Regulation of G-protein coupled adenylate cyclase in human platelets

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In memory of my Father

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Abbreviations

C	Catalytic subunit of adenylate cyclase
G_i	Inhibitory guanine nucleotide regulatory protein
G_s	Stimulatory guanine nucleotide regulatory protein
R_i	Inhibitory receptor
R_s	Stimulatory receptor
AppNHp	Adenosine 5'-(beta,gamma-imido)triphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3'5' cyclic monophosphate
EDTA	Ethylene diaminetetracetic acid
EGTA	Ethylene glyco-bis (beta-aminoethyl ether) N,N,N',N',-tetracetic acid
GppNHp	Guanosine 5'-(beta-gamma-imido)triphopshate
GTP	Guanosine triphosphate
NAD	Nicotinamide adenine dinucleotide
Treacl	Triethanolamine hydrochloride
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol

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SUMMARY

Platelet adenylate cyclase is a complex system composed of a catalytic unit coupled to stimulatory (R_s) and inhibitory (R_i) receptors through the guanine nucleotide regulatory proteins G_s and G_i respectively.

Treatment of human platelet membranes with concentrations of benzyl alcohol up to 50mM augmented adenylate cyclase activity when it was assayed in the basal state. Stimulation of enzymatic activity at R_s by PGE_1 and isoprenaline, and at G_s by NaF, was similarly enhanced. The stimulatory effect of the diterpene forskolin on the catalytic unit of adenylate cyclase was antagonised by benzyl alcohol. This inhibition was shown to occur via a non-competitive mechanism.

Benzyl alcohol did not modify the magnitude of the inhibitory response when the catalytic unit of adenylate cyclase was inhibited using low concentrations of GppNHp which acts selectively on the inhibitory G-protein, G_i. Neither was the potent inhibitory action of the $alpha_2$-adrenoceptor on the catalytic unit altered by the presence of benzyl alcohol. However, the ability of adrenaline (+ propranolol) to inhibit PGE_1-stimulated adenylate cyclase was attenuated by benzyl alcohol in a dose-dependent fashion (IC_{50} = 12.5mM). Some residual inhibitory action remained even at concentrations of benzyl alcohol as high as 50mM, thus identifying an inhibitory component which was apparently insensitive to blockade by action of benzyl alcohol. The effect of benzyl alcohol on adrenaline (+ propranolol) inhibition of PGE_1-stimulated adenylate cyclase could not be mimicked by increasing temperature which will enhance membrane fluidity. Treatment of platelet membranes with benzyl alcohol did not lead to the release of either the alpha-subunit of G_i or G-protein beta

subunits. The $alpha_2$-adrenoceptor-mediated inhibition of adenylate cyclase was abolished when assays were performed in the presence of Mn^{2+} rather than Mg^{2+} and, under such conditions, dose-effect curves for the action of benzyl alcohol on PGE_1-stimulated adenylate cyclase activity were similar whether or not adrenaline (+ propranolol) was present. It is suggested that (i) $alpha_2$-adrenoceptor- and G_i-mediated inhibition of PGE_1-stimulated adenylate cyclase may have two components, one of which is sensitive to inhibition by benzyl alcohol and (ii) the G_i-mediated inhibition of forskolin-stimulated adenylate cyclase exhibits predominantly the benzyl alcohol-insensitive component. It is proposed that the benzyl alcohol- insensitive component may reflect the direct inhibitory action of alpha-G_i on the catalytic unit of adenylate cyclase, whilst the benzyl alcohol-sensitive component may reflect an action on the beta/gamma-subunit-mediated inhibitory effect of G_i on G_s dissociation. These actions of benzyl alcohol we suggest to occur due to direct effects upon protein components rather than due to changes in membrane fluidity.

G_i-mediated inhibition of the catalytic unit of adenylate cyclase was shown to be attenuated (45% decrease) by the presence of the anionic drug pentobarbital. Positively charged prilocaine augmented the ability of G_i-alpha to interact with the catalytic unit (87% increase). It is suggested that a positive charge may be required for the efficient interaction of the alpha-subunit of G_i with the catalytic unit of platelet adenylate cyclase.

Adenylate cyclase activity is also shown to be attenuated by the presence of acidic phospholipids. Inhibition occurs by a mechanism which does not require GTP and is not Mn^{2+} sensitive. It is proposed that acidic phospholipids directly attenuate the activity of the catalytic unit and, under normal circumstances, may be excluded from the lipid annulus.

Inhibition of adenylate cyclase activity is also observed in the presence of insulin. It is shown that attenuation arises by a mechanism which differs from that mediating either $alpha_2$-adrenoceptor- or GppNHp-mediated inhibition. The ability of insulin to activate a GTPase activity in platelets suggests the mechanism of inhibition to involve a G-protein, but not G_i.

The hysteretic nature of GppNHp-mediated stimulation of platelet adenylate cyclase was shown to be a time, Mg^{2+} and guanine-nucleotide, dependent process. The rate limiting step being suggested to reflect the conversion of G_s from an inactive to an active form, due to the dissociation of the heterotrimer as:

$$G_s \text{ alpha/beta/gamma} \xrightarrow{GppNHp} G_s \text{ alpha}^{GppNHp} + \text{beta/gamma}$$

It is proposed that the ability to modulate the efficiency of cAMP production in platelets may be of importance in determining the aggregatory properties of these cells.

CHAPTER 1

INTRODUCTION

Regulation of G-Protein Coupled Adenylate Cyclase in Human Platelets

1. INTRODUCTION

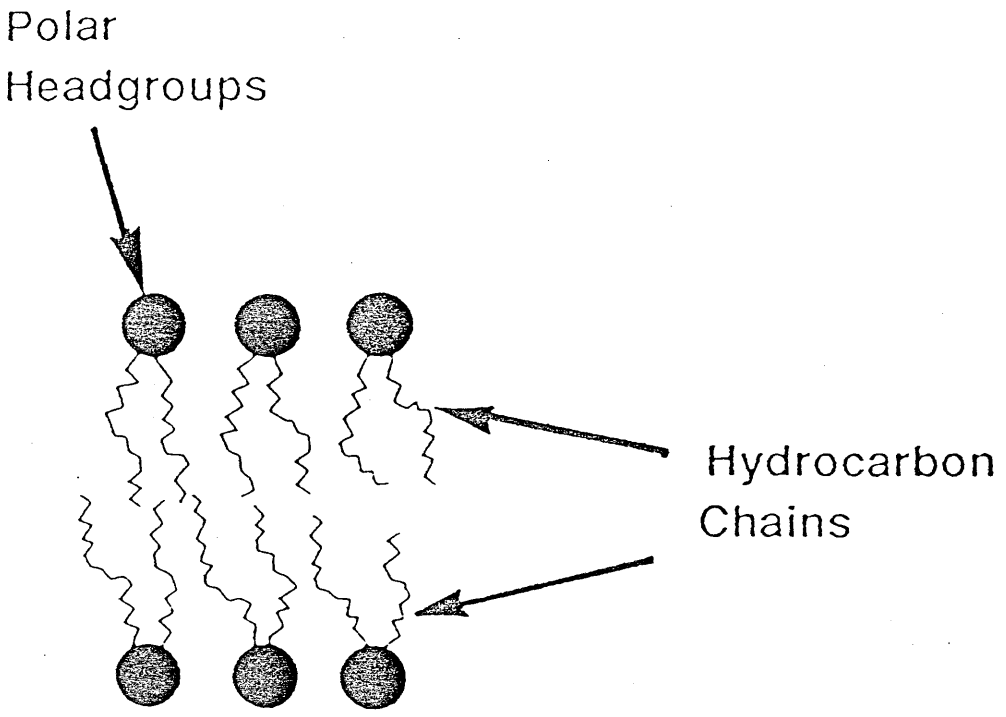
1.1 Membrane Structure

Because adenylate cyclase is primarily located in plasma membranes (Davoren & Sutherland, 1963) it was decided to study the effect of manipulating membrane integrity on the activity of this enzyme in human platelet plasma membranes. It is thought that the structure of biological membranes, and more importantly, the maintenance of these structures, is vital to the functioning of integral proteins such as adenylate cyclase.

(i) Structure and composition of biological membranes

Membranes are basically organised assemblies of lipid and protein, with some carbohydrate present in the form of glycolipids and glycoproteins. Whilst the proportions of lipid and protein vary from membrane to membrane, they are all based on the same general structure; an asymmetric bilayer. The most abundant lipids in these bilayers are the phospholipids i.e. phosphoglycerides and sphingomyelins, although cholesterol and glycolipids also exist. It is therefore apparent that lipids are numerous and quite variable. However, they are mostly amphipathic molecules of similar overall dimensions, allowing them to pack closely in a lipid bilayer. It is this amphipathic structure of the polar membrane lipids that directly determines the bilayer structure as it is the repulsion of lipid hydrocarbon chains by the water structure that drives these chains into an environment sequestered from water (Yeagle, 1989). Hence the bilayers are arranged so that polar or charged groups are positioned on the surface of the bilayer creating a hydrophobic interior of two layers of hydrocarbon moieties positioned tail-to-tail. The polar head-groups lie parallel to, rather than perpendicular with, the plane of the bilayer (Storch & Kleinfeld, 1985) (see Fig.1).

Figure 1 Diagrammatic representation of phospholipid bilayer structure



Associated with these lipid bilayers are to be found proteins. Membrane proteins can generally be categorised as being either intrinsic, or extrinsic, depending on their ease of removal from the membrane. Extrinsic proteins are loosely associated proteins, and may only partially penetrate the bilayer, or be held in place by electrostatic forces. These proteins are easily dissociated from the bilayer by mild treatment such as changes in ionic strength. Intrinsic proteins on the other hand, penetrate the bilayer to varying degrees, and may span the bilayer, or be situated in one leaflet. Disruption of the bilayer by detergents or chaotropic agents is required to release intrinsic proteins.

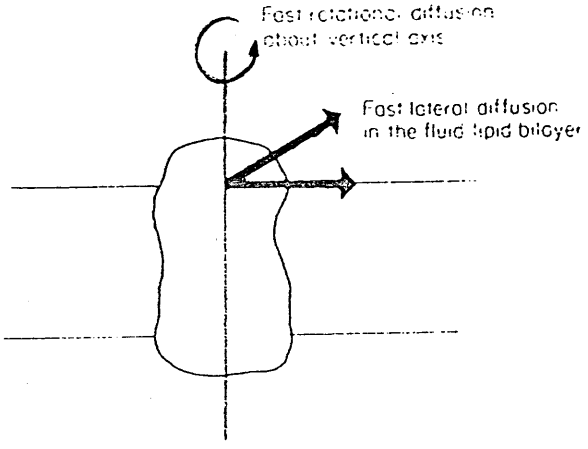
Integral membrane proteins are amphipathic molecules, with the apolar regions of their structure being buried in the bilayer, whilst extrinsic proteins have a micellar structure, i.e. charged/polar sidechains are near the surface of the protein, and hydrophobic residues are buried in the apolar core.

The model of membrane structure most widely accepted is the Fluid-Mosaic model of Singer and Nicholson (1972) where the lipids provide the structural backbone of the membrane and proteins are 'suspended' in this lipid bilayer. Both lipids and proteins are mobile in the plane of the bilayer; this permits the short range ordering of these components, and the formation of regions, or 'domains' of distinctive character. This mobility exists due to the fact that biological membranes are held together by non-covalent forces, which allows a high degree of flexibility and fluidity.

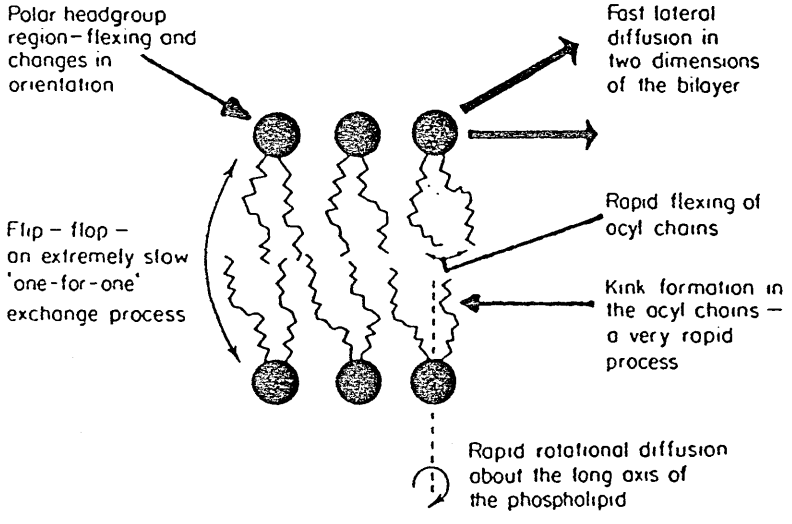
Integral membrane proteins exhibit two distinct types of mobility within the bilayer; (a) fast lateral diffusion in the bilayer, and (b) fast rotational diffusion about the vertical axis (Fig.2)

Figure 2 **Membrane proteins and lipids: permitted modes of mobility**

(A) Integral Membrane Proteins



(B) Membrane Phospholipids



Rotation through the plane of the bilayer is a prohibited event due to the thermodynamic difficulties that would have to be overcome in transferring hydrophilic constituents through the apolar core of the bilayer. By the same argument, 'flip-flop' of phospholipids is also a rare event, although it is one of four permitted modes of mobility.

(a) Fast lateral diffusion

(b) Fast axial rotation

(c) Flip-flop

(d) Intra-chain motion (Fig.2)

Not all phospholipids are able to undergo these movements at the same rate i.e. a 'shell' of lipids surrounds integral proteins, which, by virtue of their interaction with the proteins they solvate, appear to exchange with their neighbours in the bulk lipid pool at rates at least one order of magnitude slower. This ring of lipid which provides the interface between the protein and the bulk lipid pool has been termed the annular lipid domain. The composition of lipids in this annular domain need not reflect that of the bulk lipid pool, as a protein may exhibit segregation for, or even against, particular lipid species (Houslay & Stanley, 1982).

As stated previously, biological membranes are asymmetric structures, i.e. with respect to both protein and lipid distribution. In general, neutral and positively charged phospholipids are concentrated in the outer leaflet of the bilayer, whilst negatively charged phospholipids are predominantly found in the inner leaflet. This distribution of membrane components, and the maintenance of the structure which they form is important with respect to determining the activity of associated proteins, such as adenylate cyclase, as will be discussed later.

(ii) Methods of manipulating membrane structure

The physical properties of a bilayer, in particular its fluidity or rigidity, can influence not only the lateral migration of any integral proteins, but also the internal motions of groups or peptide chains within the protein connected with its function (Houslay & Stanley, 1982). Hence, the membrane structure may determine the activity of integral proteins such as adenylate cyclase. The ability to manipulate this lipid bilayer is therefore of interest, and forms the basis of the following discussion.

The lipid bilayer can be perturbed in a number of ways. One of the simplest means of achieving this is to alter the temperature, where increased temperature relates directly to increase fluidity. This has been determined in a number of membrane systems, including human platelet plasma membranes (Gordon et al., 1983), by the use of spin-labelling techniques such as e.s.r. In platelets, it has been shown from Arrhenius-type plots of order-parameter vs temperature, that a lipid phase separation occurs between 37^{o}C and 22^{o}C. The fact that the platelet plasma membrane, like all biological membranes, contains a mixture of lipids, means that it does not undergo a clear-cut lipid phase separation as do pure lipids. With pure lipid species, the transition from liquid to solid state occurs at a well-defined temperature, whereas in biological membranes, a lipid phase-separation rather than transition occurs (Houslay & Gordon, 1983).

Here, lowering the temperature causes lipids in the fluid state to form 'quasi-crystalline clusters' (QCC) which co-exist with fluid lipid. These domains consist of lipid which is more ordered than the fluid state, but less so than the solid state. Further decreases in temperature result in the lipids of these

quasi-crystalline clusters attaining the solid state. Hence, the bulk phase lipid will be transformed from liquid, to quasi-crystalline clusters, to solid state by a lowering of temperature. As integral membrane proteins are excluded from ordered lipid domains, such alterations in the composition and size of the fluid lipid environment may affect protein activities. Indeed, break-points occur in Arrhenius plots of the activities of many integral membrane proteins, and these usually coincide with a lipid phase separation, indicating that the altered lipid environment affects the activity of the biological process (see Section 1.3(vi)).

It is possible to alter membrane fluidity at more physiologically relevant temperatures in _in vitro_ experiments using a wide variety of agents, such as local and general anaesthetics (Houslay & Gordon, 1983). Much of the work in this area has entailed studies of rat liver plasma membranes, where a lipid phase separation has been observed as occurring at 28^{o}C (Dipple & Houslay, 1978). This is proposed as being confined to the external leaflet of the bilayer, i.e. no such phase separation occurs in the inner leaflet over a temperature range of 0-42^{o}C.

Examining the effect of local anaesthetics on this system (Houslay & Gordon, 1983; Friedlander _et al._, 1987; De Foresta _et al._, 1987; Cherenkevich _et al._, 1982; Dipple & Houslay, 1978; Houslay _et al._, 1981; Houslay _et al._, 1980(b)) showed that the temperature at which this phase separation occurred could be manipulated, and indeed other phase separations could be induced. Hence, the use of such anaesthetics is proposed as a method by which membrane fluidity can be altered.

The neutral, local anaesthetics are particularly useful, as their neutrality precludes any electrostatic interactions with

either the asymmetically dispersed phospholipids, or proteins, in the membrane. Benzyl alcohol (see Appendix I for structure) is one such anaesthetic which is commonly used in manipulating membrane fluidity in both model, and biological membranes (Friedlander et al., 1987; De Foresta et al., 1987; Cherenkevich et al., 1982; Dipple & Houslay, 1978). In examining the effects of such neutral anaesthetics it was found that benzyl alcohol lowers the lipid phase separation temperature by around 6^{o}C (Dipple & Houslay, 1978), as the result of a classical depression-of-freezing-point mechanism. This arises because small lipophilic molecules like benzyl alcohol can readily partition into the fluid-lipid from aqueous solution, but are excluded from the crystalline-phase lipid which forms on cooling. As benzyl alcohol molecules disrupt the ordered packing of the hydrocarbon chains, a lower temperature is required before chain interactions are strong enough to expel the alcohol from this region, and allow crystallisation to ensue (Houslay et al., 1980(b)).

Such disruption to the ordered packing of the hydrocarbon chains is a result of the way in which benzyl alcohol orientates itself in the membrane. From n.m.r. studies (Colley & Metcalfe, 1972) it was deduced that benzyl alcohol was positioned in the bilayer such that its hydroxyl group aligned with the polar head groups of the phospholipids, and the aromatic residue pointed towards the interior of the bilayer. Not only does benzyl alcohol penetrate the bulk phase lipid, but it will also enter the annulus. Such penetration leads to an increase in fluidity of this shell of lipids, and may affect enzyme activity (see Section 1.3(vi)).

Due to the asymmetric distribution of proteins and phospholipids, it is possible to exert selective manipulations on membrane fluidity. Such selective manipulation can be brought

about by the use of charged local anaesthetics. Due to the asymmetric distribution of lipids in the membrane these compounds are proposed to preferentially interact at one or other sides of the bilayer. As the external leaflet of the membrane is believed to contain a high proportion of positive/neutral lipids, it would be expected that anionic drugs would undergo preferential interactions here. In fact, studies on rat liver plasma membranes have shown the negatively charged drug phenobarbital (Houslay et al., 1981) to selectively fluidise the external leaflet (see Appendix I for structure). The lipid phase separation temperature of 28°C is depressed in accordance with this (to 16°C), whilst there is no detectable effect on the inner leaflet.

Cationic drugs such as prilocaine (Houslay et al., 1980(b)) are suggested to undergo selective interactions with the negative phospholipids of the inner leaflet (see Appendix I for structure). Indeed, the use of such positively charged anaesthetics has been shown to induce a lipid phase separation at 11°C in the inner half of the bilayer, whilst having no detectable effect on the phase separation occurring at 28°C in the external leaflet.

It is also possible to manipulate membrane fluidity by other methods, such as manipulation of fatty acids. For example, incorporation of fatty acids of various chain lengths, and degrees of unsaturation into the bilayer will alter the ability of lipids to pack together, and hence will modulate membrane fluidity (Houslay & Gordon, 1983). Similarly, the incorporation of perturbants such as the cations Ca^{2+} and La^{2+} will modulate membrane fluidity, as reflected by an elevation of phase separation temperature (Sauerheber et al., 1980). It is proposed that these ions manipulate fluidity in an asymmetric manner, binding to the cytosolic half of the bilayer (Houslay & Gordon, 1983).

Finally, one of the more widely employed methods of selectively modulating membrane fluidity, is to modify membrane composition by the incorporation of specific types of phospholipid into the bilayer. In fact, incorporation of phosphatidic acid into rat liver plasma membranes has been found to induce a lipid phase separation (at 16^{o}C) in the inner leaflet of the bilayer, i.e. where such acidic phospholipids would be expected to reside (Houslay et al., 1986(a)).

A number of these methods of manipulating membrane fluidity will be examined in this study with respect to their effect on the adenylate cyclase activity in human platelets. It was decided to examine such parameters in platelets as these elements provide an easily available system of functional significance for looking not only at signal transduction events, but at membrane perturbations. It should be noted that most of the results presented here centre on the effects of the local anaesthetic benzyl alcohol with respect to adenylate cyclase activity.

1.2 Platelets

(i) Morphology and functions

Platelets are the smallest of the formed elements of human blood, appearing as anucleate, granular bodies, 2-4uM in diameter. These disc-shaped elements arise by the cytoplasmic fragmentation of mature megakaryocytes in the bone-marrow, and typically have a life-span of 7-10 days. Each platelet has an intact plasma membrane about 7-9nm in thickness, and a complement of intracellular organelles.

These organelles include mitochondria, lysosomal-like organelles, glycogen-containing granules (from which they derive most of their energy by glycolysis) and two types of organelles that are specific for platelets; dense granules and alpha-granules.

Dense granules contain serotonin, calcium, ADP, and ATP, whilst alpha-granules contain proteins that are (a) also found in the plasma (e.g. fibrinogen) or (b) specific for alpha-granules (e.g. beta-thrombglobulin) (Crawford & Taylor, 1977).

Platelets also possess a complex canalicular network which connects with the surface membrane, and a circumferential band of microtubules which seem to be concerned in maintaining the discoid shape. A system of microfilaments also exists within platelets, which are believed to be components of a contractile system.

Physiologically, the platelet helps maintain the integrity of the vascular system by interacting with the vessel wall and other elements of the blood. The platelet also fulfils a number of other important roles, such as the storage and secretion of vasoactive compounds, and the transport of various materials. It also participates in the body's defence mechanism, by undergoing phagocytosis and chemotaxis as well as by releasing bacteriocidal substances (Crawford & Taylor, 1977; Page, 1988).

In order to fulfil any of these roles, a platelet must become activated; this entails a complex series of events, the trigger to which is the modulation of the level of some key regulatory elements within the platelet (namely cyclic AMP and/or Ca^{2+}).

(ii) Signal transduction in platelets

Because platelets are electrically non-excitable, there must exist transduction processes which link events at the cell surface, to the key intracellular reactions which control cellular responsiveness. These signal-transduction processes are centred around the production of cyclic AMP and inositol-phosphates, i.e. these compounds act as second-messengers conveying information from the cell surface to effectors within the platelet.

Inhibition of platelet responsiveness is associated with agonist-induced elevation of the intracellular concentration of cyclic AMP, whilst stimulation is associated with agonist-induced elevations in the intracellular concentraions of diacylglycerol, inositol triphosphate and Ca^{2+} (from inositol phosphate signalling pathway). How these two systems interact in platelets is quite complex and an attempt to illustrate this interaction is made in Figure 3.

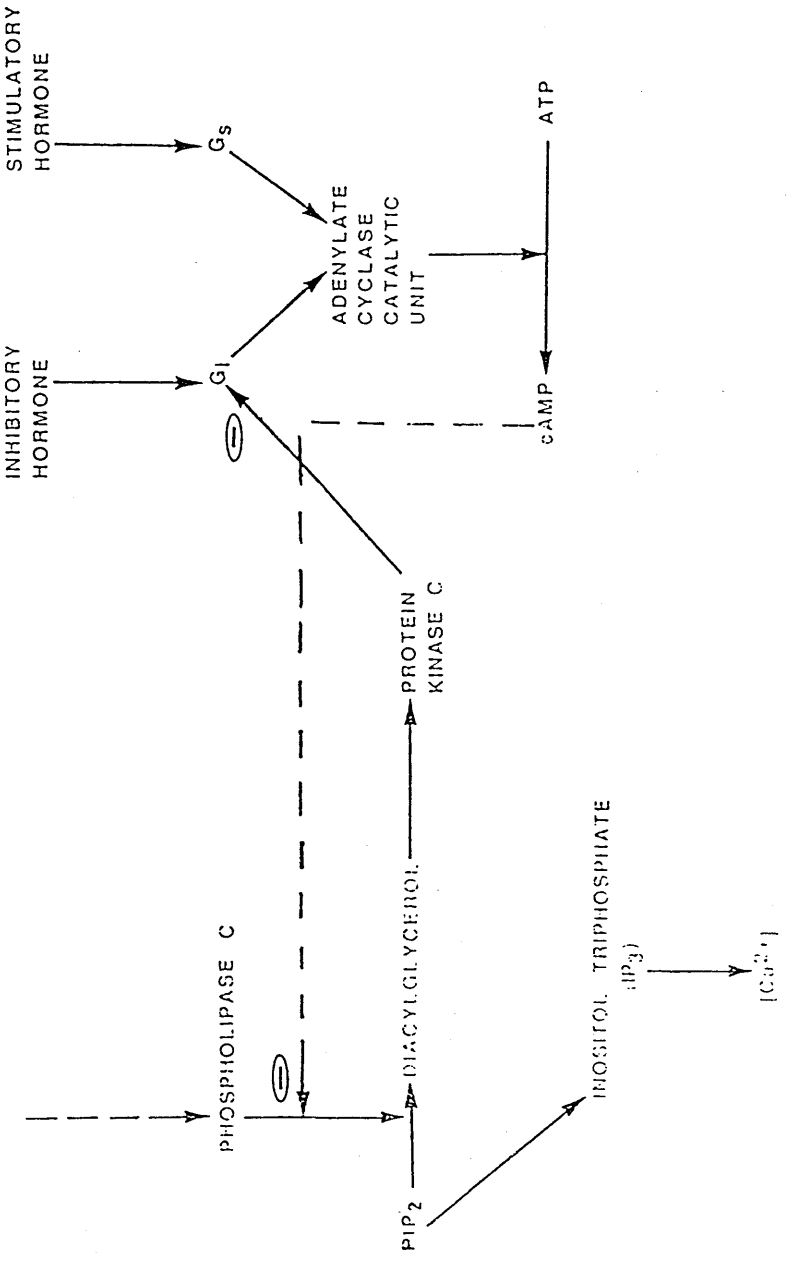
Obviously there appears to be several mutual interactions between these two signal-generating systems. On the one hand, increases in cyclic AMP levels inhibit the formation of inositol phosphates and diacylglycerol. Is is unclear whether this inhibition is due to a direct effect of cyclic AMP on the phospholipase C itself, or to an indirect mechanism. On the other hand, protein kinase C, which is activated by diacylglycerol, largely interferes with the adenylate cyclase system. This kinase, when activated by diacylglycerol, apparently phosphorylates the guanine-nucleotide binding alpha-subunit of G_i (see Section 1.3(iv) for G_i), which results in an impairment or loss of the inhibitory hormonal signal transduction to the adenylate cyclase (Jakobs et al., 1986).

Here we are looking at the adenylate cyclase system of platelets, with emphasis on the inhibitory arm of regulation in this dual controlled system.

(iii) The role of cyclic AMP in platelets

Cyclic AMP is one of the major intracellular modulators of biochemical reactions involved in the stimulation of platelets (the other being the calcium ion). This stimulatory response of platelets involves adhesion to sub-endothelial tissues, the formation of platelet aggregates, the secretion of secretory granule

Figure 3 **Signal transduction pathways in platelets**

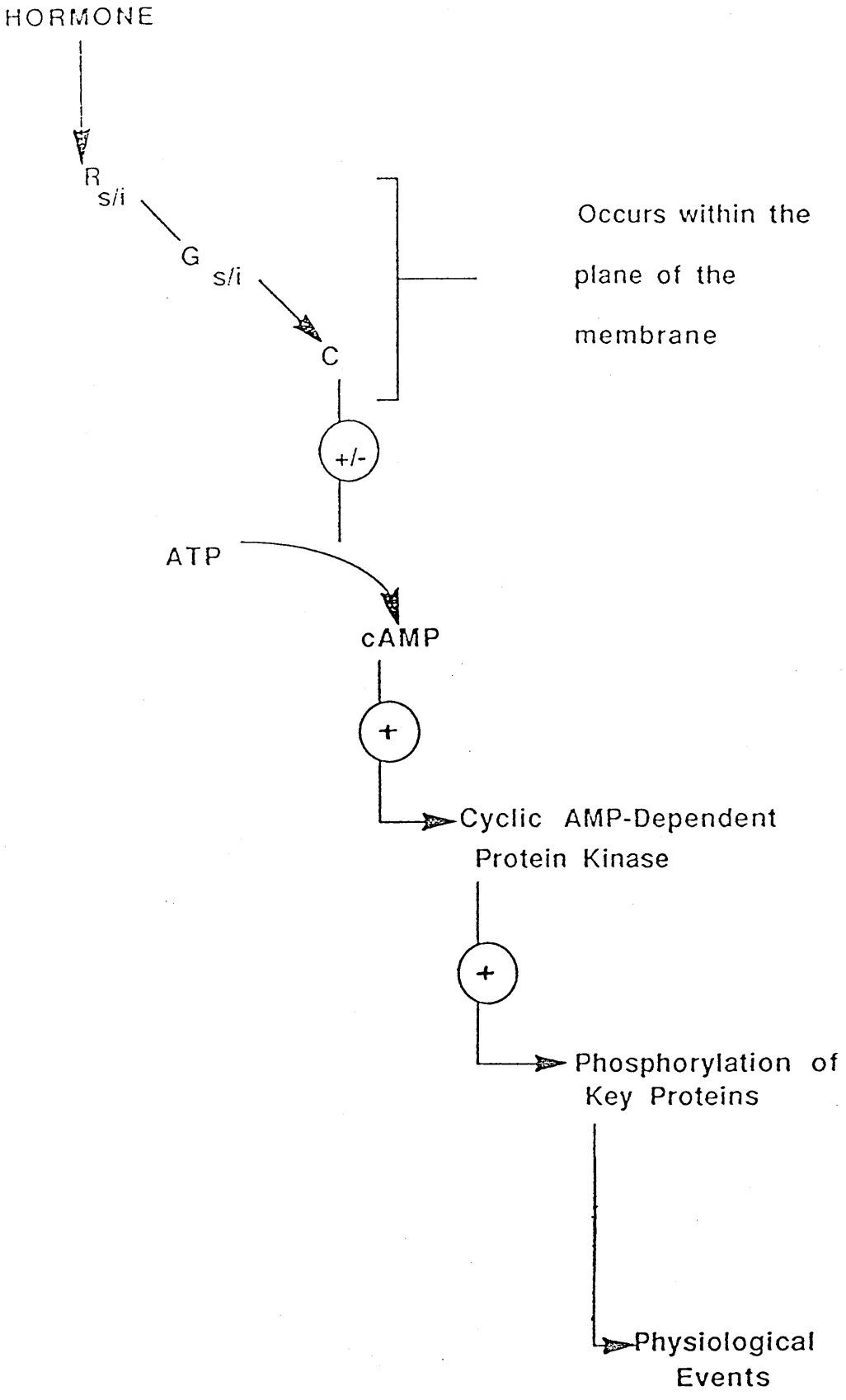


components, the rapid synthesis of oxidative metabolites of arachidonic acid, increased glycogenolysis, and the consumption of substantial amounts of ATP. All of these reactions depend in one way or another, on intracellular and/or extracellular Ca^{2+}. However, agents which increase cyclic AMP levels antagonise these responses by regulating the free Ca^{2+} level in the platelet, or by controlling the Ca^{2+}-dependent reactions that are essential for the responses. Cyclic AMP therefore represents the most important intracellular regulator which prevents cellular activation (Feinstein et al., 1981).

However, it is not possible to directly relate cyclic AMP levels to functional changes in platelets, as the level of cyclic AMP represents a parameter distal to the functions which are affected. Cyclic AMP actually activates protein kinases to catalyse phosphorylation reactions, which in turn affect various enzymes or regulator proteins. It is therefore the steady-state level, or rate and direction of change of these phosphoproteins which is more directly related to cellular responses (Booyse et al., 1976). This series of reactions, which is initiated by the production of cyclic AMP, constitutes a cascade-mechanism, whereby the production of a small amount of cyclic nucleotide can regulate a wide variety of cellular reactions. The effects of cyclic AMP are therefore mediated by protein phosphorylations catalysed by cyclic AMP-dependent proteins kinases (Fig.4).

Both soluble and membrane-bound cyclic AMP-dependent protein kinase activity has been identified in platelets. These two forms have been termed type I (membrane-bound) and type II (cytosolic) on the basis of their elution from DEAE cellulose, i.e. two principal classes of isoenzymes exist in platelets. In fact, cyclic AMP-dependent protein kinase is, thus far, the only receptor

Figure 4 **Cascade mechanism for cyclic AMP action**



protein defined for cyclic AMP in eukaryotic cells (Krebs, 1985). Each cyclic AMP-dependent protein kinase has two parts, a catalytic subunit and a regulatory subunit. Cyclic AMP binds to the regulatory subunit, thereby liberating the catalytic unit, which is then free to phosphorylate proteins (Berridge, 1985).

i.e. $R_2C_2 + 4\ cAMP \rightleftharpoons R_2 \cdot (cAMP)_4 + 2C$

It has been suggested that protein phosphorylation is involved in the platelet release reaction rather than in primary aggregation. In fact it is proposed that Ca^{2+}-mediated phosphorylation occurs prior to secretion and places the platelet in a state where secretion can occur (Feinstein, 1981).

Modulation of this phosphorylation, and therefore of platelet activation, is possible via the production of cyclic AMP by adenylate cyclase in the plasma membrane.

1.3 The Adenylate Cyclase System

(i) General introduction

The formation of cyclic AMP is platelets is therefore dependent upon the regulation of the enzyme adenylate cyclase [E.C. 4.6.1.1; ATP pyrophosphate-lyase (cyclising)]. The reaction catalysed by this enzyme can be represented by:-

$$Mg^{2+}ATP \longrightarrow 3'5'\ cyclic\ AMP + 2P_1$$

(see Fig.5)

The platelet enzyme, like the adenylate cyclase in the membranes of other eukaryotic cells, is a multicomponent system. Here, the activity of the catalytic unit (C) is regulated by stimulatory (R_s)- and inhibitory (R_i)-receptors whose actions are mediated by the guanine nucleotide-binding proteins G_s and G_i respectively. The system is therefore composed of at least five elements, the arrangement of which is shown in Figure 6.

Figure 5 **The reaction catalysed by adenylate cyclase**

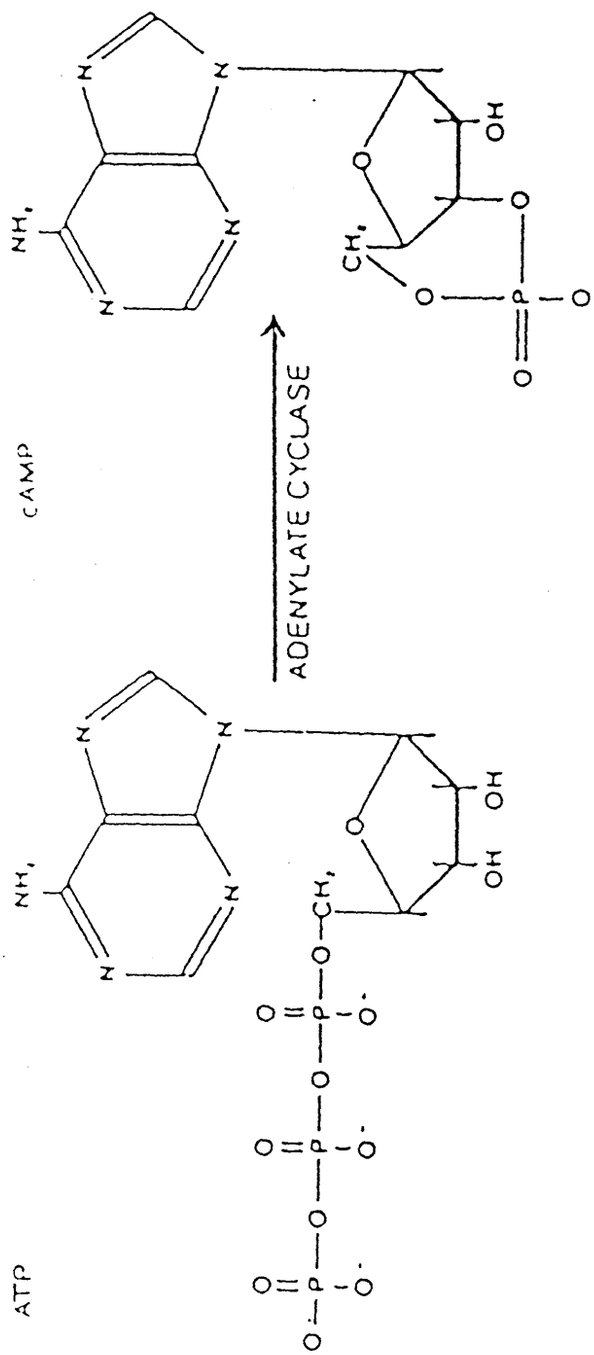
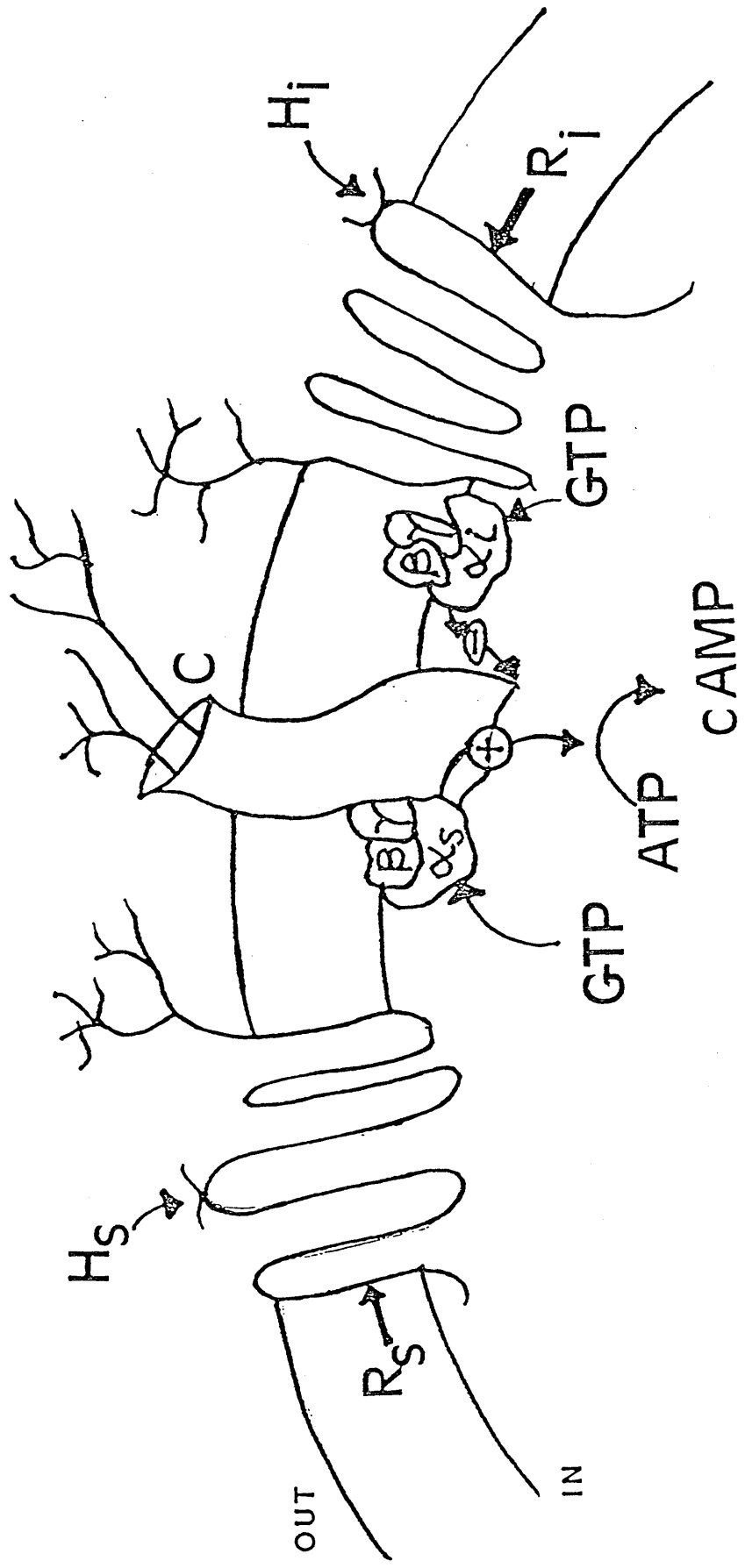


Figure 6 Arrangement of adenylate cyclase components in the membrane



(ii) Association of receptor, G-protein and catalytic unit in the membrane

Here the association of adenylate cyclase components refers largely to R_s, G_s and C interactions, as most analysis has been on the mammalian beta-adrenergic system, i.e. a stimulatory system. However, the interaction of components of the inhibitory arm of regulation will be discussed later (see section 1.3(iv)).

Although detailed kinetic studies have taken place (Levitzki, 1986; Hanski et al., 1979) the precise mechanism by which these components interact is still a matter of controversy. However, three main contenders exist with respect to a plausible model for component interaction (Arad et al., 1984).

(a) The collision coupling model

The main tenets of this hypothesis are that the G-protein and the catalytic unit are always associated with each other, and that the hormone-receptor complex only makes a transient encounter with G_s C.

$$R_s + G_s\ (GDP) \bullet C$$
$$\downarrow$$
$$H \bullet R_s \bullet G_s'\ (GTP) \bullet C' \qquad \text{Active state}$$
$$\downarrow$$
$$HR + G_s\ (GDP)\ C$$

Inactivation occurs by hydrolysis of GTP to yield inactive G_s (GDP) C.

(b) The precoupled model

Here is it assumed that all three components involved in stimulatory regulation of adenylate cyclase are associated at all times.

$$R_s \bullet G_s\ (GDP) \bullet C$$
$$\downarrow$$
$$HR_s \bullet G_s'\ (GTP) \bullet C' \qquad \text{Active state}$$
$$\downarrow$$
$$HR_s \bullet G_s\ (GDP) \bullet C$$

(c) The shuttle model

$$R_s + G_s\ (GDP) \longrightarrow H{\cdot}R_s{\cdot}G_s'\ (GTP) \longrightarrow HR_s + G_s'\ (GTP)$$

then,

$$G_s'\ (GTP) + C \longrightarrow G_s'\ (GTP){\cdot}C' \longrightarrow G_s\ (GDP) + C$$

Active state

Here all three components are separate, and interactions occur only in an ordered sequence, the 'cycle' being terminated by hydrolysis of GTP to yield free G_s.

Evidence exists to both support and disfavour each of these three models. For example, the precoupled model is not favoured mainly due to the fact that the beta-adrenergic receptor, in the absence of Mg^{2+} and GTP, exhibits an altered affinity for agonist binding, i.e. this may result from $R_s \cdot G_s$ dissociation. Other evidence which tends to disfavour this model of component interaction is the fact that receptor down regulation has been observed without concomitant movement of G_s. In other words, different lines of evidence exist for the R_s and G_s unassociated state, which is in major disagreement with the basis of the 'precoupled model'.

This last piece of evidence would not, however, be in disagreement with the tenets of the collision-coupling model. This theory could accommodate R_s and G_s being unassociated at times, as it assumes only a transient R_s-G_s-C complex formation. The major proposal of this theory is that a persistently activated G_s-C complex exists and in support of this suggestion is the fact that alpha-G_s has been copurified with the catalytic unit. In fact, high salt concentrations are required to separate these two components (Levitzki, 1986).

The final model to be considered, i.e. the shuttle model, again has conflicting evidence for and against it. In favour of this model is the fact that it would allow the activation of G_s in the absence of the catalytic unit (as has been found with non-hydrolysable GTP analogues); a condition which would not be thought to exist under the constraints of the other two models. However, the observation that R_s and G_s can form a complex in the presence of agonists and the absence of GTP, goes against the 'shuttle' model (Levitzki, 1986).

The conclusion to be drawn is therefore that the interaction of components within the membrane occurs by a mechanism which is, as yet, undefined, although kinetic analysis does tend to favour a collision-coupling type model.

(iii) Receptors

Platelets have receptors for a large number of agents which are either synthesised/secreted by the platelets themselves, or which are normally present in the circulation, e.g. adrenaline, ADP, thromboxane A_2, vasopressin, PGD_2, adenosine etc. A number of these agents act at receptors which are coupled to guanine nucleotide-regulatory proteins(G-proteins) and it is these receptors which are of interest in discussing regulation of adenylate cyclase activity (see Table 1).

Prostanoid receptors

Classification of prostanoid receptors is limited due to the lack of specificity of available antagonists. However, it is believed that receptors exist for each of the natural prostanoids. Human platelet membranes are thought to contain distinct receptors for PGD_2 and PGI_2. PGE_1 effects parallel those of PGI_2 and it is believed these prostaglandins share a receptor-type, although PGI_2 is in fact the endogenous ligand. Both the PGD_2 and the

Table 1 G-protein-coupled receptors which modulate adenylate cyclase activity in platelets

Stimulatory receptors and their agonists

PGE_1	
PGD_2	R_s linked to G_s
PGI_2	
Adenosine	
Catecholamines ($beta_2$)	

Inhibitory receptors and their agonists

Catecholamines ($alpha_2$)	R_i linked to G_i
ADP	

PGI_2/PGE_1 receptor types are linked to activation of platelet adenylate cyclase via the stimulatory G-protein, G_s (MacIntyre, 1981).

Purine receptors

Purine receptors fall into two general classes as regards their sensitivity to the purine agonists adenosine, AMP, ADP, and ATP;

(a) P_1-purinoceptors — also known as the adenosine receptor as this is the most potent agonist here.

(b) P_2-purinoceptors — also known are the ATP receptor as this is the most potent purine agonist here.

Whilst the exact role of the P_2-receptor is unclear, the P_1-receptor is known to be linked to adenylate cyclase regulation. In human platelets, adenosine exhibits a biphasic effect on cyclase activity; low concentrations (2-25uM) causing stimulation and high concentrations (>100uM) causing inhibition (Schwabe, 1982). On this basis it has therefore been proposed that two subtypes of P_1-purinoceptor exist:

A_1 — inhibition of adenylate cyclase (R_i)

A_2 — stimulation of adenylate cyclase (R_a/R_s)

(see Satchell, 1984)

Both A_1- and A_2-subtypes are located in the plasma membrane, and are linked to adenylate cyclase via the appropriate G-protein. Studies do however indicate that the predominant P_1-receptor in platelets is the R_s subtype (Schwabe, 1982).

Another adenosine regulatory site exists in platelets; this is an intracellular P-site, which inhibits adenylate cyclase (see Section 1.3(v)).

Catecholamine receptors

Platelet receptors can be grouped into two classes with respect to their effect on adenylate cyclase activity upon agonist binding, stimulatory receptors (R_s), and inhibitory receptors (R_i).

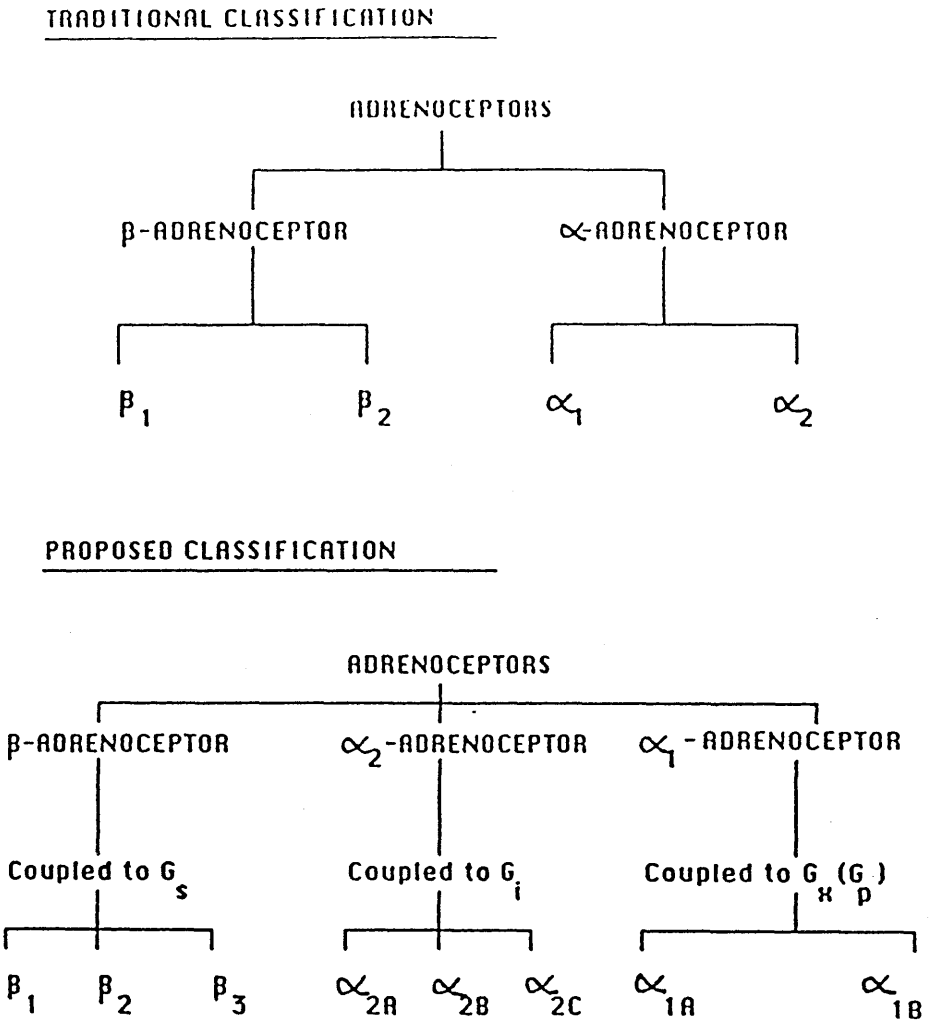
Catecholamine receptors exist in platelets which fall into each of these classes, i.e. adrenoceptor-mediated stimulation and inhibition of adenylate cyclase has been demonstrated in human platelet lysates (Jakobs et al., 1978).

Adrenoceptors can be subdivided into alpha- and beta-adrenoceptors on the basis of their pharmacological characteristics. Whilst both these classes can be further sub-divided, it has not been shown that these subtypes are all linked to regulation of adenylate cyclase activity. The classical subdivision of adrenoceptors is as shown in Figure 7.

However, from studies of pharmacological characteristics, mechanisms of signal transduction, and the molecular cloning of the cDNA encoding the receptors, it has been proposed that adrenoceptors can be subdivided as shown in Figure 7. Three major subtypes exist; beta, $alpha_2$, and $alpha_1$, and each can be further divided into two or more additional secondary subtypes.

It is assumed that both $beta_1$ and $beta_2$ are linked to adenylate cyclase activation as there is no convincing evidence to suggest differences in their stimulus-response coupling mechanism. However, the spectrum of compounds capable of exhibiting intrinsic activity through these receptors in human platelets is very narrow, suggesting that the human platelet beta-adrenoceptor differs from those found in other cell types (Jakobs et al., 1978). Radioligand binding analyses have in fact led to the proposal that the platelet beta-adrenoceptor is of the $beta_2$-subtype, and that they are

Figure 7 Classification of adrenoceptors



G_x = Not yet specifically identified G-protein, although it may be the G_p proposed to modulate inositol phosphate hydrolysis.

present in very small numbers, being approximately 1/10-1/20 of the $alpha_2$-adrenoceptor number.

Although not suggested as occurring in human platelets, a third species of beta-adrenoceptor has recently been reported (Zaagsma & Nahorski, 1990). Functional studies on adipocytes indicate the presence of this atypical receptor, i.e. in addition to the known $beta_1$- and $beta_2$-adrenoceptor subtypes. This polypeptide is thought to be encoded by a recently isolated gene whose expression product has approximately 50% homologies with the $beta_1$- and $beta_2$-adrenoceptors (Emorine et al., 1989). The occurrence of such '$beta_3$-adrenoceptors' is reported in an increasing number of tissues, and in several tissues, including atrial and ventricular heart tissues, the three separate beta-adrenoceptor subtypes may mediate the same function, depending upon the stimulation conditions used.

Studies using various catecholamine agonists and antagonists have shown platelets to possess alpha-adrenoceptors, with numbers in the region of 100-460 per platelet. It has been suggested that this receptor is exclusively of the $alpha_2$-subtype. However, whilst this is indeed the predominant platelet receptor for adrenaline, $alpha_1$-receptors have been found to exist, although they account for only a small percentage of the total alpha-adrenoceptor population (Scrutton & Wallis, 1981).

Although the $alpha_1$-receptor has been shown to exist in platelets, its exact physiological role is unclear; current evidence suggests they are coupled to regulation of calcium flux through phosphatidylinositol turnover (Limbird, 1984). On the other hand, $alpha_2$-adrenoceptors are known to be linked to inhibition of adenylate cyclase. The $alpha_2$-adrenoceptors themselves have been found to be a heterogenous population with

respect to their pharmacological characteristics. It has actually been proposed that subdivisions exist, the adrenoceptor found in human platelets being a typical alpha$_2$ A subtype. The prototype tissue for the alpha$_2$ B receptor is neonatal rat lung, whilst a third subtype, alpha$_2$C, may exist, although so far it has been found in only one (kidney-derived) cell line.

The gene encoding the platelet alpha$_2$-adrenoceptor has been localised to chromosome 10, and from its DNA sequence, is predicted to have a unique, but similar, primary amino acid sequence to the receptor from human kidneys. This receptor is encoded by a gene on chromosome 4, and may represent a fourth subtype of alpha$_2$adrenoceptor (Bylund, 1988). More recently, a human alpha$_2$-adrenoceptor has been cloned which represents the product of a gene located on chromosome 2, i.e. alpha$_2$C2 (Lomasney et al., 1990). This receptor is alpha$_{2B}$-like in that it is non-glycosylated, but it is distinct from alpha$_{2B}$, and represents a previously unidfentified alpha$_2$ adrenoceptor subtype. Indeed, other reports exist describing the cloning of alpha$_2$ adrenoceptors which are suggested to be different again from alpha$_2$ C10, alpha$_2$ C4 and alpha$_2$C2.

The platelet alpha$_2$-adrenoceptor is linked to inhibition of adenylate cyclase. However, inhibition was found to occur only upon binding of the natural catecholamines, L-adrenaline and L-noradrenaline. This would therefore suggest that the alpha-adrenoceptors of platelets, like the beta-adrenoceptors, are indeed somewhat different from those found in other cells (Jakobs et al., 1978).

So far, all G-protein coupled receptors which have been isolated and sequenced, have been found to be glycoproteins which are characterised by a common topology of seven transmembrane

regions, and by varying degrees of primary sequence similarity (Schofield & Abbott, 1989; Brooker et al., 1983; Dohlman et al., 1987). Much of the work on G-protein-coupled receptors has been performed on beta-adrenergic receptors. Using this system it was found, from primary sequence analysis, that seven hydrophobic stretches of approximately 20-25 amino acid residues existed, surrounded by eight hydrophilic stretches of variable length. It is thought that each hydrophobic domain forms a transmembrane alpha helix, with the intervening (less conserved) hydrophilic loops being alternatively exposed intra- and extra-cellularly (Strader et al., 1989). Genetic analysis of the beta-adrenergic receptor revealed the ligand binding domain to involve residues within the hydrophobic core of the protein, i.e. it is possible by deletion mutagenesis (and by proteolytic cleavage) to remove most of the connecting hydrophilic regions without affecting ligand binding to the receptor. On the other hand, lesions within the transmembrane domains interfere with ligand binding (Strader et al., 1988).

From mutagenesis experiments, it was found that aspartate 113 in helix 3 is involved in agonist/antagonist binding, amino groups of the ligand forming an ion-pair with the carboxylate side chain of this residue (Strader et al., 1989). Other interactions between specific residues of the receptor and functional groups on ligands have also been proposed e.g. photolabelling suggests tryptophan in transmembrane domain 7 forms part of the ligand binding site. A suggestion which is supported by studies involving chimeric receptors (Schofield & Abbott, 1989). However, it should be remembered that transmembrane domains all have a potential role in ligand binding.

Results therefore indicate that transmembrane (TM) domains fold around to make a pocket into which the ligands bind. It is

also thought that the cytoplasmic side of this structure has a number of contact points for G-proteins, thus providing a means of signal transduction, i.e. positive residues on the cytoplasmic side are thought to constitute the putative G-protein binding domain. This is thought to involve three domains on the cytoplasmic side of TM5, TM6 and TM7, and palmitilation of cysteine 341 of TM7 is thought to be particularly important. This post-translational modification is conserved between various members of the G-protein-coupled receptor family, and if palmitoylation cannot occur, receptor affinity for G-proteins is reduced (Schofield & Abbott, 1989).

As stated previously, these receptors have been found to be glycoproteins (1-3 consensus sites for N-linked glycosylation near the amino terminus of the protein). However, glycosylation appears to play no role in either ligand binding or functional coupling to G-proteins. It would appear that glycosylation serves a role only in trafficking the receptor through the cell, and in expression of the protein at the cell surface (Benovic et al., 1987).

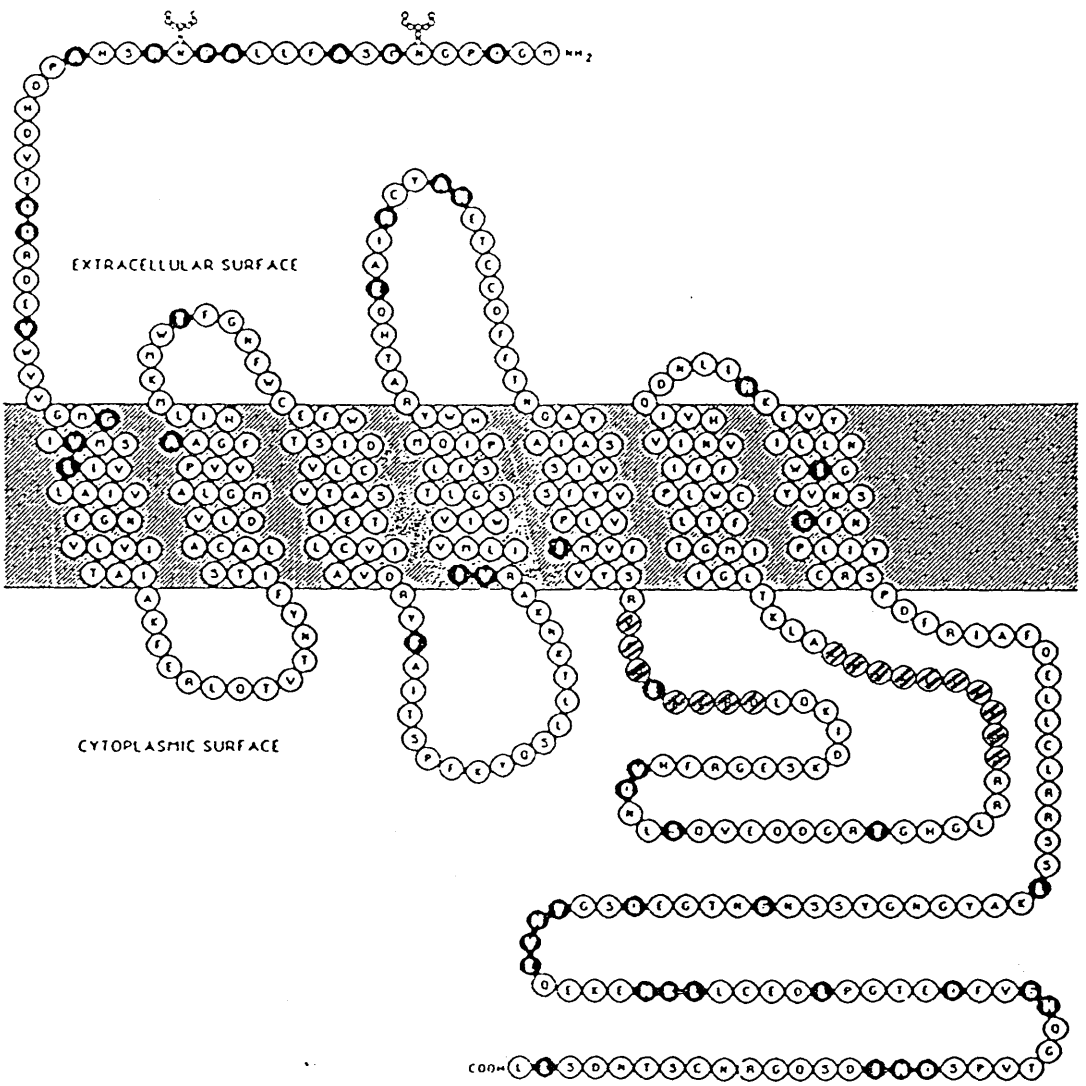
The orientation of G-protein-coupled receptors in the membrane is therefore as shown in Figure 8.

(iv) Guanine nucleotide regulatory proteins

(a) Structure and mode of activation

Guanine nucleotide regulatory proteins (G-proteins) play an important role in the regulation of adenylate cyclase activity. These proteins serve to transduce information between the catalytic unit of adenylate cyclase and the receptors in the membrane.

Figure 8 Orientation of the beta$_2$-adrenergic receptor in the membrane



▨ = Areas determined as being critical for G_s- coupling.

Figure is reproduced from Dohlman et al. 1987

In platelets, where the activity of the catalytic unit may be dually regulated, two forms of G-protein are especially important:

(1) G_s which mediates stimulatory effects, and

(2) G_i which mediates inhibitory effects.

G_s and G_i are part of a larger 'family' of G-proteins which are all believed to be structurally similar heterotrimers, consisting of a large alpha-subunit (M_r 41-45,000), and two smaller subunits, beta, M_r=approximately 35,000 and gamma, M_r approximately 10,000 (Gilman, 1984).

It has been proposed that upon hormone receptor interaction with the G-protein, the latter dissociates into free alpha subunits and beta-gamma complexes (Gilman, 1984). This is believed to involve a change in conformation at the receptor upon agonist binding being transmitted to the G-protein, probably via receptor interaction at the C-terminal domain of the alpha-chain. This results in the guanine-nucleotide binding site attaining an open conformation, such that bound GDP is released, whilst GTP can enter to cause yet another change in conformation. The end result is that the G-protein alpha-subunit's affinity for binding beta-gamma is reduced, whilst its affinity for the catalytic unit increases (Bourne et al., 1987). This, therefore, constitutes the 'on-reaction', and in the case of G_s alpha would mean that adenylate cyclase would be activated, resulting in increased formation of cyclic AMP (see page 41, for discussion of G_i activation). In fact, it has recently been suggested that activation by GTP is sequential; $alpha^{GTP}$-beta-gamma heterotrimer dissociating from the receptor before $alpha^{GTP}$ and beta-gamma separate, and subsequently interact with the catalytic unit (Miller et al., 1988). The 'off-reaction' results from the fact that G-protein alpha-subunits exhibit an intrinsic GTPase activity, which

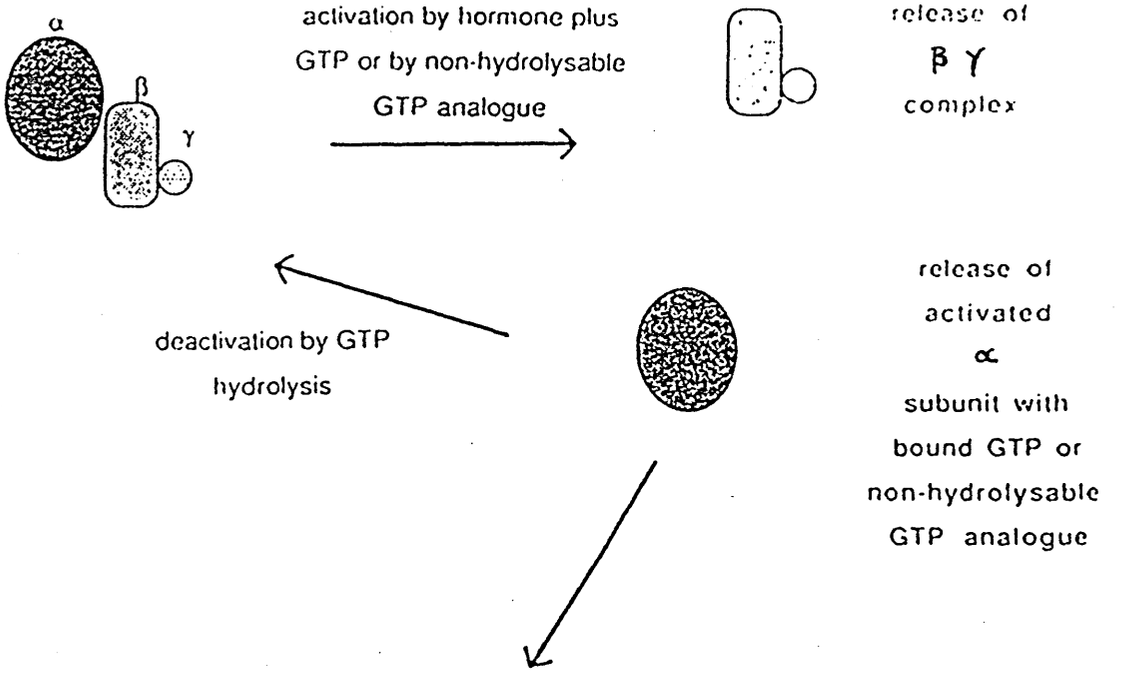
allows them to hydrolyse GTP to GDP (Bourne et al., 1987). This slow hydrolysis of GTP (4 min^{-1}) is followed by the reassociation of alpha GDP with beta-gamma to reform the inactive heterotrimer (Weiss et al., 1988); see Diagram 9.

