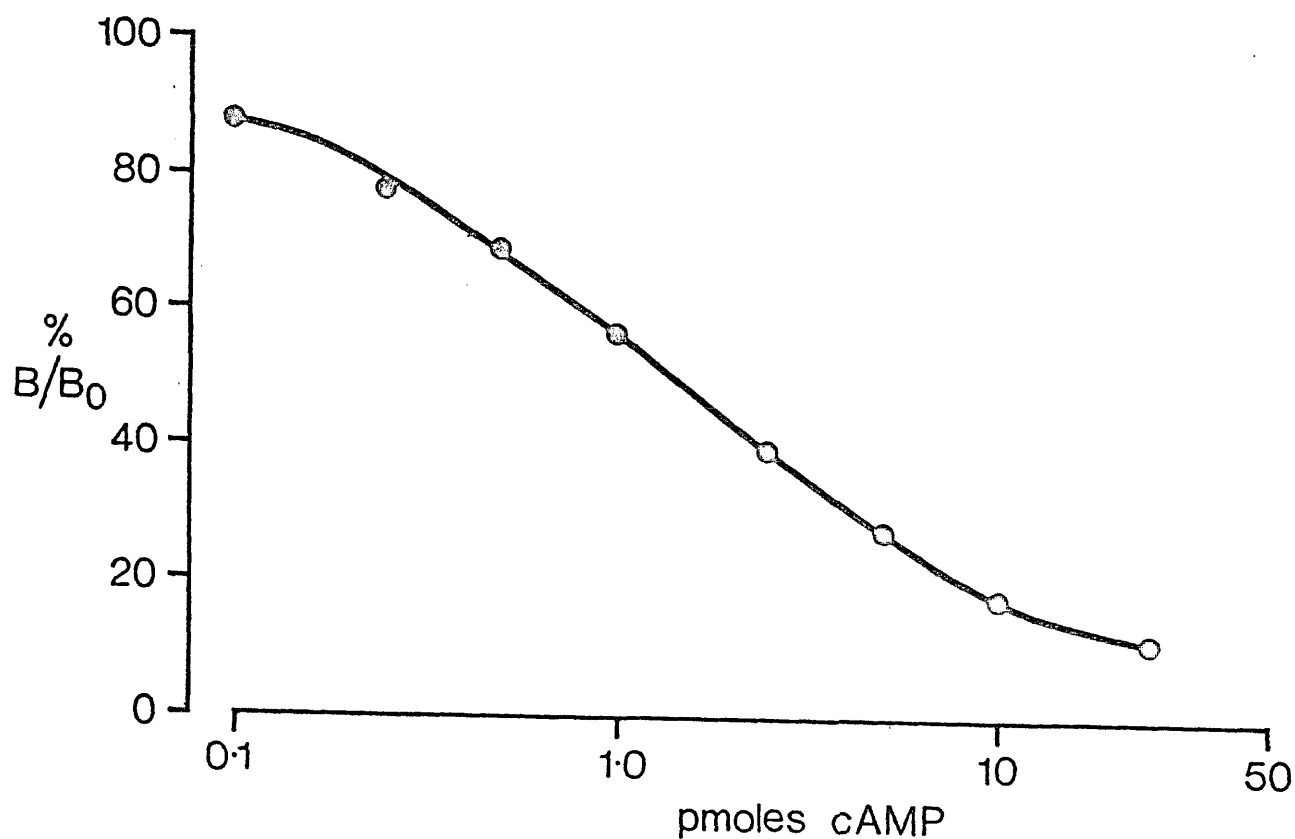


Figure 1.6 A sample standard curve showing the relationship between % B/B₀ (where B₀ = the amount of radioactive antigen bound when no cold cAMP is added, and B = the amount bound in all other cases) and picomoles cAMP as measured using the NEN kit.

using linear regression for the analysis of the results.

iv. Comparability studies of The Amersham Kit and the NEN Kit Methods of Measuring cAMP.

The purpose of these experiments was to ensure that measurements of cAMP produced in one experiment determined by different methods agree with one another within the limits of experimental error.

Method. The cAMP produced by the membranes under basal and GppNHp stimulated conditions was measured using the Amersham and NEM kits. Since guanine nucleotides were to be used, the assay procedure described in Methods B2aⁱⁱⁱ_N was used. The assay constituents were dissolved in adenylate cyclase assay buffer containing 12 mM Mg . ATP at 0.5 mM was used and 10 μ M GppNHp. The reaction was stopped by taking two 50 μ l aliquots from each tube and putting them into 'Amersham ' stopping buffer and 'NEN' stopping buffer (see previous 2 sections). These were then neutralised with 3M Tris (Amersham) or adjusted to pH 6.2 with 1M Tris (NEN) before aliquots were taken for measurement with the

appropriate kit.

Results. Preliminary experiments revealed that the stopping buffer used with the Amersham kit was unsuitable for use with the NEN kit since the counts of the zero was reduced by 20%, suggesting impaired binding of the antibody to the tubes. Nor was a stopping buffer prepared by adjusting the sodium acetate solution provided in the kit to pH 4.0 suitable for use since this also reduced the counts to a certain extent. The specially made up buffer described in section B2ci still reduced the counts by 5-10%, but this was felt to be acceptable. Table 1.2 shows the amount of cAMP measureable by the different kits, in an average of 3 experiments. As can be seen there is a reasonable agreement between the two methods.

Table 1.2 Comparasion of Amersham Kit and NEN kit measurements of cAMP.

Condition	pMoles cAMP/min/mg protein.	
	Amersham	NEN
Basal	43.7	46.6
	47.9	42.7
	69.2	54.3
+GppNHp	159.1	154.7
	181.9	158.1
	250.0	204.7

The numbers shown are the means of separate triplicate determinations.

3. Setting up of Adenosine-Free Assay Systems.

a. Addition of Adenosine Deaminase.

In order to study the effect of adenosine and its analogues on the activity of adenylate cyclase, any endogenously produced adenosine must first be removed from the assay medium. The ATP used as a substrate for adenylate cyclase is produced by Sigma from adenosine by enzymatic phosphorylation, and is therefore likely to contain traces of adenosine. In addition the nucleoside may be generated from ATP by reactions catalysed by phosphohydrolases on the sarcolemmal membranes . There are two ways of removing this endogenous adenosine. The simplest is to add adenosine deaminase to the assay medium. This converts adenosine to inosine which shows no activity at adenosine receptors. The action of adenosine deaminase-resistant analogues can now be assessed. The methodology behind this is described below. The second method which involves the use of deoxyATP is described later.

i Determination of The lowest Amount of ATP which

May be Used.

Since ATP is the major source of contaminating adenosine, it was felt that it would be advantageous to reduce the level of ATP added to the assay. A concentration response curve of adenylate cyclase activity versus ATP concentration was produced. The general assay procedure was used and solutions were made up in assay buffer containing 12 mM Mg. The different ATP concentrations were made up by serial dilution and creatine kinase added to an aliquot of each. The results are shown in figure 1.7 . A concentration of ATP at 0.5 mM was selected for use on a basis of these results , since above 0.1 mM no further increases in adenylate cyclase activity were elicited by raising the substrate concentration. A supramaximal concentration was used to prevent variations in adenylate cyclase activity between experiments.

ii. Selection of the Optimal Amount of Adenosine Deaminase For Use.

Three different types of adenosine deaminase were selected for use in this system. The crude powder proved to be too variable in its purity between batches. When a carrier control was performed for the type suspended in 3.2 M $(\text{NH}_4)_2$

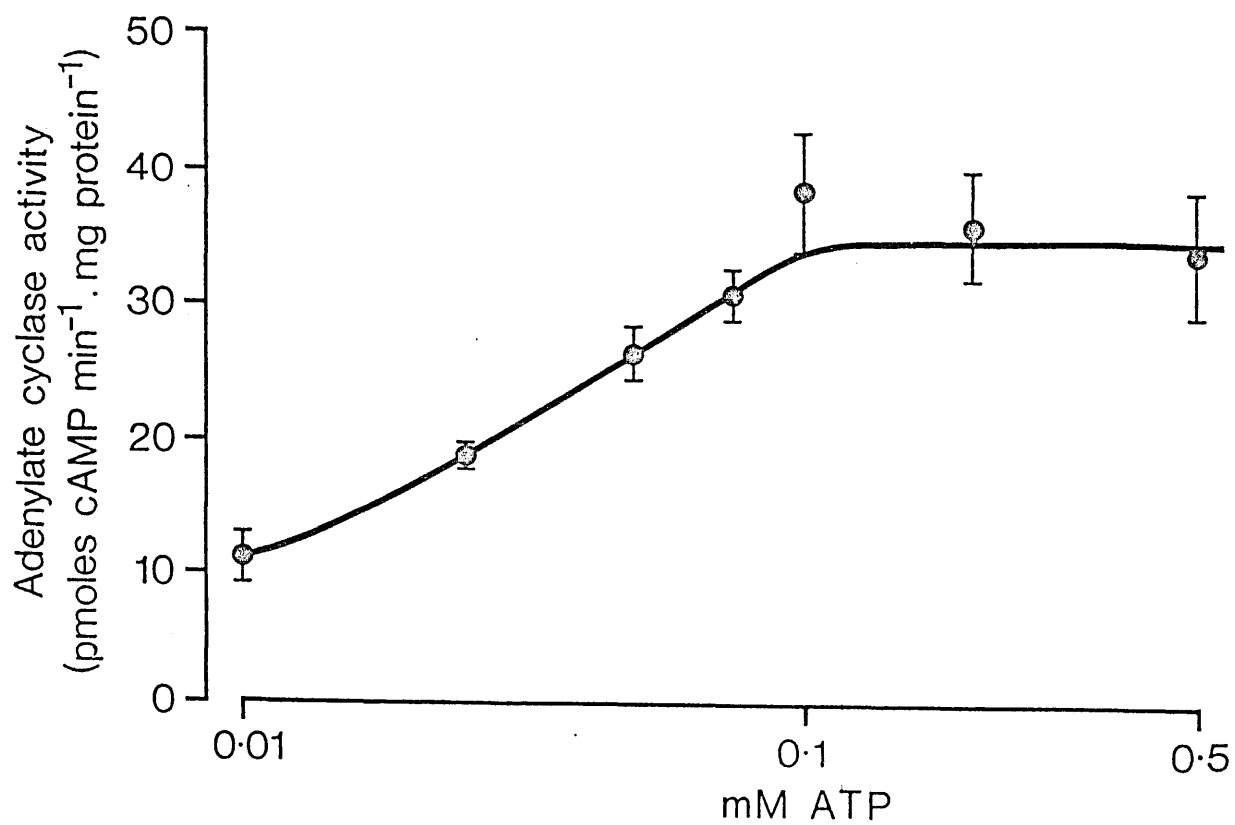


Figure 1.7 A dose response curve showing the relationship between adenylate cyclase activity and mM ATP.

SO4 it was found that the ammonium sulphate alone stimulated adenylate cyclase activity to a considerable extent. Attempts were made to desalt the enzyme by passing it down a Dowex column, but this merely caused the protein to precipitate out. Carrier controls for adenosine deaminase suspended in 50% glycerol/0.02M phosphate showed that this solvent had no effect on adenylate cyclase activity (table 1.3). Adenosine deaminase at a concentration of 1 unit ml⁻¹ was found to remove 1 mM adenosine from the assay mixture (Table 1.4).

This adenosine-free assay system used 0.5 mM ATP in conjunction with 1 unit ml⁻¹ adenosine deaminase. The cAMP generated was measured using the Amersham International assay kit.

b. Use of deoxy ATP.

The main problem with the above method is that the effects of adenosine itself cannot be determined, only the actions of the deaminase-resistant analogues. These analogues may act by displacing adenosine from the active site of adenosine deaminase, thus allowing the adenosine concentration to rise [Londos et al 1982].

Table 1.3 Carrier Controls For Adenosine Deaminase in Glycerol/Phosphate.

% Glycerol	M KOH	= U/ml Deam	Adenylate Cyclase (pMol cAMP/ min/mg protein)
0	0	0	85.5
.375	.00015	10	90.1
.75	.0003	20	86.3
1.125	.00045	30	89.7
1.5	.0006	40	91.2
1.875	.00075	50	92.3

The numbers represent the means of a single experiment performed in triplicate.

Table 1.4 The Effect of Adenosine Deaminase on Basal Adenylate Cyclase Activity in the Presence and Absence of 1mM Adenosine.

Condition	Cyclase Activity (cAMP/min/mg protein)	
	Control	+1mM Adenosine
Basal	47.2 $\pm$ 12.4	17.1 $\pm$ 4.0
+adenosine deaminase (1u/ml)	46.1 $\pm$ 9.8	50.8 $\pm$ 12.7

Each number is the mean $\pm$ S.E.M. of three separate triplicate determinations.

The second method prevents the accumulation of adenosine by the use of a system that cannot generate it. If deoxyATP (dATP) is used as the substrate, cdAMP is produced and deoxyadenosine is the byproduct. Deoxyadenosine has been reported to have little affinity for the cyclase coupled adenosine receptors [Cooper and Londos 1979]. The effects of adenosine and its analogues can now be determined.

We have determined that the Amersham kit cAMP binding protein will not bind cdAMP. Many groups use a modified 'Salomon' assay [Cooper and Londos 1979]. It was felt that it would be preferable if a less complicated and costly method could be found. New England Nuclear produce a cAMP 125I radioimmunoassay kit which they report to be more sensitive to cyclic nucleotides substituted in the 2'0 position. This raised the possibility that the antibody complex of this kit might be used to bind cdAMP. I have investigated the ability of the N.E.N. cAMP R.I.A. kit antibody to bind cdAMP. The results of this investigation are presented below.

i. cdAMP Standard Curve. Comparison with Normal cAMP Standard Curve.

To find out whether or not cdAMP can bind to

the antiserum complex and displace the antigen in a similar manner to cAMP, a comparison of cAMP and cdAMP standard curves was made.

Method. A series of standard solutions of cdAMP at the same concentrations as those used in the cAMP standard curve was prepared and treated in the way described earlier (section B2c).

Results. The two standard curves are illustrated together in figure 1.8. It will be noticed that there is close agreement between the two. 50% displacement of the radioactive antigen was brought about by 1.86 ± 0.13 pMoles cAMP ($n=9$) and by 1.88 ± 0.13 pMoles cdAMP ($n=10$). Thus cdAMP is able to displace the radioactive antigen in a manner very similar to the way that cAMP displaces the antigen. This suggested that the NEN RIA method is suitable for measuring cdAMP.

vi Cross-Reactivity of Deoxynucleotides with the cAMP Antiserum.

The purpose of these experiments was to check that the deoxynucleotides do not cross-react with the cAMP antiserum any more than do the normal nucleotides.

Method. Solutions of dAMP, dADP, and dATP were

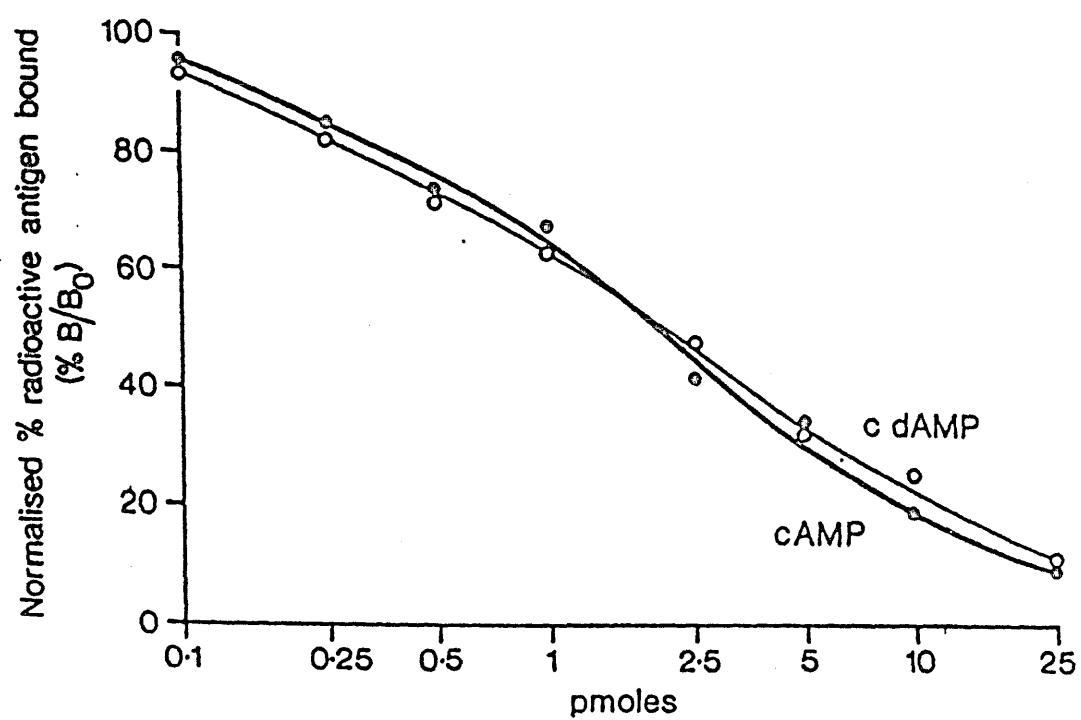


Figure 1.8 A comparason of cAMP and cdAMP standard curves as measured with the NEN I125 radioimmunoassay kit (see section B2c).

made up and diluted to give a wide range of concentrations between 100 and 100,000 pMoles per assay tube. These were reacted with the radioactive antigen and antibody in the usual way

Results. Figure 1.9 shows the average of three such determinations. At a concentration of 1 nMol/100 ul sample, none of the deoxynucleotides displaced more than 5% of the labelled antigen. Figure 1.9a shows a cross-reactivity study performed by New England Nuclear using normal nucleotides. If the two graphs are compared, it will be seen that the cross-reactivity profiles for the deoxynucleotides are similar to those for the normal nucleotides.

vii. Cross-Reactivity of the Adenosine Analogues with the cAMP Antiserum.

The adenosine analogues were dissolved in the NEN sodium acetate buffer at 0.1mM x 50/570 (PIA and NECA) or 1mM x 50/570 (all others). (See earlier study with the Amersham kit, section B2b.) The results are shown in table 1.5. Only 2'-deoxyadenosine 3' monophosphate and 2'-deoxyadenosine displaced the antigen to an appreciable extent. The kit cannot, therefore be used in studies involving these compounds.

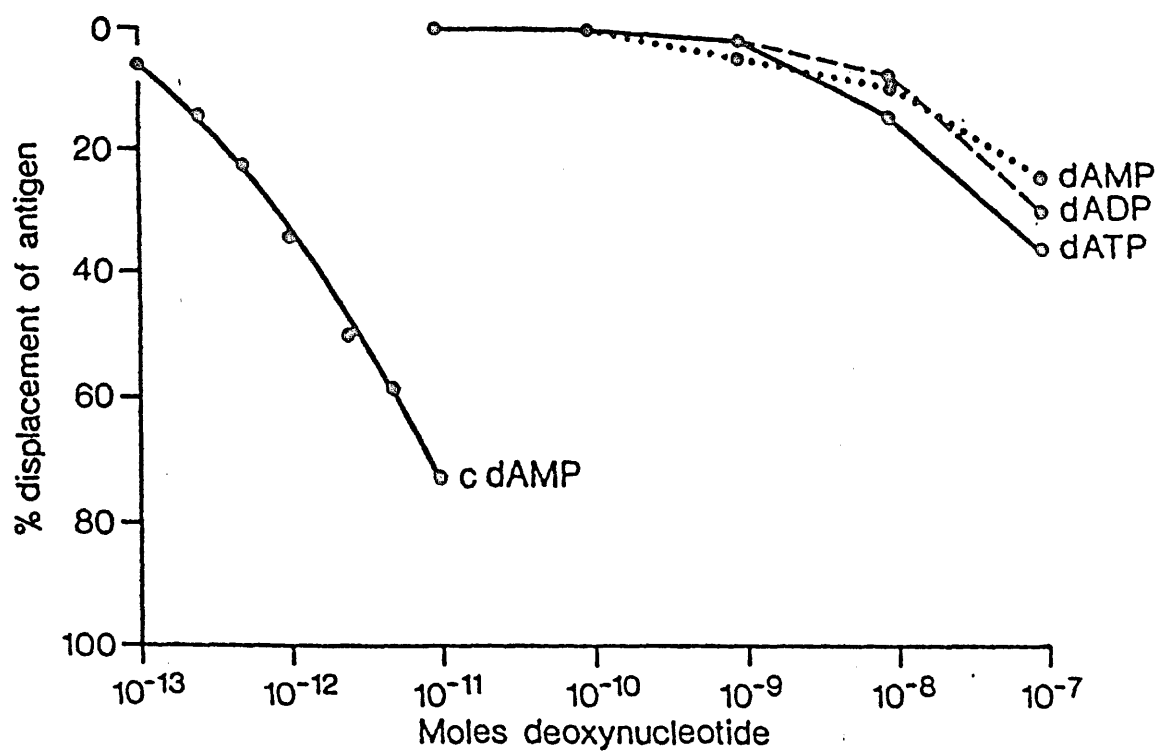


Figure 1.9 A cross-reactivity profile for the deoxyadenine nucleotides with the antibody of the NEN I 125 radioimmunoassay kit. The points shown are the average of 3 separate determinations performed in duplicate.

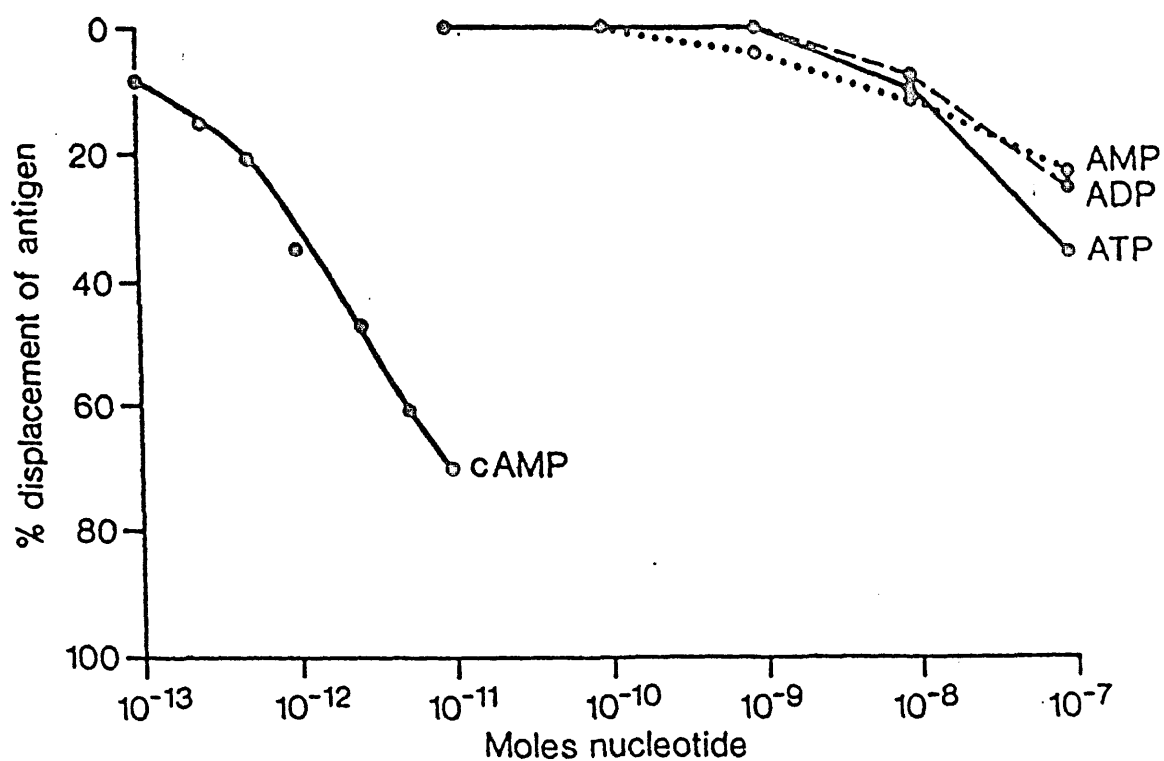


Figure 1.9a A cross reactivity profile for adenine nucleotides with the antibody of the NEN radioimmunoassay kit. This is reproduced from the NEN booklet.

Table 1.5 Cross-reactivity of NEN kit Antibody with Adenosine and its analogues.

Analogue	%Displacement
Adenosine	2.9
2-Cl Adenosine	0
2'deoxyadenosine- 3'monophosphate	69.7
2'5'dideoxyadenosine	0
2'deoxyadenosine	11.0
PIA	0
NECA	0

iii. Assessment of the Suitability of deoxy ATP as a Substrate for Adenylate Cyclase.

DeoxyATP has proven to be a suitable substrate for many adenylate cyclases [Londos et al 1981], but it remained to be seen if this held true for cardiac cyclase. Hyperbolic plots were used to determine the lowest possible concentration of dATP which can be used. Double-reciprocal Lineweaver-Burke plots were used to determine the kinetic parameters of enzyme-substrate interactions.

Methods. The procedure for assessing basal enzyme activity was used (section B2a) and the assay constituents were dissolved in assay buffer containing 12 mM Mg.

Results. A plot of cyclase activity versus mM dATP is presented in figure 1.10 This should be compared with the similar plot of cyclase activity versus mM ATP shown in figure 1.7. The graphs resemble one another. Using 0.5 mM substrate, the basal activity was 70.1 ± 7.2 pMol cAMP min⁻¹ mg protein⁻¹ (n=27) and 69.6 ± 6.5 pMol cdAMP min⁻¹ mg protien⁻¹ (n=16). Figure 1.11 shows an average of 3 separate determinations of the double-reciprocal

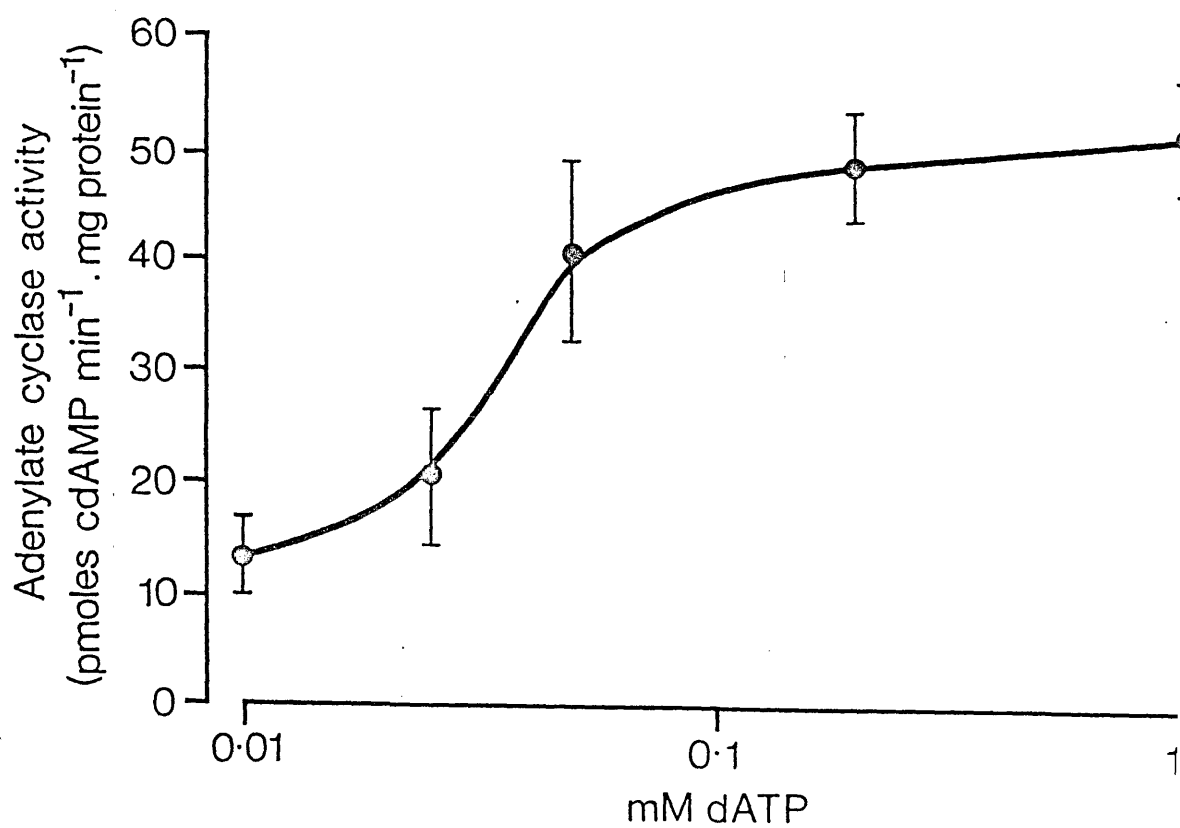


Figure 1.10 This shows the relationship between dATP concentration and adenylate cyclase activity. $n=3 \pm$ SEM.

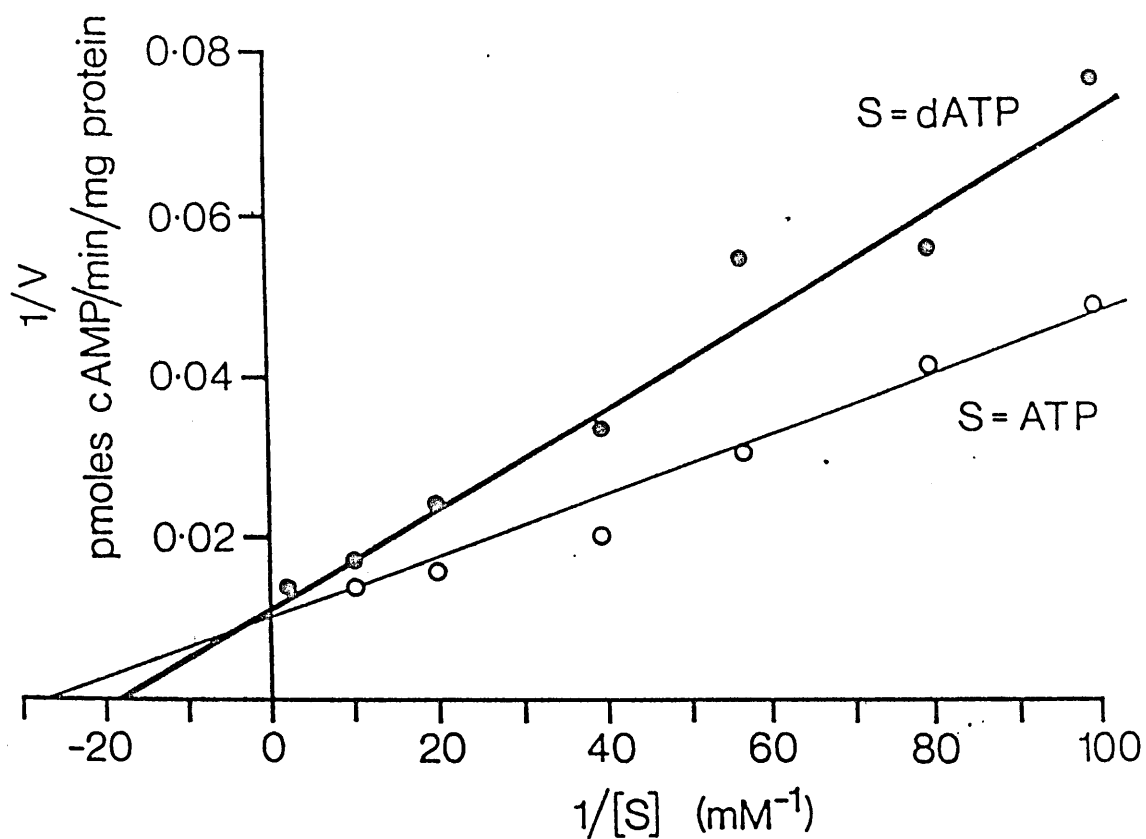


Figure 1.11. A comparison of the double reciprocal relationship between rate and substrate concentration for ATP and deoxy ATP. Each point shown is the average of 3 separate determinations.

relationship between rate and substrate concentration. The K_m of adenylate cyclase was 34 ± 4 μM for ATP and 45 ± 8 μM for dATP ($n=3$).

Thus deoxy ATP is a good substrate for cardiac adenylate cyclase, and the NEN RIA method is an appropriate one for measuring the cdAMP generated. (see Ridgeway et al 1983)

4. Non-Methylxanthine Phosphodiesterase Inhibitors.

The sarcolemmal membrane has a very active cAMP phosphodiesterase. To enable accurate measurement of cAMP formed this activity must be fully blocked. Unfortunately, the most commonly used phospho- diesterase inhibitors are methylxanthines which, at micromolar concentrations will also antagonise the effects of adenosine and its analogues. Millimolar concentrations of methylxanthines are required to inhibit the phosphodiesterase fully. In studies on adenosine receptors, a substance other than a methyl- xanthine must be used. Papaverine was an obvious candidate, but it was found that it would not go into solution at millimolar concentrations with a pH more than 6.5. It was therefore unsuitable for use. Two others were tried; ICI 63 197 and RO 20 1724. The chemical structures are shown in the appendix.

i Test of ICI 63 197 as a Phosphodiesterase Inhibitor.

