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Interaction of α_2-adrenergic receptors with G-proteins

A thesis presented for the degree of
DOCTOR OF PHILOSOPHY

by

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ABBREVIATIONS

ADP	adenosine 5' - diphosphate
AppNHp	adenylyl 5' - imidodiphosphate
ATP	adenosine 5' - triphosphate
βARK	β-adrenergic receptor kinase
BME	2 - mercaptoethanol
[^{125}I]pBABC	ρ-(bromoacetamido)benzyl-1-[^{125}I] iodocarazolol
Bromophenol blue	3',3",5',5"-Tetrabromophenolsulfonphthalein
BSA	bovine serum albumin
cAMP	adenosine 3' : 5' - cyclic monophosphate
cDNA	complementary DNA
CP	creatine phosphate
CPK	creatine phosphokinase
cpm	counts per minute
C - terminus	carboxyl terminus
DADLE	enkephalin (2-D-alanine,5-D-leucine)
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPM	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
FCS	foetal calf serum
FMLP	N -formyl-methionyl-leucyl-phenylalanine
Forskolin	7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd14-ene-11-one
Gi	inhibitory G-protein of adenylyl cyclase regulation
Go	G-protein of unknown function
Gs	stimulatory G-protein of adenylyl cyclase regulation
Gpp(NH)p	guanylyl 5'- imidodiphosphate
G - protein	Guanine nucleotide - binding protein
GTP	guanosine 5'- triphosphate
GDP	guanosine 5'-diphosphate
GDPβS	guanosine 5'-(2-O-thiodiphosphate)

GTPγS	guanosine 5´-(3-O-thiotriphosphate)
HAT	hypoxanthine-aminopterin-thymidine
Ig	immunoglobulin
kDa	kilodaltons
KLH	keyhole limpet haemocyanin
mA	milliamps
mRNA	messenger RNA
NAD^+	nicotinamide adenine dinucleotide
NEM	N - ethylmaleimide
N-terminus	amino terminus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate - buffered saline
PGE_1	prostaglandin E1
PLC	phospholipase C
PLD	phospholipase D
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TBS	tris - buffered saline
TCA	trichloroacetic acid
Td	transducin
TEMED	N ,N, N', N' - tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate

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SUMMARY

A wide variety of hormones and neurotransmitters have been shown to produce intracellular signals mediated by guanine-nucleotide binding proteins (G-proteins). These have been shown to form a link between receptors on the extracellular face of the cell membrane, and their intracellular second-message-generating effectors, such as phospholipase C and adenylyl cyclase. An increasing number of different heterotrimeric G-proteins have been isolated, with varying degrees (50-95%) of sequence homology. This similarity of sequence has raised questions of specificity of interaction of G-proteins: do individual G-proteins interact only with specific receptors and effectors, or are they more promiscuous in their interaction? This question has been addressed by examining the specificity of coupling of G-proteins to α_2-adrenergic receptors in two model systems.

The NG108-15 cell line is known to express an adrenergic receptor coupled to inhibition of adenylyl cyclase via a pertussis toxin sensitive G-protein. The identity of this G-protein has been determined by utilising antisera raised against synthetic peptides corresponding to the carboxy-terminal sequence of various G-proteins: the C-terminus of the G-protein α-subunit has been implicated as the site of receptor interaction. These antisera were used to show that NG108-15 cells expressed Gi2, Gi3, Go and Gs. Gi1 was not detected. The adrenergic receptor in NG108-15 cells was shown to bind the adrenergic antagonist, $[^3H]$-yohimbine with high affinity (Kd=2.3nM). Displacement of $[^3H]$-yohimbine binding indicated that the receptor had a high affinity for both prazosin and oxymetazoline, which is characteristic of an α_{2B} receptor. The poorly hydrolysable GTP

analogue Gpp(NH)p caused a reduction in the affinity of the receptor for the agonist (-)noradrenaline. This change in the affinity of receptors for agonists is thought to be the result of uncoupling of the G-protein from the receptor, and this was used as an assay to identify the G-protein coupling to the α_{2B} receptor in NG108-15 cells. Preincubation of membranes from NG108-15 cells with antisera raised against the C-terminal sequences of Gs, Go, and Gi3 did not affect the affinity of the receptor for (-)noradrenaline, however preincubation with the antiserum AS7, which recognises Gi2 in NG108-15 cells, caused a reduction in affinity of the receptor for (-) noradrenaline. These results suggested that the α_{2B} receptor in the NG108-15 cells was coupled specifically to Gi2.