The change in conformation of G-proteins, notably G_s, resulting from GTP-binding, are reflected in agonist binding affinities of R_s; GTP-G_s binding generally resulting in a decrease in agonist high-affinity binding at R_s. The same is also true for R_i with respect to GTP binding to G_i.

As well as containing the site of guanine nucleotide binding, the alpha subunits, which are unique gene products (Weiss et al., 1988), are therefore responsible for regulating the effector, and also confer on each G-protein its specificity for interacting with particular receptors (Bourne et al., 1987). These alpha-chains are 300-400 residues long, and from comparison of sequences from different G-proteins, an 'average' structure for the alpha-chain has been proposed, in which aspartate residues bind guanine nucleotides to a guanine-nucleotide binding domain. Other domains are thought to exist, including a short hydrophilic domain at the amino terminus which may be involved in controlling interactions of the alpha-chains with the beta-gamma complex. Contact sites for G-protein alpha-chain interaction with effector molecules also exist, whilst a carboxy-terminal domain is thought to form at least part of the contact site for receptors coupled to G-proteins (Bourne, 1988).

Whilst the alpha-subunits are distinctive from, and non-identical to each other, the beta-subunits appear to be identical in each member of the G-protein family. These subunits are functionally and structurally indistinguishable by biochemical and immunological methods (Sternweis, 1986), although molecular

Figure 9 **G-protein activation/deactivation cycle**



cloning of cDNA shows at least two forms of the beta chain exist (Fong et al., 1987). These proteins have approximately 90% homology and are denoted $beta_1$ (36,000 M_r) and $beta_2$ (35,000 M_r): they are encoded by distinct genes located on chromosomes 1 and 7 respectively. It would appear that a third form of the beta-subunit ($beta_3$), may exist: restriction mapping having shown it to be distinct from the other two forms. This species is encoded by a gene localised on chromosome 12. However, its precise molecular weight as well as its functional significance, are as yet unidentified (Levine et al., 1989).

The third subunit species present in G-proteins is the gamma-subunit, which has a low molecular weight of approximately 10kDa. This was first identified as being a component of G_s and G_i by Hildebrandt et al., 1984(b), who proposed the gamma-subunits of G_s and G_i as being indistinguishable. Recently however, studies have suggested that multiple forms of the gamma-subunit also exist (Robishaw et al., 1989). Although the total number of gamma-subunits is not yet known, immunoblotting studies have suggested the existence of at least four different types of gamma-subunit. These are the y_5 and y_6 subunits of brain G_s, G_i and G_o; the y_6 subunit of retinal G_t, and the gamma-subunit of placental G_i. Whilst all of these gamma-subunits are structurally distinct, they are all proposed as exhibiting a conserved C-terminal cysteine residue. This residue may be subject to covalent modification (palmitilation or isoprenylation) as part of the process leading to membrane-associated forms of these proteins.

Given that there are at least two beta-subunits, and at least four gamma-subunits, the possible number of combinations of these subunits is relatively large. However, whether a specific

beta-gamma complex is required to interact with a particular alpha-subunit to modify its function, is not yet known. These three components, i.e. alpha, beta and gamma, therefore combine to form what is termed a G-protein, with the alpha-subunit apparently being the more active species. In fact, the alpha-subunits are soluble in aqueous solution (Sternweis, 1986) and will only interact with phospholipid vesicles which have beta-gamma already present. On the other hand, beta-gamma subunits will associate totally with phospholipid vesicles, thereby implying that the function of the beta-gamma complex is to attach the G-protein to the membrane. Alpha-subunit association with beta-gamma-containing vesicles actually occurs in a saturable fashion, indicating a stoichiometric association between the subunits. However, whether beta-gamma's serve only to function as 'anchors' for alpha subunits is debatable, beta-gamma subunits being proposed as having other functions, as will be discussed later.

G_s and G_i are actually part of an ever-expanding family of G-proteins, which includes, amongst others, transducin, G_o, G_p, G_z, and the putative G_{ins}. Other GTP-binding proteins do exist in platelets. However, these are unrelated to the G_s/G_i family, having no beta-gamma subunits, and are termed 'small molecular weight GTP-binding proteins'. Binding studies using radioactively-labelled guanine nucleotides such as [^{35}S]GTPγS (Ohmori et al., 1989), and [α^{32}P]GTP (Bhullar & Haslam. 1987) have revealed the presence of a family of such monomeric GTP-binding proteins, with molecular weights in the range 20-30kDa (Nagata et al., 1989 : and see Table 2). Whilst it is noted that such species exist, these low molecular weight G-proteins will not be discussed here. Instead we will focus on the roles of G_s and G_i with respect to adenylate cyclase regulation.

Table 2 Low molecular weight GTP-binding proteins

Name	Comments
ARF	ADP-riblsylation factor
rab 1	Expressed in most cells
rab 2	Expressed in most cells
rab 3	Expressed in brain
rab 4	Expressed in most cells
rab 5	
ral	Expressed in most cells
rap 1A	Expressed in most cells
rap 1B	Reverts ras-transformed cells
rap 2	
BRL-ras	Expressed in most cells
Ha-ras	Involved in growth control
Ki-ras	Expressed in most cells
N-ras	
R-ras	
rho A	Botulinum toxin C_3 substrate
rho B	Expressed in most cells
rho C	Botulinum toxin C_3 substrate

(b) G_s

This G-protein stimulates the activity of adenylate cyclase, resulting in an increase in the formation of cyclic AMP. The alpha-subunit of G_s exists as two species in most cells, with M_r 45,000 and 52,000 (Robishaw et al., 1986). The relative amounts of these two species is tissue-specific, although the reason for the differences is not known. Both these products are encoded by a single 13-exon gene spanning 21Kb (Ishikawa et al., 1990) and it has been shown that alternative splicing of precursor RNA can give rise to at least four types of $alpha_s$ mRNA, which are almost identical in sequence (Bray et al., 1986). All four forms of G_s activate adenylate cyclase (and Ca^{2+} channels) with similar potencies (Carty et al., 1990).

It is probable that other forms of G_s alpha may exist; for example by the use of an alternative promoter, a 38kDa form of G_s alpha has been recorded (Ishikawa et al., 1990). whilst other groups have reported a unique $alpha_s$ mRNA to exist in haploid germ cells (Haugen et al., 1990).

The G_s alpha subunit can undergo ADP-ribosylation by cholera toxin in the presence of a membrane-bound protein factor, termed ARF (Morgan, 1989) the result being that GTP is bound and the alpha-subunit remains in this activated state due to the inhibition of GTPase activity.

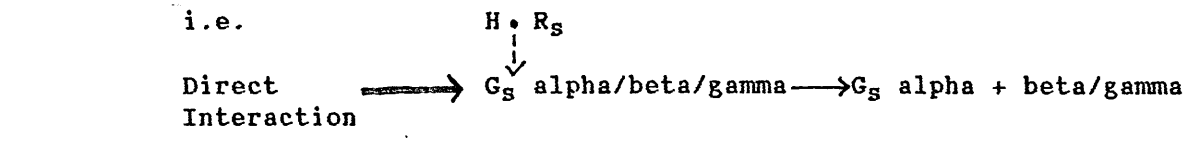
Activation of G_s by agonist-occupied receptors ($H^{\cdot}R_s$) requires a number of factors in order to occur efficiently. One such factor is the presence of Mg^{2+} (Iyengar & Birnbaumer, 1981a; Iyengar & Birnbaumer, 1981b), with the K_m for Mg^{2+} being 5-10mM. However, it has been postulated that receptor activation of G_s decreases this K_m (Iyengar & Birnbaumer, 1982), i.e. activation is primarily by decreasing the Mg^{2+} requirement such

that physiological concentrations of free Mg^{2+} (0.5mM) are sufficient to saturate the system.

The other factor required for G_s activation is the presence of guanine nucleotides. Here, physiological concentrations of GTP (approx. 0.1mM) are such that saturating levels of the nucleotide are always present; the K_m of G_s alpha for GTP being approximately 1uM.

The activation of G_s can be brought about directly at the level of the G-protein rather than via the stimulatory receptor

i.e.



This can be attained in a number of ways:

1. The use of GTP analogues such as GTP-γ-S and p[NH]ppG. These non-hydrolysable molecules activate G_s following a definite 'lag' period which is not observed with GTP. This lag reflects a distinct activation process which, once completed, leads to a steady-state of activation (Birnbaumer et al., 1980).

2. $G_s$alpha can also be activated by sodium fluoride. This requires the presence of Al^{3+}, the active species being AlF_4^-. This appears to promote persistent subunit dissociation, the AlF_4^- being thought to resemble the terminal gamma-phosphate of guanine nucleotides, i.e. it is suggested that AlF_4^- associates with the beta-phosphate of GDP and mimics the effect of non-hydrolysable GTP analogues (Chabre, 1989).

Activation of G_s by any of these routes is thought to result in dissociation of the alpha-beta-gamma heterotrimer to yield the active G_s-alpha species. G_s-alpha is then thought to directly modulate the activity of the catalytic unit. The basis of this model being the fact that the species $alpha_s{}^{GTP}-\gamma-S$ is sufficient to activate the purified catalytic subunit of adenylate cyclase (Levitzki, 1987).

(c) $\underline{G_i}$

G_i is a class of G-protein that not only inhibit adenylate cyclase but regulate other enzymes and ion channels. The existence of such an inhibitory G-protein in the adenylate cyclase system was first postulated when GTP was found to be able to inhibit the enzyme's activity in rat adipocyte membranes. As discussed previously, this protein is an alpha-beta-gamma heterotrimer, which undergoes dissociation following GTP binding. The alpha-subunit has M_r in the region 40-41,000, with cDNA studies identifying at least three forms of this polypeptide which are probably the products of at least two separate genes (Kim <u>et al</u>., 1988). These species termed $G_i$alpha-1 (M_r 41,000), G_i alpha-2 (M_r 40,000) and G_i alpha-3 (M_r 41,000), have greater than 80% sequence homology. However, distribution of these G_i subtypes varies within tissues, the sequence for G_i alpha-2 being the most common.

Studies using G_i-specific antibodies have shown that platelets contain G_i-2 and G_i-3 but not apparently G_i-1 (Simonds et al., 1989). As would be expected from the earlier statement, G_i-2 is the major G_i-subtype present. In platelets, the use of G_i-2-specific antibodies has led to the conclusion that this species is the dominant mediator of cyclase inhibition, at least in the pathway of $alpha_2$-adrenoceptor-mediated inhibition. This conclusion arose from observations that only antibodies specific for the C-terminal of G_i-alpha-2 were able to block this inhibition. The function of G_i-alpha-3 in platelets is therefore unclear. However, although these findings would rule out a predominant role for G_i-3 in this inhibitory pathway, they do not exclude an ancillary role for G_i-3 in cyclase inhibition, nor do they rule out the possibility that G_i-3 mediates inhibition of adenylate cyclase by some other ligands. It may be that G_i-3 is not actually linked to adenylate cyclase, and indeed attempts have been made to assign specific roles to each of the $G_i$alpha-subtypes. For example, $G_i$alpha-3 is proposed as a regulator of K^+-channel function. However, studies have since shown that all three forms of G_i are capable of this function (Yatani et al., 1988a). It may be that the differential expression of $alpha_i$ genes in different cell types will permit characterisation of distinct physiological roles for the G-proteins.

Recently it has been shown that whilst there is no apparent difference in effector interactions, the three forms of G_i do have different kinetics of activation, i.e. the rates of GDP-release and GTP-binding may be independently regulated for these three proteins. Hence the relative proportions of these species may be a crucial factor in determining the kinetics of signal-transduction through G_i-coupled effectors (Carty et al., 1990).

As regards G_i involvement in adenylate cyclase inhibition, $G_i$alpha, like $G_s$alpha, binds GTP upon hormonal stimulation, i.e. $H'R_i$ interacts with G_i alpha-beta-gamma resulting in the alpha chain binding GTP. The resultant change in conformation causes beta-gamma to dissociate from the holomeric complex. Once again GTP hydrolysis to GDP, via the inherent GTPase of the alpha-subunit, constitutes the 'off-reaction'. Concentrations of guanine-nucleotides required to activate G_i are lower than those required by G_s, i.e. G_i has a higher affinity for GTP and its analogues. Similarly, $G_i$alpha requires lower concentrations of Mg^{2+} (uM) for activation than does $G_s$alpha (Jakobs et al., 1985).

G_i, like G_s, can be ADP-ribosylated. Here, the bacterial toxin which mediates this ribosylation is pertussis toxin, isolated from the bacterium Bordetella pertussis. This ribosylation occurs at a cysteine residue in the C-terminal region of the alpha-chain, and requires the presence of the alpha-beta-gamma holomeric complex. Treatment with pertussis toxin, also known as IAP (Islet Activating Protein), results in the inactive [G $alpha^{GDP}$]beta-gamma complex being stabilised, with the net effect being that G_i activation cannot occur (Morgan, 1989). This toxin will also ADP-ribosylate the alpha-subunits of G_o and G_t, i.e. it is not specific for $G_i$alpha as had first been thought.

It has also been shown that $G_i$alpha can be mono(ADP-ribosyl)ated at a cysteine residue (presumed to be the same as that ribosylated by pertussis toxin) (Tanuma et al., 1988), by an endogenous mono(ADP-ribosyl) transferase enzyme. The role of these enzymes is unclear, although it has been suggested they attenuate G_i-mediated inhibition in platelets, i.e. they may have a role in regulation of adenylate cyclase activity (Tanuma & Endo, 1989).

It is possible to selectively inactivate G_i in platelets by various treatments:

1. Pretreatment of cells/membranes with low concentrations of N-ethylmaleimide (NEM). This sulphydryl agent is thought to specifically affect the receptor-G_i interaction (Garcia-Sevilla et al., 1988).
2. Low concentrations of Mn^{2+} (1mM) can similarly be used to preferentially uncouple G_i-mediated inhibition. This is thought to interfere with G_i-catalytic unit interactions (Hoffmann et al., 1981).
3. Treatment of platelets with phorbol esters which activate protein kinase C leads to attenuation of G_i-function. This is due to protein kinase C phosphorylating the alpha-chain of G_i to the impairment of its activity. This is probably a result of an increase in hydrolysis of $G_i$alpha-bound GTP to give inactive $G_i$alpha-GDP; as hormonal/GTP-dependent inhibition is attenuated, whilst the action of stable GTP analogues is unaffected (Bauer & Jakobs, 1986).

Activation of G_i either via agonist occupied receptors ($H'R_i$) or directly by guanine nucleotides, e.g. low concentrations of p[NH]ppG (0.01-1uM) (Seamon & Daly, 1982), is analogous with the activation of G_s; dissociation of the heterotrimer to yield $alpha^{GTP}$ and beta-gamma. However, beyond this point the two routes differ, for it is not clear which G_i component interacts with the effector.

It has been proposed (Gilman, 1984) that inhibition occurs by either direct $G_i$alpha interaction with the catalytic unit, and/or by G_i beta-gamma acting to 'mop up' $G_s$alpha and so prevent activation of C.

i.e.

G_s alpha•beta•gamma $\rightleftharpoons$ G_s alpha + beta-gamma

Reaction would be driven to the left by presence of excess beta-gamma from G_i; therefore inhibition by mass action.

It is probable that $G_i$alpha regulates the activity of the catalytic unit, at least to some degree. This conclusion arises from the fact that hormonally-mediated inhibition of C has been demonstrated in cyc^- S49 cell membranes, which lack functional $G_s$alpha (Katada et al., 1984a). This interaction of $G_i$alpha with C is thought to occur at a site distinct from that at which G_s interacts, as G_s and G_i affect catalytic unit activity in a non-competitive manner (Hildebrandt et al., 1984(a)).

However, because beta(gamma)'s are highly homologous, and functionally interchangeable, they do have the potential to modulate the activity of different signalling pathways within the same cell. The role of $G_i$beta-gamma in attenuating stimulation of adenylate cyclase has been studied in a number of systems, including platelets and S49 lymphoma cells. Here beta-gamma was observed to cause a dose-dependent inhibition of G_s-stimulated adenylate cyclase activity by reassociation with free $alpha_s$ (Katada et al., 1984a,b). It has also been observed that hormone-independent adenylate cyclase activity is inhibited to a greater extent by beta-gamma subunits than is the hormone-dependent activation (Im et al., 1987). This suggests that whilst beta-gamma probably does play an important role in inhibiting G_s-controlled adenylate

cyclase, that this role cannot be directly extrapolated to hormonal inhibition (Birnbaumer, 1987), i.e. here alpha$_i$ will no doubt play a role (Katada et al., 1984a), whilst the extent of the contribution from beta-gamma is as yet, unclear. Evidence has also been put forward which implicates beta-gamma as having a role to play in directly inhibiting the catalytic unit i.e. direct beta-gamma-C interaction (Enomoto & Asakawa, 1986; Katada et al., 1986).

However, whilst its precise role remains unclear, it has been proposed that beta-gamma acts as a general attenuator of the actions of all G-proteins operating in the normal membrane environment i.e. it will lower the noise of systems coupled by G-proteins, and thereby improve the 'signal-to-noise' ratio of hormonal effects (Cerione et al., 1985). It has even been suggested that some systems contain excess free beta-gamma which suppresses adenylate cyclase activity (Bokoch, 1987).

One function of beta-gamma which is agreed upon, is that its association with the alpha-subunit is essential for efficient receptor-catalysed guanine nucleotide exchange, i.e. the alpha-beta-gamma heterotrimer must be present. It is therefore possible that free beta-gamma might regulate the functioning of different G-proteins at two stages:

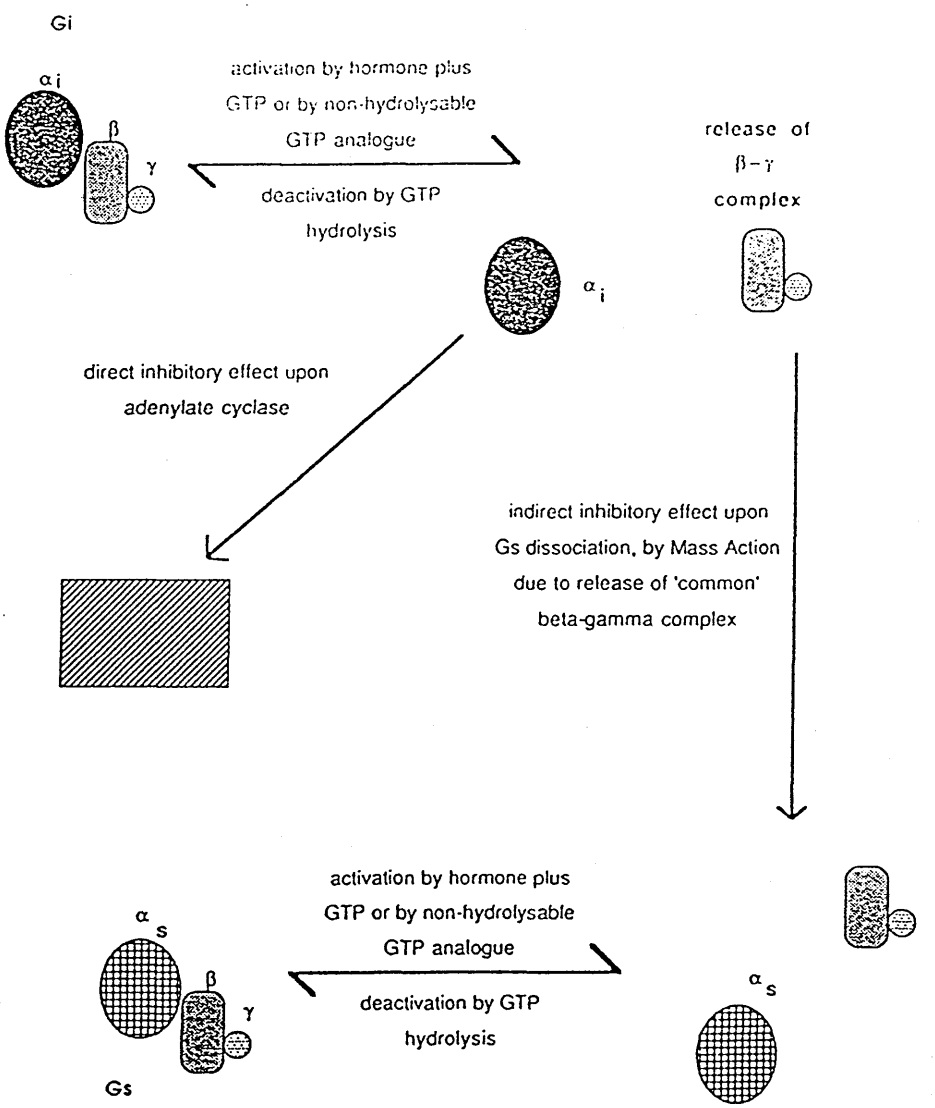
1. by binding free alpha-subunits and preventing activation of C, and
2. by stabilising the interaction of the alpha-subunit with the receptor, and stimulating guanine-nucleotide exchange (Weiss et al., 1988).

It has been proposed that these G-proteins, G_i and G_s, may be involved in the regulation of effector systems other than adenylate cyclase. There is evidence for a direct role of $G_i alpha_{1-3}$ in potassium-channel regulation (Yatani et al.,

1988b), with conflicting evidence for $G_i$beta-gamma in the same role (Kim et al., 1989). Other roles for beta-gamma include the proposed inhibition of calmodulin-stimulated phosphodiesterase activity (Asano et al., 1986), as well as involvement in the regulation of phospholipase A_2 activity (Jelsema & Axelrod, 1987). $G_i$alpha has also been proposed as having a role to play in thrombin regulation of phospholipase C activity in platelets (Crouch & Lapetina, 1988). Although not as many roles have been implicated for $G_s$alpha, this too has been suggested as a regulator of other systems, such as Ca^{2+}-channels (Yatani et al., 1988a). No doubt, other roles will be found in the near future.

Finally, it should be noted that the G-protein, G_z, exists in platelets. This protein is most closely related to the $G_i$alpha family, although it is distinct, having only 66-67% homology with G_i1-3 as regards amino acid sequence (Premont et al., 1989). It has an M_r of 41,000 and whilst its function is, to date, unknown, it is presumed to be involved in mediating transduction in signalling systems which are insensitive to pertussis toxin, i.e. $G_z$alpha cannot be ADP-ribosylated by pertussis toxin presumably as it lacks the appropriate C-terminal cysteine residue. In fact, a recent study has shown an isoleucine residue to replace this cysteine residue (Casey et al., 1990). Preliminary work also suggests it is not ribosylated by any other known bacterial toxin. As G_z is implicated as playing some role in platelet signal transduction, it may yet materialise that G_z, or perhaps some other, as yet, unidentified, G-protein, is involved in the regulation of platelet adenylate cyclase activity, i.e. in addition to G_s and G_i (Fig.10).

Figure 10 **Scheme for G_s/G_i interaction in regulation of platelet adenylate cyclase activity**



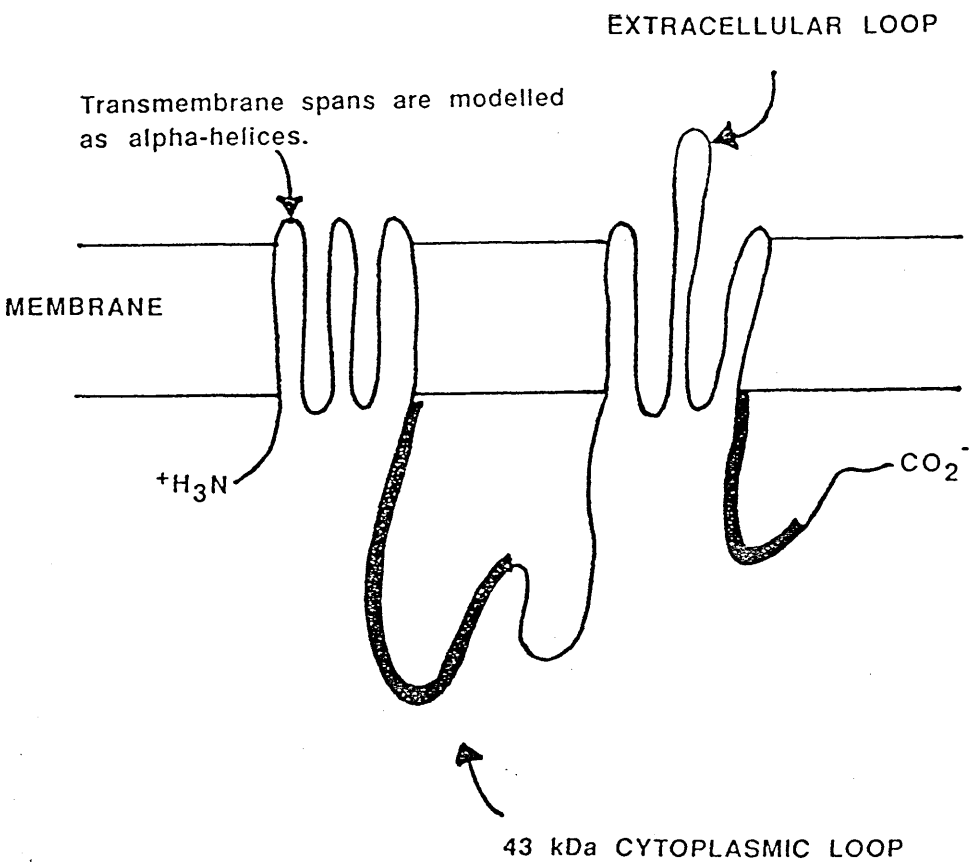
(v) The catalytic unit

The catalytic unit of adenylate cyclase binds MgATP and converts this molecule to cyclic AMP. However, whilst the reaction catalysed by this component has been known about for many years, it is only quite recently that the catalytic unit itself has been identified and isolated.

Catalytic units isolated from a number of sources, including rabbit heart, bovine brain and rat synaptosomes, yield similar molecular weights, i.e. approximately 150kDa (Pfeuffer et al., 1985; Monneron et al., 1987; Bender & Neer, 1983). This is in agreement with the M_r estimation by target size theory of membrane-bound rat liver adenylate cyclase i.e. M_r=150,000 (Schlegel et al., 1979). Also, the finding that adenylate cyclase specifically binds to wheat-germ lectin (Pfeuffer et al., 1985) suggests that the catalytic unit is a glycoprotein. This fact, coupled with the large M_r of the catalytic unit suggests that this component of the adenylate cyclase system may traverse the membrane.

Such an orientation of the catalytic unit in the bilayer has very recently been confirmed via cloning studies, i.e. the primary amino acid sequence of adenylate cyclase has finally been deduced (Schofield & Abbott, 1989). From this information it is believed that the protein has a pair of six membrane-spanning segments separated by a large (43kDa) cytoplasmic loop, and one major extra-celllular loop containing one of the four possible glycosylation sites in the sequence. There is also thought to be a long (36kDa) cytoplasmic tail, and a short cytoplasmic amino-terminal sequence. Thus, most of the protein is located on the cytoplasmic side of the membrane (Fig.11). This is in agreement with earlier studies on rat liver plasma membranes, which by the use of charged anaesthetics to selectively manipulate

Figure 11 **Orientation of the catalytic unit in the membrane**



membrane fluidity, predicted that the bulk of the catalytic unit was located in the cytoplasmic side of the membrane, i.e. adenylate cyclase was sensitive to fluidity changes in the cytoplasmic half of the bilayer only (Houslay & Gordon, 1983). Although the catalytic unit is a very minor component of the plasma membrane, accounting for less than 0.01% of its proteins (Monneron et al., 1987), it does have very specific requirements for its activity.

The catalytic unit has an absolute requirement for magnesium: Mg^{2+}-ATP being the true substrate for adenylate cyclase. However, Mg^{2+} ions also fulfil a regulatory role, as a second allosteric site exists on the catalytic unit. The existence of this divalent cation binding site was deduced from the fact that the catalytic unit is much more active in the presence of manganese than magnesium, whilst the affinity of the catalytic unit for its substrate, Me'ATP is the same with either cation. Also, free Mn^{2+} activates the enzyme at concentrations greater than that required to convert all the ATP to Mn^{2+}.ATP. This is consistent with Mn^{2+} being able to interact with the catalytic unit both at the active site (with ATP) and at a separate divalent cation site (Bender & Neer, 1983).

Not only is the activity of the catalytic unit regulated by divalent cations, but it is also subject to regulation by the calcium-binding protein calmodulin. This is true only in some tissues; in fact, in tissues other than the central nervous system, the stimulatory effect of calmodulin is weak or absent. Evidence actually suggests that in neuronal tissues there are distinct calmodulin-sensitive and insensitive forms of adenylate cyclase (Cooper & Caldwell, 1988). However, reports that platelet cyclase is stimulated by calmodulin (Resink et al., 1986) are actually believed to reflect calmodulin antagonising inhibition caused by low

Ca^{2+} concentrations, i.e. calmodulin does not mediate a direct stimulatory effect on the catalytic unit in platelets.

Direct interaction at the level of the catalytic unit is also one of the modes of action of adenosine. This compound inhibits adenylate cyclase activity in detergent-solubilised cyclase preparations, indicating that it acts at an internal site, and not at an external receptor (Londos, 1977). It is believed that this GTP-independent inhibition is mediated by adenosine binding directly at the so-called 'P'-site on the catalytic unit.

However, probably the most widely used means of directly modulating catalytic unit activity is via the use of the diterpene forskolin. This compound was discovered in root extracts of the plant Coleus Forskohlii(Seamon & Daly, 1981b), and has been found to stimulate adenylate cyclase activity in intact cells and membrane preparations, including human platelets, where it is a potent inhibitor of aggregation (Siegl et al., 1982).

The precise mechanism of action of forskolin is unknown, although a number of lines of evidence suggest that it causes activation via an allosteric conformational change in the catalytic unit (Seamon & Daly, 1986). This direct interaction with the catalytic unit is consistent with the enhanced thermal stability of the enzyme in the presence of forskolin (Seamon & Daly, 1981b; Awad et al., 1983).

It has also been shown that activation by forskolin does not require guanine nucleotide regulatory proteins (Seamon & Daly, 1981a) nor does it require adenylate cyclase to be associated with a lipid bilayer. This latter point is suggested by the fact that forskolin can activate solubilised adenylate cyclase in the presence of ionic and non-ionic detergents. This also suggests that this compound does not activate the enzyme by causing perturbations of

either membrane structure, or of the interactions between the catalytic unit and phospholipids (Seamon & Daly, 1986). In fact, forskolin only begins to have any effect on membrane fluidity at concentrations greater than those required to maximally activate adenylate cyclase (Whetton et al., 1983).

As yet, C from eukaryotic tissues is the only enzyme which has been demonstrated to be directly affected by forksolin (Seamon & Daly, 1984). This activation is rapid and reversible, resulting in an increased V_{max}, without significantly altering the enzyme's K_m for MgATP (Birnbaumer et al., 1983). This activation does not desensitise the enzyme with respect to subsequent activation by forskolin (Seamon & Daly, 1981a). Low concentrations of forskolin, which by themselves have little effect on cyclic AMP levels, markedly potentiate the effects of various hormones. However, the precise mechanism behind this is unclear (Seamon & Daly, 1984). It has been suggested that forskolin acts at two different sites in the cyclase system; a low affinity site which is responsible for activation of adenylate cyclase, and a high affinity site which is responsible for forskolin's synergistic action with hormones (Nelson & Seamon, 1986). It is thought that the low affinity sites represent binding to C, and that these sites are numerous, i.e. low affinity/high capacity (Shi et al., 1986), whilst the high affinity sites are thought to reflect a ternary complex of C, G_s, and forskolin. In fact, it is thought that forskolin may act to stabilise the complex between G_s and C. This is reflected by an increase in the sedimentation co-efficient of solubilised adenylate cyclase in the presence of forskolin (Bouhelal et al., 1985).

It has also been reported that low concentrations of forskolin have, in some cases, been found to inhibit adenylate cyclase (Watanabe & Jakobs, 1985). This inhibition is again suggested to be due to a direct interaction with the catalytic unit.

It should be emphasised again that forskolin may not bind directly to C, and in fact recent evidence has shown forskolin can modulate a number of membrane transport processes via a cyclic AMP-independent mechanism. For example, the diterpene has been shown to directly bind to the glucose transporter (Laurenza et al., 1989). It may, therefore, come to light in the near future that forskolin interaction with adenylate cyclase is via a specific, as yet unidentified, binding protein (Brooker et al., 1983).

(vi) The effect of manipulating membrane fluidity on adenylate cyclase activity

As stated previously (Section 1.1(ii)), the fluidity of the plasma membrane is important as regards the activity of integral membrane proteins. In the case of adenylate cyclase, a number of studies have been undertaken (Gordon et al., 1980; Houslay & Gordon, 1983; Friedlander et al., 1987; De Foresta et al., 1987; Cherenkevich et al., 1982; Dipple & Houslay, 1978; Houslay et al., 1981; Houslay et al., 1980(b)) which show that enhanced membrane fluidity augments adenylate cyclase activity. Obviously the sensitivity of such penetrant proteins to modulations in fluidity, and the degree to which activity is subsequently altered, is a property peculiar to an individual protein and its particular conformation in the bilayer.

In studies where membrane fluidity is enhanced by the local anaesthetic benzyl alcohol (Gordon et al., 1980; Houslay & Gordon, 1983; Friedlander et al., 1987; De Foresta et al., 1987; Cherenkevich et al., 1982; Dipple & Houslay, 1978), it has been shown that the increase in lipid fluidity (both bulk phase and annular) effects an increase in adenylate cyclase activity. This activation applies to both the receptor-coupled and receptor-uncoupled states, and is presumed to result from the

increase in membrane fluidity removing some conformational constraint imposed on the enzyme by the membrane (Gordon et al., 1980).

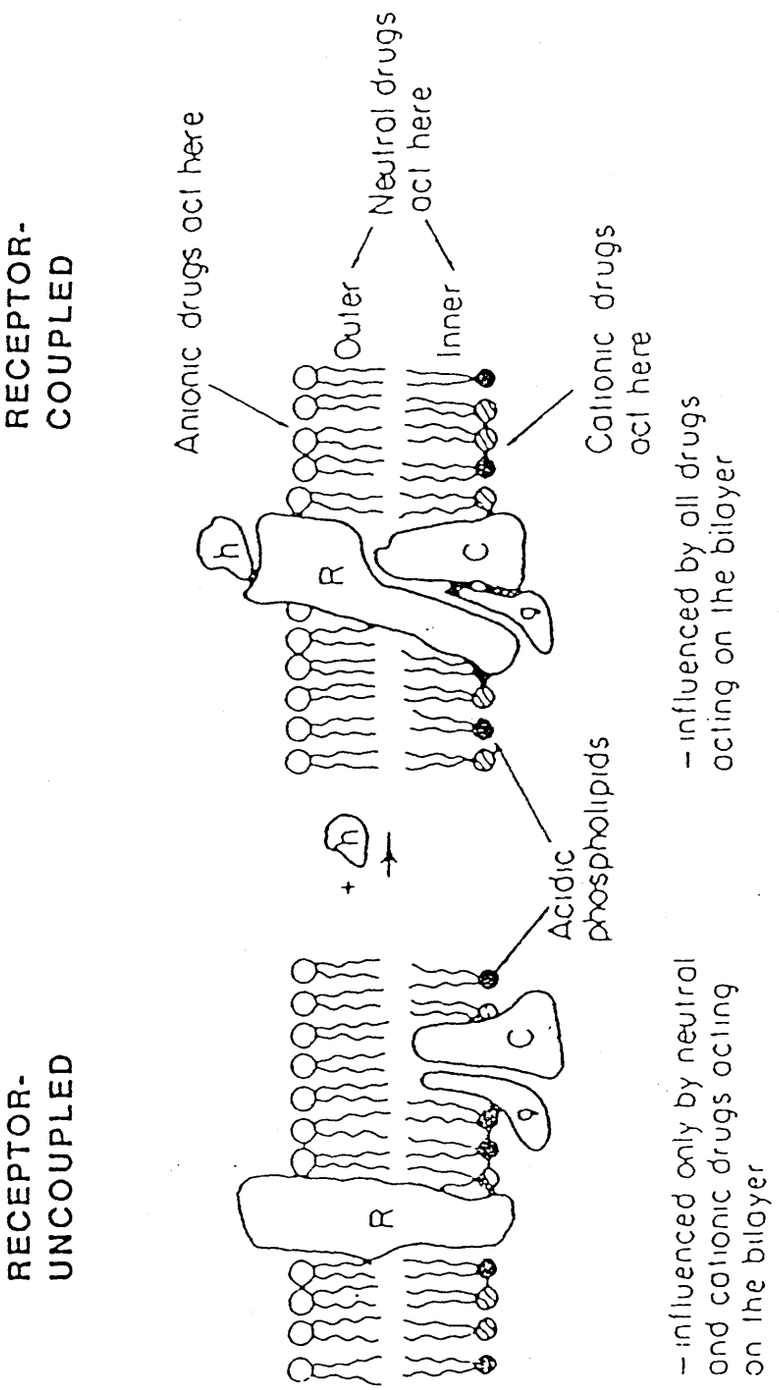
However, it should be noted that enzymes such as adenylate cyclase, whose activity is augmented by low concentrations of benzyl alcohol (40-50mM), are also inhibited at higher concentrations of anaesthetic. This inhibition is not a direct result of the anaesthetic's ability to manipulate fluidity, i.e. it is not due to the formation of a 'too fluid' lipid bilayer. Rather, such inhibition is believed to arise from alcohol competing for sites on the protein previously occupied by annular lipid. The displacement of such annular lipid would, by this hypothesis, give rise to inhibition of activity either because the displaced lipid species were essential for activity, or the alcohol when occupying these sites is itself inhibitory. This inhibition is characteristic of a number of neutral and charged anaesthetics, and is of a reversible nature. Clearly, the susceptibility of a protein to such inhibition depends upon the strength of lipid-protein interaction, and the affinity of the sites for anaesthetic (Houslay & Stanley, 1982).

In examining the effects of selective perturbations in membrane fluidity by charged anaesthetics (Houslay & Gordon, 1983; Houslay et al., 1981; Houslay et al., 1980(b)), the coupling state of adenylate cyclase will determine whether or not the enzyme activity is enhanced by this manipulation of fluidity. In studies with rat liver plasma membranes (Dipple & Houslay, 1978), receptor-uncoupled activity (NaF-stimulated), was found to exhibit a linear Arrhenius plot. This is presumably because in this conformation adenylate cyclase experiences the environment of the inner half of the bilayer, where no lipid phase separation has been

found to occur (see Section 1.1(ii)). This is because the functional, globular, domains of both the stimulatory guanine nucleotide regulatory protein, and the catalytic unit are postulated as residing within this leaflet. Indeed, this is consistent with structural predictions which show the active site of adenylate cyclase to exist on a loop at the cytoplasmic surface of the bilayer (Schofield & Abbott, 1989). In the presence of the appropriate hormone, in this case glucagon, the receptor couples, structurally and functionally, with the G-protein to form a transmembrane complex. As a result of this conformation, the activity of the catalytic unit of adenylate cyclase will be modulated by the lipid environment in both hemileaflets. Arrhenius plots of such receptor-coupled activity are therefore found to exhibit a break at 28^{o}C reflecting the lipid phase separation of the external leaflet (Dipple & Houslay, 1978).

The effects of selective modulations in fluidity can therefore be observed by examining hormone-stimulated (receptor-coupled) activity in comparison to basal, or fluoride-stimulated (receptor-uncoupled) activities (see Diagram 12). It has been shown that this increased membrane fluidity will only amplify the stimulation caused by NaF, if the lipid environment of the inner leaflet is altered, i.e. as G_s and C are postulated as residing here. With receptor-coupled activity, the presence of the transmembrane complex means adenylate cyclase activity is regulated by the lipid environment of both halves of the bilayer. Amplifying the fluidity of only the external leaflet would have a selective effect here, as only the receptor-coupled state would experience such changes in the outer half of the bilayer, i.e. there would be no effect on basal, or fluoride-stimulated activities (Houslay & Gordon, 1983).

Figure 12 **Selective modulations of membrane fluidity**



Hence, perturbation of the external leaflet by the anionic drug phenobarbital, results in a reduction in the temperature at which a break occurs in Arrhenius plots of receptor-coupled activity, whilst having no effect on the linear Arrhenius plot of receptor-uncoupled activity (Houslay et al., 1981). Similarly, the cationic anaesthetic prilocaine selectively amplifies the fluidity of the inner leaflet of the bilayer. This preferential manipulation of fluidity is reflected in Arrhenius plots of adenylate cyclase activity. Here a break is observed in the activity plots of both the fluoride- and glucagon-stimulated states, reflecting the induction of a lipid phase separation at 16^{o}C in the inner leaflet. The break at 28^{o}C observed in Arrhenius plots of receptor-coupled (glucagon-stimulated) activity is unaltered, as would be expected of a phase separation of the external leaflet (Houslay et al., 1980(b)).

It is therefore apparent that adenylate cyclase activity is not only sensitive to the fluidity of its membrane environment, but that it is possible to selectively modulate this parameter and thereby to preferentially alter the activity of chosen adenylate cyclase states.

(vii) Insulin and its interaction with the adenylate cyclase system

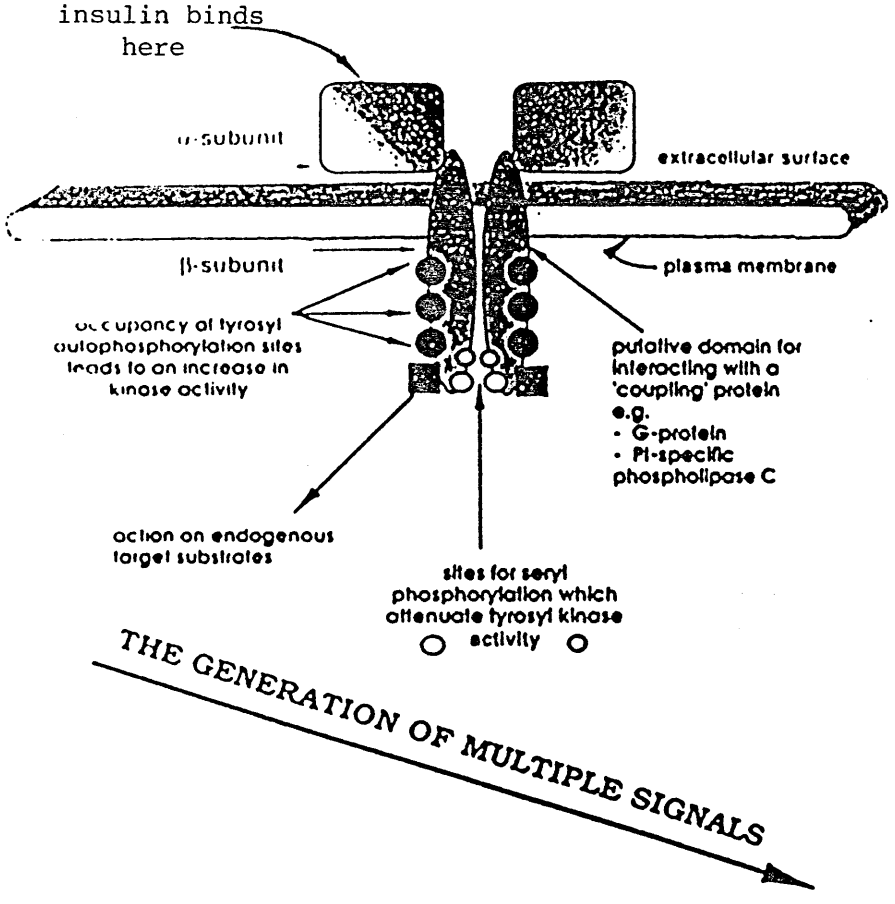
Insulin has been shown to reduce the levels of cyclic AMP in a number of tissues, including human platelets. This effect is mediated by insulins ability to stimulate phosphodiesterase activity, and attenuate adenylate cyclase activity. Insulin has no effect on the rate of cyclic AMP efflux from cells. Exactly how insulin alters the activity of these enzymes is unclear; the regulation of adenylate cyclase being more open to question, and of particular interest here.

The effects of insulin depend upon the recognition and binding of the hormone by specific plasma membrane receptors. These receptors have been shown to exist in human platelets at levels similar to those exhibited by more conventional target tissues such as liver and adipose tissue, i.e. in the region of 25 sites/uM^2 platelet surface area (Falcon et al., 1988).

Insulin receptors are heterologous molecules, consisting of two types of polypeptide chain (alpha and beta) cross-linked by disulphide bridges. The holomeric structure is actually a complex of around 450kD, consisting of two alpha-subunits of 135kD and two beta-subunits of 90kD (Fig.13). Both types of subunit are glycosylated. However, whether or not the platelet insulin-receptor exhibits this characteristic structure is unclear, as some reports have suggested that although the beta-subunit is unaltered, a typical alpha-subunit may not exist (Falcon et al., 1988).

In the classical model of receptor activation, the alpha-subunit exhibits insulin-binding properties, being exposed at the extracellular surface of the plasma membrane. The transmembranous beta-subunit exhibits no such activity, but anchors the alpha-subunit to the bilayer. However, as the beta-subunit has a cytosolic domain, it is involved in eliciting the intracellular signal(s) produced upon insulin binding. In fact, insulin binding activates an endogenous tyrosyl kinase activity, such that the beta-subunit undergoes autophosphorylation (Houslay, 1990). Exactly how such tyrosine phosphorylation relates to the generation of intracellular effects of insulin in unclear. No endogenous cellular substrates have been identified, and indeed the bulk of cellular phosphorylations occur on serine and threonine residues, as opposed to tyrosine.

Figure 13 **Structure of the insulin receptor**

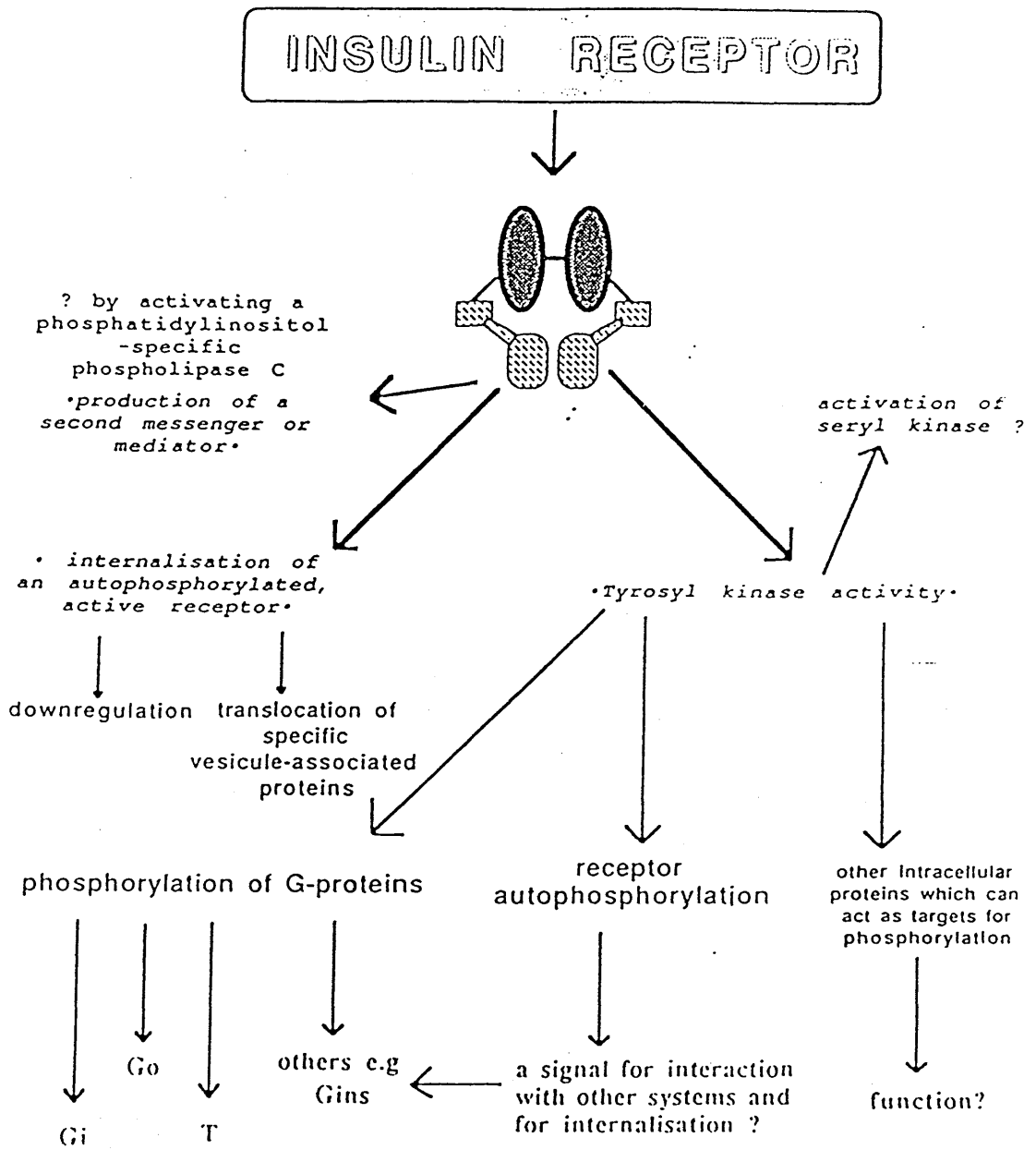


It is therefore, apparent that the mechanism by which insulin elicits its effects subsequent to receptor-binding are unclear. Various methods have been proposed as to how the hormone is able to exert such varied effects, both with respect to time and variety of substrate (Fig.14).

Amongst these suggestions is the possibility that the insulin-receptor tyrosyl-kinase activity is capable of phosphorylating the components of the adenylate cyclase system. In fact, it has been demonstrated that the alpha and beta-subunits of holomeric G-proteins (G_i and G_o) are capable of being phosphorylated in this manner (O'Brien et al., 1987; Krupinski et al., 1988; Zick et al., 1986) as is transducin. However, the functional significance of this tyrosine phosphorylation is unclear, although if may alter the stability of the inactive, holomeric states. Insulin may therefore interact and modify signal transduction through the G-protein system.

It has also been proposed that insulin-interaction with the G-protein system may occur via a novel G-protein, termed G_{ins}. Indeed, it has been shown in human platelets that insulin actually stimulates a high affinity GTPase (Gawler & Houslay, 1987). This novel putative G-protein has an alpha-subunit of M_r 25kD, and exhibits sensitivity to cholera toxin, whilst it is unable to undergo ADP-ribosylation by pertussis toxin. The actual effector/signal generating mechanism which G_{ins} couples to is unknown, although it has been postulated as mediating the insulin-inhibition of adenylate cyclase activity. Such inhibition could result from direct interaction of G_{ins}alpha with the catalytic unit of adenylate cyclase, or via the beta-subunit of G_{ins} attenuating dissociation of G_s (Houslay, 1990: Houslay, 1986), i.e. analogous to G_i-mediated inhibition. Alternatively,

Figure 14 **A multipathway mechanism for the action of insulin**



central metabolic and growth promoting actions

insulin may inhibit adenylate cyclase activity by more direct interaction with G_s, for example by phosphorylating one of its components.

Other propositions as to how insulin mediates its effects within the cell have included the suggestion that at least some of its effects, including the attenuation of adenylate cyclase activity, arise due to the production of a chemical mediator-substance(s) (Houslay, 1990). Such mediators were originally thought to be peptides, although it is now believed that they are inositol phosphate oligosaccharides (Mato, 1989). The precise composition of these small molecular weight (1-3K) mediators remains to be established, but it appears to contain glucosamine or galactosamine, galactose and further phosphate linked to the inositol phosphate moiety (Houslay & Siddle, 1989).

As these substances can be produced by insulin action on membrane fractions, as well as whole cells, this suggests all the necessary components are located in the plasma membrane. It is possible that the precursor from which these substances arise is a glycolipid analogous to the phosphatidylinositol phosphate anchor described for a number of membrane-associated proteins (Saltiel & Cautrecasas. 1986). Generation of the mediator from such a precursor is thought to involve the action of an insulin-stimulated phospholipase. How insulin would stimulate such a selective activity is again a matter of conjecture (Houslay & Siddle, 1989; Saltiel & Cuatrecasas, 1986), as is the mechanism by which such a mediator would attenuate adenylate cyclase activity. It has been proposed that the mediator could modulate the phosphorylation state of adenylate cyclase, as has been found for various other proteins, or that the mediator could interact with the G-proteins of the adenylate cyclase system (Shirong et al., 1988).

In conclusion, the method(s) by which insulin mediates its effects is unclear, although various mechanisms have been proposed. Amongst these suggestions are insulin stimulation of phosphorylation, interaction with G-proteins, including the novel G_{ins}, or the production of a chemical mediator substance. It may be that insulin modulates adenylate cyclase activity by one or more of these methods, or by a method as yet unidentified.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Solutions

Double-distilled deionised water was used in the preparation of all solutions. pH values were determined using a Horiba F.8L pH meter with temperature compensator.

2.2 Chemicals

Chemicals used, and their suppliers, are listed in Appendix II.

2.3 Source of human blood for platelet membrane preparations

Blood was obtained from volunteers not undergoing medication for at least one month prior to donation. 500ml volumes were removed from the anticubital vein. Blood was obtained via the co-operation of the Blood Transfusion Service, St. Vincent Street, Glasgow and Law Hospital, Carluke, Lanarkshire, Scotland.

2.4 Preparation of crude membrane pellets from human platelets

Membrane pellets were prepared from platelets using the method outlined by Jakobs *et al*. (1982).

This entailed collecting 10ml aliquots of fresh blood into 1ml of 3.8% (w/v) trisodium citrate and centrifuging at $1,000g_{av}$ for 5 minutes at room temperature in a Centaur 2 bench centrifuge. The supernatant was aspirated and retained and this pooled liquid was then centrifuged at $30,000g_{av}$ for 15 minutes using an MSE-21 Hi-spin centrifuge and an 8x50ml rotor at $4^{o}C$.

The resulting pellet was resuspended in 15ml of 10mM Tris/1mM EDTA, pH7.4, using ten up/down strokes with a glass Potter-Elvejm homogeniser and teflon pestle and then recentrifuged at $30,000g_{av}$ as before. A wash step was then carried out with the pellet being resuspended in 15ml of 10mM Tris, pH7.4 and centrifuged once more. The final 'crude membrane pellet' was resuspended in 10mM Tris; 2ml of buffer for every 100ml of fresh

blood used. This membrane suspension was stored frozen at -80°C until required.

2.5 Protein estimations

A modified-form of the method of Lowry et al. (1951) was used to determine protein concentrations of the platelet membranes. This procedure is as detailed by Peterson et al. (1977) and permits the removal of interfering substances from the assay. This is done by the inclusion of a solubilisation/ precipitation step preceding the colourimetric estimation of protein concentration.

Standard solutions of bovine serum albumin were prepared covering the range 0-100ug protein ml^{-1}. These, and unknown protein solutions, were made up to a 1ml volume using distilled water and 0.1ml of 0.15% (w/v) deoxycholate was then added to each tube. Samples were mixed and incubated at room temperature for 10 minutes. 0.1ml of 72% (w/v) trichloroacetic acid was then added and samples were again mixed. This was followed by centrifugation at $3,000g_{av}$ for 15 minutes at room temperature in a Centaur 2 bench centrifuge. Supernatants were then decanted and discarded, with any excess liquid being removed by aspiration.

Each sample was then made up to a 1ml volume using distilled water. A 'Lowry C' solution consisting of 50ml of 4% (w/v) Na_2CO_3 containing 0.4% (w/v) sodium dodecyl sulphate and 1ml of 1% (w/v) $CuSO_4.5H_2O$ in 2% (w/v) sodium citrate was then prepared. 1ml of this solution was then added to each tube and samples were vortexed and left at room temperature for 10 minutes. 0.5ml of Folin and Ciocalteu's reagent (diluted 1 to 5 with distilled water) was added. Samples were left at room temperature for 30 minutes to allow colour to develop and absorbance was read at 750nm using an LKB spectrophotometer. Samples were measured in triplicate and protein concentration was determined using an LKB 'Wavescan' linear regression curve-fit programme (LKB, Surrey, UK).

2.6 Preparation of cyclic AMP binding protein from bovine cardiac muscle

The method employed here was essentially as detailed by Rubin et al. (1974) and can be considered as 3 distinct stages.

i) Homogenisation

Fresh bovine heart was dissected into small cubes, which were then finely minced. The resultant 'homogenate' was mixed with 4 litres of 40mM KPO_4 buffer, pH6.1, containing 2mM beta-mercaptoethanol and further homogenised in a Waring blender. This step was performed using small batches of the minced heart and homogenisation lasted for approximately 1 minute. The homogenate was then centrifuged at 4°C for 10 minutes at $10,000g_{av}$ and the supernatant was filtered through Whatman No.54 filter paper using a Buchner funnel and flask under vacuum. The pellet was further washed with 1 litre of the KPO_4 buffer and the supernatant again filtered. This step was repeated once more and the resultant supernatants were combined.

ii) $(NH_4)_2SO_4$ precipitation

Solid $(NH_4)_2SO_4$ was added to the combined supernatants until 55% saturation was obtained. (320g $(NH_4)_2SO_4$/L) pH was maintained at 7-8 by adding concentrated $(NH_4)OH$ solution. After 2.5-3 hours at 4°C, protein precipitation had occurred and the precipitate was collected by centrifugation at $10,000g_{av}$ for 10 minutes at 4°C. Having discarded the supernatant, the precipitate was dissolved in 500ml of 50mM Tris/HCl, pH7.6, containing 4mM beta-mercaptoethanol and 10mM NaCl. This solution was dialysed overnight against 5 litres of Tris buffer (4°C) to remove the $(NH_4)_2SO_4$.

iii) DE-50 cellulose purification

a. Pre-treatment of DE-50 cellulose

50g of DE-50 cellulose (in place of DEAE-Sephadex in Rubin's method) was added to 2 litres of 0.5M HCl and left for 30 minutes. This was then washed with distilled water until the pH rose to 4 units. The resin was then stirred into 2 litres of 0.5M NaOH and left for a further 30 minutes. Once again the DE-50 cellulose was washed, this time until the eluate was neutral. The whole acid/alkali cycle was repeated once more and the resin was then equilibrated with 50mM Tris/HCl, pH7.6 containing 10mM NaCl and 4mM beta-mercaptoethanol. A final slurry was prepared to give a wet settled volume: final volume ratio of 2:1.

b. Absorption of the binding protein

The dialysed preparation was mixed with 800ml of equilibrated DE-50 cellulose and stirred for 1 hour. Under these conditions cyclic AMP-dependent protein kinase activity should be absorbed. The mixture was filtered using Whatman No.54 filter paper and a Buchner funnel and flask under vacuum. The resin collected was then washed with 3 litres of Tris/HCl pH7.6 containing 10mM NaCl and 4mM beta-mercaptoethanol, until the filtrate was colourless. The resin was then mixed with 200ml of 50mM Tris-HCl pH7.6 containing 0.3mM NaCl and 4mM beta-mercaptoethanol for 45 minutes. The DE-50 cellulose was collected by washing through filter paper using 50mM Tris/HCl buffer and a Buchner flask and funnel.

c. Isolation of the cyclic AMP binding protein

Filtrates from the previous step were combined and $(NH_4)_2SO_4$ added to give 35% saturation. pH was maintained at 7-8 units using NH_4OH solution and after 60 minutes the mixture was centrifuged for 10 minutes at $10,000g_{av}$, 4^oC. The

precipitate was discarded and the supernatant was brought to 75% saturation by adding 258g/litre $(NH_4)_2SO_4$. Again this mixture was left for 1 hour and the resultant precipitate was collected by centrifuging at 10,000g_{av} as before. This time the pellet was retained, and suspended in a minimal volume of 50mM KPO_4 pH7 containing 4mM beta-mercaptoethanol. Following overnight dialysis at 4°C, against 2 litres of this KPO_4 buffer, the purified protein kinase was stored at -80°C in 250ul aliquots. Each preparation was found to be stable for at least 6 months under these conditions.

2.7 Assay of adenylate cyclase activity

Adenylate cyclase (E.C. 4.6.1.1) was assayed by 2 methods as described:

i) Activity was measured as described by Houslay et al. (1976). Here an assay cocktail was prepared consisting of an ATP-regenerating system (1.5mM ATP, 7.4mg/ml creatine phosphate, 0.2mg/ml creatine kinase and 0.8mg/ml BSA) in a buffer of 25mM triethanolamine, 5mM $MgSO_4$, 10mM theophylline, 1mM EDTA and 1mM dithiothreitol, pH7.4. Drugs and membranes (20-100ug) were then added as appropriate to give a final assay volume of 100ul.

Samples were then incubated at 30°C for 10 minutes, and reactions terminated by boiling samples at 90°C for 3 minutes. Centrifugation at 14,000g_{av} for 5 minutes caused the precipitated protein to pellet, and the supernatants were then removed for cyclic AMP determination (see Section 2.8).

ii) Here activity was measured using the method of Jakobs and Aktories (1983), which is an adaptation of Salomon et al. (1974). The reaction mixture consisted of 50uM ATP, 5mM creatine phosphate, 0.4mg/ml creatine kinase, 2mg/ml BSA and 0.1mM cyclic AMP in a buffer of 50mM triethanolamine, pH7.4, containing 2mM $MgCl_2$, 0.1mM

ethylene glycol bis(beta-amino ethyl ether) N,N'-tetracetic acid, 1mM isobutylmethylxanthine and 1mM dithiothreitol.

Reactions were initiated by the addition of platelet membranes (20-100ug protein) and continued for 15 minutes at 25°C. [α-^{32}P]ATP (2uCi/tube) was then added and reactions continued for a further 10 minutes. Again final volumes were 100ul. Incubations were terminated by the addition of a 'stopping' solution containing 2% sodium dodecyl sulphate, 40mM ATP, and 1.4mM cyclic AMP at pH7.5. Cyclic AMP was determined as described by Salomon et al. (1974).

2.8 Cyclic AMP determination

Again, 2 methods of determination were employed:

i) Determination of cyclic AMP content in samples assayed for adenylate cyclase activity by the method of Houslay et al. (1976) (Section 2.7(i)), was based on the saturation assay of Brown et al. (1972), as described by Whetton et al. (1983).

This depends upon the specific binding of the cyclic AMP nucleotide to a cyclic AMP-binding protein isolated and purified from bovine heart, as described in Section 2.6. Samples and binding protein are incubated together until equilibrium occurs and bound cyclic AMP is then separated from unbound by the use of a charcoal/BSA suspension. The charcoal absorbs free nucleotides and therefore estimation of ^{3}H-cyclic AMP bound to the protein can be made.

Using a range of known cyclic AMP concentrations, it is possible to construct a standard displacement curve from which estimations of cyclic AMP content can be made. This is done by incubating increasing amounts of unlabelled cyclic AMP with a fixed amount of the tritiated nucleotide, and binding protein. The portion of cyclic AMP can in this way be reduced, as the unlabelled

and tritiated species compete for a finite number of binding sites on the protein.

Total cyclic AMP binding to the protein is determined by incubating the tritiated cyclic AMP in the absence of the unlabelled species, whilst non-specific binding is estimated by the incubation of tritiated cyclic AMP in the absence of binding protein as well as unlabelled cyclic AMP.