The ability of this compound to block cAMP breakdown was tested. (See assay procedures B2a, 12 mM Mg assay Buffer was used). Percentage

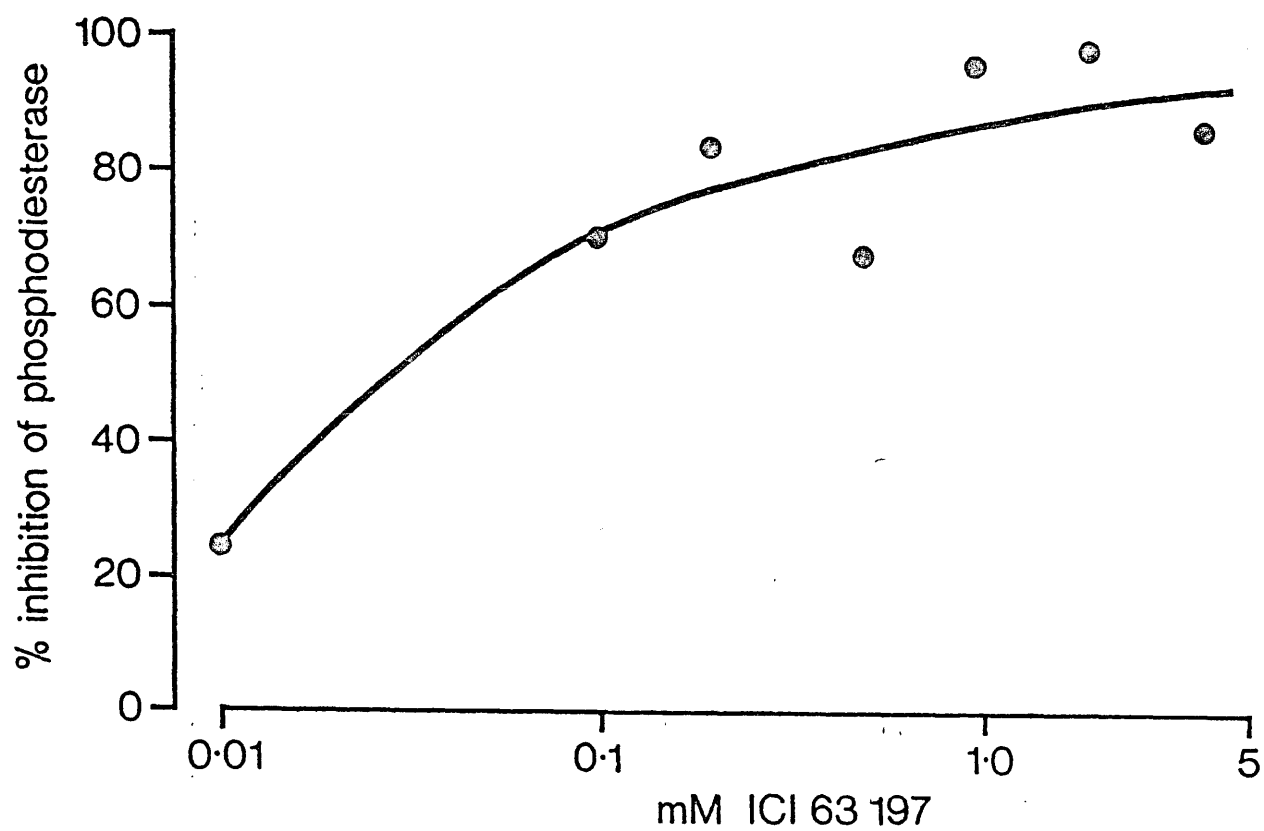


Figure 1.12 A plot of % inhibition of phosphodiesterase activity versus ICI 63 197 concentration. Results shown are the average of 2 determinations performed in triplicate.

12 mM Mg assay Buffer was used). Percentage inhibition of phosphodiesterase activity is plotted against milimoles of ICI 63 197 in figure 1.12. Inhibition of phosphodiesterase activity was calculated as follows. The zero time control represents the maximum amount of cAMP which can be measured by this method. The tubes which contain no phosphodiesterase inhibitor will give the amount of cAMP remaining when the cAMP phosphodiesterase is fully active. The first value is equivalent to 100% inhibition of phosphodiesterase. The second value is subtracted from all other values, which are then plotted as a percentage of the zero time control. A concentration of ICI63 197 at 2 mM was selected for use in adenylate cyclase assays, since above 1 mM no further increases in phosphodiesterase inhibition were obtained.

ii. Test of Ro20 1724 as a Phosphodiesterase Inhibitor.

This compound is rather more widely used. However its solubility in aqueous buffers is extremely limited. It was found that the compound had initially to be dissolved in propylene glycol. Warmed assay buffer at 30°C could then be added to this to give a solution containing 16% propylene glycol.

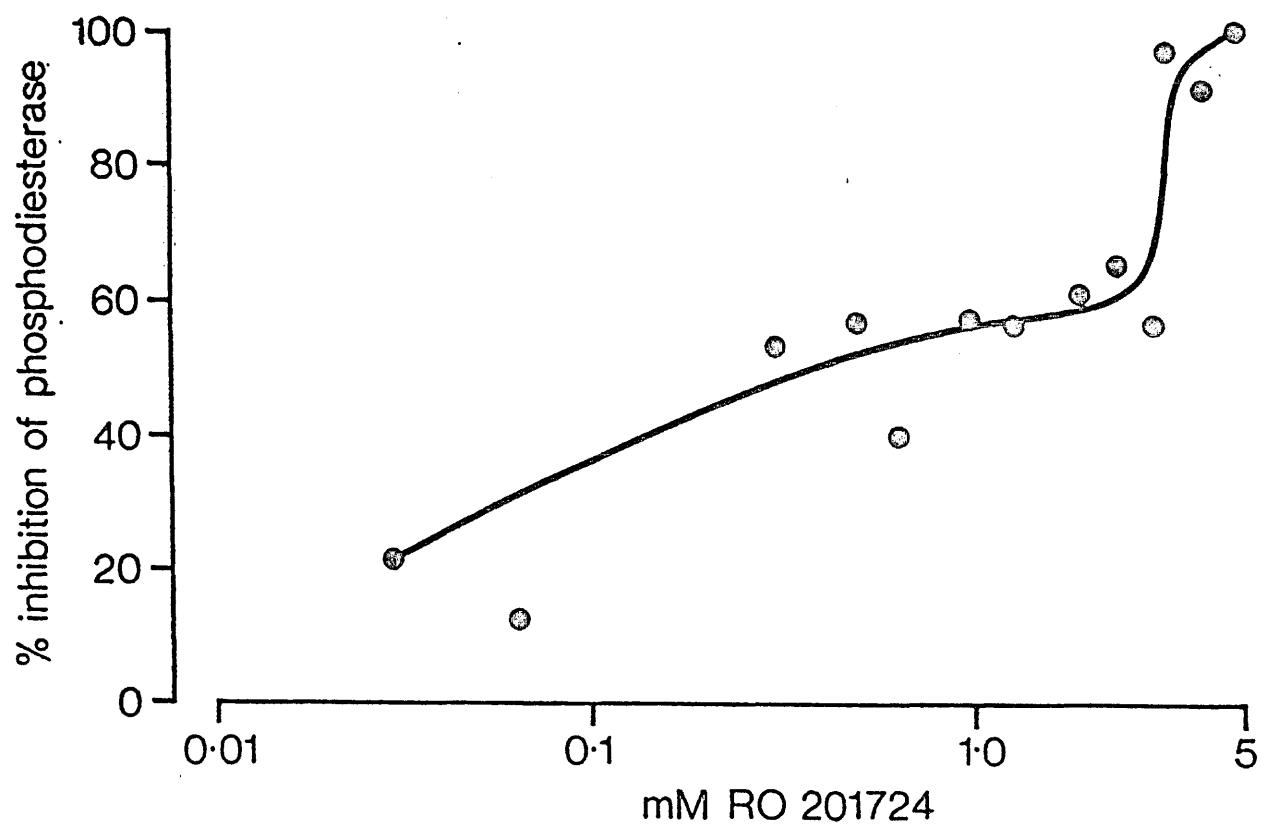


Figure 1.13 A plot of % inhibition of phosphodiesterase activity versus RO 20 1724 concentration. Points shown are the average of 2 determinations performed in triplicate.

As this concentration of propylene glycol may adversely affect adenylate cyclase activity, a control experiment was performed using theophylline as the phosphodiesterase inhibitor. Basal activity was 48.1 ± 9.4 pMoles cAMP min⁻¹ mg protein⁻¹ while with 16% propylene glycol the activity was 43.4 ± 6.0 pMoles cAMP min⁻¹ mg protein⁻¹. Thus propylene glycol at this concentration does not appear to have much effect on activity.

The percentage inhibition of phosphodiesterase activity is expressed as a function of RO 20 1724 concentration in figure 1.13. A concentration of 3.5 mM was selected for use since this provided a good compromise between effective phosphodiesterase inhibition and economy.

CHAPTER 2

The Effect of Adenosine on Basal and Isoprenaline
Stimulated Tension in Isolated Langendorff Perfused
Guinea-Pig Hearts and Papillary Muscles.

Introduction.

Schrader in 1977 showed that with guinea pig Langendorff perfused hearts, adenosine could attenuate the response to isoprenaline while having no effect on basal tension. The reversal of isoprenaline stimulated tension was almost complete at 10 μ M adenosine, where a 90% inhibition of the positive effect of a bolus injection of 40 pmoles isoprenaline was seen. This contrasts with the rather modest (40%) inhibition seen by Endoh and Yamashita [1980] working with rabbit papillary muscle. The difference may represent species variation between guinea-pig and rabbit or could be artefactual in nature, because of differences in experimental technique.

In this chapter I report the results of experiments performed using both the guinea pig papillary muscle preparation and the isolated Langendorff-perfused guinea pig heart preparation. The effect of adenosine on the increase in force stimulated by isoprenaline was examined with both preparations in an attempt to find out the reasons for the differences. The major points under investigation are;

1. Some of the effects on whole heart might be due to haemodynamic complications caused by the vasodilatory effects of adenosine, in which case a smaller effect on papillary muscles might be expected. By dilating the coronary arteries, adenosine would tend to have an erectile effect on the muscle, this turgidity indirectly reducing the amount of stimulation of force by positive inotropes such as isoprenaline. Therefore I compared the effects on whole heart with papillary muscles where no haemodynamic effects are possible

2. Superfusion of muscles may be insufficient to prevent hypoxia. As adenosine is thought to be released under hypoxic conditions [Berne et al 1974] this would tend to mask the effect of added adenosine. To test this, the effect of adenosine deaminase on isoprenaline-stimulated tension was investigated in papillary muscles.

3. If an effect of adenosine is observed with papillary muscles, it needs to be established whether or not this action is mediated by an extracellular A-type receptor or the intracellular P-site. If the response can be blocked by methyl xanthines, then it can be stated, with a degree of

certainty, that the effect is mediated by extracellular A-type receptors. This was not demonstrated by Endoh and Yamashita [1980].

4. In order to show that a given response is mediated by external A-type receptors, it must be shown that the response can be blocked by methyl xanthines. However most methylxanthines are also potent phosphodiesterase inhibitors and will tend to raise cAMP levels. Since (as I will show) the effect of adenosine is smaller at higher isoprenaline levels (and therefore when cAMP levels are elevated), the possibility remains that methyl xanthines may exert their adenosine antagonism by inhibiting phosphodiesterase and raising cAMP. To allow for this possibility, papaverine, a non-methyl xanthine phosphodiesterase inhibitor, was used in place of 8-phenyltheophylline to show that the antagonism was not at the level of cAMP.

5. To determine the effects of isoprenaline and adenosine on cAMP levels, hearts were freeze clamped and tissue cAMP measured.

Methods.

Hearts were perfused by the method of Langendorff as described in methods A.1. Isolated papillary muscles were treated as described in section A.2.

Langendorff Perfused Hearts. a.Dose Response to Isoprenaline $\pm$ a Fixed dose of Adenosine.

The addition of adenosine in the high micromolar range was found to bring about a small alteration in the mechanical tracing simulating a small negative inotropic response. This alteration is probably due to the haemodynamic effects of coronary vasodilation. A steady trace to adenosine alone was therefore obtained before isoprenaline plus adenosine was given. Isoprenaline was perfused into the heart for a fixed period of one minute and the maximum response attained in this time measured. To work out the percentage increases in tension, the developed tension immediately before the addition of adenosine was used as the denominator in this calculation.

Langendorff-perfused hearts; b. freeze clamping and measurement of tissue cAMP.

Hearts were freeze clamped and the tissue cAMP measured as described in methods A1g. Hearts were perfused with Krebs-Henseleit solution for 20 minutes before the addition of isoprenaline or freeze clamping. As far as was possible, a control heart, and hearts treated with isoprenaline, adenosine and isoprenaline plus adenosine were perfused on the same day. Adenosine treated hearts were perfused with the nucleoside after 15 minutes of control perfusion.

Papillary Muscles. a. Dose Response to Isoprenaline ± a Fixed Dose of Adenosine.

After the equilibration period, a control dose of adenosine was administered. If a positive effect was seen (see later, this chapter), this dose was repeated until no further increase could be elicited, since it was found that the increase in force declined with repeated administration. Isoprenaline, or isoprenaline plus adenosine was infused into the organ bath until the response obtained reached a maximum. The order of doses with respect to isoprenaline alone and isoprenaline plus

adenosine was varied between experiments.

Papillary Muscles b. Antagonism of the Effect of Adenosine by 8-Phenyltheophylline.

To increase the stimulation of developed tension by isoprenaline, the amount of calcium in the perfusate was reduced from 2.5 to 1.25 mM. This increased the response of the muscle to 10 nM isoprenaline from 17 to 82% in one test preparation. After the one hour equilibration period, responses to adenosine, isoprenaline and isoprenaline plus adenosine were determined. 8-Phenyltheophylline was added to the superfusing solution and allowed to act on the muscle for 30 minutes. The above measurements were repeated. The tissue was then returned for 30 minutes of superfusion with control Krebs-Hensleit after which the original measurements were taken. Finally, after 30 minutes exposure to papaverine, the responses to isoprenaline and isoprenaline plus adenosine were once more determined. The order in which the muscle was exposed to 8-PhenylTheophylline and papaverine was alternated between experiments.

Results

Guinea pig Langendorff; a. Dose Response to Isoprenaline $\pm$ 15 μ M Adenosine

The average resting tensions for the isolated perfused guinea pig hearts was 13.7 ± 0.57 g (n=19). Figure 2.1 shows a dose response curve of the effect of increasing concentrations of isoprenaline on developed tension in the presence and absence of 15 μ M adenosine. Adenosine produced a complete block of the increase in tension due to 0.1nM and 1nM isoprenaline. Above these concentrations the effect of adenosine was negligible.

Adenosine alone brought about a slight alteration in the mechanical tracing mimicking a small negative inotropic effect, the average response being -21.2 ± 5.9 % (n=8). This was significant at a level of $P < 0.01$, (Student's t-test). This could be due to haemodynamic effects of coronary vasodilation or it may be that adenosine does have a direct effect on ventricular myocardium. As this is not easily tested on this

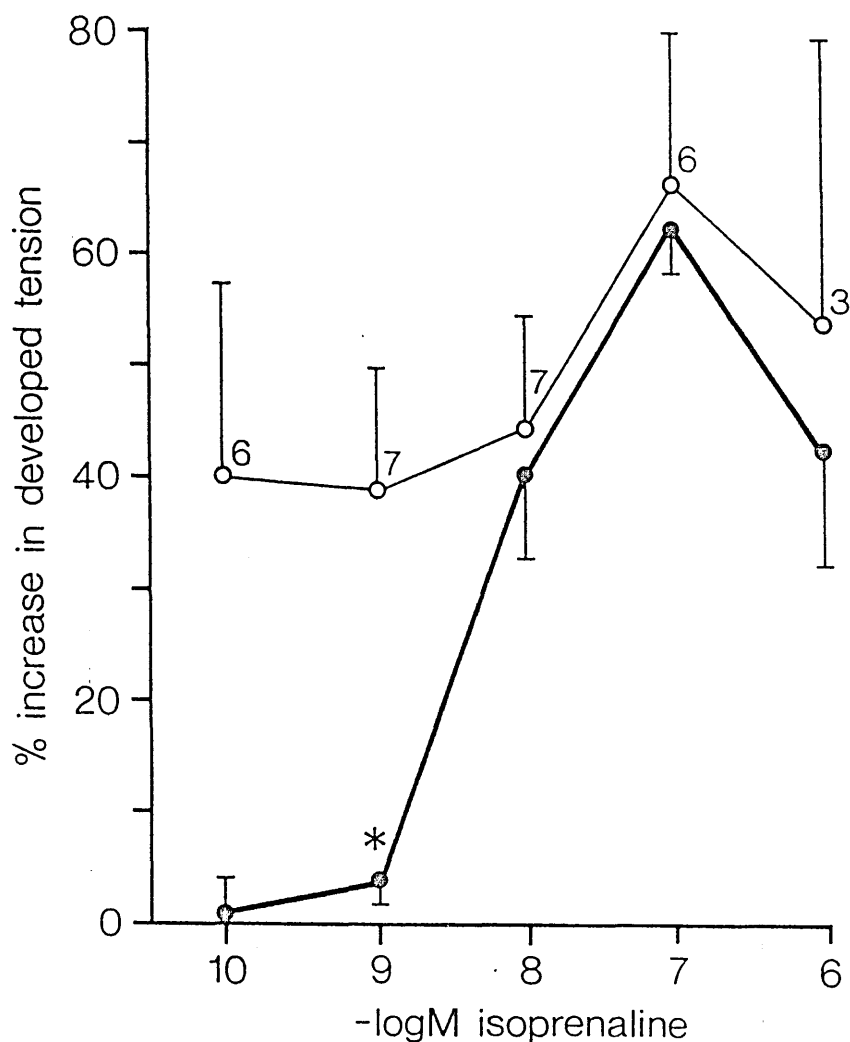


Figure 2.1 Dose response of guinea pig langendorff perfused heart to isoprenaline + 15 uM adenosine. Isoprenaline alone = $\circ$ — $\circ$, + 15uM adenosine = $\bullet$ — $\bullet$. The number of experiments at each concentration is indicated by a number by the appropriate point in the isoprenaline alone curve. the points shown are the mean $\pm$ S.E.M. of the indicated number of separate determinations. * = $P < 0.005$ (paired Student's t-test).

preparation, I have designed experiments using papillary muscles aimed at answering this question. They are described in chapter 3.

Guinea pig Langendorff; b. Measurement of tissue cAMP.

The amount of cAMP measured in 6 control hearts was 6.2 ± 0.8 pMoles/mg protein. This value is in the range of levels reported by Dobson [1983] (2.6), Linden et al [1985] (7.5), and Belardinelli et al [1982] (25). Since other workers have expressed their results in pMol cAMP/g tissue it is difficult to compare such values with those shown here. In hearts stimulated with 1nM isoprenaline, the amount of cAMP measured was raised to 7.8 ± 1.2 (n=6). Although this was not statistically significant by the grouped t-test or by the Wilcoxon rank test, it was noted that in every group, the heart perfused with isoprenaline had a higher level of cAMP than was seen in the control heart. Other workers used rather higher concentrations of isoprenaline ranging from 10 nM to 1 μ M, but no antiadrenergic effects were seen at these higher concentrations of isoprenaline with isolated guinea pig hearts (see figure 2.1). The six hearts perfused with 15 μ M adenosine also showed a slightly elevated cAMP level on average

(7.65 ± 1.3 pMoles cAMP/mg protein). One of the latter hearts showed a lower cAMP level than the appropriate control. Dobson [1983] and Schrader et al [1977] also noted a slightly higher level of cAMP in the presence of $10 \mu\text{M}$ adenosine. When hearts were perfused with isoprenaline and adenosine, the cAMP levels were raised to 10.6 ± 1.4 pMoles cAMP/mg protein. This was statistically significant by the grouped t-test and the Wilcoxon rank test, both at a level of $p < 0.05$. All these six hearts showed a higher cAMP level than the relevant control. This result is in direct contradiction to Schrader et al [1977], Linden et al [1985], Belardinelli et al [1982], Bauman et al [1981], and Dobson [1983] who all found that adenosine reduced levels of cAMP raised by isoprenaline.

Guinea-pig Papillary; a. Dose Response to Isoprenaline + $15 \mu\text{M}$ Adenosine.

Guinea pig papillary muscles exerted an average resting tension of 0.67 ± 0.58 g ($n=38$). The dose response curve performed with whole heart was repeated with guinea pig papillary muscle. The observations are presented in figure 2.2. There was no complete block of the isoprenaline-induced rise in tension at any dose of isoprenaline, but an

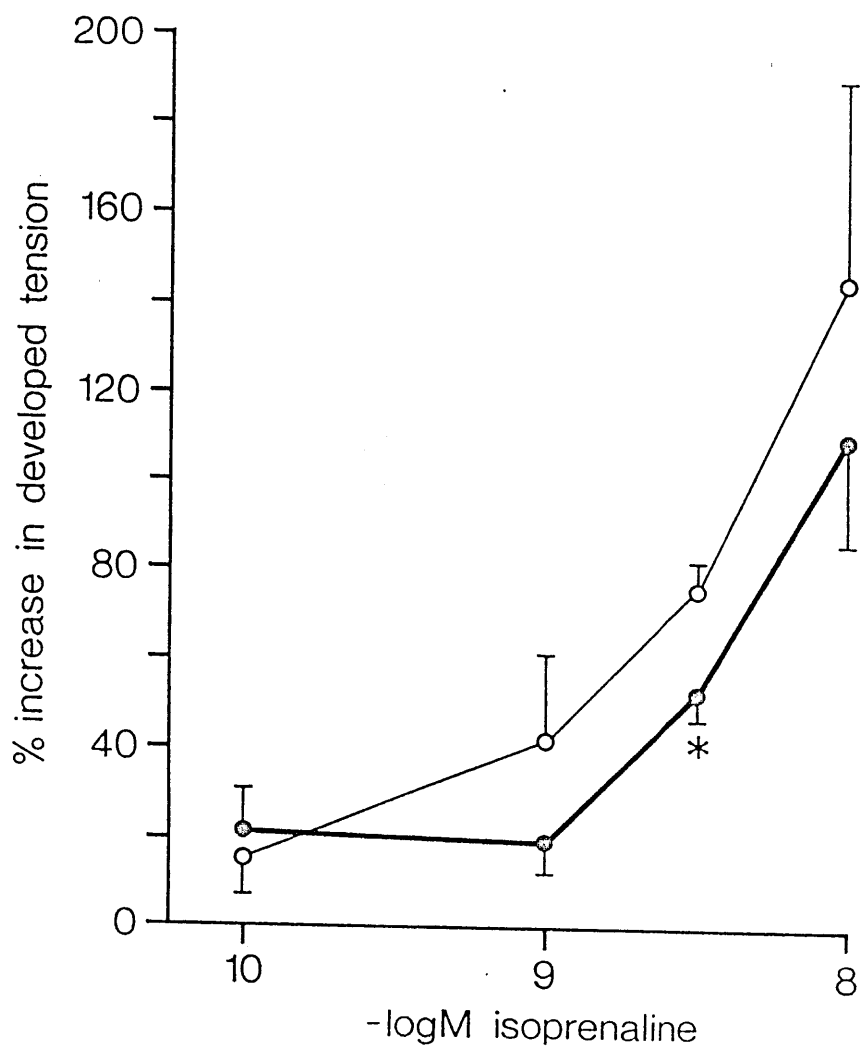


Figure 2.2 Dose response of guinea pig papillary muscle to isoprenaline + 15 μ M adenosine. Isoprenaline alone = $\circ$ — $\circ$, + adenosine = $\bullet$ — $\bullet$. The points shown are the mean $\pm$ S.E.M. of 5 separate determinations. * = $p < 0.05$ (paired Student's t-test).

inhibition of around 40 % between 1 and 10 nM. At 0.1 μ M isoprenaline, the muscles tended to go into arrhythmias, but in the two cases where measurement of tension was possible, there was no noticeable effect of adenosine.

b. Positive effects of high doses of 2-chloroadenosine and adenosine.

Both 2-chloroadenosine and adenosine at doses exceeding 10 μ M sometimes produced a positive inotropic effect. This was prevented by the administration of atenolol (10 μ M) (figure 2.3) suggesting that adenosine and 2-chloroadenosine could be releasing endogenous catecholamines from adrenergic nerve endings in the preparation. Since the effect was seen with 2-chloroadenosine as well as adenosine, this suggests that adenosine itself rather than inosine (formed by deamination of adenosine) was responsible for the stimulation. The positive inotropic effect decreased with successive doses of adenosine consistent with the release of catecholamines from nerve endings.

c. Antagonism of the Anti-Adrenergic Effect of Adenosine By 8-Phenyltheophylline.

The effect of adenosine at its receptors is

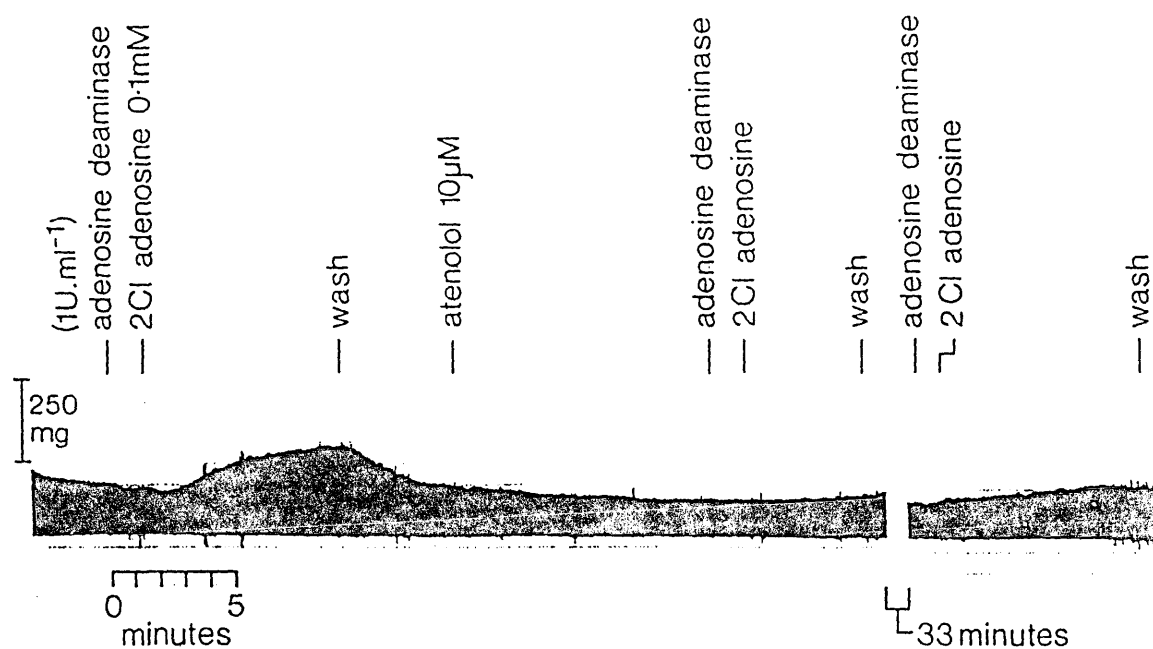


Figure 2.3 The positive effect of high doses of 2'-chloroadenosine, and antagonism of the effect by atenolol. Shown is a copy of a tension trace illustrating the size of this positive effect, the fact that it is absent in the presence of atenolol, and may be seen again (much reduced) after washing off the B-blocker.

antagonised by methylxanthines. However, most methylxanthines are also phosphodiesterase inhibitors. 8-phenyltheophylline was used since it has been reported to have good adenosine receptor antagonism properties with little phosphodiesterase activity [Griffiths et al 1981]. In five ^{inhibitory} experiments, 10 μ M 8-phenyltheophylline increased tension by $33.8 \pm 10.6 \%$, showing that at this concentration 8-phenyltheophylline might inhibit the phosphodiesterase to a small extent. It is also possible that the methylxanthine could be antagonising an inhibitory effect of endogenous adenosine.

To determine whether any block of the effect of adenosine by 8-phenyltheophylline was an indirect effect of this phosphodiesterase inhibition and the resulting rise in force, experiments were repeated using papaverine, which has no action at adenosine receptors but blocks phosphodiesterase activity. In three experiments, 10 nM papaverine increased the tension by $22.5 \pm 13.1 \%$. The positive inotropic effect of these agents made no difference to the response to 5 nM isoprenaline; control = $92.8 \pm 16.3 \%$ (n=10), + 8-phenyltheophylline = $97.8 \pm 15.2 \%$ (n=5), + papaverine = $87.9 \pm 13.2 \%$ (n=3).

Adenosine at a concentration of 15 μ M reduced the tension stimulated by 5nM isoprenaline by $31.5 \pm 8.7 \%$ (n=5). In the presence of 8-phenyl theophylline, this inhibition was reduced to $11.5 \pm 10.9 \%$ (n=5). With papaverine in the perfusate, the reduction in tension was $31.8 \pm 17.9 \%$ (n=3). However due to the small effect of adenosine and the large experimental error, these values were not significantly different from each other. So, from these results it is not possible to say whether or not the effect of adenosine is mediated by external A-type receptors.

d. Potentiation of the Response to Isoprenaline by Adenosine Deaminase.

One of the reasons for the difference between the response of whole heart and of papillary muscle to isoprenaline may lie in the method of perfusion. Langendorff perfused hearts are perfused through the capillary bed and are likely to be well oxygenated. With a superfused muscle, the middle is likely to be hypoxic and adenosine may be released in response to this. Such endogenously produced adenosine will mask the effect of any added adenosine and will also tend to make the response to isoprenaline smaller.

Therefore the ability of adenosine deaminase to potentiate the response to isoprenaline was tested. In the four cases where this was tested, 5nM isoprenaline increased developed tension by $63.8 \pm 23.9 \%$ and in the presence of adenosine deaminase (1 unit/ml) by $141.1 \pm 26.7 \%$. This suggests that there was endogenous adenosine present which could have had an antiadrenergic effect, thereby masking the effect of added adenosine.

Discussion.

The results presented in this chapter provide evidence for the theory that the disparity between the observations of Schrader et al [1977] with guinea pig Langendorff and the findings of Endoh and Yamashita [1980] with rabbit papillary muscle may be due in part to differences in experimental technique.

The first reason for the differences may lie in the concentration of isoprenaline used to elicit the positive inotropic effect, since, as can be seen in figure 2.1, the effect of adenosine is only seen when the heart is stimulated with isoprenaline at concentrations of 10 uM or lower. This is also true for the papillary muscle, but here the

antiadrenergic effect of adenosine is smaller and the apparent dependency on isoprenaline concentration not so marked.

The experiment using adenosine deaminase shows that some of the reason for the meagre response to added adenosine in papillary muscle may be due to endogenously produced adenosine. Adenosine is thought to be produced in response to hypoxia [Rubio et al 1974], and superfusion of muscles may allow them to become hypoxic internally. This is not likely to happen with the Langendorff preparation as the perfusion technique directs the oxygenated solutions through the capillary bed.

A further reason for the reduced antiadrenergic effect with the papillary muscle preparation could be due to the fact that at high concentrations adenosine has a positive inotropic effect. This effect is shared by 2-chloroadenosine and is therefore not likely to be due to a degradation product, since the analogue is deaminase resistant.

The experiments described here on tissue cAMP do not support the currently held hypothesis that the antiadrenergic effect of adenosine is mediated by a lowering of adenylate cyclase activity. Most other groups have found that adenosine lowered the

cAMP levels raised by isoprenaline [Schrader et al 1977, Linden et al 1985, Belardinelli et al 1982, Bauman et al, 1981, and Dobson 1983]. However all of the above groups used hydrochloric acid or perchloric acid for denaturation of the heart proteins. Bates et al [1978], have shown that certain enzymes, notably adenylate kinase and creatine kinase, are not destroyed by acid extraction, although they are inactivated by acid/alcohol extraction at -10°C . Thus where the heart is extracted in perchloric or hydrochloric acid alone, the values reported for adenine nucleotides must be regarded as uncertain. Using the same extraction procedure described by Bates et al [1978], I have noted that both adenosine and isoprenaline raise the tissue levels of cAMP. This overall elevation of cAMP by adenosine may be due to opposing effects of adenosine on the adenylate cyclases of the myocytes and the smooth muscle cells lining the coronary circulation. (see Introductory chapter, part E for further discussion). Thus as far as the total tissue levels of cAMP are concerned, a small increase is seen.

Because the effect on papillary muscles was so small. I was unable to show whether it is mediated by A-type receptors or not, although the results obtained tended to support this conclusion. In the

following chapter I describe experiments performed using the deaminase resistant analogue, 2-chloroadenosine. Using this compound, adenosine deaminase may be added to remove any endogenous adenosine. The antiadrenergic effect of 2-chloroadenosine would be expected to be enhanced, allowing experiments to be performed with greater accuracy.