In order to examine the factors which govern specificity of interaction between G-proteins and receptors, the coupling of G-proteins to the α_2-C10 receptor transfected into Rat-1 fibroblasts was assessed. One clone was isolated which was shown to express a high level of α_2-C10 receptor, as defined by [^{3}H]-yohimbine binding. Clone 1C bound [^{3}H]-yohimbine with high affinity (Kd=1nM), and displacement of yohimbine binding indicated that the receptor possessed a high affinity for oxymetazoline and a low affinity for prazosin, which is typical of an α_{2A} receptor.

The coupling of the α_2-C10 receptor to G-proteins in clone 1C cells was examined by using anti-G-protein antisera. The α_2-adrenergic agonist UK14,304 was shown to stimulate a pertussis toxin-sensitive high affinity GTPase activity in membranes from clone 1C cells. Preincubation of membranes from clone 1C with anti-G-protein antisera raised against the C-termini of Gi1, Gi2, and Gi3 attenuated the UK14,304 stimulated GTPase activity. Preincubation with antisera against Gs or Go did not have this effect. These data suggest that the α_{2A} receptor in clone 1C cells

couples to Gi1, Gi2, and Gi3, although the relative levels of expression of the G-proteins suggest that the role of Gi1 may be minimal.

The α_{2A} receptor in the human platelet has been shown to mediate adrenergic inhibition of adenylyl cyclase via Gi2. It was therefore of interest to assess the α_{2A} receptor in clone 1C cells for inhibition of adenylyl cyclase. UK14,304 was shown to inhibit forskolin-amplified adenylyl cyclase activity in membranes from clone 1C cells. This inhibition was pertussis toxin sensitive. Incubation of membranes from clone 1C cells with the antiserum AS7 completely attenuated the adrenergic inhibition of forskolin stimulated adenylyl cyclase, indicating that the α_{2A} receptor in clone 1C cells was coupled to inhibition of adenylyl cyclase activity via Gi1 and Gi2. Hence, although the α_{2A} receptor in clone 1C cells clearly activated Gi1, Gi2 and Gi3 (as assessed by GTPase uncoupling), only Gi1 and Gi2 mediate inhibition of adenylyl cyclase.

These results indicate a high degree of specificity of G-protein coupling to the α_{2B} receptor in NG108-15 cells, but a loss of specificity in the case of the α_{2A} receptor when transfected into the Rat-1 fibroblast. The implications are discussed, and the suggestion is made that G-protein specificity of interaction with receptors and intracellular effectors depends on factors other than the identity of the G-protein. It is proposed that factors such as tissue-specific expression of G-proteins and G-protein compartmentalisation within the cell membrane all play a part in determining the specificity of interaction of G-proteins.