For the assay, a series of unlabelled-cyclic AMP solutions were prepared ranging from 0-320pmoles ml^{-1}. This was done by dissolving cyclic AMP in a buffer containing 50mM Tris/5mM EDTA, pH7.4. Tritiated-cyclic AMP (5.8-^{3}H-adenosine 3'5' cyclic phosphate) in 50% ethanol was diluted in this Tris/EDTA buffer to give approximately 500,000cpm/ml. 100ul of this solution was added to tubes containing 50ul of assay buffer, and 50ul of standard cyclic AMP solution or 50ul of supernatant from samples with unknown cyclic AMP content was then added. After mixing, 100ul of binding protein, (see Section 2.6, diluted 1:70 with assay buffer) was added to the samples, which were mixed once again. Following incubation at 4^{o}C for 2 hours, the binding reaction was terminated by addition of 0.25ml of a BSA/charcoal suspension. This solution consisted of 2% GSX-100 charcoal and 1% BSA suspended in ice-cold assay buffer. The charcoal was pelleted from the samples by centrifuging at 14,000g_{av} for 5 minutes at a temperature of 4^{o}C. 400ul of supernatant was removed into 'Ecoscint' scintillation fluid, and the samples counted in an LKB scintillation counter. This counter had an RIA curve fitting programme and hence cyclic AMP content was automatically calculated from the standard curve. The binding assay gave optimum sensitivity between 0.25 and 8pmoles/sample.

ii) To determine cyclic AMP content in samples from the adenylate cyclase assay of Jakobs and Aktories (1983) (Section 2.7(ii)), the cyclic AMP was isolated using the procedure detailed by Salomon (1974).

Here, 100ul of tritiated cyclic AMP (approx. 20,000 cpm) was added to samples in order to monitor cyclic AMP recovery. Reaction tubes were then processed following the addition of 0.75ml of distilled water. This entailed passing the samples over 1ml Dowex-50 AGWX4 resin in 0.4 x 15cm columns. The eluate from this and two successive 1ml water washes were discarded. Three millilitres of water were then added to each column and the eluate from these columns was then passed directly over 0.6g neutral alumina (again in 0.4 x 15cm columns). After discarding the subsequent eluate, these columns were then washed with 4ml of 0.1M imidazole, pH7.5, and the eluate was collected directly into scintillation vials. After the columns were completely drained, 12ml of 'Ecoscint' scintillation fluid was added to each vial, and the samples were counted using an LKB scintillation counter. This counter measured both ^{32}P and ^{3}H counts in all samples, results being expressed in cpm.

Dowex columns could be reused by regenerating with 2 x 1ml 1N HCl and washing with 10ml of distilled water prior to use. Alumina columns could also be reused if equilibrated with 0.1M imidazole (8ml) prior to use.

2.9 Lubrol-solubilisation of adenylate cyclase

Membrane protein was solubilised using the method of Swislocki and Tierney (1975). This entailed adding approximately 30mg of protein to 4ml of a solubilising solution containing Lubrol-PX (40mM), 0.25M sucrose and 1mM EDTA, in 50mM Tris/HCl, pH7.4. This suspension was then mixed by inversion and left on ice

for 10 minutes. The sample was then centrifuged for 1 hour at 165,000g_{av} at 5^{o}C on a Beckman TL-100 benchtop ultra-centrifuge. Adenylate cyclase activity was present in the supernatant fraction produced. The supernatant was therefore retained and used in assays of adenylate cyclase activity. This fraction was prepared afresh for each experiment.

2.10 Phosphodiesterase assay

Phosphodiesterase activity in platelet membranes was measured by the method of Rutten et al. (1973). Here ^{3}H-cyclic AMP (8-^{3}H-adenosine 3'5'-cyclic phosphate) was diluted in 20mM Tris/HCl, pH7.4, containing 5mM $MgCl_2$ and 1uM cyclic AMP. 50ul of this solution (approx. 0.2uCi) was then added to tubes containing 15-30ug membrane protein, and the volume made up to 100ul using 20mM Tris/HCl, pH7.4, containing 5mM $MgCl_2$ and 1mM cyclic AMP. Samples were then incubated at 30^{o}C for 10 minutes, and boiled for 2 minutes to terminate the reaction.

After being allowed to cool, 25ug of snake venom (Ophiphagus hannah-cobra, 1mg/ml in H_2O) was added, and the samples were again incubated at 30^{o}C for 10 minutes. 400ul of Dowex AG I-X2 in ethanol/water (1:1:1) was then added and following incubation on ice for 15 minutes, the samples were then spun at 15,000g_{av} for 2 minutes on a Beckman benchtop microcentrifuge. 150ul of supernatant was then removed into 4ml of 'Ecoscint' scintillation fluid. The amount of ^{3}H-adenosine present was determined by counting on an LKB scintillation counter.

2.11 Preparation of gamma-^{32}P GTP

GTP was radioactively labelled at the gamma-phosphate position using the method detailed by Maxim and Gilbert (1980).

The principle here can be outlined as:

a) Glycerate-3-phosphate + GTP $\xrightleftharpoons{\text{phosphoglycerate kinase}}$ 1,3 diphosphoglycerate + GDP

b) 1,3 diphosphoglycerate $\xrightleftharpoons{\text{Glyceraldehyde-3-phosphate dehydrogenase}}$ Glyceraldehyde-3-P + P_i

3-phosphoglycerate kinase [E.C. 2.7.2.3] catalyses the reversible conversion of glycerate-3-phosphate to 1,3 diphosphoglycerate using GTP (or ATP) as the phosphate donor. The 1,3-diphosphoglycerate can be further converted to glyceraldehyde-3-phosphate in the presence of the enzyme glyceraldehyde-3-phosphate dehydrogenase [E.C. 1.2.1.12]. ^{32}P-labelled inorganic phosphate is also produced in this reaction, but under the conditions employed, the position of equilibrium is shifted so far to the left that this $^{32}P_i$ is incorporated into the 1,3 diphosphoglycerate and subsequently into GTP at the gamma position. Theoretically, a 78% conversion of the $^{32}P_i$ to [^{32}P]GTP is possible; 70-75% conversion was actually obtained.

The method employed involves 4 major stages, which entail washing commercially bought enzymes, incubation of the reaction mixture, PEI-cellulose thin-layer chromatography to identify and quantify products, and finally purification of the gamma[^{32}P]GTP by ion-exchange chromatography.

i) Removal of $(NH_4)_2SO_4$ from commercial enzyme preparations

This step is included in order to remove traces of inorganic phosphate which would reduce the percentage conversion of [^{32}P]P_i into gamma[^{32}P]GTP, and also to remove residual $(NH_4)_2SO_4$ which would inhibit the reaction.

Here 12.5ul of yeast 3-phosphoglycerate kinase (specific activity approx. 4.5U/ul) was combined with 32.5ul of glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, SA approx. 0.8U/ul) in a 1.5ml Eppendorf tube on ice. This was then sedimented by centrifugation at 14,000g for 5 minutes at 4^{o}C, and the supernatant was removed and discarded. The sediment was very gently resuspended in 50ul of 'wash buffer' i.e. 100mM Tris/HCl, pH8, containing 1mM EDTA (pH8), 0.1mM NAD^{+}, 3.2M $(NH_4)_2SO_4$ and 10mM beta-mercaptoethanol. This was then sedimented by centrifugation as before, and washed and recentrifuged again. The walls of the Eppendorf tube and the top of the pellet were then carefully washed with 12.5ul of distilled water, at all times trying not to disturb the pellet. Finally, the enzymes were centrifuged once more, and all supernatant was discarded. The pellet was very gently resuspended in 37.5ul of distilled water and placed on ice.

ii) Incubation of the reaction mixture

Before using [^{32}P]P_i, the inorganic phosphate (specific activity 1mCi/100ul) was neutralised by adding 10ul of 500mM NaOH and mixed using a heat-sealed glass capillary tube. 50ul of a 10 x concentrated solution of (final concentrations) 500mM Tris/HCl (pH8), 50mM $MgCl_2$, 20mM reduced glutathione, 10mM glycerate-3-phosphate, 1mM EDTA (pH8), and 0.1M NAD^{+}, was added to 500ul of neutralised [^{32}P]-inorganic phosphate.

5ul of 4mM GTP and 2ul of the washed enzyme suspension were added to initiate the reaction, and the mixture was gently mixed and incubated at room temperature for 20 minutes. The reaction was terminated by placing the sample on ice.

iii) PEI-cellulose thin layer chromatography

A 10x20cm sheet of PEI-cellulose was pre-run in distilled water to take most of the yellow soluble material to the top. The plate was then dried, and marked in pencil into 10x5cm rectangles, with an origin line 1.5cm from the bottom. These rectangles were cut out and stored at $4^{o}C$ prior to use.

1ul reaction mixture samples were spotted onto these plates immediately prior to initiation of the reaction, and immediately before its termination. The plates were left to dry at room temperature for 5 minutes, and the placed in a chromatography tank containing 10ml of 0.75M KH_2PO_4 buffer (pH3.5). The buffer was allowed to travel about three-quarters of the way up the plate (approx. 30 minutes), allowing excellent separation of GTP from P_i; P_i runs near the front, and GTP has an R_f in the region 0.15-0.20.

The relative amounts of radioactive GTP and P_i were determined by developing an autoradiograph of the dried PEI-cellulose plate, and cutting out the appropriate areas for Cerenkov counting using a wide-open channel setting on an LKB scintillation counter. From these counts it was possible to calculate the %-conversion of ^{32}P-P_i to gamma [^{32}P]GTP.

It was also possible to ensure that [^{32}P]-labelled inorganic phosphate had actually been converted to gamma[^{32}P]-labelled GTP by using a charcoal slurry. Here, 1ul samples were removed from the reaction mixture at 0 and 20 minute incubation times into 200ul of 50mM $NaPO_4$, pH7. 100ul of this mixture was removed into an Eppendorf tube, and 0.5ml of a 5% slurry of Norit A charcoal (in 50mM $NaPO_4$, pH7) was added. After vortexing, the suspension was centrifuged for 5 minutes at $14,000g_{av}$ at $4^{o}C$. 0.4ml of supernatant was added to 3ml of 'Ecoscint' scintillation

fluid, and counted in an LKB scintillation counter. Under these conditions the charcoal is saturated with unlabelled inorganic phosphate, therefore it is possible to separate radioactive GTP from radiolabelled inorganic phosphate by specific absorption to the charcoal.

The 0 minute reaction mixture sample allows the determination of non-specific binding to the charcoal by P_i. This 'blank' can then be subtracted from all other samples. Use of this relatively quick method enables continuation of the preparation to the purification stage without having to wait for the PEI-cellulose chromatography quantification to have reached completion.

iv) Purification of the [^{32}P]GTP

The [^{32}P]-labelled GTP was purified by anion exchange using Dowex 1-X2 (100-200 mesh, Cl^- form) as the chromedia. The resin was washed before use. This entailed suspending 50g of Dowex resin in 1.5L of 1M NaOH for 30 minutes, and then filtering on a large Buchner funnel. The resin was washed with 5L of distilled water to remove NaOH, and then suspended in 1.5L of 1M HCl for 30 minutes. The Dowex was then filtered and washed as before.

A 3cm column of this washed resin was then poured in a glass Pasteur pipette which had been 'plugged' with some siliconised glass wool. After washing the column with 5ml of 1M NaCl, and 10ml of distilled water, the sample was applied. A further 5ml of water was then added, followed by 10ml of 0.02M NH_4Cl/0.02M HCl to elute inorganic phosphate, GMP and GDP. Any ammonium ions were removed from the column by washing with 5ml of water, and the GTP was then eluted by applying 15 aliquots of 0.25M HCl (0.75ml). These eluted fractions were collected in Eppendorf tubes containing 0.25ml of 1M Tris (pH9).

Fractions were counted by Cerenkov counting, and the peak fractions (usually tubes 2-14) were pooled, aliquoted, and stored at -20°C. Using the Norit A charcoal method outlined previously, samples of the final GTP were found to be 95% absorbable.

The specific activity of the [^{32}P]-labelled GTP produced was calculated by the following equation:-

% conversion x mCi [^{32}P] used

= Radioactivity/20nmoles GTP

2.12 Assay of GTPase activity

GTPase activity was measured as described by Houslay et al. (1986(b)), which is a modification of the original method described by Cassel and Sellinger (1976).

The assay system contained 0.1uM [^{32}P]GTP, 5mM $MgCl_2$, 100mM NaCl, 1mM dithiothreitol, 0.1mM EGTA, 1mM ouabain and 1mM App[NH]p in a buffer of 50mM Tris/HCl, pH7. A regeneration system of 1mM ATP, 5 units of creatine kinase, and 12mM creatine phosphate was also present, in order to suppress the transfer of gamma-^{32}P from GDP to ADP. The final assay volume was 100ul, with drugs being added as appropriate. Reactions were initiated by the addition of platelet membranes, and after vortexing, the cocktail was then incubated for 5 minutes at 30°C. The assay was terminated by the addition of 500ul of a 2% slurry of Norit A charcoal in 50mM KPO_4 buffer, pH7. Samples were then vortexed, and spun at 14,000g_{av} for 5 minutes.

0.4ml of supernatant was removed from the samples, and counted by Cerenkov counting on an LKB scintillation counter using a wide-open channel setting.

2.13 SDS-PAGE electrophoresis

Electrophoretic separation of proteins was carried out in 10% polyacrylamide gel slabs as described by Laemmli (1970). Firstly, samples were dissolved in a buffer of 0.0625M Tris/HCl, pH6.8 containing 2% SDS, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol and 0.1% (v/v) bromophenol blue as the tracking dye. They were then boiled for 1 minute prior to application, and electrophoresis was carried out at room temperature in a 'running buffer' of 0.025M Tris/HCl, pH8.3, containing 0.192M glycine and 0.1% SDS. When the dye front reached the edge of the gel, electrophoresis was stopped, and proteins transferred to nitrocellulose paper by Western Blotting (Section 2.14).

2.14 Western Blotting

Proteins were transferred from gel to nitrocellulose paper using constant current, and 100 volts for 90 minutes in a Bio-Rad transblot apparatus. This method is essentially as detailed by Huff et al. (1985). Buffer used here is 0.025M Tris with 0.192M glycine/methanol (4:1 v/v). The nitrocellulose paper was then incubated at room temperature in 20mM Tris/HCl, pH7.5, containing 500mM NaCl (i.e. TBS) and 3% dried milk powder. After 90 minute incubation to block non-specific protein binding, the dried milk powder/TBS is removed, and the 'blot' washed thoroughly with distilled water. The paper was then incubated overnight in the same buffer containing 1% dried milk powder, 0.05% thimerasol, and a 1:200 dilution of the appropriate antisera.

The antisera used in these studies (Section 3.1) were AS7 and RV6, which detect alpha-G_i, and G-protein beta-subunits respectively. Antiserum AS7 was raised in rabbits against a synthetic decapeptide (KENLKDCGLF) representing the carboxy-terminus of the alpha-subunit of rod transducin. This peptide was

conjugated with keyhole limpet hemocyanin. The resultant antiserum was affinity purified and is specific for the alpha-subunits of transducin and G_i. Transducin does not occur in platelets. RV6 antiserum was prepared in rabbits using a purified beta-subunit polypeptide. This antiserum reacts strongly with all known G-protein beta-subunits in platelets.

Next day, the blot was washed with distilled water, Tris/HCl with 500mM NaCl, and 0.5ml/L Tween 20, (i.e. TTBS) and finally Tris/HCl with 500mM NaCl. Following these washes, the paper was incubated with a second antibody (1ug of peroxidase-conjugated goat, antirabbit immunoglobulin/ml) for 2-3 hours at room temperature.

After repeated washings (as before), papers were stained in 10mM Tris/HCl, pH7.5, 0.025% hydrogen peroxidase, and a small volume of 1% orthodianisidine-HCl. Following the appearance of protein bands, the colour reaction is stopped by rising with distilled water.

2.15 Concentrating protein samples

Where the protein content of samples was too dilute, i.e. would require use of large volume of material, these samples were made more concentrated using the following procedure.

First, the volume of sample containing the required amount of protein was made up to 150ul with distilled water. 6.25ul of a 2% solution of deoxycholate was then added to solubilise proteins, and this was followed by the addition of 0.75ml of distilled water. 250ul of a 24% (w/v) solution of trichloroacetic acid was then added to precipitate proteins, and samples were spun at 2,500 rpm on a benchtop microcentrifuge. The supernatant was removed from the tubes, and pellets were then resuspended in Tris base (1M) to give the volume of sample required.

2.16 Release of G-protein subunits from platelet membranes

Here platelet membranes, prepared as described previously (Section 2.4), were incubated in a buffer of 20mM Tris/HCl, pH7.5, 20mM $MgCl_2$, 1mM dithiothreitol and 100uM EDTA, at 30°C for 10 minutes. Ligands were added as detailed elsewhere (Section 3.1). At termination of incubation, 25ug of soyabean trypsin inhibitor was added as a soluble carrier protein. Samples were then spun at 20 psi (approx. 126K x g) for 2 minutes, using a TL100 (30°) rotor in a Beckman airfuge. This was to resolve them into supernatant and particulate fractions.

The protein in the supernatant fraction was collected by deoxycholate/trichloroacetic acid precipitation (Section 2.15), and then resolved by SDS-PAGE as described previously (Section 2.13). Protein from membrane pellets was solubilised with Laemmli 'loading buffer' (Section 2.13) and added directly to the gels. Immunoblotting of the resolved proteins was as described previously (Section 2.14).

Essentially this method is as described by McArdle et al. (1988).

2.17 Fusion of acidic phospholipid-liposomes with platelet membranes

Fusion of dioleoylphosphatidic acid liposomes, dioleoyl-phosphatidylcholine liposomes, and dioleoylphosphatidylserine liposomes was performed using the following procedure, based on the method described by Houslay et al. (1986(c)).

Firstly, a sonicated phospholipid suspension was made. This involved taking 0.5-4mg of solid phospholipid and dissolving it in 0.5ml of chloroform/methanol (2:1 v/v) in an acid-washed glass scintillation vial. The solvent was then evaporated off under a stream of nitrogen, and 1ml of 50mM-triethanolamine/HCl

(N_2-saturated), pH7.4, was added. The vial was sealed with parafilm, capped, and shaken for 20 minutes at 8°C. Following this, the sample was then sonicated to optical clarity using a bath sonicator, kept below 20°C, for 2 hours. The suspension was centrifuged at 15,000g_{av} for 1 minute, and this suspension was then used to incubate with the platelet membranes.

Pre-incubation of membranes at 8°C for 20 minutes was performed prior to addition of the liposomes. After the liposome suspension was added to the membranes this mixture was then incubated at 8°C for 45 minutes, following which this was used in the determination of adenylate cyclase activity.

2.18 Cholera toxin catalysed ribosylation of platelet membranes

(a) Pre-activation of cholera toxin

100ul of cholera toxin (1mg/ml) was added to 100ul of 40mM dithiothreitol. Following mixing, this solution was incubated at 30°C for 20 minutes. Incubation was terminated by placing the sample on ice.

(b) Cholera toxin ribosylation

500ul of ribosylation cocktail (1mM GTP, 2mM NAD^+, 30mM thymidine and 100mM KPO_4, pH7.4) was added to the pre-activated cholera toxin sample. 300ul of platelet membranes (approx. 2.5mg of protein) were then added, mixed, and incubated at 30°C for 10 minutes. Centrifugation at 14,000g for 15 minutes (4°C) was followed by washing of the pellet in 500ul distilled water. This procedure was repeated, and the pellet finally resuspended to the required volume in 10mM Tris/HCl, pH7.4.

2.19 Pertussis toxin catalysed ribosylation of platelet membranes

(a) Pre-activation of pertussis toxin

100ul of pertussis toxin (500ug/ml) was added to 100ul of 40mM dithiothreitol. Following mixing, this solution was incubated at 37^{o}C for 45 minutes. Incubation was terminated by placing the sample on ice.

(b) Pertussis toxin ribosylation

500ul of ribosylation cocktail (1mM GTP, 2mM ATP, 2mM NAD^{+}, 30mM thymidine and 100mM KPO_4, pH7.4) was added to the pre-activated pertussis toxin sample. 300ul of platelet membranes (approx. 2.5mg of protein) were then added, mixed, and incubated at 37^{o}C for 30 minutes. Centrifugation at 14,000g_{av} for 15 minutes (4^{o}C) was followed by washing of the pellet in 500ul distilled water. This procedure was repeated, and the pellet finally resuspended to the required volume in 10mM Tris/HCl, pH7.4.

CHAPTER 3

PLATELET ADENYLATE CYCLASE AND THE MEMBRANE ENVIRONMENT

General Introduction

This chapter describes the characterisation of the platelet adenylate cyclase system with respect to stimulation and inhibition by various agents. The effects of manipulating membrane fluidity on ligand-regulated enzyme activity is investigated. Such modulations in fluidity were achieved by various means, namely through the use of charged anaesthetics, neutral anaesthetics, acidic phospholipids, and dimethylnitrosamine. However, the bulk of this work centres around studies employing the local anaesthetic benzyl alcohol.

3.1 The platelet adenylate cyclase system and benzyl alcohol

(i) Introduction

Stimulatory aspects of adenylate cyclase regulation have been examined in detail in other systems (Ruiz et al., 1986; Nelson & Seamon, 1985), most notably, rat liver plasma membranes. Here such studies have been extended to the human platelet, where regulation at the level of each component is examined. This is achieved via the use of different ligands:

- activation at the level of the catalytic unit was determined by the use of the diterpene forskolin (10^{-4}M), which is believed to augment basal adenylate cyclase activity by direct interaction with C (see Section 1.3(v)).

- sodium fluoride (15mM) is thought to mimic the terminal phosphate moiety and so, in combination with GDP bound in the active site of G_s, will stimulate adenylate cyclase. Such activation via G_s is not the only mode of NaF action; this ligand is also capable of attenuating hormone-stimulated adenylate cyclase, as will be discussed.

- stimulation at the level of the receptor. R_s, was examined by two routes: (a) the PGI_2/PGE_1 prostanoid receptor, through the use of prostaglandin E_1 ($2x10^{-5}$M), and to a lesser extent through (b) the beta-adrenoceptor, by the use of the beta-agonist isoproterenol (10^{-4}M).

In each case, the effect of adding these ligands was determined by monitoring cyclic AMP production. By similar means, the effect of adding inhibitory ligands was also determined. Much less is known about inhibitory aspects of regulation, to the point that the actual mechanism of inhibition is unclear, i.e. is it direct G_i alpha interaction with the catalytic unit, or is it via G_i beta-gamma association with G_s alpha? (see Section 1.3 (iv)). It is this aspect of adenylate cyclase regulation which forms the basis of this chapter.

Inhibition was investigated both at the level of the inhibitory G-protein G_i, using low concentrations of the non-hydrolysable GTP-analogue Gpp(NH)p (10^{-8}M or 10^{-6}M), and at the level of the inhibitory $alpha_2$-adrenoceptor, through the use of adrenaline (10^{-4}M) in the presence of propranolol (10^{-6}M) to block beta-adrenoceptor functioning.

As all of the stimulatory/inhibitory effects upon adenylate cyclase activity are believed to involve the interactions of distinct components within the platelet membrane, it was decided to extend the characterisation of this adenylate cyclase system to include the influence of the membrane environment on its activity. In this section, the membrane environment has been manipulated mainly by the use of the local anaesthetic benzyl alcohol (0 -50mM). This neutral compound was previously shown to enhance fluidity, and augment stimulatory aspects of adenylate cyclase regulation (Houslay & Gordon, 1983; Dipple & Houslay, 1978). The effect on benzyl alcohol as regards inhibitory regulation of adenylate cyclase activity has not been reported. Here we attempt to determine this effect, and in subsequent chapters, to look at the effects of other means of manipulating the membrane environment.

(ii) Results

Adenylate cyclase activity was assayed by the method of Houslay et al., 1976 and cyclic AMP production was measured as detailed by Brown et al., 1972 (see Materials & Methods Section 2.7 and 2.8 respectively).

Any changes in the level of cyclic AMP detected here are believed to reflect modulations in adenylate cyclase activity, and not platelet phosphodiesterase (PDE) activity. This is because all experiments were performed in the presence of the phosphodiesterase inhibitors theophylline (8mM) and isobutylmethylxanthine (IBMX, 1mM). Such concentrations gave 97.9 $\pm$ 0;84% and 99.4 $\pm$ 1.06% inhibition of phosphodiesterase activity. (n=3 separate experiments using different membrane preparations).

In examining the stimulation of adenylate cyclase activity, it was found that activation by the various ligands employed here exhibited the same trends as observed in other systems. Figures 15-18 show dose effect curves for ligands which interact with the catalytic unit, G_s and R_s, respectively and illustrate that saturation is obtained. This suggests that, in each case, a finite number of binding sites exists, whilst the sigmoidal nature of the curves suggest single-types of binding site. The EC_{50s} for activation by these ligands are shown in Table 3. It should be noted that NaF was also capable of attenuating adenylate cyclase activity: IC_{50} for inhibition of PGE_1 is 3.02mM. In all further experiments, saturating concentrations of these ligands were used.

A more extensive characterisation of the stimulatory aspects of adenylate cyclase regulation is shown in Table 4. Here, it is apparent that the ability of these various ligands to stimulate adenylate cyclase activity is markedly different, with

PGE_1 and forskolin being the most potent activators. This is in agreement with other studies (Insel et al., 1982).

In order to determine that the activation observed with forskolin was indeed due to a direct interaction at the level of the catalytic unit, the effect of detergent-solubilisation of adenylate cyclase was examined. Following the solubilisation procedure, detailed in Materials and Methods Section 2.9, the activities shown in Table 5 were obtained. High specific activities observed here are due to the fact that experiments were performed in the presence of manganese rather than magnesium. From these results it can be seen that forskolin is the only ligand able to stimulate the solubilised enzyme significantly. This suggests that forskolin does not mediate its effects via binding to some other membrane component, (i.e. other than the catalytic unit), nor does it activate adenylate cyclase by a perturbation of membrane structure.

It should also be noted that another ligand, namely adenosine (10^{-5}M) was able to modulate the activity of the solubilised enzyme. In the absence of GTP, adenosine was found to inhibit forskolin-stimulated adenylate cyclase activity by 46.3% $\pm$ 8.8, for the membrane-bound state. Lubrol-solubilised adenylate cyclase was found to be similarly inhibited, i.e. 48.3% $\pm$ 3.4 inhibition, suggesting that adenosine inhibition is at the level of the catalytic unit P-site.

The effect of manipulating the membrane environment through the use of benzyl alcohol is reflected in the activity profiles shown in Figures 19-23. Concentrations of up to 50mM benzyl alcohol were employed, as over this range the anaesthetic has been shown to partition into biological membranes. At circa 50mM benzyl alcohol, it appears to elicit an increase in bilayer fluidity which is equivalent to a temperature rise of approximately 6-8^{o}C

in various model and biological membranes, including human platelets (Houslay & Gordon, 1983). Here the effect of the anaesthetic is examined at each level of stimulation.

Figure 19 shows that benzyl alcohol enhanced basal adenylate cyclase activity, such that 300% activity was observed at optimal anaesthetic concentrations i.e. a 3-fold increase in activity. An almost identical profile was obtained for NaF-activated adenylate cyclase, i.e. at the level of G_s (Fig.20). However, examining the effects of benzyl alcohol on receptor-stimulated adenylate cyclase yielded different profiles depending on which hormone was used. Figure 21 shows that where beta-adrenoceptor-stimulated activity was observed, through isoprenaline-stimulation, a similar profile to that observed previously was obtained. On the other hand, activation via the PGE_1-receptor, did not exhibit such a profile. Here activity was enhanced to only approximately 150% of original values (Fig.22). Indeed, looking at the effect of benzyl alcohol on the fold-stimulation these ligands exerted showed that the anaesthetic had little effect on the fold-stimulation (stimulated/basal activity ratio) of adenylate cyclase activity elicited by either isoprenaline or NaF (Table 6). However, the presence of benzyl alcohol decreased the fold-stimulation of adenylate cyclase activity by PGE_1. Examining the EC_{50} for PGE_1-activation showed it to be altered by the presence of benzyl alcohol; being 0.28 ± 0.09uM in the absence of benzyl alcohol, and 0.85 ± 0.02uM in the presence of benzyl alcohol. (Figures are mean $\pm$ S.D., for n=3 separate experiments using different membrane preparations).

From Figure 23 it can be seen that the profile obtained for benzyl alcohol action on forskolin-stimulated adenylate cyclase was different from that obtained for all other stimulatory

ligands. Here, increasing concentrations of benzyl alcohol led to a progressive inhibition of enzyme activity. When a lubrol-solubilised extract of platelet membrane was used, benzyl alcohol at concentrations up to 50mM, failed to affect basal adenylate cyclase activity (less than 5% change). In contrast, forskolin-stimulated soluble adenylate cyclase was still inhibited by the anaesthetic; a 31% reduction in activity was observed at 50mM. Benzyl alcohol exerted little effect on the EC_{50} value for activation of platelet adenylate cyclase by forskolin, which was 25 $\pm$ 7uM in the absence of benzyl alcohol, and 23 $\pm$ 10uM in its presence. (n=3, separate experiments using different membrane preparations). Figure 24 illustrates this point.

The effect of benzyl alcohol on forskolin-stimulated adenylate cyclase is thought to reflect a direct action of the local anaesthetic. This is because other methods of manipulating fluidity, e.g. by increasing temperature, gave rise to a forskolin profile which was identical to other stimulatory ligands (compare Figs.25,26).

It should be noted that benzyl alcohol, at the concentrations used in this study, had no effect on linearity (See Figs.27-29).

G_i-mediated effects on adenylate cyclase were examined under conditions where this inhibitory G-protein was activated either directly, through low concentrations of GppNHp, or indirectly, via occupancy of the $alpha_2$-adrenoceptor. Thus, in one instance, adenylate cyclase was first stimulated by PGE_1 ($2x10^{-5}$M), and inhibition was then achieved through the $alpha_2$-adrenoceptor route by the use of adrenaline (100uM) in the

presence of propranolol (10uM) to block beta-adrenoceptor functioning. This resulted in a 79.5 ± 1.1% inhibition of activity (n=5; results are mean ± S.D.). Alternatively, forskolin was used to enhance the activity of the catalytic unit, and inhibition was elicited by using either adrenaline/propranolol (50.0 ± 3.55% inhibition), or low concentrations of GppNHp (0.01uM), which selectively activate G_i (53.5 ± 5.0% inhibition).

When either GppNHp or adrenaline (+ propranolol) was used to inhibit forskolin-stimulated adenylate cyclase, then increasing benzyl alcohol concentrations had no discernible effect on the degree of inhibition elicited (Figs.30,31 respectively). Indeed, the IC_{50} for inhibition of forskolin-stimulated adenylate cyclase by GppNHp was 0.20 ± 0.04uM in the absence of benzyl alcohol, and 0.28 ± 0.05uM in the presence of 50mM anaesthetic (n=3; results are mean ± S.D.). In contrast to this however, increasing benzyl alcohol concentrations diminished the ability of adrenaline (+ propranolol) to inhibit PGE_1-stimulated enzyme activity (Fig.32). The attenuating effect of benzyl alcohol exhibited an IC_{50} of 12.5 ± 4.2mM (n=6; results are mean ± S.D.). At 50mM benzyl alcohol, the inhibitory effect of adrenaline (+ propranolol) was decreased from a 79% inhibition of activity to approximately 52% inhibition of activity. Examining the effect of benzyl alcohol on adrenaline-stimulated GTPase activity showed that no detectable change in activity could be recorded at concentrations of anaesthetic in the range 0-50mM (Fig.33). The IC_{50} was observed to increase from 57 ± 15uM in the absence of benzyl alcohol, to 130 ± 38uM in its presence (n=4 separate experiments using different membrane preparations. Figures are mean ± S.D.; results are significantly different at $p < 0.01$).

The presence of manganese (in excess of 1mM) in an assay is believed to prevent G_i from inhibiting adenylate cyclase (Hoffmann et al., 1981). This condition is employed here with regard to the effect of benzyl alcohol on adrenaline (+ propranolol) inhibition of PGE_1, i.e. to ensure that the observed phenomenon is due to an effect at the inhibitory G_i component. When adenylate cyclase assays were performed in the presence of Mn^{2+} (5mM) rather than Mg^{2+} (5mM), the PGE_1-stimulated activity was increased by some 2.01-fold (Table 7). Here the presence of adrenaline (+ propranolol) in the assay elicited only a 16 ± 4% inhibition of PGE_1-stimulated activity, compared with 79 ± 5% seen in its absence (Fig.32). Both of these observations are compatible with a loss of functional G_i. Indeed, when Mn^{2+} was used in the assay, the dose-effect curves for PGE_1-stimulated activity to benzyl alcohol were very similar, whether or not adrenaline (+ propranolol) was present (Fig.34). Such observations not only apply to adrenaline-mediated inhibition of PGE_1 but can be extended to adrenaline- and GppNHp-mediated inhibition of forskolin also. In fact, on average, the degree of inhibition elicited by these ligands was reduced by 75% ± 16.8 (results are mean ± S.D., for n=8 separate experiments using different membrane preparations) by replacing the divalent cation in this manner. Similarly, from Table 7 it can be seen that the Mn/Mg ratio for forskolin was augmented to a similar extent to that witnessed with PGE_1. The addition of benzyl alcohol altered the Mn/Mg ratios significantly; PGE_1 ratio was reduced, whilst that for forskolin-stimulated activity was enhanced (see Table 7).

Once again, to be certain that the effects of benzyl alcohol on inhibitory aspects of regulation were due to the anaesthetic, it was attempted to manipulate the membrane environment

through increasing temperature by an amount equivalent to that change expected to be exerted by benzyl alcohol, i.e. 6-8^{o}C. Here, no such decrease in the ability of adrenaline to attenuate PGE_1-stimulated activity was observed (Table 8).

All of the actions of benzyl alcohol were reversible upon dilution or washing the membranes to remove this ligand.

It has been proposed that treatment with benzyl alcohol may lead to a loss of components of the G_i system from the membrane. Here the result of immunoblotting is shown in Figure 35. No change (less than 8%) occurred in the amount of alpha-G_i present in platelet membranes treated with 50mM benzyl alcohol in the presence or absence of PGE_1 and adrenaline (+ propranolol). Similarly, no immunoreactive material was found to be released into the supernatant fractions of platelet membranes treated with 50mM benzyl alcohol. Essentially identical results were found by using the antiserum to detect G-protein beta-subunits.

Figure 15 Forskolin-stimulated activation of adenylate cyclase

Values are mean ± S.D. of a single representative experiment employing triplicate observations.

Activity is shown as pmoles of cAMP/min/100ul assay.

n=3 separate experiments using different membrane preparations.

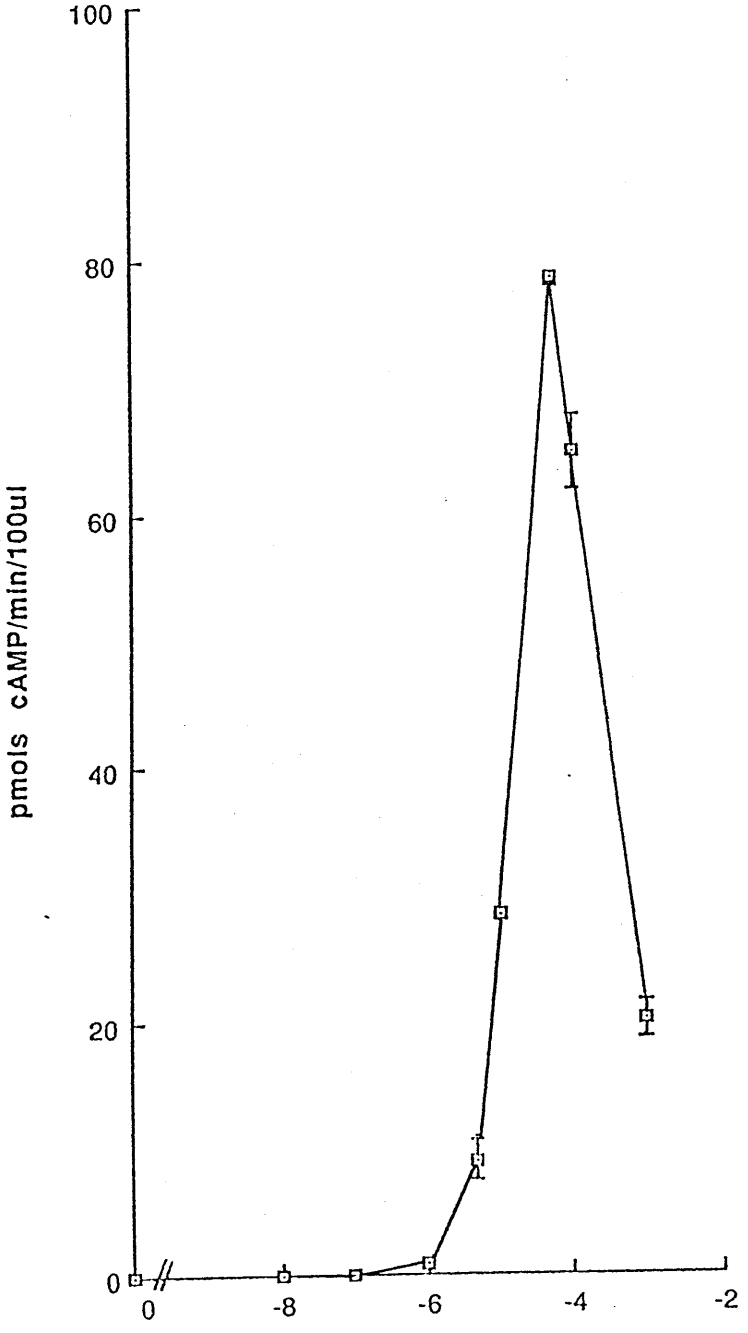

100
80
60
40
20
0
pmols cAMP/min/100ul
0
-8
-6
-4
-2
Log[Forskolin] M

Figure 16 Sodium-fluoride modulation of basal adenylate cyclase activity

Figures are mean ± S.D. of a single representative experiment employing triplicate observations.

Activity is shown as pmoles cAMP/min/100ul assay.

n=3 separate experiments using different membrane preparations.

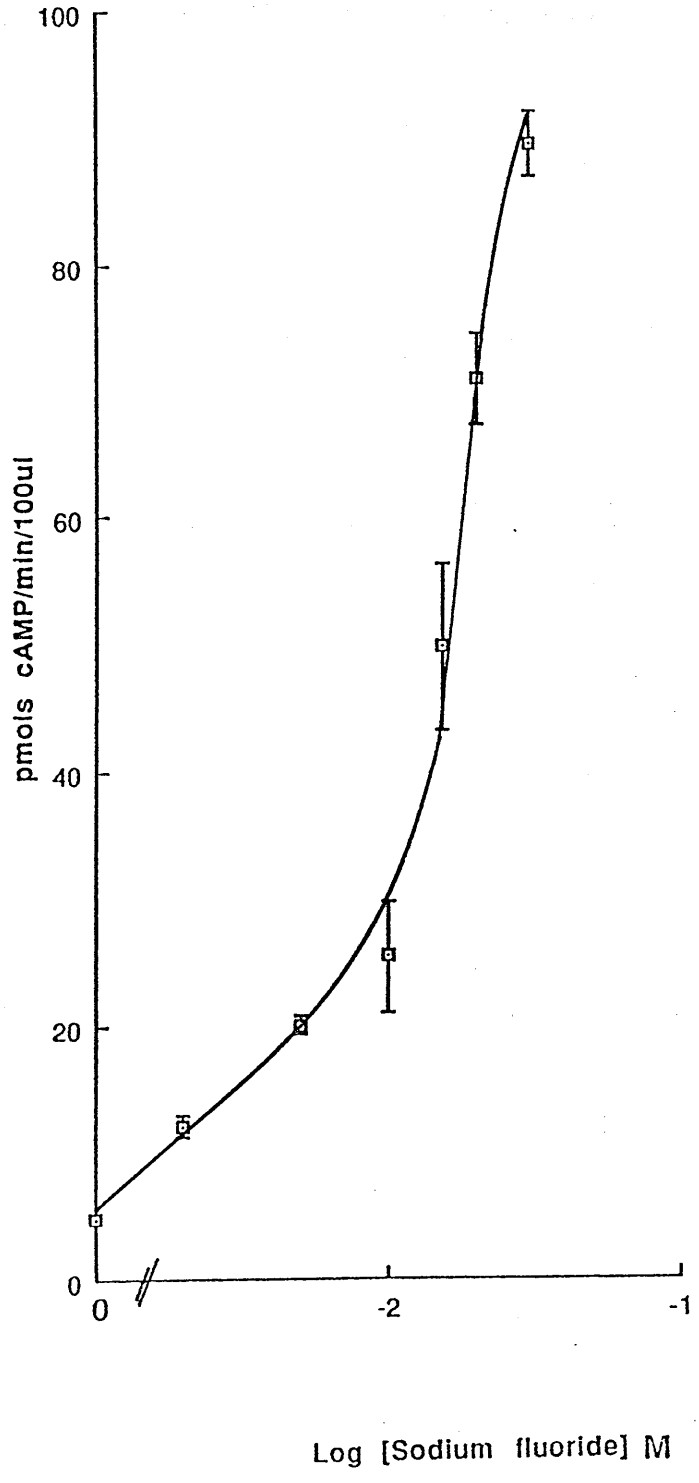

100
80
60
40
20
0
pmols cAMP/min/100ul
0
-2
-1
Log [Sodium fluoride] M

Figure 17 Sodium-fluoride modulation of prostaglandin-E_1-stimulated adenylate cyclase activity

Figures are mean ± S.D. of a single representative experiment employing triplicate observations.

Activity is shown as pmoles cAMP/min/100ul assay.

n=3 separate experiments using different membrane preparations.

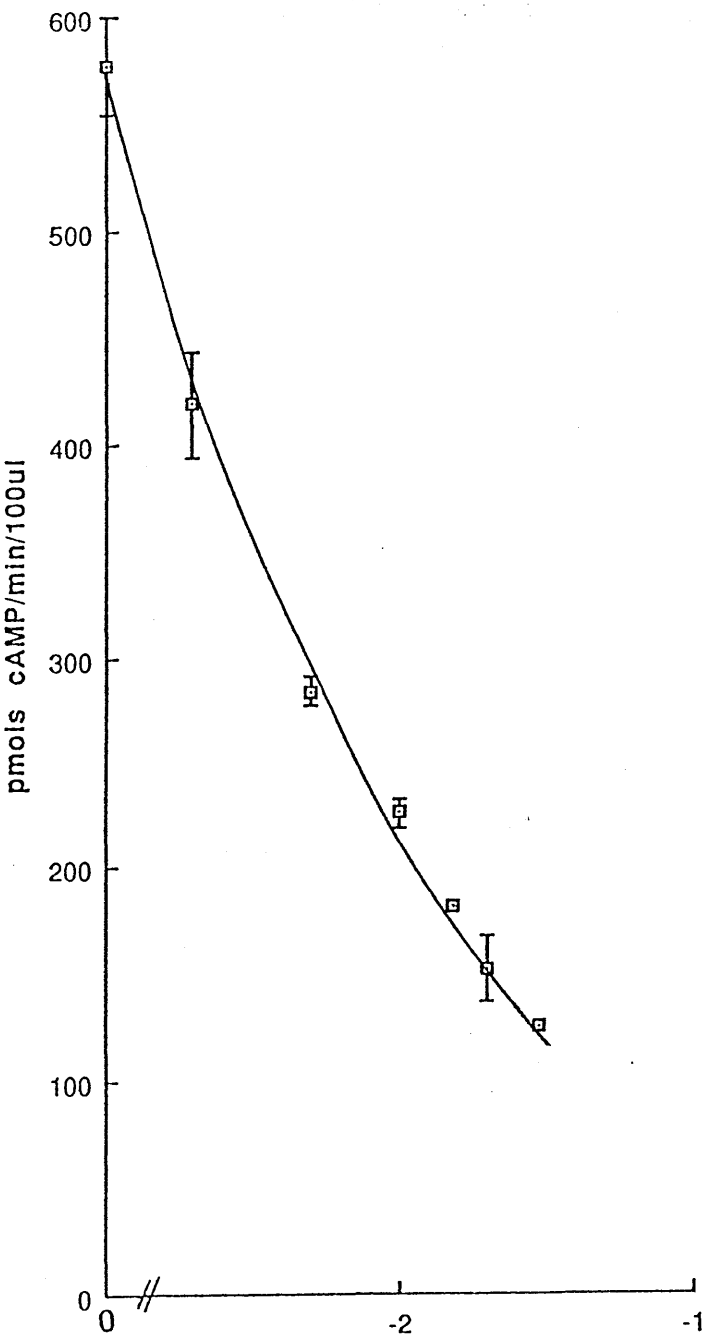

600
500
400
300
200
100
0
pmols cAMP/min/100ul
0
-2
-1
Log [Sodium fluoride] M

Figure 18 Prostaglandin-E_1-mediated activation of adenylate cyclase

Figures are mean ± S.D. of a single representative experiment employing triplicate observations.

Activity is shown as pmoles cAMP/min/100ul assay.

n=3 separate experiments using different membrane preparations.

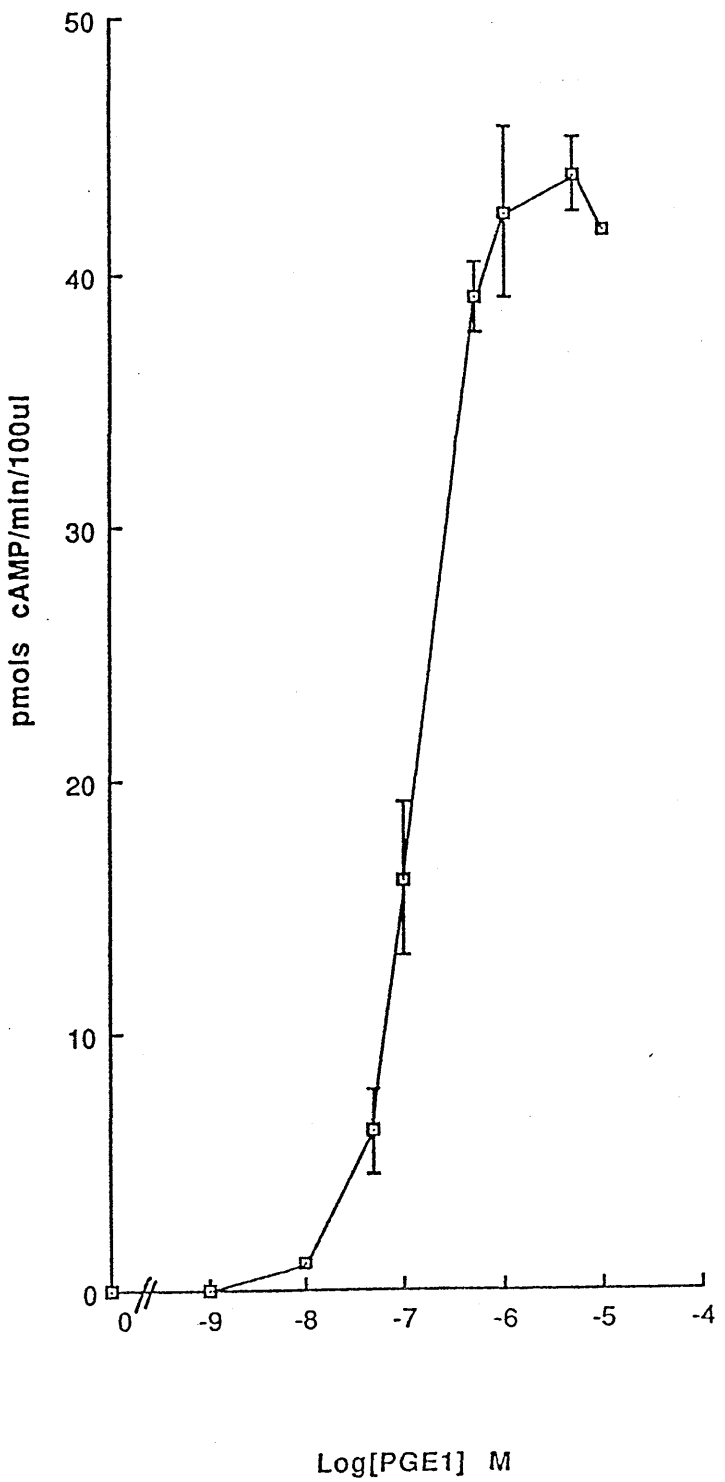

50
40
30
20
10
0
pmols cAMP/min/100ul
0
-9
-8
-7
-6
-5
-4
Log[PGE1] M

Figure 19 Action of benzyl alcohol on basal adenylate cyclase activity

Results are means ± S.D. for n=6 separate experiments using different platelet membrane preparations. Specific activity is given in Table 4.

Activity is shown as percentage of that observed in the absence of benzyl alcohol (control) i.e. 100%.

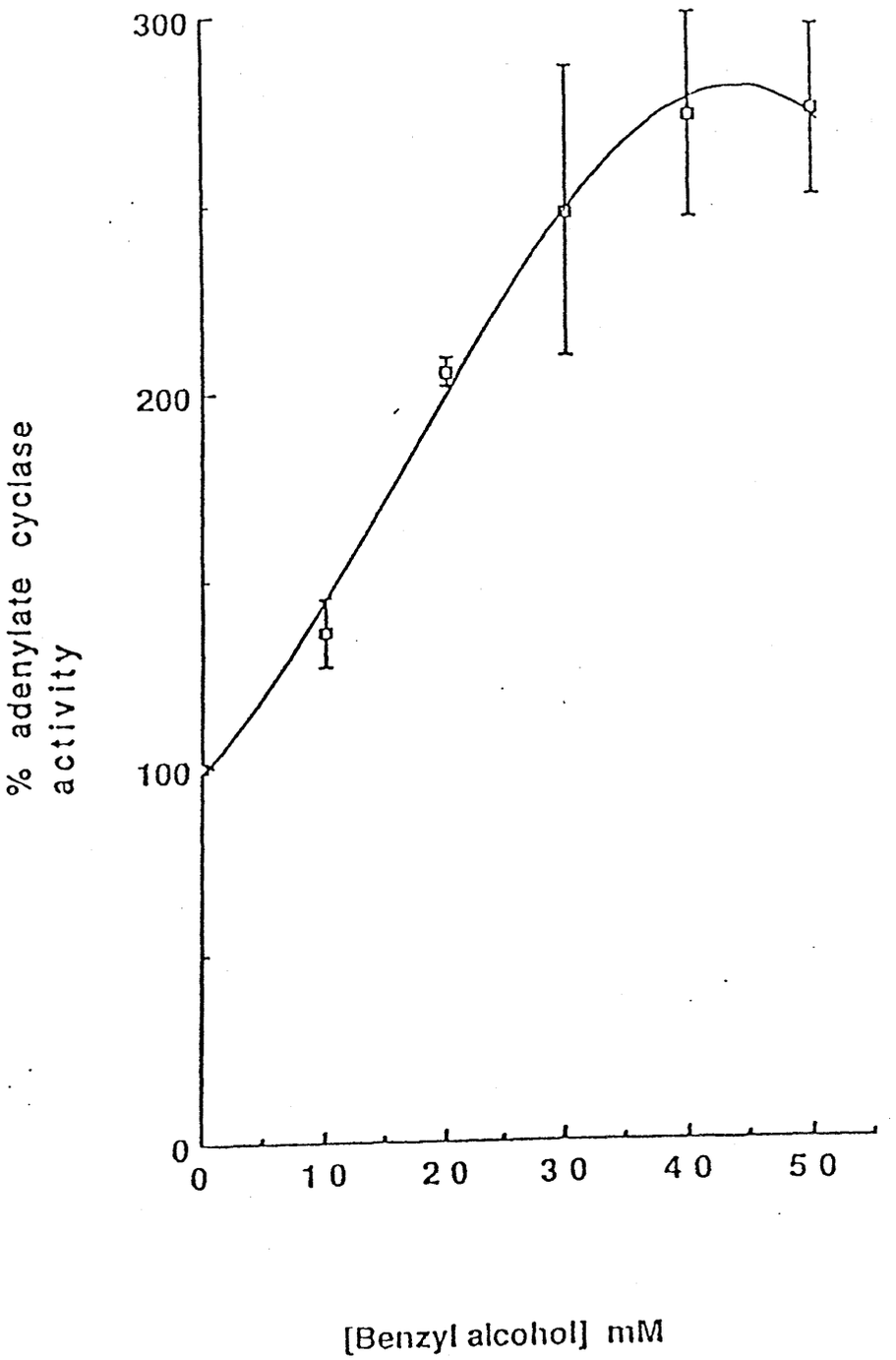

300
200
100
0
% adenylate cyclase activity
0
10
20
30
40
50
[Benzyl alcohol] mM

Figure 20 Action of benzyl alcohol on NaF-stimulated adenylate cyclase activity

Results are means ± S.D. for n=6 separate experiments using different platelet membrane preparations. Specific activity is given in Table 4.

Activity in the absence of added benzyl alcohol (control) is shown as 100%.

Concentration of NaF is 15mM.

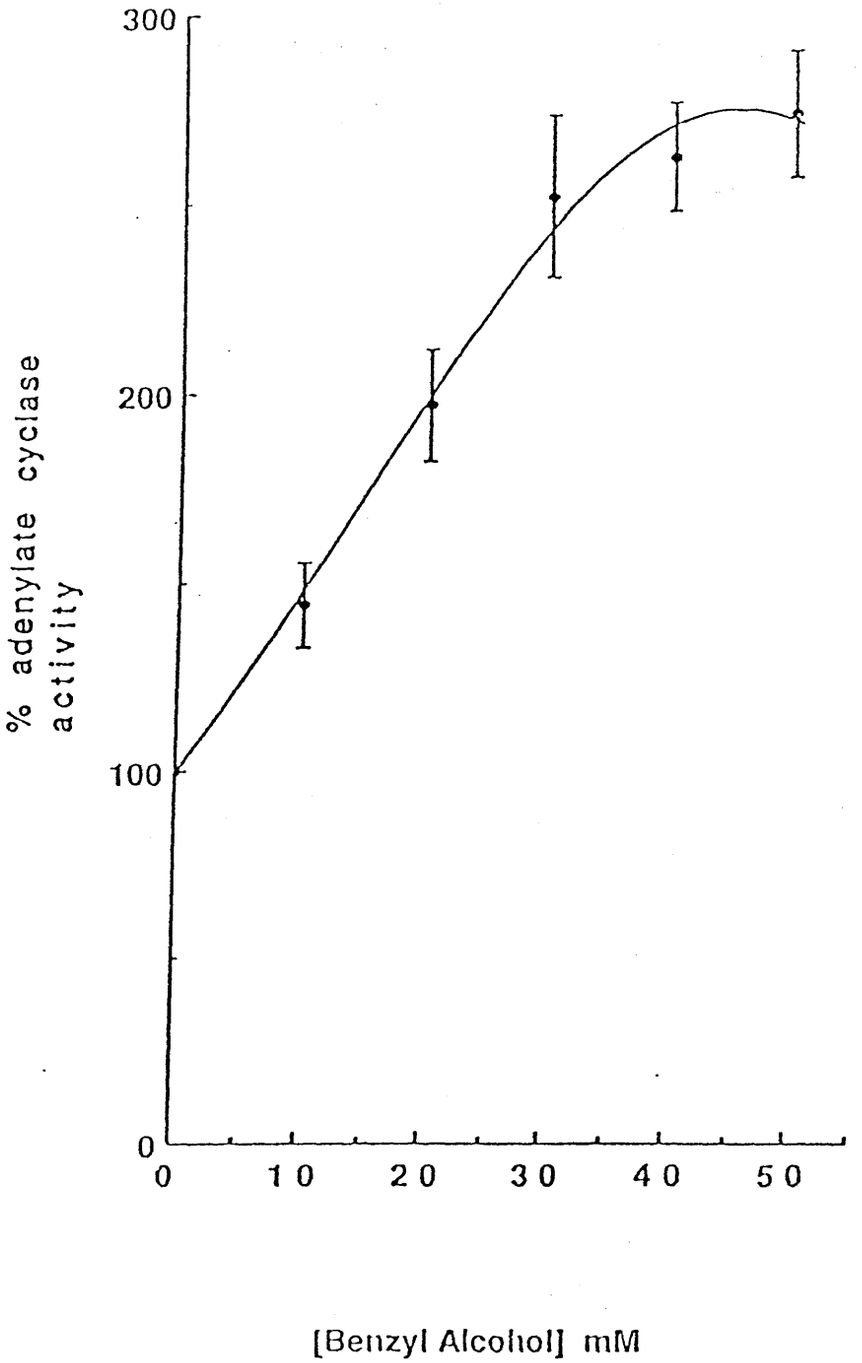

300
200
100
0
% adenylate cyclase activity
0
10
20
30
40
50
[Benzyl Alcohol] mM

Figure 21 Action of benzyl alcohol on isoprenaline-stimulated adenylate cyclase activity

Results are means ± S.D. for n=6 separate experiments using different platelet membrane preparations. Specific activity is given in Table 4. Activity in the absence of added benzyl alcohol (control) is shown as 100%.

Concentration of isoprenaline is 10^{-4}M.

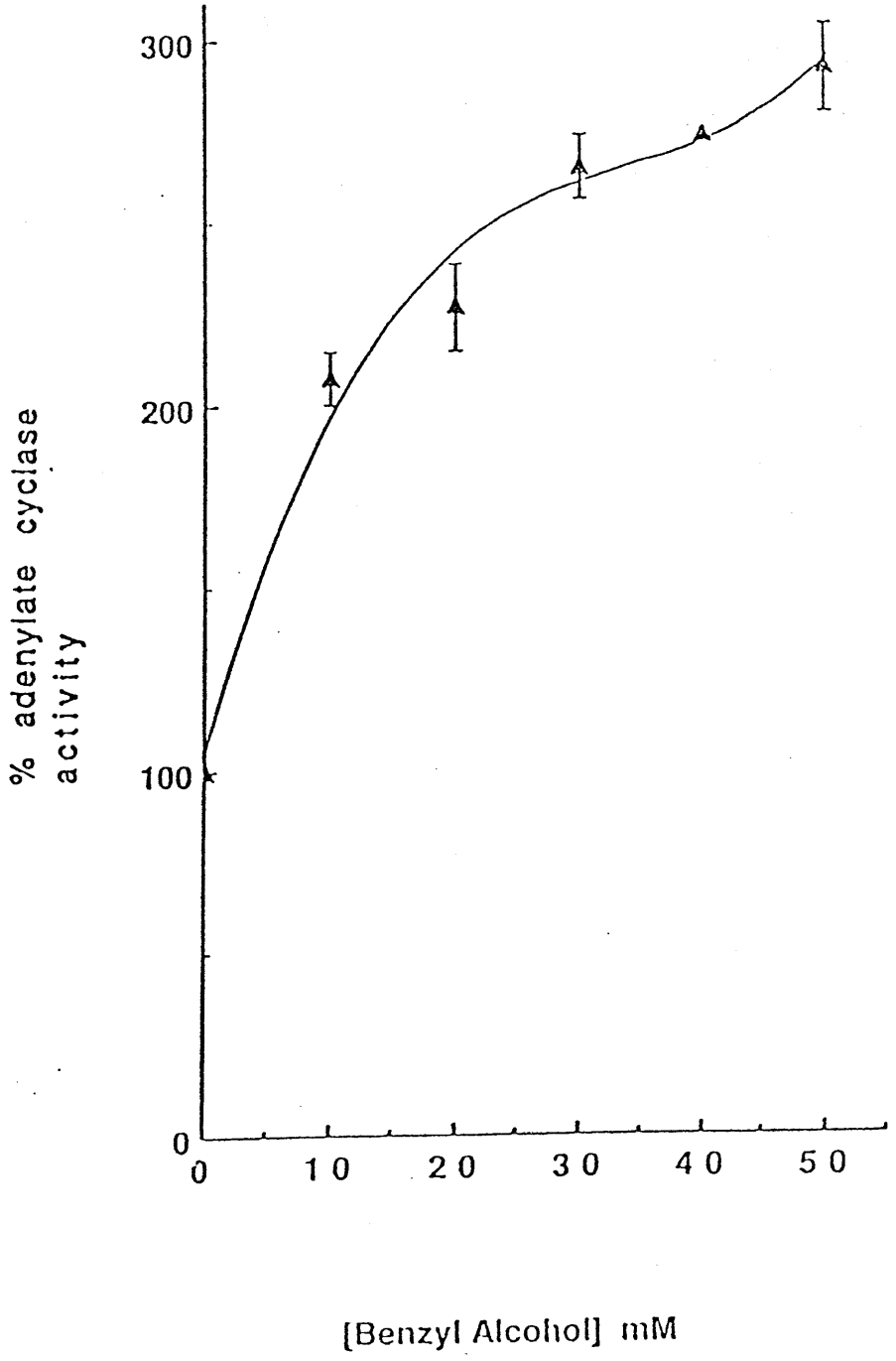

300
200
100
0
% adenylate cyclase activity
0
10
20
30
40
50
[Benzyl Alcohol] mM

Figure 22 Action of benzyl alcohol on prostaglandin-E_1-stimulated adenylate cyclase activity

Results are means ± S.D. for n=6 separate experiments using different platelet membrane preparations. Specific activity is given in Table 4. Activity in the absence of added benzyl alcohol (control) is shown as 100%.

Concentration of PGE_1 is 2×10^{-5}M.

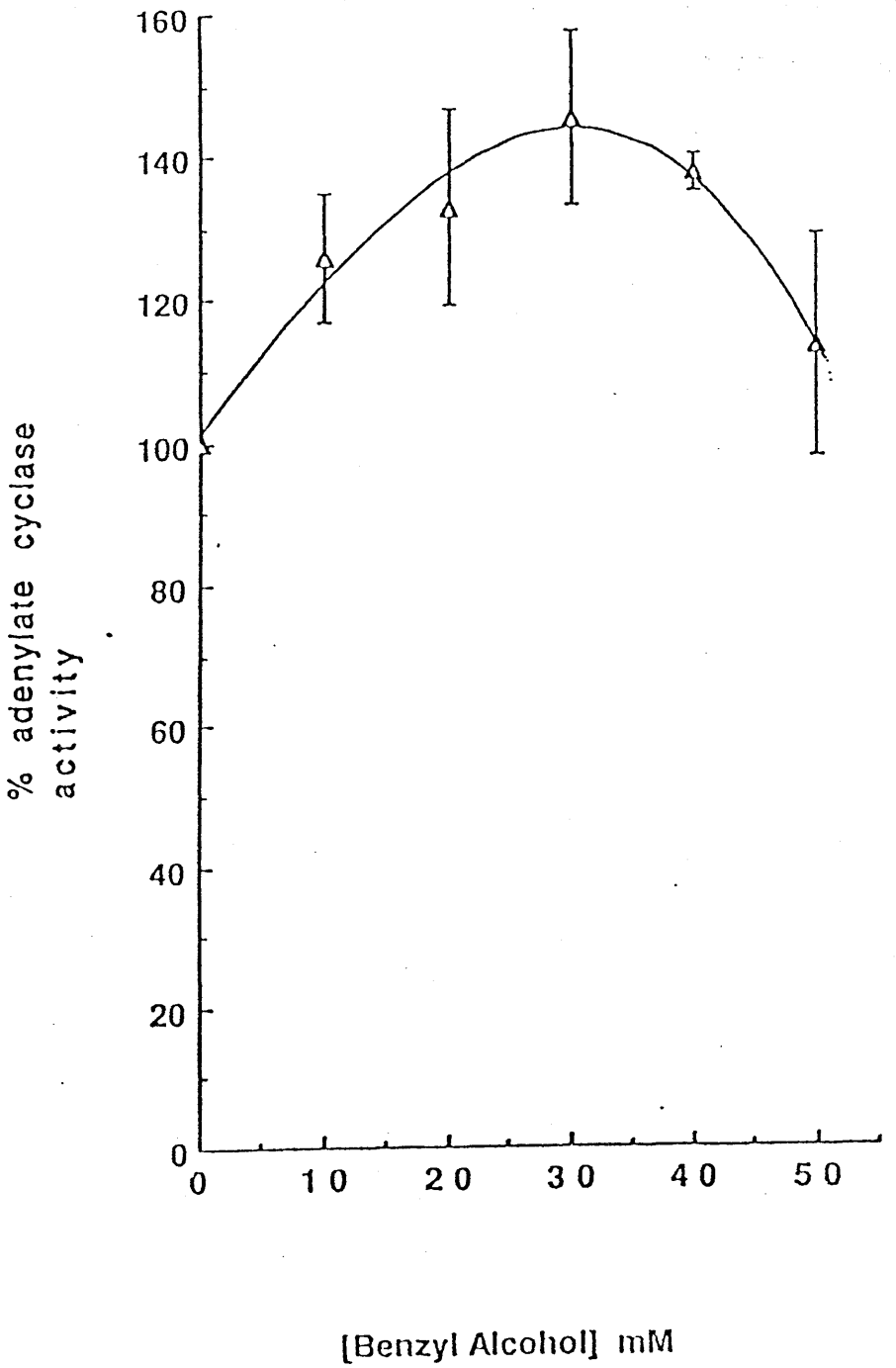

160
140
120
100
80
60
40
20
0
% adenylate cyclase activity
0
10
20
30
40
50
[Benzyl Alcohol] mM

Figure 23 Action of benzyl alcohol on forskolin-stimulated adenylate cyclase activity

Results are means ± S.D. for n=6 separate experiments using different platelet membrane preparations. Specific activity is given in Table 4. Activity in the absence of added benzyl alcohol (control) is shown as 100%.

Concentration of forskolin is 10^{-4}M.