CHAPTER 3

The Effect of 2-chloroadenosine on the Positive
Inotropic Response of Guinea-pig Papillary Muscle
to Isoprenaline.

Introduction

In chapter 2 I mentioned that the antiadrenergic effect of added adenosine may be masked by endogenously produced nucleoside. In this chapter I have set out to test this. As has already been shown, adenosine deaminase potentiates the response to isoprenaline, suggesting that adenosine produced in response to hypoxic conditions has an antiadrenergic effect on added isoprenaline. If 2-chloroadenosine (an adenosine deaminase resistant analogue) is used in conjunction with adenosine deaminase, then a greater antiadrenergic effect should be seen. The reasons for this are twofold. Firstly, the removal of endogenous adenosine should allow the added analogue to exert the maximum possible antiadrenergic effect, secondly, the receptors will see the actual amount of 2-chloroadenosine added since it is not metabolised by added or endogenous adenosine deaminase. Nor is it removed into the cells since it is not a substrate for the uptake system [Sturgill et al 1975]. The difference between the effect of adenosine and that of 2-chloroadenosine (plus adenosine deaminase) should be more pronounced at higher concentrations of isoprenaline since the

muscle will be working harder and releasing more adenosine [Berne 1980].

Using the concentrations of both 2-chloroadenosine and isoprenaline which give the maximum (and most reproducible) response, the action of 2-chloroadenosine was characterised with respect to antagonism by methylxanthines.

Methods

Guinea pig papillary muscles were set up as described earlier (methods A.2.) and superfused with Krebs-Hensleit solution containing 1.25 mM Ca^{++} . In these experiments adenosine deaminase was perfused into the organ bath for two minutes or until a stable trace was regained, before the addition of isoprenaline (plus deaminase) or isoprenaline plus 2-chloroadenosine (plus deaminase). Experimental procedures for concentration/response curves as well as antagonism by 8-phenyltheophylline were as described in the previous chapter.

Results.a. Dose Response of the Antiadrenergic Effect of 2-chloroadenosine in The Presence of Adenosine Deaminase.

In the presence of 1 unit/ml adenosine deaminase, 2-chloroadenosine was found to attenuate the rise in tension due to 10 nM isoprenaline. This inhibition was found to be dose dependent (figure 3.1). The maximum effect was at 5 μ M 2-chloroadenosine, when no significant rise in tension was seen. This inhibition was found to be completely reversible after the removal of 2-chloroadenosine from the bathing solution (figure 3.2). Adenosine deaminase at a concentration of 1 unit/ml will completely remove up to 0.1 mM adenosine (see figure 8.3, Chapter 8)

b. Dose Response To Isoprenaline $\pm$ 10 μ M 2-chloroadenosine plus adenosine deaminase.

Figure 3.3 shows the effect of 10 μ M 2-chloroadenosine on the increase in tension by isoprenaline in guinea pig papillary muscle. 2-chloroadenosine causes a parallel shift to the right of the isoprenaline dose response curve. There is a greater percentage inhibition by 2-chloroadenosine at lower concentrations of

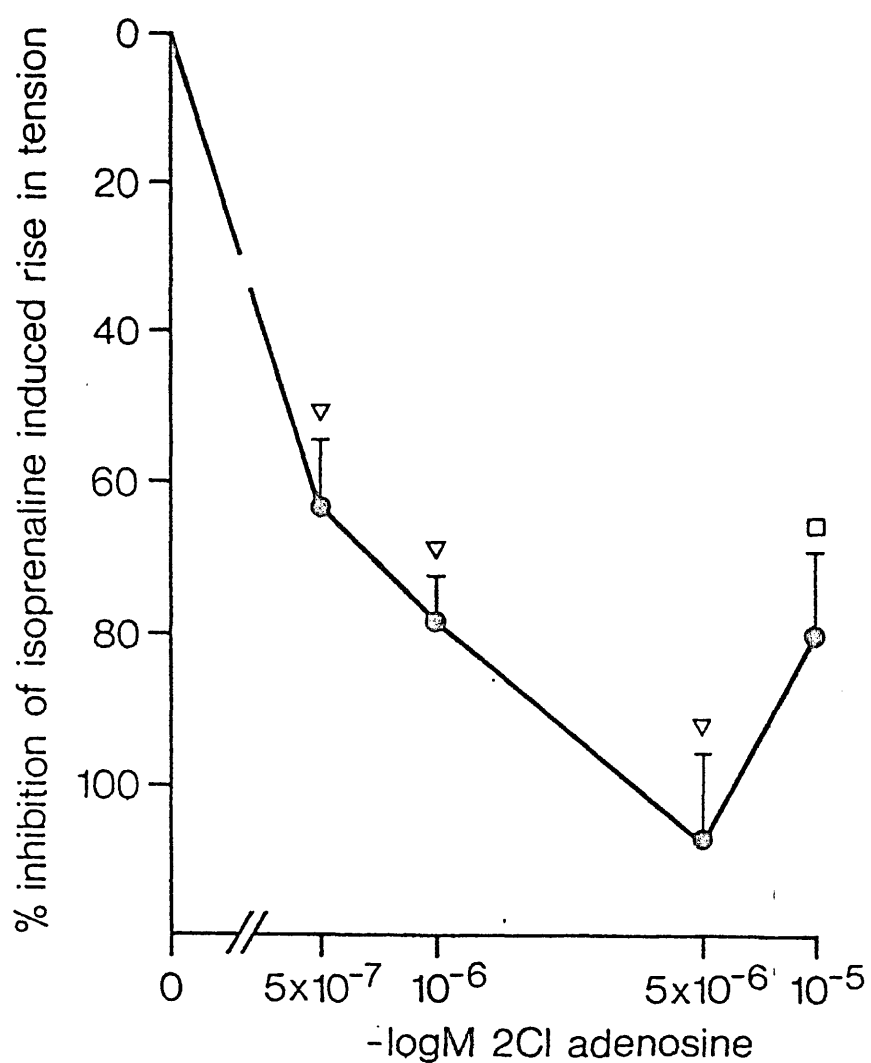


Figure 3.1 Dose response of guinea pig papillary muscle to 2chloroadenosine in the presence of 1 unit/ml adenosine deaminase. Developed tension was stimulated by 10 nM isoprenaline . The points shown are the mean $\pm$ S.E.M. of 5 separate determinations. $\nabla = P < 0.002$, $\square = P < 0.01$. (paired Student's t-test)

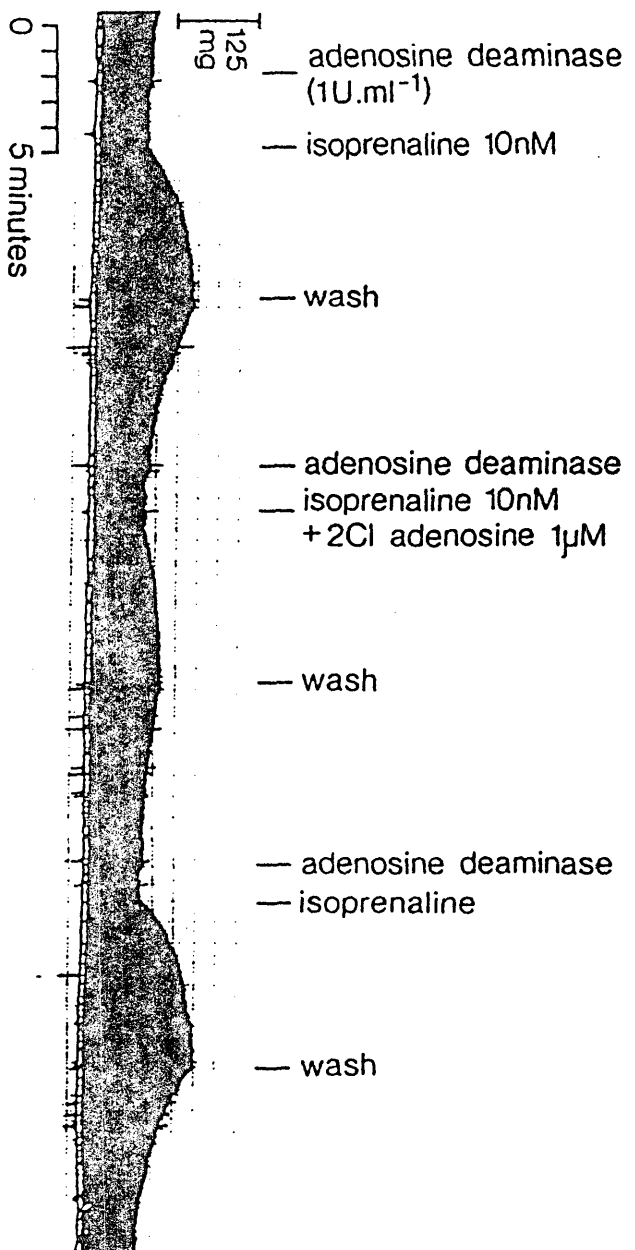


Figure 3.2 Effect of 2-chloroadenosine on the positive inotropic effect of isoprenaline. Shown is a copy of a tension trace illustrating the extent of, and reversibility of the effect of 1 µM 2-chloroadenosine on the positive inotropic effect of 10 nM isoproterenol.

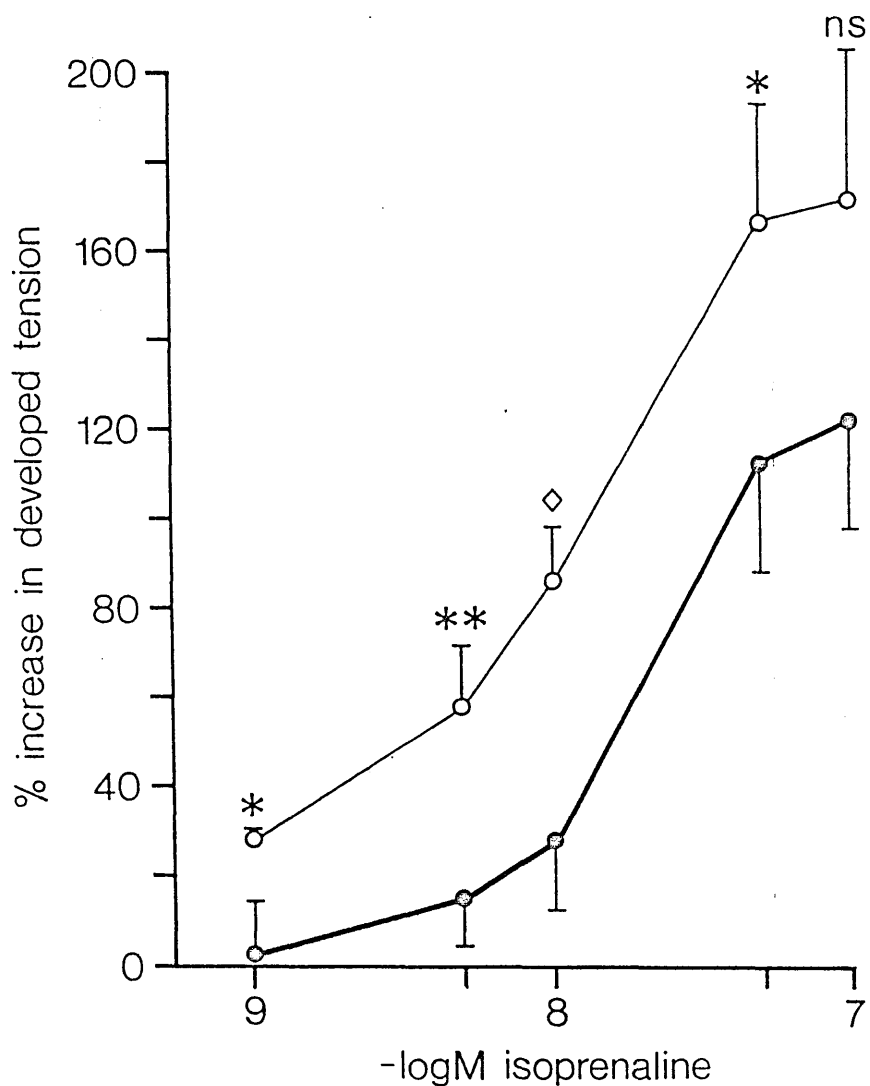


Figure 3.3 Dose response of guinea-pig papillary muscle to isoprenaline + 10 uM 2-chloroadenosine in the presence of 1 unit/ml adenosine deaminase.

Isoprenaline alone = ○—○, + 2-chloroadenosine = □—□

The points shown are the mean + S.E.M. of 5 separate determinations. * = $P < 0.05$, ** = $P < 0.002$, ◇ = $P < 0.001$. (paired Student's t-test)

isoprenaline than at higher ones. However, there is also significant block at 50 nM isoprenaline which was not seen with the Langendorff preparation.

c. Antagonism of the Antiadrenergic Effect of 2-chloroadenosine by 8-Phenyltheophylline.

The experiments described in the previous chapter were now repeated using 2-chloroadenosine together with 1 unit/ml adenosine deaminase. The results of these experiments are summarised in figure 3.4. Note that 10 μ M 8-phenyl theophylline almost completely blocks the antiadrenergic effect of 10 μ M 2-chloroadenosine. A sample trace from these experiments is shown in figure 3.5. A control was performed using papaverine as described in the previous chapter. Although papaverine raised developed tension to a similar extent as 8-phenyltheophylline, no inhibition of the antiadrenergic effect of 2-chloroadenosine was seen. It was found that 2-chloroadenosine reduced basal tension by approximately 35% (fig. 3.4) this was prevented by the prior administration of reserpine to the animals to deplete catecholamine stores.

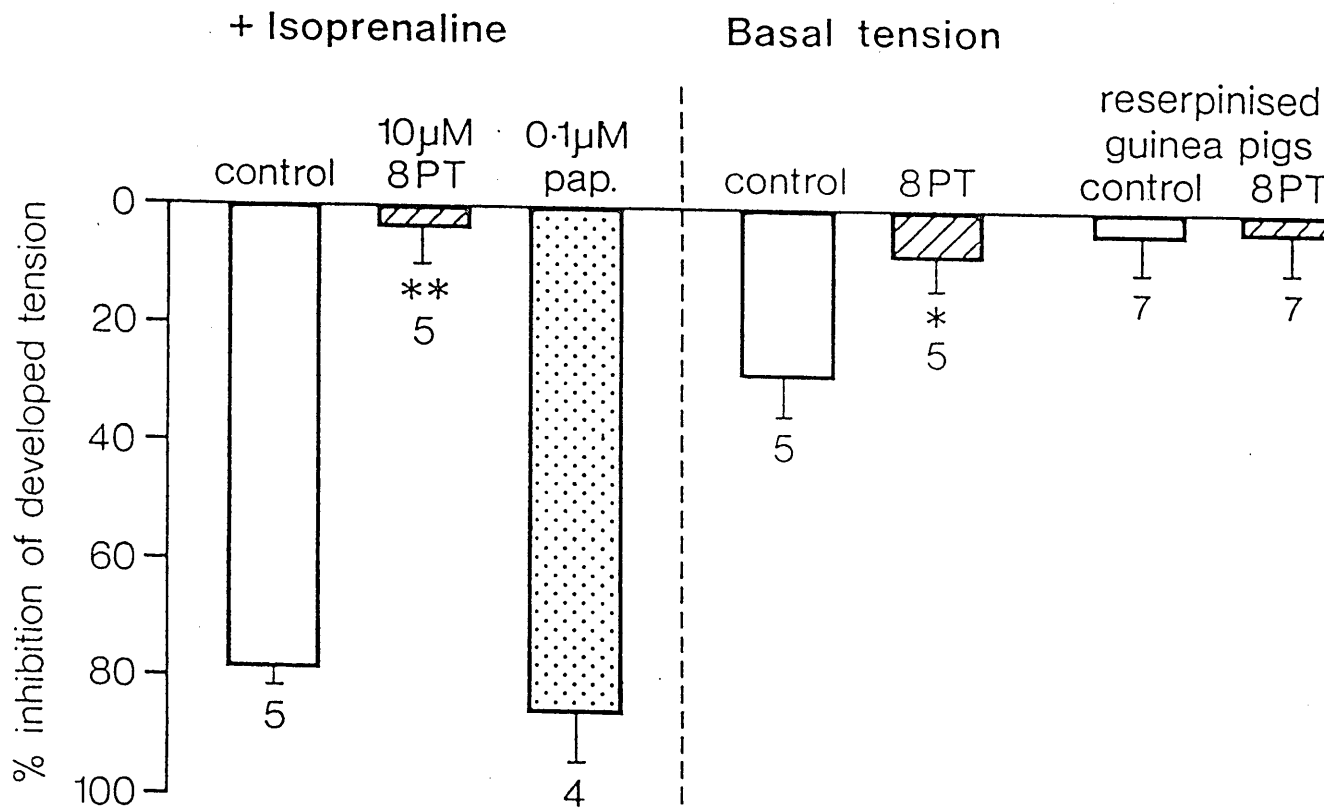
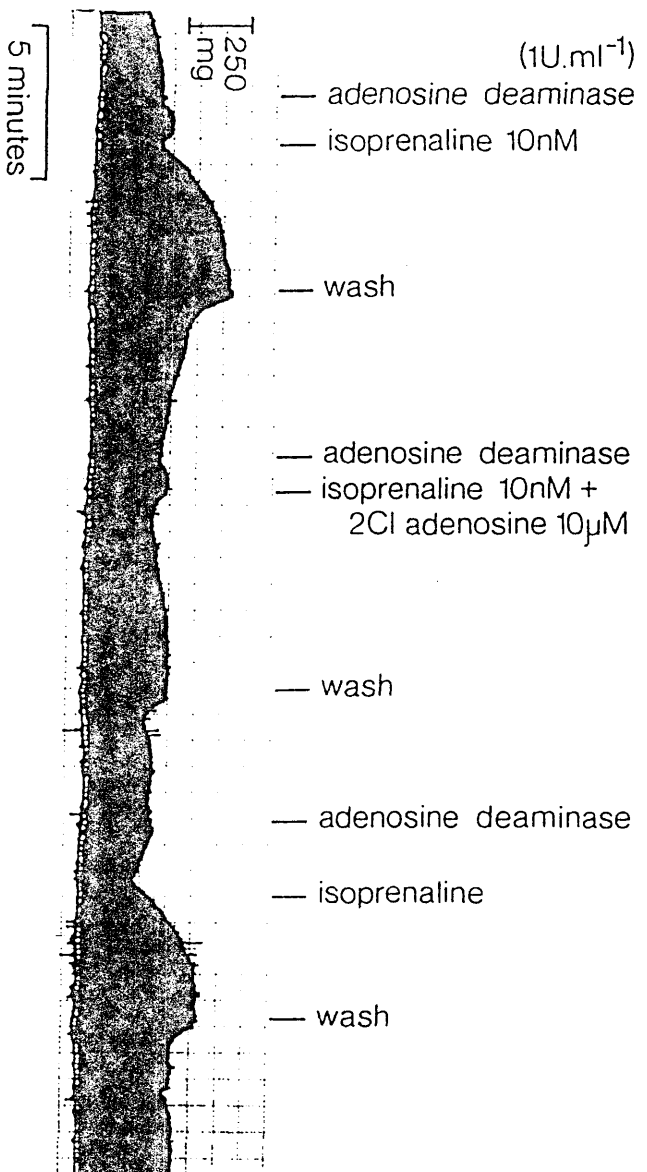


Figure 3.4 The antagonism of the antiadrenergic effect of 2-chloroadenosine in the presence of adenosine deaminase by 8-phenyltheophylline (8PT). Also shown is the effect of papaverine (PAP). In these experiments 10 μ M 8PT stimulated tension by 42.1 ± 16.1 %, and 0.1 μ M papaverine stimulated tension by 21.5 ± 2.5 %. n for each experiment is as indicated on the figure. * = $p < 0.05$, ** = $P < 0.001$ (paired Student's t-test.)

Control



In the presence of 10μM 8-phenyltheophylline

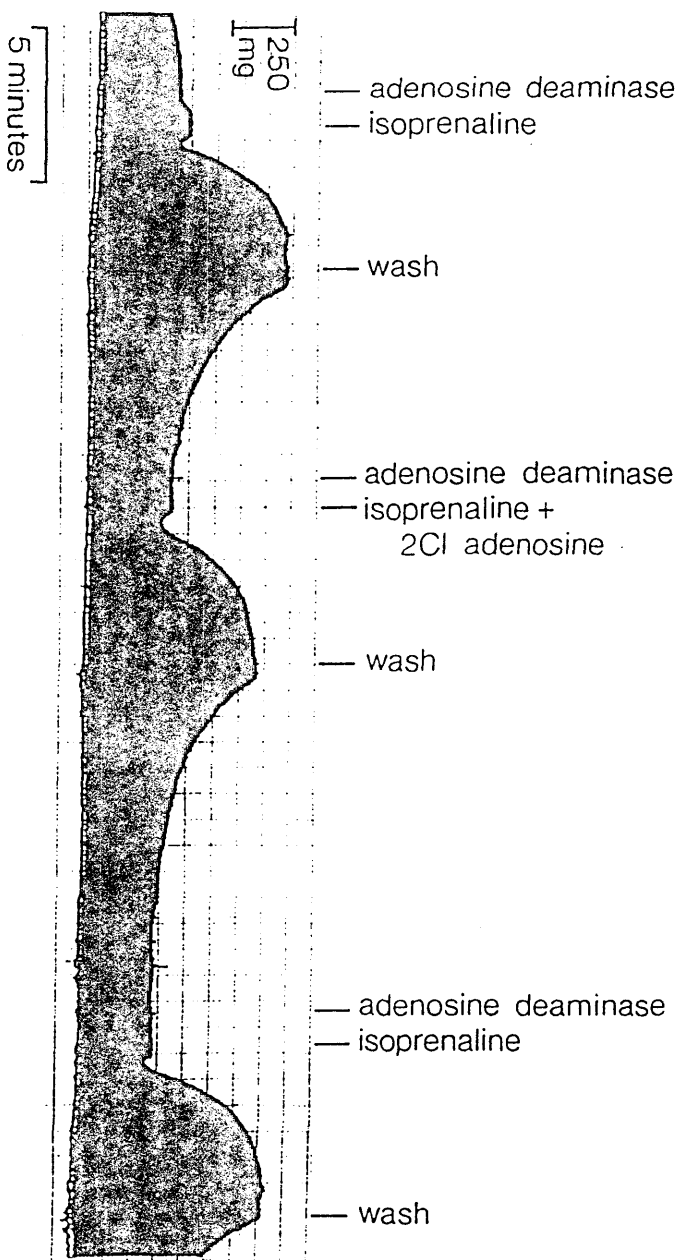


Figure 3.5. Sample traces illustrating the antagonism of the antiadrenergic effect of 2-chloroadenosine in the presence of adenosine deaminase by 8-phenyltheophylline.

Discussion

The use of 2-chloroadenosine has a twofold advantage. It is not broken down by adenosine deaminase, (present in the muscle or added exogenously), nor is it removed from the extracellular medium as it is a poor substrate for the uptake system. This makes it a good probe of adenosine actions at cell surface receptors, despite its reported in vitro P-site actions [Londos et al 1981]. The two most commonly used 'R-site' modified analogues, PIA and NECA were also considered for use in this study. However there were problems encountered with both compounds. PIA is lipophilic and is not easily washed off, indeed in preliminary experiments a satisfactory reversibility of action was not obtained even after 40 minutes of continuous washing. NECA is more potent at A₂ than A₁ receptors, which makes it unsuitable for use in this study, where the receptors are most likely to be A₁ in nature.

The complete block of isoprenaline-stimulated tension by 5 μ M 2-chloroadenosine suggests that the modest effect of adenosine on papillary muscles was due to a masking effect by endogenously produced adenosine. This is a more likely reason for the disparity between whole heart and papillary muscle

than the hypothesis that some of the effects of adenosine on whole heart are due to additional haemodynamic effects.

2-chloroadenosine is able to inhibit the positive effect of isoprenaline at all concentrations of isoprenaline tested. Approximately the same amount of isoprenaline-stimulated tension was inhibited by 2-chloroadenosine in each case. This type of parallel dose response curve displacement is reminiscent of antagonist-agonist interactions at a discrete receptor site. However it has been shown that the action of adenosine is not at the β -receptors but at separate adenosine receptors [Drummond and Severson 1979].

The displacement can be explained if the following assumptions are made;

1. The actions of adenosine are primarily on cAMP levels in cardiac myocytes. Up to 40-60% inhibition of maximum cAMP production can be attained. This is the most inhibition observed in vitro [Leung et al 1983a & b].

- ii. Basal cAMP does not contribute to tension.

- iii. The relationship between cAMP concentration and tension is hyperbolic. At low

concentrations of isoprenaline, the increase in cAMP is approximately linearly related to the increase in tension, but at the higher concentrations more than enough cAMP is produced to account for the observed increase in tension. Experiments with forskolin, an agent which greatly stimulates adenylate cyclase activity, support this view [Rodger and Shahid 1984].

Thus at lower concentrations of isoprenaline the inhibition of adenylate cyclase by adenosine can match the increase in cAMP levels elicited by the B agonist. At intermediate concentrations a percentage inhibition is seen because the amount of cAMP produced is higher than the inhibitory adenosine receptors can cope with, ie. after the 40-60% inhibition of adenylate cyclase some cAMP still remains to stimulate tension. At 10^{-7} M isoprenaline, tension and cAMP are no longer linearly related and the % inhibition by adenosine is that much smaller.

This is an example of functional antagonism. At certain concentrations of agonist and antagonist, the parallel displacement of the dose response curves can closely resemble competitive behavior. The main difference between the two types of antagonism, is that the maximum is depressed

with functional but not competitive antagonism. The maximum stimulation of tension by isoprenaline appears to be depressed by 2-chloroadenosine in these experiments. However I was unable to determine whether a maximum could be re-attained with a higher concentration of isoprenaline since this compound tends to have an arrhythmic effect on the heart at concentrations above $0.1 \mu\text{M}$ (see chapter 2). Although the above scheme cites inhibition of adenylate cyclase as the mechanism for the antiadrenergic effect, the actual site for the functional antagonism between 2-chloroadenosine and isoprenaline could be one (or more) of many. These include stimulation of the cAMP phosphodiesterase, alterations of Ca^{++} gating or alterations in phosphatidylinositol breakdown.

The effects of adenosine and its purine modified analogues at the receptor are antagonised in a competitive manner by methylxanthines. This provides a good indication that the antiadrenergic effect of adenosine effect is mediated by extracellular A-type receptors rather than the intracellular P-site. However, as noted in the previous chapter, methylxanthines also inhibit the phosphodiesterase and thus raise cAMP levels. 8-phenyltheophylline was used since it has good adenosine receptor antagonism properties with

little phosphodiesterase inhibitor activity. As has been demonstrated in the previous chapter, at a concentration of 10^{-5} M, this compound increases developed tension, possibly by increasing cAMP levels. Therefore, as a control, papaverine (a non-methylxanthine phosphodiesterase inhibitor with little effect at adenosine receptors) was also used at a concentration which brings about approximately the same rise in tension. It was important to design a proper control for this since I have observed that adenosine has a smaller percentage effect at higher isoprenaline concentrations, and therefore higher cAMP levels. Such an effect could make it appear that 8-phenyltheophylline was antagonising the effect of adenosine by competition at the receptor when all it was doing was raising the level of cAMP. 8-phenyltheophylline at 10^{-5} M can almost entirely block the effect of 2-chloroadenosine. This is strong evidence for the idea that all of the effects of 2-chloroadenosine are mediated by extracellular A-type receptors and not by the internal P-site. The lack of effect of papaverine on the antiadrenergic effect of 2-chloroadenosine shows that the block by 8-phenyltheophylline is not an artefact due to blocking of the phosphodiesterase.

When 2-chloroadenosine was used, a marked

reduction of basal tension was noted. This was not apparent in papillary muscles obtained from reserpinised animals, suggesting that 2-chloroadenosine was having an antiadrenergic effect on tension stimulated by endogenous catecholamines. These catecholamines are probably released in response to the electrical stimulation used. That 8-phenyltheophylline blocked this effect suggests that it was mediated by the same receptors that mediate the antiadrenergic effect.

Summary of Main Points

2-chloroadenosine reduced the tension stimulated by 10 nM isoprenaline in a dose dependent manner reaching a maximum of 100 % inhibition at 5 μ M. This effect becomes progressively smaller at higher concentrations of isoprenaline, possibly due to the small fixed effect that 2-chloroadenosine has on cAMP levels in cardiac myocytes and also to the proposed non-linearity of the relationship between cAMP and tension at high cAMP levels. This antiadrenergic effect of 2-chloroadenosine has been shown to be mediated by A-type receptors, as illustrated by the ability of 8-phenyltheophylline to abolish the response. Any effects on basal tension can be attributed to an antiadrenergic effect on released

endogenous catecholamines.

CHAPTER 4

The effect of Adenosine and 2-chloroadenosine on
Tension in Rabbit Papillary Muscles.

Introduction

Although the disparity between the observations of Schrader et al [1977] on guinea pig Langendorff and the results reported by Endoh and Yamashita [1980] have been partially explained on the basis of different experimental procedures (chapter 2), it still remains to be seen whether there is also a real species difference between guinea pig and rabbit in the way that ventricular myocardium reacts to adenosine. The rabbit might be expected to show a smaller response since it has been shown to differ from some other species in that coronary flow is not sensitive to adenosine [Day and Johnson 1959]. The following experiments were performed to see if there is any detectable species difference in the antiadrenergic effect of adenosine and 2-chloroadenosine.

Methods.

Rabbit papillary muscles were set up as described in methods A2. and perfused with Krebs Hensleit solution containing 2.5 mM Ca^{++} . Dose response curves to isoprenaline and isoprenaline plus adenosine were performed as described for guinea pig papillary muscle (Chapter 2). In the experiment with 2-chloroadenosine, adenosine deaminase was infused into the bath for 2 minutes before the addition of 2-chloroadenosine or isoprenaline as described before (Chapter 3).

The dose-response to adenosine in the presence of a fixed concentration of isopenaline was performed as follows; After the initial equilibration period, 5×10^{-8} M isoprenaline was given and the tension developed allowed to plateau. With isoprenaline continually in the perfusion fluid, doses of adenosine in the range 0.1 μM to 1 mM were given. The muscle was stimulated at 0.5 Hz and maintained at a temperature of 37 °C. These conditions are the same as those described by Endoh and Yamashita [1980].

Results a. Dose Response to Isoprenaline $\pm$ 15 μ M Adenosine.

Rabbit papillary muscles had a resting tension of 1.25 ± 0.17 g ($n=25$). The response of the muscle to adenosine when stimulated by 1-10 nM isoprenaline was examined (figure 4.1). As can be seen, there was no noticeable effect. The effect of adenosine on 10-100 nM isoprenaline was then investigated, but no significant effect was seen (Figure 4.2).

b. Dose response to adenosine in the presence of 50 nM isoprenaline.

When the experiment was performed as described by Endoh and Yamashita [1980], a maximum of 15 % inhibition by 100 μ M adenosine could be seen. (figure 4.3). Above this concentration only the positive inotropic effect noted in Chapter 3 was observed. In control experiments, in the absence of isoprenaline it was found that below 0.1 mM a negligible, non-significant effect on basal tension was seen. At 1 mM, adenosine stimulated tension by 75.8 ± 17 4% ($n=3$).

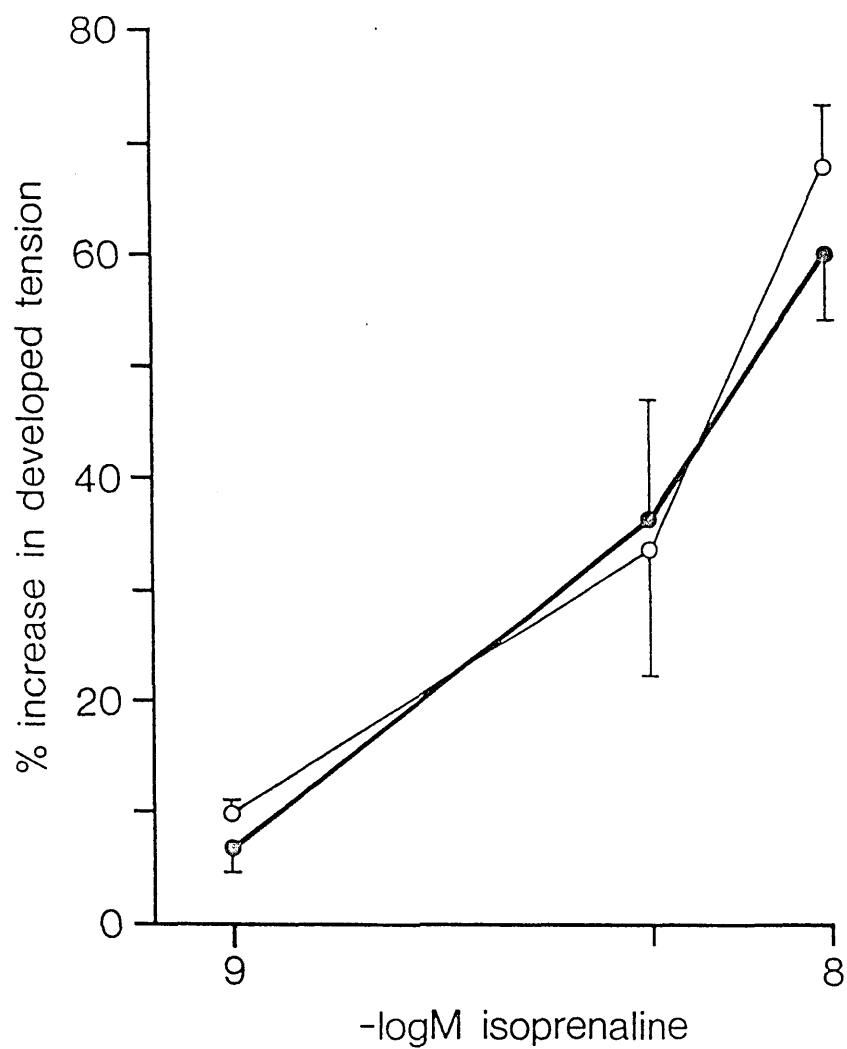


Figure 4.1 Dose response of rabbit papillary muscle to low doses of isoprenaline + 15 uM adenosine. Isoprenaline only = $\bigcirc$ — $\bigcirc$, + 15 uM adenosine = $\bullet$ — $\bullet$. The points shown are the mean $\pm$ S.E.M. of 5 separate determinations.