CHAPTER 1

INTRODUCTION

HISTORICAL

It is now axiomatic that most hormones and neurotransmitters interact with cell-surface receptors, and that this interaction generates an intracellular signal which ultimately results in the appropriate cellular response. These ideas were established in the late 1950s and early 1960s, and have become refined over the last twenty years (for review, see Birnbaumer, 1990). The field of signal transduction research began with the discovery of a 'heat stable factor' which was generated in response to catecholamine hormones and which could activate liver phosphorylase. (Rall *et al.* 1957). This factor was later identified as 3′,5′-cyclic AMP (cAMP), and the agent of its generation as the enzyme adenylyl cyclase, which was localised to the cell membrane (Rall and Sutherland, 1961). It was initially thought that the cyclase enzyme itself contained the site for binding hormone, albeit through a separate regulatory subunit referred to as the 'discriminator' (Robison *et al.* 1967). However it was shown in the 1960s that different hormones could stimulate adenylyl cyclase activity in a non-additive fashion, implying that hormones, by combining with separate and independent binding sites, could activate a single adenylyl cyclase (Rodbell *et al.* 1969). Finally, the distinction between receptor and adenylyl cyclase was made clear when the β-adrenergic binding site of frog erythrocytes was separated from the enzyme by gel exclusion chromatography (Limbird and Lefkowitz, 1977). The full purification of the β-adrenergic receptor was not achieved until 1981 (Shorr *et al.* 1981) and purification of adenylyl cyclase itself was achieved in 1985 (Pfeuffer *et al.* 1985).

It was during 1970 that the discovery was made that receptor and ligand alone were insufficient to activate adenylyl cyclase. Martin Rodbell

and his group found that glucagon stimulation of adenylyl cyclase was dependant on the presence of the guanine nucleotide, guanosine triphosphate (GTP) (Rodbell *et al.* 1971). Curiously, this discovery was made because the batches of ATP they had used as the substrate for adenylyl cyclase were substantially contaminated with GTP, and when new, purer batches of ATP were used, their assays stopped working!

Subsequently it was found that nonhydrolysable analogues of GTP such as guanylylimidodiphosphate (GppNHp) caused a persistent activation of adenylyl cyclase (Schramm and Rodbell, 1975). This contrasted with the transient activation due to GTP and suggested the presence of a GTP binding site which could both bind the guanine nucleotide and cleave it to GDP, ie that the GTP regulatory component of adenylyl cyclase stimulation was the active site of a GTPase. The demonstration that a high affinity GTPase activity in turkey erythrocytes was stimulated by the presence of catecholamine hormones which activated adenylyl cyclase confirmed this idea (Cassel and Selinger, 1976). Cassel and Selinger, by observing the kinetics of [^{3}H]-GDP release from the regulatory subunit, further showed that receptor occupancy increased the rate of release of GDP (Cassel and Selinger, 1978). They also demonstrated that a protein product of the bacterium *Vibrio cholerae*, cholera toxin, inhibited catecholamine stimulated GTPase activity in turkey erythrocytes, and that this resulted in an enhanced activation of adenylyl cyclase (Cassel and Selinger, 1977). They suggested that GTP hydrolysis at the regulatory GTP binding subunit constituted an essential 'turn-off' mechanism for activation of adenylyl cyclase.

Thus the framework for the role of G-proteins in activation of adenylyl cyclase was laid. A GTPase regulatory cycle was proposed, in which GDP bound to a regulatory subunit of adenylyl cyclase and was driven off and replaced by GTP following the binding of a hormone to its

receptor. In the GTP bound state, the regulatory component could activate adenylyl cyclase. This activation of adenylyl cyclase would then be terminated by a GTPase activity .

The isolation of the GTP binding factor from adenylyl cyclase was achieved by using GTP-Sepharose to isolate a 42kDa protein identified by a GTP-photoaffinity probe from detergent-solubilised pigeon erythrocyte membrane preparations (Pfeuffer, 1977). At the same time a report appeared which noted a mutant murine lymphoma cell line, S49 AC^- (now called *cyc* $^-$), in which the communication between receptor and adenylyl cyclase appeared to be disrupted. Initially, it was thought that these cells lacked the cyclase enzyme, however it was shown by Ross and Gilman (Ross and Gilman, 1977) that addition of a detergent extract from wild-type membranes which had their adenylyl cyclase inactivated could restore β-adrenergic stimulated cyclase activity. Ross and Gilman showed that the *cyc* $^-$ cells possessed adenylyl cyclase, but were lacking the GTP-binding protein (Ross *et al.* 1978). They called this component G/F, as it mediated both guanine nucleotide and fluoride ion stimulation of adenylyl cyclase, but it is known today as G_S, the stimulatory G-protein of adenylyl cyclase.