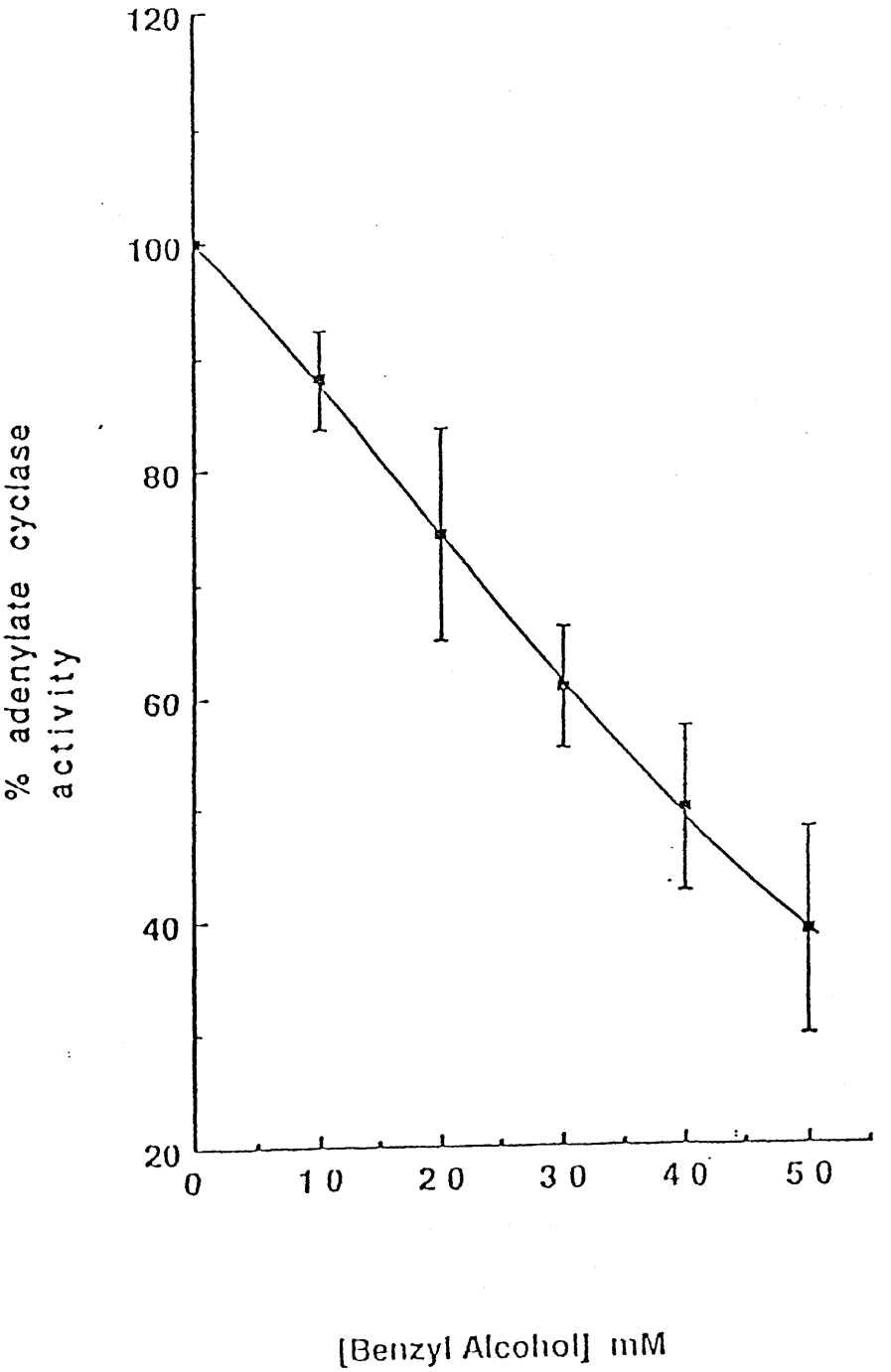

120
100
80
60
40
20
0
10
20
30
40
50
% adenylate cyclase activity
[Benzyl Alcohol] mM

Figure 24 Effect of benzyl alcohol on forskolin dose effect curve

Figures are mean ± S.D. of a single representative experiment employing triplicate observations.

Activity is shown as pmoles cAMP/min/100ul assay.

n=3 separate experiments using different membrane preparations.

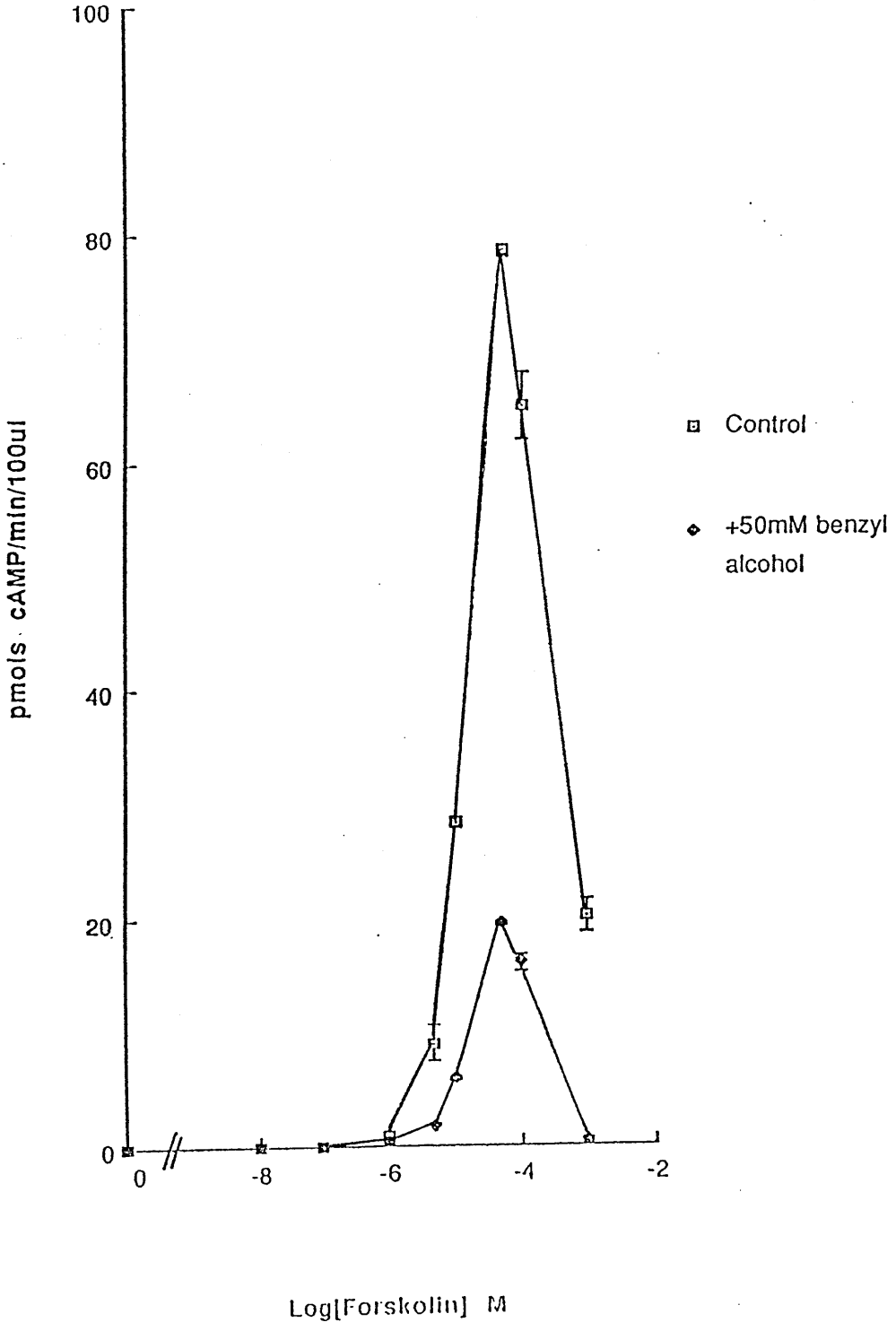

100
80
60
40
20
0
pmols cAMP/min/100ul
0
-8
-6
-4
-2
Log[Forskolin] M
Control
+50mM benzyl alcohol

Figure 25 Effect of increasing temperature on forskolin-stimulated adenylate cyclase activity

Figures are mean ± S.D. for n=3 separate experiments employing different membrane preparations.

Activity is shown as pmoles cAMP/min/100ul assay.
Concentration of forskolin is 10^{-4}M.

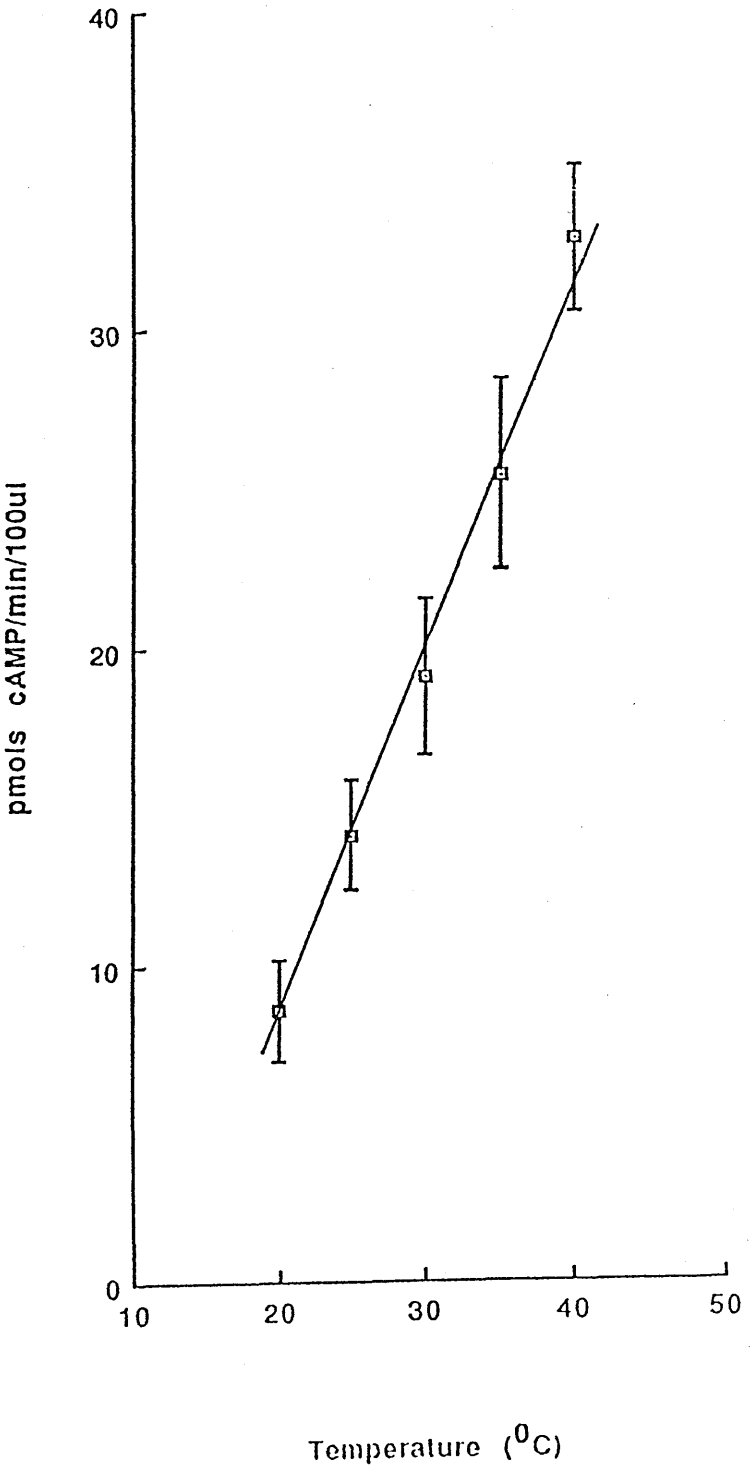

40
30
20
10
0
pmols cAMP/min/100ul
10
20
30
40
50
Temperature (°C)

Figure 26 Effect of increasing temperature on prostaglandin-E_1-stimulated adenylate cyclase activity

Figures are mean ± S.D. for n=3 separate experiments employing different membrane preparations.

Activity is shown as pmole cAMP/min/100ul assay.
Concentration of PGE_1 is 2×10^{-5}M.

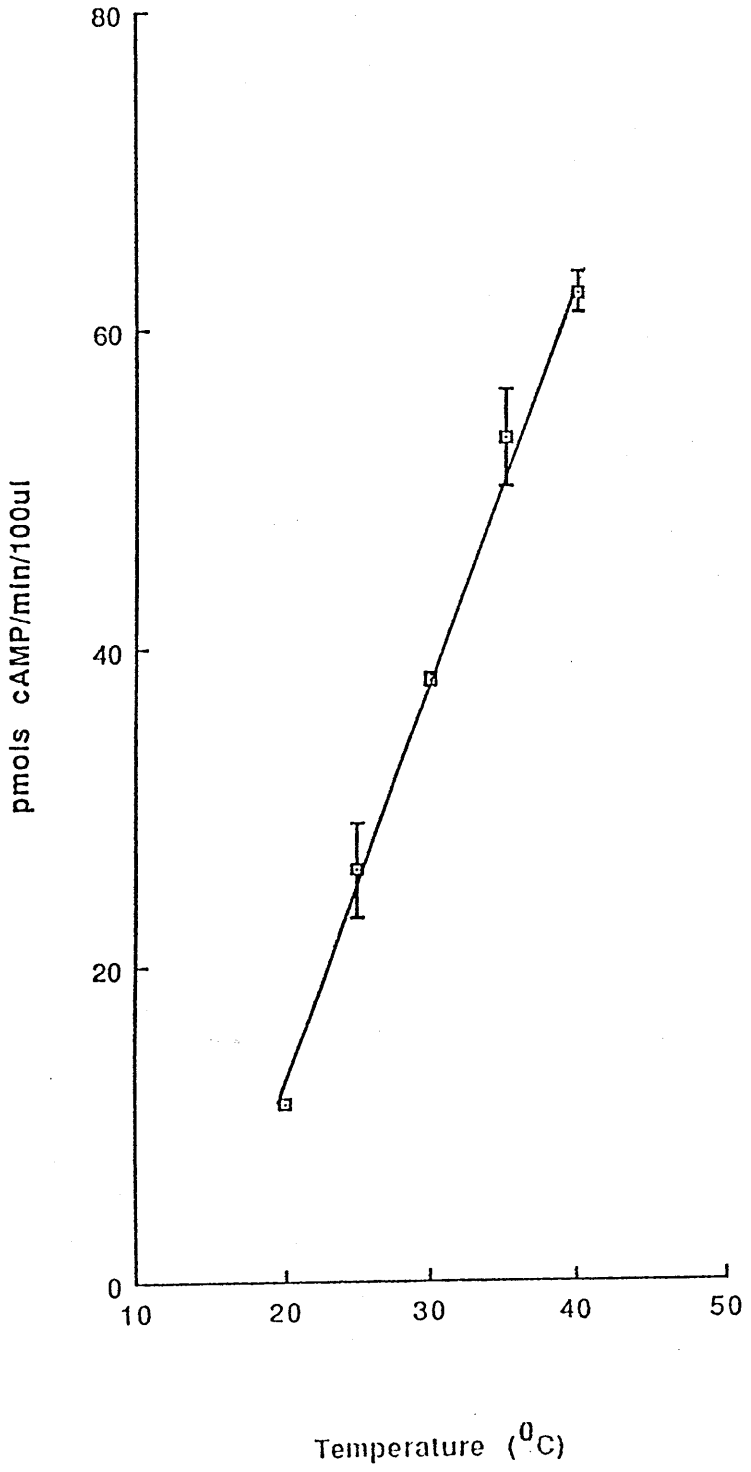

80
60
40
20
0
10
20
30
40
50
pmols cAMP/min/100ul
Temperature (^{0}C)

Figure 27 Effect of benzyl alcohol (50mM) on the linearity of the adenylate cyclase reaction

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Activity is shown as pmoles/min/100ul assay.

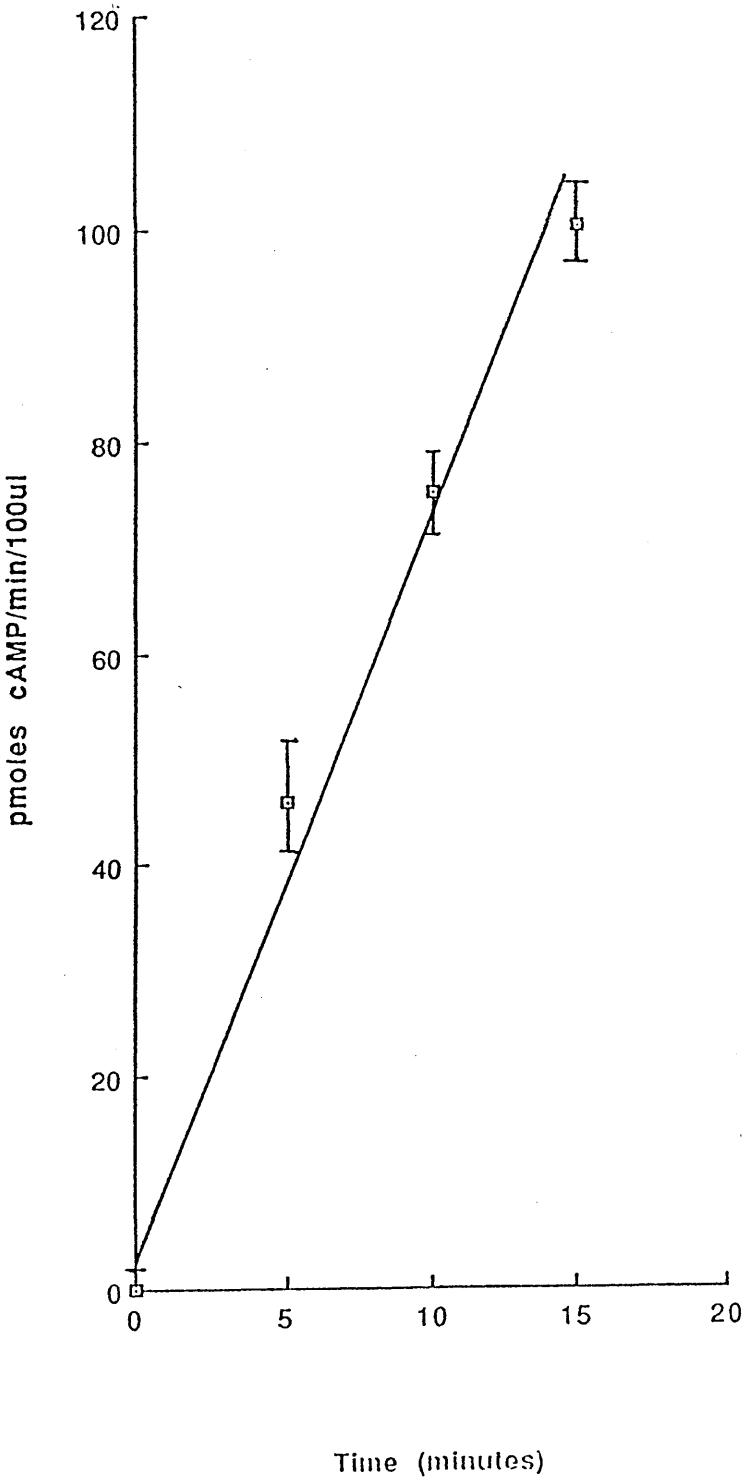

120
100
80
60
40
20
0
pmoles cAMP/min/100ul
0
5
10
15
20
Time (minutes)

Figure 28 Effect of benzyl alcohol (50mM) on the linearity of G_s-coupled adenylate cyclase activity

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Activity is shown as pmoles/min/100ul assay.

Concentration of NaF is 15mM.

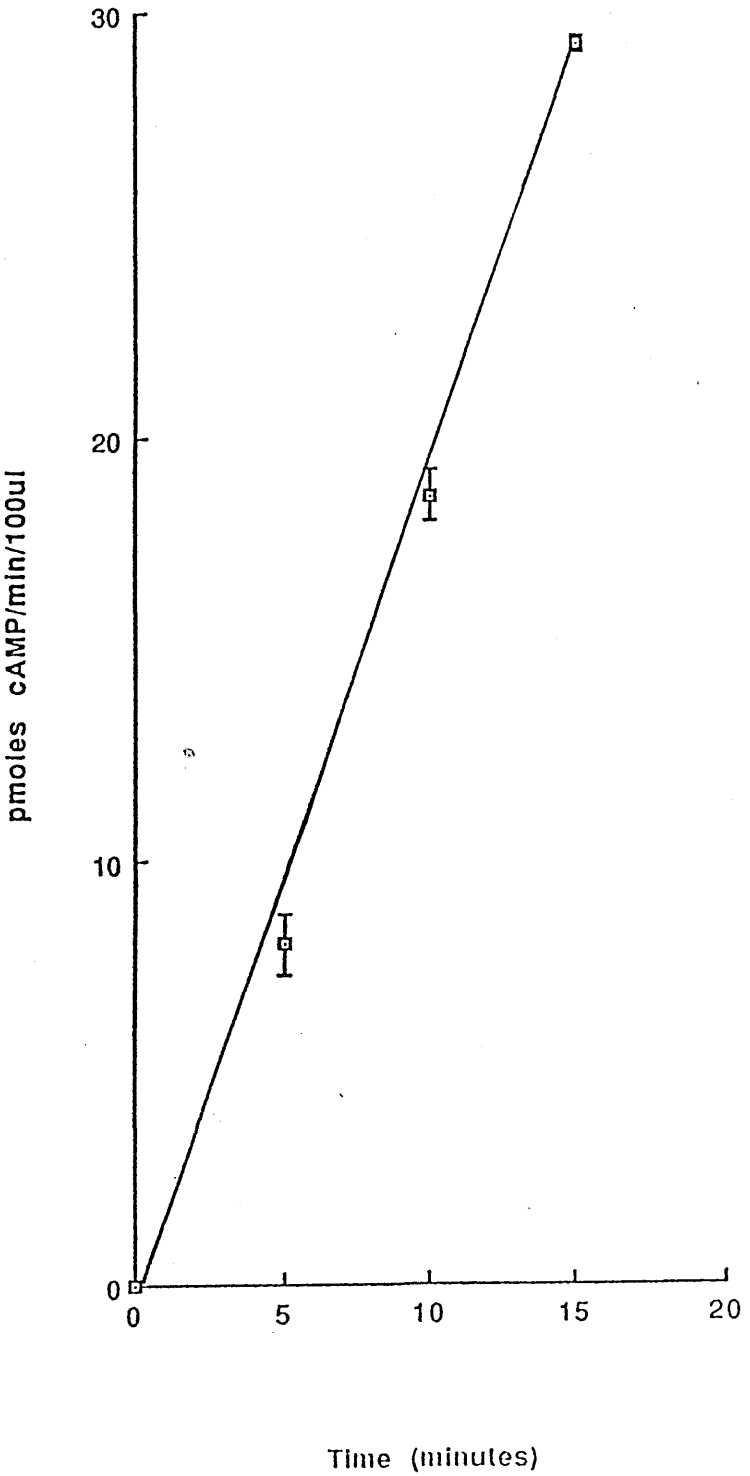

30
20
10
0
pmoles cAMP/min/100ul
0
5
10
15
20
Time (minutes)

Figure 29 Effect of benzyl alcohol (50mM) on the linearity of R_s-G_s-coupled adenylate cyclase activity

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Activity is shown as pmoles cAMP/min/100ul assay.

Concentration of PGE_1^- is 2 x 10^{-5}M.

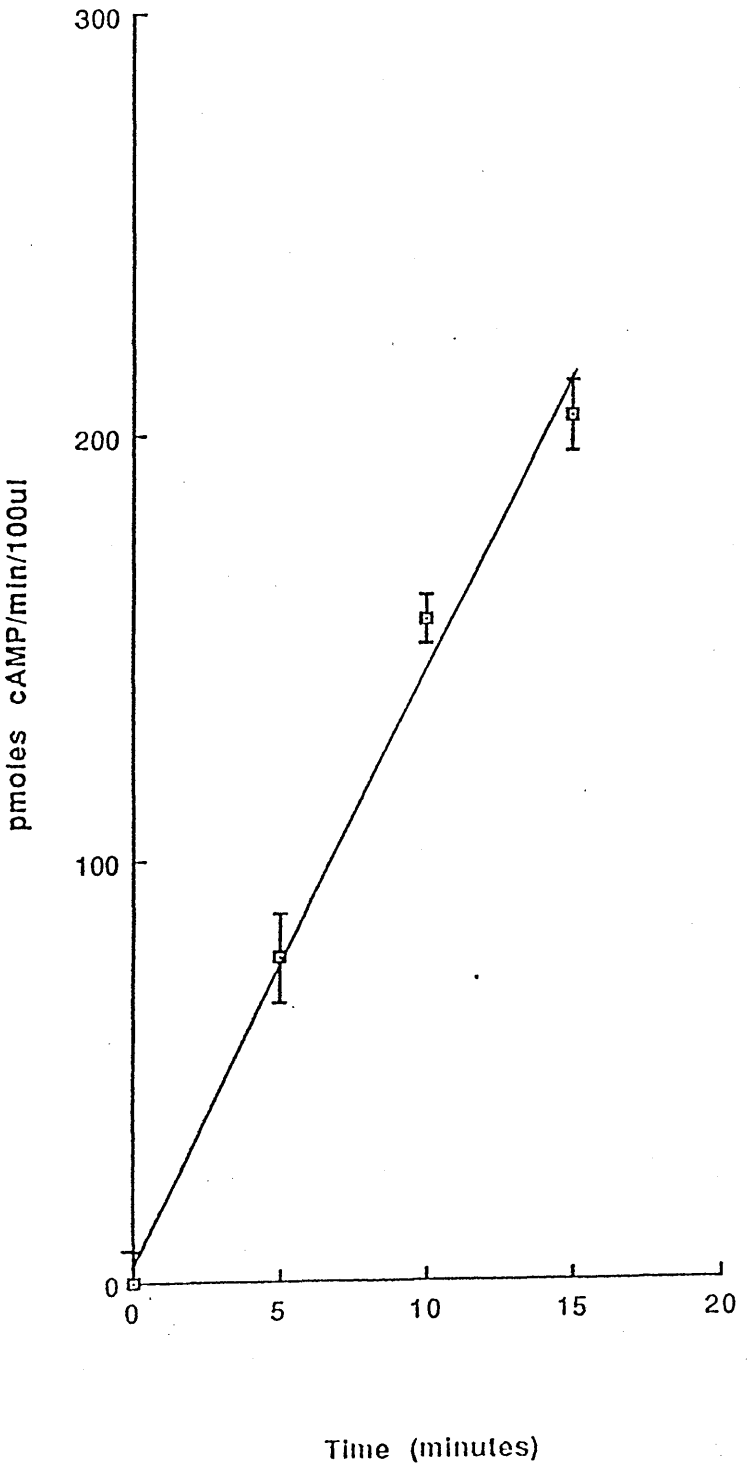

300
200
100
0
pmoles cAMP/min/100ul
0
5
10
15
20
Time (minutes)

Figure 30 Action of benzyl alcohol on GppNHp-mediated inhibition of forskolin-stimulated adenylate cyclase activity

Effect of benzyl alcohol is shown on the percentage inhibition of forskolin (10^{-4}M)-stimulated activity elicited by low concentrations (10^{-8}M) of p[NH]ppG.

Results are means $\pm$ S.D. for n=6 separate epxeriments using different membrane preparations.

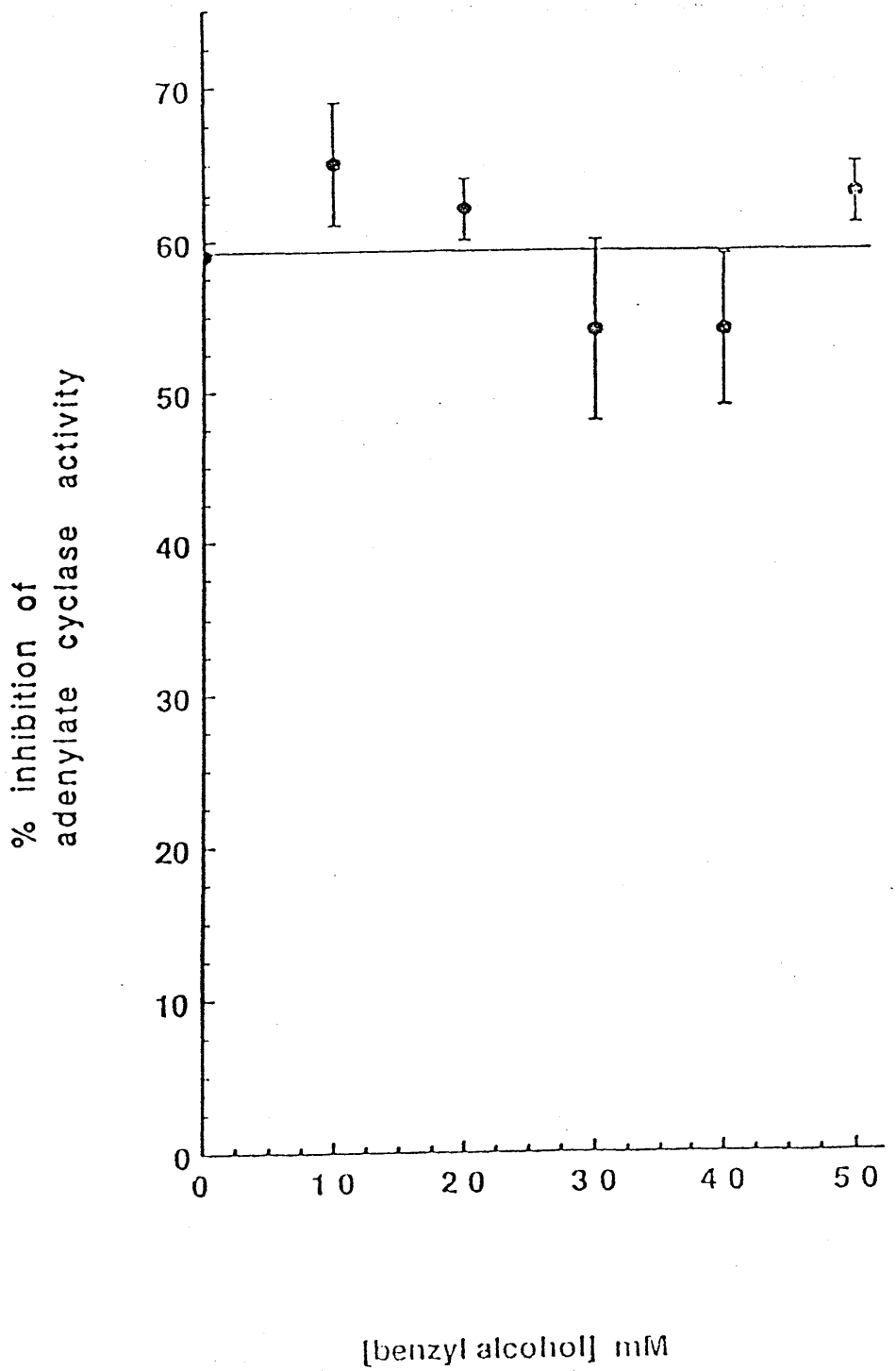

% inhibition of
adenylate cyclase activity
70
60
50
40
30
20
10
0
0
10
20
30
40
50
[benzyl alcohol] mM

Figure 31 Action of benzyl alcohol on adrenaline (+ propranolol)-mediated inhibition of forskolin-stimulated adenylate cyclase activity

Effect of benzyl alcohol is shown on the percentage inhibition of forskolin (10^{-4}M)-stimulated activity elicited by adrenaline (10^{-4}M)in the presence of propranolol (10^{-5}M).

Results are means $\pm$ S.D. for n=6 separate experiments using different membrane preparations.

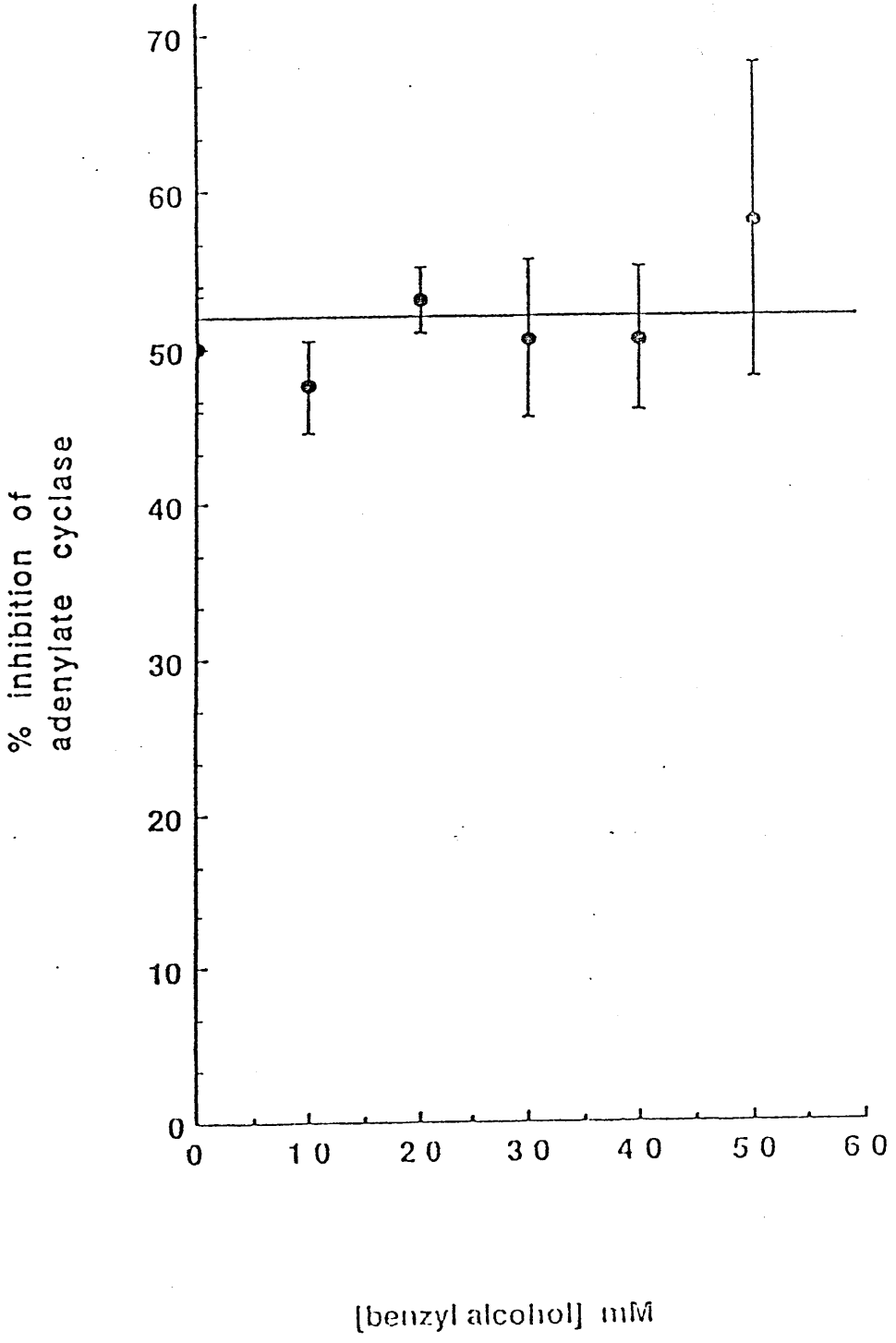

70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
% inhibition of adenylate cyclase
[benzyl alcohol] mM

Figure 32 Action of benzyl alcohol on adrenaline (+ propranolol)-mediated inhibition of PGE_1-stimulated adenylate cyclase activity

Effect of benzyl alcohol is shown on the percentage inhibition of PGE_1 (2×10^{-5}M)-stimulated activity elicited by adrenaline (10^{-4}M) in the presence of propranolol (10^{-5}M).

Results are means $\pm$ S.D. for n=6 separate experiments using different membrane preparations.

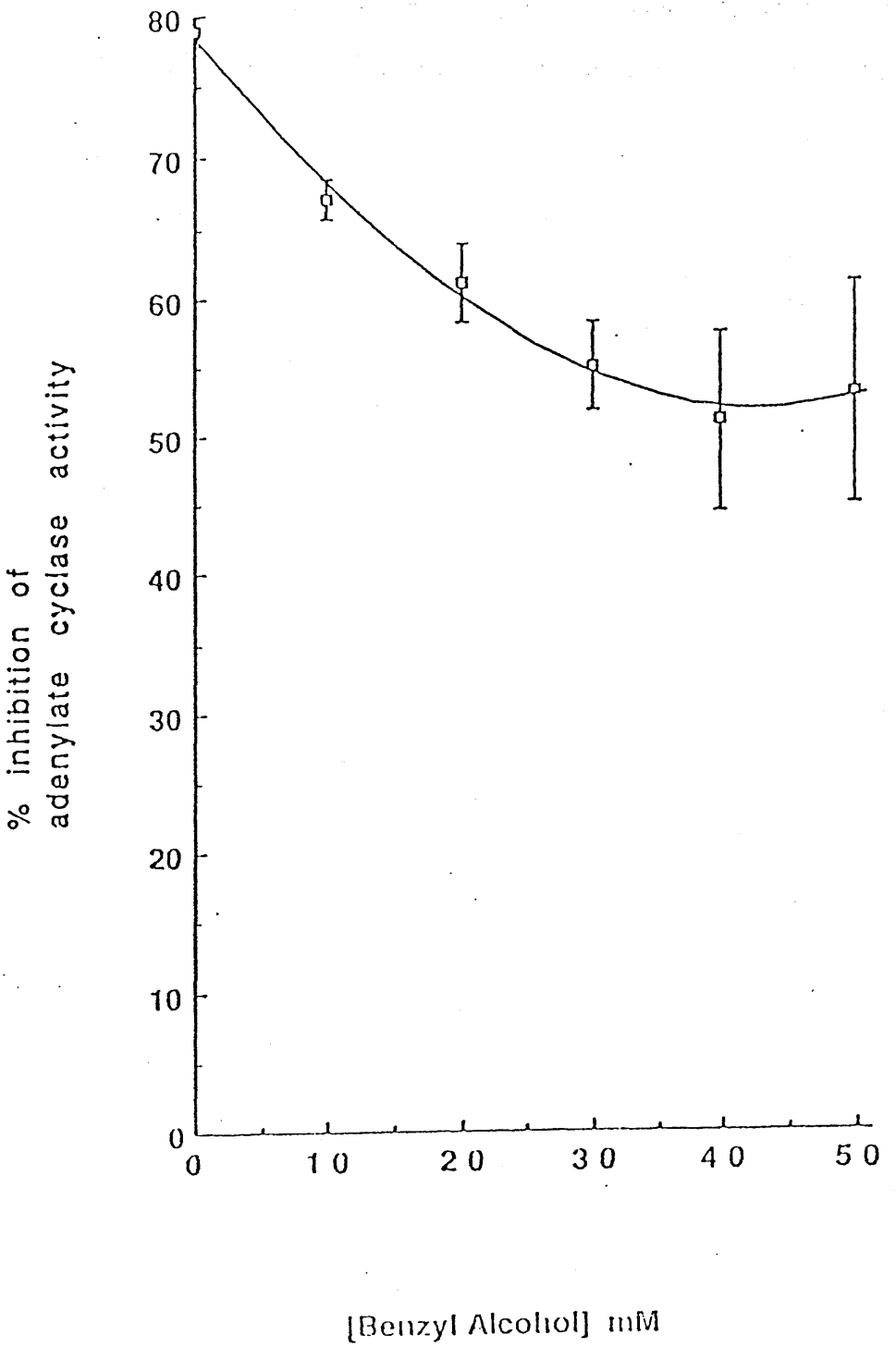

% inhibition of adenylate cyclase activity
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
[Benzyl Alcohol] mM

Figure 33 Effect of benzyl alcohol on adrenaline-stimulated GTPase activity in human platelets

Figures are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Adrenaline (10^{-4}M) is in the presence of 10^{-5}M propranolol

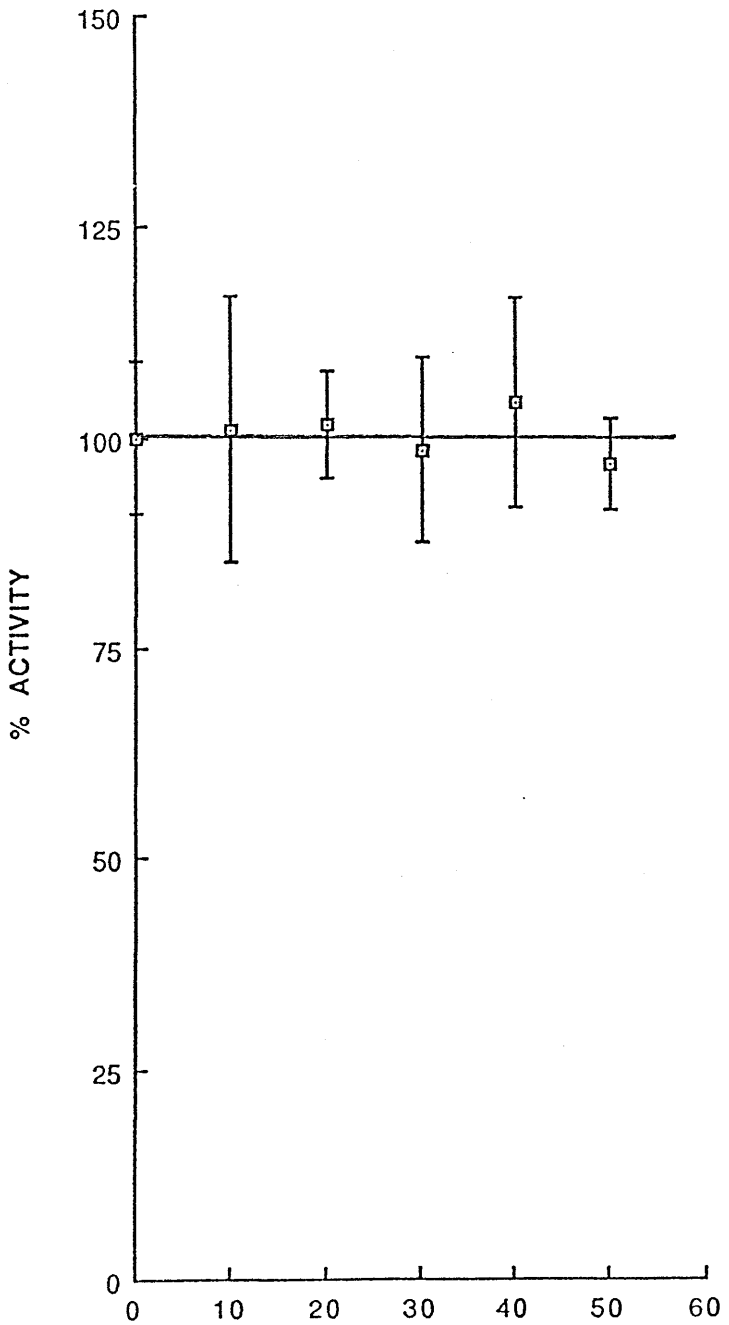

150
125
100
75
50
25
0
% ACTIVITY
0
10
20
30
40
50
60
[BENZYL ALCOHOL] mM

Figure 34 Action of benzyl alcohol on adrenaline (+ propranolol)-mediated inhibition of PGE_1-stimulated adenylate cyclase activity, in the presence of manganese

Effect of benzyl alcohol on the adenylate cyclase activity (pmol/min/100ul assay) profiles for PGE_1-stimulated activity in the presence of either Mg^{2+} (○,□) or Mn^{2+} (●,■) is shown for experiments done in either the presence (□,■) or absence (○,●) of adrenaline (10^{-4}M) + propranolol (10^{-5}M).

Results are means ± S.D. for n=6 separate experiments using different membrane preparations.

Concentration of PGE_1 is 2 x 10^{-5}M.

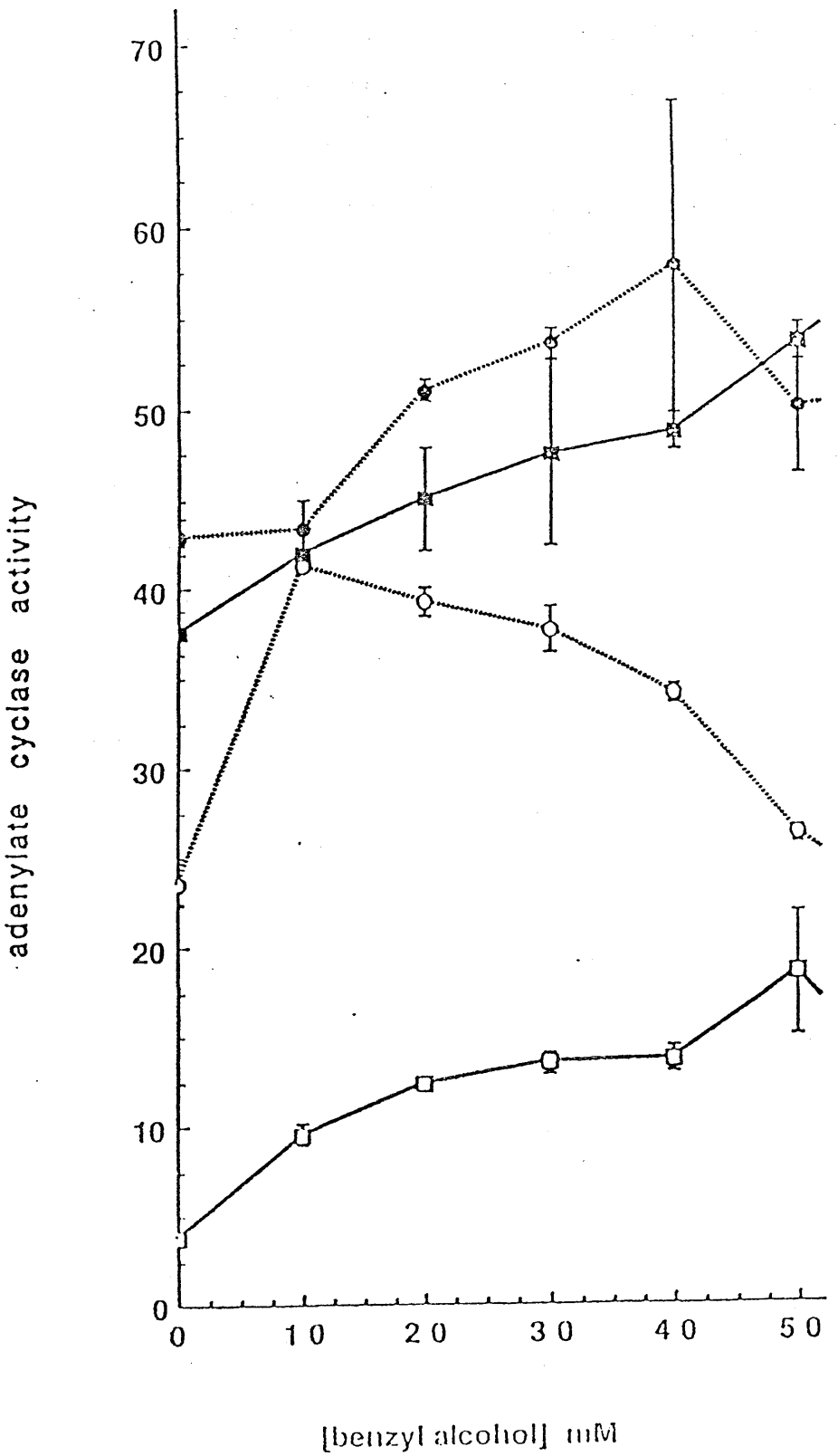

adenylate cyclase activity
70
60
50
40
30
20
10
0
0
10
20
30
40
50
[benzyl alcohol] mM

Figure 35 Immunoblotting of platelet membranes

After incubation of platelet membranes either with or without benzyl alcohol (50mM) under the conditions specified in Materials and Methods, Section 2.17, membranes and the supernatant fraction were isolated. Immunoblotting of these fractions was performed using antiserum RV6 for G-protein beta-subunits and antiserum AS7 for alpha-G_i subunits. Data shown is a typical experiment of one done three times with different membrane preparations. In the experiments shown, incubations were done in the presence of PGE_1 ($2x10^{-5}M$), adrenaline ($10^{-4}M$) and propranolol ($10^{-5}M$).

Abbreviations: P, pellet; SN, supernatant; BA, benzyl alcohol

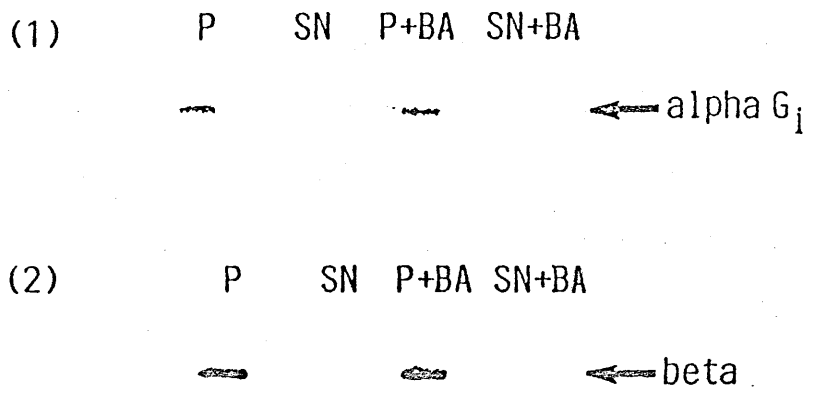

(1)
P
SN
P+BA
SN+BA
alpha G_i
(2)
P
SN
P+BA
SN+BA
beta

Table 3 EC_{50}'s for ligand-mediated activation of platelet adenylate cyclase

Ligand	Component involved	EC_{50}	No. of observations
Forskolin	C	25.0 ± 7.00uM	3
Sodium fluoride	G_s	14.80 ± 0.50mM	3
PGE_1	R_s	0.20 ± 0.04uM	3

n= No. of separate experiments using different membrane preparations.

Errors are S.D. for the given number of observations.

EC_{50} is the concentration of ligand giving 50% activation.

Table 4 Activity of adenylate cyclase in human platelets

Assays were done in the presence of forskolin (10^{-4}M), NaF (15mM), PGE_1 (10^{-5}M) and isoprenaline (10^{-4}M). Specific activity is given in pmol/min/mg protein. Fold stimulation is the fold increased in activity over that seen in the absence of any added ligand (basal). C-catalytic unit of adenylate cyclase; G_s-stimulatory guanine nucleotide regulatory protein. Results are means ± S.D. for the given number of different membrane preparations.

Ligand	Components involves	No. of observations	Specific activity	fold stimulation
none (basal)	C	6	8.7 ± 1.3	(-)
Forskolin	C	6	270.4 ± 41.1	31.0
NaF	G_s-C	4	71.6 ± 7.9	8.2
PGE_1	PGE_1 receptor-G_s-C	14	234.5 ± 35.5	26.7
Isoprenaline	beta-adrenceptor	5	14.1 ± 4.1	1.6

Table 5 Comparison of membrane-bound and lubrol-solubilised adenylate cyclase activities

Ligand	Component	Specific activities Control	Solubilised
none (basal)	C	193.3 ± 11.4	61.5 ± 3.6
GTP (10^{-4}M)	G_s	213.0 ± 17.0	79.5 ± 12.2
PGE_1 ($2x10^{-5}$M)	R_s	1310.6 ± 65.6	74.7 ± 3.0
forskolin (10^{-4}M)	C	3412.5 ± 101.3	1763.8 ± 93.8

Data is from typical experiment : each figure is the mean of 3 samples ± S.D.

No. of separate experiments = 6; all give similar trends.

Specific activity is expressed as p moles cAMP/mg/min

Table 6 Stimulatory action of benzyl alcohol on platelet adenylate cyclase activity

Maximum stimulation of adenylate cyclase activity is given compared with activity monitored in the absence of this agent (control - 100%). The ligand-stimulated increase in adenylate cyclase activity is given as a fold-increase over that of the basal for experiments done in either the absence of benzyl alcohol or in the presence of maximally stimulating concentrations of this ligand. Results are means ± S.D. for n=6 experiments using different membrane preparations. Specific activities are given in Table 4.

regulatory ligand	maximal stimulation by benzyl alcohol (%)	ligand-mediated stimulation (fold increase over basal)	
		absence of benzyl alcohol	presence of benzyl alcohol
none (basal)	311 ± 7%	(1)	(1)
NaF (15mm)	262 ± 22%	8.2	6.9
PGE_1 ($2x10^{-5}M$)	144 ± 5%	26.7	12.5
isoprenaline ($10^{-4}M$)	294 ± 6%	1.6	1.5

Table 7 Effect of manganese (5mM) on PGE_1- and forskolin-stimulated adenylate cyclase activities

	Mn/Mg ratio	
Ligand	Absence of Benzyl Alcohol	Presence of 50mM Benzyl Alcohol
PGE_1 ($2x10^{-5}M$)	2.01 ± 0.66 (6)	1.44 ± 0.33 (7)
forskolin ($10^{-4}M$)	2.48 ± 0.93 (6)	6.34 ± 2.13 (5)

Results are shown as mean ± S.D.

n= No. separate experiments using different membrane preparations.

Table 8 Effect of temperature on inhibitory ligands

% Inhibition

Temperature

Inhibitory Condition	30°C	35°C	40°C
forskolin + low GppNHp	66.7 ± 13.1 (n=4)	66.7 ± 6.5 (n=3)	46.3 ± 3.2 (n=3)
forskolin + Adrenaline	68.7 ± 3.8 (n=3)	63.5 ± 13.5 (n=4)	66.0 ± 5.3 (n=3)
PGE_1 + Adrenaline	71.7 ± 4.0 (n=3)	77.3 ± 8.9 (n=4)	82.3 ± 8.2 (n=4)

Results shown as mean ± S.D.

n= No. separate experiments using different membrane preparations

Adrenaline (10^{-4}M) is in the presence of propranolol (10^{-6}M)

Concentration of forskolin is 10^{-4}M.

Concentration of PGE_1 is 2×10^{-5}M.

(iii) Discussion

It is apparent from this study that the adenylate cyclase system of human platelets is 'typical' in a number of respects, although some new aspects to regulation of this enzyme will be discussed.

Stimulatory ligands were observed as being able to activate the enzyme at various levels, namely receptor (PGE_1, or beta-adrenoceptor), stimulatory G-protein, G_s (NaF) and directly at the level of the catalytic unit (forskolin). This is as reported for the adenylate cyclase system of other tissues/cells (Ruiz et al., 1986; Nelson & Seamon, 1985), and exhibits the same order of potency; PGE_1 and forskolin being the most powerful stimulants tested. Once again this was as reported by others (Insel et al., 1982). The platelet adenylate cyclase enzyme was found to retain its activity in the solubilised form, and was able, in this state, to be stimulated by forskolin and attenuated by adenosine. This provides evidence for the routes by which these ligands mediate their effects. For example, forskolin activation of adenylate cyclase must be via a direct interaction of the diterpene with the catalytic unit. It also provides support for the existence of the 'p-site' for adenosine interaction with the catalytic unit i.e. adenosine interaction with adenylate cyclase is not solely at the level of the receptor.

It was also observed that both NaF and GppNHp exhibited their characteristic dual regulation of adenylate cyclase, being able to both stimulate basal activity and inhibit stimulated activities. This property reflects the different affinities of G_i and G_s for these ligands. G_s binding GppNHp with a much lower affinity than G_i (Gawler et al., 1987; Hildebrandt et al., 1982), whilst the dual effect of NaF arises due to the fact that in

combination with the GDP bound to the G-proteins it mimics the GTP in a manner analogous to that observed with GppNHp.

Inhibitory aspects of platelet adenylate cyclase regulation are again similar to those reported by others (Morgan, 1989). Here too, activation was possible at different levels: receptor ($alpha_2$-adrenoceptor), inhibitory G-protein, G_i (GppNHp), and directly at the catalytic unit (adenosine). Although very little is reported about adenylate cyclase inhibition beyond those details, in this chapter the inhibitory arm of regulation will be covered in detail.

Having determined that the basic stimulatory and inhibitory aspects of platelet adenylate cyclase regulation were as found elsewhere, attempts were then made to examine the effects of benzyl alcohol on this system. As has been shown in a variety of membrane systems (Friedlander et al., 1987; De Foresta et al., 1987), the activity of adenylate cyclase in the basal and ligand-stimulated states is enhanced in the presence of this local anaesthetic. Here it was found that platelet adenylate cyclase is no exception. The augmentation of activity is seen when monitoring the basal catalytic unit activity, and also when G_s is stimulated directly with sodium fluoride, and when stimulated by receptor-G_s coupling invoked by either PGE_1, or by the beta-adrenoceptor agonist isoprenaline. In hepatocytes, however, basal adenylate cyclase activity was relatively insensitive to the action of benzyl alcohol, whilst the glucagon-stimulated activity (R_s) was markedly enhanced (Dipple & Houslay, 1978). Thus benzyl alcohol profoundly amplified the ability of glucagon to stimulate the activity of adenylate cyclase (i.e. fold stimulation increased). Here, however, benzyl alcohol was observed to exert a potent stimulatory effect on the basal activity of human platelet adenylate

cyclase. This may reflect tissue and species differences in either the enzyme itself, or its incorporation into the membrane bilayer. It is presumed that the stimulatory effect of benzyl alcohol was related to its ability to decrease membrane order (Insel et al., 1982; Dipple & Houslay, 1978; Hildebrandt et al., 1982: Gordon et al., 1980), as no stimulatory effect of benzyl alcohol was observed with the detergent-solubilised enzyme. Benzyl alcohol also enhanced the degree of sodium fluoride-stimulation of adenylate cyclase to a similar extent to that seen for basal activity. This suggests that the amplification seen is due to an effect predominantly on the catalytic unit of the enzyme, and indicates that coupling between G_s and adenylate cyclase was not influenced by the increase in disorder elicited by the anaesthetic. Indeed, despite mobile collisions between the protein components being required for this process (Houslay et al., 1980a), it has been shown (Houslay & Gordon, 1983; Henis et al., 1982) that at the temperatures of assay (30^{o}C) diffusion is not a rate-limiting step in the reaction, and thus any decrease in membrane order is unlikely to elicit any significant facilitation of the rate of reaction. Thus, the membrane environment does not appear to exert any constraint on the conformational flexibility of G_s, which is not entirely unexpected, as at least the alpha-subunit of G_s is not an integral membrane protein (Gilman, 1984; Houslay, 1984; Birnbaumer et al., 1985). As with NaF-stimulation, the presence of benzyl alcohol did not augment the net (fold) stimulation of basal adenylate cyclase by isoprenaline (Table 6). Thus, the enhanced activity seen here may again be due predominantly to an effect on the catalytic unit of adenylate cyclase. In contrast, the fold increase in stimulation of adenylate cyclase by PGE_1 was attenuated by the presence of benzyl alcohol (Table 6). Such

experiments imply that there are profound differences in the ability of benzyl alcohol to modify the efficiency of coupling of various stimulatory receptors to adenylate cyclase. This does not appear to be mediated by any change in affinity of the ligands for the receptor, but rather, in their potency to couple and activate G_s. Although in other experiments benzyl alcohol was seen to elicit a change in the EC_{50} for PGE_1 activation this would not account for the effects of benzyl alcohol seen here i.e. concentration of PGE_1 employed is saturating.

Benzyl alcohol, like ethanol and other short-chain alcohols (Whetton et al., 1983; Robberecht et al., 1983; Huang et al., 1982) was seen to inhibit adenylate cyclase when the enzyme was activated by the diterpene forskolin (Fig.23). This effect was noted as occurring with the forskolin-stimulated lubrol-solubilised enzyme also. It has been suggested that such inhibition (Whetton et al., 1983; Robberecht et al., 1983) may arise by the alcohol competing for the hydrophobic site on adenylate cyclase to which forskolin binds. However, in this study it was noted that the EC_{50} value for activation of adenylate cyclase by forskolin was not altered by benzyl alcohol. This suggests that benzyl alcohol is acting as a non-competitive, rather than competitive, inhibitor of forskolin action, and thus interacting at a different site(s) to forskolin on the catalytic unit of human platelet adenylate cyclase.

Further evidence that this reduction in forksolin-stimulated adenylate cyclase activity is a result of properties of the local anaesthetic comes from studies where other methods of manipulating membrane fluidity were employed. For example, manipulation of temperature by approximately $8^{o}C$ should elicit the same effects as the addition of 50mM benzyl alcohol. In fact, it was observed that such temperature manipulation not only

enhanced the activity of the PGE_1-stimulated state as would be expected, but also enhanced the activity of the forskolin-stimulated state. Activation profiles were almost identical (Figs.25,26), thereby supporting the proposal that the attenuation of forskolin-stimulated activity, seen with benzyl alcohol, was a direct reflection of the properties of the local anaesthetic.

G_i-mediated effects on adenylate cyclase can be elicited and monitored in two distinct ways. Firstly, one can determine any direct action on the catalytic unit of adenylate cyclase by monitoring action on the forskolin-amplified basal activity. In this instance inhibition can be engendered by selectively activating G_i with low concentrations of GppNHp, as this G-protein has a much higher affinity for GppNHp than does G_s (Gawler et al., 1987; Hildebrandt et al., 1982). Direct inhibitory effects on the catalytic unit can also be measured by assessing the ability of the $alpha_2$-adrenoceptors, which couple to G_i, to inhibit the forskolin-stimulated activity (Jakobs et al., 1985; Houslay et al., 1986a). For both receptor- and GppNHp-mediated inhibitory effects on the catalytic unit of adenylate cyclase activity it was observed that a complete insensitivity to benzyl alcohol at concentrations up to 50mM existed. In contrast to this, the $alpha_2$-adrenoceptor-mediated inhibition of receptor-stimulated adenylate cyclase was attenuated on addition of benzyl alcohol. This occurred in a saturable fashion, with a maximal attenuation of approximately 34% being achieved, and was not due to any interference with the G-protein GTPase activity. It may be that this inhibitory effect of benzyl alcohol resulted from its ability to modulate the IC_{50} for adrenaline-mediated inhibition. However, this would not appear to be the case here, as it was noted that saturating concentrations of adrenaline were used in these

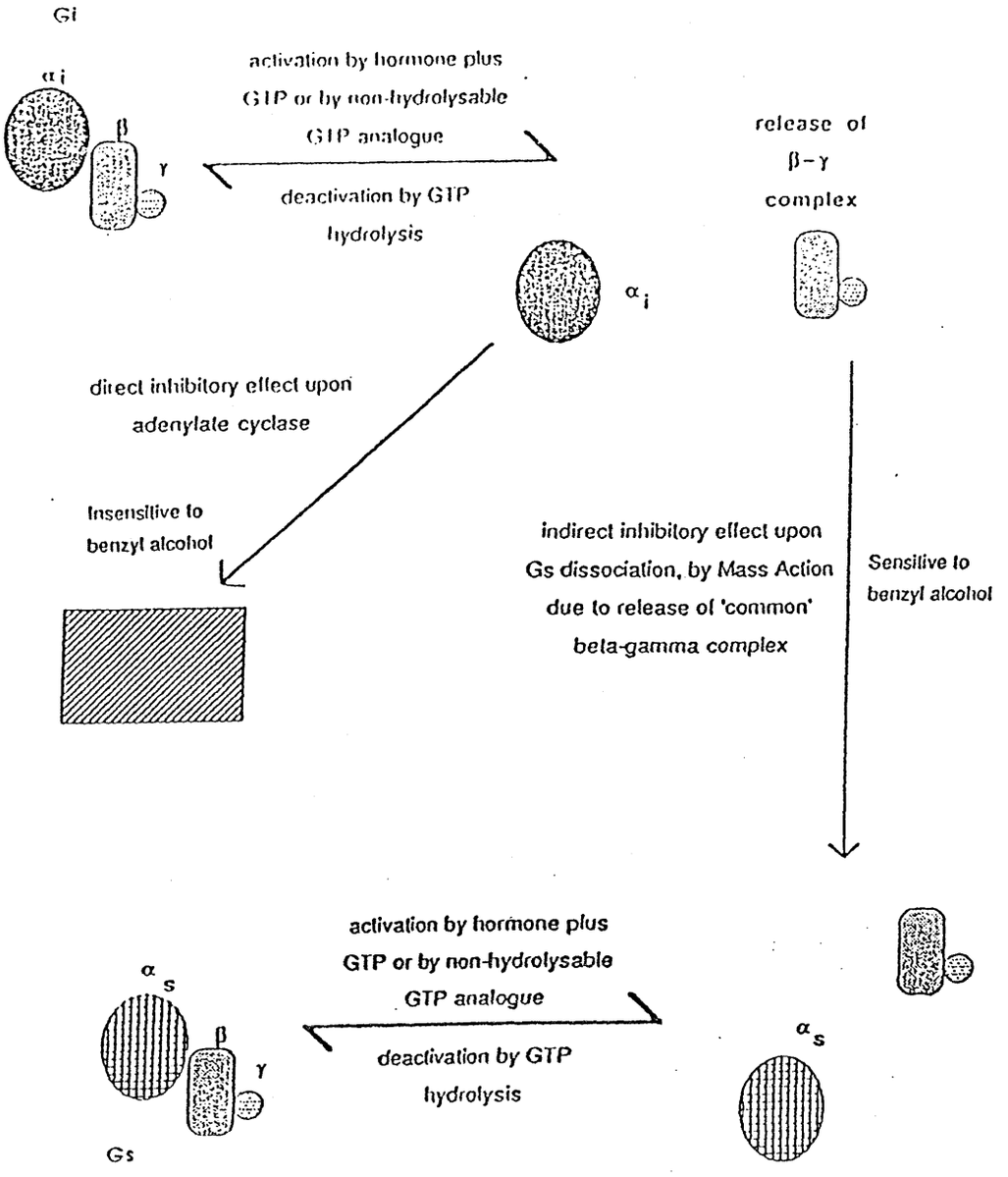
benzyl alcohol experiments. Also, such an explanation would not explain why adrenaline-mediated inhibition of forskolin was unaffected by the presence of the anaesthetic. (The IC_{50} for GppNHp-mediated inhibition of adenylate cyclase was unaltered by benzyl alcohol).