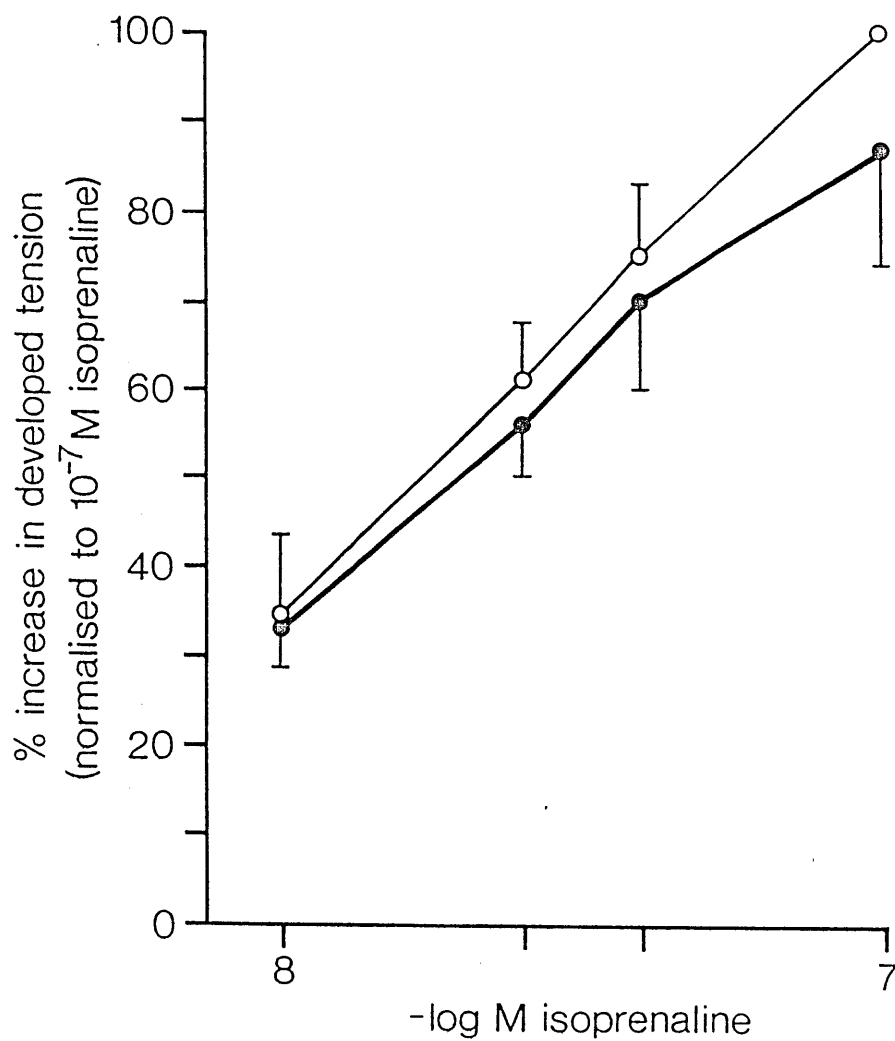


Figure 4.2 Dose response of rabbit papillary muscle to high doses of isoprenaline + 15 μ M adenosine. Isoprenaline only = $\bigcirc$ — $\bigcirc$, + 15 μ M adenosine = $\bullet$ — $\bullet$. The points shown are the mean $\pm$ S.E.M. of 5 separate determinations.

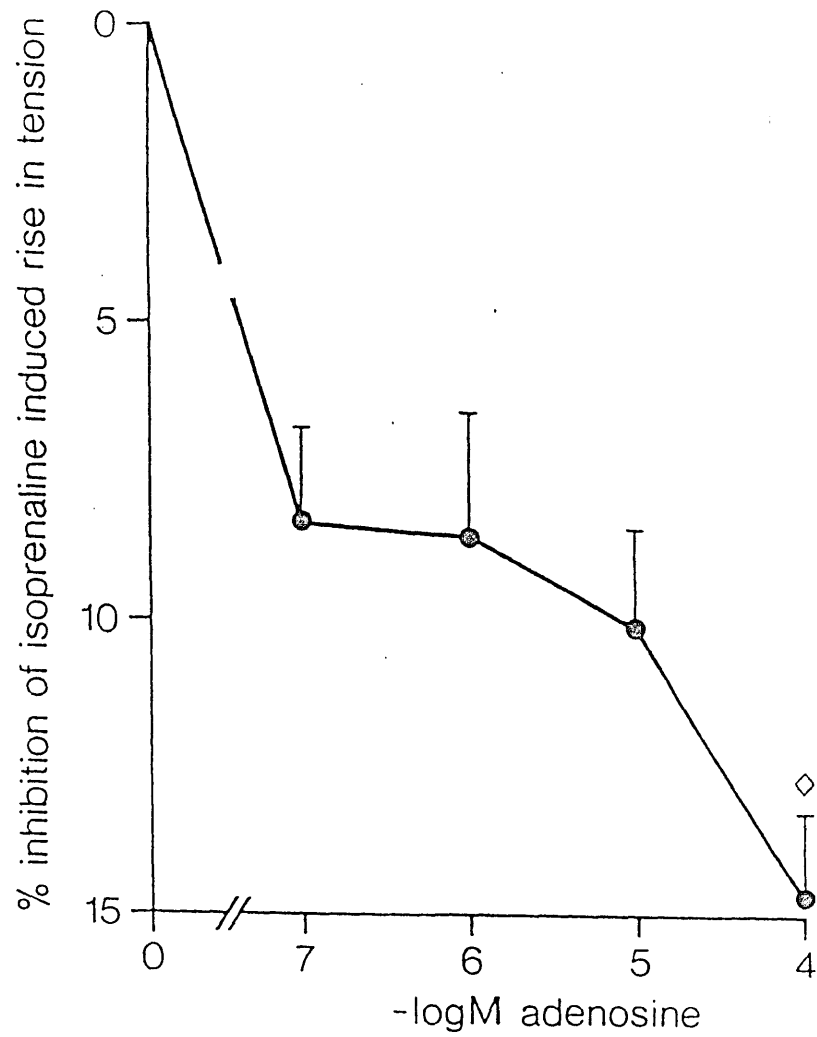


Figure 4.3 Dose response of rabbit papillary muscle to adenosine in the presence of 50 nM isoprenaline. The muscle was exposed to isoprenaline for one hour before addition of adenosine. $\diamond = P < 0.001, n=5$ (Student's t-test, paired against zero adenosine).

c. Antiadrenergic Effect of 2-chloroadenosine in the Presence of Adenosine Deaminase.

10 nM isoprenaline stimulated tension by $34.2 \pm 10.2 \%$ (n=6). 10 μ M 2-chloroadenosine had very little action on this positive inotropic effect, the rise in developed tension being $31.1 \pm 2.8 \%$ (n=6). Note that these are the conditions under which I was able to see a 100% antiadrenergic effect with guinea pig papillary muscles (see previous chapter). A sample trace from these experiments is shown in figure 4.4.

Discussion.

Under the conditions where a small but significant antiadrenergic effect of adenosine was seen with guinea pig papillary muscles, no such response was seen with rabbit muscles. The idea that rabbits might have a similar response to adenosine as rats, ie. that they could show a greater inhibition by adenosine at higher isoprenaline concentrations [Dobson 1983] was tested but also proved fruitless. If however the experiment was performed as described by Endoh ie adding adenosine after a stable response to

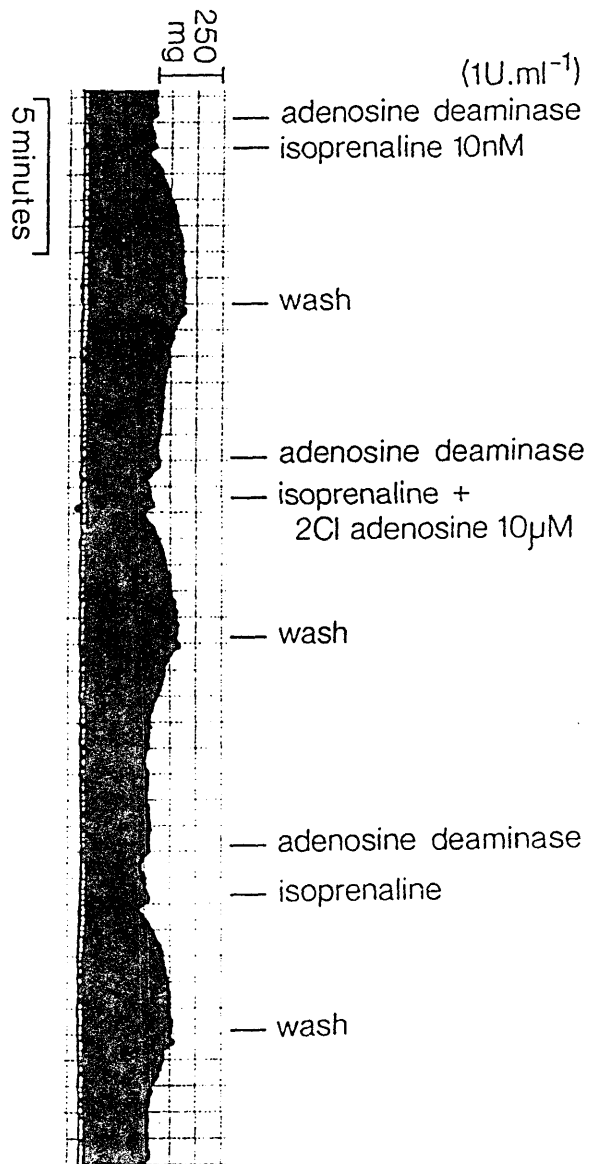


Figure 4.4 A sample trace showing the lack of effect of 2-chloroadenosine on the positive inotropic effect of isoprenaline on rabbit papillary muscles.

isoprenaline had been established, then a small but statistically significant inhibitory effect of adenosine was seen. However Endoh and Yamashita reported a rather larger response- 45%. I initially thought that the discrepancy between my results and those of Endoh and Yamashita [1980] could be explained by assuming that the latter used smaller less hypoxic muscles. Adenosine is thought to be released in response to hypoxia, [Berne 1980] and the more hypoxic the muscle, the greater the production of adenosine. However, I was unable to see a more pronounced effect using 2-chloro adenosine in conjunction with adenosine deaminase, which suggests that this explanation is incorrect.

These results imply that there is a real species difference between guinea pig and rabbit in the way that the ventricular myocardium reacts to isoprenaline and adenosine.

CHAPTER 5

The Effect of Adenosine and its Analogues on the
Adenylate Cyclase Activity of Rabbit and Guinea-pig
Ventricular Myocardium.

Introduction.

Schrader et al in 1977 and Dobson [1983] have been able to show that adenosine lowers cAMP levels in whole tissue. This is contrary to my findings (chapter 2), where I showed that adenosine had a small stimulatory effect on cAMP levels. The experiments of both Dobson^b_A [1983] and Schrader's Group [1977] on homogenates from ventricles are inconclusive in that the inhibition exhibited is more characteristic of the P-site. This is especially apparent with Dobson's studies, since he was unable to antagonise the attenuation of adenylate cyclase activity with theophylline. He also found that the reduction in adenylate cyclase activity was more pronounced at higher Mg ion concentrations, which is characteristic of the P-site [Cochaux et al 1982, Wolff et al 1978, Welton and Simko 1980 and de Vente and Zaagsma 1981]. The possible involvement of the P-site is discussed in the next chapter.

Biochemical studies of the effects of adenosine and its analogues on the adenylate cyclase activity of ventricular preparations have done little to clarify this picture. Schutz and

Tuisl [1981] (guinea pig and rat) and Avdonin et al [1982] (rabbit) report that there is no effect of adenosine mediated through A-type receptors -only a P-site effect. Anand-Strivasrava and Cantin [1983] (neonatal rat myocytes), Webster and Olsson [1981] (dog ventricle) and Huang and Drummond [1976, 1978] (rat and guinea pig ventricular slice, cAMP levels) report a stimulatory effect of adenosine on cardiac adenylate cyclase. Leung et al [1983] report that PIA can inhibit the adenylate cyclase of guinea pig myocardium in the absence of B-stimulation, whereas Hosey et al [1984] working with chick heart homogenate, and Dobson [1978] (rat ventricular slices, cAMP levels) found that only an antiadrenergic effect could be seen. Further evidence that the adenosine receptors on the myocytes might be negatively coupled came from the studies of Hazeki et al [1981] who found that IAP treatment prevented adenosine or muscarinic receptor mediated inhibition of adenylate cyclase in adult rat myocytes.

Some of these disparities can be attributed to species variations, but others may be a consequence of differences in experimental design. As discussed in the introductory chapter, if both A1 and A2 receptors are present in a given homogenate, manipulation of the assay conditions will tend to

favour the expression of one or the other of the receptors.

In this chapter I have attempted to find the optimal conditions for the expression of A1 receptors on membranes from guinea pig and rabbit heart.

In 1983 Leung et al reported that the optimal Na^+ concentration was at 50 mM. No appreciable inhibition was seen at 100 mM, the generally reported ideal concentration of sodium for expression of inhibitory adenosine and other receptors. [Anand-strivaatava 1983, Blume et al 1979, Bockaert et al 1984, Cooper and Londos 1979, Cooper et al 1979, Cooper et al 1980, de Mazancourt et al 1982, Hosey et al 1984, Londos et al 1978, Onali et al 1983]. In this chapter the dependency of A1 receptor expression on Na^+ was studied using guinea pig and rabbit membranes.

The Mg dependency of these receptors was recently brought to light by the findings of Bockaert et al [1984]. They observed that high concentrations of Mg^{++} would tend to suppress hormonal inhibition of adenylate cyclase. In this chapter the optimal Mg ion concentration was therefore established for guinea pig and rabbit ventricular membranes.

Hsia et al [1984] found that with opiate receptors, the relationship between GTP concentration and receptor mediated inhibition of adenylate cyclase was biphasic, the optimum concentration being around 10 μ M, with less inhibition at concentrations above this. Using the best Na⁺ and Mg⁺⁺ levels, I determined the optimal GTP concentration for both rabbit and guinea pig ventricle.

Using the best conditions for both species, the effect of a concentration of isoprenaline which gave half maximal stimulation of cyclase activity on A1 receptor mediated inhibition was studied. Hosey et al [1984] found isoprenaline stimulation to be necessary for receptor expression.

De Mazancourt and Guidicelli in 1984[a&b] reported that adenosine could stimulate the phosphodiesterase in fat cells and brain in a GTP dependent manner. As this would lead to an indirect lowering of cAMP levels and might be seen in assays where the phosphodiesterase was not fully blocked, [Leung et al 1983, Hosey et al 1984], the effect of adenosine on the breakdown of cAMP in the absence of a phosphodiesterase inhibitor was investigated.

Methods.

General. GTP was present in all the experiments described in this Chapter, so in each case the membranes were preincubated with GTP before the addition of ATP (see Methods, section B2). RO 20 1724 at 3.5 mM was present as a phosphodiesterase inhibitor in all but the experiments on cAMP breakdown.

Rabbit sarcolemmal membranes were prepared by the method described in Methods section B1a and crude membranes from guinea pig and rabbit prepared by the method described in B1b.

Both the adenosine free systems were utilised in this chapter and the use of one or the other is noted in the relevant experiment.

1. Preliminary studies with rabbit membranes.

The dATP (as described in Methods section B3b) system was used in these experiments. The concentrations of effectors were; GTP=0.1 mM, Na+=100 mM, Mg=12 mM. A concentration of theophylline was selected which did not inhibit adenylate cyclase (see Chapter 7.)

2.Optimisation of Conditions.

The experiments involved in studying the effect of Na^+ and Mg^{++} used the dATP system. The optimisation of GTP and the experiments described in the last section were performed using adenosine deaminase (Methods section B3). For optimisation of Na^+ , Cyclase Assay Buffer with 4mM Mg was used with 0.1 mM GTP. Studies on Mg influence used buffer containing no Mg with 50 mM Na^+ , and 0.1 mM GTP. The optimal GTP was determined using assay buffer containing 3.53 mM Mg and 58 mM Na (to allow for the extra dilution of the solvent for RO 20 1724).

When the contamination of the assay medium by adenosine was investigated, the NEN kit was used for the measurement of cAMP and cdAMP. Cyclase Assay Buffer containing 4 mM Mg was utilised together with 50 mM Na^+ and 0.1 mM GTP.

3.To test whether homogenisation speed influences receptor expression.

The heart was removed from a guinea pig in the usual way, cut into small pieces and divided into two portions. One half of the heart was homogenised for 3 seconds at speed 9 with the Polytron homogeniser. The other fraction was homogenised at

speed 5 for 3 seconds. The rest of the preparation was carried out as described in Methods, section B1b. Buffer containing 3.53 Mg and 58 mM Na was used in the assay.

4. Effect of PIA on adenylate cyclase activity and cAMP breakdown in the presence and absence of isoprenaline.

In both cases assay buffer containing 3.53 mM Mg and 58 mM Na was used. Half maximal isoprenaline was utilised, since it has been thought that the effect of adenosine and its analogues is more likely to be expressed at the lower doses of catecholamine. GTP at 10 μ M was present. In the experiment on breakdown of cAMP, a cAMP solution was made up and diluted so that 5.5 pMoles cAMP would be the maximum cAMP measurable in the 50 μ l sample taken for assay. The amount of cAMP broken down was calculated as described in Methods, section B4.

Results.

1. Preliminary Studies With Rabbit Sarcolemma.

a. Dose response to adenosine + 0.05 mM

theophylline. These experiments were performed prior to those described in chapters 2-4 in an attempt to find if there was any evidence for negatively coupled adenosine receptors in rabbit ventricle. Figure 5.1 shows a dose response to adenosine $\pm$ 0.05 mM theophylline. The addition of theophylline brings about a small shift to the right of the dose response curve at the submillimolar concentrations of adenosine. Although this shift is not statistically significant, it is suggestive of inhibitory adenosine receptors.

b. Inhibition of Adenylate cyclase In the Presence and Absence of Isoprenaline. Since Schrader et al [1977] and Dobson [1983] have produced evidence that stimulation of adenylate cyclase by isoprenaline is a necessary prerequisite for inhibition by adenosine, the effect of PIA on activity in the presence and absence of isoprenaline was studied. Isoprenaline at a

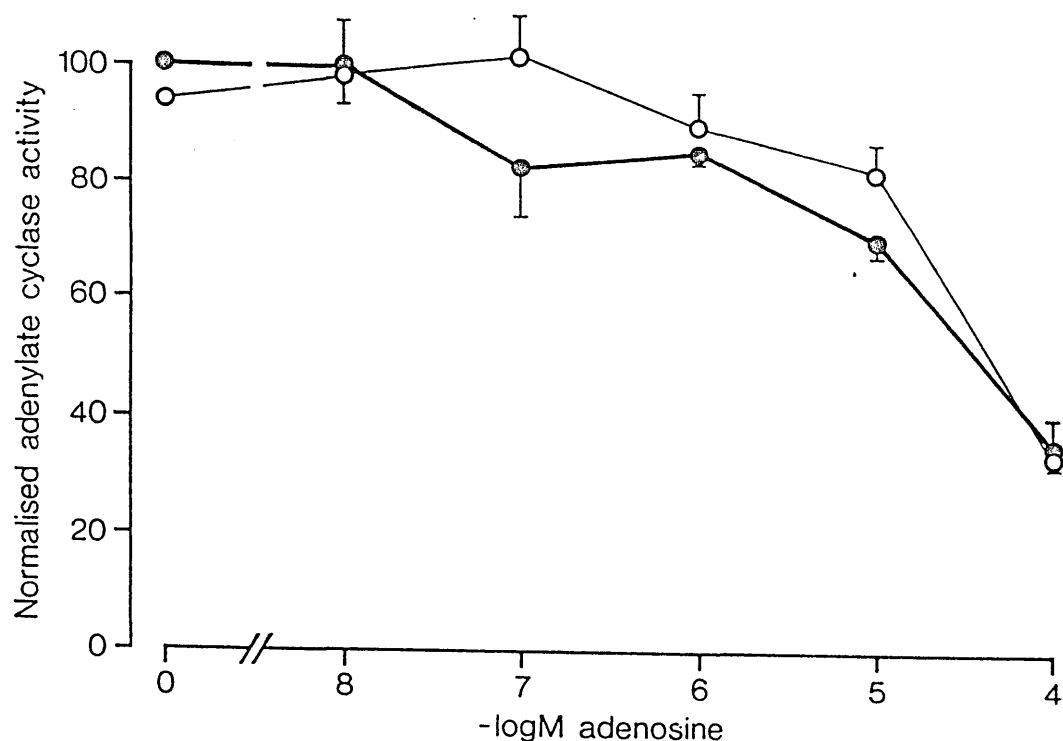


Figure 5.1 Concentration-response relationship between adenosine concentration and rabbit cardiac adenylyate cyclase activity in the absence ■—■, and presence of 0.05 mM theophylline. Actual activity at 0 M adenosine = 161.5 ± 37.3 pMoles cdAMP/min/mg protein. $n=5$, each point = mean $\pm$ S.E.M.

concentration of 0.1 mM stimulated adenylate cyclase activity by $49.6 \pm 14.1 \%$ (n=6). 0.1 mM PIA inhibited basal adenylate cyclase activity by $4.9 \pm 1.1 \%$ and isoprenaline stimulated activity by $10.9 \pm 7.3 \%$ (n=6). This is much smaller than the 40 % inhibition reported by Leung et al [1983], and is not statistically significant.

c. Effect of Method of Preparation of Membranes on Receptor Expression. Schutz and Tuisl [1981] felt that their membrane preparation could be at fault in that they were losing receptors at the 'high salt' step. To test whether this could be a factor in this case, the action of PIA on adenylate cyclase activity in a sarcolemmal preparation (methods B1a) and a crude preparation (B1b) was examined. The results are shown in table 5.1. There is no improvement in inhibition seen in a crude preparation. β -stimulation is the same in both cases, suggesting that few beta-receptors are lost at this stage.

2. Optimisation of Conditions For the Expression of Inhibitory Adenosine Receptors.

a. Determination of optimal sodium. The influence of Na⁺ concentration on A1 receptor expression was

Table 5.1 Percentage inhibition by 0.1 mM PIA, of adenylate cyclase from rabbit sarcolemma and crude membranes in the absence and presence of 0.1 mM isoprenaline.

Membrane Prep.	Percentage inhibition by PIA	
	-isoprenaline	+isoprenaline
Sarcolemma	8.2	14.7
Crude prep.	0.0	4.4

Isoprenaline at 0.1 mM stimulated adenylate cyclase of sarcolemma by 57 % and that of the crude prep by 62%. Results are the mean of a single determination, performed in triplicate.

Table 5.2 The effect of 25-150 mM NaCl on the percentage inhibition of adenylate cyclase of rabbit sarcolemma and guinea pig crude membranes by 0.1 mM PIA .

mM NaCl	% change in A.C. activity	
	Guinea pig	Rabbit
25	+5.7	+1.0 $\pm$ 6.2
50	-12.6	-12.7 $\pm$ 7.4
75	-3.5	+3.5 $\pm$ 4.8
100	-7.0	-9.2 $\pm$ 4.0
125	-7.4	-1.9 $\pm$ 6.1
150	-9.3	-3.1 $\pm$ 2.7
	(n=2)	(n=3 $\pm$ SEM)

A '+' = a stimulation of adenylate cyclase activity. Actual adenylate cyclase activities at 150 mM NaCl = 352.6 $\pm$ 120 pmoles cdAMP/min/mg protein for rabbit and 53.7 pmoles cdAMP/min/mg protein for guinea pig.

studied in both guinea pig and rabbit membranes. The results are shown in Table 5.2. None of the inhibitions by PIA are statistically significant. Since the best numerical inhibition in both cases was at 50 mM NaCl, this concentration was selected for use in further experiments.

b.Determination of optimal Magnesium. The optimal Mg^{++} concentration for A1 receptor expression was determined for both species. The results are shown in table 5.3. The best inhibition was seen at 3 mM Mg, although (in the case of the rabbit where it was tested) this was not statistically significant

c.Determination of optimal GTP concentration. The effect of GTP concentration on the inhibition of rabbit and guinea pig ventricular adenylate cyclase by PIA was investigated. The findings are shown in Table 5.4. At 10 μ M GTP a statistically significant inhibition was seen in the case of rabbit membranes. No more than 13% inhibition of activity was seen with guinea pig membranes. 10 μ M GTP was selected for use in the following experiments.

d.Contamination of the assay medium by adenosine. Adenosine could be present as an impurity in the membranes or in one of the other constituents such

Table 5.3 The effect of 2-12 mM Mg on the percentage inhibition by PIA of adenylate cyclase activity of rabbit sarcolemma and guinea pig crude membrane preparation.

mM Mg Acetate	Percentage inhibition by PIA	
	Guinea pig	Rabbit
2	0.0	0.0
3	13.0	27.1 $\pm$ 6.2
3.5	4.8	12.2 $\pm$ 10.4
4	0.0	5.8 $\pm$ 4.1
6	/	8.9 $\pm$ 2.8
12	5.1	8.9 $\pm$ 2.6
	(n=2)	(n=3 $\pm$ SEM)

Actual activity at 12 mM Mg = 289.8 $\pm$ 127.5 pMoles cdAMP/min/mg protein for rabbit sarcolemma and 15.6 cdAMP/min/mg protein for guinea pig crude membrane preparation.

Table 5.4 The effect of 0-0.1 mM GTP on the inhibition of the adenylate cyclase activity of rabbit sarcolemmal and guinea pig crude membranes by 0.1 mM PIA.

$\mu\text{M GTP}$	Percentage inhibition by PIA	
	Guinea pig	Rabbit
0	12.9 ± 13.5	2.0 ± 5.3
0.1	5.0 ± 2.1	1.4 ± 6.0
1.0	8.1 ± 4.2	8.7 ± 2.9
10.0	9.0 ± 5.5	23.4 ± 5.6 **
50.0	3.2 ± 0.4	14.2 ± 6.1
100.0	<0	14.2 ± 4.2 *
	(n=3 + SEM)	(n=5 + SEM)

Actual activities at 100 $\mu\text{M GTP}$ were 148.3 ± 21.4 pMoles cAMP/min/mg protein for rabbit and 54.0 ± 11.5 pMoles cAMP/min/mg protein for guinea pig.

** = $P < 0.01$, * = $P < 0.05$. (Student's t-test, tested against control).

as creatine phosphate. The effect of adenosine deaminase was tested on basal activity of rabbit sarcolemma membranes, when ATP or dATP was used as the cyclase substrate. Table 5.5 shows the results of this experiment. In both cases there was a slight stimulation of adenylate cyclase activity by adenosine deaminase. This suggests that there might be some contamination by adenosine, although the extent of the stimulation is not sufficient to account for the 'missing' 20% inhibition that might be expected. Since it seems advisable to add adenosine deaminase to remove all endogenous adenosine, the deoxyATP method was abandoned in favour of the 'addition of Deaminase' system, and the effects of adenosine deaminase resistant analogues only were studied.

e. Possible receptor loss due to ultrasonic component of Polytron noise. The influence of homogenisation speed on receptor loss was studied using guinea pig heart. As will be seen from Table 5.6, all effects of PIA are small and not improved by slower homogenisation.

Table 5.5 The effect of adenosine deaminase on the activity of rabbit adenylate cyclase when dATP or ATP were used as substrate.

Substrate	Adenylate cyclase Activity pMoles cNT/min/mg protein		%stimulation by deaminase
	-deaminase	+deaminase	
ATP	84.8 $\pm$ 0.92	91.8 $\pm$ 6.4	8.3 $\pm$ 8.5
dATP	102.1 $\pm$ 14.3	111.6 $\pm$ 22.4	9.5 $\pm$ 5.1

n=3 $\pm$ SEM. (NT =Nucleotide, adenosine monophosphate or deoxyadenosine monophosphate)

Table 5.6 The effect of lower homogenisation speed on expression of A1 and B receptors on membranes from guinea pig myocardium.

Condition	Adenylate cyclase Activity pMoles cAMP/min/mg protein		% inhibition by PIA	
	speed of homogenisation		fast	slow
	fast	slow		
control	124.8 $\pm$ 12.3	105.8 $\pm$ 14.5	/	
+ PIA (0.1 mM)	113.1 $\pm$ 11.3	100.5 $\pm$ 13.7	-9.4	-5.0
+ ISO. (1 μ M)	140.3 $\pm$ 17.3	124.2 $\pm$ 15.0	/	
ISO+ PIA	133.0 $\pm$ 13.1	119.4 $\pm$ 14.5	-5.2	-3.9

n=3 $\pm$ SEM. ISO = isoprenaline. Concentration of effectors were as follows; GTP= 10 μ M, Mg = 3 mM, Na = 50 mM.

3. The effect of PIA on cAMP formation and Breakdown In the Presence and Absence of Isoprenaline.

a. The effect of PIA on the adenylate cyclase activity of rabbit and guinea pig ventricular membranes in the presence and absence of isoprenaline. The effect of PIA on the adenylate cyclase activity of membranes from rabbit and guinea pig ventricle was studied in the presence and absence of isoprenaline. Isoprenaline at 1 μM was used as this is half maximal with respect to stimulation of rabbit and guinea pig adenylate cyclase activity. (Figure 5.2). Examination of Table 5.7 will reveal that all the effects of PIA were small and not statistically significant.

b. The effect of PIA on cAMP breakdown by rabbit and guinea pig membranes. Table 5.8 shows the effect of PIA on cAMP breakdown in the presence and absence of isoprenaline. PIA has a slight inhibitory effect, possibly due to the inhibition of the phosphodiesterase. However, the inhibition of phosphodiesterase activity seen in these studies was not as great as reported by Meyer et al [1984] for guinea-pig heart. It should be noted that Meyer

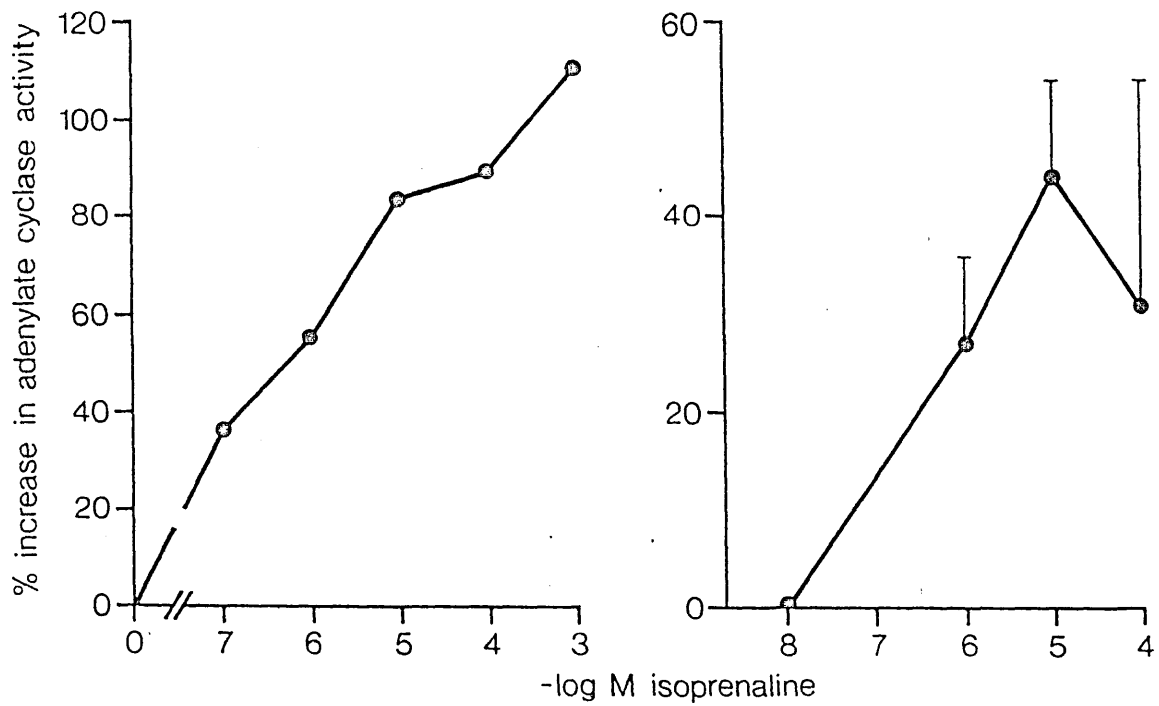


Figure 5.2 Concentration response relationships between isoprenaline concentration and adenylate cyclase activity, for rabbit sarcolemma (right) and guinea pig crude membranes (left). For rabbit the average cyclase activity at zero isoprenaline was 240.6 pMoles cAMP/min/mg protein (n=2) and for guinea pig 79.0 ± 25.1 (n=3).