Thus, the $alpha_2$-adrenoceptor-mediated inhibition of receptor-G_s-stimulated adenylate cyclase appears to exhibit two components, one of which is insensitive to benzyl alcohol and the other which is attenuated by benzyl alcohol.

The attenuating effect of benzyl alcohol was clearly targetted at a fraction of the inhibitory G_i component. This could be shown by the experiments performed in the presence of manganese, rather than magnesium, which serves to uncouple $G_i$mediated responses (Gilman, 1984; Hoffmann et al., 1981). Under such conditions, an increase in PGE_1-stimulated activity was observed (as shown by Mn/Mg ratios), owing to a loss of inhibitory input, i.e. the removal of tonic inhibition. This loss of functioning G_i, results in a near obliteration of the $alpha_2$-mediated inhibition of PGE_1-stimulated adenylate cyclase, and a response of PGE_1-stimulated adenylate cyclase to benzyl alcohol which is similar, whether or not adrenaline (+ propranolol) is present to interact with the $alpha_2$-adrenoceptors (see Fig.34).

Having established that the observed effect was indeed a result of benzyl alcohol action on a fraction of the inhibitory G_i component, the next step was to propose exactly which fraction of this component was being affected. Hence, it is proposed that the two inhibitory components (see Fig.36) may reflect, (i) a benzyl alcohol-insensitive component, which is suggested as reflecting the direct inhibitory action of alpha-G_i on the catalytic unit of adenylate cyclase, and (2) a benzyl alcohol-sensitive component,

Figure 36 Modulation of G_i-mediated inhibition by benzyl alcohol



which is suggested may reflect an action on the beta,gamma-subunit-mediated inhibitory effect of G_i on G_s-dissociation. Thus, in the absence of ligands stimulating G_s, only the benzyl alcohol-insensitive response may be observed. This is proposed as being the case with benzyl alcohol insensitive GppNHp- and $alpha_2$-adrenoceptor-mediated inhibition of the forskolin-stimulated enzyme state.

At this point, the effect of benzyl alcohol on the Mn/Mg ratios for PGE_1- and forskolin-stimulated activities should be mentioned. As stated previously, the substitution of magnesium with manganese resulted in an increase in PGE_1-stimulated activity. A similar increase was detected with forskolin-stimulated adenylate cyclase. However, the addition of benzyl alcohol to these systems elicited different effects depending upon the stimulatory ligand used. With PGE_1-stimulated adenylate cyclase, the Mn/Mg ratio was reduced in the presence of 50mM benzyl alcohol. This is proposed as arising due to the fact that magnesium-stimulated PGE_1 activity is enhanced to a larger degree by benzyl alcohol than is the manganese-stimulated state. From this result it may be deduced that part of the method by which benzyl alcohol activates adenylate cyclase is by removing tonic inhibition. Of course, with the manganese-stimulated state this cannot occur, and so the stimulatory effect of benzyl alcohol is lessened. With forskolin-stimulated adenylate cyclase the opposite is true, i.e. the addition of benzyl alcohol enhanced the Mn/Mg ratio. This arises due to the fact that the actual reduction in forskolin-stimulated activity elicited by benzyl alcohol, in the presence of manganese, is lessened, as specific activities are higher in the presence of this cation, relative to those seen with magnesium.

It cannot be defined unequivocally whether the attenuating effect of benzyl alcohol on PGE_1-stimulated activity is due either to enhanced bilayer fluidity, or to a direct action of benzyl alcohol itself. However, the 'fluidising' effect of 50mM benzyl alcohol can be mimicked by increasing the temperature by some 6-8^{o}C, as mentioned previously. Here, such an increase in temperature did not diminish the potency of adrenaline (+ propranolol) to inhibit PGE_1-stimulated adenylate cyclase. Nor in fact did it alter $alpha_2$-adrenoceptor- or GppNHp-mediated inhibition of forskolin-stimulated adenylate cyclase (Table 8). This suggests that the ability of benzyl alcohol to attenuate the inhibitory effect of $alpha_2$-adrenoceptor occupancy on PGE_1-stimulated activity is by a direct, albeit unknown, action. However, it can be stated that such an attenuation of inhibitory action was not due to benzyl alcohol causing a loss of components of the G_i system from the membrane. Immunoblotting studies (Fig.35) clearly show that both alpha-G_i and G_i beta-subunits were not displaced by benzyl alcohol under the conditions of the assay.

(iv) Conclusion

In conclusion, it would appear that the adenylate cyclase system of human platelet membranes provides a readily available model in which to study the dual regulation of this enzymatic activity. Whilst it would seem that the platelet enzyme is 'typical' as regards its ability to be regulated at various levels, it would seem that in benzyl alcohol, we have identified an 'atypical reagent', which may be able to differentiate between the regulatory pathways involved in inhibition.

3.2 Effect of charged anaesthetics on platelet adenylate cyclase activity

(i) Introduction

It has been proposed that, due to the asymmetry of the lipid composition of the plasma membrane, that charged anaesthetics are able to preferentially modulate the fluidity of one, or other halves of the bilayer (Houslay & Gordon, 1983; Houslay et al., 1981; Houslay et al., 1980b). Positively charged drugs are suggested to enhance the fluidity of the inner leaflet, whilst negatively charged drugs are thought to affect the outer leaflet in a similar manner.

Here, the effects of the anionic pentobarbital, and the cationic prilocaine, are examined as regards the modulation of adenylate cyclase activity through such perturbations.

(ii) Results

(a) Anionic drugs

Following an initial period of activation, increasing concentrations of these negatively charged drugs appeared to attenuate both forskolin- and PGE_1-stimulated adenylate cyclase activity in a dose-dependent manner.

With pentobarbital, low concentrations led to an increase in adenylate cyclase activity: forskolin-stimulated activity (Fig.37) being enhanced by 180% in the presence of 5mM pentobarbital, whilst PGE_1-stimulated activity (Fig.38) was augmented by a much smaller degree, namely 112%. In both cases, increasing the concentration of pentobarbital beyond these levels led to a loss of adenylate cyclase activity, such that activity was effectively abolished at concentrations in excess of 30mM (Figs.37,38)

Similarly, with phenobarbital, low concentrations augmented adenylate cyclase activity. (n=4 separate experiments using different membrane preparations). Here, 1mM phenobarbital led to 127% and 129% increases in PGE_1- and forskolin-stimulated activities respectively. Once again, increasing concentrations of drug led to a dose-dependent reduction in adenylate cyclase activity.

When these two anionic compounds were examined with regard to their effects on inhibitory aspects of adenylate cyclase regulation it was found that pentobarbital at concentrations up to 10mM apparently had no effect on the adrenaline (+ propranolol)-mediated inhibition of PGE_1 (Fig.39). However, adrenaline mediated inhibition of forskolin-stimulated activity (Fig.40) was slightly reduced by 1mM pentobarbital (34.9% reduction), as was the GppNHp-mediated inhibition of this state (45.3% reduction) (Fig.41).

Once again, similar trends were obtained with phenobarbital, although not to such marked degrees (n=4 separate experiments, using different membrane preparations).

(b) Cationic drugs

Studies with the positively charged drug prilocaine showed that increasing concentrations of the anaesthetic led to attenuation of both PGE_1-(receptor-coupled), and forskolin-(receptor-uncoupled)-stimulated adenylate cyclase activities.

It would appear that PGE_1-stimulated activity is inhibited by concentrations of prilocaine in excess of 10mM (Fig.42). Such attenuation of activity appeared to occur in a dose-dependent manner, such that at 50mM prilocaine, activity was attenuated by 55%. Forskolin-stimulated adenylate cyclase activity was affected in a similar manner, although here inhibition occurred at lower concentrations than with PGE_1, and again activity was diminished by increasing concentrations of prilocaine. At 50mM prilocaine, forskolin-stimulated activity was reduced by some 76% (Fig.43).

Such effects of prilocaine on receptor-coupled and -uncoupled activities were mirrored in studies using carbocaine (another cationic drug) over the same concentration range; 0 - 50mM. (n=3 separate experiments using different membrane preparations). Carbocaine induced similar effects to prilocaine with respect to the action of positively charged drugs on inhibitory aspects of adenylate cyclase regulation. Here, adrenaline-mediated inhibition of PGE_1-stimulated adenylate cyclase was found to be slightly reduced by low concentrations ($<$30mM) of the drug, although inhibition was restored by further increases in the concentration of prilocaine (Fig.44). In contrast to this, the ability of adrenaline (+ propranolol) to inhibit forskolin-stimulated activity

was augmented by increasing concentrations of prilocaine. At 50mM prilocaine, a 78% increase in the ability of adrenaline to inhibit the forskolin-activated state was observed (Fig.45). From Figure 46, it is apparent that the GppNHp-mediated inhibition of forskolin-activated adenylate cyclase was similarly affected, i.e. the efficiency of GppNHp to inhibit adenylate cyclase was enhanced by prilocaine in a dose-dependent manner. The 38.5% inhibition observed in the absence of prilocaine was increased to 72%-inhibition in the presence of 50mM prilocaine.

Figure 37 The effect of pentobarbital on forskolin-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=3 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.

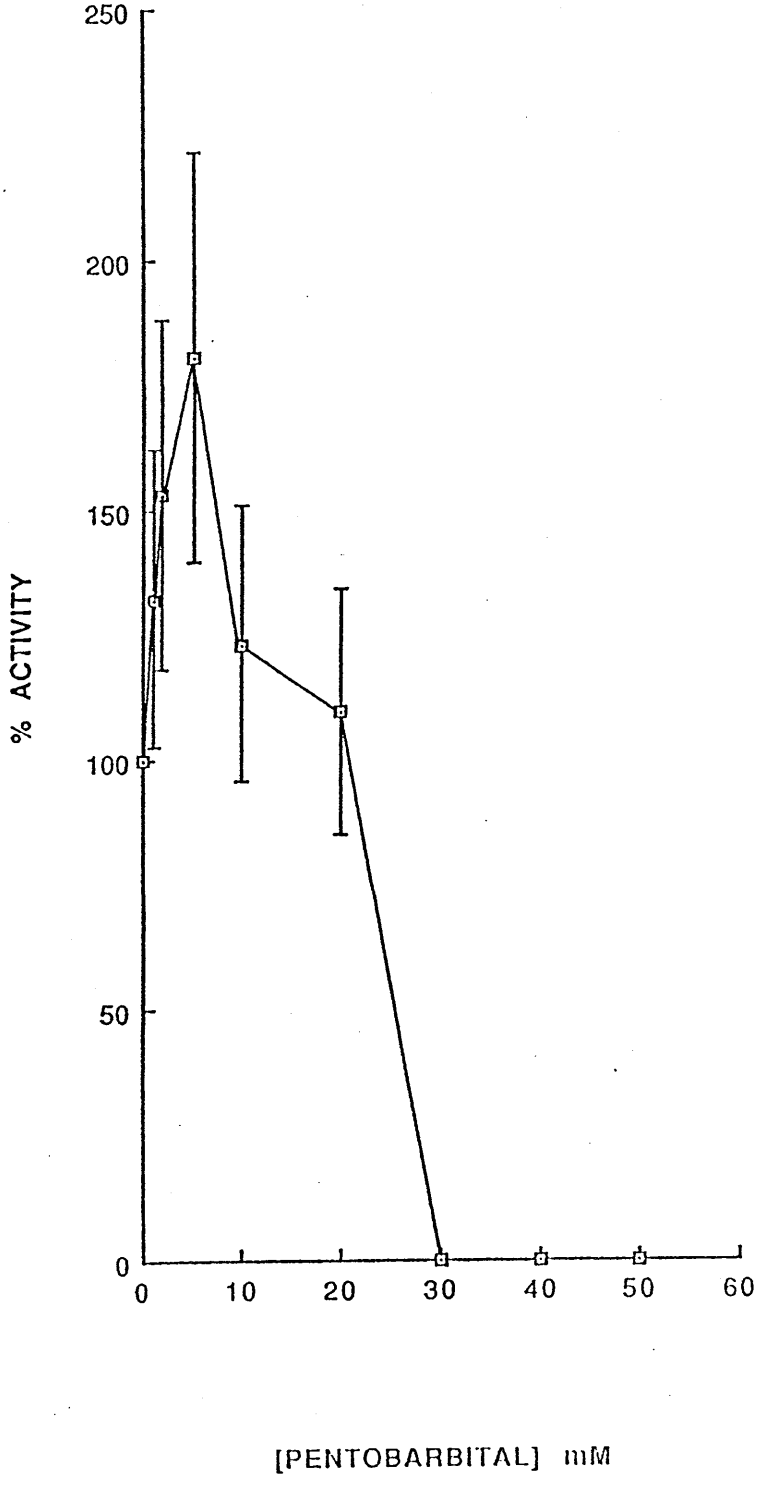

250
200
150
100
50
0
% ACTIVITY
0
10
20
30
40
50
60
[PENTOBARBITAL] mM

Figure 38 Effect of pentobarbital on PGE_1-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=3 separate experiments using different membrane preparations.

Concentration of PGE_1^- is 2×10^{-5}M.

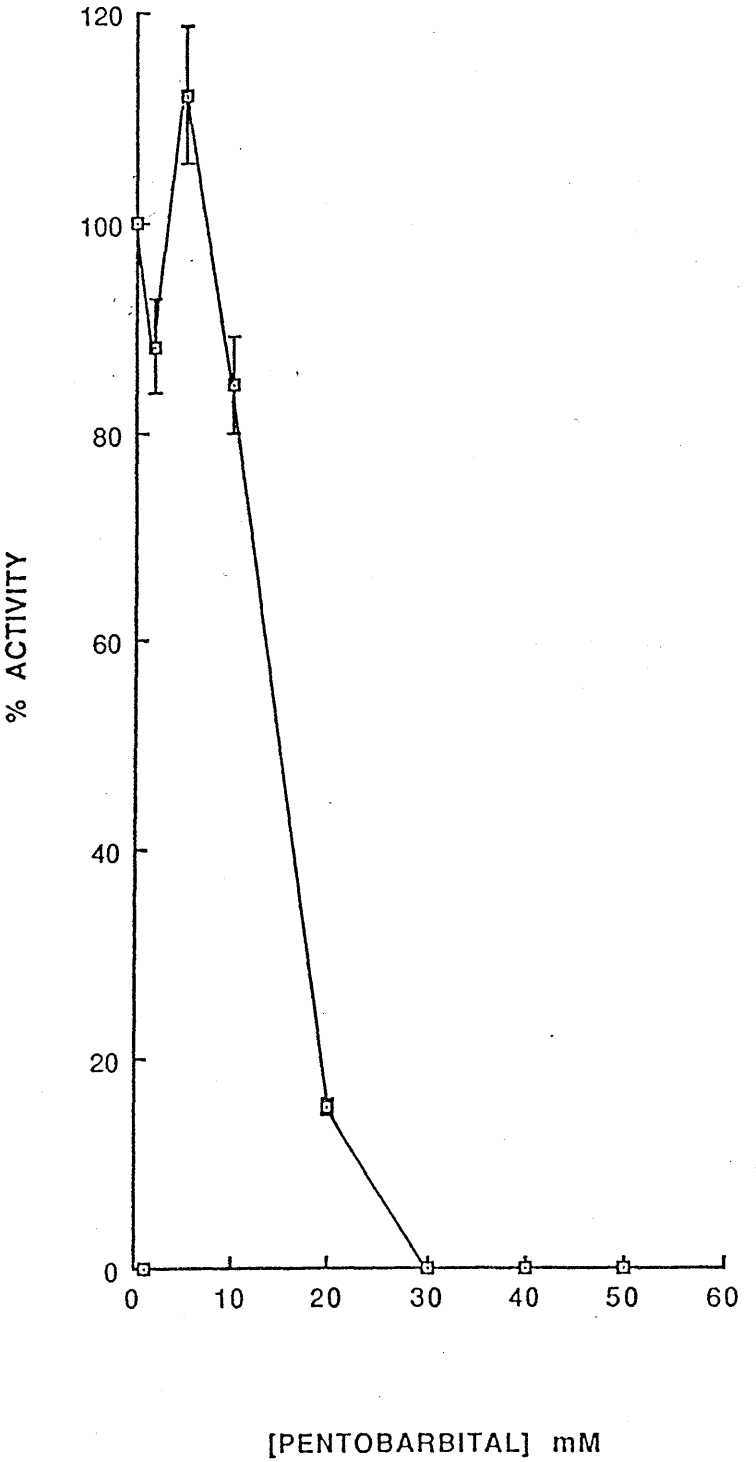

120
100
80
60
40
20
0
% ACTIVITY
0
10
20
30
40
50
60
[PENTOBARBITAL] mM

Figure 39 Effect of pentobarbital on adrenaline (+ propranolol)-mediated inhibition of PGE_1-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=3 separate experiments using different membrane preparations.

Concentration of PGE_1 is 2×10^{-5}M.
Adrenaline (10^{-4}M) is in the presence of 10^{-5}M propranolol

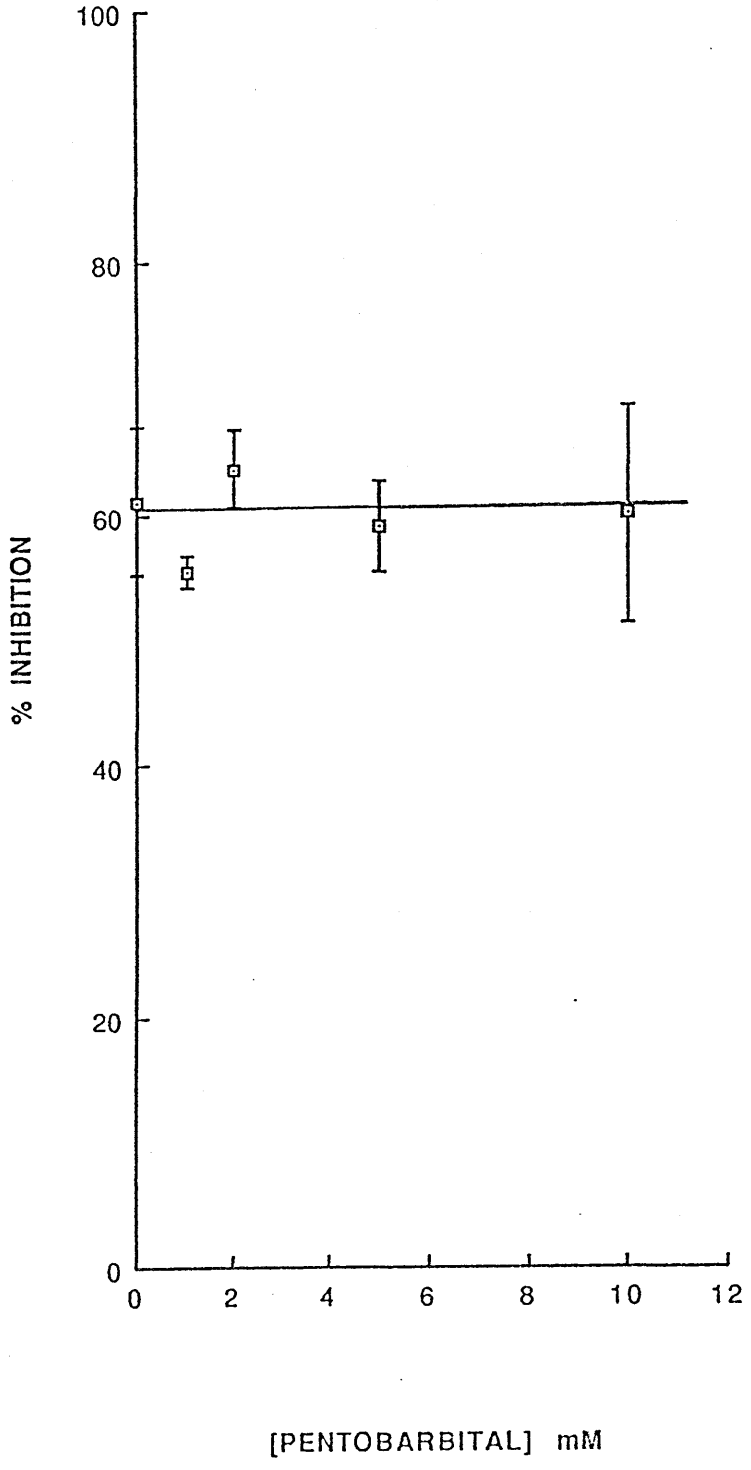

100
80
60
40
20
0
% INHIBITION
0
2
4
6
8
10
12
[PENTOBARBITAL] mM

Figure 40 Effect of pentobarbital on adrenaline (+ propranolol)-mediated inhibition of forskolin-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=3 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.
Adrenaline (10^{-4}M) is in the presence of 10^{-5}M propranolol

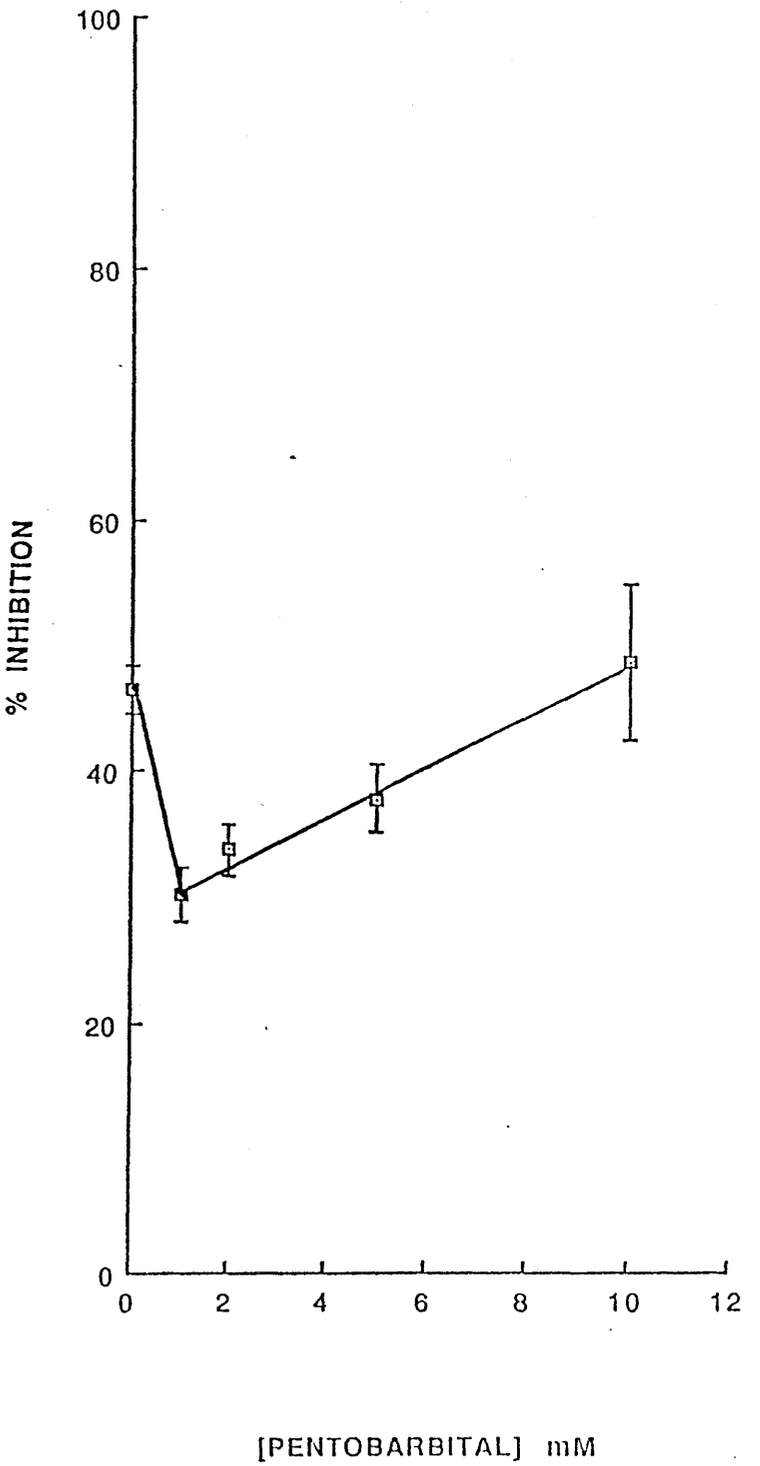

100
80
60
40
20
0
% INHIBITION
0
2
4
6
8
10
12
[PENTOBARBITAL] mM

Figure 41 Effect of pentobarbital on GppNHp-mediated inhibition of forskolin-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=3 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.

Concentration of GppNHp is 10^{-8}M

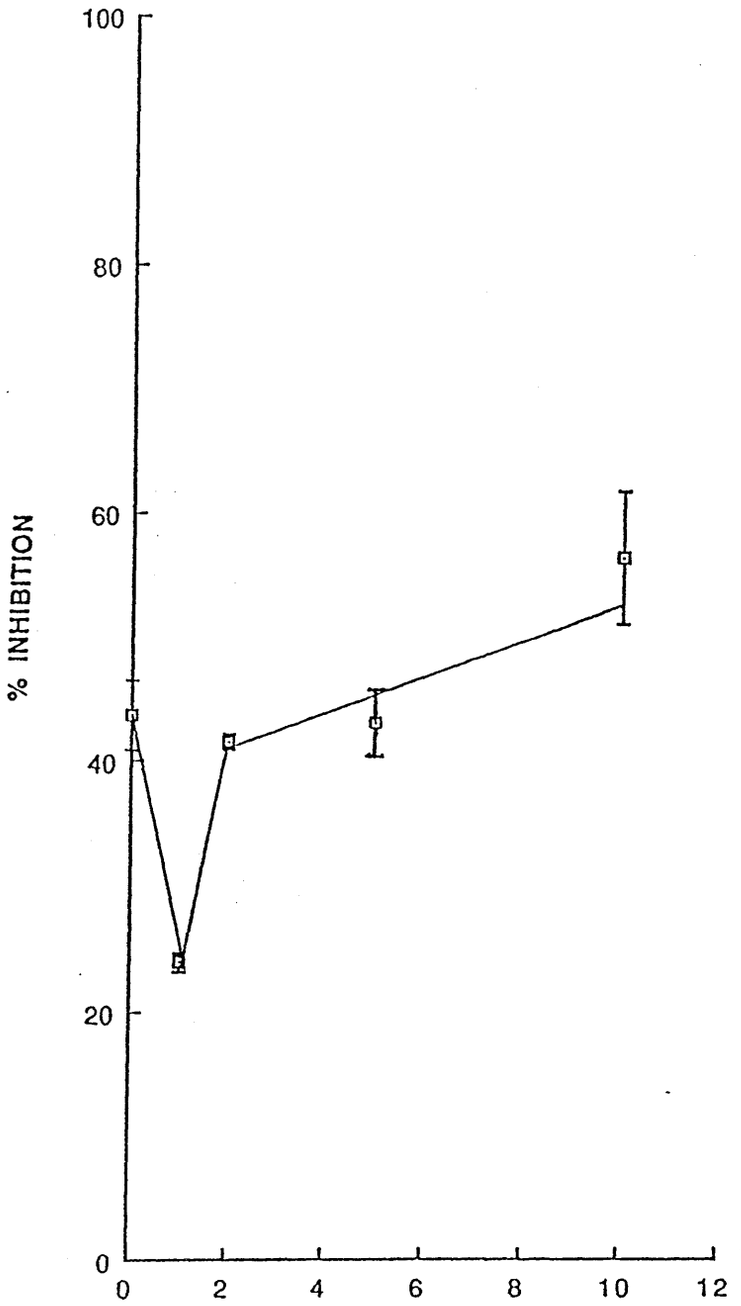

100
80
60
40
20
0
% INHIBITION
0
2
4
6
8
10
12
[PENTOBARBITAL] mM

Figure 42 Effect of prilocaine on PGE_1-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=4 separate experiments using different membrane preparations.

Concentration of PGE_1^- is 2×10^{-5}M.

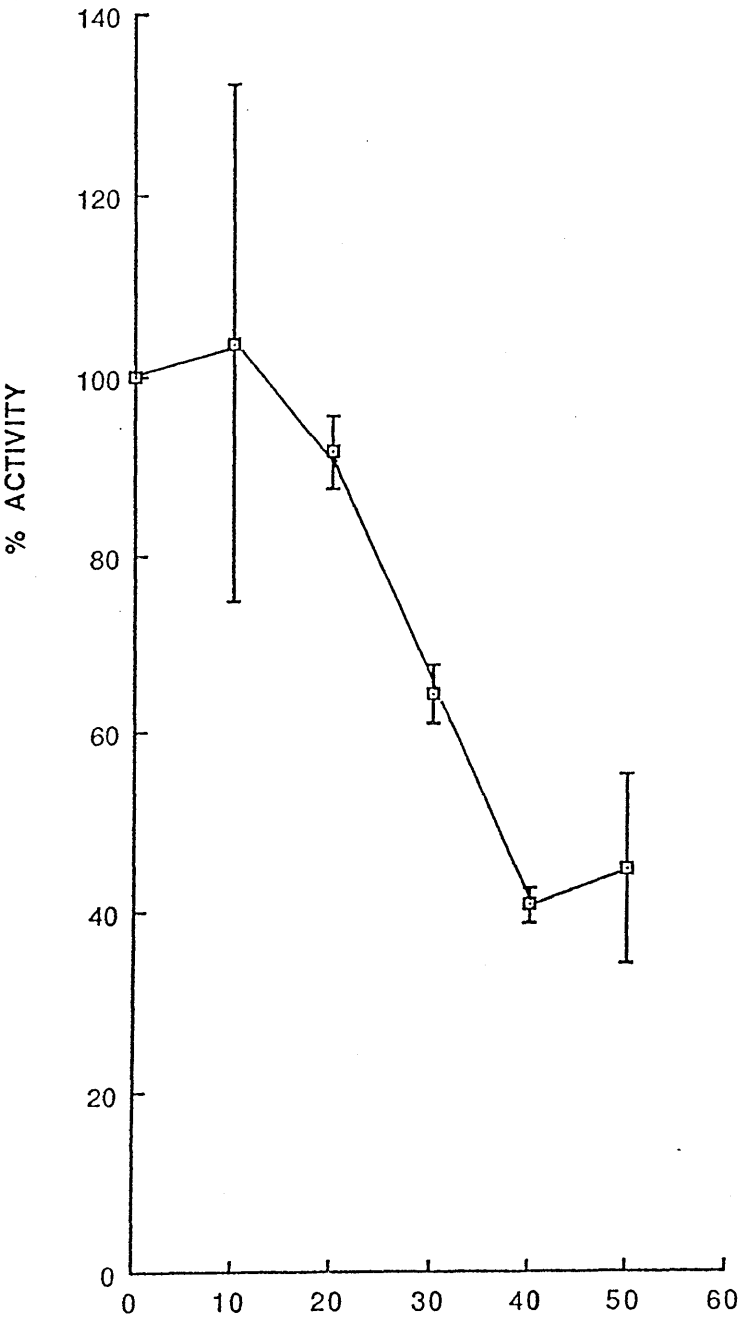

140
120
100
80
60
40
20
0
% ACTIVITY
0
10
20
30
40
50
60
[PRILOCAINE] mM

Figure 43 Effect of prilocaine on forskolin-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=4 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.

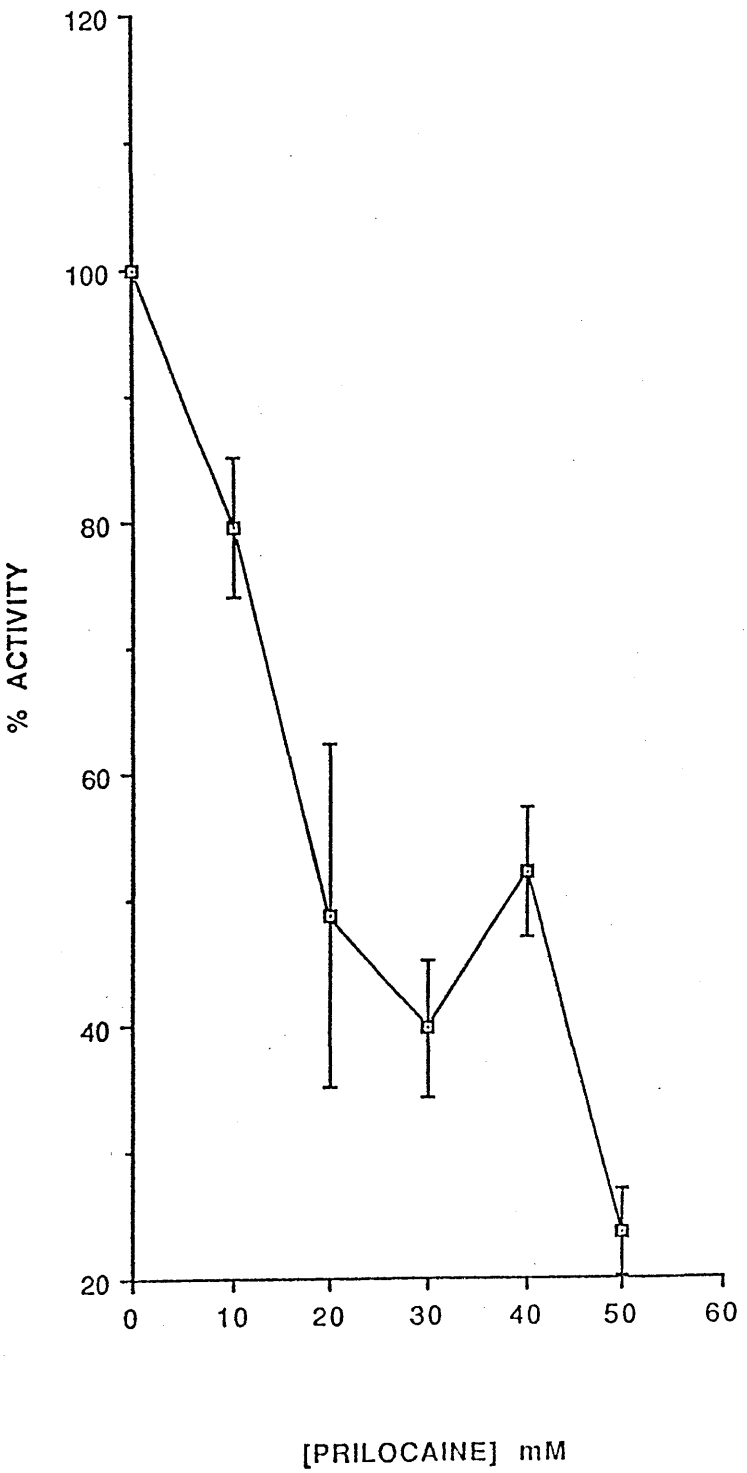

120
100
80
60
40
20
% ACTIVITY
0
10
20
30
40
50
60
[PRILOCAINE] mM

Figure 44 Effect of prilocaine on adrenaline (+propranolol)-mediated inhibition of PGE_1-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=4 separate experiments employing different membrane preparations.

Concentration of PGE_1 is 2×10^{-5}M.

Adrenaline (10^{-4}M) is in the presence of 10^{-5}M propranolol

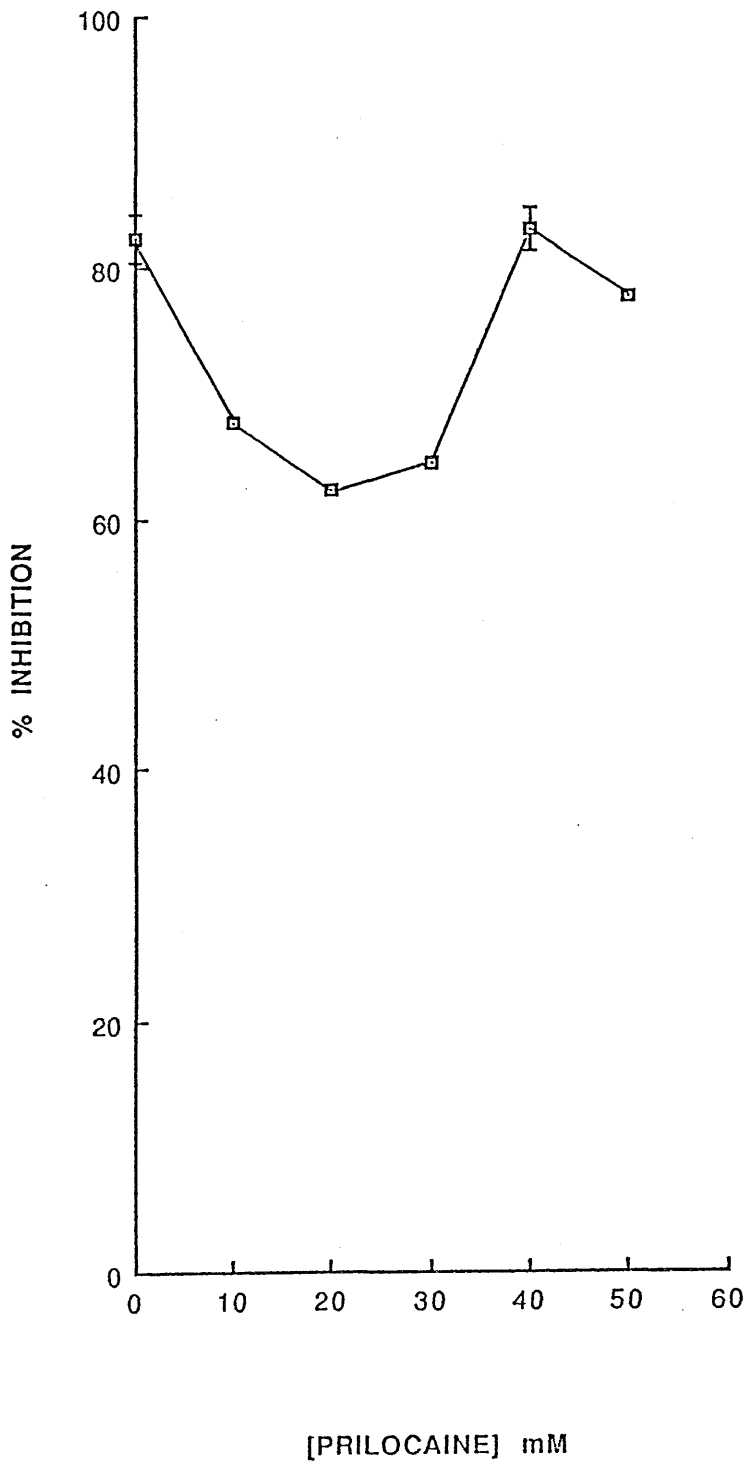

100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
[PRILOCAINE] mM

Figure 45 Effect of prilocaine on adrenaline (+ propranolol)-mediated inhibition of forskolin-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=4 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.
Adrenaline (10^{-4}M) is in the presence of 10^{-5}M propranolol

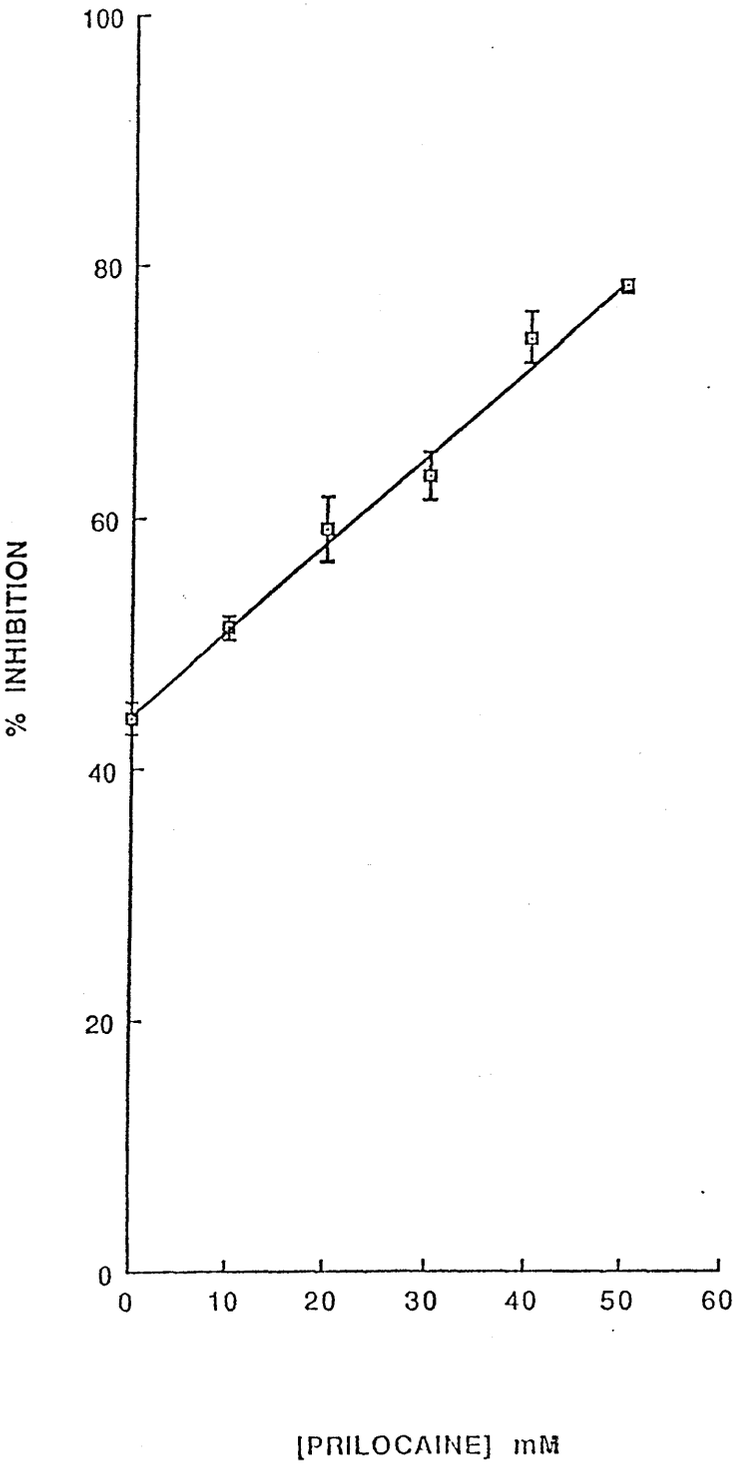

100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
[PRILOCAINE] mM

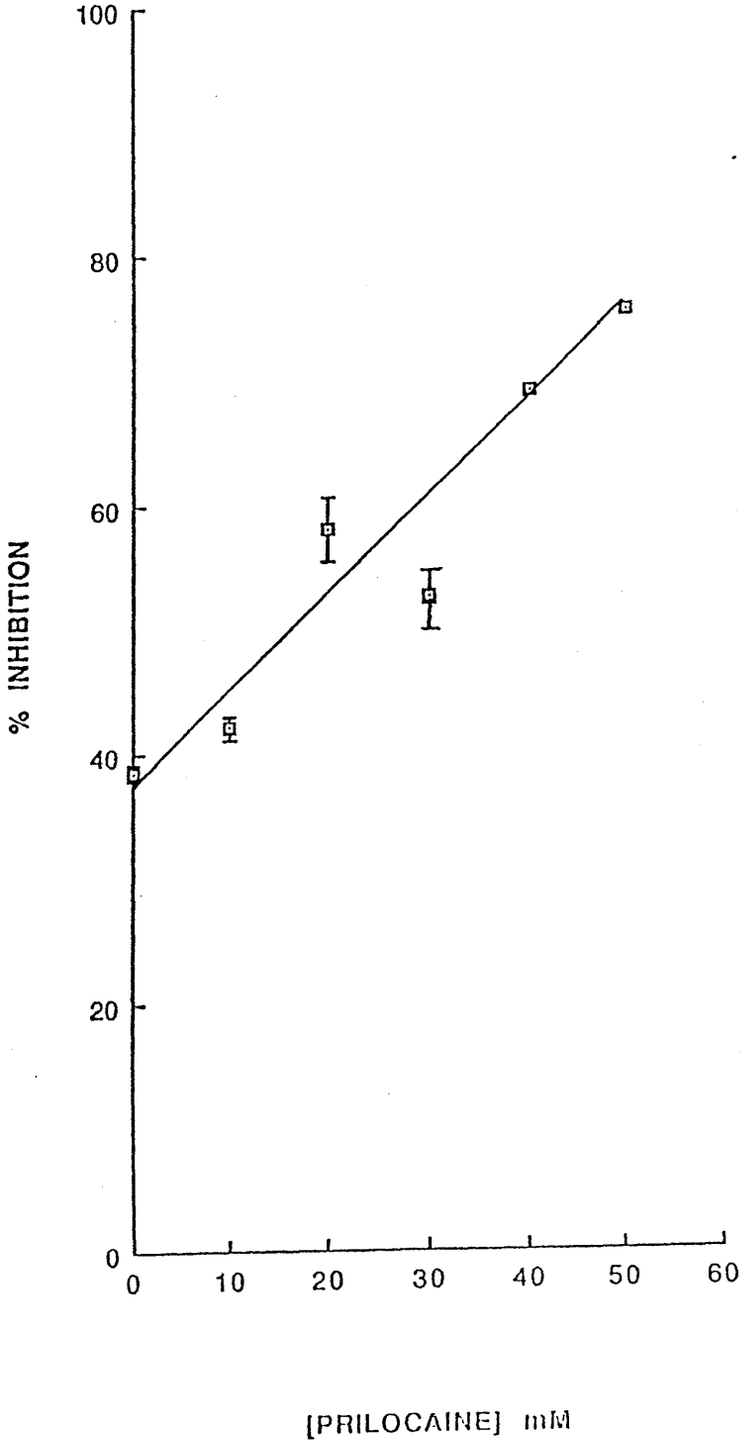
Figure 46 Effect of prilocaine on GppNHp-mediated inhibition of forskolin-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=4 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.

Concentration of GppNHp is 10^{-8}M


100
80
60
40
20
0
% INHIBITION
0
10
20
30
40
50
60
[PRILOCAINE] mM

(iii) Discussion

(a) Anionic Drugs

From experiments using the anionic drug pentobarbital, it was observed that both receptor-coupled (prostaglandin E_1-stimulated) and -uncoupled (forskolin-stimulated) adenylate cyclase activities were enhanced at low concentrations (1mM) of pentobarbital. It is presumed that this reflects an increase in fluidity due to the presence of the anaesthetic. However, as others have proposed pentobarbital as acting mainly in the external half of the membrane (Houslay & Gordon, 1983) it would have been expected that receptor-coupled activity (PGE_1) (which experiences the environment of this leaflet, as well as the inner leaflet), would have been enhanced to a much larger degree than the uncoupled, forskolin-stimulated state. In fact, in rat liver plasma membranes, anionic drugs were observed as having no effect on receptor-uncoupled activities (Houslay et al., 1981). In platelets, it was found that not only was receptor-uncoupled activity enhanced by pentobarbital, but it was actually augmented to a larger degree than was receptor-coupled adenylate cyclase; forskolin-stimulation being enhanced by some 80% over control, with PGE_1-stimulation being enhanced by only 12% over control.

Although receptor-coupled activity is enhanced by pentobarbital in agreement with the rat liver plasma membrane system (albeit to a smaller degree - see later), the effect of pentobarbital on receptor-uncoupled activity is in total contrast with such studies. This suggests that differences exist in the sensitivity of platelet- and rat liver-adenylate cyclase to changes in fluidity; a fact that was suggested by studies with benzyl alcohol (see Section 3.1). Along similar lines, the fact that high concentrations of pentobarbital ($>$30mM) can effectively abolish

platelet adenylate cyclase activity, is in contrast to the rat liver system, where maximal attenuation of adenylate cyclase activity was in the region of 60% at high concentrations of anaesthetic.

This inhibition may result from anaesthetic molecules displacing components of the lipid annulus which surrounds integral membrane proteins such as cyclase. By this theory, inhibition ensues either because the displaced lipid was essential for activity, or alternatively, because occupancy of such lipid-obscured sites by the anaesthetic allows it to be inhibitory. Such inhibition has been observed for both charged (Houslay et al., 1981; Houslay et al., 1980b) and neutral (Houslay & Gordon, 1983) anaesthetics (see Section 1.3.(vi)).

Assuming the inhibition observed here with pentobarbital does reflect this phenomenon of annular displacement, then it is apparent that anionic drugs are able to displace platelet annular lipid more readily than rat liver annular lipid. The reasons for this are unclear, either reflecting differences in the strength of lipid-protein interaction or the affinity of the sites for anaesthetic (Houslay & Stanley, 1982).

The small increase in PGE_1-stimulated adenylate cyclase activity seen with pentobarbital is in contrast to the 170% increase in receptor-coupled (glucagon-stimulated) activity seen in rat liver. However, this may not be due to differences in the sensitivity of platelet and rat liver stimulatory receptors (R_s) to pentobarbital, rather it may reflect a specific property of the platelet PGE_1-receptor. This conclusion arises due to studies on the effect of benzyl alcohol on PGE_1-stimulated activity (see Section 3.1). Here it was observed that PGE_1-stimulated adenylate cyclase was enhanced to a much lesser degree than other ligand-stimulated states, R_s-coupled inclusive. It can therefore

only be presumed that pentobarbital would also augment other ligand-stimulated states to a larger extent, as seen with forskolin.

Activation of the forskolin-stimulated state by pentobarbital in this manner, is in direct contrast to the inhibitory effect of benzyl alcohol on this state (see Section 3.1). This lends support to the proposal that the benzyl alcohol-induced inhibition is a direct result of the properties of that particular anaesthetic i.e. that the properties of benzyl alcohol and pentobarbital are sufficiently different that this selective inhibition of forskolin was not observed.

When the effect of pentobarbital was examined with regard to the inhibitory aspects of adenylate cyclase regulation, different effects were observed depending upon whether the stimulatory state being inhibited was receptor-coupled or not, i.e. the effects of inhibitory ligands were examined on adenylate cyclase activities which were already enhanced, through either prostaglandin E_1 (receptor-coupled), or forskolin (receptor-uncoupled). Such studies showed that adrenaline-mediated inhibition of PGE_1-stimulated adenylate cyclase was unaltered by the presence of the anaesthetic. In contrast to this, both adrenaline- and GppNHp-mediated inhibition of the forskolin-stimulated state were attenuated at low concentrations of pentobarbital.

If these effects are a result of pentobarbital-induced modulations in membrane fluidity, then it may be that such modulations alter the efficiency of interaction between the components of the adenylate cyclase system. It would therefore appear that the interaction in question is at the level of G_i-C coupling as effects at the level of the receptor (R_i) would not account for the attenuation of GppNHp-mediated inhibition, i.e. involves direct activation of G_i. However, the absence of any

detectable effect of pentobarbital on adrenaline-mediated inhibition of PGE_1 would appear to invalidate G_i-C interaction as being the site of anaesthetic action, i.e. any modulation in this site would be reflected in adrenaline-inhibition of PGE_1 also. It may be that the inability to detect any anaesthetic-mediated effect in this situation results from the presence of a beta-gamma component to inhibition here; adrenaline-mediated inhibition of PGE_1 being proposed to involve G_i alpha and beta-gamma as discussed in Sections 3.1 and 1.3(iv). It is therefore suggested that the effect of pentobarbital as regards inhibitory ligands, is to modulate the efficiency of the G_i-alpha-C interaction. Such reduction in the efficiency of this coupling leads to attenuation of inhibition. However, this is not observed with PGE_1-stimulated adenylate cyclase due to the fact that a beta-gamma inhibitory component exists to mask this.

Although the effects of pentobarbital with regard to both inhibitory- and stimulatory-aspects of adenylate cyclase regulation are proposed as being a result of changes in membrane fluidity, it is apparent that the expected selective manipulation of fluidity did not arise, i.e. no preferential effects of pentobarbital were observed on receptor-coupled activities, hence the likelihood of selective fluidity modulation can be discounted.

It may be that pentobarbital modulates adenylate cyclase activity by a more direct means than enhancing fluidity. For example, the possibility cannot be ruled out that pentobarbital is acting directly at the G_i-C coupling interface rather than modulating its efficiency through membrane fluidity. It may be that G_i alpha-C interaction is charge sensitive, or that the anaesthetic may disrupt the interaction of the components with specific lipids that are required for activity, or indeed

pentobarbital may affect the species of phospholipid recruited into lipid clusters and hence removed from the bulk lipid pool (Houslay et al., 1981). Due to their charge properties, anionic drugs such as pentobarbital may be expected to exert a variety of effects on integral proteins, such as adenylate cyclase, through perturbing the bilayer in ways other than a simple fluidising effect.

(b) Cationic Drugs

When examining the effects of cationic drugs on adenylate cyclase activity, it was observed that both receptor-coupled and uncoupled activities were attenuated by increasing concentrations of prilocaine (Figs.42,43). No stimulation of either activity was observed. This is in contrast to studies on rat liver plasma membranes (Houslay et al., 1980b), where receptor-uncoupled states were found to be augmented by low concentrations of prilocaine. The inhibition of both PGE_1- and forskolin-stimulated adenylate cyclase activities at concentrations above 10mM, was similar to that observed in the rat liver plasma membrane system and may be due to annular displacement. This has been observed for both charged and neutral anaesthetics. In the rat liver plasma membrane system it was observed that high concentrations of prilocaine led to a progressive inhibition of activity, whilst e.s.r. studies showed bilayer fluidity to be enhanced at such concentrations. As no direct interaction of prilocaine with the protein was detected i.e. no effect on detergent-solubilised adenylate cyclase, it was suggested that the observed inhibition was indeed due to annular displacement.

Although it has been shown that prilocaine, at these concentrations, is able to enhance membrane fluidity, it may be that this charged compound exerts its effects via a more direct interaction with the adenylate cyclase system. For example, it may

be that prilocaine directly interferes with ligand binding; although the saturating concentrations of ligands used here tend to rule out any detectable changes in the adenylate cyclase activity through a shift in ligand-binding.

Adrenaline-mediated inhibition of PGE_1-stimulated adenylate cyclase (Fig.44) was found to be reduced at low concentrations of the anaesthetic. This may be a result of prilocaine perturbing coupling interactions. Coupling has been suggested to occur at the cytosol surface, and to involve acidic phospholipids, which tend to be localised here. As there is strong evidence (from n.m.r. studies) for interaction between cationic anaesthetics and negative phospholipids (Houslay & Gordon, 1983), an interaction between prilocaine and the acidic phospholipids involved in coupling might be expected to yield an inhibitory response. The site of this interaction must be at the level of G_i beta/gamma-G_s alpha; this conclusion arises because interference at any other level would be reflected in the activities of other coupling states. The return of inhibition to original levels at higher concentrations of prilocaine (i.e. $>$ 30mM) may be due to prilocaine-induced increases in fluidity overcoming the effect of anaesthetic at the coupling interface.

Any increase in fluidity which prilocaine induces would be expected to enhance other activities as well. Adrenaline-mediated inhibition of forskolin was enhanced by prilocaine, presumably due to such an increase in membrane fluidity. Almost identical results were observed with GppNHp-mediated inhibition of forskolin, and presumably reflect increased efficiency of G_i-C coupling also. It may be that the enhanced inhibition seen with ligands on forskolin-stimulated activity is a result of enhanced fluidity augmenting the efficiency of G_i-alpha-C coupling. With

inhibition of PGE_1-stimulated adenylate cyclase it may be that although G_i-alpha-C coupling was enhanced, the attenuation of the beta/gamma component of inhibition resulted in the effects observed here. This attenuation of beta/gamma-inhibition may be a direct effect of prilocaine, as discussed earlier.

It is therefore apparent that the incorporation into the bilayer of a cationic, hydrophobic molecule such as prilocaine, which can also displace membrane-bound calcium, is likely to have profound effects on lateral lipid distribution. As a consequence of this, the effects observed with prilocaine are not as 'simple' as those observed with the neutral, benzyl alcohol.

(iv) Conclusions

The concentration ranges used here were the same as employed in studies on rat liver plasma membranes (Houslay & Gordon, 1983). From studies done on rat liver plasma membranes and other systems, we can assume that similar concentrations of these anaesthetics will be able to partition into and perturb platelet membranes also. It is likely that prilocaine and pentobarbital will be able to act to increase membrane fluidity. However, it is apparent that although experiments in other systems suggested that charged drugs exhibited a greater tendency to interact with one or other halves of the bilayer, and that this was reflected in modulations of receptor-coupled or -uncoupled adenylate cyclase activities, no such selective effects are observed in human platelet membranes.

The question here is whether the observed effects are due to modulations in membrane fluidity at all. It would be necessary to perform experiments on solubilised adenylate cyclase in order to rule out any direct effects of the anaesthetic on the enzyme. However, such experiments would still leave the possibility that prilocaine and pentobarbital act to interfere with coupling. In fact, taking the results of these experiments together, it is apparent that the $G_i$alpha-C interaction is attenuated by the negatively charged pentobarbitol, whilst the positively charged prilocaine is able to enhance the efficiency of this coupling state. From such observations it can be postulated that the interaction of the G_i-alpha subunit with the catalytic unit involves positive charge to the extent that modulations in this charge alter the ability of these two components to interact effectively.

Although the exact mechanism by which these anaesthetics modulate adenylate cyclase activity is unclear, it has been possible to suggest points at which they may affect the adenylate cyclase system, and changes in fluidity could indeed explain the majority of these effects. From these experiments using charged anaesthetics it can be seen that the effects elicited by such drugs (positively or negatively charged) are different from those seen with the neutral benzyl alcohol.

3.3 Effect of dimethylnitrosamine on platelet adenylate cyclase activity

(i) Introduction

Dimethylnitrosamine is a carcinogenic compound, which has previously been used to manipulate the membrane environment of rat liver plasma membranes (Whetton et al., 1984). In these studies it was found that this compound was capable of reducing the fluidity of the membrane, and that this was reflected in a decreased adenylate cyclase activity.

Here, the action of dimethylnitrosamine was investigated on adenylate cyclase activity in human platelets.

(ii) Results

From Figure 47 it can be concluded that forskolin-stimulated adenylate cyclase activity (10^{-4}M) was attenuated by the presence of dimethylnitrosamine. Such attenuation occurred in a dose-dependent manner, such that at 50mM dimethylnitrosamine, this receptor-uncoupled activity was diminished by approximately 30%. Increasing drug concentrations beyond this led to still further reductions in adenylate cyclase activity: at 100mM dimethylnitrosamine only 43.4% $\pm$ 3.9 of the original forskolin-stimulated adenylate cyclase remained.

Receptor-coupled adenylate cyclase activity was affected in an almost identical manner by dimethylnitrosamine. Figure 48 shows PGE_1-stimulated adenylate cyclase to be reduced by dimethylnitrosamine; a 40% decrease occurring over the first 10mM. At 50mM dimethylnitrosamine a 54% decrease in PGE_1-stimulated activity is observed.

Figure 47 Effect of dimethylnitrosamine on forskolin-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate determinations.

n=3 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.

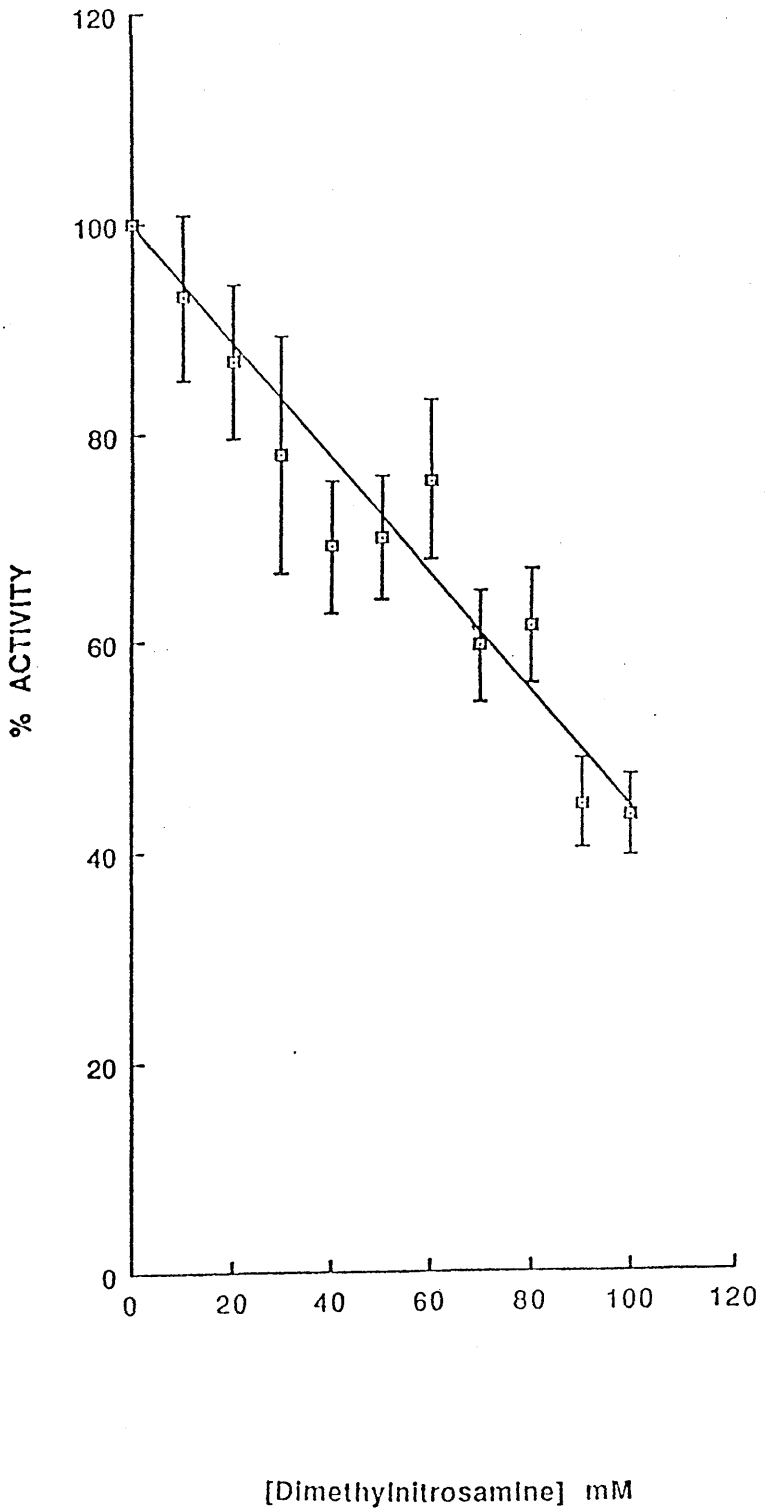
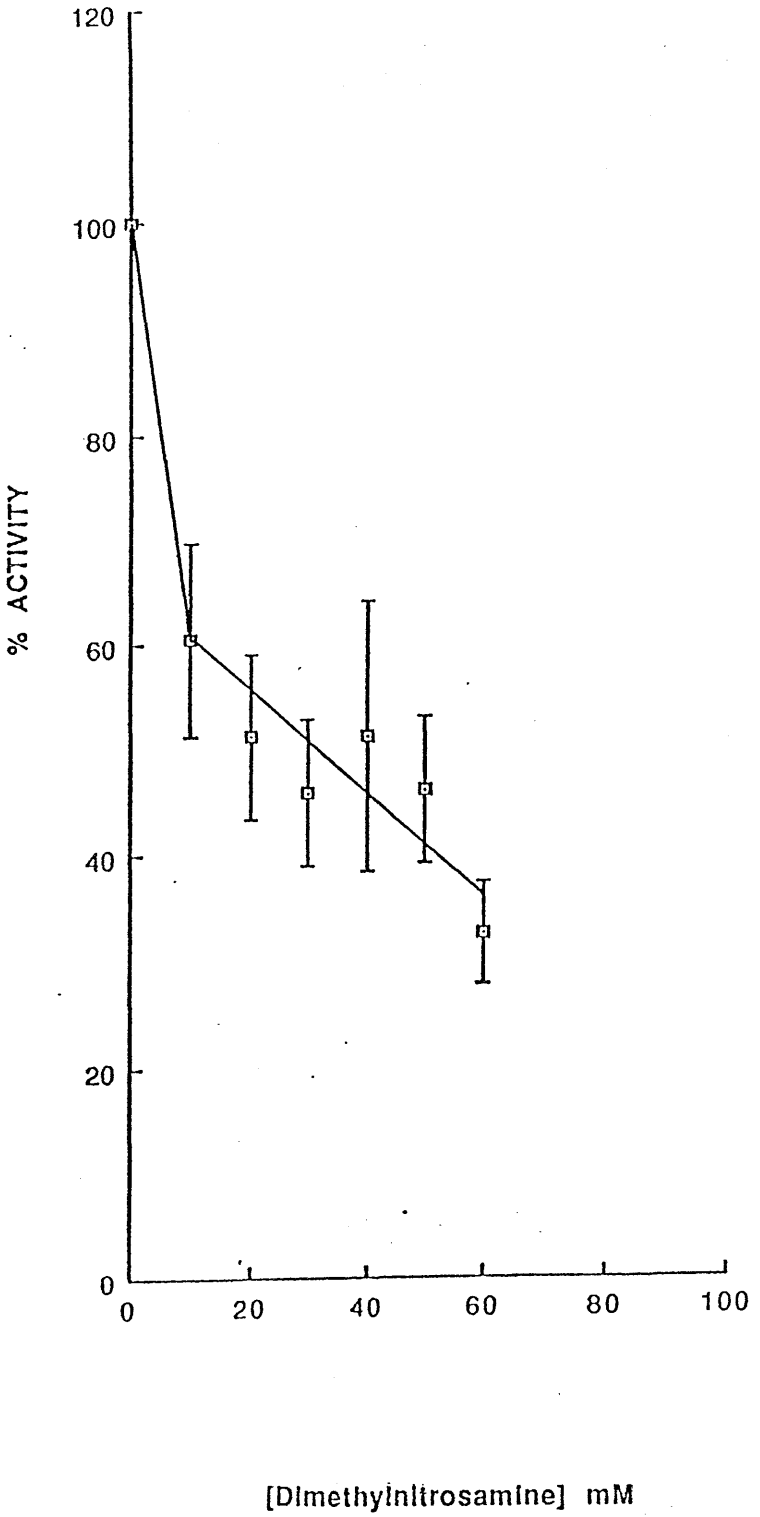

120
100
80
60
40
20
0
% ACTIVITY
0
20
40
60
80
100
120
[Dimethylnitrosamine] mM

Figure 48 Effect of dimethylnitrosamine on PGE_1-stimulated adenylate cyclase activity

Values are mean ± S.D. of a single representative experiment employing triplicate observations.

n=3 separate experiments using different membrane preparations.

Concentration of PGE_1 is 2×10^{-5}M.


120
100
80
60
40
20
0
% ACTIVITY
0
20
40
60
80
100
[Dimethylnitrosamine] mM

(iii) Discussion and conclusions

From results shown here it is obvious that dimethylnitrosamine affects receptor-coupled and -uncoupled adenylate cyclase activities in the same manner. The presence of dimethylnitrosamine appears to attenuate these activities in platelet membranes in an analogous fashion to that observed in rat liver membranes. Hence, it is assumed that such attenuation is a result of dimethylnitrosamine inducing rigidity in the membrane as this was shown to occur in the studies on rat liver plasma membranes (Whetton et al., 1984).

Comparison of PGE_1-stimulated adenylate cyclase and forskolin-stimulated adenylate cyclase at 50mM dimethylnitrosamine, showed the receptor-coupled and -uncoupled states to exhibit slight differences in their ability to be attenuated by this drug. At 50mM dimethylnitrosamine, the receptor-coupled activity was reduced by approximately 54%, whereas the receptor-uncoupled activity was reduced by only 30%. The reason for this large difference may lie in the fact that PGE_1-mediated stimulation of adenylate cyclase requires the interaction of a number of components in the membrane, whereas forskolin stimulates adenylate cyclase by direct interaction with the catalytic unit. As a result of this difference it may be that any reduction in bilayer fluidity mediated by dimethylnitrosamine would lead to reduced efficiency of component interaction, i.e. R_s-G_s-C, such that adenylate cyclase activity would be diminished. The reduction in forskolin-stimulated adenylate cyclase induced by dimethylnitrosamine can be attributed solely to reduction in fluidity increasing the physical constraint of the bilayer on the catalytic unit. As no mobile collisions are required for forskolin-activation, the effect of dimethylnitrosamine-induced rigidity is not as marked here.

In conclusion, it would appear that a decrease in membrane fluidity could indeed lead to the observed diminishment of adenylate cyclase activity. Hence, this would lend support to fluidity modulation of adenylate cyclase, whereby increased fluidity is porposed to enhance activity.

3.4 Effect of acidic phospholipids on platelet adenylate cyclase activity

(i) Introduction

Acidic phospholipids such as the negatively charged, phosphatidic acid, have been shown to modulate adenylate cyclase activity in a number of systems, including rat liver plasma membranes (Houslay et al., 1986c), and cultured cell lines (Clark et al., 1980; Murayama & Ui, 1987; Schimmel et al., 1980; Proll et al., 1985). In each case, it would appear that the presence of acidic phospholipids leads to attenuation of adenylate cyclase activity, although the precise mechanism by which this occurs is unclear.

However, it has been postulated that manipulation of membrane phospholipid content may result in changes in membrane fluidity (Houslay et al., 1986c) whether or not this leads to the reduction in adenylate cyclase activity observed with acidic phospholipids is questionable. This possibility will be examined here as will the proposals that acidic phospholipids mediate their effects via specific phospholipid receptors (Murayama & Ui, 1987), or G-proteins (Murayama & Ui, 1987; Schimmel et al., 1980; Proll et al., 1985)

(ii) Results

From Table 9 it can be seen that the acidic phospholipids, phosphatidic acid and phosphatidylserine were able to attenuate platelet adenylate cyclase. This inhibition of activity occured at all levels of regulation; these negative-charged species were capable of attenuating activity which had previously been stimulated at the level of the receptor (PGE_1; 2×10^{-5}M), the stimulatory G-protein, G_s, (NaF; 15mM), and directly at the level of the catalytic unit either basal, or forskolin-stimulated (forskolin; 10^{-4}M).

The actual percentage-inhibition mediated by phosphatidic acid appeared to differ depending on the stimulatory-state of the enzyme. Forskolin- and PGE_1-stimulated activities were inhibited by 64.5% $\pm$ 5.4, and 64.9% $\pm$ 8.2 respectively, with basal and NaF-stimulated activities inhibited by 32.8% $\pm$ 7.6 and 30.6% $\pm$ 9.1. However, with phosphatidylserine it would seem that this species was capable of attenuating the forskolin-stimulated state (64.7% $\pm$ 4.7) to a greater extent than either the receptor-activated (PGE_1), G_s-activated (NaF), or basal states, which were all inhibited by 43-48%. The neutral phospholipid, phosphatidylcholine, exhibited no tendency to inhibit any of these cyclase states i.e. either basal or stimulated.

It should be noted that phosphatidic acid was able to inhibit adenylate cyclase to a lesser degree if smaller concentrations of the phospholipid were employed: approximately 30-35% inhibition of the forskolin- and PGE_1-stimulated states was observed with concentrations of phosphatidic acid lower than 1mg. Hence, although inhibition by acidic phospholipids is not clearly dose-dependent, it can be generalised that lower concentrations of phospholipid tend to give lower percentage-inhibitions.

The fold-stimulation exerted by these stimulatory ligands was reduced in the presence of phosphatidic acid, and phosphatidylserine (Table 10). In agreement with the inhibitions detailed in Table 9, it was observed that the fold-stimulation exerted by both PGE_1 (receptor-mediated activation) and forskolin (direct activation at the catalytic unit) was attenuated by phosphatidic acid. Phosphatidylserine, whilst reducing the fold-stimulation mediated by forskolin (22.8 fold $\rightarrow$ 8.9 fold), had virtually no effect on the stimulation mediated by PGE_1. The neutral phosphatidylcholine had no detectable effect on either of these cyclase activities.

Table 11 illustrates the fact that the presence of manganese in the system had no significant effect on the phosphatidic acid-induced inhibition of any of these cyclase states. It would appear that phosphatidylserine-mediated inhibition was similarly unaffected by the presence of 5mM manganese; should uncouple G_i-C at this concentration.

Other attempts to modify the cyclase system in the presence of phosphatidic acid included the addition of benzyl alcohol to manipulate membrane fluidity. Table 12 shows that the presence of 50mM benzyl alcohol apparently had no significant effect on the degree of inhibition elicited by phosphatidic acid. However, there is apparently an exception to this statement; forskolin-stimulated adenylate cyclase did appear to be inhibited to a lesser extent by phosphatidic acid if benzyl alcohol was present. This reduction from 41.4% $\pm$ 4.9 to 25.9% $\pm$ 0.2 inhibition, may be due to an anomalous effect of benzyl alcohol on the forskolin-stimulated state.

It should be noted that the effects of these phospholipids were examined regarding inhibitory aspects of adenylate cyclase

regulation. Table 13 shows that phosphatidylserine mediated inhibition of PGE_1-activated adenylate cyclase was not additive with the adrenaline-mediated inhibition of this state. This is true of phosphatidic acid-mediated inhibition also, although here a slight degree of co-operativity is apparent.

With adrenaline-mediated inhibition of forskolin, no additivity or synergy was seen with either phosphatidic acid or phosphatidylserine and adrenaline. On the other hand, a slight co-operativity may be observed with both phospholipids and GppNHp-mediated inhibition of this forskolin-stimulated state.

Table 9 Acidic phospholipid-mediated inhibition of platelet adenylate cyclase

Results are shown as percentage-inhibitions; mean ± S.D. for n= between 2 and 6 separate experiments as indicated. Each experiment used different membrane preparations and employed triplicate observations in each case. Amount of acidic PL present is 1 - 4mgs

	Percentage-inhibition by phospholipids	
	Phospholipid	
Ligand	Phosphatidic Acid	Phosphatidyl-serine
None (basal)	32.8 ± 7.6 (n=5)	43.0 ± 5.6 (n=2)
Forskolin (10^{-4}M)	64.5 ± 5.4 (n=6) [34.5 ± 5.8] (n=3)	64.7 ± 4.7 (n=2)
PGE_1 (2×10^{-5}M)	64.9 ± 8.2 (n=6) [31.1 ± 5.4] (n=4)	48.7 ± 4.8 (n=3)
NaF (15mM)	30.6 ± 9.1 (n=3)	47.5 ± 3.5 (n=2)

Table 10 Effect of acidic phospholipids on fold stimulations

Results are shown as mean ± S.D. for n= given number of separate experiments. Each experiment employs triplicate observations, and uses different membrane preparations.

Stimulatory Ligand	Control	Fold Stimulation PA	PS	PC
PGE_1 (2×10^{-5}M)	23.4 ± 2.2 (n=14)	16.4 ± 2.3 (n=7)	21.5 ± 1.5 (n=3)	24.1 ± 2.1 (n=3)
Forskolin (10^{-4}M)	22.8 ± 1.7 (n=15)	16.1 ± 2.7 (n=8)	8.9 ± 4.2 (n=3)	22.2 ± 2.4 (n=3)

PA = phosphatidic acid
PS = phosphatidylserine
PC = phosphatidylcholine

Table 11 Acidic phospholipid-mediated inhibition of adenylate cyclase in the presence of manganese

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Concentration of manganese present is 5mM.

Ligand	Percentage-inhibition by phospholipid			
	Phosphatidic Acid		Phosphatidyl Serine	
	Mg	Mn	Mg	Mn
None (basal)	55 ± 9	68 ± 3	47	58
Forskolin (10^{-4}M)	63 ± 4	65 ± 6	ND	ND
PGE_1 ($2x10^{-5}$M)	66 ± 4	55 ± 1	47	48
NaF (15mM)	63 ± 4	65 ± 14	45	60

ND = not determined

Table 12 Effect of benzyl alcohol on phosphatidic acid-mediated inhibition of adenylate cyclase

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Benzyl alcohol is present at a final concentration of 50mM.

Stimulatory Ligand	Percentage-inhibition by phosphatidic acid	
	Absence of Benzyl alcohol	Presence of Benzyl aocohol
None (basal)	37.2 ± 1.3	34.4 ± 2.6
Forskolin (10^{-4}M)	41.4 ± 4.9	25.9 ± 0.2
PGE_1 (2 x 10^{-5}M)	35.7 ± 1.0	42.9 ± 0.6
NaF (1.5 x 10^{-2}M)	39.3 ± 3.1	44.3 ± 1.9

Table 13 Effect of acidic phospholipids on ligand-mediated inhibition of adenylate cyclase

Results shown are from preliminary experiments.

	Percentage inhibition
PGE_1/adrenaline	56
PGE_1/PS	54
PGE/Adrenaline + PS	56
PGE_1/PA	57
PGE_1/Adrenaline + PA	72
Forskolin/Adrenaline	25
Forskolin/PS	61
Forskolin/Adrenaline + PS	67
Forskolin/PA	29
Forskolin/Adrenaline + PA	32
Forskolin/GppNHp	43
Forskolin/PS	61
Forskolin/GppNHp + PS	73
Forskolin/PA	29
Forskolin/GppNHp + PA	51

PS = phosphatidylserine

PA = phosphatidic acid

(iii) Discussion

As the membrane bilayer is asymmetric with respect to lipid distribution, (acidic phospholipids being found predominantly in the cytosol half of the bilayer), it has been suggested that the addition of exogenous acidic phospholipids may preferentially manipulate this half of the bilayer. Indeed, it has actually been shown that phosphatidic acid is able to induce a lipid phase separation (at 16^{o}C) in the inner half of the rat liver plasma membrane in accordance with this (Houslay et al., 1986c). It may, therefore, arise that the activity of integral membrane proteins, such as adenylate cyclase, will be affected by such modulations.

From results reported here it would appear that acidic phospholipids are indeed capable of modulating adenylate cyclase activity in human platelet plasma membranes. Such modulation of enzymatic activity has been reported by others (Clark et al., 1980; Murayama & Ui, 1987; Schimmel et al., 1980; Proll et al., 1985), although in these studies it was suggested that the ability to manipulate adenylate cyclase activity was specific to phosphatidic acid, and did not apply to acidic phospholipids in general. In human platelets, it would seem that the ability of phosphatidic acid to attenuate adenylate cyclase activity is mirrored in studies employing phosphatidylserine. Hence, it would appear that this attenuating effect is common to other acidic phospholipids. The fact that the neutral phosphatidylcholine is unable to evoke such an inhibitory effect supports this proposal. These trends are in agreement with those reported by Houslay et al., 1986c in studies on rat liver plasma membranes.

Exactly how these acidic phospholipid species mediate the inhibition of platelet adenylate cyclase is unclear. It has been suggested that phosphatidic acid inhibits adenylate cyclase by a

process mediated by G-proteins (Murayama & Ui, 1987; Schimmel et al., 1980; Proll et al., 1985). In fact, in fibroblasts it has been reported that not only is phosphatidic acid-mediated inhibition GTP-dependent, but such inhibition is attenuated by pertussis toxin. This would suggest that G_i is the specific G-protein involved in this process. However, in the same study, phosphatidic acid was found to be incapable of inhibiting adenylate cyclase in other cell types (including platelets), and hence it was proposed that a direct effect of phosphatidic acid on G_i was unlikely. Instead, it was suggested that inhibition relied on the interaction of the G-protein with cell-specific phosphatidic acid receptors (Murayama & Ui, 1987). In this study of human platelets, it would appear that the addition of exogenous GTP is not required in order for acidic phospholipid-mediated inhibition to be observed. However, it is not possible to discount the fact that a G-protein may play a role in mediating these effects. It may be that a high-affinity G-protein exists, such that enough GTP is already present in the platelet membrane to support this G-protein activity. Although not ruling out the involvement of a G-protein, the lack of requirement for exogenous GTP does suggest that the inhibitory G-protein, G_i, is not the mediator of these effects. This is supported by the fact that replacement of magnesium with manganese had no significant effect on the degree of inhibition elicited by phosphatidylserine. Had this inhibition been G_i-mediated, it would have been expected that the addition of manganese would uncouple G_i-C interactions (Hoffman et al., 1981), and that acidic phospholipid-mediated inhibition would be attenuated.

In conflict with this theory of G_i-independent inhibition, is the apparent lack of additivity between acidic phospholipid-mediated inhibition and that known to be mediated by

G_i (i.e. $alpha_2$-adrenoceptor and GppNHp-mediated). This would imply that acidic phospholipid-mediated inhibition occurs via the same route, i.e. G_i. However, the contrasting results of the experiments discussed previously would tend to dispel this notion, and it may be that the interaction of G_i with the catalytic unit, and the method by which acidic phospholipids cause inhibition are mutually exclusive. It should, however, be remembered that 'additivity' experiments were very much preliminary results, and would require further characterisation before definite conclusions could be deduced from them.

Having eliminated G_i as being the mode by which these phospholipid species exerted their inhibitory effects on the platelet adenylate cyclase system, other methods were considered. Indeed, comparing phosphatidylserine-mediated inhibition of receptor-coupled (PGE_1-stimulated) and receptor-uncoupled (forskolin-stimulated) states suggests that the modulation by phospholipid is at the level of a component common to these states, namely the catalytic unit. The forskolin-stimulated state is particularly sensitive to manipulation of membrane phospholipid composition.

Examining phosphatidic acid-mediated inhibition reveals the same trends, although here an enhanced effect is seen with inhibition of the PGE_1-stimulated state also. This may tell us something of the nature of the PGE_1-receptor interaction with the adenylate cyclase system; namely that the presence of phosphatidic acid in particular is detrimental to efficient coupling. Perhaps phosphatidic acid may be excluded from the coupling interface in native membranes, and the addition of exogenous phosphatidic acid would therefore lead to inhibition of this state in particular.

If the interaction of acidic phospholipids in general is at the level of the catalytic unit, it may be that activation of this component by forskolin leads to a conformational change which renders the catalytic unit even more susceptible to inhibition by these phospholipid species. This is reflected in the degree of inhibition elicited by phospholipids on this state relative to both coupled states, and to basal activity (Table 9). As would be expected from the fact that the forskolin-stimulated state is inhibited to a greater degree than the basal cyclase state, acidic phospholipids are able to attenuate the fold-stimulation exerted by this ligand (as is phosphatidic acid able to reduce the fold-stimulation exerted by PGE_1) (Table 10). Such manipulation of the ability of ligands to enhance adenylate cyclase is important. It may be that acidic phospholipids mediate their inhibitory effects by preventing stimulation of adenylate cyclase, as well as, or perhaps instead of, directly inhibiting the enzyme.

Whilst it is unlikely that acidic phospholipids act to modulate membrane fluidity (Houslay et al., 1986c), the effect of benzyl alcohol on the cyclase system was examined in the presence of these lipid species. It was thought that the addition of benzyl alcohol would influence enzymatic activity in a different manner to that observed in native membranes (see Section 3:1). Addition of 50mM benzyl alcohol was, in fact, found to have no obvious effect on the degree of inhibition elicited by either phosphatidic acid or phosphatidylserine (Table 12). There is, however, one notable exception to this statement; namely phosphatidic acid-mediated inhibition of the forskolin-stimulated state, which was apparently attenuated by the presence of the local anaesthetic. No obvious reason for this effect can be suggested, although it may arise from an anomalous effect of benzyl alcohol on the forskolin-stimulated

state, as has been noted elsewhere (Section 3:1). On the other hand, it may be that this effect arises from the particular susceptibility of this stimulated state to inhibition by the acidic phospholipid species (Table 9), i.e. benzyl alcohol may induce a change in conformation such that this state is not as sensitive to phosphatidic acid. This phosphatidic acid-'insensitive' state may arise due to benzyl alcohol modulation of membrane fluidity, or by a more direct prevention of phosphatidic acid interaction with the enzyme.

One aspect of acidic-phospholipid inhibition which can be deduced from experiments involving benzyl alcohol, is that phosphatidic acid-mediated inhibition (of receptor-coupled states) does not involve G-protein beta/gamma components which we have suggested as being benzyl alcohol-sensitive (Section 3:1). Whilst telling us nothing about the involvement of G-protein alpha-subunits, these results, in combination with those from manganese experiments, as well as the lack of requirement for GTP, tend to suggest that a G-protein is not the route by which acidic phospholipids inhibit platelet adenylate cyclase.

It has also been proposed that acidic phospholipids inhibit rat liver adenylate cyclase by a specific headgroup interaction, and that this perturbation is at the level of G_s (Houslay et al., 1986c). Obviously, whilst such specific headgroup interaction cannot be discounted, it would appear that in human platelets G_s is not the site of interaction, as the basal and G_s-coupled states are inhibited to the same extent.

It should be noted that the effects observed here are not likely to have arisen through a loss of cholesterol from the platelet membranes during the incubation. Liposome incubation was under conditions which minimise such loss (short time, low

temperature, and low concentrations). Also, experiments in rat liver plasma membranes showed that the inclusion of cholesterol in liposomes, to counteract any cholesterol depletion, had no appreciable effect on the degree of inhibition elicited (Houslay et al., 1986c). It would, therefore, appear that the observed effects of acidic phospholipids are mediated at the level of the catalytic unit.

It may be that acidic phospholipids mediate such changes in adenylate cyclase activity by virtue of their negative charge. If this is so, then it might be expected that the negatively charged anaesthetic, pentobarbital, would exert similar effects. Comparing the results described in this chapter with those obtained in the presence of pentobarbital (Section 3.2) does show some similarities. For example, both these negative species are able to attenuate the activity of the platelet enzyme. This effect must be at the level of the catalytic unit in order to explain pentobarbital-mediated attenuation of the forskolin-stimulated state and phospholipid-mediated inhibition of the basal state. At this stage it is not possible to eliminate the possibility that both pentobarbital and acidic phospholipids inhibit cyclase by the same mechanism. However, when comparing the effect of these negative species on inhibitory aspects of enzyme regulation, differences arise. It has been proposed that pentobarbital modulates the efficiency of the G_i-alpha-C interaction, and it is possible that this arises by the negative anaesthetic altering the catalytic unit such that G_i-alpha cannot interact effectively. Hence, it may be possible to argue that this conforms to the mechanism whereby negatively charged species modulate cyclase activity at the level of the catalytic unit. However, upon examining the effects of acidic phospholipids on inhibitory aspects of regulation, it becomes

apparent that these species do not adhere to this theory. Both phosphatidic acid and phosphatidylserine were found to have no effect on the ability of either adrenaline or GppNHp to inhibit adenylate cyclase. This suggests that these phospholipids do not modulate the ability of $G_i$alpha to interact with the catalytic unit.

Hence, although pentobarbital and acidic phospholipids exhibit the same charge characteristics, their effects on the inhibitory arm of regulation are quite different. It is therefore likely that pentobarbital manipulates this aspect of regulation by modulating fluidity (i.e. as opposed to charge sensitivity), whilst acidic phospholipids have a more direct effect on the catalytic unit, which does not extend to modulations in the ability of G_i (or G_s) to interact with this component.

(iv) Conclusion

Although it has been proposed that the inhibitory effects of phosphatidic acid are mediated at the level of the G-protein (Murayama & Ui, 1980; Proll et al., 1985), this is apparently not the case in human platelet membranes, i.e. neither G_i nor G_s appear to be involved. It would seem that the ability to attenuate adenylate cyclase activity is not restricted to phosphatidic acid, but appears to apply to acidic phospholipids in general. It is proposed that this inhibition results from an effect at the level of the catalytic unit, although the precise mechanism by which this attenuation occurs is by no means clear.

CHAPTER 4

INTERACTION OF INSULIN AND THE PLATELET ADENYLATE CYCLASE SYSTEM

4. INTERACTION OF INSULIN AND THE PLATELET ADENYLATE CYCLASE SYSTEM

4.1 Introduction

Insulin modulation of platelet cyclic AMP levels may occur by two routes; either increased phosphodiesterase activity or reduced adenylate cyclase activity. Phosphorylation (Pyne & Houslay, 1988), involvement of a chemical mediator (Shirong et al. (1988)), and interaction of the G-protein system (Heyworth & Houslay, 1983) have all been proposed as mediating the effects of insulin on both these enzymes. However, here we are able to rule out insulin modulation of phosphodiesterase activity, as studies are performed in the presence of phosphodiesterase inhibitors. Instead, we will concentrate on the interaction of insulin with the adenylate cyclase system, and attempt to determine whether this occurs via the processes described above.

4.2 Results

Figures 49 and 50 show that PGE_1 and forskolin-stimulated adenylate cyclase activities were inhibited by insulin in an almost identical manner. IC_{50} for this inhibition was approximately 8.3uM for PGE_1-stimulated adenylate cyclase (Fig.49) and approximately 10uM for inhibition of the forskolin-stimulated state (Fig.50). Examining the effect of insulin on the ability of these ligands to bind and activate adenylate cyclase showed the EC_{50} for activation by PGE_1 to be unaltered by 10^{-8}M insulin; being 0.30uM $\pm$ 0.17 in the absence of insulin and 0.33uM $\pm$ 0.17 in the presence of insulin (n=3 separate experiments using different membrane preparations). Forskolin-mediated activation was similarly unaltered, exhibiting an EC_{50} of 15uM $\pm$ 7.07 in the absence, and 8.5uM $\pm$ 2.1 in the presence of insulin. Figure 51 illustrates this point, i.e. that the dose effect curve for these ligands (in this case PGE_1) was not shifted by the presence of insulin.

Attempts were made to manipulate insulin-mediated inhibition by denaturing the insulin molecule. Heat denaturation, through boiling the insulin for 5-10 minutes before use, resulted in complete removal of the ability of insulin to attenuate either PGE_1 or forskolin-stimulated adenylate cyclase, e.g. approximately 34% inhibition seen with non-heat treated insulin was reduced to 0% with boiled insulin.

The requirement of guanine-nucleotides for insulin-mediated inhibition was examined using both GTP (10^{-4}M) and the non-hydrolysable guanine nucleotide analogue GppNHp (10^{-4}M). Table 14 shows that the presence of GTP had no effect on the percentage-inhibition mediated by insulin on either the PGE_1 or forskolin-stimulated states. Similarly, the presence of the

non-hydrolysable GppNHp did not influence the percentage-inhibition, nor did it alter the IC_{50} for insulin-mediated inhibition. PGE_1-stimulated adenylate cyclase was inhibited by insulin with an IC_{50} of 8uM in the absence of GppNHp, and an IC_{50} of 9uM in its presence. The forskolin-stimulated state was inhibited by insulin with an IC_{50} of 10uM in both the presence and absence of GppNHp.

In order to determine if a G-protein was involved in insulin-mediated inhibition, maximally inhibitory concentrations of ligands were employed in the presence and absence of insulin to see if additivity could be detected. Table 15 shows that adrenaline- (10^{-4}M, plus 10^{-6}M propranolol) and insulin-mediated inhibitions were not additive. However, it may be that a slight co-operativity exists when considering adrenaline- and insulin-mediated inhibitions of both the PGE_1 and forskolin-stimulated states. In the same manner, co-operativity may be observed with GppNHp (10^{-8}M) and insulin-mediated inhibitions of forskolin-stimulated adenylate cyclase. It should be noted that in human platelets, insulin was observed as being capable of stimulating a GTPase activity; 124.4% $\pm$ 11.4 activity being observed in the absence of benzyl alcohol. In the presence of 50mM benzyl alcohol no significant change in this activity was detected, being 132.8% $\pm$ 18.8 activity (n=3 separate experiments using different membrane preparations).

If the effect of benzyl alcohol was examined with respect to insulin-induced inhibition of both receptor-coupled (PGE_1-stimulated) and -uncoupled (forskolin-stimulated) states, it was found that inhibition was insensitive to the presence of the anaesthetic in both cases. Figure 52 showed insulin was able to inhibit forskolin-stimulated adenylate cyclase by approximately 40% over the range of benzyl alcohol concentrations studied; 0-50mM.

From Figure 53 it can be seen that although insulin-attenuated the PGE_1-stimulated state to a larger degree (approximately 75% inhibition), this too was unaffected by benzyl alcohol. This is in contrast to the effects of benzyl alcohol on adrenaline-mediated inhibition of these states; adrenaline-mediated inhibition of PGE_1 being attenuated by the anaesthetic, whilst inhibition of forskolin was unaffected (see Section 3.1). Another way in which insulin-mediated inhibition differs from adrenaline-mediated inhibition is in it sensitivity to manganese. Figure 54 shows that whilst replacing magnesium with manganese (5mM) resulted in a 74.9% $\pm$ 7.4 attenuation in the ability of adrenaline to inhibit adenylate cyclase, insulin-mediated inhibition showed no significant change.

In order to examine the possibilities that insulin may mediate inhibition of adenylate cyclase by a route other than the G-protein system, a number of other aspects of inhibition were considered. As portrayed in Figure 55, the degree of inhibition elicited by insulin is apparently independent of the time of incubation. This applies not only to the inhibition of forskolin as illustrated, but to inhibition of PGE_1-stimulated adenylate cyclase also i.e. 26.2% $\pm$ 4.8 at 12 minute incubation, and 32.4% $\pm$ 5.1 at 24 minutes. Nor apparently was insulin-mediated inhibition dependent upon the amount of protein present; protein levels ranging from 5ug to 60ug yielded almost identical levels of inhibition (Fig.56). This lack of dependence on protein concentration was observable at other insulin concentrations too i.e. not only was 10^{-6}M insulin capable of inhibiting forskolin in a manner independent of protein levels, but so too was 5×10^{-8}M insulin. Here, with 1.5ug of portein, insulin led to a 35.3% reduction in adenylate cyclase activity, and at 30ug of protein, this inhibition was observed as 32.4% $\pm$ 4.0 (n=3).

The replacement of the physiological enzyme substrate ATP, with its non-hydrolysable analogue AMP-PNP, apparently had no effect on the degree of inhibition elicited by insulin (Table 16). Once again this applies to inhibition of both the PGE_1- and forskolin-stimulated states.

Finally, it should be noted that the insulin-like growth factor, IGF-I, was found to inhibit platelet adenylate cyclase. Inhibition of both PGE_1- and forskolin-stimulated activities was observed, being 31.1% and 22.0% respectively. This inhibition occurred at approximately the same concentration as insulin-mediated inhibition i.e. 10^{-7}M IGF-I, and 5 x 10^{-7}M insulin (see Table 17).

Figure 49 Insulin dose effect curve for inhibition of PGE_1-stimulated adenylate cyclase activity

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Figures are expressed as pmoles cAMP/min/100ul assay.

Concentration of PGE_1^- is 2×10^{-5}M.

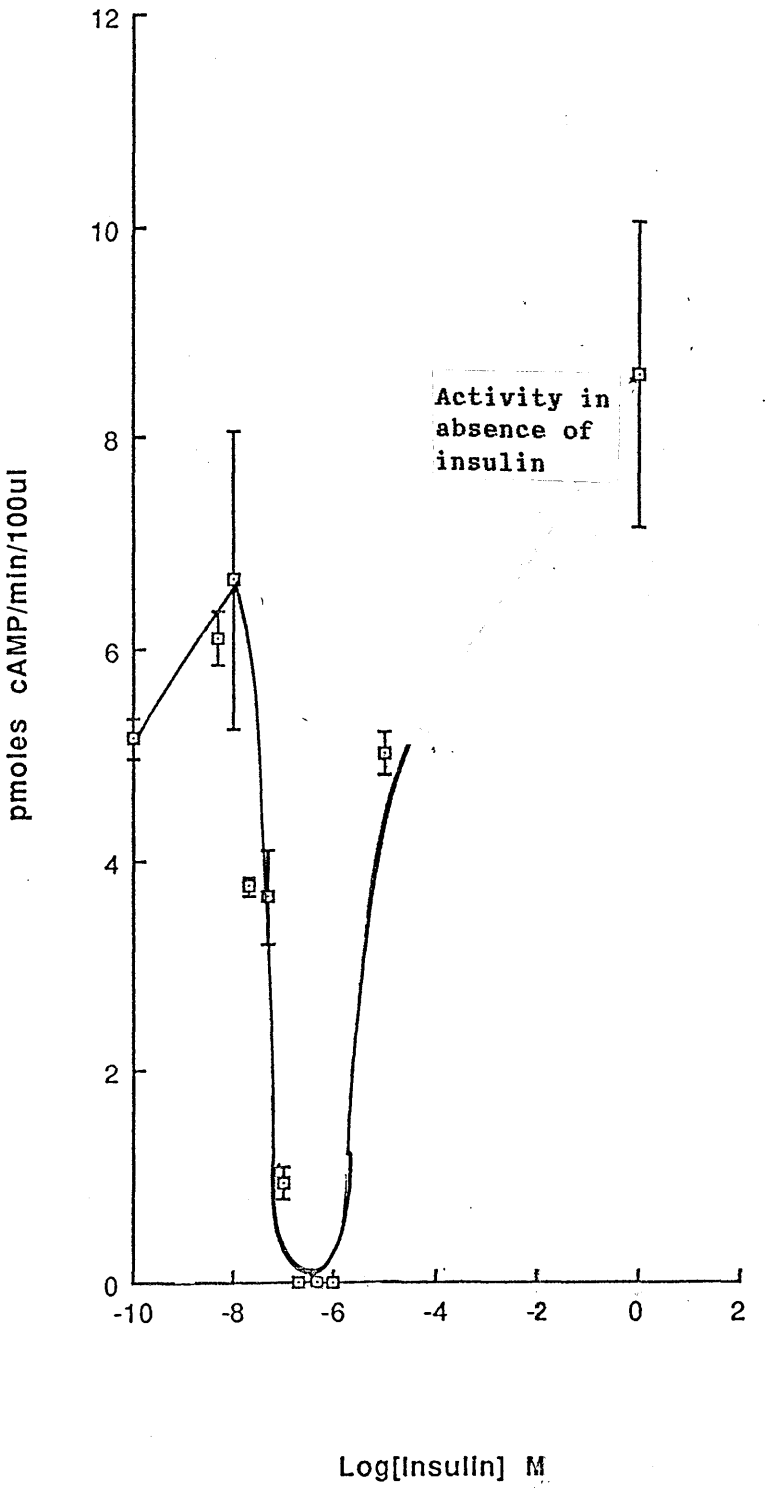

12
10
8
6
4
2
0
pmoles cAMP/min/100ul
Activity in absence of insulin
-10
-8
-6
-4
-2
0
2
Log[Insulin] M

Figure 50 Insulin dose effect curve for inhibition of forskolin-stimulated adenylate cyclase activity

Results are mean ± S.D for n=4 separate experiments using different membrane preparations.

Figures are expressed as pmoles cAMP/min/100ul assay.

Concentration of forskolin is 10^{-4}M.

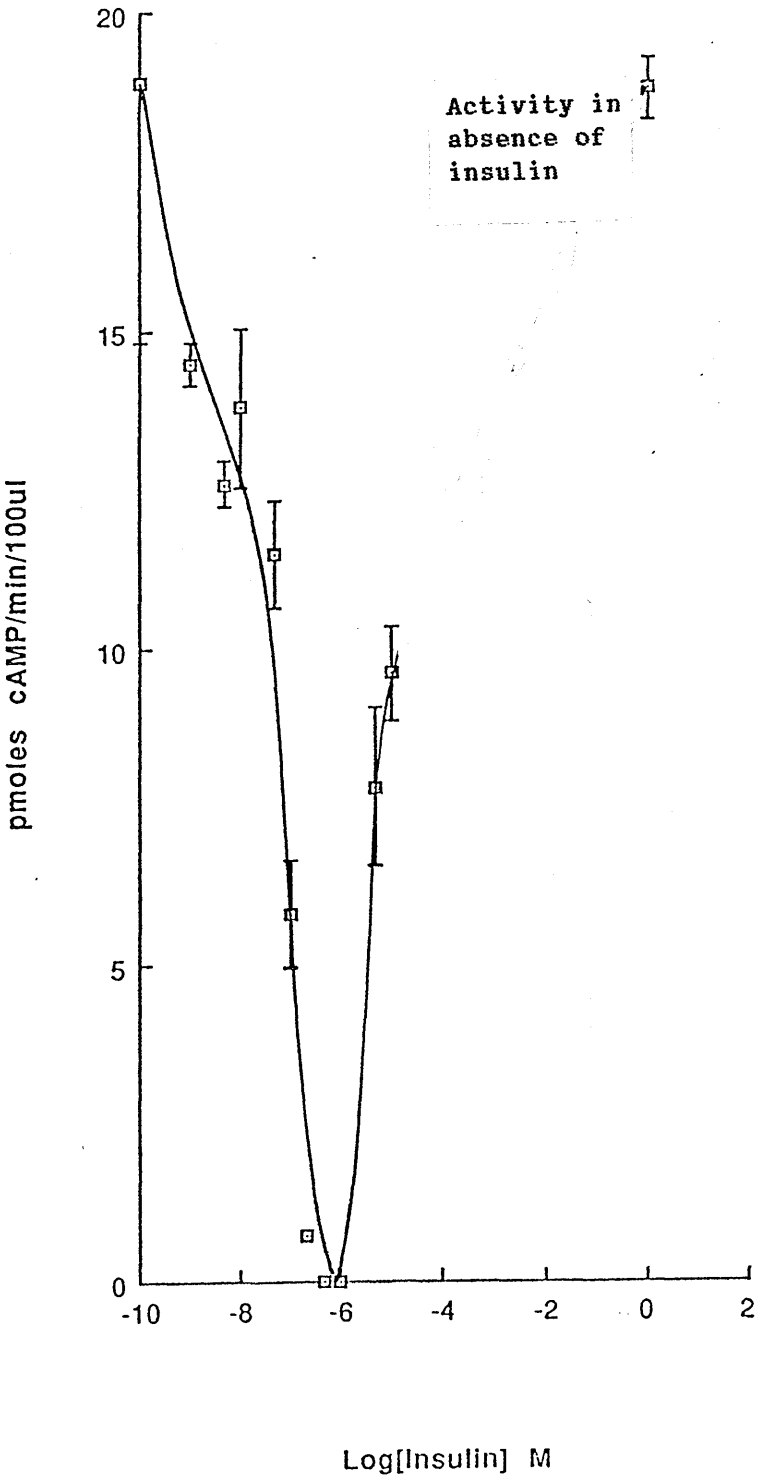

20
15
10
5
0
-10
-8
-6
-4
-2
0
2
Activity in absence of insulin
pmoles cAMP/min/100ul
Log[Insulin] M

Figure 51 Effect of insulin (10^{-8}M) on PGE_1-mediated-stimulation of adenylate cyclase activity

Results are mean ± S.D for n=3 separate experiments using different membrane preparations.

Figures are expressed as pmoles cAMP/min/100ul assay.

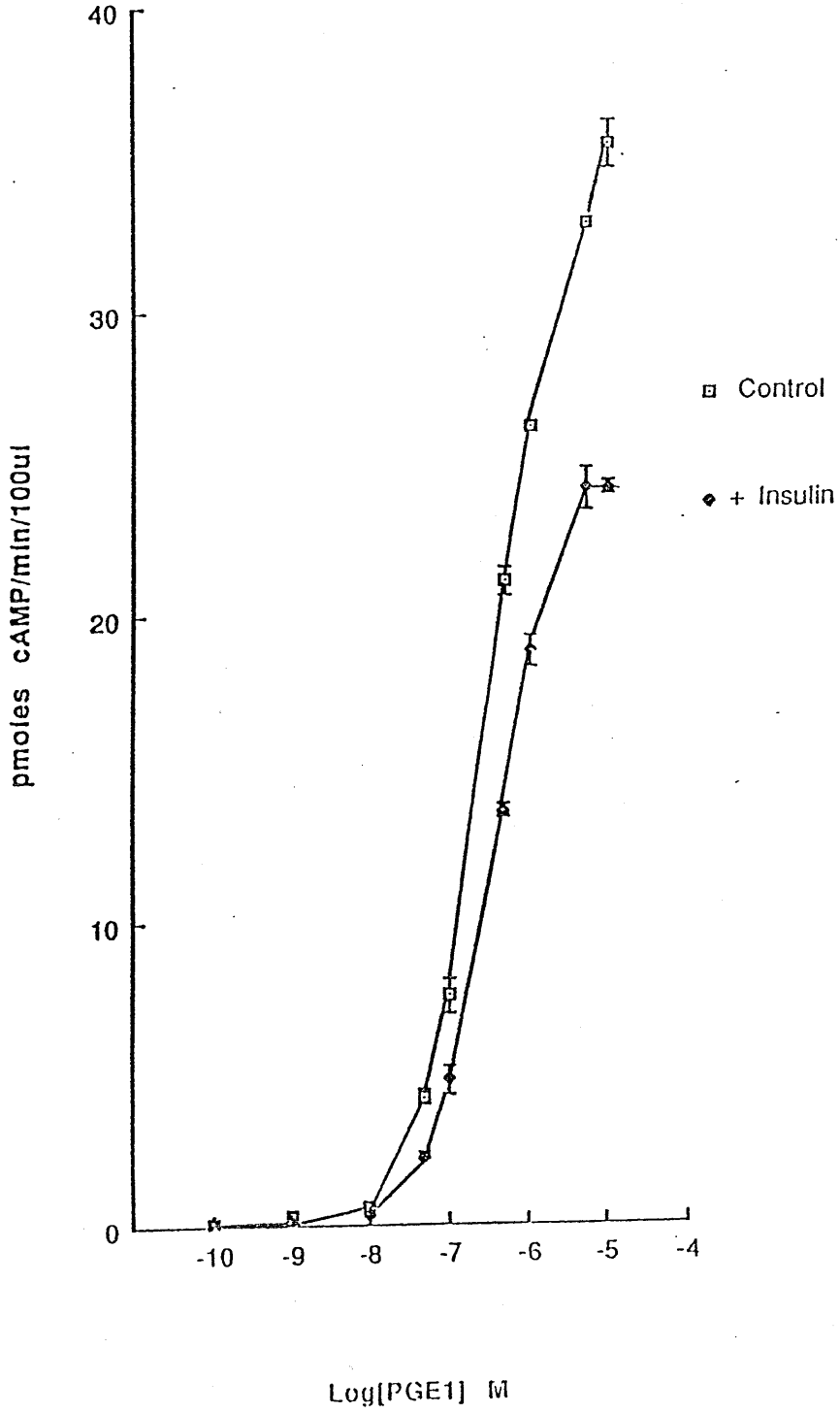

40
30
20
10
0
pmoles cAMP/min/100ul
Control
+ Insulin
-10
-9
-8
-7
-6
-5
-4
Log[PGE1] M

Figure 52 Effect of benzyl alcohol on insulin-mediated inhibition of forskolin-stimulated adenylate cyclase activity

Results are mean ± S.D for a single representative experiment, employing triplicate observations.

n=3 separate experiments using different membrane preaprations.

Concentration of forskolin is 10^{-4}M.

Insulin is present at a final concentration of 10^{-8}M.

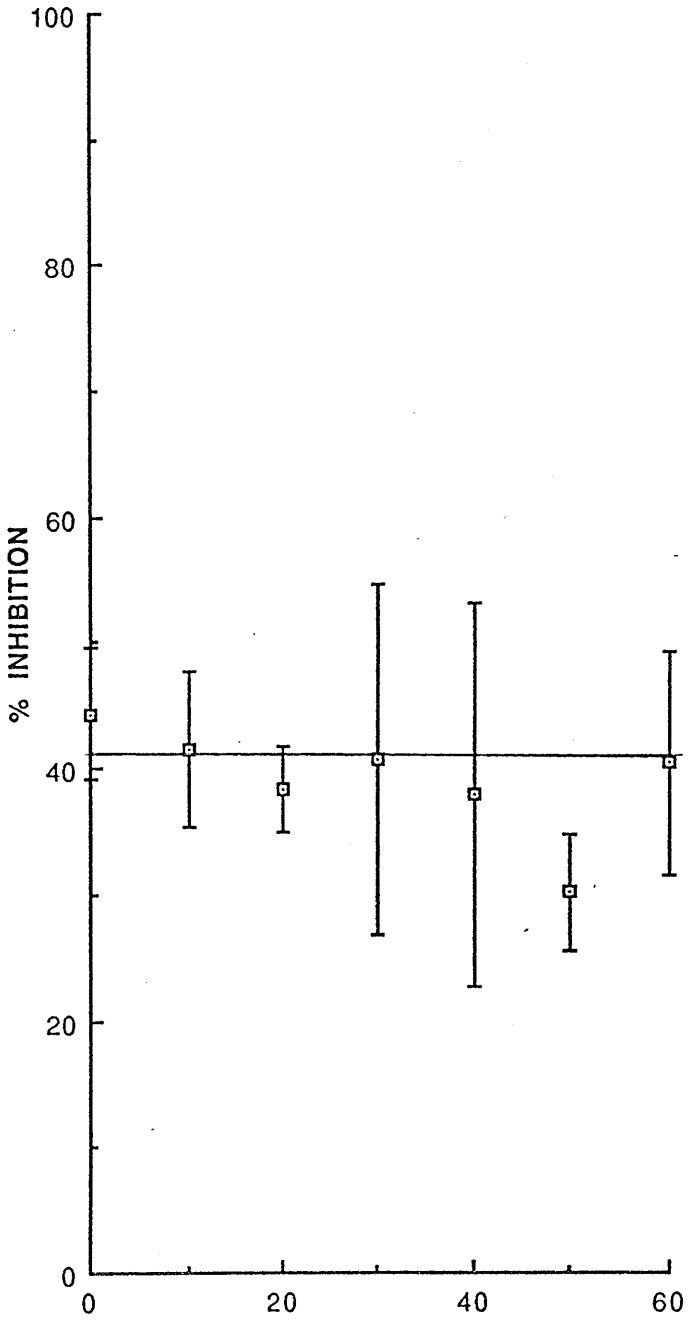

100
80
60
40
20
0
% INHIBITION
0
20
40
60
[Benzyl alcohol] mM

Figure 53 Effect of benzyl alcohol on insulin-mediated inhibition of PGE_1-stimulated adenylate cyclase activity

Results are mean ± S.D for a single representative experiment, employing triplicate determinations.

n=3 separate experiments using different membrane preparations.

Concentration of PGE_1^- is 2×10^{-5}M.

Insulin is present at a final concentration of 10^{-8}M.

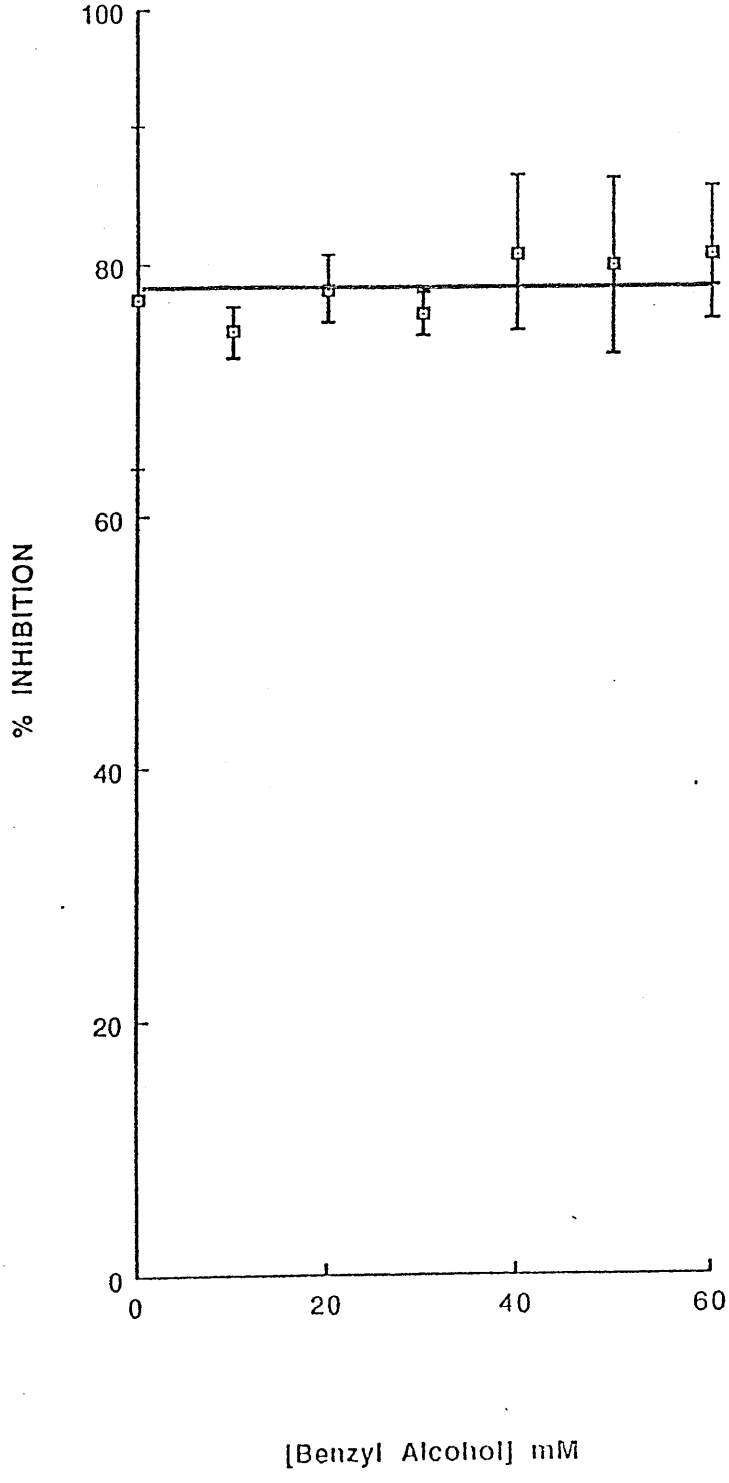

100
80
60
40
20
0
% INHIBITION
0
20
40
60
[Benzyl Alcohol] mM

Figure 54 Modulation of insulin-mediated inhibition by manganese (5mM)

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Condition 1 = Insulin inhibition + Mg

Condition 2 = " " + Mn

Condition 3 = Adrenaline inhibition + Mg

Condition 4 = " " + Mn

Inhibition is shown as percentage of that observed in the presence of 5mM Mg^{2+} (control) i.e. 100%.

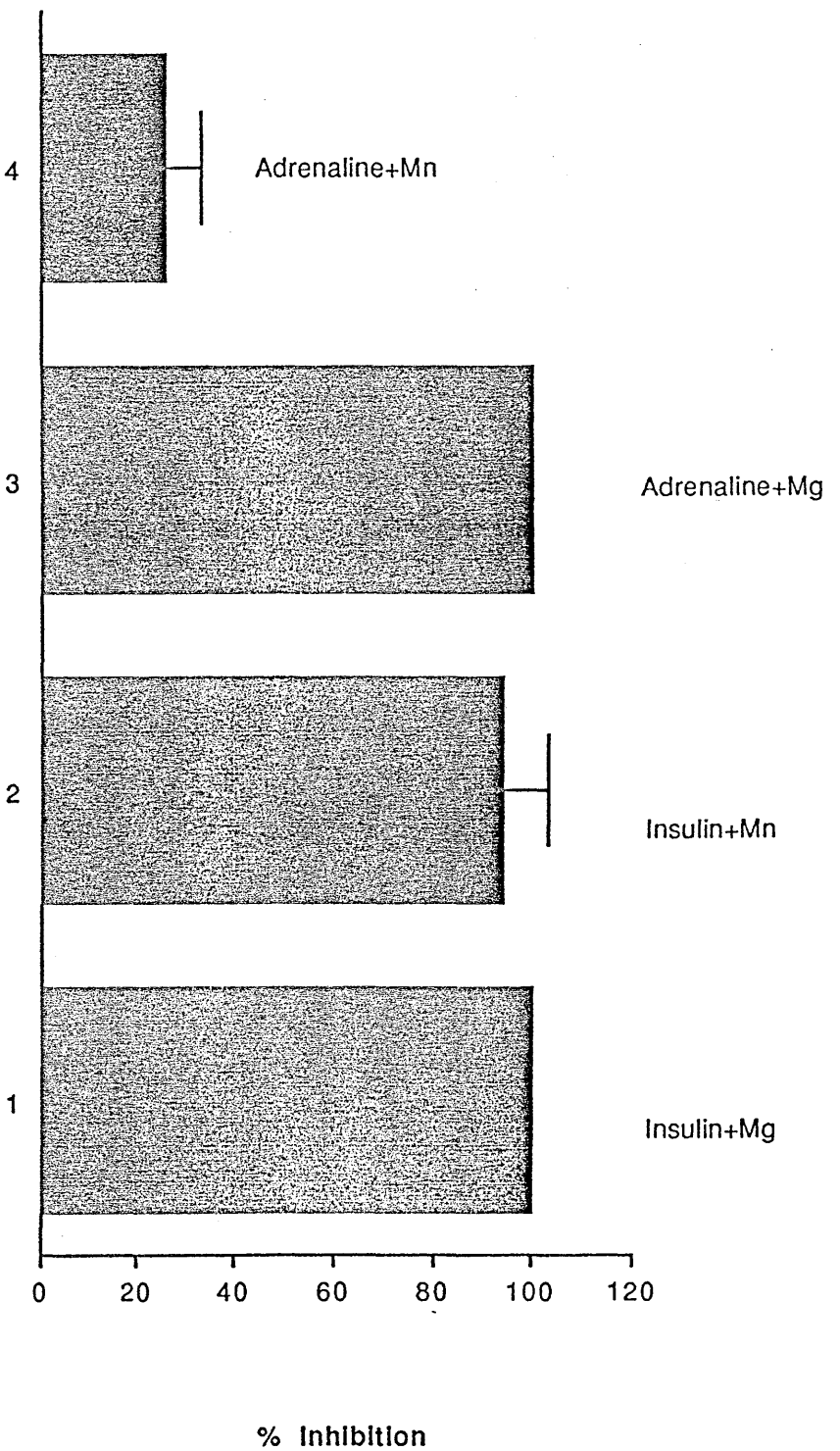

4
Adrenaline+Mn
3
Adrenaline+Mg
2
Insulin+Mn
1
Insulin+Mg
0
20
40
60
80
100
120
% Inhibition

Figure 55 Insulin-mediated inhibition of forskolin-stimulated adenylate cyclase as a function of time

Results are mean ± S.D. for n=3 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.
Insulin is present at a final concentration of 10^{-6}M.

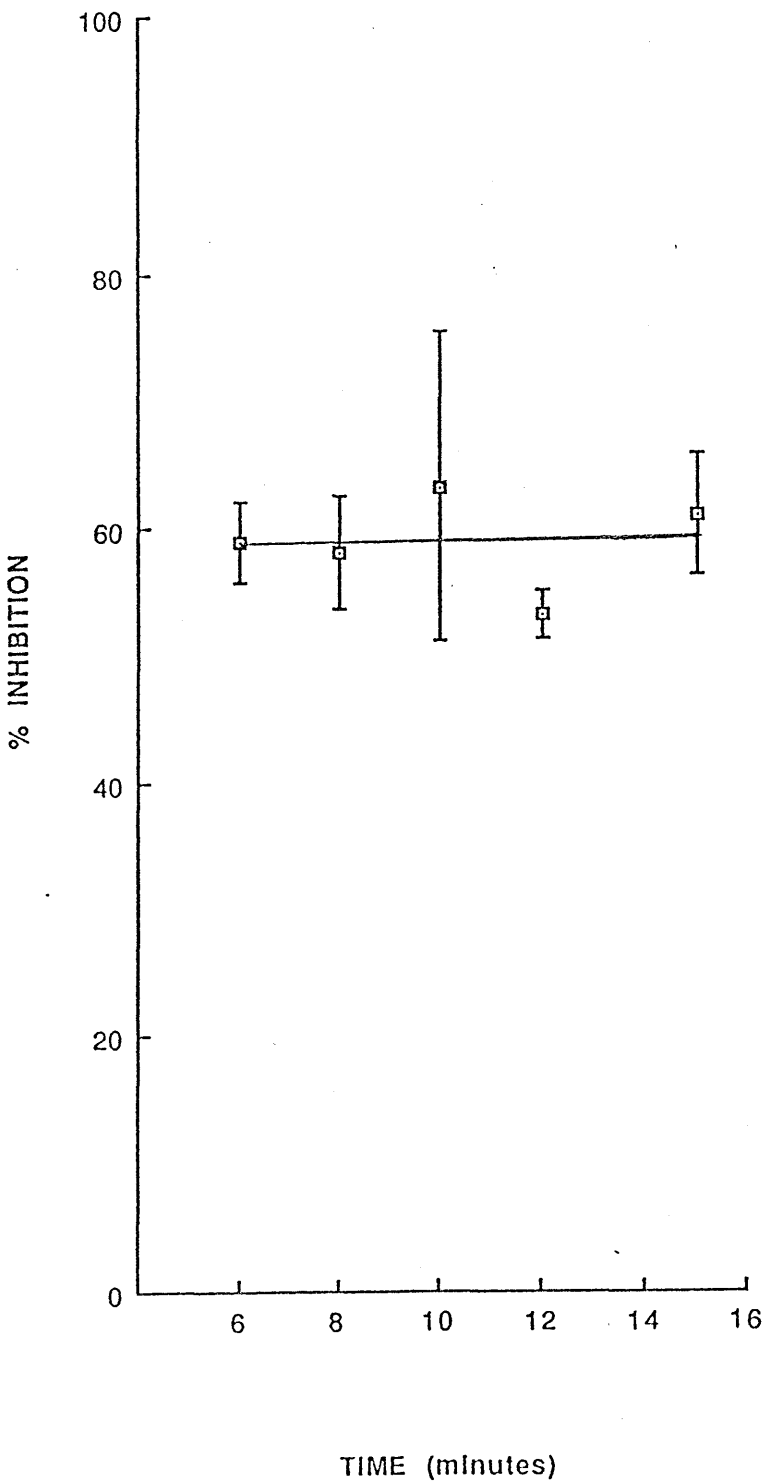

100
80
60
40
20
0
% INHIBITION
6
8
10
12
14
16
TIME (minutes)

Figure 56 Insulin-mediated inhibition of forskolin-stimulated adenylate cyclase: effect of protein concentration

Results are mean ± S.D. for n=4 separate experiments using different membrane preparations.

Concentration of forskolin is 10^{-4}M.
Insulin is present at a final concentration of 10^{-6}M.

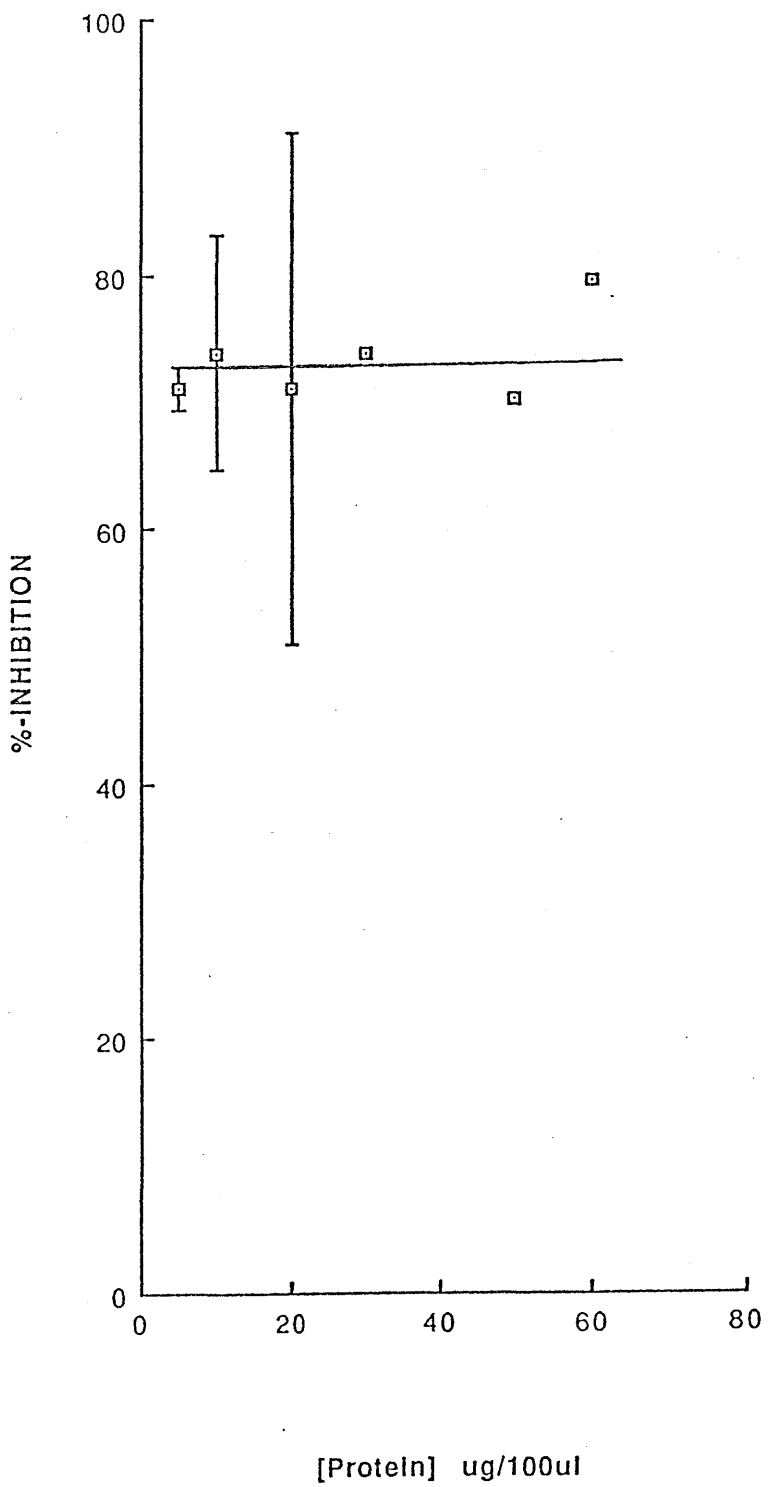

100
80
60
40
20
0
%-INHIBITION
0
20
40
60
80
[Protein] ug/100ul

Table 14 Effect of 10^{-4}M GTP/GppNHp on insulin-mediated inhibition of adenylate cylase

Results are mean ± S.D. for n=4 separate experiments using different membrane preparations.

		Percentage-inhibition		
Stimulatory Ligand	[insulin]	Absence of added guanine nucleotide	Presence of 10^{-4}M GppNHp	Presence of 10^{-4}M GTP
PGE_1 (2×10^{-5}M)	10^{-6}M	61.7 ± 4.8	64.5 + 8.2	49.4 ± 3.9
	10^{-7}M	24.4 ± 9.1	21.9 ± 6.8	N.D.
Forskolin (10^{-4}M)	10^{-6}M	55.9 ± 12.9	54.6 ± 10.0	66.6 ± 2.0
	10^{-7}M	28.5 ± 5.2	20.5 ± 0.2	N.D.

Table 15 Additivity of insulin-mediated inhibition of adenylate cyclase

Stimulatory Ligand	Inhibitory Ligand	%-inhibition
PGE_1 (2×10^{-5}M)	Adrenaline (10^{-4}M)	44.0%
	Insulin (10^{-6}M)	40.0%
	Adrenaline + Insulin	57.6%
Forskolin (10^{-4}M)	Adrenaline (10^{-4}M)	41.8%
	Insulin (10^{-6}M)	32.2%
	Adrenaline + Insulin	62.7%
	GppNHp (10^{-8}M)	56.7%
	GppNHp + Insulin	77.2%

Table 16 Insulin-mediated inhibition of adenylate cyclase in the presence of AMP-PNP

Figures are mean ± S.D. of n=3 separate experiments using different membrane preparations.

Stimulatory Ligand	Inhibitory Ligand	Percentage-inhibition ATP	AMP-PNP
PGE_1 (2×10^{-5}M)	Adrenaline (10^{-4}M)	69.0 ± 4.0	66.9 ± 12.9
	Insulin (10^{-8}M)	25.1 ± 6.1	25.6 ± 5.7
	Insulin (10^{-6}M)	52.8 ± 9.6	44.9 ± 1.8
Forskolin (10^{-4}M)	Adrenline (10^{-4}M)	66.5 ± 9.5	77.6 ± 3.5
	Insulin (10^{-6}M)	54.8 ± 0.6	45.9 ± 0.4

AMP-PNP is 1.5mM
ATP is 1.5mM

Table 17 Comparison of insulin- and IGF-I-mediated inhibitions of adenylate cyclase activity

Stimulatory Ligand	Insulin (5×10^{-7}M)	IGF-I (10^{-7}M)
	Percentage inhibition	
PGE_1 (2×10^{-5}M)	31.9%	31.2%
Forskolin (10^{-4}M)	30.3%	22.0%

4.3 Discussion

In this study, the ability of insulin to mediate inhibition of both receptor-coupled (prostaglandin E_1-stimulated) and receptor-uncoupled (forskolin-stimulated) states was characterised. This inhibition is in agreement with studies by Heyworth & Houslay (1983) on rat liver plasma membranes; but is in contrast to reports by a number of other groups (Falcon et al., 1988). A recent report by Kahn and Sinha (1990), suggests that at physiological concentrations (nM), insulin is able to stimulate prostaglandin E_1 binding to human platelet membranes, and to lead to activation of adenylate cyclase. This report was based on studies employing intact platelets, and proposed that the insulin-mediated enhancement of prostaglandin E_1-stimulated adenylate cyclase activity arose from insulin increasing the availabiltiy of 'spare' prostaglandin E_1 receptors in the membrane bilayers. No effect of insulin was noted on isolated platelet membranes. Although populations of such 'spare' receptors may have occurred in our membrane preparations, the lack of ability to detect this activation phase illustrates the complexity of insulin's effects on the platelet adenylate cyclase system. It may be that the full mechanism behind insulin's ability to activate PGE_1-stimulated adenylate cyclase is more complex than noted by Kahn et al., and remains unidentified.