Table 5.7 The effect of 1 μ M isoprenaline on the inhibition of adenylate cyclase activity of rabbit and guinea pig membranes by 0.1 mM PIA.

Presence of isoprenaline	% change in A.C. activity	
	guinea pig	rabbit
No	-10.9 $\pm$ 12.5	+19.8 $\pm$ 14.1
Yes	-13.5 $\pm$ 10.0	-1.3 $\pm$ 5.9

n=5 $\pm$ SEM in both cases. Actual activity under control conditions = 146.0 $\pm$ 39.0 pMoles cAMP/min/mg protein for rabbit sarcolemma and 62.8 $\pm$ 16.0 pMoles cAMP/ min/mg protein for guinea pig crude membranes. Percentage stimulation by 1 μ M isoprenaline = 34.6 $\pm$ 8.2 for rabbit and 35.5 $\pm$ 19.0 for guinea pig.

Table 5.8 The effect of 0.1 mM PIA on cAMP breakdown with rabbit sarcolemma and guinea pig crude membranes.

Condition	CAMP Removed/20 min/mg protein	
	Guinea pig	Rabbit
Control	748 $\pm$ 24.6	519 $\pm$ 160
+GTP	756 $\pm$ 70.0	584 $\pm$ 94
+PIA	634 $\pm$ 64	506 $\pm$ 154
+ISO	760 $\pm$ 84	568 $\pm$ 42
ISO+PIA	668 $\pm$ 86	510 $\pm$ 120

ISO = isoprenaline, GTP = 10 μ M n=3 $\pm$ SEM.

at all required concentrations of PIA in excess of 100 μ M to achieve significant inhibition of phosphodiesterase activity. Whether such concentrations would be found in a physiological situation is less than certain.

Discussion.

The fact that theophylline is able to displace the dose response curve of adenylate cyclase activity to adenosine concentration to the right is evidence that there are negatively coupled adenosine receptors on rabbit sarcolemma. However, experiments with PIA (an adenosine analogue modified in such a way as to render it more potent at A1 adenosine receptors) showed that this effect was very small, even in the presence of isoprenaline. Since Schultz and Tuisl [1981] suspected that steps in the preparation of their sarcolemmal membranes could have led to receptor loss, experiments were performed to determine whether a crude homogenate showed better receptor expression. However, this proved not to be the case.

As literature became available on the

influence of Na^+ , Mg^{++} and GTP on receptor expression, I investigated whether the extent of inhibition could be improved by modulating the concentrations of these effectors. In agreement with Leung et al [1983], the optimal Na^+ concentration proved to be 50mM, however I was unable to see the 40% inhibition of adenylate cyclase reported by these workers. Mg^{++} at a concentration of 4mM was used in these experiments since an extensive review of the literature on inhibitory receptors of all kinds showed that most other groups used less than 5mM [Belardinelli et al 1982, Hazeki and Ui 1981, Leung et al 1983 & 1983b, Aktories et al 1982, Bockaert 1984, Bockaert and Sebbes Perez 1983, Cooper et al 1979, 1980, Garcia-Sainz et al 1981, Londos et al 1978, deMazancourt et al 1982, Aktories and Jakobs 1981, Anand-Strivastava 1983, Blume et al 1979, Jakobs et al 1978, Koski et al 1982, Onali et al 1983, Sabol and Nireberg 1979, and Lichenstein 1979]. The publication of Bockaert's group in 1984 laid more importance on the Mg^{++} concentration, so I decided to see whether there existed a sharp optimum for Mg^{++} . Although 3mM proved to be the best, the improvement was not startling.

I had been aware of the possibility of adenosine contamination from the breakdown of ATP

and had developed and used an assay method for adenylate cyclase which involved the use of deoxy ATP as a substrate for adenylate cyclase. The 2'-deoxyadenosine which may be generated has no effect at the external A-type receptors. However it was brought to my attention that there could be adenosine contamination from other sources such as in the membranes themselves or in creatine phosphate. It was found that adenosine deaminase stimulated the activity of adenylate cyclase when ATP or dATP was used as substrate, however this stimulation was small (<10%) and certainly not enough to account for the 'missing' 20% inhibition that might be expected for A1 adenosine receptors. Since there seemed to be a small amount of contamination by adenosine even with the deoxyATP system, the latter adenosine-free system was abandoned in favour of the less costly method of adding adenosine deaminase to remove endogenous adenosine and measuring the cAMP produced with the Amersham kit. This meant that it was possible to determine the effects of deaminase-resistant analogues, but not adenosine.

When Hsia et al [1984] presented evidence for a definite GTP optimum for opiate receptor action, I investigated whether adenosine receptors showed a similar preference for a lower GTP concentration.

In the case of rabbits this proved to be true with a small but statistically significant inhibition occurring at 10 μ M GTP. With guinea pigs there was no real improvement over the whole dose response range.

Since the effect was still only half of what was expected (particularly in the case of guinea pig cardiac membranes), further experiments were performed to try to find the reason for this diminished response. Dr Riemersma (personal communication) suggested that the ultrasound component of Polytron homogenisation could account for some receptor loss. However a slower homogenisation speed made no difference to the inhibition by PIA.

The amount of inhibition in the presence and absence of isoprenaline in both rabbit and guinea pig heart was studied. Isoprenaline does not improve the amount of inhibition by PIA. This is in contrast to the findings of Dobson [1978] and Hosey et al [1984] who found that stimulation of the cyclase by isoprenaline was necessary for inhibition by A1 receptors. It may be noted that the effect of PIA was very variable, sometimes a 25% inhibition being seen and on other occasions a stimulatory action.

Since it is possible that adenosine could have effects on the breakdown of cAMP by accelerating the activity of the cAMP phosphodiesterase, the effects of PIA on cAMP lability was studied. It was found that PIA slightly inhibited cAMP breakdown by guinea pig membranes, but had no effect with rabbit sarcolemma. This reduction in breakdown possibly reflects an inhibition of phosphodiesterase activity, as reported by Meyer et al [1984]. Teo and co-workers [1981] found that adenosine could stimulate the phosphodiesterase of whole fat cells but tended to cause inhibition in broken cells. If this is the case in heart, it could explain some of the discrepancies between cAMP levels measured in whole heart and the effects seen with isolated membranes. With whole heart adenosine could decrease cAMP levels by stimulating the phosphodiesterase via a receptor mediated event. In most studies with isolated membranes, such an action would be masked by the presence of a high concentration of phosphodiesterase inhibitor which must be included to ensure accurate measurement of cAMP.

Summary.

The effect of adenosine and its analogues on adenylate cyclase activity in rabbit and guinea pig

myocardial membranes was small. Modulation of Na^+ , Mg^{++} and GTP over wide ranges gave no markedly improved or sustained inhibition. The assay medium may be slightly contaminated with adenosine from other sources besides the substrate. Slow homogenisation did not increase A_1 mediated inhibition. The expression of the receptors was not improved by the presence of isoprenaline. PIA was found to inhibit slightly the breakdown of cAMP in guinea pig but not rabbit membranes.

Chapter 6

The effect of 2'-deoxyadenosine 3'-monophosphate (a P-site agonist) on the activity of rabbit and guinea pig ventricular adenylate cyclase.

Introduction.

The P-site is an adenosine sensitive site present on all adenylate cyclase systems so far investigated, with the exception of sea urchin sperm [Garbers 1977]. The main features of this site are; it always mediates inhibition, it faces the inside of the cell, the site is distinct from the active site of the enzyme, stimulation of the enzyme is not necessary for the expression of its effects, although such stimulation may lead to increased potency of P-site agonists.

Aviado et al [1980] and Webster and Olsson [1980] have investigated the P-site of cardiac tissues, but not extensively. After examining the available literature I set out to characterise the inhibition of rabbit and guinea pig ventricular adenylate cyclase by 2'-deoxyadenosine 3' monophosphate (3MP).

I tested to see whether 3MP inhibited adenylate cyclase of these two species non-competitively with respect to Mg.ATP, as reported by most groups, [Welton and Simko 1980, Braun and Levitzki 1979, Johnson et al 1979 and Haslam et al 1978]. Others have found this inhibition to be

mixed [Wolff et al 1978, Glynn and Cooper 1978].

The main feature of interest in the P-site is that it has been reported to be regulated by divalent cations such as Mg^{++} and Mn^{++} [Cochaux et al 1982, Wolff et al 1978, Bregman et al 1980, Welton and Simko 1980, de Vente and Zaagsma 1981, Premont et al 1979, Londos and Wolff 1977, Johnson et al 1979, Peck et al 1976 and Webster and Olsson 1980]. It has been suggested that an increase in inhibitory potency might be associated with an activation of the enzyme by metal ions [Londos and Preston 1977, Wolff et al 1978, and Glynn and Cooper 1978]. The effect of raising the concentration of Mg or replacing it with Mn was investigated in this chapter in relation to the sensitivity of the P-site and activation of adenylate cyclase of both rabbit and guinea pig heart.

The interest of this site as far as my investigations are concerned relates to the possibility that it may be involved in the antiadrenergic response. Many investigators find that the activity of the P-site is enhanced under conditions of hormonal stimulation [Cochaux et al 1982, Wolff et al 1978, Glynn and Cooper 1978, Bregman et al 1980, McKenzie and Bar 1973, Rodbell and Londos 1976 and

Johnson and Welden 1977]. In 1980 Dobson provided evidence that this might be the case with rat heart since adenosine would only inhibit adenylate cyclase when the enzyme was stimulated by isoprenaline. He also noted that the inhibition could not be blocked by methylxanthines and was enhanced at higher Mg concentrations, making the inhibition characteristic of P-site behavior. Bearing in mind the species differences noted in the earlier chapters between rabbit and guinea pig in relation to the antiadrenergic effect, I investigated the effect of isoprenaline stimulation of adenylate cyclase on the sensitivity of the P-site with both types of animal.

As mentioned above, the P-site is reported to be unaffected by methylxanthines [Cochaux et al 1982, Wolff et al 1978, Bregman et al 1980]. However I have found that theophylline inhibits adenylate cyclase in its own right, the possible locus for this action being the P-site (see following chapter). It is possible that methylxanthines could antagonise the effect of P-site agents at concentrations too low to elicit the inhibitory effect. Since this would invalidate the use of methylxanthines to provide evidence that a given response is mediated by the A-type receptors, I felt that it was important to determine the

effect of 8-phenyl theophylline on the response of the P-site of rabbit and guinea pig myocardium. I have used 8-phenyl theophylline as an antagonist of the A-type responses of Guinea pig atrium and papillary muscle (chapters 8, 2 and 3).

Methods

General. The standard assay buffer containing 12 mM Mg was used in most experiments. Where the effects of divalent cations were investigated, assay buffers were made up containing the appropriate amount of the relevant ion. 2mM ICI 63 197 was used as the phosphodiesterase inhibitor. Rabbit sarcolemmal membranes were prepared as described in section B1a and guinea pig crude membranes as described in section B1b.

1. Lineweaver-Burk double reciprocal plots.

100 mM NaCl was added to increase activity, and thus accuracy of measurement of cAMP. A series of solutions of ATP in the range 0.1-0.0025 mM was made up by serial dilution and an aliquot taken from each dilution and added to the appropriate amount of creatine kinase. The reaction was started by the addition of ATP/creatine kinase in the usual way. These experiments were performed in duplicate.

2. Influence of divalent cations.

Separate buffers were made up containing 3mM Mg, 3mM Mn or 20 mM Mg as required. All other components of the assay were dissolved in these buffers. These experiments were performed in triplicate, using rabbit or guinea pig membranes on separate occasions.

3. The influence of GTP and isoprenaline stimulation of adenylate cyclase activity.

GTP and isoprenaline both at a concentration of 0.1 mM were preincubated with the membranes for 20 minutes, before the reaction was started by the addition of ATP. These experiments were performed in duplicate, both rabbit and guinea pig on the same occasion.

4. Test for antagonism by 8-phenyltheophylline.

In a preliminary experiment, a concentration of 8-phenyltheophylline was selected which did not have any effect on activity. Since 8-phenyltheophylline must initially be dissolved in 80% methanol containing 0.2 M NaOH, a carrier control was included in all other tubes to allow for any effect of this solvent.

Results.

1. Lineweaver-Burk plots.

The effect of increasing concentrations of 3MP on the slope and intercept of the double reciprocal rate versus substrate plot for rabbit sarcolemmal adenylate cyclase is shown in figure 6.1. The position of the lines was calculated using the Wilkinson programme [Wilkinson 1961]. This programme gives the reciprocals of the points of intercept on the y and x axes (ie the k_m and V_{max}). These values are given in table 6.1. Both k_m and V_{max} can be seen to be reduced, though the effect on rate is rather more pronounced. This behavior most closely resembles noncompetitive inhibition.

This study was repeated with guinea pig crude membranes. As can be seen from an examination of figure 6.2 and table 6.2 a similar alteration of kinetic parameters was seen.

2. Effect of divalent cations.

a. MgATP vs MnATP.

With rabbit membranes, substitution of 3mM Mn

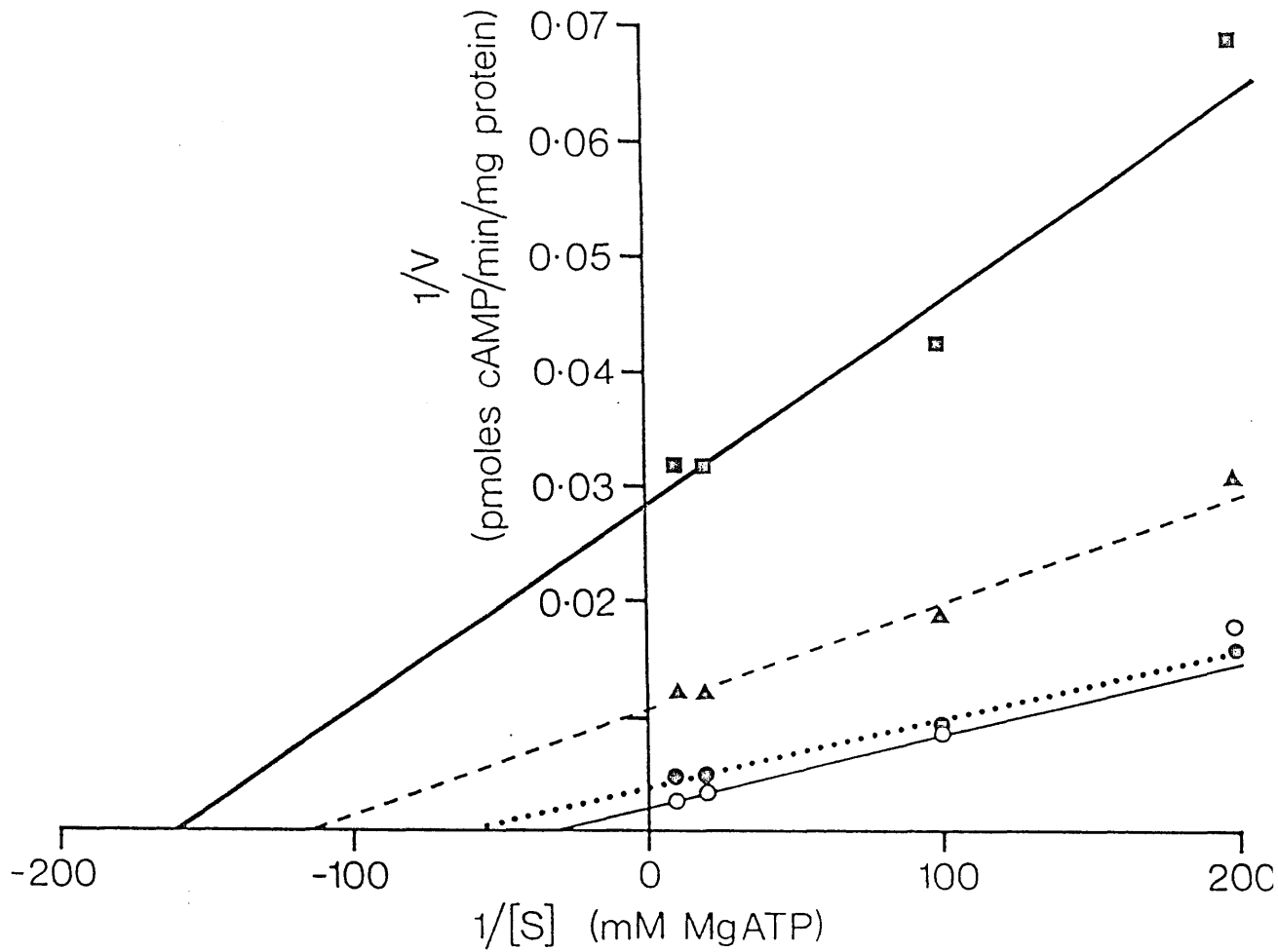


Figure 6.1 Double reciprocal plot of rabbit adenylate cyclase activity versus Mg.ATP concentration. $\circ-\circ$ = control, $\circ\cdots\circ$ = 1 μM 3MP, $\Delta--\Delta$ = 10 μM 3MP, $\square-\square$ = 50 μM 3MP. $n = 3$. Adenylate cyclase activity was stimulated by 100 mM NaCl.

Table 6.1 Effect of 2'deoxyadenosine 3'monophosphate on the kinetic parameters of rabbit cardiac adenylate cyclase.

Conc. 3MP (M)	Km mM ATP	Vmax. pMol cAMP/min/mg protein.
0	0.032 $\pm$ 0.003	481 $\pm$ 16
10 ⁻⁶	0.016 $\pm$ 0.004	281 $\pm$ 19
10 ⁻⁵	0.008 $\pm$ 0.002	93 $\pm$ 4
5x10 ⁻⁵	0.006 $\pm$ 0.001	35 $\pm$ 2

n=3 $\pm$ SEM.

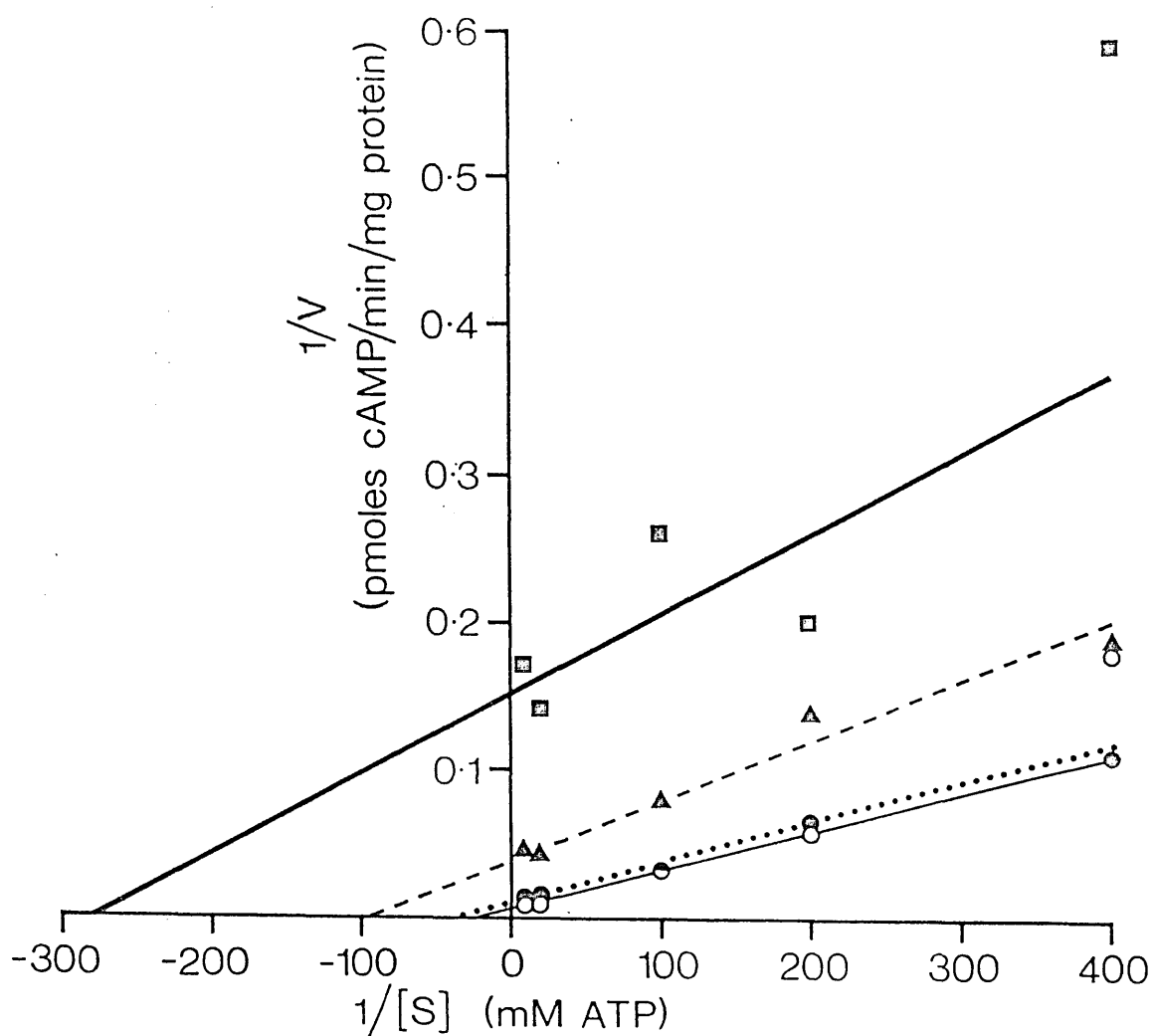


Figure 6.2 Double reciprocal plot of guinea pig adenylate cyclase activity versus Mg.ATP concentration. $\circ-\circ$ = control, $\circ\cdots\circ$ = 1 μM 3MP, $\Delta--\Delta$ = 10 μM 3MP, $\square-\square$ = 50 μM 3MP. $n=3$. Adenylate cyclase activity was stimulated by 100 mM NaCl.

Table 6.2 Effect of 2'-deoxy adenosine 3'-monophosphate on the kinetic parameters of guinea pig cardiac adenylate cyclase.

Conc 3MP (M)	Km mM ATP	Vmax. pMol cAMP/min/mg protein.
0	0.034 $\pm$ 0.006	144 $\pm$ 10
10 ⁻⁶	0.024 $\pm$ 0.003	101 $\pm$ 4
10 ⁻⁵	0.010 $\pm$ 0.003	25 $\pm$ 2
5 $\times$ 10 ⁻⁵	0.004 $\pm$ 0.002	7 $\pm$ 1

n=3 $\pm$ SEM.

for 3mM Mg made little difference to either the activity of adenylate cyclase or the potency of 3MP (figure 6.3 right hand graph). With guinea pig membranes, substitution of Mn for Mg made little difference to the potency of 3MP, (figure 6.3 left), but in this case Mn increased the activity of adenylate cyclase by 3.5 fold over that seen with Mg.

b. 20 mM Mg vs 3mM Mg.

With rabbit, increasing the concentration of Mg in the assay buffer made little difference to the potency of 3MP but increased the activity of adenylate cyclase by 2.4 fold (see legend to figure 6.4) With guinea pig the effect on activity was rather more pronounced, 20 mM Mg raising the activity by 6.2 fold, but this was not accompanied by an increase in the potency of 3MP.

3. Potency of P-site agonist when adenylate cyclase is stimulated by isoprenaline and GTP.

The results shown in figure 6.5 illustrate the fact that both GTP and isoprenaline enhance the effects of 3MP on the adenylate cyclase activity of both rabbit and guinea pig membranes. The effect of GTP is small and does not reach statistical

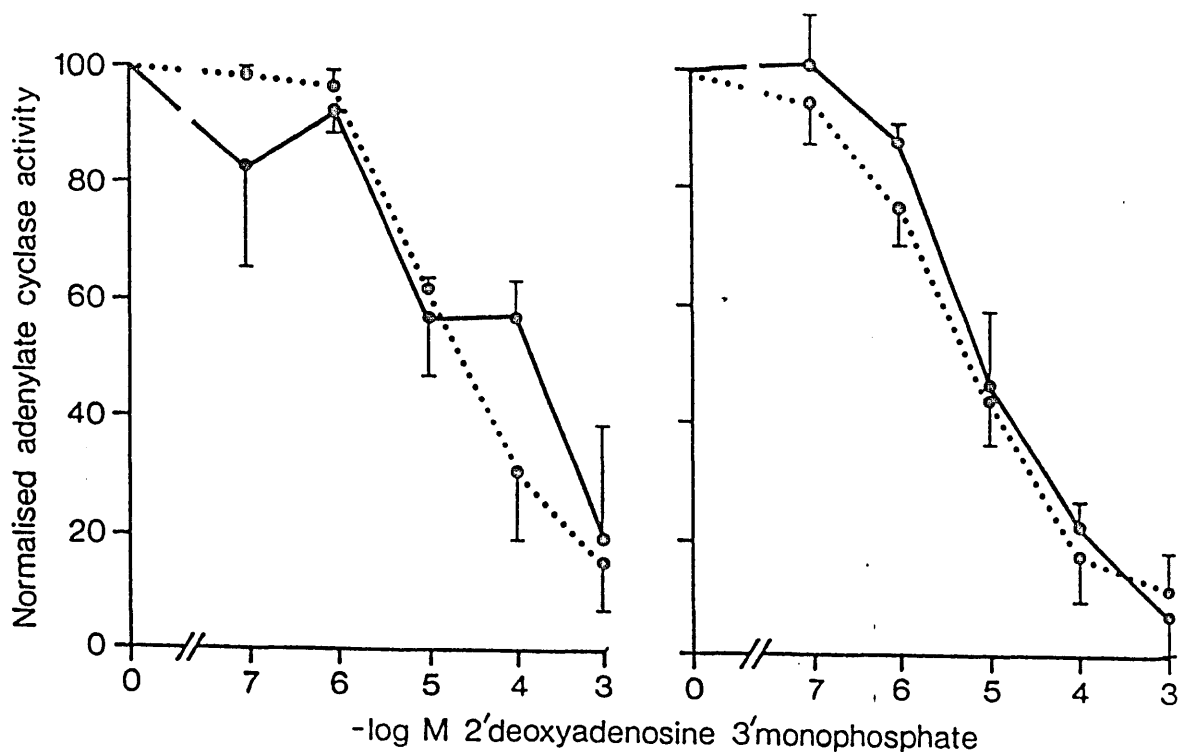


Figure 6.3. The effect of substituting 3mM Mn for 3mM Mg on the activity of adenylate cyclase and the potency of 2'deoxy adenosine 3' monophosphate.

The right hand graph shows the effect of increasing concentrations of 3MP on the normalised activity of rabbit sarcolemmal adenylate cyclase
 o—o = 3mM Mg (activity at 0 3MP = 53.0 ± 10.8),
 o...o = 3mM Mn (activity at 0 3MP = 77.7 ± 7.3).

The left hand graph shows the results of the same experiment performed with guinea pig membranes. With Mg the activity was 16.7 ± 3.9 , and with Mn, 58.8 ± 10.2 .

Activities quoted are expressed in pMoles cAMP/min/mg protein. $n=3 \pm \text{SEM}$. Experiments with guinea pig and rabbit were performed on separate occasions.

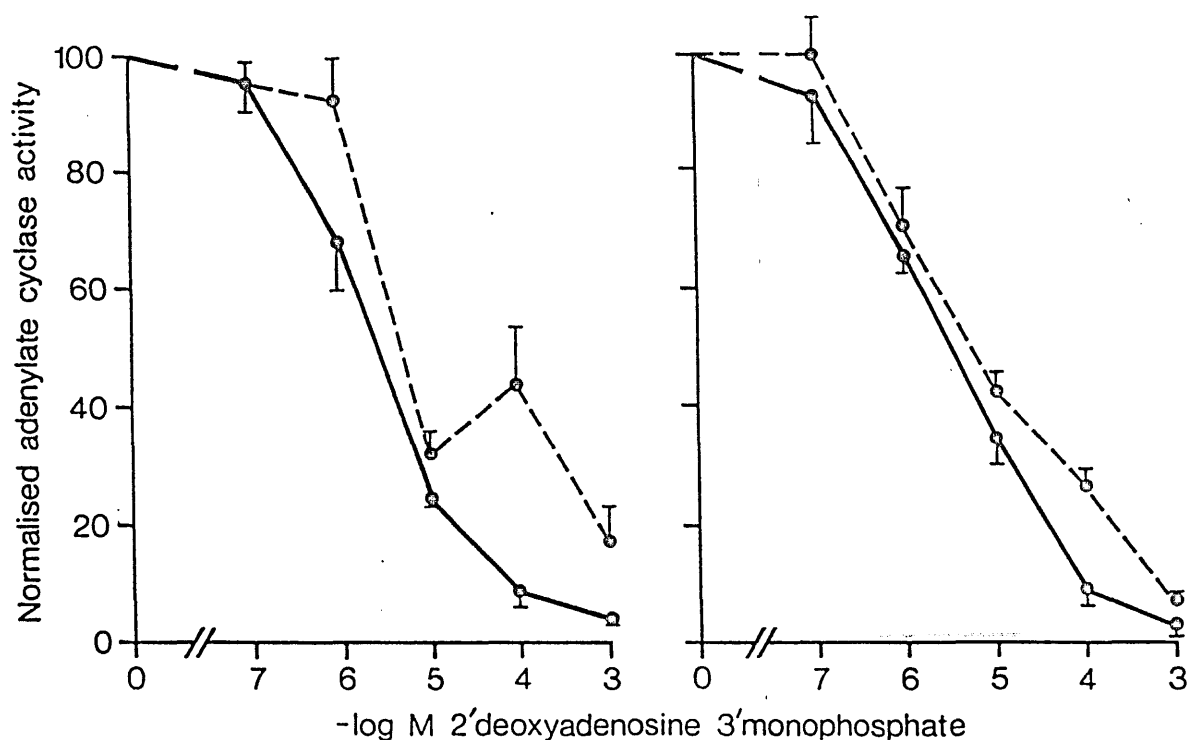


Figure 6.4 The effect of increasing the concentration of Mg from 3 to 20 mM on the activity of adenylate cyclase and the potency of 2'deoxy adenosine 3'monophosphate.

The right hand graph shows the effect of increasing concentrations of 3MP on the normalised adenylate cyclase activity of rabbit cardiac sarcolemma. $\circ-\circ$ = 3mM Mg, (activity at 0 3MP = 26.1 ± 2.2), $\circ--\circ$ = 20 mM Mg (activity at 0 3MP = 62.8 ± 9.0).

The left hand graph shows the same experiment repeated with guinea pig ventricle. Activity with 3 mM Mg = 10.2 ± 3.5 , and with 20 mM = 63.1 ± 13.3 .

The activities are expressed in pMoles cAMP /min/mg protein. $n=3 \pm$ SEM. Experiments with guinea pig and rabbit were performed on separate occasions.