The ability to detect insulin-mediated inhibition of adenylate cyclase is also a matter of conflict, although such inhibition was routinely detected here. The concentration of insulin required to mediate this inhibition of platelet adenylate cyclase (10^{-6}M) was much greater than that noted to cause inhibition in the rat liver plasma membrane system (10^{-9}M) (Heyworth & Houslay, 1983). The higher levels of insulin required

to exert inhibition may reflect a difference in the platelet insulin receptor relative to those expressed in more conventional target tissues (Falcon et al., 1988).

One reason which has been proposed as contributing to the conflicting reports on the ability of insulin to attenuate adenylate cyclase activity is the method of analysis applied. In the majority of cases where insulin-mediated inhibition was not detectable, the method by which cyclic AMP was detected involved the use of a 'trap' of unlabelled cyclic AMP (cf. Salomon assay/$\propto$ [^{32}P]-assay). It has been suggested that the presence of this pool of exogenous cyclic AMP stimulates any cyclic AMP-dependent protein kinase which may be present in the membranes. The result of this stimulation is that the kinase phosphorylates the insulin-receptor to attenuate its functioning: insulin-mediated inhibition is thereby negated (Houslay, 1990). Indeed, insulin-mediated inhibition of platelet adenylate cyclase was undetectable employing the cyclic AMP determination method of Salomon et al. (1974) over a concentration range of 10^{-10} to 10^{-4}M insulin. For this reason, the method employed to assay adenylate cyclase here utilised the cyclic AMP binding assay of Brown et al. (Brown et al., 1972) as detailed in Materials and Methods, Section 2.8.

As stated previously, both PGE_1- and forskolin-stimulated states were inhibited by insulin. The fact that these states were inhibited with similar IC_{50}'s would suggest that this insulin-mediated inhibition may occur by the same route. Whichever route insulin takes to mediate this inhibition is unclear. However, it does not involve insulin acting to prevent ligand binding: EC_{50} for stimulatory ligands is unaltered by the presence of insulin, as shown in Figure 51.

As other groups have suggested that insulin inhibits adenylate cyclase via interaction with the G-protein system (Gawler & Houslay, 1987; Heyworth & Houslay, 1983), various methods were employed to see if this was the route of insulin inhibition of the platelet adenylate cyclase system. First of all, to ensure that the inhibition detected was due to an action of insulin, it was decided to denature the insulin molecule, and thereby remove the inhibition of adenylate cyclase. Boiling insulin for 5-10 minutes before use resulted in heat denaturation, and as expected, this species was unable to elicit inhibition of the enzyme. Attempts to break the disulphide bonds of the insulin molecule by treatment with thiol reagents such as mercaptoethanol, were unsuccessful when applied to this system. This is thought to result from the fact that thiol reagents have previously been shown to interfere with activity of this enzyme. For example, dithiothreitol (DTT) has been shown to directly activate rabbit skeletal muscle adenylate cyclase. It is presumed that this effect arises due to interaction of DTT with an essential sulphdryl moiety in the enzyme (Wright & Drummond, 1983).

Having established the the observed inhibition was indeed due to insulin, the requirement of GTP/GppNHp was then examined. Table 14 shows that these guanine-nucleotides neither modulate the IC_{50} for insulin-inhibition, nor altered the percentage-inhibition mediated by this ligand. This would tend to rule out the involvement of a G-protein in the route by which insulin inhibits platelet adenylate cyclase. However, it could be that the G-protein involved has a high affinity for GTP, such that saturating levels of the nucleotides are already present in the membranes i.e. before exogenous guanine-nucleotide is added. Other groups have reported the involvement of a novel G-protein, G_{ins}, which has an

affinity for GTP of K_m=0.6uM (Gawler & Houslay, 1987) and indeed it was noted that insulin was capable of activating such an activity in human platelets (124.4% ± 11.4 activity).

Another means by which the involvement of G-proteins can be examined is by the use of ligands known to inhibit adenylate cyclase via the G-protein system. Here maximally inhibitory concentrations of these ligands were employed, and then insulin added to see if further inibition could be detected. Any further inhibition should, in theory, occur by a route other than G_i, as this route is already functioning to maximal capacity. In this study on platelets, full additivity was not found with insulin and either adrenaline, or GppNHp. This would suggest these species must inhibit adenylate cyclase by the same route, namely, G_i. However, some co-operativity was observed with insulin and adrenaline as well as insulin and GppNHp. This co-operativity suggests that insulin is indeed capable of mediating inhibition by a route other than G_i. Whilst it may be that part of the insulin-mediated inhibitory pathway is via G_i, the results shown here illustrate the existence of another route, which can account for up to two-thirds of the observed adenylate cyclase inhibition. What this other route may be is the subject of investigation here.

Comparison of insulin-mediated inhibition with other ligand-mediated inhibitions of adenylate cyclase should suggest a route(s) by which insulin acts. Not only did insulin inhibit forskolin-stimulated adenylate cyclase to a similar degree to that observed with adrenaline and GppNHp, but this inhibition was also insensitive to the local anaesthetic benzyl alcohol. This suggests that insulin may inhibit cyclase via G_i-alpha interaction with the catalytic unit. However, insulin-mediated inhibition of prostaglandin E_1 was also benzyl alcohol-insensitive (and

therefore differed from adrenaline-mediated inhibition of prostaglandin E_1) suggesting that although a G_i-alpha component may be involved in inhibition, a beta/gamma component apparently is not, i.e. beta/gamma-mediated inhibition is reported as being benzyl alcohol-sensitive (Section 3.1). If insulin inhibition of adneylate cyclase is a G_i-mediated process, it is unclear why such inhibition of the prostaglandin E_1-stimulated state does not involve a beta/gamma component. Also, such a condition would raise the question, what accounts for the difference in percentage-inhibition mediated by insulin on the forskolin- and PGE_1-stimulated states? Perhaps insulin does inhibit adenylate cyclase via a G-protein, but not G_i. If insulin-mediated inhibition was through the putative G_{ins}, it may be that G_{ins}alpha-inhibition was analogous to $G_i$alpha-inhibition, being benzyl alcohol-insensitive, whilst G_{ins} beta/gamma mediated inhibition was different from G_i beta/gamma, i.e. the latter is benzyl alcohol-sensitive and the former is insensitive. Exactly why this would be so is unclear, although the beta/gamma 'family' of molecules is being shown to be much more heterologous than previously thought (Robishaw et al., 1989).

A final attempt to determine directly whether or not G_i was involved in the actions of insulin entailed the use of manganese in the assay medium. At 5mM, manganese is believed to uncouple G_i interaction with the catalytic unit, and indeed, adrenaline-mediated inhibition was attenuated in accordance with this (Fig.54). However, it would appear that the replacement of magnesium with manganese has no effect on insulin-mediated inhibition, suggesting the G_i-component is not involved in insulin-mediated inhibition of adenylate cyclase.

The possibility that insulin might mediate its effects via the production of a chemical mediator was considered next. As it has been suggested that these mediators arise from the breakdown of inositol glycolipids in the membrane (Houslay, 1986) it might be expected that their production would vary with time and with the amount of membrane (and therefore substrate) present. Here, however, it was found that length of incubation (Fig.55) was not a factor in determining the degree of inhibition elicited by insulin. This would tend to suggest that the production of a mediator probably is not a step in insulin's actions on adenylate cyclase. This suggestion receives further support from the observation (Fig.56) that the amount of protein present in the assay is not a factor either. Again, however, the possibility arises that insulin may stimulate a specific phospholipase in a much shorter time span than that considered here, and that the efficiency of this phospholipase may be such that a very small amount of substrate is required, i.e. that insulin produces a highly effective mediator in small quantities, over a short time, and that this could not be detected by the limitations of these experiments.

Another possible route of insulin-mediated inhibition which was considered was the possibility that the insulin-receptor acted to phosphorylate some component of the adenylate cyclase system. This suggestion arises because the insulin-receptor is known to exhibit an endogenous tyrosyl kinase activity (Houslay & Siddle, 1989). It may be that insulin stimulates the receptor such that this kinase activity is employed either to phosphorylate cyclase directly, or to initiate a phosphorylation cascade which results in phosphorylation of the enzyme. In either case it may be that phosphorylation of G_i occurs such that it attains a more active conformation, or, that phosphorylation of G_s or C occurs such that

activation of adenylate cyclase cannot arise. The use of the non-hydrolysable AMP-PNP, whilst not distinguishing between these possibilities will rule out the involvement of phosphorylation in general as the mechanism of insulin-induced inhibition. Indeed, experiments here (Table 16) show that insulin-inhibition was equally effective in the presence of AMP-PNP as with ATP. This suggests that insulin inhibition cannot be via phosphorylation, i.e. the terminal phosphate moiety cannot be transferred to some component of the adenylate cyclase system.

Finally, the action of insulin-like growth factor I was determined on the platelet adenylate cyclase system. It was observed (Table 17) that this growth promoting polypeptide was capable of inhibiting both receptor-coupled (PGE_1-stimulated) and receptor-uncoupled (forskolin-stimulated) adenylate cyclase activities. This inhibition occurs to approximately the same degree as that observed with similar concentrations of insulin, and raises the question, are IGF-I and insulin acting through the same receptor, and if so, is it the IGF-I or insulin receptor?

It has been reported that IGF-I may simply act as a low-affinity insulin analogue and mediate many of its insulin-like activities by binding to the insulin receptor. However, it is proposed that the IGF-I-mediated inhibition observed here does not arise by this route but, rather, that IGF-I acts to attenuate platelet adenylate cyclase activity via interaction with its own receptor. This proposal arises due to the fact that IGF-I can mediate inhibition of adenylate cyclase at concentrations of the same order of magnitude as insulin, whilst it has been reported that IGF-I is less than 1% as potent as insulin at the insulin receptor (Baxter & Williams, 1983). It would therefore appear that insulin and IGF-I either bind to their own specific receptors, or both act through the IGF-I receptor.

By analogy to the previous argument, it is unlikely that insulin acts by binding to the IGF-I receptor. Binding of this polypeptide to the IGF-I receptor again occurs with a much lower affinity than the binding of the natural substrate. Here the order of binding affintiy is IGF-I$\gg$IGF-II$\gg$Insulin (Hollenberg, 1985) and hence binding of insulin at a receptor other than its own would not explain the insulin-mediated inhibition of adenylate cyclase observed here.

Even though IGF-I and insulin are related both structurally and functionally, it would appear from their ability to inhibit platelet adenylate cyclase by similar degrees at the same concentrations, that each does so by acting as its own specific receptor.

4.4 Conclusion

In conclusion, it would appear that insulin is capable of inhibiting the platelet adenylate cyclase system via an interaction with its own specific receptors. However, the route by which these receptors mediate the action of this peptide within the platelet is unknown. From this study it would appear that no possible route can be eliminated, although some appear more probable than others.

It would appear that insulin does not cause inhibition via preventing ligand binding, and so inhibition must arise by a more direct interaction of insulin with the adenylate cyclase system. Whether this interaction involves a G-protein is debatable. On one hand, insulin-mediated inhibition apparently does not require the presence of exogenous GTP, although insulin was shown to activate a specific GTPase activity. The possibility that the G-protein in question may be G_i is remote, as not only does manganese fail to attenuate this inhibition, but examining the effect of benzyl alcohol on insulin-mediated inhibition suggests that it occurs by a different route to the known G_i-mediated, adrenaline inhibition. It may be that the G-protein in question is actually G_{ins}; this would explain the observed GTPase activity, and perhaps also the apparent lack of requirement for GTP, i.e. enough GTP is already present in the membranes, as G_{ins} has a high affinity for the nucleotide. Hence, it may be that at least some of the insulin-mediated inhibition of platelet adenylate cyclase arises via the action of G_{ins}.

From the studies with the non-hydrolysable AMP-PNP, it would seem unlikely that phosphorylation plays a major role in inhibition of the platelet enzyme system. This is in agreement with a recent study, where hepatocyte G_i alpha was reported as being unable to be phosphorylated by insulin treatment of either

whole cells or isolated membranes (Pyne et al., 1989). It was suggested that a change in G_i alpha conformation may arise by such insulin treatment rather than a stable (covalent) modification, such as phosphorylation. It would also appear that the production of a mediator-substance is not a major inhibitory route either. However, caution should be taken not to disregard these possibilities, especially with respect to the production of the mediator, as it may be that formation of such compounds is not detectable within the constraints of detection methods employed here.

Finally, it should be noted that the insulin-like compound IGF-I is also capable of attenuating the platelet adenylate cyclase system; apparently via its own specific receptors. Again, the method of inhibition is unclear.

In summary, insulin-mediated inhibition of adenylate cyclase was detected in human platelet membranes. It would appear that the route by which this inhibition occurs involves, at least to some degree, a G-protein, possibly G_{ins}. It may be that inhibition results by a combination of routes, or by some route, as yet, unidentified, e.g. it has been proposed that other distinct low molecular weight subunits may be associated with the insulin receptor, and that they may prove to be part of the signal generating mechanism.

Indeed the route by which insulin mediates inhibition of adenylate cyclase in human platelets appears to differ from that which mediates inhibition in hepatocytes. This conclusion arises by the following observations. (a) higher concentrations of insulin were required to mediate inhibition in platelets, (b) no exogenous GTP was required to mediate inhibition of the platelet enzyme, and (c) insulin had no effect on the ability of GppNHp to attenuate forskolin-stimulated adenylate cyclase, whilst it is reported to

diminish such inhibition in hepatocytes (Pyne et al., 1989). the possibility that platelet insulin-mediated inhibition was different from that expressed in hepatocytes was suggested by work from other groups; platelet insulin-stimulated GTPase being reported as being cholera-toxin sensitive/pertussis toxin insensitive, whilst both bacterial toxins were reported to suppress insulin-mediated inhibition of hepatocyte adenylate cyclase (Gawler & Houslay (1987); Morgan (1989)). The differences in these mechanisms remain to be determined, a problem which will be solved when the exact mechanism of insulin-mediated inhibition is defined.

CHAPTER 5

GppNHp-MEDIATED ACTIVATION OF THE PLATELET ADENYLATE CYCLASE SYSTEM

5. GppNHp-MEDIATED ACTIVATION OF THE PLATELET ADENYLATE CYCLASE SYSTEM

5.1 Introduction

Activation of adenylate cyclase at the level of the stimulatory guanine nucleotide regulatory protein G_s, is possible by a number of means (see Section 1.3(iv)), including the use of non-hydrolysable GTP analogues such as GppNHp. In a number of systems where such direct activation has been studied, it has been noted that hysteresis occurs; a period of slow activation preceding a phase of linear activity. Although this effect has been observed in rat liver membranes (Iyengar & Birnbaumer, 1981), reconstituted cyclase systems (Iyengar, 1981) and human platelet membranes (Farndale et al., 1987) amongst others, its exact cause has been a matter of debate.

Extensive kinetic studies on GppNHp-mediated activation of rat liver adenylate cyclase have ruled out a number of possible causes of this slow activation phase. Amongst these is the proposal that this period arises due to the slow dissociation of resident GDP molecules from G_s. The finding that the rate of activation varies between GTP and its various analogues disproved the theory that GDP-dissociation is the rate-limiting step (Iyengar & Birnbaumer, 1981a), as did the observation that NaF-mediated activation of G_s exhibits hysteresis at low Mg^{2+} concentrations, i.e. NaF exhibited a lag phase in its activation of G_s, yet this species does not require GDP dissociation (Iyengar, 1981). Likewise, the theory that the cause of the rate limiting step lay in the difference in the susceptibility of the gamma phosphate bond to hydrolysis was similarly disproved. Although GppNHp is indeed insensitive to terminal phosphatases, that this is not the basis of the rate limiting step was confirmed by studies in rat liver plasma

membranes. Here it was found that hysteresis could be observed in the action of GTP, if the Mg^{2+} ion concentration was lowered to below 1mM (Iyengar & Birnbaumer, 1981).

The existence of this lag period is in fact, believed to reflect the conversion of G_s from an inactive to an active form; G_s inactive-G_s active (Iyengar & Birnbaumer, 1981; Iyengar, 1981). The active form produced is believed to reflect a reversibly activated state, which can undergo further conversion to give the quasi-irreversible state, i.e.

$$G_s \text{ active(reversible)} \longrightarrow G_s \text{ active(irreversible)}$$

Conversion of G_s(rev) to G_s(irrev) is not rate limiting and follows first-order kinetics (Farndale et al., 1987). Activation of G_s is therefore thought to involve at least two steps, and can be summarised as:

$$G_s \text{ inactive} \longrightarrow G_s \text{ active (rev)} - - \rightarrow G_s \text{ active (irrev)}$$

The conversion of G_s from its inactive form to its active form is thought to involve dissociation of the alpha/beta/gamma holomer:

$$G_s \text{ alpha/beta/gamma} \longrightarrow G_s\text{alpha} + \text{beta/gamma}$$

This equilibrium reaction actually accounts for the formation of the G_s active (rev) species, which in human platelets has been proposed to be less effective than G_s (irrev) in activating the catalytic unit of adenylate cyclase. However, in other systems such as rat liver, G (rev) and G (irrev) are reported as being equipotent with regard to their ability to activate C.

Here we attempt to manipulate the equilibrium:

$$\underset{\text{inactive}}{G_s \text{ alpha/beta/gamma}} \rightleftharpoons G_s + \underset{\text{active}}{\text{beta/gamma}}$$

by addition of guanine nucleotides, beta/gamma subunits etc. In doing so, it is hoped that the lag phase in GppNHp-mediated

activation of adenylate cyclase can be manipulated, i.e. proof that the conversion of G_s from its inactive form to its active form is the rate limiting step in GppNHp-mediated activation of the platelet adenylate cyclase system.

5.2 Results

From Figure 57 it can be seen that activation of human platelet adenylate cyclase by the non-hydrolysable GTP analogue GppNHp occurred following a lag period. Indeed, activation was linear with time only after an initial period of slow activation lasting approximately 6.4 minutes. However, the actual duration of this lag phage varied between membrane preparations and reagent batches. The average lag was 5.7 minutes $\pm$ 2.7 in length, where n=17 separate experiments using different membrane preparations.

The duration of this lag phase was found to be independent of the concentration of guanine nucleotide analogue employed (Fig.58). GppNHp at concentrations of 0.01, 1.0 and 100uM, elicited a lag phase lasting approximately 3.3. minutes. However, although this period of slow activation was unaffected, increasing GppNHp concentrations did enhance the steady-state rate of activity. Similarly, the actual extent of activation was augmented by increasing levels of the nucleotide. This is in agreement with studies of adenylate cyclase activation in rat liver membranes (Iyengar & Birnbaumer, 1981; Iyengar, 1981).

Previously it has been shown that the activation of G_s requires the simultaneous presence of guanine nucleotides and divalent metal ions (Iyengar & Birnbaumer, 1982). In the presence of saturating GppNHp concentrations (100uM), the presence of a lag phase in the activation of G_s was found to be dependent on the concentration of divalent metal ion present (Fig.59). Increasing the concentration of magnesium ion from the normal 5mM present in the assay, to 25mM, or replacing it with 5mM of manganese ion, resulted in a reduction in the lag period. Typically, steady state activation occurred following a much shorter length of time, the lag being reduced to less than one-quarter of its original duration.

For example, the lag of 3.6 minutes depicted in Figure 59 in the presence of 5mM Mg^{2+}, could be seen to be reduced to less than 1 minute with 25mM Mg^{2+} (0.95 min), or 5mM Mn^{2+} (0.90 min). Here, although the actual extent of activation was enhanced by manipulation of the divalent cation species/concentration, the steady-state velocity was essentially unaltered; 32.5 with 5mM Mg^{2+} and 36.8 with 5mM Mn^{2+}.

It was possible to reduce the duration of this lag phase by pre-incubating platelet membranes with GppNHp. Figure 60 shows that a 10 minute pre-incubation in the presence of 100uM GppNHp/5mM Mg^{2+} resulted in the lag time being reduced from 5.5 minutes to 2.3 minutes. Adenylate cyclase activity was also increased with pre-incubation time, being typically forty percent higher than that observed in the absence of such pre-incubation. Steady-state rates also showed a slight increase with pre-incubation, being 92.8 in the absence of such treatment, and 118.9 following a 20 minute pre-incubation.

Further attempts to manipulate the equilibrium between G_s inactive and G_s active included examining the effect of the local anaesthetic benzyl alcohol on GppNHp-treated platelet membranes. Addition of benzyl alcohol was found to shorten the length of the period of slow activation from 6.3 to 2.1 minutes at 50mM anaesthetic and to 1 minute at 80mM anaesthetic (Fig.61). Similar results were found each time this experiment was performed; in some cases, 80mM benzyl alcohol totally abolished the lag phase, i.e. activation was linear with time. Assuming this effect of benzyl alcohol was due to the ability of the anaesthetic to manipulate membrane fluidity (see Section 1.3(vi)), performing the experiment at higher temperatures should mimic this effect. Indeed, at 35^{o}C the initial lag period seemed to be shorter (3.83 $\pm$ 0.1 min; n=3

separate experiments employing different membrane preparations) and lower concentrations of benzyl alcohol were required to abolish this phase, i.e. 50mM benzyl alcohol was sufficient to reduce the lag to zero (Fig.62). At both normal assay temperature (25^{o}C) and at higher temperatures (35^{o}C) benzyl alcohol led to an increase in steady state velocities, and to an increase in the extent of adenylate cyclase activation.

Assuming that G_s activation by GppNHp does involve the dissociation of the alpha/beta/gamma heterotrimer, the effect of adding purified beta/gamma subunits to the equilibrium reaction was examined. Figure 63 shows that the presence of these subunits appeared to lengthen the duration of the lag phase. It should be noted that in some experiments excess beta/gamma had no detectable effect on the adenylate cyclase activity measured. Here, however, the original lag of 4.5 minutes was increased to 6.2 minutes duration.

The presence of exogenously added beta/gamma subunits also reduced the steady-state velocity and led to an overall inhibition of adenylate cyclase activity. In fact, at 10 minute incubation with beta/gamma, GppNHp-stimulated adenylate cyclase activity could be inhibited by up to 95%. This inhibition may be dependent upon the concentration of beta/gamma-subunits present, e.g. 94.5% $\pm$ 3.3 inhibition observed with 3.72ug's of the dimer was reduced to 77.6% inhibition with 0.93ug's beta/gamma. Addition of benzyl alcohol to this system would appear to go some way to overcoming this beta/gamma-mediated inhibition, and to shorten the duration of the beta/gamma-lengthened lag phase. This is seen in Figure 64 where the original 6.5 minute lag was lengthened to 7.3 minutes by the addition of beta/gamma subunits. By adding 50mM benzyl alcohol to this system, the lag was reduced to 3.2 minutes. The anaesthetic

did not therefore completely overcome the inhibitory effect of beta/gamma, as benzyl alcohol alone could reduce the GppNHp-induced lag to 2.7 minutes duration.

Other attempts to manipulate this GppNHp-induced lag were made by employing the use of bacterial toxins. Treatment of platelet membranes with cholera toxin, which leads to a persistently activated G_s state, resulted in a 2.5-3.0 fold augmentation of adenylate cyclase activity. The length of the lag phase was also reduced; in fact, in some cases actually being abolished (Fig.65). A slight increase in steady state velocity was also observed under these conditions.

Pertussis-toxin treatment of membranes which ADP-ribosylates the inhibitory guanine nucleotide regulatory protein G_i, also augmented adenylate cyclase activity. The increase in enzymic activity seen here was not as great as that observed with cholera toxin, being only 1.5-fold (Fig.66). Again, the duration of the lag phase was reduced (from 4.4 minutes to 1.8 minutes), but was never totally removed; maximal reduction being 59%. Pertussis-toxin treatment of platelet membranes had an even smaller effect on steady state velocity than did cholera toxin mediated ADP-ribosylation.

The addition of high salt concentrations to GppNHp-stimulated adenylate cyclase, resulted in an overall inhibition of activity. Here there was a decrease in steady state velocities, as well as a lengthening of the lag period. This is illustrated in Figure 67, where a 1.1 minute lag was increased approximately 3-fold, to give a 3.5 minute lag in the presence of 500mM sodium chloride.

Finally, the effect of adding an inhibitory hormone to the system was examined with regard to its effect on the GppNHp-induced

lag. Addition of 10^{-4}M adrenaline (+ 10^{-6}M propranolol) would appear to lengthen this lag period (Fig.68). Here a 2.8 minute lag was increased to 5 minutes duration by the presence of this hormone which stimulates G_i via the inhibitory $alpha_2$-adrenoceptor.

Figure 57 Activation of platelet adenylate cyclase by the non-hydrolysable guanine nucleotide analogue, GppNHp

Results are mean ± S.D. of a single representative experiment employing triplicate observations. n=17 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = 6.4 minutes.

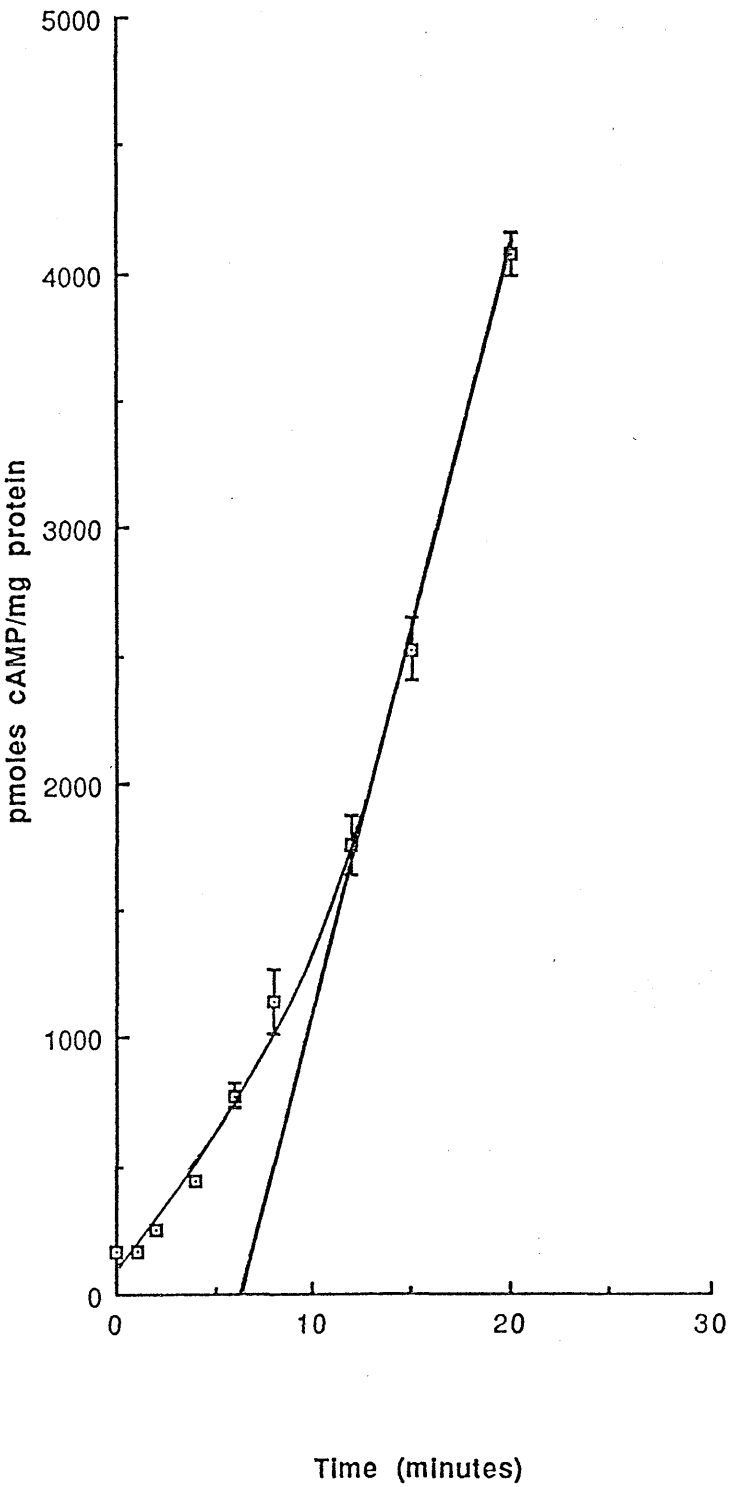

5000
4000
3000
2000
1000
0
pmoles cAMP/mg protein
0
10
20
30
Time (minutes)

Figure 58 Effect of varying GppNHp concentrations on G_s activation of adenylate cyclase

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=3 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = 3.3 minutes.

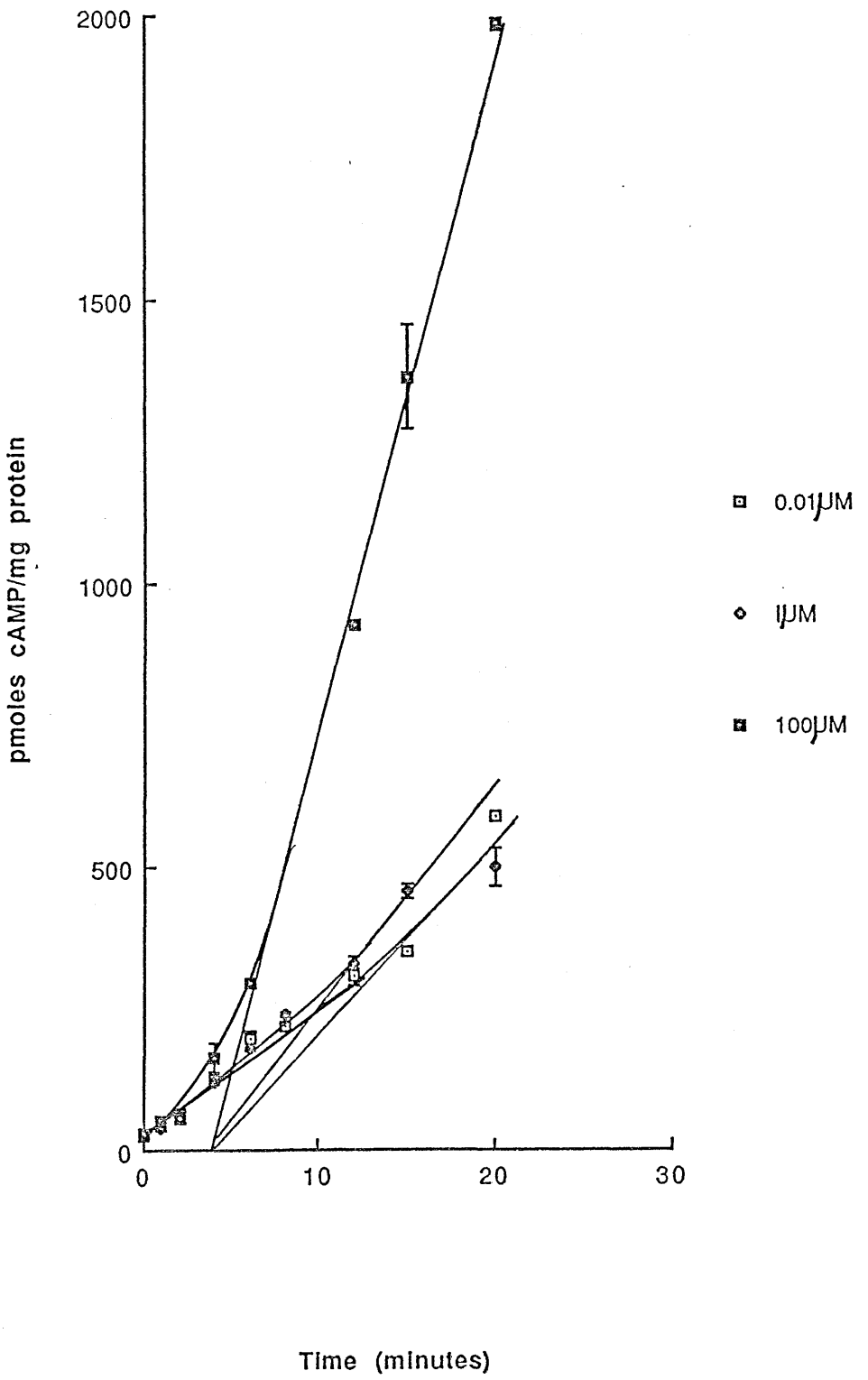

2000
1500
1000
500
0
0
10
20
30
pmoles cAMP/mg protein
Time (minutes)
0.01μM
1μM
100μM

Figure 59 Effect of varying divalent cations on GppNHp-stimulated adenylate cyclase activity

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=3 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 3.6 minutes with 5mM Mg^{2+}

ii) 0.96 minutes with 25mM Mg^{2+}

iii) 0.90 minutes with 5mM Mn^{2+}

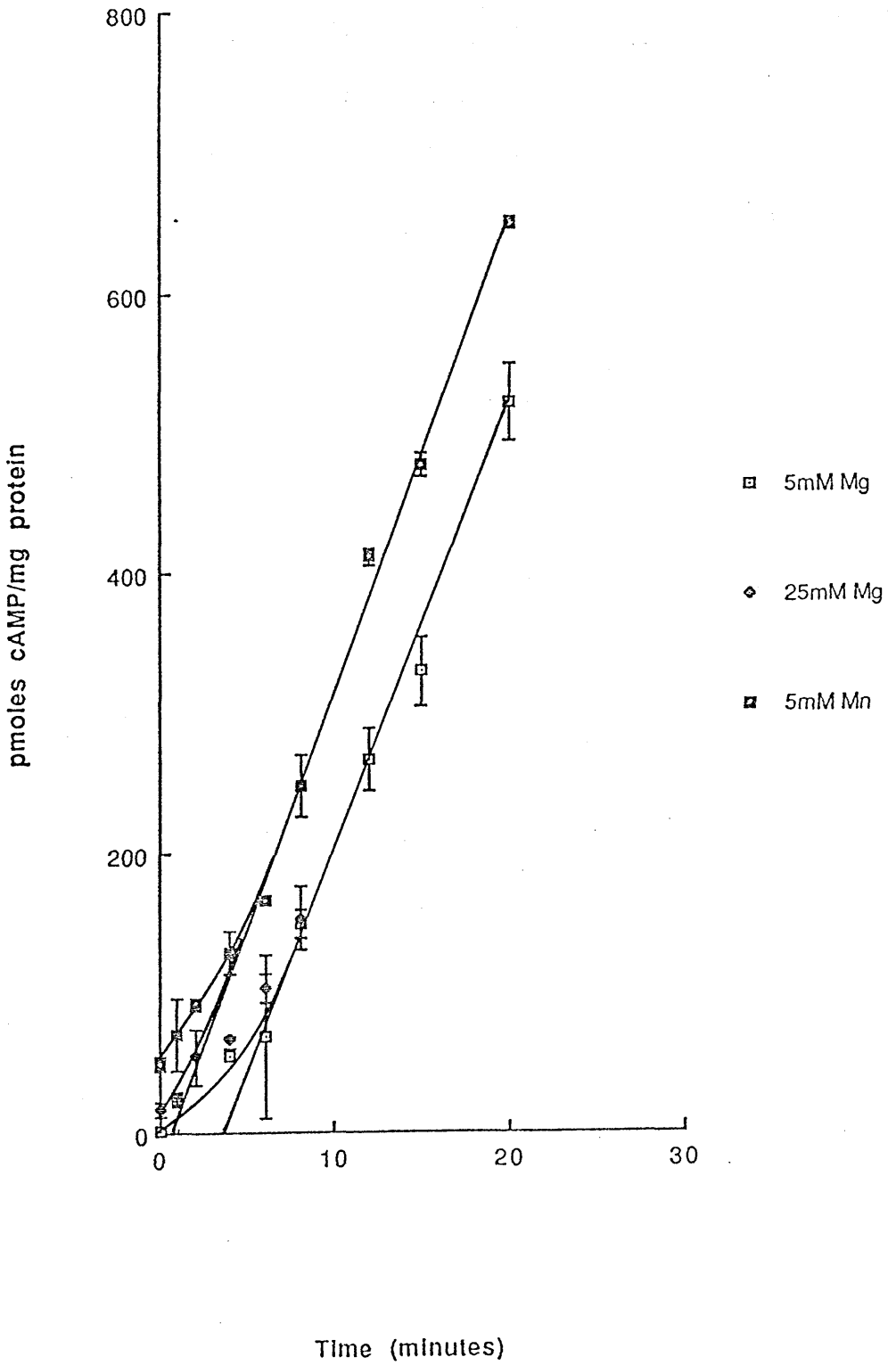

800
600
400
200
0
0
10
20
30
pmoles cAMP/mg protein
Time (minutes)
5mM Mg
25mM Mg
5mM Mn

Figure 60 GppNHp-mediated activation of adenylate cyclase following a pre-incubation period

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=3 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 5.5 minutes without pre-incubation

ii) 2.3 minutes with 10 minute pre-incubation

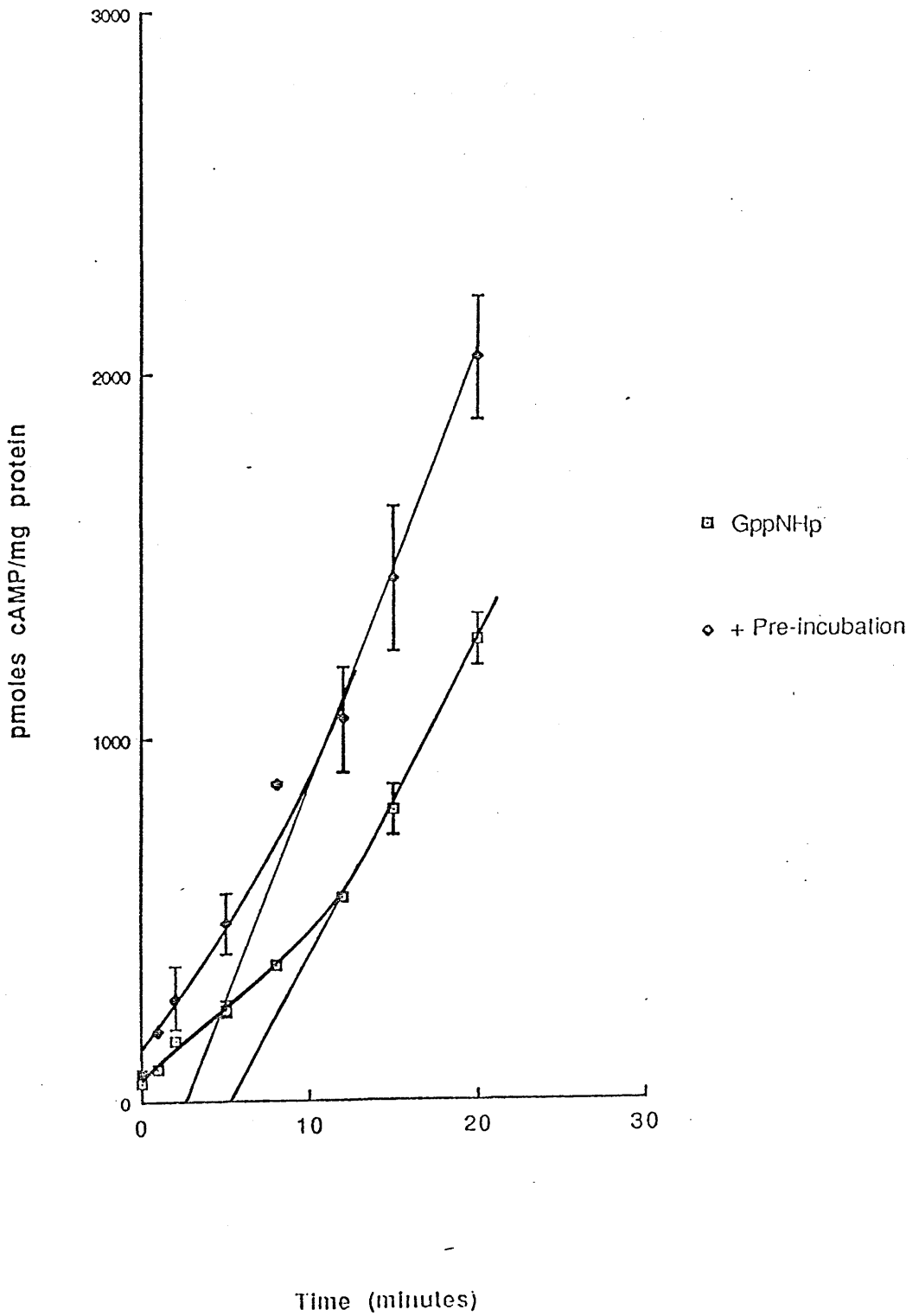

3000
2000
1000
0
0
10
20
30
pmoles cAMP/mg protein
Time (minutes)
GppNHp
+ Pre-incubation

Figure 61 Effect of benzyl alcohol on GppNHp-mediated stimulation of adenylate cyclase activity (25°C)

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=14 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 6.3 minutes with 0mM benzyl alcohol

ii) 2.1 minutes with 50mM benzyl alcohol

iii) 1.0 minutes with 80mM benzyl alcohol

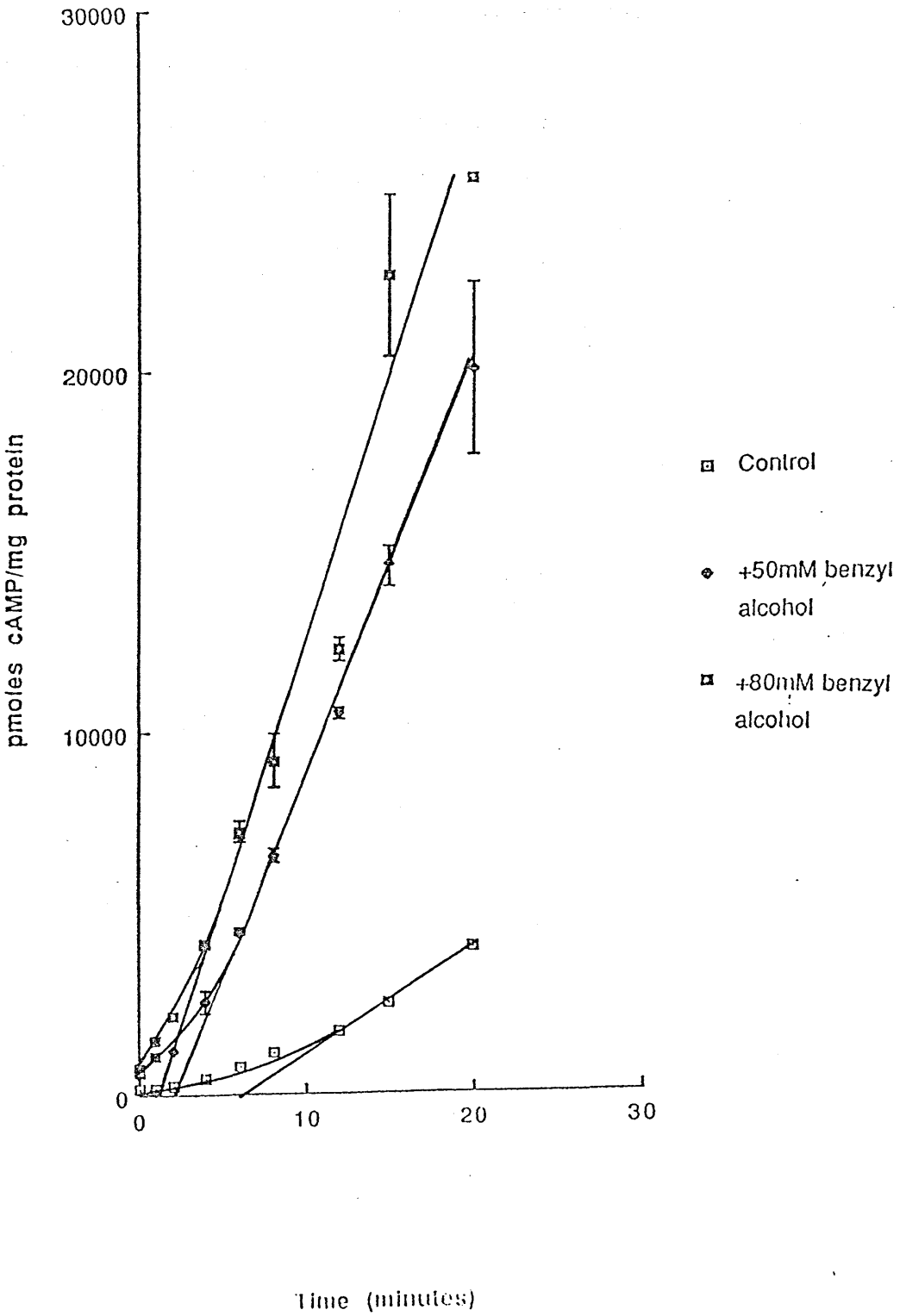

30000
20000
10000
0
pmoles cAMP/mg protein
0
10
20
30
Time (minutes)
Control
+50mM benzyl alcohol
+80mM benzyl alcohol

Figure 62 Effect of benzyl alcohol on GppNHp-mediated stimulation of adenylate cyclase activity (35°C)

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=3 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 3.8 minutes with 0mM benzyl alcohol

ii) 0 minutes with 50mM benzyl alcohol

iii) 0 minutes with 80mM benzyl alcohol

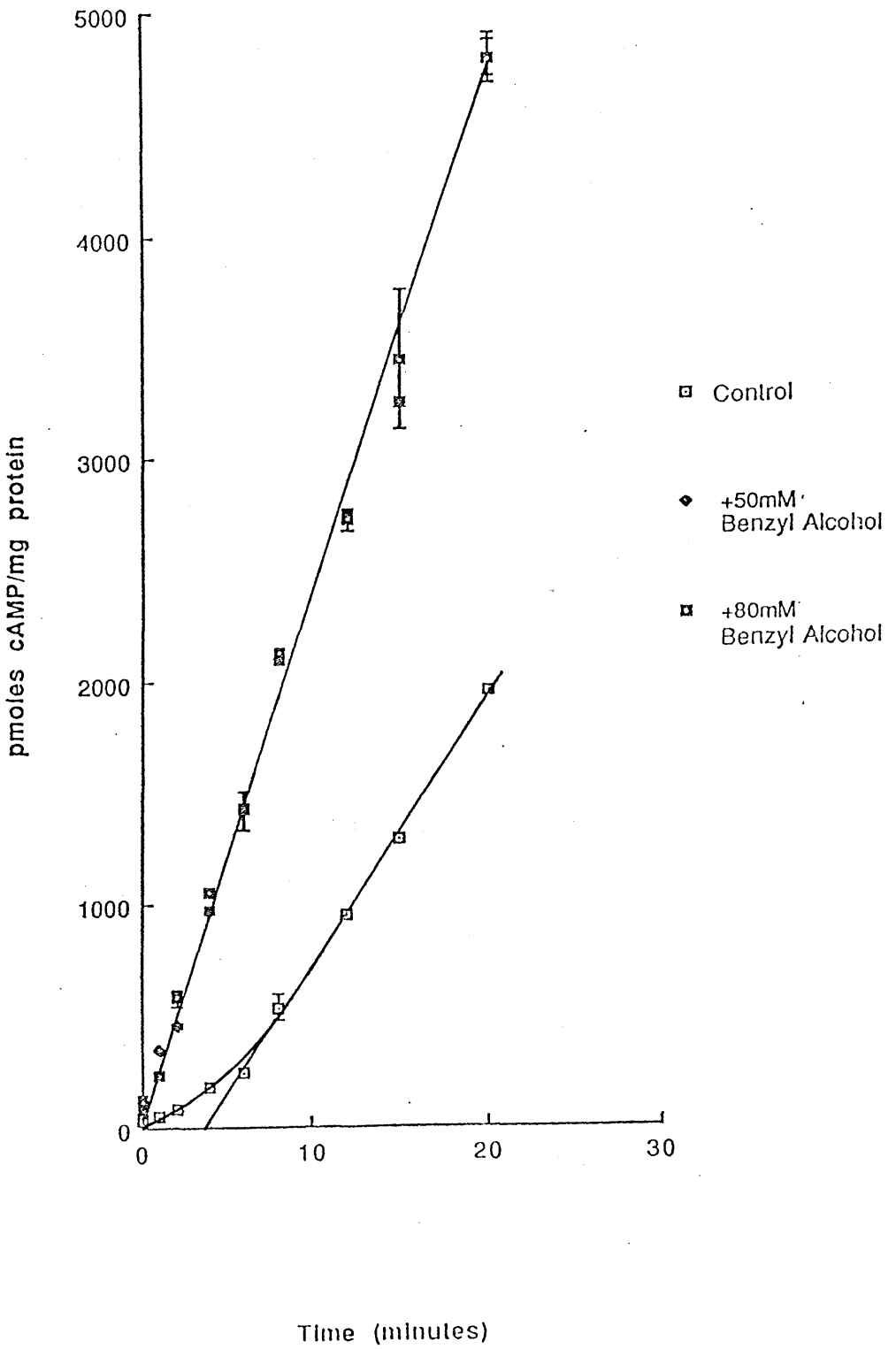

5000
4000
3000
2000
1000
0
pmoles cAMP/mg protein
0
10
20
30
Time (minutes)
Control
+50mM Benzyl Alcohol
+80mM Benzyl Alcohol

Figure 63 Addition of purified beta/gamma subunits to the GppNHp-activated adenylate cyclase system

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=3 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/100ul assay volume.

Lag period = i) 4.5 minutes in the absence of beta/gamma

ii) 6.2 minutes in the presence of beta/gamma

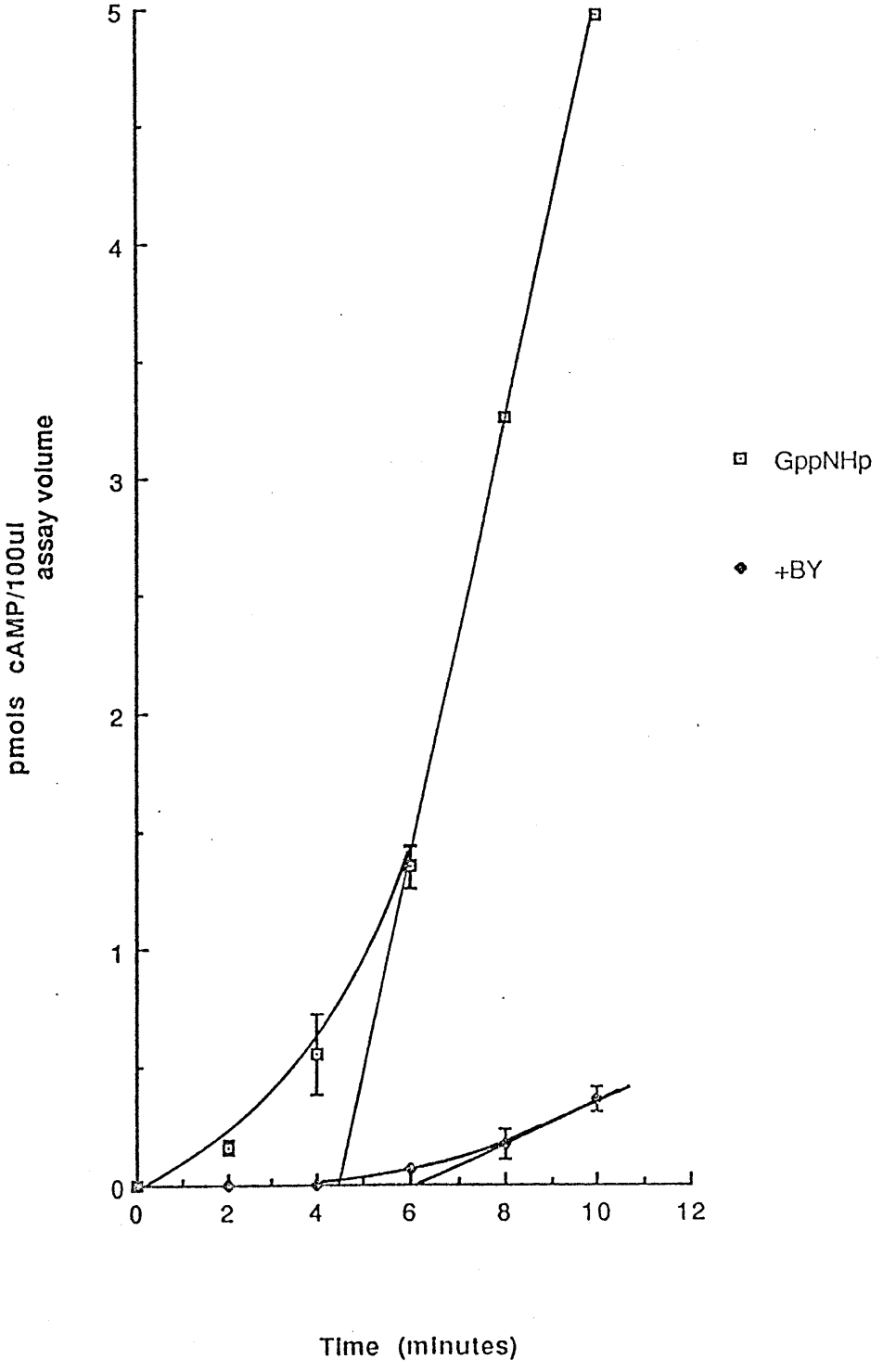

5
4
3
2
1
0
0
2
4
6
8
10
12
pmols cAMP/100ul assay volume
Time (minutes)
GppNHp
+BY

Figure 64 Modulation of the GppNHp-induced lag by benzyl alcohol and purified beta/gamma-subunits

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=2 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/100ul assay volume.

Lag period = A) 6.5 minutes

B) 7.2 minutes in the presence of beta/gamma

C) 2.7 minutes in the presence of beta/gamma and 50mM benzyl alcohol

D) 3.2 minutes in the presence of 50mM benzyl alcohol

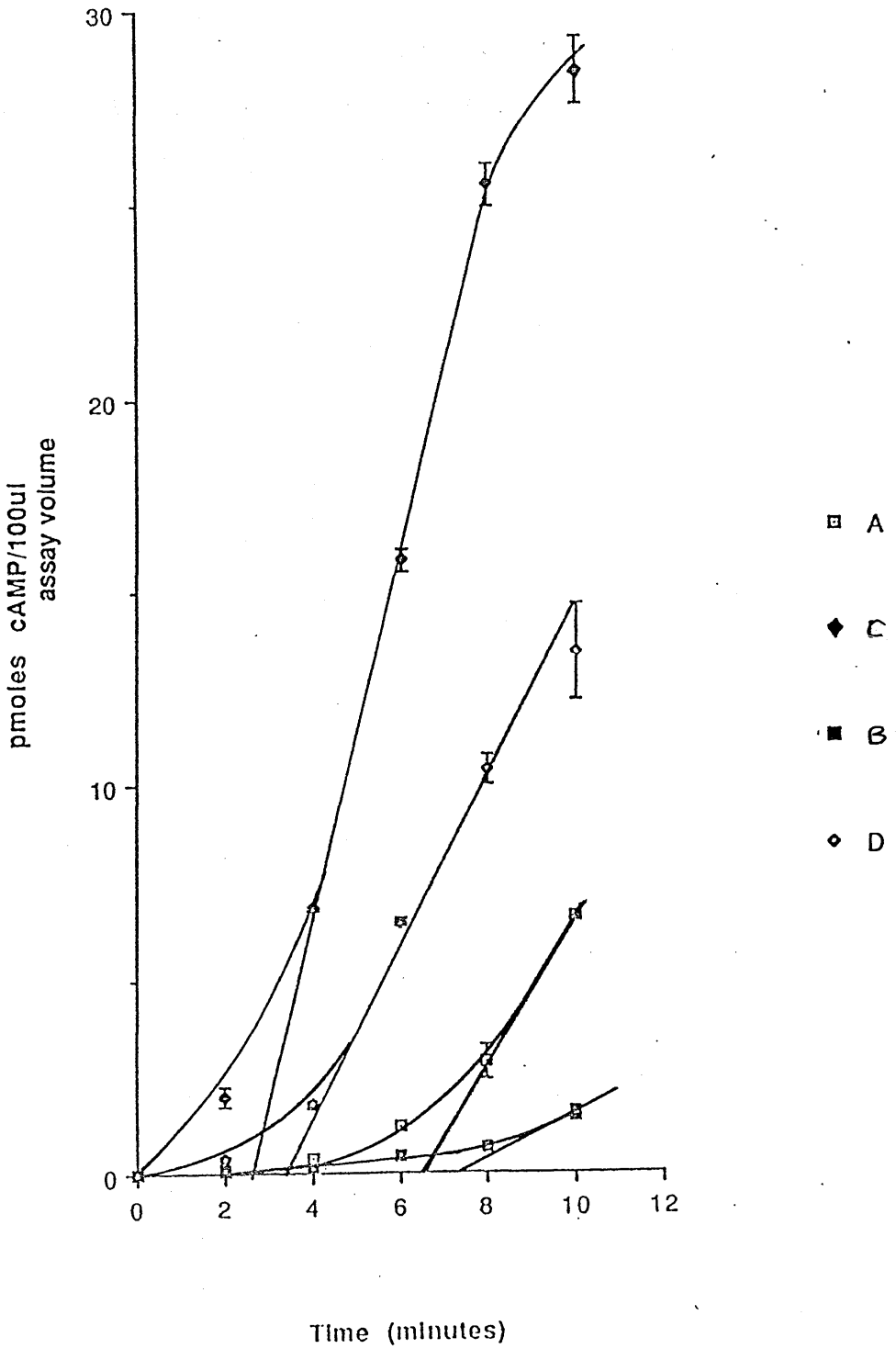

30
20
10
0
pmoles cAMP/100ul
assay volume
0
2
4
6
8
10
12
Time (minutes)
A
C
B
D

Figure 65 Effect of cholera toxin on GppNHp-mediated stimulation of adenylate cyclase activity

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=2 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 3.0 minutes before cholera toxin treatment

ii) 0 minutes after cholera toxin treatment

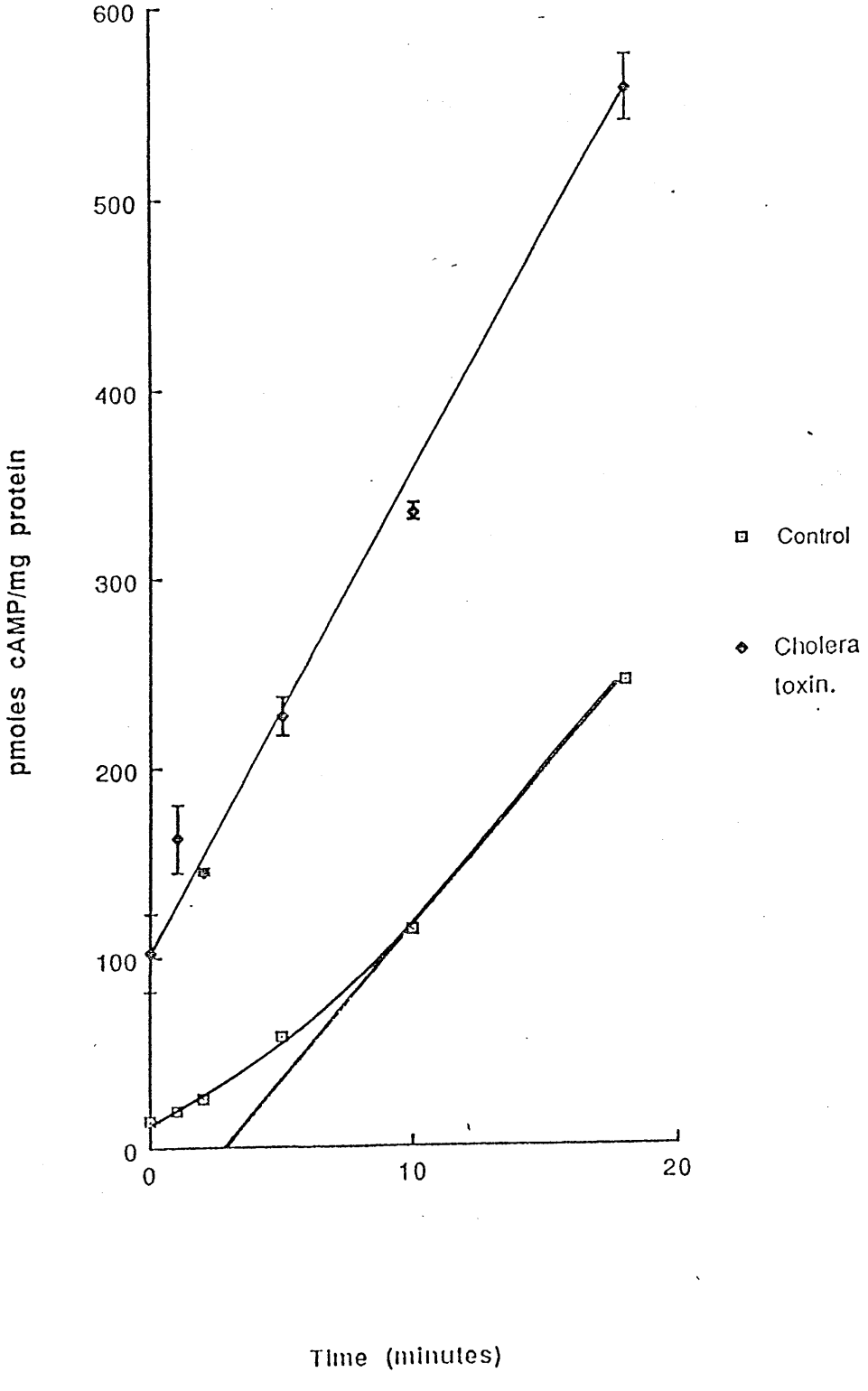

600
500
400
300
200
100
0
0
10
20
pmoles cAMP/mg protein
Time (minutes)
Control
Cholera toxin.

Figure 66 Effect of pertussis toxin on GppNHp-mediated stimulation of adenylate cyclase activity

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=2 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 4.4 minutes before pertussis toxin treatment

ii) 1.8 minutes after pertussis toxin treatment

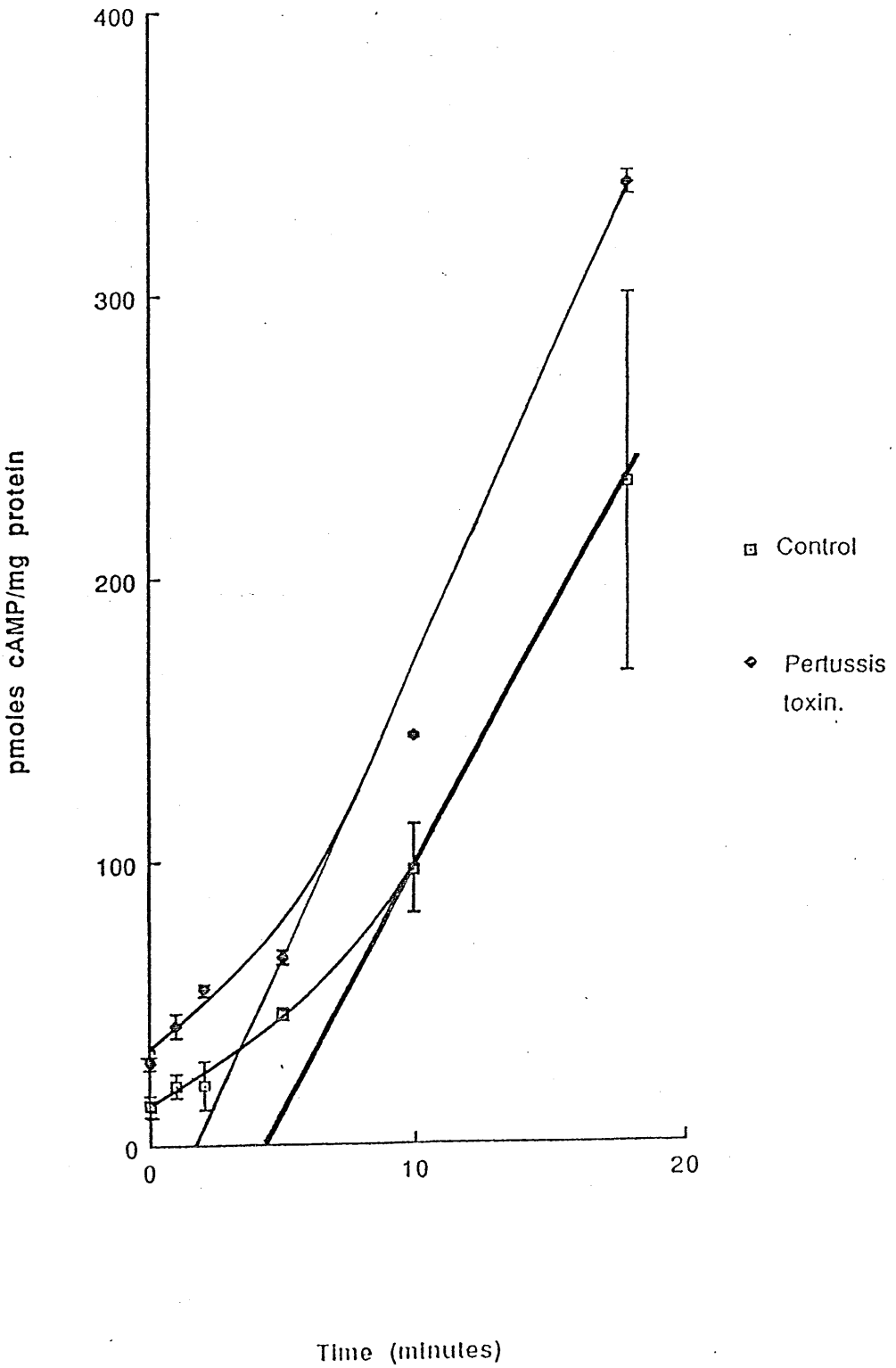

400
300
200
100
0
0
10
20
pmoles cAMP/mg protein
Time (minutes)
Control
Pertussis toxin.