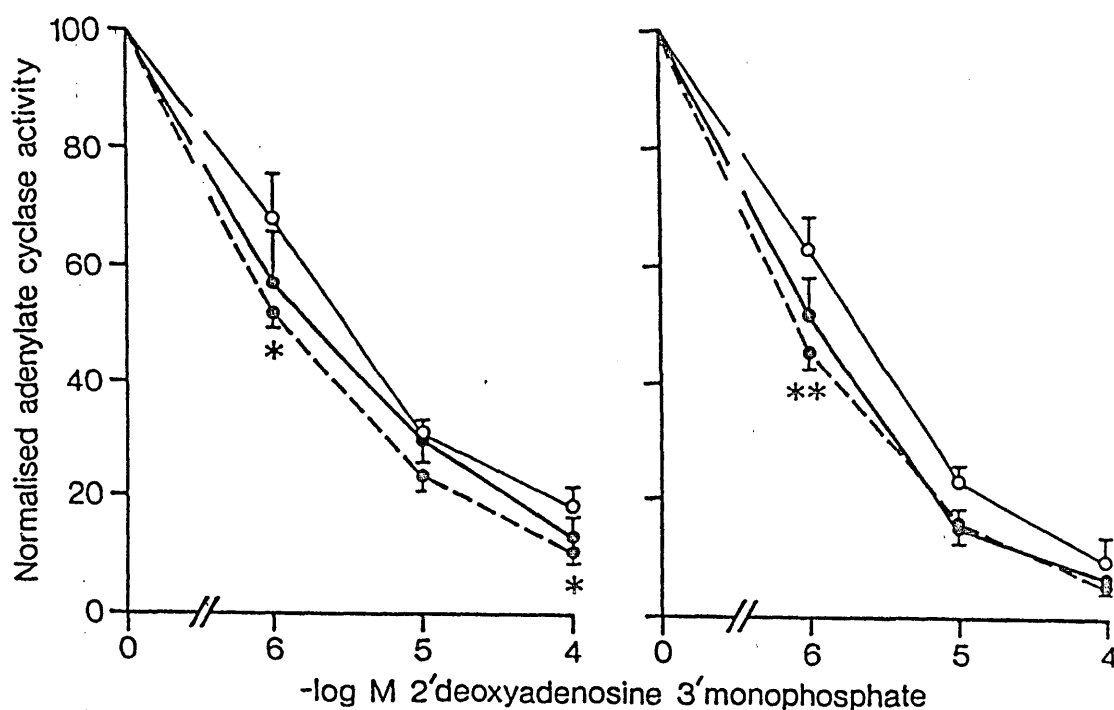


Figure 6.5. The effect of activation of adenylate cyclase by isoprenaline and GTP on the potency of 2'-deoxyadenosine 3' monophosphate.

The right hand graph shows the effect of increasing concentrations of 3MP on the normalised adenylate cyclase activity of rabbit sarcolemma, in the absence o—o, and presence o—o of 0.1 mM GTP and GTP plus 0.1 mM isoprenaline o--o. Basal cyclase activity = 64.6 ± 10.7 , % stimulation by GTP = 167%, and by isoprenaline = 53.4%.

The left hand graph shows the results of the same experiment performed with guinea pig ventricular membranes. Basal activity = 43.8 ± 4.5 , % stimulation by GTP = 22.6 and by GTP + isoprenaline = 73.4 %.

Adenylate cyclase activities are expressed in pMoles cAMP/min/mg protein. $n=5 \pm \text{SEM}$.

significance (paired Student's t-test). However when isoprenaline is used in conjunction with GTP the increase in potency becomes statistically significant at some concentrations of 3MP (see figure 6.5)

4. Antagonism of the P-site response by 8-phenyl-theophylline.

Since I had found that theophylline inhibits adenylate cyclase (chapter 7), a preliminary experiment was performed to determine the highest concentration of 8-phenyltheophylline which did not inhibit adenylate cyclase (table 6.3). With rabbit membranes a stimulation was seen. With guinea pig membranes, 0.1 mM 8 phenyl-theophylline was found to be the highest concentration which had no appreciable effect on activity.

Using this concentration of 8-phenyl-theophylline, the effect on the inhibition of adenylate cyclase was investigated. The results in figure 6.6 (left) show that 8-phenyltheophylline has no effect on the P-site activity of guinea pig membranes. However with rabbit membranes (right hand graph), some antagonism of the higher doses of 3MP can be seen. This effect is likely to be due to the partial agonist effect of methylxanthines at

Table 6.3 The effect of 8-phenyltheophylline on the adenylate cyclase activity of rabbit sarcolemmal and guinea pig ventricular membrane adenylate cyclase.

mM 8PT	Adenylate cyclase activity pmoles cAMP/min/mg protein.	
	Rabbit	Guinea pig
0	34.5 $\pm$ 3.3	41.6 $\pm$ 5.8
0.01	34.9 $\pm$ 2.8	49.6 $\pm$ 11.8
.05	43.2 $\pm$ 3.7	41.7 $\pm$ 7.9
0.1	48.0 $\pm$ 3.1	43.4 $\pm$ 3.0
0.5	58.2 $\pm$ 7.3	34.6 $\pm$ 2.5
1.0	48.0 $\pm$ 11.0	18.9 $\pm$ 0.9

Mean of single triplicate determination, + SD. 08pt (ie control) contained a carrier control for methanol/NaOH (see methods).

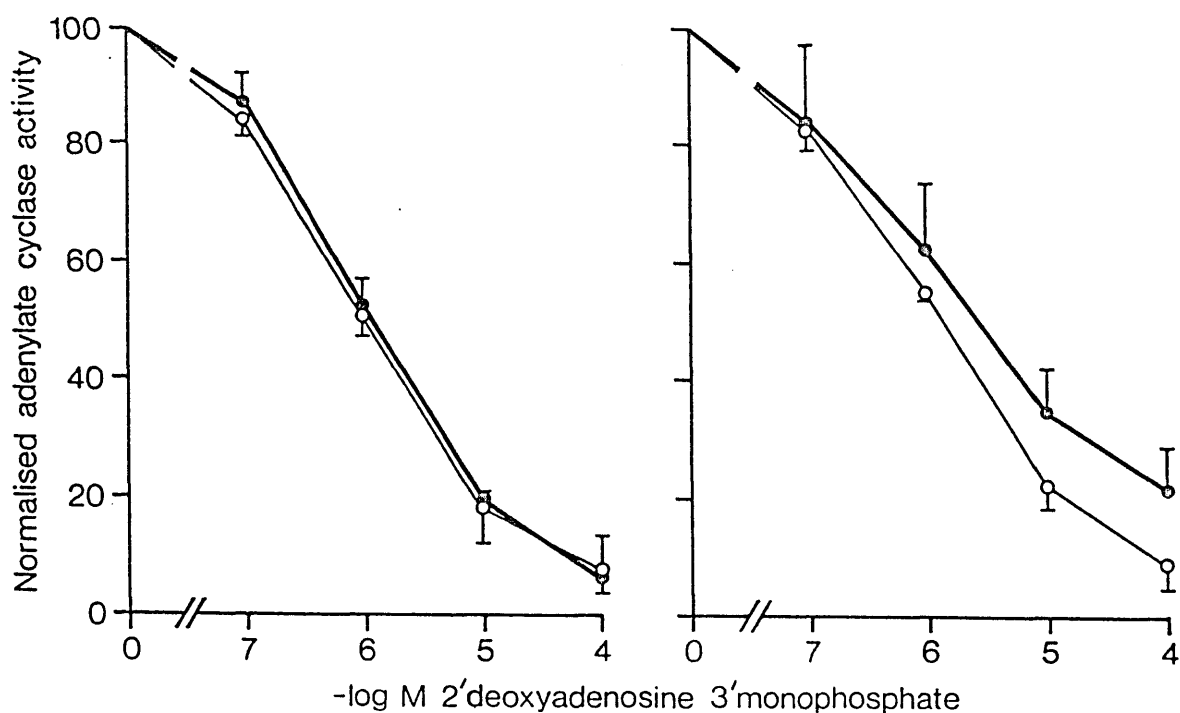


Figure 6.6 The effect of 8-phenyltheophylline on the activity of 2'-deoxyadenosine 3'-monophosphate at the P-site.

The right hand graph shows the effect of increasing concentrations of 3MP on the normalised cyclase activity of rabbit cardiac adenylate cyclase, in the absence of $\circ-\circ$, and the presence of $\circ-\circ$ 1 μ M 8-phenyltheophylline. Basal activity = 44.8 ± 5.6 , and in the presence of 8-phenyltheophylline = 45.1 ± 2.2 .

The left hand graph shows the results of the same experiment performed with guinea pig ventricle. Basal activity = 52.1 ± 9.0 , with 8-phenyltheophylline = 53.5 ± 8.9 .

Adenylate cyclase activities are expressed as pMoles cAMP/min/mg protein. $n=3 \pm$ SEM.

the P-site and is examined more thoroughly in the next chapter.

Discussion.

The inhibition seen with both guinea pig and rabbit membranes was mixed in nature and not simple non-competitive as reported by Welton and Simko [1980], Braun and Levitski [1979], Johnson et al [1979] and Haslam et al [1978]. Wolff et al [1978] also reported the inhibition to be of a mixed nature. It is possible that the type of inhibition seen reflects the analogue used. 2'-deoxy adenosine 3' monophosphate closely resembles AMP in structure and could, in addition to its P-site activities interfere with reactions at the active site of adenylate cyclase.

Very little effect was seen with variations of divalent cations, although with guinea pig membranes replacement of Mg with Mn or raising the concentration of Mg from 3 to 20 mM activated adenylate cyclase appreciably. Londos and Preston [1977], Wolff et al [1978] and Glynn and Cooper [1978] suggested that an increase in inhibitory potency might be associated with an activation of

the enzyme with metal ion. However I did not find this to be the case with guinea pig ventricular membranes. This suggests that perhaps the P-site and the divalent cation binding site are unrelated in cardiac membranes.

Both Schrader's group [1977] and Dobson [1983] found that adenosine could inhibit the adenylate cyclase of a particulate preparation in an antiadrenergic manner. However the conditions used in their experiments would tend to favour expression of the P-site (ie high Mg, no Na, no steps taken to reduce endogenous adenosine). In fact Dobson was unable to see any antagonism of the effect of adenosine by theophylline and noted that the inhibitory effect of adenosine was greater at higher Mg concentrations. Some investigators state that hormonal activation of adenylate cyclase results in increased sensitivity of the cyclase to P-site agents, [Cochaux et al 1982, Wolff et al 1978, Glynn and Cooper 1978, Bregman et al 1980, McKenzie and Bar 1973, Rodbell and Londos 1976 and Johnson and Welden 1977]. Others [deVente and Zaagsma 1981 and Welton and Simko 1980], see no increased sensitivity with hormonal stimulation, although they find that divalent cations and guanine nucleotides increase the potency of P-site agonists. If the heart adenylate cyclase is

rendered more sensitive to P-site agonists when stimulated by isoprenaline, then this would provide a possible mechanism for the antiadrenergic mechanical effect. The effect of P-site stimulation on muscle mechanics could depend on an absolute reduction in cAMP concentration. I have found that both GTP and isoprenaline increase the potency of 3MP. Although the effect is small, it may be great enough to allow the P-site to make some contribution to the antiadrenergic effect of adenosine.

A reported characteristic of the P-site is that it is not affected by methylxanthines, (Cochaux et al 1982, Wolff et al 1978, Bregman et al 1980]. However I have found that methylxanthines inhibit adenylate cyclase in their own right, possibly by interacting at the P-site. The possibility remains that at concentrations too low to elicit the inhibitory effect, methylxanthines could antagonise the P-site response, by binding to the P-site and blocking the action of more efficacious agents. With guinea pigs this was not found, but with rabbits I was able to see a small antagonism of the action of 3MP at higher concentrations of the latter. This is not characteristic of standard agonist/antagonist interactions where a parallel shift to the right

might be expected.

Thus in summary, it can be stated that both guinea pig and rabbit membranes have a very active P-site which has some features in common with the P-site from other tissues, ie it displays mainly non-competitive inhibition, and the action of P-site agonists are not antagonised by methylxanthines. However it differs from most others in one important way. The sensitivity of this site appears not to be affected by activation by divalent cations. With rabbit membranes only a modest activation was produced by substituting Mn for Mg or by raising the Mg concentration from 3 to 20 mM. However with the guinea pig, where there was a more pronounced activation of adenylate cyclase as a result of these changes, no major increase in sensitivity was achieved. I was also able to show that the addition of GTP and isoprenaline increased the potency of P-site agonists, suggesting a possible role for the P-site in the antiadrenergic action of adenosine.

Chapter 7

Inhibition of rabbit cardiac adenylate cyclase by
theophylline.

Introduction.

As I mentioned in chapter 5, I found that theophylline, in addition to its usual effects on phosphodiesterase and adenosine receptors, could inhibit adenylate cyclase in its own right. Examination of the literature revealed that this has been noted for many tissues including rat erythrocyte ghosts [Shepperd 1970], lung [Weinryb and Michel 1971, Welton and Simko 1980], guinea pig auricles [Kruse and Sholtz 1978], hepatic parenchymal cells [Pohl et al 1969], Kidneys [Jakobs et al 1972], fat cell [Shonhoffer and Skidmore 1971], rabbit heart [Avdonin et al 1982] and sperm [Garbers 1977]. The purpose of the experiments described in this chapter was to further investigate the nature of this inhibition in rabbit cardiac membranes.

Methods (General).

Rabbit membranes were prepared as described in methods chapter B1a. In all experiments the membranes were preincubated for 20 minutes with the assay components before the addition of ATP. The general cyclase assay buffer containing 12 mM Mg was used in the experiments described in this chapter, together with 2 mM ICI 63 197 as an inhibitor of the phosphodiesterase activity.

Results

1. Inhibition of adenylate cyclase activity by theophylline under various conditions of cyclase activation.

Theophylline at a concentration of 5mM inhibited adenylate cyclase in its basal state as well as when stimulated by 0.1 mM GTP, 0.1 mM GppNHp, 100 mM NaCl or 10 mM NaF (see table 7.1). As the most reproducible inhibition by theophylline was seen with adenylate cyclase stimulated by 0.1 mM GTP plus 100 mM sodium chloride, these conditions were used in the further characterisation of the theophylline effect.

Table 7.1 Inhibition of rabbit cardiac adenylate cyclase by 5 mM theophylline under various conditions

Condition	Cyclase activity (pMoles cAMP/min/ mg protein.)	
	Control	+ 5mM Theophylline
Basal (8)	74.9 $\pm$ 21.9	57.0 $\pm$ 17.0
100 mM NaCl (8)	231.2 $\pm$ 33.6	169.3 $\pm$ 30.2 *
0.1 mM GTP + 100 mM NaCl (8)	336.6 $\pm$ 27.8	227.7 $\pm$ 30.7**
0.1 mM GTP (4)	102.4 $\pm$ 10.1	58.7 $\pm$ 20.6
0.1 mM GppNHp +100 mM NaCl (4)	837.3 $\pm$ 110.0	717.1 $\pm$ 110.5
10 mM NaF (4)	328.7 $\pm$ 50.6	265.4 $\pm$ 41.3

Mean $\pm$ SEM. Number of experiments shown in brackets.* = $p < 0.002$, ** = $p < 0.001$, paired t-test. Theophylline inhibited activity in 34 out of 36 cases.

2.Dose dependancy.

Figure 7.1 shows the concentration-response relationship between increasing concentrations of theophylline and adenylate cyclase activity. Inhibition is apparent at 10 μ M, but is more marked at concentrations above 100 μ M, when it becomes statistically significant ($P < 0.05$). Isobutylmethylxanthine at a concentration of 0.5 mM also inhibited adenylate cyclase activity by 18.2 ± 8.7 % ($n=3$)

3.Kinetic analysis.

Figure 7.2 shows a double reciprocal plot of adenylate cyclase activity versus Mg ATP concentration. Theophylline is clearly not competing with Mg.ATP for the active site and the behavior most closely resembles a noncompetitive interaction. Table 7.2 shows the alteration in kinetic parameters brought about by theophylline. The values were calculated using the Wilkinson programme [Wilkinson 1961].

4.Effect of Adenosine deaminase.

Adenosine deaminase at a concentration of 1 unit/ml had no effect on the inhibition by

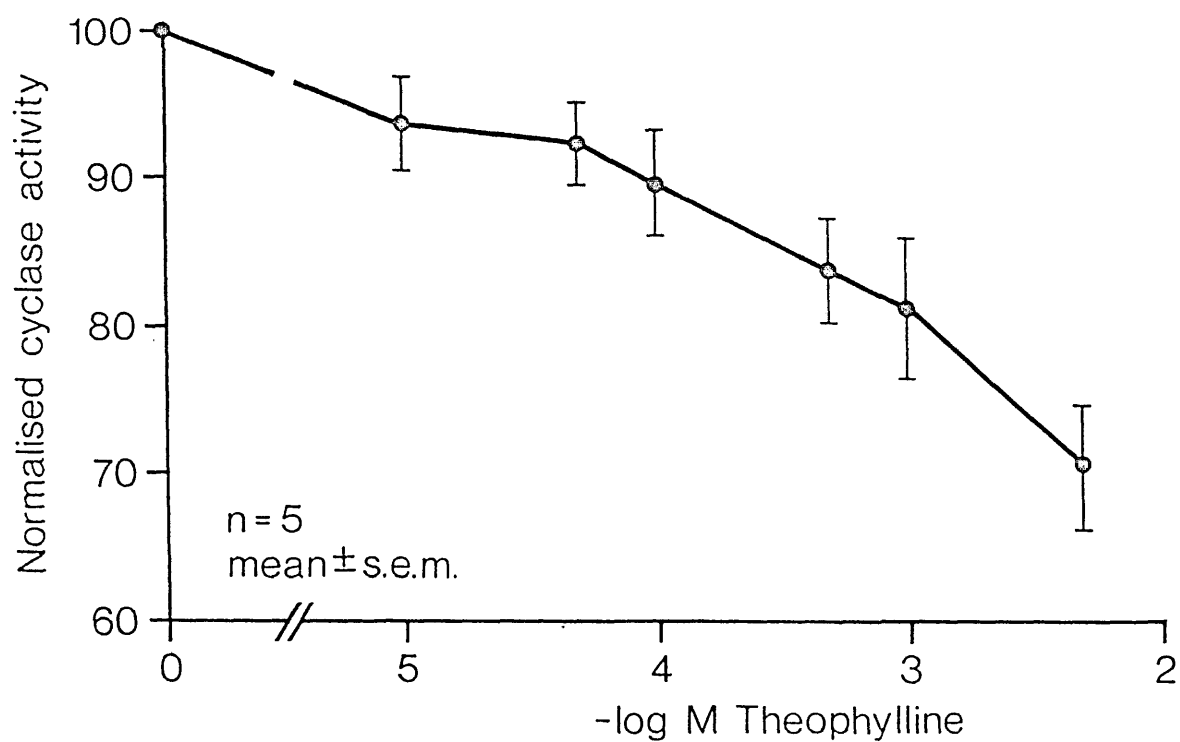


Figure 7.1 Effect of increasing concentrations of theophylline on the activity of rabbit cardiac adenylate cyclase stimulated by 100 mM Na⁺ and 0.1 mM GTP. n=5, mean ± SEM.

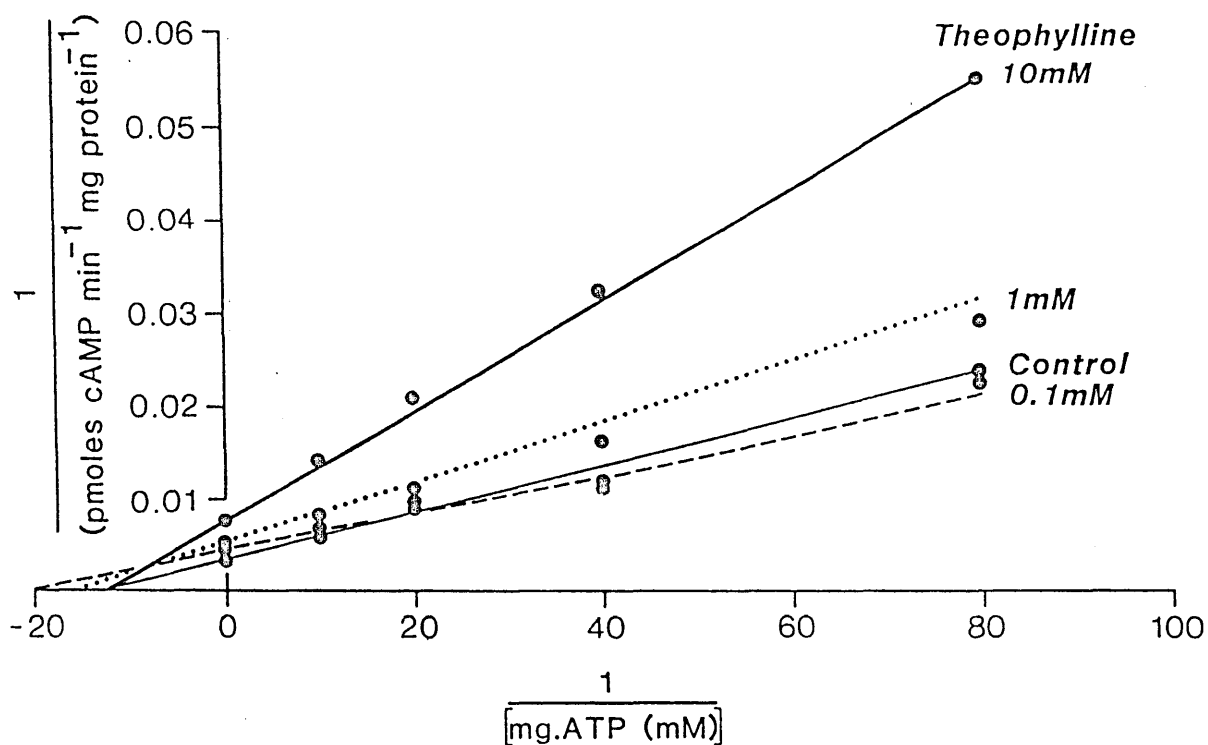


Figure 7.2 Lineweaver-Burk plots showing the inhibition of rabbit cardiac adenylate cyclase by theophylline. Adenylate cyclase activity was stimulated by 100 mM Na⁺ and 0.1 mM GTP. The lines shown are the average of 3 experiments.

Table 7.2 Effect of Theophylline the kinetic parameters of rabbit cardiac adenylate cyclase.

Conc Theo (mM)	Km (mM ATP)	Vmax (pmolcAMP/min mg protein)
0	0.070 ± 0.024	281.4 ± 53.6
0.1	0.050 ± 0.007	236.0 ± 15.8
1.0	0.060 ± 0.004	207.8 ± 7.9
10.0	0.078 ± 0.007	125.5 ± 5.8

n=3 ± SEM Adenylate cyclase activity was stimulated by the addition of 0.1 mM GTP and 100 mM NaCl. The values shown in the table were calculated using the 'Wilkinson' programme.

Table 7.3 Effect of adenosine deaminase on the inhibition of rabbit cardiac adenylate cyclase (stimulated by 100 mM Na+ and 0.1 mM GTP) by 5 mM theophylline.

Condition	Cyclase activity (cAMP/min/mg protein).	
	control	+theophylline
GTP/Na stimulated	377.5 $\pm$ 22.3	265.3 $\pm$ 20.3 **
+ adenosine deaminase (1U/ml)	367.2 $\pm$ 36.0	229.2 $\pm$ 21.8 *

Mean $\pm$ SEM of 5 experiments. * = $p < 0.05$, ** = $p < 0.01$, paired t-test, against control.

theophylline (Table 7.3) thus ruling out the possibility that theophylline may be exerting its action by blocking stimulation of adenylate cyclase by endogenous adenosine. Adenosine deaminase at this concentration is effective in removing up to 1mM adenosine (see Methods B3a).

5. Interaction between 2'deoxyadenosine 3'mono-phosphate and theophylline.

Dose response curves to 2'deoxyadenosine 3'monophosphate (a P-site agonist), in the absence and presence of 1, 5 and 10 mM theophylline are shown in figure 7.3. At 10 μ M 2'deoxyadenosine 3'monophosphate, addition of theophylline produces no further reduction in adenylate cyclase activity and there is only partial additivity of the inhibition by theophylline with that of 2'deoxyadenosine 3'monophosphate at low concentrations of the latter. A similar though not so pronounced non-additivity was seen between 2'deoxyadenosine 3'monophosphate and 2'deoxyadenosine; two compounds which are known to act at the P-site. (Figure 7.4).

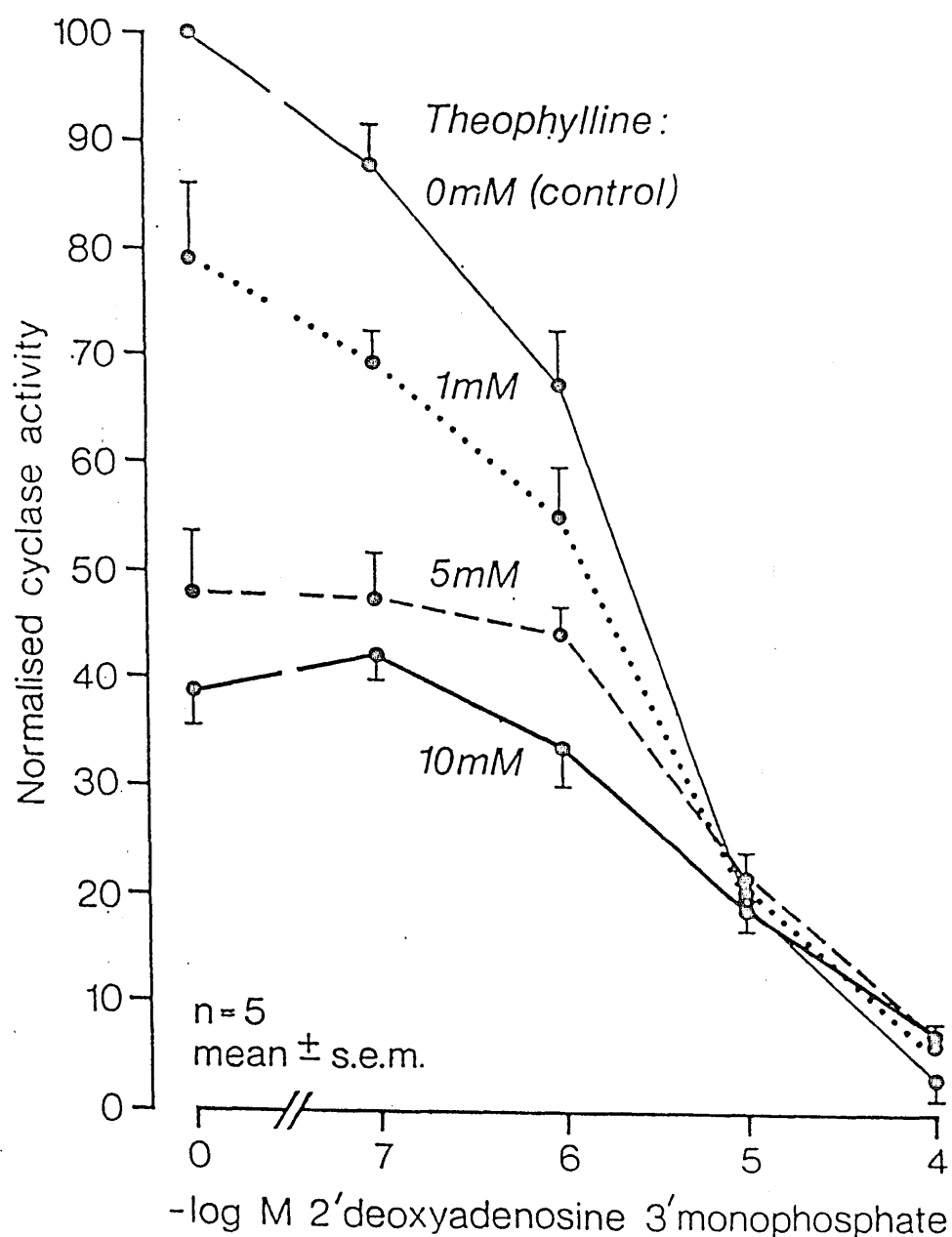


Figure 7.3 Effect of increasing concentrations of 2'-deoxy adenosine 3' monophosphate on the activity of rabbit cardiac adenylate cyclase stimulated by 100 mM Na⁺ and 0.1 mM GTP in the absence and presence of 1, 5 and 10 mM theophylline. n=5, mean $\pm$ SEM.

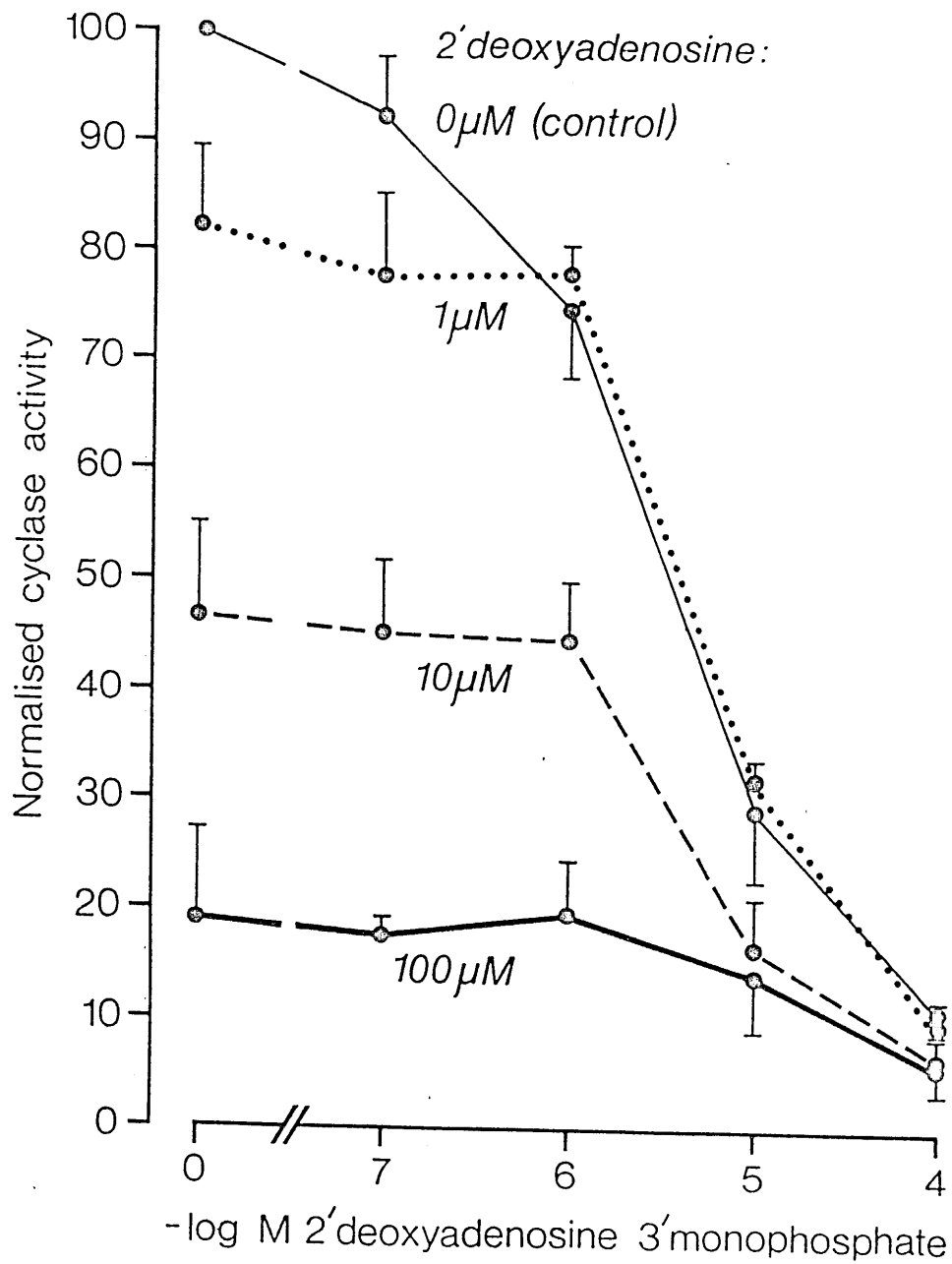


Figure 7.4 Effect of increasing concentrations of 2'-deoxyadenosine 3'-monophosphate on the activity of rabbit cardiac adenylate cyclase stimulated by 100 mM Na⁺ and 0.1 mM GTP in the absence and presence of 1, 10 and 100 μ M 2'-deoxyadenosine. $n=3$ mean $\pm$ SEM.

Discussion.

The results show that theophylline inhibits rabbit adenylate cyclase in a dose dependent manner. The non-competitive nature of the inhibition shows that theophylline is not directly competing with Mg.ATP for binding at the active site. In sea-urchin sperm, however, theophylline was shown to inhibit the adenylate cyclase in a competitive manner, with respect to Mn.ATP [Garbers 1977]. Whether this difference reflects different sites of action, for Mn.ATP and Mg.ATP, or is due to species and/or tissue difference is not known.

The inhibitory effect of theophylline is seen under basal conditions and when adenylate cyclase is stimulated with NaCl. In neither of these cases are guanine nucleotides included in the assay medium. This action of theophylline does not, therefore depend on an activation of the guanine nucleotide binding proteins (Ns and Ni).

The non-competitive nature of the inhibition resembles that seen with P-site agonists [Welton and Simko 1980, Braun and Levitski 1970, Johnson et al 1979 and Haslam et al 1978] (see also Chapter 6). The results presented in chapter 6 show that the inhibition of rabbit cardiac adenylate cyclase

by 2'-deoxyadenosine 3'-monophosphate is mixed. This may reflect the fact that the latter compound more closely resembles the structure of AMP than do other commonly used P-site agonists. The lack of additivity of inhibition by theophylline with that due to 2'-deoxyadenosine 3'-monophosphate provides evidence that the two may be acting at the same site. As the effect is also seen with isobutylmethylxanthine (this chapter) caffeine and methylisopropylxanthine [Jakobs et al 1972], the effect is likely to be common to all methylxanthines. If methylxanthines can indeed inhibit adenylate cyclase through the P-site this could give some insight into the structure of the site.