Figure 67 Modulation of GppNHp-stimulated adenylate cyclase activity by high salt concentrations

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=4 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 1.1 minutes with 0mM NaCl

ii) 2.7 minutes with 100mM NaCl

iii) 3.5 minutes with 500mM NaCl

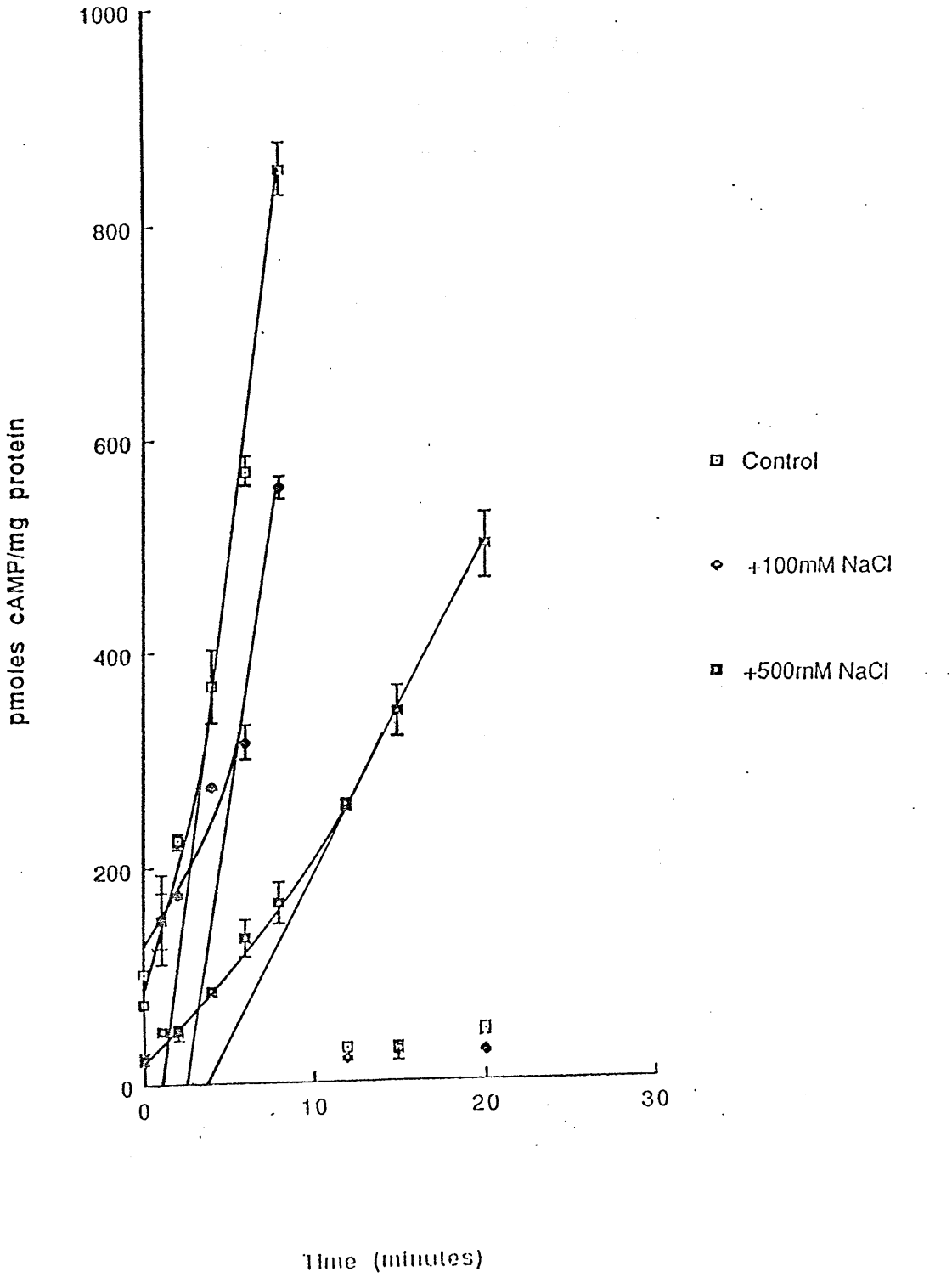

1000
800
600
400
200
0
0
10
20
30
pmoles cAMP/mg protein
Time (minutes)
Control
+100mM NaCl
+500mM NaCl

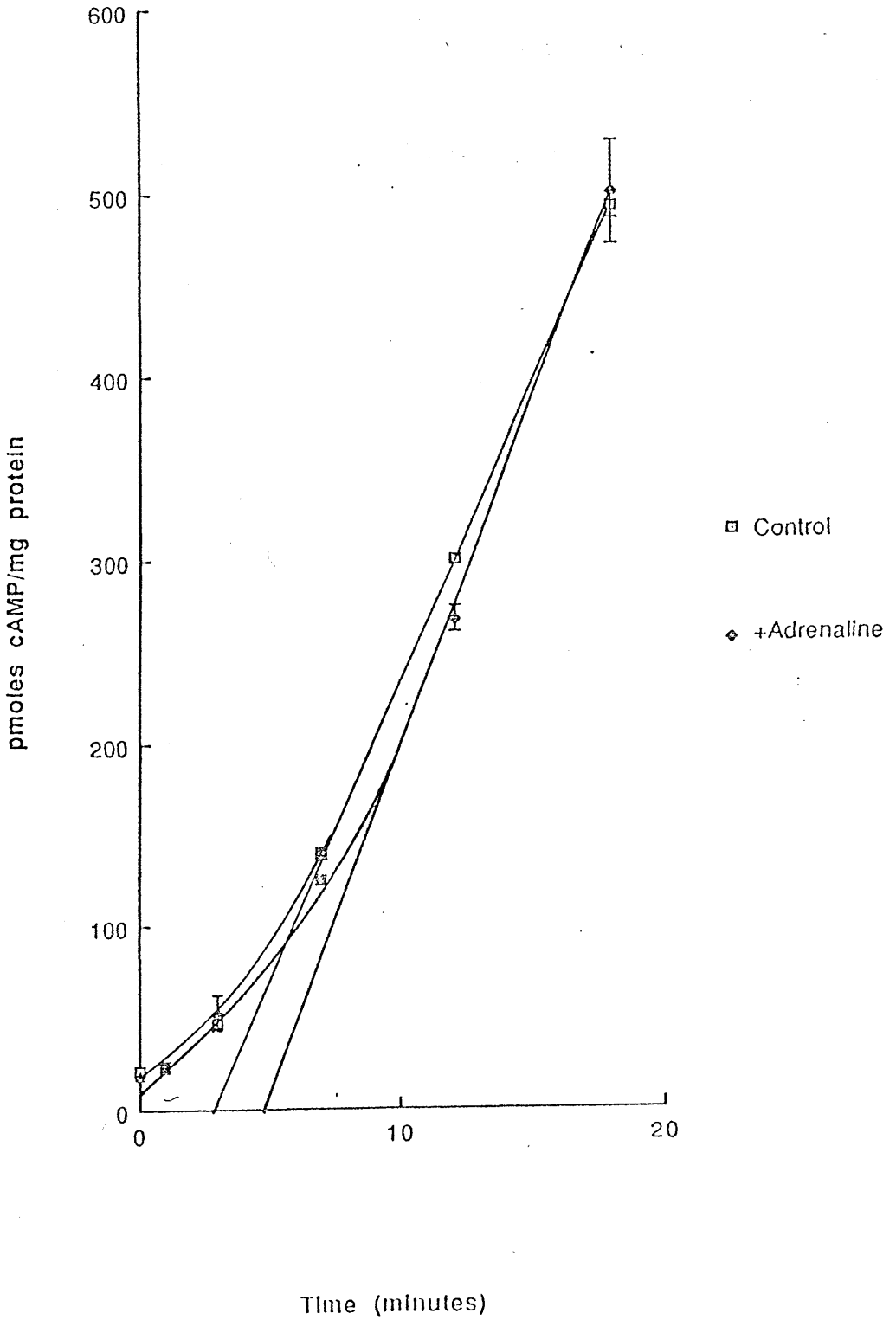
Figure 68 Modulation of GppNHp-stimulated adenylate cyclase activity by adrenaline (+ propranolol)

Results are mean ± S.D. of a single representative experiment employing triplicate determinations. n=2 separate experiments using different membrane preparations.

Results are shown as pmoles cAMP/mg protein.

Lag period = i) 2.8 minutes in the absence of adrenaline/ propranolol

ii) 5.0 minutes in the presence of adrenaline/ propranolol


600
500
400
300
200
100
0
0
10
20
pmoles cAMP/mg protein
Time (minutes)
Control
+Adrenaline

5.3 Discussion

Activation of adenylate cyclase by the non-hydrolysable guanine nucleotide analogue GppNHp, has previously been shown to exhibit hysteresis (Iyengar & Birnbaumer, 1981). Here it was observed that the platelet adenylate cyclase system is no exception, exhibiting a lag phase of average duration 5.7 minutes. This is in agreement with the magnitude of the lag detected by other groups studying platelet adenylate cyclase (Farndale et al., 1987). Manipulating the concentration of guanine nucleotide present apparently did not affect the lag time, suggesting that the rate limiting step is independent of the concentration of GppNHp. This supports the theory that it is the conversion of the nucleotide bound form of G_s from its inactive to its active state which constitutes the rate limiting step. Hence, as long as there is sufficient nucleotide to activate G_s, further increases in concentration have no effect on the rate at which this reaction occurs, i.e.

$$G_s\text{alpha}\ \overset{\text{GppNHp}}{\text{beta/gamma}} \xrightarrow[\text{Independent of GppNHp concentration}]{\text{RLS}} G_s\text{alpha}\ \overset{\text{GppNHp}}{} + \text{beta/gamma}$$

That the rate limiting step involves dissociation of the G_s heterotrimer has been suggested by a number of groups, including Gilman, 1987 who reported binding of the non-hydrolysable guanine nucleotide analogue GTPγS to be negatively co-operative with the binding of beta/gamma to G-protein alpha subunits. Thus, GTPγS (or GppNHp as used here) promotes G-protein subunit dissociation. This is supported by studies where activation of G_s has been shown to result in a decrease in its size, as assessed by sucrose density ultracentifugation and gel filtration. Indeed, many enzymes that display long-term hysteresis, such as seen here

with adenylate cyclase, have subunit association or dissociation reactions as their rate limiting step (Iyengar & Birnbaumer, 1982).

Although the duration of the rate limiting step was unaltered by increasing concentrations of nucleotide, total enzyme activity was altered. This is as expected, the final level of adenylate cyclase activity having been reported to be dependent upon the concentration of nucleotides in a saturable manner (Tolkovsky, 1986). Similarly, the increase in steady-state velocities which was noted here has been recorded with increasing GppNHp concentrations in other systems (Iyengar & Birnbaumer, 1981).

Such activation of G_s by guanine nucleotides requires the simultaneous presence of Mg^{2+}. Neither GppNHp, nor Mg^{2+} by itself will produce an active G_s. However, once G_s has been activated only one of these ligands is required to sustain the activate state, i.e. Mg^{2+} (Iyengar & Birnbaumer, 1982). If the Mg^{2+} concentration is manipulated in the presence of saturating levels of GppNHp, it is reported to modify the lag phase. The lag becoming progressively shorter with increasing concentrations of Mg^{2+} ion, and concentrations greater than 20mM being reported as abolishing the lag in rat liver plasma membranes (Iyengar & Birnbaumer, 1981). In platelets it would appear that this is also the case (Fig.59), not only does altering the Mg^{2+} ion concentration modify the lag, but so too does replacing the divalent cation with Mn^{2+}. The observed reduction in the duration of the lag is thought to arise by Me^{2+} activation of adenylate cyclase through direct interaction with a cation-binding site on the G-protein. Effects are independent of the ratio of free ATP to MeATP (see Section 1.3(v)). Binding of Me^{2+} at this G-protein site results in the observed decrease in lag time presumably by modulating the conversion of G_s (inactive) to G_s (active).

Exactly how Me^{2+} modulates this rate limiting step is not entirely clear. Whilst increasing Mg^{2+} may be expected to activate more regulatory components at higher divalent cation concentrations than at lower concentrations, this would account for the observed increase in total activity, but not for the variation in the rates of activation, i.e. the rate limiting step, in the presence of saturating guanine nucleotide is regulated by Mg^{2+} ion in terms of both rate and extent. This is in agreement with studies on rat liver plasma membrane (Iyengar, 1981). For Me^{2+} to modulate the lag phase, it may be that upon binding of Me^{2+}, the G_s alpha species changes its conformation, regulating in its dissociation from the beta/gamma dimer. Higher concentrations of Mg^{2+}, or replacing the cation species with Mn^{2+}, could encourage this; increasing the affinity of G_s alpha for the guanine nucleotide whilst decreasing its affinity for beta/gamma (Tolkovsky, 1986). Hence, Mg^{2+} would shift the equilibrium reaction:

G_s alpha/beta/gamma $\rightleftharpoons$ G_s alpha + beta/gamma far to the right

This observed reduction in lag time seen at high Mg^{2+} concentrations is not a result of modulation of inhibitory aspects of adenylate cyclase regulation (see Section 1.3(vi)). High Mg^{2+} is reported to prevent G_i alpha interaction with the catalytic unit, but not to prevent G_i alpha/beta/gamma dissociation. Hence, G_i beta/gamma would still be available to participate in the equilibrium reaction of G_s dissociation. Instead, the effect of Me^{2+} on the lag phase of G_s activation is likely to be due to the altered affinity of G_s alpha for guanine nucleotides and G beta/gamma. However, the attenuation of G_i by high Mg^{2+}/Mn^{2+} probably does contribute to the increased final activity observed under these conditions i.e. removing tonic inhibition by suppressing the G_i alpha inhibitory component. Mg^{2+} therefore behaves as

an antihysteretic agent for the stimulation of platelet adenylate cyclase by GppNHp, by regulating the rate and extent of activation of G_s, i.e. the rate limiting step.

If indeed the rate limiting step is the conversion of G_s inactive, to G_s active by subunit dissociation, then it may be that altering the membrane environment would influence this dissociation and hence the lag phase. Benzyl alcohol treatment is suggested to increase membrane fluidity and to augment stimulatory aspects of adenylate cyclase regulation (see Section 3.1). Applying this anaesthetic to studies of G_s activation showed 50mM benzyl alcohol to have this same effect on GppNHp-mediated stimulation of platelet adenylate cyclase, i.e. total activity was enhanced (Fig.61). However, here the duration of the lag phase was also reduced, suggesting that benzyl alcohol somehow affects the rate limiting step. As G_s is not an integral membrane protein its functioning per se is unlikely to be fluidity modulated. However, it is possible that a more fluid bilayer would modify the dissociation of holomeric G_s, as it is linked to the membrane by some unknown means, e.g. via association with an integral protein or by lipid linkage. It may be that changing fluidity alters the dissociation capability of G_s. If G_s alpha/beta/gamma was to dissociate more readily in the presence of benzyl alcohol, this would indeed explain the shortened lag phase observed here. That this is not a direct effect of benzyl alcohol on G_s is suggested by the findings of two subsequent experiments. In the first of these, increasing temperature was used to manipulate membrane fluidity. A 6-8^{o}C increase in temperature is reported to mimic the effect of adding 50mM benzyl alcohol with respect to manipulation of membrane fluidity. The observed reduction in the length of the lag phase which accompanied this increase in

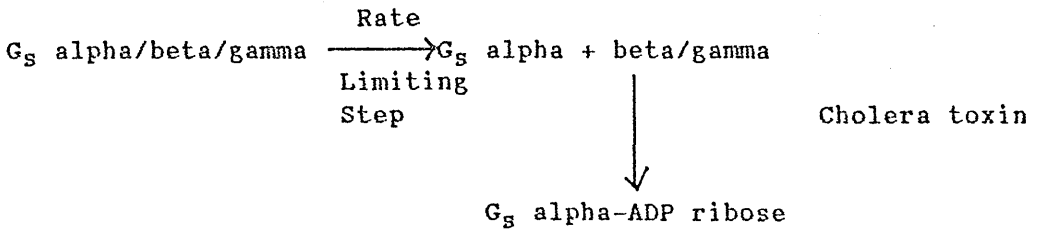
temperature suggests that the effect seen with benzyl alcohol is due to its ability to modulate membrane fluidity rather than any direct effect of the anaesthetic on G_s. The second experiment which lends support to this theory is the addition of purified beta/gamma units to the enzyme system. Here the benzyl alcohol-induced reduction in lag time was seen to be reversed by beta/gamma - i.e. the lag time was increased again. In fact, the addition of these subunits could increase the duration of this slow activation phase to longer than its original length. This suggests that not only is the benzyl alcohol-mediated effect reversible, but that the G_s dissociation reaction is the site of modulation, i.e.:

$$G_s \text{ alpha/beta/gamma} \underset{\text{beta/gamma}}{\overset{\text{benzyl alcohol}}{\rightleftharpoons}} G_s \text{ alpha} + \text{beta/gamma}$$

Benzyl alcohol drives this equilibrium to the right, encouraging subunit dissociation and decreasing lag time, whilst the addition of beta/gamma drives the reaction to the left and lengthens the lag. Indeed, results from Gilman's laboratory indicate that addition of beta-subunits to activated G-proteins results in faster deactivation, in agreement with this (Iyengar & Birnbaumer, 1982). Although it would appear that the effects of benzyl alcohol and beta/gamma do not cancel each other out, this may arise from incorrect proportions rather than any permanent effect of these ligands on the adenylate cyclase system.

If the rate limiting step for adenylate cyclase activation is, as appears, the conversion of G_s from its inactive to its active form, then the effect of bacterial toxins is of interest. The presence of cholera toxin, which ADP-ribosylates G_s alpha, is seen to attenuate the lag time in human platelets. Cholera toxin, which stabilises G_s alpha-GppNHp dissociated from G beta/gamma, would be expected to lead to an increase in total adenylate cyclase

activity as observed here, as the enzyme is persistently activated under such conditions. However, as cholera toxin modifies G_s alpha, it would seem that the rate limiting step would be completed before cholera toxin-mediated activation occurred, i.e.:



Cholera toxin would therefore be expected to have no effect on the lag time. However, by modifying G_s alpha, cholera toxin removes this species from the equilibrium reaction, i.e. G_s alpha-ADP ribose cannot recombine with beta/gamma to give the inactive G_s holomer (Gilman, 1987). The result of this is that the equilibrium reaction is drive to the right as G_s alpha is removed, and so we get the observed reduction in lag time until activation by GppNHp is linear from time zero, i.e. cholera toxin treatment can totally abolish the lag period. Pertussis toxin treatment is observed to have a similar effect, reducing the duration of the lag in the platelet system, although the means by which this arises is slightly different. This treatment should fix G_i alpha/beta/gamma in the holomeric form as it stabilises the G_i $alpha^{GPD}$/beta/gamma complex. If G_i cannot dissociate and release beta/gamma subunits, this should remove the possibility that G_i beta/gamma combines with G_s alpha to extend the lag and so we get the observed reduction in lag time, i.e.

$$G_s \text{ alpha/beta/gamma} \rightleftharpoons G_s \text{ alpha} + \text{beta/gamma}$$

This equilibrium cannot be driven to the left by G_i beta/gamma and so the dissociation of G_s alpha/beta/gamma to G_s alpha is encouraged. However whether it will be actively encouraged i.e. to a greater degree than that seen normally, is

debatable, being dependent upon the extent to which G_i beta/gamma affected this equilibrium under normal conditions. Under such conditions it would be expected that G_i beta/gamma would play some role in determining where equilibrium lay in the dissociation of G_s, as G_i occurs in vast excess relative to G_s. Hence, the treatment of membranes with pertussis toxin to eliminate this beta/gamma input should encourage dissociation of the G_s holomer, and so lead to the reduced lag period.

Na^+ ions, at high concentrations, have previously been reported as augmenting the inhibition of adenylate cyclase in a number of membrane systems, including human platelets (Jakobs et al., 1985). The mechanism underlying this inhibition is unclear as it is not obvious as to whether the ability of Na^+ to inhibit G-protein activation is at the level of guanine nucleotide binding, or at a reaction distal to this in the activation process.

Here it is seen that at concentrations sufficient to release peripheral proteins (500mM), NaCl leads to a reduction in total activity when added to the GppNHp-activated state. This is in agreement with the observations recorded in the preceding paragraph. However, it is also noted that NaCl increased the duration of the lag phase for GppNHp activation of cyclase. In fact, Figure 67 showed the lag to be extended to as much as three times its original length in the presence of 500mM Na^+ ion, i.e. from 1.1 minutes to 3.5 minutes. By comparison to the observations made earlier, this would suggest that Na^+ is acting to drive the equilibrium reaction of G_s association/dissociation to the left.

$$G_s \text{ alpha/beta/gamma} \xleftarrow{Na^+} G_s \text{ alpha} + \text{beta/gamma}$$

Hence, the lower activity observed here is due to the reduced ability of G_s to form the active G_s alpha species. Such

modulation of the lag phase by NaCl is not unrecorded sodium ions having been reported to increase the lag phase of GTPγS's action on G_s in various membrane systems (Jakobs et al., 1985). In this study it is noted that this effect occurs in the action of GppNHp on platelet adenylate cyclase, and it is suggested that this arises by Na^+ ion-modulation of G_s subunit dissociation rather than by any modification of nucleotide binding.

Finally, the effect of a known inhibitory agonist on the G_s equilibrium was examined. The presence of adrenaline (+ propranolol) was found to extend the lag phase. This is in contrast to other studies on platelet adenylate cyclase, where the activation of the enzyme by GppNHp was unaltered by the presence of adrenaline (Steer & Wood, 1979). However, if adrenaline (+ propranolol) causes inhibition via $alpha_2$-adrenoceptor activation of G_i, then we would expect it to release beta/gamma subunits whilst promoting G_i dissociation. The indirect result of such G_i dissociation would be that the G_s equilibrium would be driven in the direction:

$$G_s \text{ alpha} + G_i \text{ beta/gamma} \longrightarrow G_s \text{ alpha/beta/gamma}$$

As this reaction constitutes the rate limiting step, then the lag phase would be extended. Indeed this is what was observed here, the 2.8 minute lag being extended to 5 minutes duration.

5.4 Conclusions

In platelets, the activation of adenylate cyclase by the non-hydrolysable guanine nucleotide GppNHp increases with a lag time of approximately 5.7 minutes and thereafter constant activity exists. This activation of G_s is Mg^{2+}, time, and GppNHp dependent, and its hysteretic nature is in agreement with other membrane systems (Iyengar & Birnbaumer, 1981; Iyengar, 1981).

Activation of G_s by GppNHp is therefore thought to involve at least the following steps:

(a) G_s alpha^{GDP}/beta/gamma $\longrightarrow$ G_s alpha^{GppNHp}/beta/gamma

(b) G_s alpha^{GppNHp}/beta/gamma $\longrightarrow$ G_s alpha^{GppNHp} + beta/gamma (reversible)

(c) G_s alpha^{GppNHp} (reversible) $\longrightarrow$ G_s alpha^{GppNHp} (irreversible)

The dissociation of the nucleotide-bound holomer (step b) is believed to constitute the rate-limiting step. The position of this equilibrium and hence the duration of the lag phase, can be altered by various means, as described within this chapter.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 6

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From the results presented in the preceding chapters, it is apparent that the platelet adenylate cyclase system is an enzyme subject to a variety of complex controls. As defined classically, the basic system consists of at least five distinct types of components. These receptors are stimulatory receptors (R_s), inhibitory receptors (R_i), a catalytic unit (C) and stimulatory (G_s)- and inhibitory (G_i)-guanine nucleotide-regulatory proteins. However, it is possible that considerable heterogeneity could exist within adenylate cyclase enzymes given the findings that there are multiple R_s and R_i, multiple forms of G_s (from differential splicing) (Bray et al., 1986), multiple forms of G_i (G_i, 1,2,3 with G_i2 probably mediating inhibition of platelet adenylate cyclase (Simonds et al., 1989; Bushfield et al., 1990)) and more than one species of catalytic unit (see e.g. Krupinski et al., 1989).

In the system analysed here I have tried to investigate regulation at each of these levels. Regulation at the catalytic unit was followed by examining basal adenylate cyclase activity and also activity enhanced by the diterpene forskolin (which acts directly at the level of the catalytic unit). G_s-C interaction was followed by studying enzyme activity in the presence of sodium fluoride which mimics the gamma-phosphate of GTP and thus activates G_s when GDP is bound. The interaction of stimulatory receptors with the enzyme system was studied by the use of either prostaglandin-E_1 or the beta-adrenergic agonist isoprenaline. Both these substances activate their own specific receptors in the platelet membrane. As well as examining these stimulatory aspects of adenylate cyclase regulation I also tried to gain some insight

into the mechanisms of cyclase inhibition which is much less well defined. This study was undertaken by examining the ability of 'G_i' to inhibit the activity of either the receptor-coupled (PGE_1-stimulated) or the receptor-uncoupled (forskolin-stimulated) enzyme. Hence, in one case enzymatic activity would be enhanced by treatment with forskolin and then G_i would be activated either directly (via low concentrations of GppNHp) or indirectly, by the use of the $alpha_2$-adrenergic agonist adrenaline (in the presence of the beta-adrenoceptor antagonist propranolol to block any possible action of adrenaline on beta-adrenoceptors). This approach allowed the examination of G_i-C interactions and R_i-G_i-C interactions respectively. Alternatively, adenylate cyclase was activated by the use of PGE_1 with inhibition being elicited via $alpha_2$-adrenoceptors employing adrenaline (plus propranolol) as before. Under such conditions it was possible to study inhibition in the presence of receptor (R_s)-mediated stimulatory input. All known components of the enzyme system were thus involved in such circumstances i.e. $R_sG_sCG_iR_i$.

The outlined approaches forms the basis of the work detailed in Chapters 3-5. From these studies it became apparent that the platelet adenylate cyclase system exhibited a number of similarities with other adenylate cyclase systems (Ruiz et al., 1986; Dipple & Houslay, 1978). Interestingly, a number of unusual regulatory aspects were observed. In this chapter I will concentrate on these latter aspects and speculate as to how and why they might have arisen.

In studying the stimulatory control of the human platelet enzyme, it was found that this enzyme, in common with that of rat liver, adipocytes and red blood cells (Ruiz et al., 1986), amongst others, could be stimulated directly at the level of the catalytic

unit, or via G_s or R_s. Hence, the diterpene forskolin, at similar concentrations (10^{-4}M) to that which was effective in other tissue systems (Gawler et al., 1987) was observed to be a potent activator of the platelet enzyme. In fact, of the compounds studied, it was the most potent activator; again in agreement with studies on other systems. Similarly, sodium fluoride was observed as exerting stimulation (via G_s) of the platelet enzyme at concentrations (15mM) and with similar efficiencies (8.2-fold increase over basal) to that observed by others (Robberecht et al., 1983). The ability of the beta-adrenergic agonist isoprenaline to enhance adenylate cyclase activity has also been studied in other systems (Jakobs et al., 1978) and, indeed, was noted to augment enzyme activity by only 1.2 - 1.5-fold. Once more this was in accordance with the effects observed here, i.e. a 1.6-fold increase over basal activity. Finally, stimulation of platelet adenylate cyclase by PGE_1 is believed to occur via occupancy of the stimulatory PGE_1/PGI_2 receptor. The circa 27-fold stimulation of activity observed here with 20uM PGE_1 suggests that binding to functionally active receptors occurs in the platelet membranes.

As well as demonstrating that levels of adenylate cyclase activity elicited by these various agonists were similar to those previously reported by others, Chapters 3-5, showed that it was possible to manipulate such stimulatory control by modulation of the membrane environment. The effect of the local anaesthetic benzyl alcohol on adenylate cyclase activity has been studied extensively in rat liver plasma membranes, where the increased fluidity it induces is paralleled by an increase in stimulation (Houslay & Gordon, 1983 (refs. therein)). This effect has been studied to a lesser degree in a number of other systems including bovine adrenal cortex (de Foresta et al., 1987), macrophages (Cherenkevich et al., 1982) and renal epithelial cells (Friedlander et al., 1987). Here

this was extended to include human platelet membranes, where benzyl alcohol was observed to enhance stimulatory aspects of regulation at all levels.

At the catalytic unit, basal adenylate cyclase activity was seen to be augmented by the presence of benzyl alcohol. This suggested that increasing fluidity, via benzyl alcohol perturbing the lipid order of the bilayer, exerted its primary effect on adenylate cyclase by releasing the physical constraint of the bilayer on this enzyme. That is, presumably a higher degree of conformational flexibility ensued allowing a more biologically active conformation to be attained. In contrast to the platelet enzyme, the basal adenylate cyclase state as expressed in hepatocytes, was found to be relatively insensitive to the action of benzyl alcohol (Dipple & Houslay, 1978). This may reflect tissue and/or species differences in either the enzyme itself, its incorporation into the membrane bilayer, or relate to differences in physical properties of the bilayer of these two membranes this exerted different degrees of constraint. Indeed, biochemical and genetic evidence indicate that there are multiple forms of adenylate cyclase (Krupinski et al., 1989) It may be that the platelet and hepatocyte enzymes are two different enzymatic species. Similarly, the interaction of the enzyme with the membrane bilayer is almost certainly different, whether or not the enzymes themselves differ. This could arise from the fact that the membrane composition of rat hepatocytes and human platelets differ. The finding that a lipid phase separation exists in platelets at 37^{o}C but at 28^{o}C in rat liver, illustrates this point, as does comparison of the cholesterol/phospholipid (C/P) ratios of these membranes. A C/P value of 0.5. has been determined for human platelet plasma membranes, whilst rat liver membranes exhibit a C/P ratio of 0.71

(Gordon et al., 1983). Hence, these membranes may well present differing fluidities. In fact, domains of varying fluidity are believed to co-exist within the same membrane (Houslay & Stanley, 1982). It is, therefore, reasonable to assume that the platelet and hepatocyte enzymes exist in environments of differing fluidities. Hence, it would be expected that modulations in fluidity would have (slightly) different effects on these two species.

Along similar lines, it was noted that the adenylate cyclase activity of platelets was attenuated to a greater degree at high concentrations of benzyl alcohol, than was the enzyme in rat liver plasma membranes. This inhibition at high concentrations of benzyl alcohol is believed to be a consequence of the phenomenon of annular lipid displacement. Here the lipid species directly surrounding the protein are displaced by the benzyl alcohol molecules. The displacement of these lipids from the 'shell', or annulus, results in inhibition of enzymatic activity either because the lipids were essential for activity or because the benzyl alcohol molecules are themselves inhibitory (Houslay & Stanley, 1982). Whichever mechanism accounts for the observed inhibition, it is apparent that the concept of annular displacement depends upon the ability of benzyl alcohol to displace the lipid from around the catalytic unit of adenylate cyclase. From the attenuation noted here it would, therefore, seem that high concentrations of benzyl alcohol can displace annular lipid more easily from the platelet enzyme that from the rat liver enzyme. This may arise as a result of these two tissues either expressing different adenylate cyclase species or the annular lipid composition is sufficiently different between the two tissues to account for the observed differences.

Another somewhat unusual aspect of regulation of the platelet catalytic unit was brought to light in studying the effect of benzyl alcohol on the forskolin-stimulated activity of this enzyme. Here it was noted that benzyl alcohol led to a dose-dependent attenuation of this activity. Whilst this is in agreement with studies on other adenylate cyclase systems (Robberecht et al., 1983), a difference arises as to the mechanism by which this inhibition occurs. The general consensus has been that inhibition arises by a competitive mechanism; presumably resulting from the alcohol competing for hydrophobic sites on adenylate cyclase to which forskolin binds (Whetton et al., 1983). However, I noted that dose-effect curves of forskolin-activation of adenylate cyclase revealed a progressive decrease in the maximal efficacy of the diterpene with increasing concentrations of benzyl alcohol. However, there was no parallel shift to the right of log dose-effect curves in the concentration required for half-maximal enzyme stimulation, which would have been indicative of competitive inhibition, i.e. that V_{max} was unaltered. Whilst K_m increased the result here, i.e. V_{max} decreased by K_m unaltered, suggested that in platelets, benzyl alcohol attenuated the ability of forskolin to simulate adenylate cyclase by a non-competitive inhibitory process. It is possible that benzyl alcohol binds to a distinct site which alters the conformation of the catalytic unit such that the hydrophobic site to which forskolin binds is masked in some way. The nature of this site is unknown. Although, given the earlier observation that benzyl alcohol can displace annular lipid, it may be that this is a phospholipid binding site. It is not possible, however, to completely rule out mechanisms whereby benzyl alcohol alters protein structure either directly or indirectly through affects on membrane fluidity. However, the

finding that forskolin's ability to activate the lubrol-solubilised enzyme was still attenuated by benzyl alcohol (Section 3.1) would seem to indicate that this ligand may interact directly with the protein. The possibility that benzyl alcohol may displace essential detergent or detergent-phospholipid complexes from the enzyme cannot be discounted as well.

Other aspects of benzyl alcohol-regulation of platelet adenylate cyclase which were examined included regulation at the level of stimulatory receptors. Whilst it was noted that agonist-occupation of the PGE_1-receptor led to activation of the enzyme, it was proposed that the interaction of this receptor with the adenylate cyclase system may differ from that of other stimulatory receptors, notably the beta-adrenoceptors. Studies with benzyl alcohol (Section 3.1) showed that the fold-stimulation by PGE_1 was markedly attenuated by the presence of benzyl alcohol (26.7 fold - 12.5 fold), whilst that mediated via isoprenaline occupancy of the beta-adrenoceptor was unaltered. This apparently anomalous regulation of the PGE_1-receptor was enhanced by findings of previous studies, done in liver, where glucagon-(R_s)-mediated stimulation of adenylate cyclase was augmented by benzyl alcohol treatment of rat liver plasma membranes. Exactly why the various stimulatory receptors should differ in this respect, at least within the same membrane system, is unclear. This may arise as a consequence of a receptor requiring specific phospholipid species for efficient coupling to the adenylate cyclase system, as has been discussed elsewhere (Houslay & Gordon, 1983). Also, studies in this system with acidic phospholipids would tend to support the proposal that certain phospholipids are more important than others in determining the efficiency of component interactions. Such a proposal arises from the observation that PGE_1-stimulated

adenylate cyclase activity could be attenuated by the introduction of phosphatidic acid into the membrane (Section 3.4). That this is not a general phenomenon of membrane disruption is shown by the fact that phosphatidylcholine (neutral) had no effect on enzyme activity. Nor was it an effect common to all acidic phospholipids, as phosphatidylserine was similarly ineffective. Hence, phosphatidic acid may, under normal circumstances, be excluded from the coupling interface or if it does enter it will exert an inhibitory effect, attenuating activity. As proposed earlier, benzyl alcohol-induced manipulation of the membrane may remove a lipid essential for activity or allow a previously excluded lipid to enter the coupling interface. In the case of the prostanoid receptor, the manipulation of lipid species may be more potent than the benzyl alcohol-induced augmentation of fluidity. Hence, we observed inhibition rather than enhanced enzyme activity. Alternatively, it could be that the PGE_1-receptor and the beta-adrenoceptor interact with two different catalytic unit subtypes and so the effect of benzyl alcohol might differ. However, this possibility seems unlikely to account for the observed effects given that basal cyclase activity was always seen to be enhanced by benzyl alcohol to the same degree. This suggests a single population of catalytic unit or at least populations which were similarly affected by changes in the membrane environment.

I also examined direct stimulation at the level of the guanine nucleotide regulatory protein G_s. In such studies it was noted that both sodium fluoride and GppNHp could enhance the activity of platelet adenylate cyclase. This is as reported by others previously, in both platelets (Insel et al., 1982) and other membrane systems (Iyengar & Birnbaumer, 1981). However, stimulation by GppNHp exhibited hysteresis (Chapter 5) whilst that

exerted by sodium fluoride, in my hands, did not. This 'lag' in GppNHp-mediated activation was found, upon more detailed investigation, to be time, Mg^{2+} and guanine-nucleotide dependent. As a consequence of detailed manipulation of these parameters, amongst others, it was proposed that, in platelets, this hysteresis reflected the conversion of G_s from an inactive to an active form. This is proposed as being dependent upon subunit dissociation, hence -

$$G_s \text{ alpha}^{GppNHp}\text{beta/gamma} \longrightarrow G_s \text{ alpha}^{GppNHp} + \text{beta/gamma}$$

is proposed as the rate limiting step. Guanine nucleotide binding, hydrolysis and dissociation having been ruled out (see Chapter 5 for detailed discussion). The addition of benzyl alcohol to this system resulted in a reduction in the duration of the lag phase, suggesting that increases in fluidity enhance the dissociation of the stimulatory G-protein. Presumably this accounts to some degree for the enhanced enzyme activity observed with benzyl alcohol on other stimulatory conditions, i.e. for R_s-mediated stimulation as well as NaF-mediated stimulation. It is not suggested that G_s functioning per se is fluidity modulated as G_s is not an integral membrane protein. Rather, that it is possible that enhanced membrane fluidity could modify the dissociation of holomeric G_s, albeit indirectly.

Hence, benzyl alcohol may modulate (a) receptor interaction with adenylate cyclase, at least in the case of PGE_1 and/or (b) G_s dissociation and/or (c) relieve constraint of the bilayer on the catalytic unit. Each or all of these effects may contribute to the observed alterations in adenylate cyclase activity seen under such conditions. Whether or not this effect of benzyl alcohol arises purely as a result of its ability to perturb membrane fluidity is questionable. However, the lack of stimulatory effect

on solubilised preparations of the enzyme would tend to rule out any direct effect on the protein. Similarly, the ability to mimic many of these effects by increasing temperature, e.g. G_s dissociation, supports the proposal that events witnessed here arose as a consequence of altered membrane fluidity. Nevertheless, upon examination of the effects of charged anaesthetics on the platelet enzyme, it appears that these compounds had very different effects.

The use of pentobarbital, which is a negatively charged compound, showed that both receptor-coupled and uncoupled activities were initially enhanced by the presence of the drug, before being attenuated by further increases in concentration. Previously it had been suggested that such manipulation would preferentially enhance the fluidity of the outer leaflet of the bilayer, resulting in enhanced activity of the receptor-coupled state only (Houslay et al., 1981). That such changes in activity were not observed here, may reflect a direct effect of pentobarbital on the enzyme. Indeed, examination of the inhibitory-regulatory components revealed that this may indeed hold true. For, upon such examination, the alpha$_2$-adrenoceptor-mediated inhibition of the PGE_1-stimulated state was found to be unaffected by pentobarbital. In contrast, both alpha$_2$-adrenoceptor- and GppNHp-mediated inhibition of the forskolin-stimulated state, were found to be attenuated at low concentrations of pentobarbital. This suggested that at these concentrations pentobarbital was exerting some sort of selective effect. By comparing these results and taking into account the mechanisms of inhibition underlying these states, it is possible to speculate as to the site of 'interaction' between the drug and the inhibition system. In this case, it is tentatively suggested that pentobarbital may act to manipulate G_i-alpha interaction with the catalytic unit. This effect appeared to be masked in the case of

alpha$_2$-adrenoceptor-mediated inhibition of the PGE$_1$-stimulated state, perhaps as a consequence of the presence of a beta/gamma-inhibitory component. However, as pentobarbital is a negatively charged compound, it may be that the effect noted here resulted from charge rather than as a consequence of the compounds ability to perturb membrane fluidity. Hence, G$_i$-alpha may require the presence of a positive charge in order to interact effectively with the catalytic unit. In examining the effect of introducing negatively charged phospholipid species to the membrane, it was noted, in preliminary experiments, that adrenaline-mediated inhibition of PGE$_1$-stimulated adenylate cyclase was not affected by the presence of such acidic phospholipids. Neither was adrenaline- or GppNHp-mediated inhibition of the forskolin-stimulated state. Such findings further complicate the role of 'charge' in component coupling, as do the findings from experiments done using the cationic drug prilocaine.

In platelets the local anaesthetic prilocaine showed different effects from those reported in rat liver plasma membranes (Houslay et al., 1980(b)), where this compound altered the activity of both receptor-coupled and uncoupled states by inducing a lipid phase separation at 16^{o}C in the inner leaflet. It can be expected that prilocaine may selectively interact with the lipids of the inner leaflet of the membrane resulting in enhanced fluidity. A corresponding increase in the activity of both the receptor-coupled and -uncoupled states would then occur (see Section 3.2 for discussion of why). However, here it was noticed that both these activity states were attenuated in the presence of increasing concentrations of prilocaine. Moreover, the ability of adrenaline and GppNHp to inhibit these R$_s$-coupled and -uncoupled states were differentially affected. Adrenergic-mediated inhibition of the

PGE_1-stimulated enzyme was attenuated at low concentrations of prilocaine, although increasing concentrations of the drug did appear to restore this inhibitory ability to normal levels. On the other hand, adrenergic- and GppNHp-mediated inhibition of the forskolin-activated enzyme were enhanced by increasing levels of the anaesthetic. This led to the suggestion that $G_i$alpha-C interaction may be enhanced by the presence of such positive species. This would support the earlier proposal that $G_i$alpha-C interaction involves positive charge.

A proposal that gains some support from the recent observations that compounds such as mastoparan and the synthetic polyamine compound 48/80 are able to interact directly with the C-terminal domains of G alpha and lead to alterations in its activity (Mousli et al., 1990; Higashijima et al., 1988). This is presumed to arise due to a combination of positive charge and domains of hydrophobic character, which allow them to interact with G_{alpha}. Hence, the observation that prilocaine (positive charge) and pentobarbital (negative charge) are able to stimulate and inhibit G_i-alpha-C interactions respectively may reflect their interaction with such domains (see Appendix I for comparison of structures).

That enhancement of inhibition was not seen with adrenaline/PGE_1, in the presence of prilocaine, can only lead to the proposal that the presence of the beta/gamma component of the inhibition may mask this effect here. Indeed, the reduction in inhibition noted here raises the suggestion that G_i beta/gamma interaction with $G_s$alpha may be attenuated by prilocaine i.e. that the presence of the positively charged anaesthetic is detrimental to efficient G_i beta/gamma-$G_s$alpha interaction. Hence, the degree of inhibition elicited by adrenaline on PGE_1-stimulated adenylate

cyclase was actually reduced rather than augmented. That increasing concentrations of prilocaine eventually led to enhanced inhibition of this state is of interest.

Benzyl alcohol experiments suggested beta/gamma-mediated inhibition to be the minor of the two inhibitory components of alpha$_2$-adrenoceptor-mediated inhibition of the PGE$_1$-stimulated state. As the concentration of prilocaine increased, the contribution of this component and hence its regulation would become masked by the anaesthetic enhancing G_i-alpha-C-mediated inhibition. This would account for the apparent increase in the ability of adrenaline to inhibit PGE$_1$-activated adenylate cyclase at high concentrations of prilocaine. Whilst such attenuation of the G_i-beta/gamma-$G_s$alpha interaction may indeed arise as a result of appropriately charged anaesthetic, it may be that this particular interaction is fluidity 'hypersensitive'. This would be in accordance with the results obtained in the presence of benzyl alcohol, a local anaesthetic which displays no net charge. Indeed. the ability of charge compounds to manipulate the platelet adenylate cyclase system cannot be explained solely on the basis of their charge. This follows from the finding that introducing acidic phospholipids into the system did not affect G_i-mediated inhibition. Neither can the effects of these charged anaesthetics be explained solely on the basis of their ability to perturb membrane fluidity, as the results with receptor-coupled and -uncoupled states showed. It would therefore, seem reasonable to conclude that pentobarbital and prilocaine act to manipulate the platelet adenylate cyclase system via both their ability to perturb membrane fluidity and by their charge characteristics.

Examination of the effects of charged lipid species also showed that the catalytic unit itself could be inhibited by the

presence of acidic phospholipids. This is presumed to be via a direct effect on the enzyme rather than mediated through G-proteins, as the process was neither GTP-dependent nor altered by the presence of Mn^{2+}. It may be that such negatively charged phospholipids are normally excluded from the lipid annulus (see earlier discussion) but when presented at high concentrations equilibrate in and serve to inhibit activity. No such direct effect of acidic phospholipids (phosphatidic acid and phosphatidylserine) was observed upon the catalytic unit of rat liver adenylate cyclase (Houslay et al., 1986c). This again suggests that either the annular lipid encasing these two catalytic units are very different and/or that the two species of adenylate cyclase are themselves different.

Another 'unusual' aspect of adenylate cyclase regulation which was demonstrated during this study, was the ability of insulin to attenuate the activity of the platelet enzyme. Whilst this observation in itself is by no means novel (Heyworth & Houslay, 1983), it is a matter of some dispute as to whether such inhibition occurs as whilst some groups observe it (Hepp, 1971; Heyworth & Houslay. 1983), other groups have been unable to record it in isolated membranes (Pilkis et al., 1974; Falcon et al., 1988). In this study inhibition was clearly observed and it appeared to occur through a route seemingly different, at least in some respects, from that whereby insulin mediates inhibition of hepatocyte adenylate cyclase (Pyne & Houslay, 1983; Heyworth & Houslay, 1983).

Insulin mediated attenuation of both receptor-coupled (PGE_1-stimulated) and -uncoupled (forskolin-stimulated) states was achieved. That this was not purely G_i-mediated, was suggested by the lack of requirement for exogenous GTP and confirmed by the lack of attenuation by Mn^{2+}. This divalent cation having been shown

to uncouple the selective, G_i-mediated inhibition of the catalytic unit (Hoffman et al., 1981). Whilst the precise mechanism underlying insulin-mediated inhibition remains undefined, it may be that the putative G-protein, G_{ins}, has some role to play. If this protein is involved, then experiments with benzyl alcohol suggest that the beta/gamma subunits expressed with G_{ins} are not the same as those expressed with $G_i$alpha. Insulin-mediated inhibition of forskolin, like that mediated by other inhibitory ligands, was benzyl alcohol insensitive. Inhibition of the PGE_1-stimulated state was similarly unaffected by the presence of the local anaesthetic. This latter point is in direct contrast to adrenaline-mediated inhibition of this same state, which was attenuated by increasing levels of benzyl alcohol. By comparison to $alpha_2$-adrenergic inhibition, it is therefore suggested that insulin-mediated inhibition of the R_s-activated state may entail two components (i) G_{alpha} interaction with C is proposed as being benzyl alcohol insensitive, in agreement with the $G_i$alpha-C interaction observed with adrenaline inhibition of PGE_1 and/or (ii) G beta/gamma interaction with $G_s$alpha; a benzyl alcohol insensitive step and therefore different from previous observations with adrenaline. Alternatively G beta/gamma may play no role in insulin-mediated inhibition of PGE_1. However, the finding that insulin attenuated the R_s-coupled state (75% inhibition) to a larger degree than the -uncoupled state (40% inhibition) might suggest that an inhibitory component other than direct G alpha interaction with C does exist. Hence, it is likely that G beta/gamma-G_s alpha mediates insulin inhibition as seen with $alpha_2$-adrenoceptor inhibition of the R_s-coupled state. Nevertheless, it would appear that the beta/gamma expressed with 'G_{ins}' are different from those expressed with $G_i$alpha. Why

and how they differ is unclear although both beta and gamma subunits are now known to exhibit a much higher degree of heterology than was first thought (Fong et al., 1987; Robishaw et al., 1989).

If a G-protein is involved in mediating inhibitory actions of insulin, it is unclear as to whether it is directly linked to the insulin receptor or not, i.e. it could occur at some point distal to this in the pathway. It is also likely that some other components exist in the signalling pathway due to the pleiotropic effects which this hormone exerts. Some of these may be linked to the hormone's ability to attenuate adenylate cyclase. In this study it was possible to go some way to eliminating the involvement of phosphorylation in the inhibition of the platelet enzyme and to reducing the likelihood that a chemical mediator was involved (see Chapter 4 for discussion). However, no definitive route as to how insulin attenuates platelet adenylate cyclase can be suggested and so caution should be exercised in disregarding any of the possible inhibitory mechanisms discussed above. It is highly probable that insulin, upon binding to its receptors, activates a number of pathways within the cell. These either alone or in combination with others, may thus lead to the attenuation of adenylate cyclase activity which was observed here.

The platelet adenylate cyclase enzyme therefore forms a highly complex system due to (a) the number of factors involved in its regulation and (b) the fact that it is possible to manipulate each of the known levels of regulation by modulation of the membrane environment. It can only be presumed that other adenylate cyclase systems would be regulated in a similar manner. Whether this is true or not is open to debate, given the differences between the platelet and hepatocyte enzyme systems illustrated here. As well as the differences in membrane composition and fluidity, the

ever-expanding families of the components, i.e. multiple forms of R_s, R_i, G_s, G_i and C, it should also be remembered that regulatory components in addition to those already identified may exist. For example, cytoskeletal elements are candidates for supporting roles in the inhibitory regulation of adenylate cyclase (Rodbell, 1980). It seems highly probable that the existence of as yet unidentified components of the insulin-signalling pathway will come to light. Presumably these too may be subject to regulation by modulation of the membrane environment.

Not only do platelets provide a valuable system for the analysis of the complexities of the adenylate cyclase enzyme, but such examination also merits some importance as the level of cyclic AMP within the platelet is suggested to be important in determining the aggregatory properties of these cells. Indeed, correlation of the effects of adrenergic agonists and antagonists on adenylate cyclase activity in platelet lysates and on aggregation of the intact cells are reported to be very close. For example, adenylate cyclase inhibition was only seen in platelets from species in which catecholamines induced aggregation (Jakobs et al., 1978). It should be recalled that it is the level of cAMP-dependent phosphorylation ensuing from changes in the production of this cyclic nucleotide which are believed to be important. However, the possibility of manipulating aggregation via altering cyclic AMP levels is evident. The observation that increased fluidity augmented adenylate cyclase's ability to produce cyclic AMP is therefore of much interest. By the previous argument, this could lead to attenuated platelet activation and reduced aggregation. Indeed, a recent study has shown the concentration of benzyl alcohol which leads to increased platelet membrane fluidity (bovine), also inhibits platelet aggregation (Kitagawa et al., 1990). In fact, the IC_{50} for benzyl alcohol mediated inhibition

of aggregation was 12mM ± 1, whilst in the present study the IC_{50} for attenuation of $alpha_2$-adrenoceptor inhibition of receptor-coupled adenylate cyclase was 12.5mM ± 4.2 (see Section 3.1). This ability of fluidity to modulate cyclic AMP production could prove to be of special interest in diabetes given the recent observation that such subjects exhibit altered platelet membrane fluidity (Winocour et al., 1990) as well as pronounced vascular disease. Although the precise pathogenesis of such disease remains obscure, its importance is well established, with blood platelets being known to play a particularly important role in thrombus formation. In fact, it has been suggested that retinal infarctions and peripheral neuropathy associated with diabetes mellitus arise, at least in part, by increased platelet aggregation (Dalsgaad et al., 1982). Hence, elucidation of the biochemical regulatory processes controlling platelet aggregation - including synthesis of cyclic AMP by adenylate cyclase - may provide a basis for understanding how their pathophysiologic or pharmacologic disruption can affect platelet-mediated disorders of haemostasis and thrombosis. It may be that the manipulation of the membrane environment and of adenylate cyclase will prove to be a route to better control of platelet functioning (primarily aggregation).

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APPENDIX I : CHEMICAL STRUCTURES

Figure A1 Benzyl alcohol

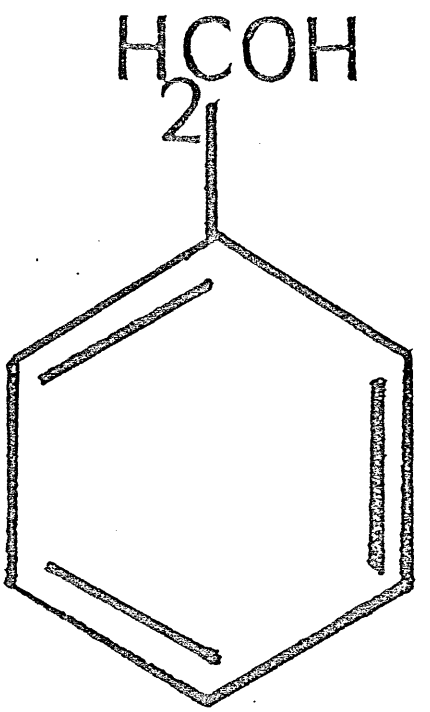
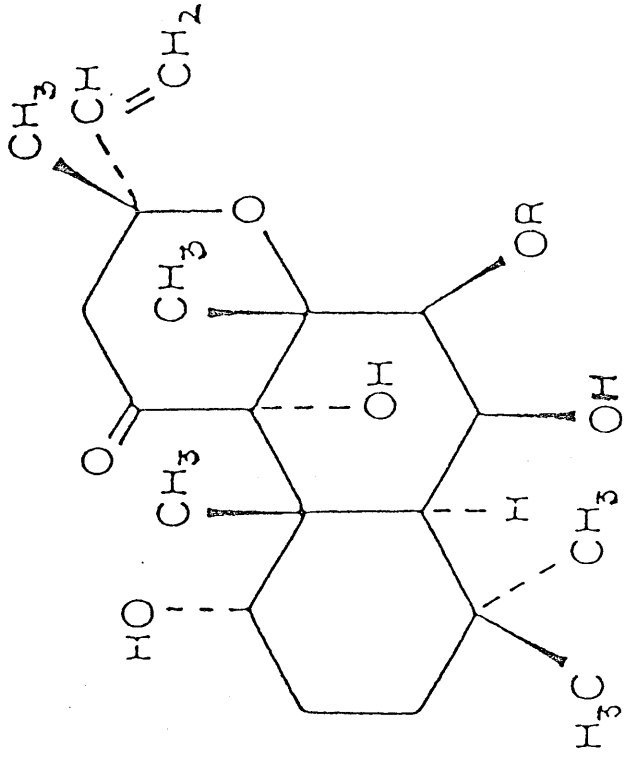


Figure A2 Forskolin



Forskolin: R = $O=C-CH_3$

Figure A3 Dimethylnitrosamine

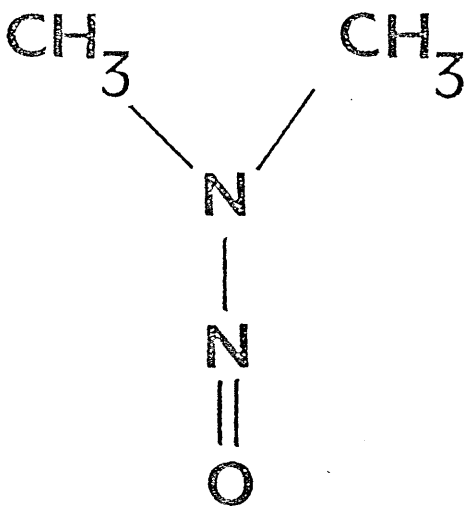
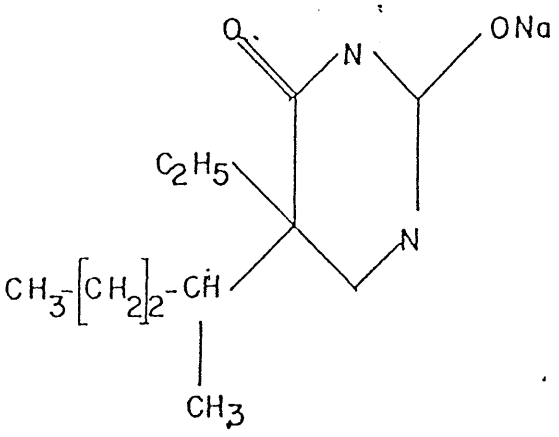
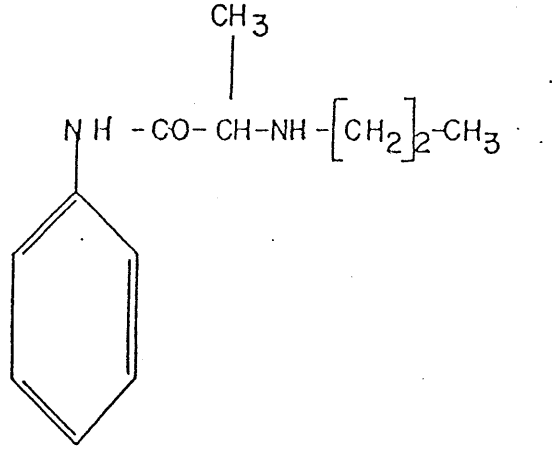


Figure A4 Charged compounds

PENTOBARBITAL



PRILOCAINE



COMPOUND 48/80

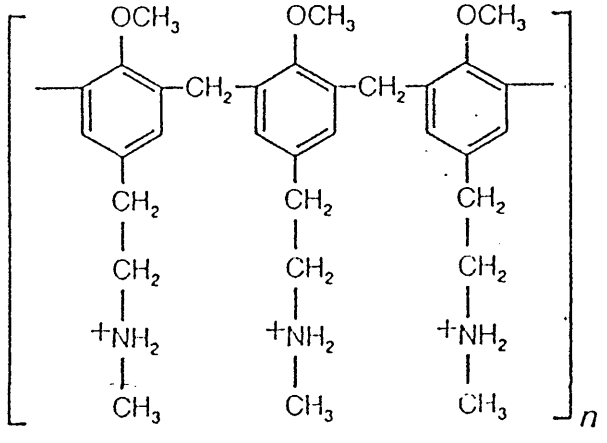
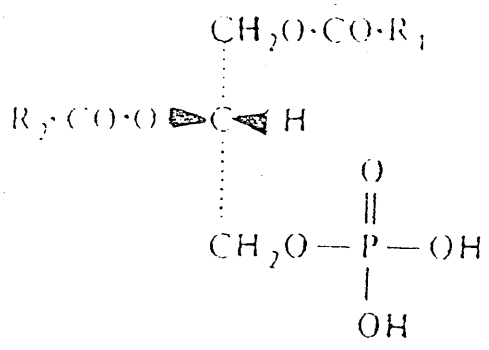
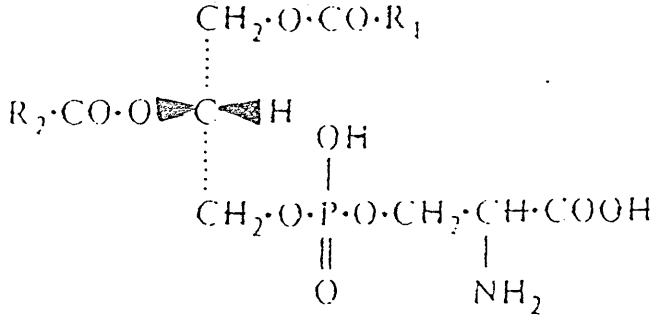


Figure A5 Phospholipid structures

(A) PHOSPHATIDIC ACID



(B) PHOSPHATIDYLSERINE



(C) PHOSPHATIDYLCHOLINE

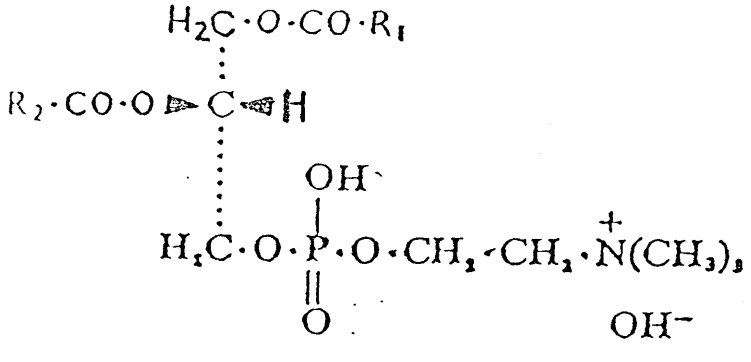
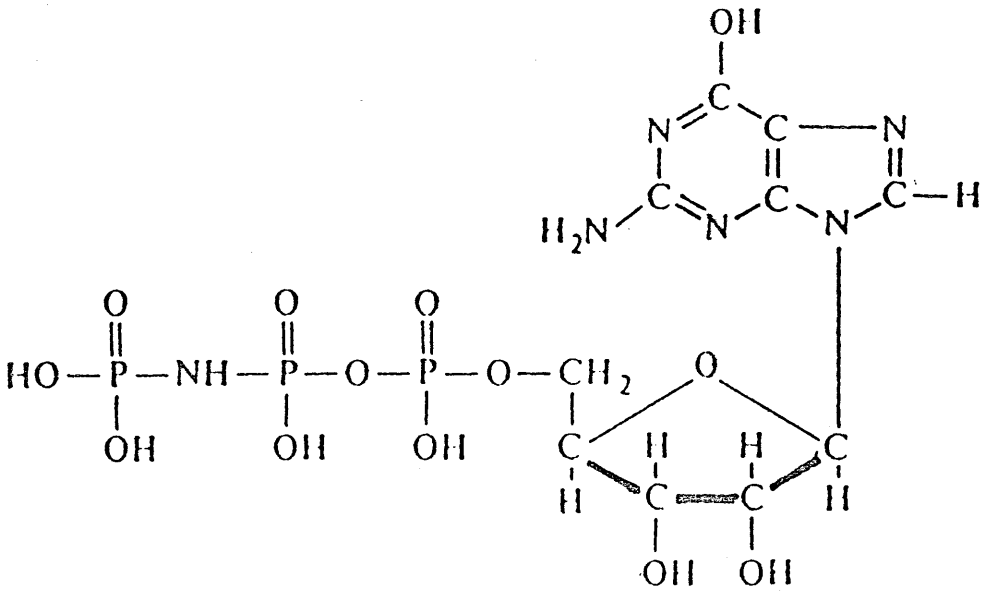


Figure A6 GppNHp



APPENDIX II : CHEMICALS

Supplier	Chemical
Amersham International Amersham, Bucks, England U.K.	- 5;8-^{3}H-adenosine 3'5' cyclic phosphate - 8-^{3}H-adenosine 3'5' cyclic phosphate - [alpha-^{32}P] Adenosine triphosphate - ^{32}P-carrier free
B.D.H. Ltd., Poole, Dorset, England, U.K.	All other chemical reagents not listed here
Bio-Rad Hemel Hempstead Herts, England	- Nitrocellulose
Boehringer Mannheim (UK) Lewes, East Sussex, England, U.K.	- App(NH)p - ATP - Creatine kinase - Glyceraldehyde-3-phosphate dehydrogenase - Glycerate-3-phosphate - Gpp(NH)p - GTP - NAD^{+} - 3-phosphoglycerate kinase - Triethanolamine (HCl) - Tris
Calbiochem Cambridge, England, U.K.	- Forskolin
Eli Lilly & Co. Indianapolis, IN, U.S.A.	- Insulin (porcine)
National Diagnostics Aylesbury, Bucks, England, U.K.	- Ecoscint

Supplier		Chemical
Porton Products Porton Down, Salisbury, Wilts, England, U.K.	-	Pertussis toxin
Scottish Antibody Prod. Unit Wishaw, Scotland, U.K.	-	Goat-antirabbit IgG (peroxidase-conjugated)
Sigma Chemical Co., Poole, Dorset, England, U.K.	-	Adenosine
	-	Adrenaline
	-	Alumina
	-	Benzyl alcohol
	-	Bovine serum albumin
	-	Cholera toxin
	-	Creatine phosphate
	-	Cyclic AMP
	-	Deoxycholate
	-	Dimethylnitrosamine
	-	Dithiothreitol
	-	Dowex 50AGWX4
	-	Dowex AG I-X2
	-	EGTA
	-	Glutathione
	-	Imidazole
	-	Isobutylmethylxanthine
	-	Isoprenaline
	-	Lubrol-PX
	-	Orthodianisidine (HCl)
	-	Ouabain
	-	Pentobarbita
	-	Phosphatidic acid
	-	Phosphatidylcholine
	-	Phosphatidylserine
	-	Prilocaine
	-	Propranolol
	-	Prostaglandin E_1
	-	Snake venom
	-	Temed
	-	Theophylline
	-	Thimerasol
	-	Thymidine
	-	Trypsin inhibitor (soyabean)
	-	Tween 20

Supplier		Chemical
Whatman	-	DE-50 Cellulose
Maidstone, Kent,	-	PEI cellulose plates
England, U.K.	-	Whatman No.54 filter paper