Chapter 8

The effect of adenosine and 2-chloroadenosine on
the developed tension of isolated guinea pig atria.

Introduction.

The depressant effects of adenosine on atrial myocardium were noted by Drury and Szent-Gyorgi in 1929. Schrader et al [1975b] and Belardinelli et al [1979], studying the action of adenosine on guinea pig and rat atrial muscle found that the depressant actions of adenosine could be antagonised by calcium. It was suggested that the effects of adenosine were mediated by a depression of the permeability of the membrane to calcium. More recently, Belardinelli and Isenberg [1983] found that potassium channels were activated by adenosine in a cAMP-independent manner in atrial myocytes. Increased movement of K^+ is felt to be the primary response of atrial myocardial cells to adenosine. The receptor mediating these events has been shown to be of the A_1 subtype by Evans et al [1982] and Collis [1983].

In this chapter I have partially characterised the action of adenosine and 2-chloroadenosine on guinea pig atrial muscle, so that the effect of pertussis toxin on atria and papillary muscles from the same heart could be compared (see chapter 9). Firstly I have compared the responses of the muscle to adenosine and 2-chloroadenosine and examined the

effect of the uptake inhibitor dipyridamole, and the adenosine deaminase inhibitor EHNA on the potency of both these compounds. The effect of lowering the calcium concentration from 2.5 to 1.25 mM was investigated, since I intended to use the lower concentration of calcium in the studies involving pertussis toxin. Lastly, I tested to see if 8-phenyltheophylline could antagonise the response to 2-chloroadenosine.

Methods.

Atria were removed and set up as described in methods A3. They were usually bathed with Krebs-Henseleit solution containing 1.25 mM Ca^{++} .

a. Comparison of the effect of adenosine and 2-chloroadenosine on atrial force. When the actions of adenosine and 2-chloroadenosine were compared, the muscles were halved from apex to base and set up in separate organ baths. One half was then treated with adenosine and the other with 2-chloroadenosine. Doses of adenosine or 2-chloroadenosine were added to the organ bath in a cumulative manner. Each successive dose was added at two minute intervals, this being sufficient time for the effects of both compounds to reach a maximum. Control dose response curves to adenosine

or 2-chloroadenosine were obtained, and the muscles washed, before EHNA at $5\mu\text{M}$ was added to the organ bath. The dose response curves were repeated after 30 minutes had elapsed. After washing and recovery, a further control dose response to the nucleosides was obtained. Finally, after the usual washing and recovery period, $1\mu\text{M}$ dipyridamole was added to the organ bath and left for 30 minutes before the final set of dose responses was obtained. The order of exposure of the muscle to EHNA and dipyridamole was varied between experiments.

b. The influence of Ca^{++} on the response to 2-chloroadenosine. Halved atria were also used in these experiments. Dose response curves to 2-chloroadenosine were prepared in either 1.25 or 2.5 mM calcium.

c. Antagonism of the response to 2-chloroadenosine by 8-phenyltheophylline. Whole atria were used in these experiments. A control dose response curve to 2-chloroadenosine was first obtained, and the muscle allowed to recover from this before $10\mu\text{M}$ 8-phenyltheophylline was added to the organ bath. After 30 minutes, the dose response curve was repeated. Control experiments, where either the solvent for 8-phenyltheophylline (80% meOH containing 0.2 M NaOH) or papaverine (a

non-methylxanthine phosphodiesterase inhibitor) were substituted for 8-phenyltheophylline were performed with separate muscles.

Results.

a. Comparison of the effects of adenosine and 2-chloroadenosine on developed tension of atrial muscle.

Figure 8.1 shows a sample dose response trace of a guinea pig atrium to adenosine. Note that the effect of adenosine was immediate in onset but tended to recover slightly during the two minutes before the next dose. The minimum point reached was used in the construction of the dose response curves. If the maximum dose of adenosine was allowed to remain in contact with the muscle for longer than two minutes, the developed tension tended to recover, suggesting metabolism or adaptation of the muscle to the inhibitory stimulus. After washout of adenosine, the developed tension tended to overshoot the original control tension before returning to pre-adenosine control values.

A similar dose response to 2-chloroadenosine is shown in figure 8.2. Notice that the threshold for the effect is an order of magnitude lower than

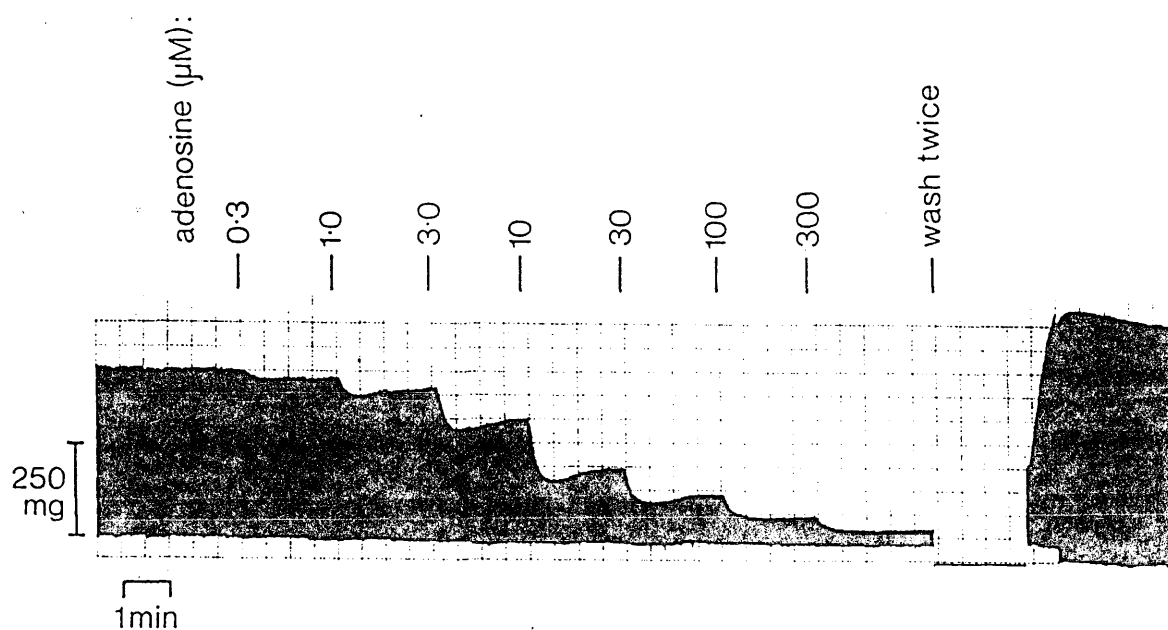


Figure 8.1 A sample trace showing the effect of adenosine on the developed tension of electrically driven left atria.

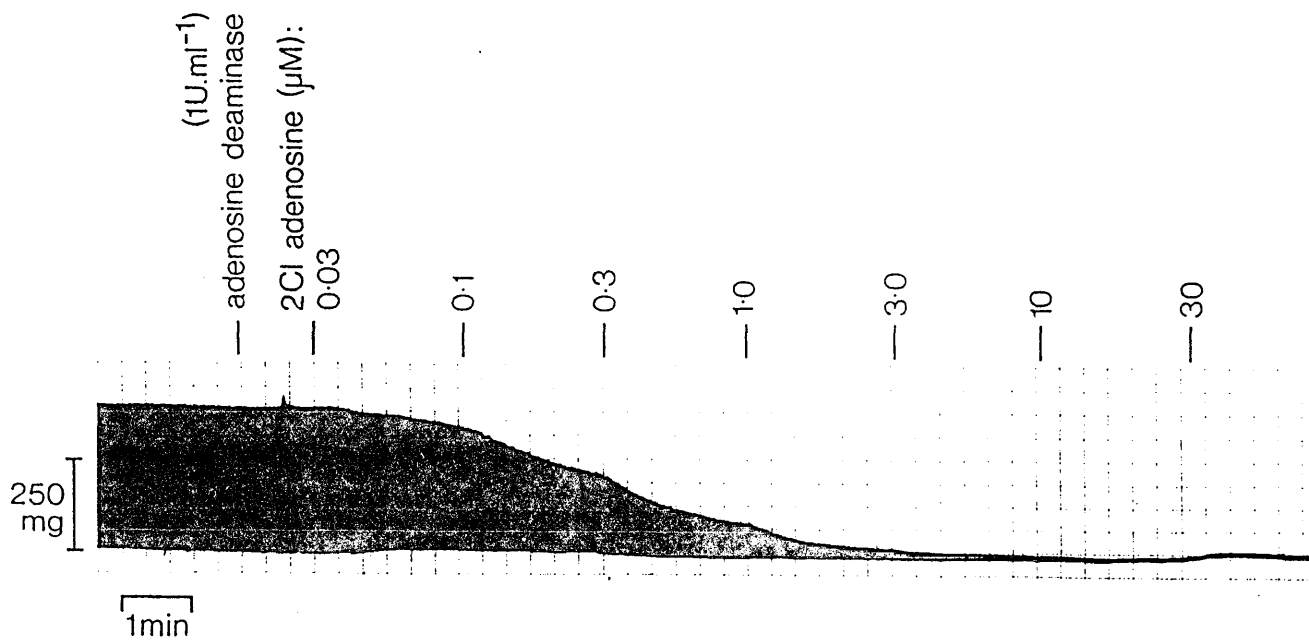


Figure 8.2 A sample trace showing the effect of 2-chloroadenosine on the developed tension of electrically driven left atria.

that for adenosine and the maximal effect is greater. The response of the muscle to 2-chloro-adenosine was not immediate in onset but was more gradual. In this case after washout, it was noted that the recovery was also more gradual, taking 30-40 minutes to reach pre-drug values.

Since the experiments with 2-chloroadenosine were performed in the presence of adenosine deaminase, it was determined that 1 unit/ml adenosine deaminase could indeed remove adenosine from the drug bath. Figure 8.3 shows that adenosine deaminase is effective in removing up to 0.1 mM adenosine. Notice also the recovery of the muscle from the initial maximal effect, and the overshoot of tension on removal of adenosine. Tension gradually returned to pre-drug values. Adenosine deaminase at 1 unit/ml had little effect on tension on average (tension was increased by $0.64 \pm 0.7\%$ $n=49$). Generally (30 out of 49 cases) there was no change in tension at all, but occasionally developed tension was increased by up to 16%.

b. Effect of EHNA and dipyridamole on the responses of atria to adenosine and 2-chloroadenosine.

As can be seen from an examination of figure 8.4, EHNA significantly potentiated the response to

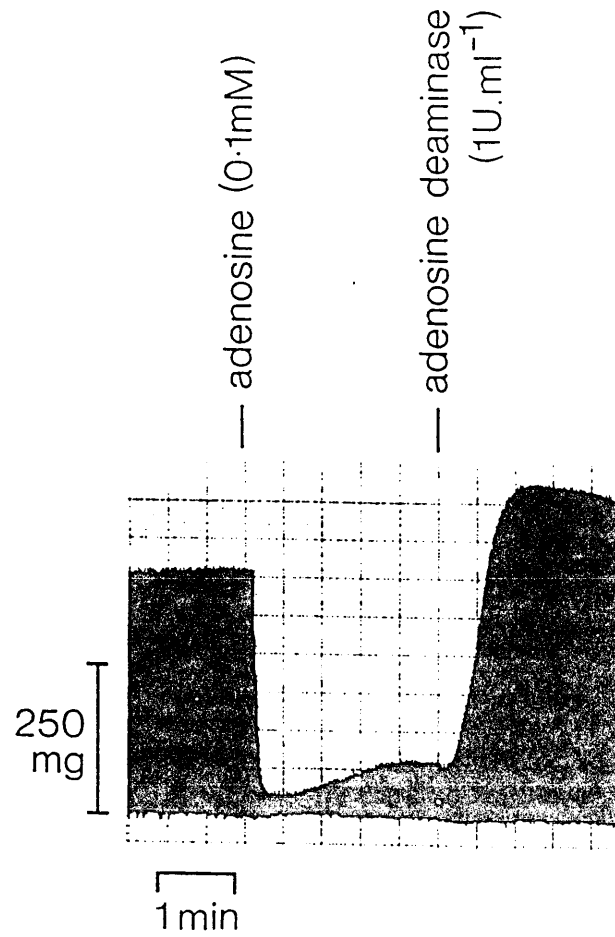


Figure 8.3 A trace illustrating the fact that adenosine deaminase at 1 unit/ml is effective in removing up to 1 mM adenosine.

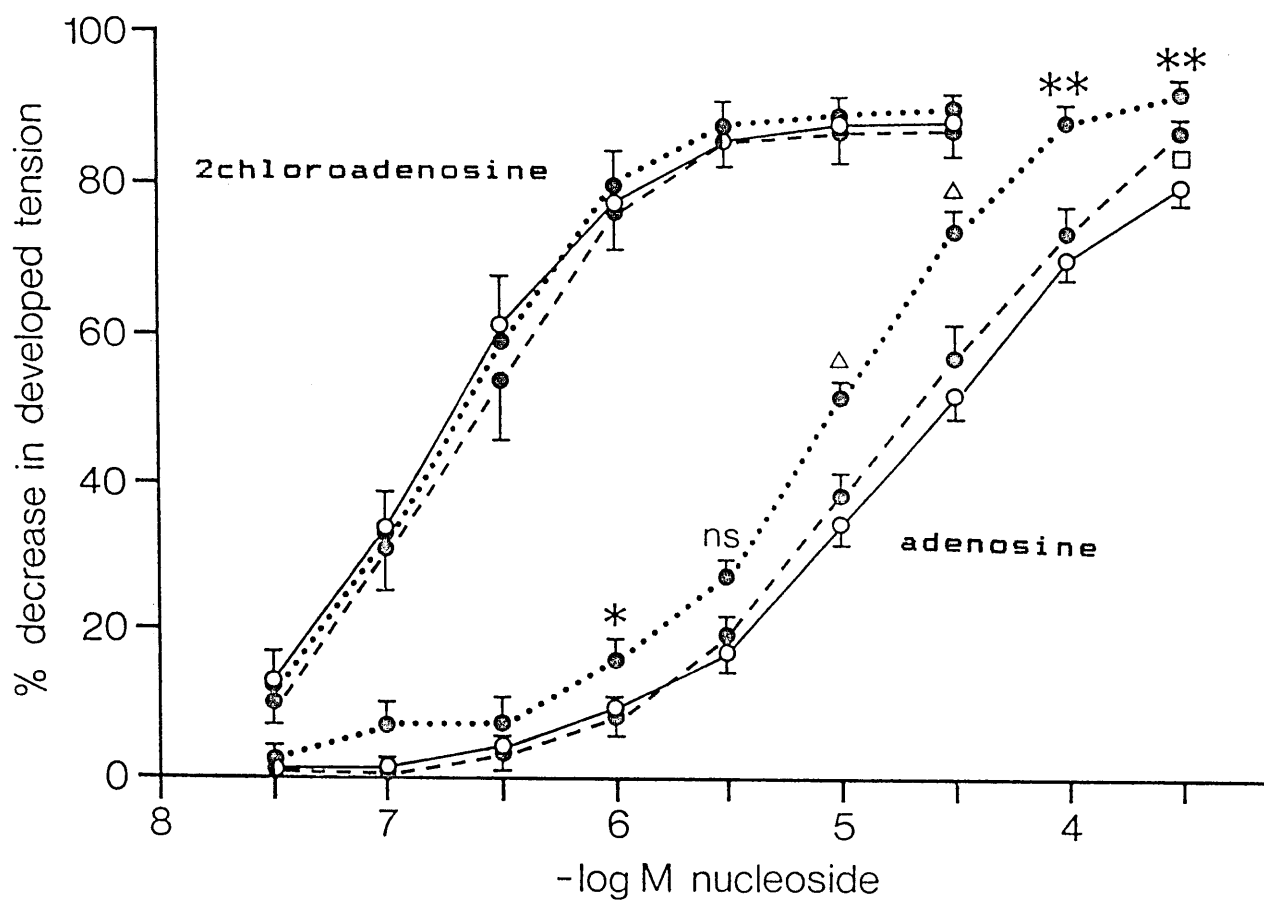


Figure 8.4. Dose response curves of paired half atria to adenosine and 2-chloroadenosine. $\text{O}—\text{O}$ = control, $\text{O}--\text{O}$ = + dipyridamole, $\text{O}...\text{O}$ = + EHNA. P values; * = <0.05 , ** = <0.01 , Δ = <0.001 , $\square$ = <0.002 . (paired t-test, $n=5$ + S.E.M.)

adenosine but not to 2-chloroadenosine. The effect of dipyridamole on the response to adenosine was small and only reached statistical significance at 0.3 mM adenosine. The uptake blocker did not have any influence on the response of the atrium to 2-chloroadenosine. The concentrations of EHNA (5 μ M) and dipyridamole (1 μ M) were those determined as maximal by Moody et al [1984] for guinea pig atria. Neither EHNA nor dipyridamole significantly affected tension on average.

c. Effect of calcium on the responses of atria to 2-chloroadenosine.

Lowering the calcium concentration from 2.5 to 1.25 mM had very little effect on the response of the muscle to 2-chloroadenosine (see figure 8.5). There was a small increase in sensitivity of the muscle to the purine at the upper end of the dose range. The average developed tension was reduced from 0.89 ± 0.32 g to 0.31 ± 0.18 g by lowering the calcium from 2.5 to 1.25 mM.

d. Antagonism of the response of guinea pig atrium to 2-chloroadenosine by 8-phenyltheophylline.

8-phenyltheophylline effectively antagonised the response of atria to 2-chloroadenosine,

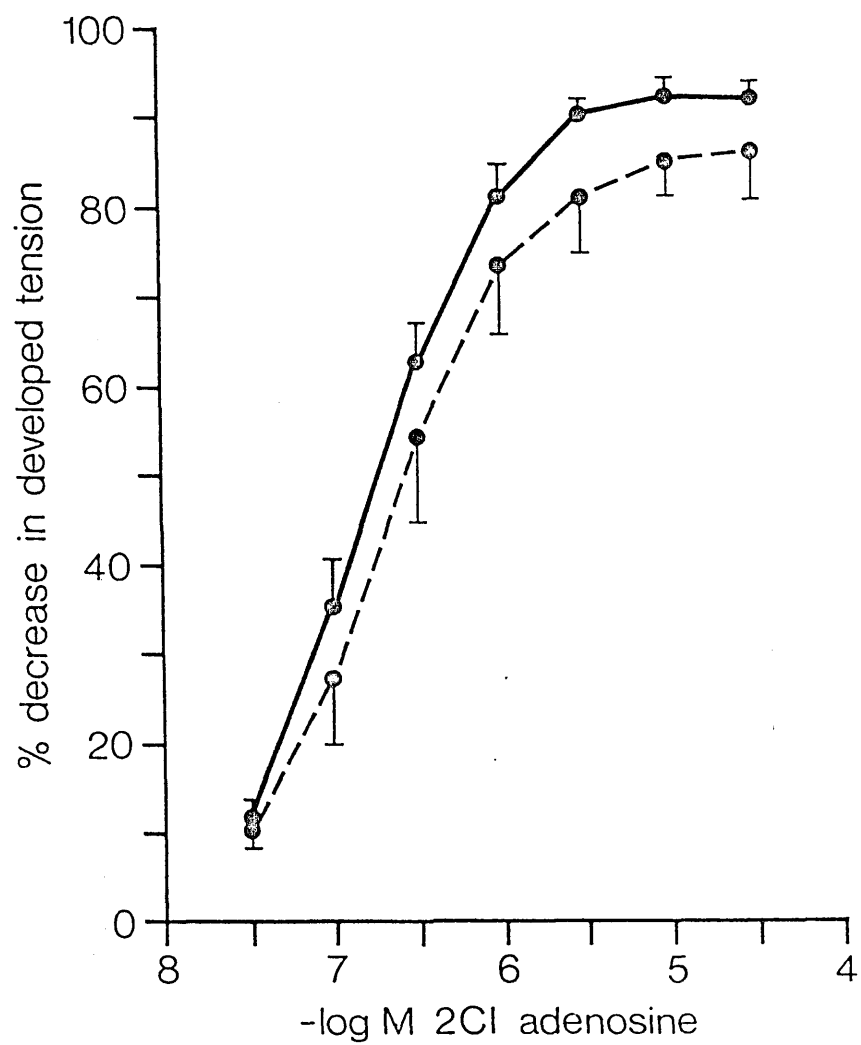


Figure 8.5. Dose response curves of paired halved atria to 2-chloroadenosine when the muscles were bathed in either 2.5 mM Ca⁺⁺ O—O or in 1.25 mM Ca⁺⁺. O—O

producing a rightwards shift of the dose response curve. This antagonism was overcome at the higher doses of 2-chloroadenosine, suggesting the antagonism to be competitive in nature, characteristic of the type of inhibition usually exhibited by methylxanthines. (figure 8.6a) Figure 8.6b shows that the solvent for 8-phenyltheophylline (80% MeOH containing 0.2 M NaOH) has no effect on the response to 2-chloroadenosine.

Since 8-phenyltheophylline increased tension by $52.1 \pm 20.4\%$, control experiments were performed using papaverine at a concentration of 3 μ M. Although tension was increased by 29.5 ± 5.3 (n=5), an examination of figure 8.6c will reveal that this had very little influence on the response to 2-chloroadenosine.

Discussion.

Since the action of 2-chloroadenosine was not affected by uptake inhibitors or by adenosine deaminase inhibitors, this strongly suggests that it is stable to metabolism by the myocardium. Adenosine was removed from the bathing solution by the action of adenosine deaminase. This, coupled with the fact that there is likely to be a variable

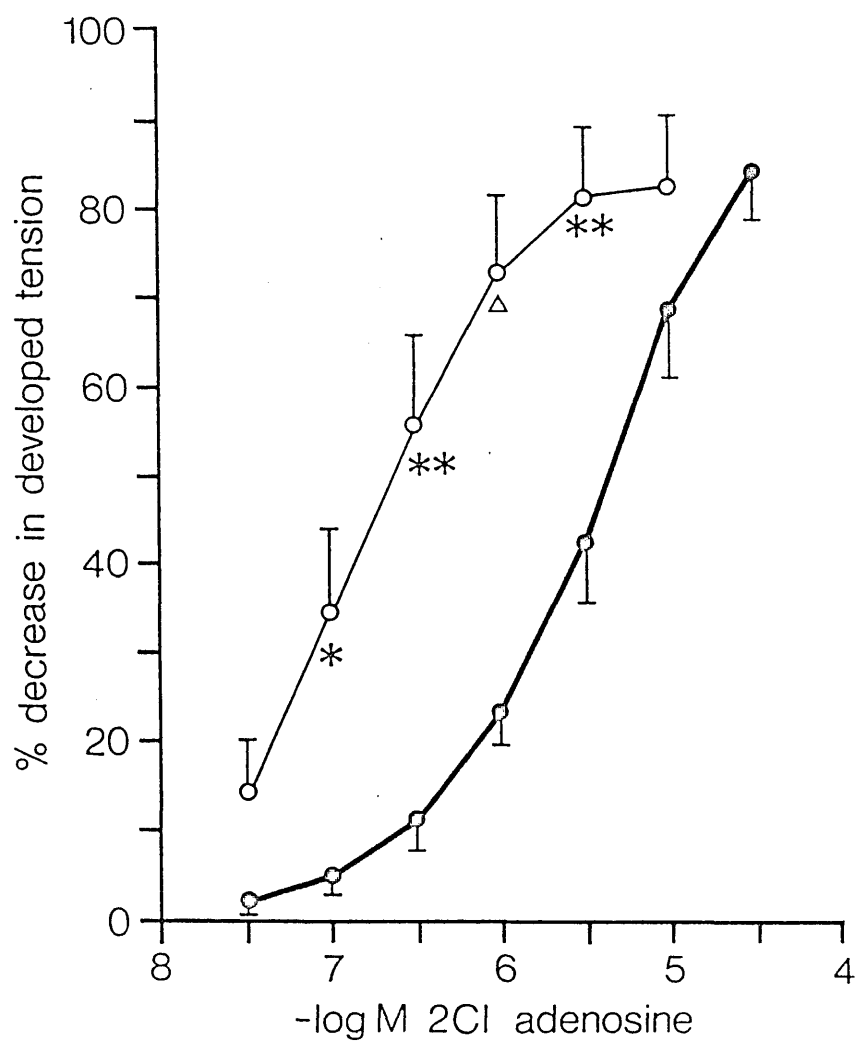


Figure 8.6a. Dose response curve of left atrium to 2-chloroadenosine in the absence O—O, and presence of 10 μ M 8-phenyltheophylline. P values; * = <0.05 , ** = <0.01 , Δ = <0.002 . (paired t-test, $n=5 \pm$ SEM)

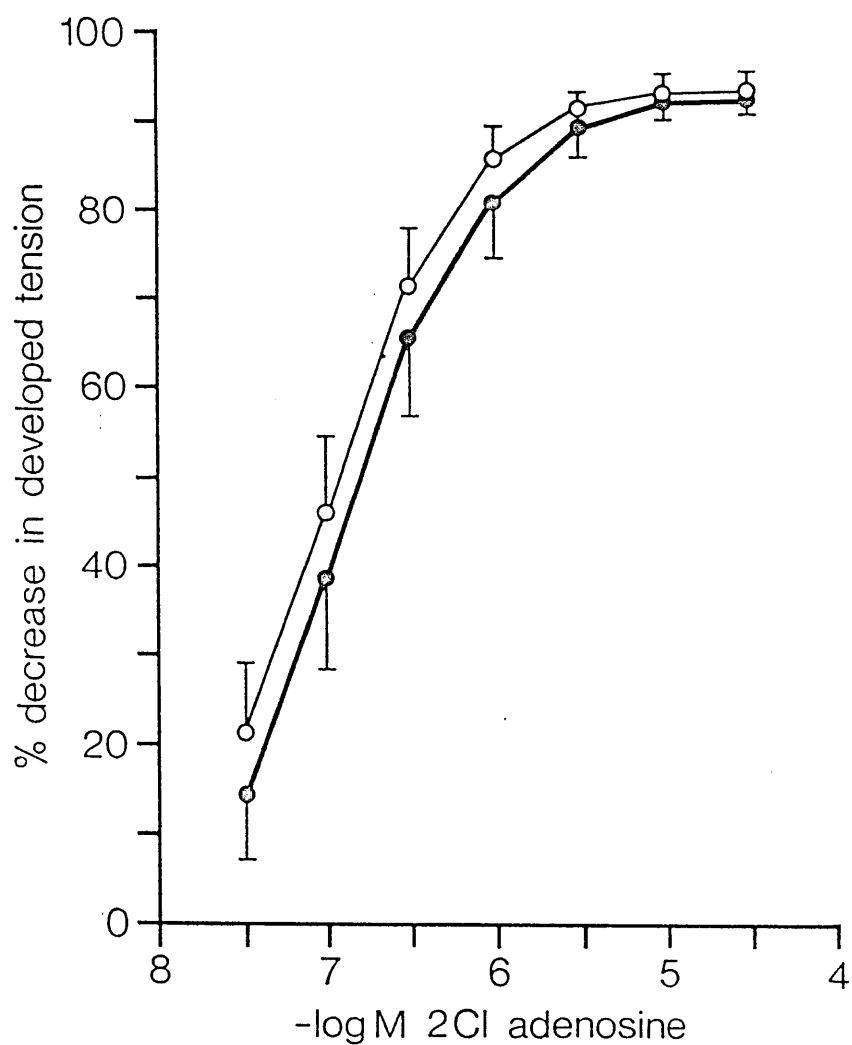


Figure 8.6b. Dose response curve of left atrium to 2-chloroadenosine, in the absence O—O, and presence of NaOH/MeOH (the carrier for 8-phenyl theophylline. ●—●. $n=5$ mean $\pm$ SEM.

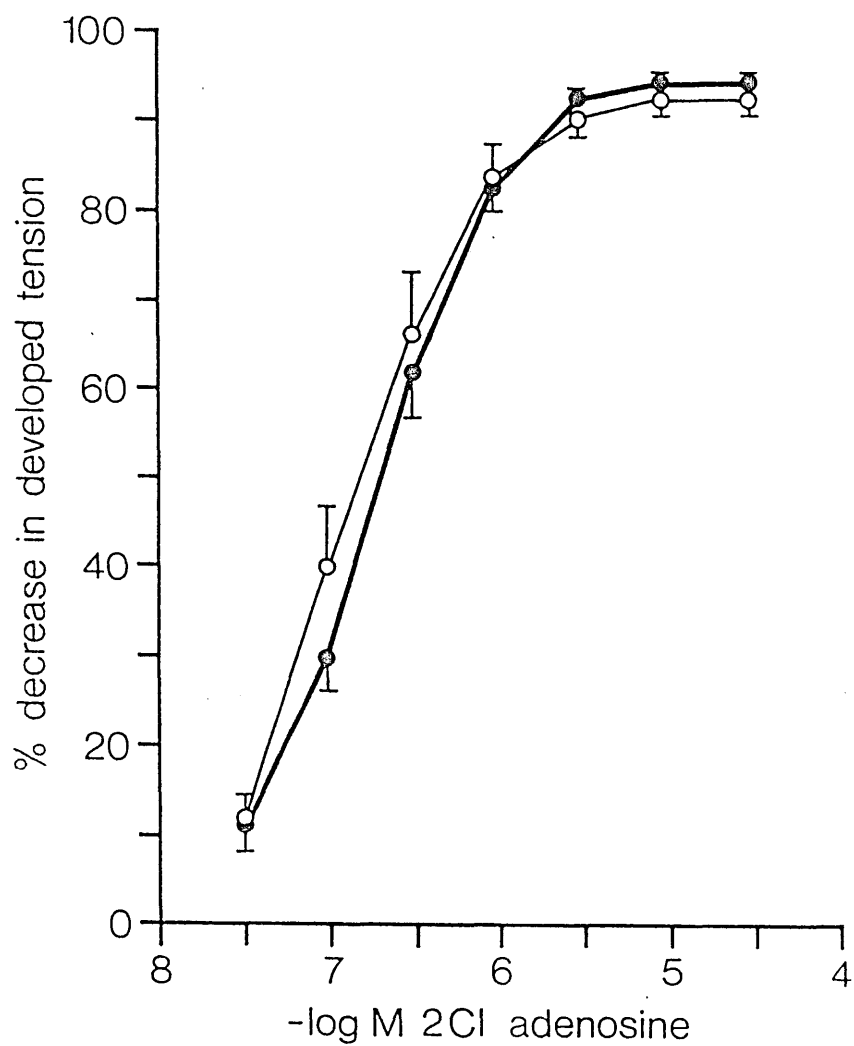


Figure 8.6c. Dose response curve of left atrium to 2-chloroadenosine in the absence O—O, and presence of 3μM papaverine ●—●. n=5, mean $\pm$ SEM.

amount of endogenous adenosine present in the bath, means that the actual concentration of adenosine presented to the muscle is only approximately the dose given. The use of 2-chloroadenosine in conjunction with adenosine deaminase circumvents these difficulties. Neither the action of 2-chloroadenosine nor that of adenosine was appreciably affected by the adenosine uptake inhibitor, dipyridamole. This suggests that the action of adenosine and this derivative are mediated by externally located receptors rather than the internal P-site.

Since I intended to perform the experiments described in the following chapter using 1.25 mM calcium in the Krebs-Henseleit solution, I investigated the effect of lowering the calcium from the 'standard' 2.5 mM to 1.25 mM. This change made little difference to the response of the atrial myocardium to 2-chloroadenosine. It is possible that by using a wider range of Ca^{++} concentrations, this difference between high and low Ca^{++} might be more pronounced, but this was not investigated here.

The potent adenosine receptor antagonist, 8-phenyltheophylline competitively antagonised the responses of atrial myocardium to 2-chloro-

adenosine. This shows that the receptors mediating the depression of atrial contraction were of the A-type. At 10 μ M, 8-phenyltheophylline increased the developed tension by around 50%. It might be argued that by inhibiting the cAMP phosphodiesterase and increasing the cAMP content of the atrium (and therefore the developed tension), the sensitivity to 2-chloroadenosine might be altered. Control experiments where papaverine, which has no effect on the adenosine receptors, was substituted for 8-phenyltheophylline showed that this was not the case.

Chapter 9

The effect of pertussis toxin on the direct and indirect actions of adenosine and 2-chloroadenosine on Guinea pig heart.

Introduction

The experiments described in chapters 2,3 and 8 illustrate the fact that the action of adenosine on atrial and ventricular myocardium is negative in character. If the actions of adenosine on ventricular myocardium are mediated through the adenylate cyclase system, one would expect to see an inhibition of adenylate cyclase activity. However the experiments described in chapters 5&6 provide little support for this hypothesis. As was stated in these chapters, the lack of repeatable inhibition may be due to factors not investigated here. These include loss of G-protein function by proteases, and contributions to whole heart enzyme activity from both sarcolemmal and smooth muscle adenylate cyclase (which would tend to have opposing effects on cAMP levels).

An alternative way of demonstrating the participation of adenylate cyclase would be to show the involvement of components of this system, such as the N-proteins. Receptors which inhibit adenylate cyclase are functionally linked to the catalytic subunit through a guanine nucleotide binding protein, Ni. The discovery of IAP (Islet Activating Protein) by Yajima et al in 1978

provided a tool for the investigation of Ni protein function. IAP (pertussis toxin) catalyses the ADP ribosylation of the inhibitory guanine nucleotide binding protein, leading to loss of its function. This effectively prevents the action of inhibitory hormones. Thus, if treatment with pertussis toxin interferes with or prevents the action of a given hormone, it may be deduced that this hormone's effects are mediated via Ni.

The experiments described in this chapter were aimed at determining the involvement of Ni in the antiadrenergic action of 2-chloroadenosine on guinea pig papillary muscles. Comparisons with atria and ileum were also made, since the involvement of Ni in both these tissues has been demonstrated [Endoh et al 1983, Tucker and von Uexküll 1985].

Methods.

Papillary muscles were set up in the flow through system (Methods A2), or in organ baths as described in methods A3. Atria were set up in organ baths as described in Methods A3. Dose-response curves of the effects of 2-chloroadenosine and adenosine on atrial tension were prepared as described in Chapter 8. Dose-response curves of

the antiadrenergic effect of 2-chloroadenosine on papillary muscles were prepared as described in chapter 3. Guinea pigs were injected with pertussis toxin or carrier plus saline as described in methods A4. A control and a pertussis toxin treated animal were injected at the same time and used on the same day. This ensures that animals were not being selected from different populations.

Results.

a. The effect of pertussis toxin on the direct effect of 2-chloroadenosine on guinea-pig isolated atria.

Treatment of guinea-pigs with 125 µg/kg pertussis toxin decreased the potency of 2-chloroadenosine on the isolated atria. The concentration response curve was shifted to the right but the maximum was unaltered (figure 9.1). The IC₅₀ for 2-chloroadenosine was increased from 57.7 ± 12.2 nM to 187.3 ± 44.1 nM (n=7 p<0.01, Wilcoxon test). There was no evident effect of 100 µg/kg pertussis toxin. Those atria which showed the most marked effect of pertussis toxin on the direct action of 2-chloroadenosine tended to beat spontaneously and, in two cases, the stimulation frequency had to be increased from the normal 1Hz

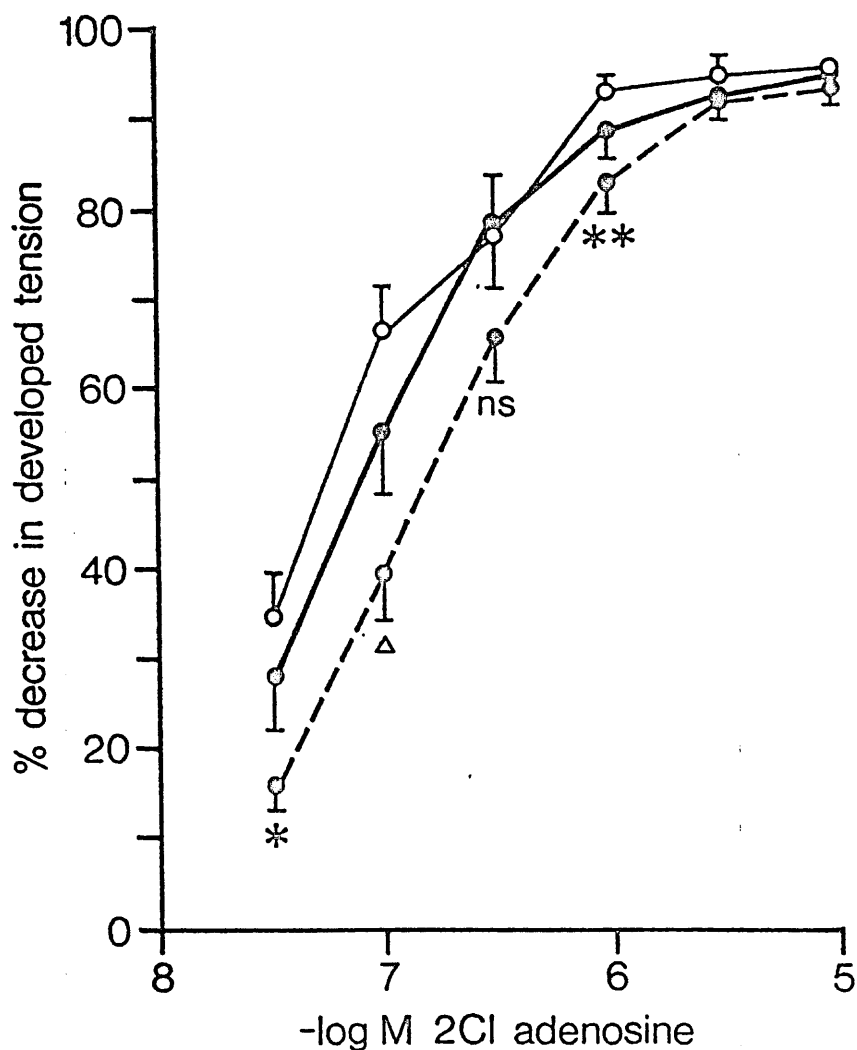


Figure 9.1 Dose response relationship between the % decline in developed tension of paced left atria versus the concentration of 2-chloroadenosine. $\circ-\circ$ = control (n=11), $\circ-\circ$ = animals treated with 100 µg/kg pertussis toxin (n=5), $\circ--\circ$ = animals treated with 125 µg/kg pertussis toxin (n=7). * $p < 0.02$, ** $p < 0.01$, $\Delta p < 0.002$ (grouped Student's t-test).

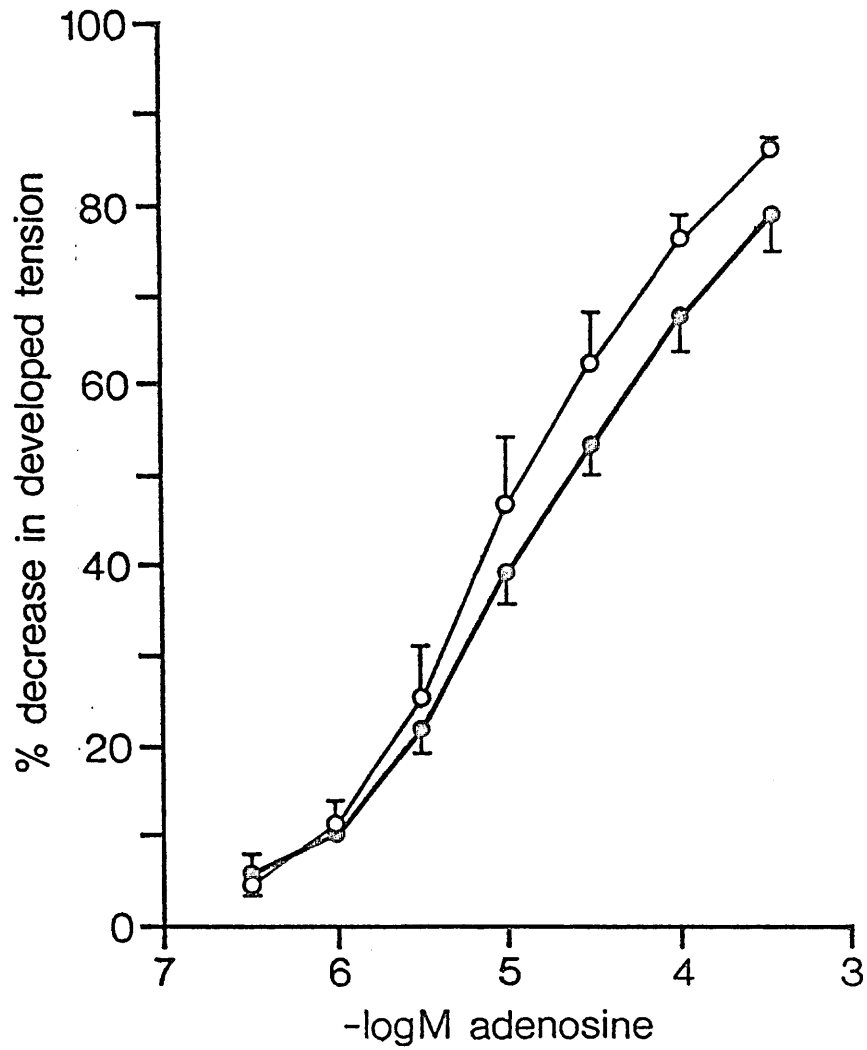


Figure 9.2. Dose response relationship between adenosine concentration and % decline in developed tension of paced left atria. $\circ$ — $\circ$ = control, $\bullet$ — $\bullet$ = animals treated with 125 μ g/kg pertussis toxin, $n=6 \pm$ SEM.

to 2.5 Hz to counteract this. Controls performed on the same day were subjected to the same conditions. Pacing at 2.5 Hz rather than 1 Hz did not appreciably affect the IC₅₀ to 2-chloroadenosine.

b. The effect of 125 ug/Kg Pertussis toxin on the direct effect of adenosine on guinea pig atria.

Since the effect of 2-chloroadenosine was not reduced by a large extent by pertussis toxin, the effect on the direct action of adenosine was studied. Figure 9.2 shows the effect of 125 µg/kg pertussis toxin on the response to adenosine. Again the extent of the effect of pertussis toxin was small.

c. The effect of pertussis toxin on the antiadrenergic effect of 2-chloroadenosine on guinea-pig papillary muscles.

Treatment with pertussis toxin reduced the antiadrenergic effect of 2-chloroadenosine in a dose dependent fashion (figure 9.3). No significant effect was seen with 60 µg/kg toxin. With 100 µg/kg toxin, the reduction was statistically significant, and the antiadrenergic effect was approximately half that in controls. Papillary muscles from animals treated with 125 µg/kg pertussis toxin beat

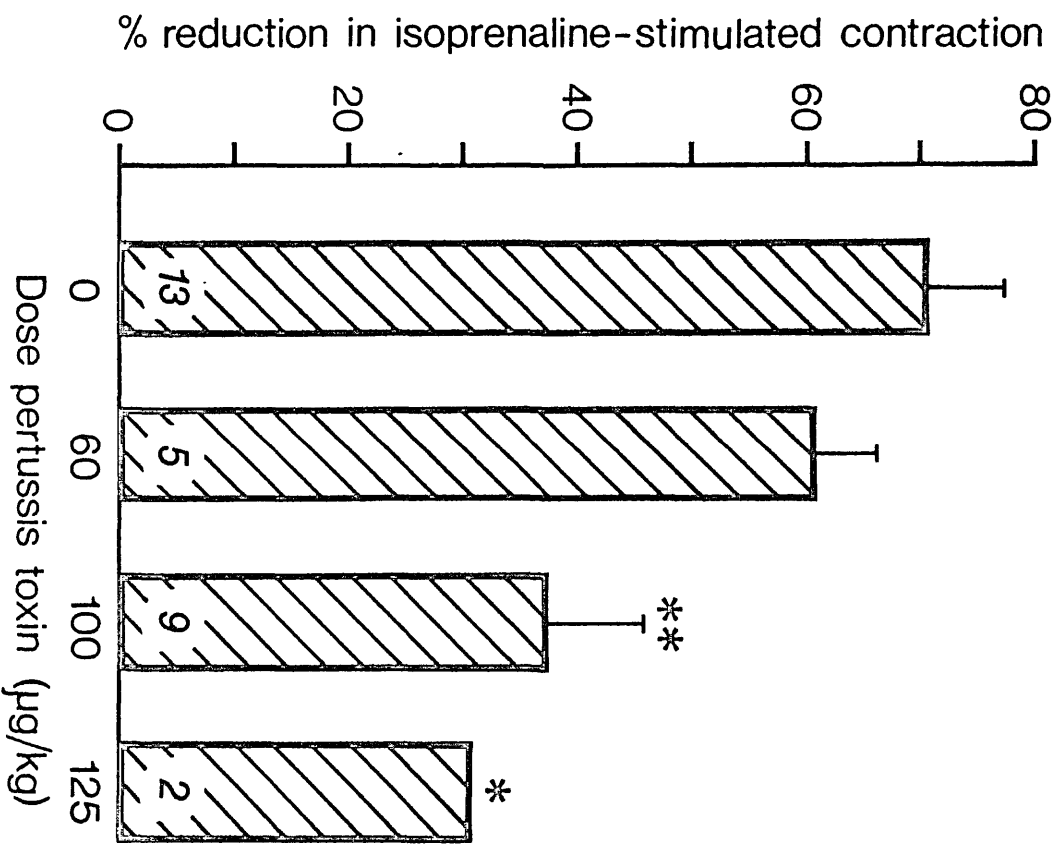


Figure 9.3 The effect of increasing concentrations of pertussis toxin on the antiadrenergic effect of 1 μM 2-chloroadenosine. * = $p < 0.05$, ** = $p < 0.01$ (Wilcoxon rank test.) Numbers of animals are shown on the columns.

weakly, and most were unusable. In two where results were obtained the potency of 2-chloroadenosine was further reduced.

Pertussis toxin pretreatment did not affect the response to isoprenaline alone. 10 nM isoprenaline raised developed tension by $95.5 \pm 15\%$ (n=9) in papillary muscles from control and by 91.1 ± 19.8 (n=6) from toxin treated animals (100 ug/kg).

c. Effects of pertussis toxin on the slow twitch in guinea pig ileum.

Alexa von Uexküll (of King's College, Chelsea, working with Dr J Tucker) took the ilea from some of the pertussis treated guinea pigs and examined the effect of pertussis toxin on the inhibition of twitch in transmurally stimulated ileum by 2-chloroadenosine. In the group of animals treated with 125 ug/kg pertussis toxin Miss von Uexküll found that there was very little alteration in the response to 2-chloroadenosine (Table 1). More pronounced effects on the 2-chloroadenosine dose response curves in ileum were seen with the group of animals treated with 100 ug/kg pertussis toxin, with a suppression of the maximum effect of 2-chloroadenosine from 100% to around 65% in two

Table 9.1 The effect of 125 ug/kg pertussis toxin on the effects of 2-chloroadenosine on ileum twitch and developed tension of atria.

Animal No	Ileum IC50 nM	Atrium IC50 nM
1	350	71
2	52	160
3	66	100
4	250	180
5	/	390

Average Ic50 for inhibition of slow ileal twitch in untreated animals = 68.7 ± 4.1 nM
2-chloroadenosine. ($\pm$ SEM, n=34)

Average IC50 for inhibition of developed tension in paced left atrium of untreated animals = 57.7 ± 12.2 nM (n=11. mean $\pm$ SEM)

cases out of four. However the effect of this concentration of pertussis toxin on the responses of atria were small.

In previous experiments with pertussis toxin, Miss Von Uexküll found that using 125 ug/kg pertussis toxin, the maximum effect of 2-chloroadenosine was reduced from 100 % to 47.2 ± 5.2 % (n=8) on average. These results suggest a variability in the potency of the pertussis toxin preparation, the batches used by us in these experiments being less effective than those obtained earlier in the year.

d. Other observations.

As stated earlier, toxin-treated atria tended to be more arrhythmic than control atria. Miss Von Uexküll noted that the ilea from such animals tended to be paler, thinner, and would contract spontaneously.

Discussion

The results presented in this chapter confirm that pertussis toxin treatment reduces the sensitivity of guinea-pig atria to adenosine analogues. It also shows, for the first time, that

the antiadrenergic effect of adenosine analogues on the contractile force of ventricular muscle is similarly sensitive to pertussis toxin. Previous work by Hazeki and Ui [1981] demonstrated that pertussis toxin treatment reduced the antiadrenergic action of adenosine on cAMP levels of isolated myocytes in culture, but no parallel measurements of contraction were made.

The antiadrenergic effect was more sensitive to toxin since it was affected by a dose of 100 ug/kg toxin, while the direct effect was only reduced with 125 ug/kg. The effect of pertussis toxin on the atria in this study was not as pronounced as that observed by Endoh et al [1983]. These authors found that 5ug/kg toxin produced a considerable suppression of the maximum effect of adenosine, as well as an increase in IC_{50} , in rat atria. This may represent a species difference in susceptibility to pertussis toxin; it has been shown that rat is more sensitive to the leucocytotic effects of the toxin than guinea-pigs [Yajima et al 1983]. I have also noted a variability in the effectiveness of different batches of toxin

The positive inotropic effect of isoprenaline was unaffected by toxin pretreatment, indicating

that Ni does not modify beta-adrenergic responsiveness in the absence of an inhibitory receptor agonist. However a role for Ni in basal contractility of the heart may be implied by the fact that both atria and papillary muscles from toxin-treated animals functioned poorly.

Does an involvement of Ni mean that the antiadrenergic effect of adenosine is mediated by adenylate cyclase? It has recently become clear that Ni is not exclusively linked to adenylate cyclase, but also to a variety of other systems including the cAMP phosphodiesterase [Elks et al 1983] and the enzymes involved in phosphatidyl-inositol turnover [Moreno et al 1983, Shefcyk et al 1985, and Nakamura and Ui 1985]. Molski et al [1984] have noted that pertussis toxin will inhibit the calcium mobilisation linked to inositol phospholipid breakdown. However, it is unlikely that the antiadrenergic effect involves this mechanism, since calcium mobilisation would tend to enhance cardiac contractility, rather than reduce it. Thus in the ventricular myocardium, it is most likely that Ni mediates the antiadrenergic effect of adenosine by an inhibitory effect on adenylate cyclase activity and/or a stimulatory effect on cAMP phosphodiesterase activity.

Concluding Remarks.

A detailed discussion of my results in relation to those of other workers in this field has been given at the end of the appropriate chapters. The purpose of this chapter is to give an overview and summary of the work presented in this thesis and how it relates to the work of other groups.

1. Influence of endogenous adenosine

The data presented in chapter 2 indicate that the antiadrenergic effect of adenosine differs between the Langendorff-perfused heart and the superfused guinea-pig papillary muscle. The reason for this difference appeared mainly to be the high levels of endogenous adenosine present in the papillary muscle. However, changes in perfusion pressure resulting from administration of adenosine may cause artefacts in the tension trace leading to further differences between measurements of contraction made with the whole heart or with papillary muscles.

2. Use of 2-chloroadenosine

Having established that endogenously produced adenosine was masking the effect of the added

adenosine, I took steps to circumvent this problem. The use of adenosine deaminase together with a deaminase resistant analogue (2-chloroadenosine) resulted in a rather greater adenosine receptor mediated effect (Chapter 3). Some of the increased potency of 2-chloroadenosine may be attributed to the fact that it is not removed from the medium surrounding the myocyte by either uptake [Sturgill et al 1975] or deamination by membrane bound adenosine deaminase. Other deaminase resistant analogues were available for use, most notably PIA, but this compound was found to have a low solubility in aqueous solvents and proved to be very lipophilic. Since I was unable to wash this compound off easily, I decided to continue with 2-chloroadenosine for the rest of these studies.

3. Antagonism by 8-phenyltheophylline

I showed that the antiadrenergic effect of 2-chloroadenosine is mediated by A-type receptors by the fact that 8-phenyltheophylline could effectively antagonise this response (Chapter 3). I noted that the extent of the antiadrenergic effect was smaller at higher isoprenaline concentrations and therefore higher cAMP levels. Since 8-phenyltheophylline is also a cAMP phosphodiesterase inhibitor, it is possible that

the raising of cAMP levels could contribute to the antagonism of the antiadrenergic effect of adenosine. However, using papaverine (a non-methylxanthine phosphodiesterase inhibitor) to raise cAMP levels I was able to show that the inhibition of the antiadrenergic effect of adenosine by 8-phenyltheophylline was not due to a raising of cAMP levels.

4. Effects of 2-chloroadenosine on basal tension

When the more potent 2-chloroadenosine was used, an effect of this purine on basal tension of guinea pig papillary muscle was unmasked. However, since this was absent in animals treated with reserpine, this suggests that the direct effect of 2-chloroadenosine was in fact an antagonism of the positive inotropic effects of endogenous noradrenaline.

5. Species Differences.

Turning my attentions to rabbit (Chapter 4), I studied the effects of adenosine and 2-chloroadenosine on the tension stimulated by isoprenaline. The antiadrenergic effect of adenosine itself was very small, even smaller than that reported by Endoh and Yamashita [1980]. Even

under conditions where a good antiadrenergic effect could be observed on guinea pig papillary muscles, little or no effect was seen with rabbit. I therefore concluded that there exists a real species difference between the way that guinea pig and rabbit myocardium reacts to the administration of adenosine. It has been noted by Day and Johnson [1959] that adenosine has little involvement in the relaxation of coronary arteries of rabbit heart.

6. Tissue cAMP

It was reasoned that if the actions of adenosine oppose those of isoprenaline, then it is likely that adenosine could be exerting its effects by inhibiting adenylate cyclase. Measurements made of tissue cAMP in whole heart did not lend support to this hypothesis (Chapter 2). However the concentration of isoprenaline I used to stimulate the hearts was very low (1 nM), and consequently the cAMP levels in isoprenaline treated hearts were only a little higher than the control hearts. Other workers [Schrader et al 1977, Linden et al 1985, Dobson and Schrader 1984, Belardinelli et al 1982, Bauman et al 1982 and Dobson 1983] used higher concentrations of isoprenaline than this (generally 10-100 nM), but as I did not see an antiadrenergic effect of adenosine at such concentrations of

isoprenaline, it did not seem valid to use higher than 1nM when measuring tissue concentrations of cAMP. I also noticed that adenosine itself stimulated cAMP levels. This was not noted by the other groups, but all of these groups used simple acid extraction of the heart. In this case such enzymes as adenylate kinase and creatine kinase would not be inactivated and the levels of the various adenine nucleotides measured would tend to be uncertain, depending on the degree of inactivation of the enzymes. The acid/alcohol extraction used by myself was that recommended by Bates et al [1978] to ensure inactivation of all the enzymes involved in purine metabolism. In doing this I have perhaps unmasked a small stimulatory effect of adenosine on cAMP levels. This small stimulation may be due to the sum of a inhibitory effect of adenosine on the adenylate cyclase of myocytes [Schutz et al 1985, Hazeki and Ui 1981] and a slightly larger stimulation of the adenylate cyclase of smooth muscle cells lining the blood vessels [Anand-Strivastava et al 1982, Kukovetz et al 1978, Goldman et al 1983]. The stimulations due to adenosine and isoprenaline were additive, no antiadrenergic effect being seen here.

7.Effects of PIA on adenylate cyclase activity of rabbit and guinea-pig ventricle

It thus appears that heart tissue has both stimulatory A₂ and inhibitory A₁ adenosine receptors. Brain hippocampus also has A₁ and A₂ adenosine receptors coupled to adenylate cyclase. Yeung and Green [1984] have shown that either one or the other may be expressed depending on the experimental conditions used. In chapter 5 I described experiments designed to enhance the expression of the A₁ receptors on the myocytes. Although by modulating the concentrations of Na⁺, Mg⁺⁺ and GTP, I was able to see an improvement in inhibitory receptor expression overall, this proved to be very variable. I was unable to see the 40% inhibition of basal adenylate cyclase activity reported by Leung et al [1983a&b], nor was I able to see the antiadrenergic effect on adenylate cyclase activity shown by Hosey et al [1984]. The findings of deMazancourt and Guidicelli [1984a&b] prompted me to carry out some preliminary experiments to test if PIA could stimulate cAMP breakdown. Only a very slight inhibitory effect was seen, which was not as great as that reported by Meyer et al for rat heart [1984].

Although the effects of PIA on adenylate cyclase activity were generally inhibitory in character, they were not sufficiently repeatable to allow further characterisation. Therefore I am unable to state with certainty that the effect that PIA has on adenylate cyclase activity is mediated by similar receptors to those that mediate the effects on myocardial contraction.

8. The P-site.

I noticed that the P-site activity of both rabbit and guinea-pig cardiac membranes was high, and could perhaps contribute to the antiadrenergic effect (Chapter 6). Characterisation experiments showed this site to be similar in most respects to the P-site of other membranes and species excepting the fact that the potency of the P-site agonists was not affected by activating adenylate cyclase with Mn or high concentrations of Mg. Many workers have found that activation of adenylate cyclase by hormones increased the potency of the P-site agonists. When both rabbit and guinea-pig adenylate cyclases were stimulated by GTP and isoprenaline, the P-site agents were rendered slightly more potent, perhaps increasing the sensitivity of the P-site sufficiently for adenosine to exert some of its effects on the internal site. During hypoxia

and isoprenaline stimulation, adenosine is released from the myocytes [Schrader et al 1975], possibly originally from the mitochondria [Bukoski et al 1983]. It is possible that some of this adenosine may bind to the P-site and inhibit adenylate cyclase internally before passing out of the cell to affect the external A-type receptor.

9. Direct effect of Theophylline

During the course of these studies I found that theophylline could inhibit adenylate cyclase in its own right [Chapter 7]. This effect is in addition to the previously reported actions of phosphodiesterase inhibition and adenosine receptor antagonism. The fact that the inhibition was not additive with that of the known P-site agonist 2'-deoxyadenosine 3'-monophosphate suggests that theophylline may act via the P-site [Hopwood et al 1985].

10. Pertussis toxin

Pertussis toxin (IAP) catalyses the ADP ribosylation of Ni, thus preventing the action of inhibitory hormones. I have been able to show that the action of 2-chloroadenosine on the positive inotropic effect of isoprenaline was reduced when

animals were treated with pertussis toxin. Comparisons were also made using atrium and ileum from toxin-treated animals since preliminary experiments showed that there was a large variability in how much animals were affected by pertussis toxin. It had already been established that pertussis toxin reduces the direct effect of 2-chloroadenosine on guinea-pig atrium [Halliday, personal communication] and guinea-pig ileum [Tucker and von Uexküll 1985]. These two preparations were therefore used to determine whether the small effect on ventricular tissue was due to limited contribution of Ni to the antiadrenergic effect of adenosine on guinea-pig ventricle or merely to a low potency of the toxin used. The extent of the effect on papillary muscles was similar to that observed in atria and greater than that observed in ileum. This suggests that the larger effects reported elsewhere for these tissues were due to a greater effect of the pertussis toxin on these animals. Guinea-pigs would be expected to show a smaller response to pertussis toxin since they have a greater resistance to pertussis toxin [Yajima et al 1983].

11. The coupling site

The final coupling site for the A-receptor-Ni complex is not certain. It is unlikely to be the

enzymes responsible for phosphatidylinositol formation for two reasons. Firstly Movesian et al [1985] have shown that phosphatidylinositol is not involved in calcium mobilisation in heart cells. Secondly Ni has been shown to couple to the phosphatidylinositol system in a positive manner—that is in such a way as to increase Ca influx and therefore contraction [Nakamura and Ui 1985]. In 1981 Hazeki and Ui performed a study on rat myocytes which complements the findings reported here. They found that pertussis toxin inhibited the adenosine induced decrease in adenylate cyclase activity. Thus even allowing for species differences, the final coupling site for the A-receptor-Ni complex is likely to be adenylate cyclase.

11.Future studies

Further studies in this field may be best performed using isolated guinea-pig myocytes. A single heart could yield many cells, some of which could be used for measurements of sarcomere shortening while others could be used for the biochemical estimation of adenylate cyclase activity. A preparation of myocytes would be free from contamination of smooth muscle and endothelial cells which may have positively coupled adenosine

receptors. A more detailed investigation into the mechanism of action of adenosine on adenylate cyclase could be made, together with a firmer correlation between the effects on contraction and the action which the nucleoside has on adenylate cyclase activity. Also myocytes would not be hypoxic, unlike papillary muscle, so complications due to release of endogenous adenosine would be eliminated. There would also be no release of endogenous catecholamines from nerve terminals. All these points considered, the isolated myocyte would be the perfect system to bridge the gap between the whole tissue pharmacology and the membrane biochemistry presented here.

Appendix.

Materials used.

1. List of compounds.

a. Materials purchased from Sigma.

Adenosine 5' monophosphoric acid (AMP),
Adenosine, Adenosine deaminase, Type 3, Ascorbate,
Atenolol, Adenosine 5' triphosphate (ATP) sodium
salt, prepared by phosphorylation of adenosine,
2-Cl adenosine, creatine phosphate, cyclic AMP ,
2'deoxyadenosine, 2'deoxyadenosine 3'
monophosphate, deoxy ATP, cyclic
deoxyAMP, dipyridamole, dithiothreitol, GppNHp,
GTP(sodium salt), isoproterenol, papaverine,
8-phenyl theophylline, propanolol, theophylline,

PIA was purchased from Boehringer Mannheim, as
was creatine kinase.

2'5'dideoxyadenosine was purchased from PL
Biochemicals.

EHNA was purchased from Wellcome
pharmaceuticals.

Pertussis toxin was purchased from Dr Irons of
The public health laboratory services, Porton down,

Salisbury

NECA was the kind gift of Dr Schick of Byk
Gulden Pharmazeutica

RO 20 1724 was the kind gift of Dr T Collins
of Hoffman la Roche, Welwyn garden city.

ICI 63 197 was the kind gift of Dr M.G. Collis
of ICI pharmaceuticals, macclesfield.

All other chemicals were of 'analar grade' and
obtained from BCL.

Special notes on solubility.

Some of the adenosine analogs and related compounds were difficult to dissolve. Below are notes on how this may be achieved.

1. 2'-deoxy adenosine, 2'5' dideoxy adenosine, IBMX, NECA and Theophylline are only soluble upto millimolar concentrations. They require stirring.

2. Papaverine will not dissolve in aqueous buffers such as those used in the assay of cAMP above pH 6.5. It will however dissolve in Krebs-Henseleit solution.

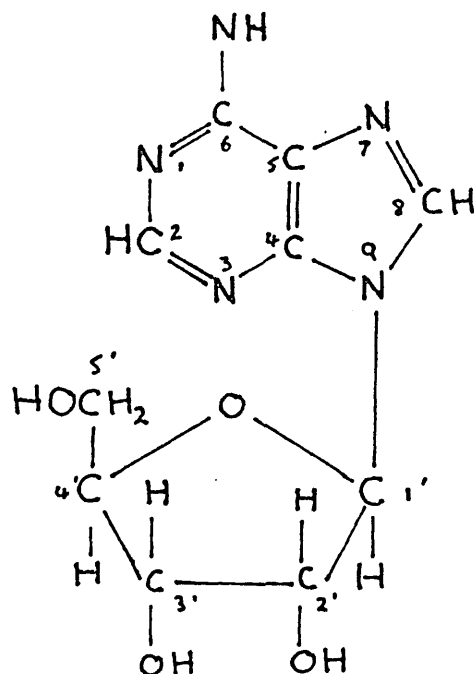
3. A 10 mM stock of 8-phenyl theophylline must be made up in 80% methanol v/v containing 0.2 M NaOH.

4. PIA may first be dissolved in propylene glycol or DMSO. However, DMSO inhibits adenylate cyclase.

5. RO 20 1724 must first be dissolved in propylene glycol (sufficient to give a 16% solution when the buffer is added) when the compound is fully in solution, warmed buffer may be added.

Structures of adenosine and its analogues

Shown below is the structure of adenosine.



Analogues

2Cl-Adenosine, The hydrogen at the 2' position is replaced with a chlorine atom.

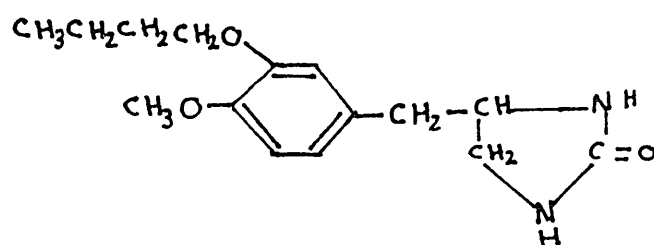
PIA, full name (-)-N⁶(R phenyl-isopropyl)-adenosine. A substitution is made at the nitrogen attached to C6. A 1-methyl 2-phenylethyl group replaces the hydrogen.

2'-deoxyadenosine 3'-monophosphate, a hydrogen replaces the OH group at position 2'. A single phosphate is joined to the 3' position.

Structures of non-methylxanthine phosphodiesterase inhibitors.

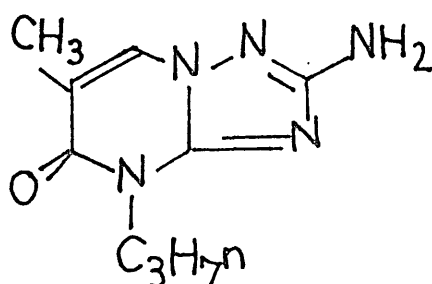
1. RO 20 1724, full name;

d,1-4-(3-butoxy-4-methoxybenzyl)-2-imiazolininone.



2. ICI 63 197, full name;

2-amino-6-methyl-5-oxo-4-n-propyl-4,5-dihydro-s-triazolo[1,5-a]pyrimidine.



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