Identification of Gs

The *cyc* $^-$ strain proved to be an invaluable tool for the study of interaction between adenylyl cyclase and its G-protein. Gilman's group were able to purify G_S essentially to homogeneity from rabbit liver plasma membranes by assaying for reconstitution of *cyc* $^-$ adenylyl cyclase stimulation (Northup *et al.* 1980). Cassel and Pfeuffer, while investigating the mechanism of action of cholera toxin, had used radiolabelled [^{32}P] NAD^+ as a substrate for the toxin, and had shown that this caused incorporation of a radiolabelled [^{32}P] ADP-ribose group into a 42 kDa

protein in turkey erythrocytes (Cassel and Pfeuffer, 1978). Using the same approach, Gilman's group had found incorporation of [^{32}P]-ADP-ribose into two proteins of Mr 52 and 45 kDa. In addition, they purified a protein of 35kDa with their factor G/F. They suggested that G_s was a dimer consisting of either the 52 or 45 kDa protein, associated with the 35kDa protein. It was later shown that G_s was in fact a heterotrimer composed of α, β, and γ subunits of Mr 45, 35 and 8 kDa (Hildebrandt *et al.* 1984). Why, then, were there two apparent substrates for cholera toxin in rat liver membranes ? It was only in 1986 that the answer was found: the mRNA for Gsα was shown to be differentially spliced, resulting in expression of two proteins, different forms of $G_{s\alpha}$, one with apparent Mr 45kDa, the other with apparent Mr 52kDa (Robishaw *et al.* 1986).

Discovery of Gi

It became clear quite early on that hormonal inhibition of adenylyl cyclase also showed a requirement for guanine nucleotides. Rodbell and colleagues showed that, in a rat adipocyte preparation, inhibition of adenylyl cyclase occured in the absence of a stimulatory hormone and in the presence of low concentrations of GTP (Harwood *et al.* 1973). In an influential review in 1980, Rodbell formulated the concept of different forms of G-proteins (or N-proteins, as they were called); G_s, responsible for stimulation of adenylyl cyclase, and G_i, responsible for its inhibition. He also suggested that different receptors would have different G-proteins to transduce their various intracellular effects (Rodbell, 1980).

Several lines of investigation suggested that the GTP-binding site coupling inhibitory receptors to adenylyl cyclase was different from that coupling to stimulatory receptors, notably the observations that the inhibitory site was preferentially susceptible to sulfhydryl alkylation with

N-ethylmaleimide (Aktories *et al.* 1982) and to trypsin (Yamamura *et al.* 1977). The work of Katada and Ui on pertussis toxin showed conclusively that "G_i" existed, and that it was a distinct moiety from G_s. They had shown that α_2-adrenergic receptors inhibited adenylyl cyclase in pancreatic islet cells, and that this inhibition was attenuated by a toxin from the bacterium *Bordetella pertussis*, the causative organism of whooping cough (Katada and Ui, 1979). They further showed that the target of pertussis toxin was a membrane protein of 41 kDa, which was the substrate for the toxin's ADP-ribosyl -transferase activity (Katada and Ui, 1982a), and that this protein was distinct from G_s (Katada and Ui, 1982b). Studies using the *cyc*$^-$ cell line showed that receptor-mediated inhibition of adenylyl cyclase occurred even in the absence of G_s, and that pertussis toxin could prevent this inhibition, indicating that the 41 kDa protein identified by Katada and Ui was "Gi", the inhibitory G-protein of adenylyl cyclase (Hildebrandt and Birnbaumer, 1983; Hildebrandt *et al.* 1983). It was also in 1983 that the first reports of "Gi" purification appeared, showing it to be a multisubunit protein with the α-subunit being the site for pertussis toxin catalysed ADP-ribosylation. Bokoch *et al.* purified "Gi" from rabbit liver, and found it to be a 41 kDa protein (Bokoch *et al.* 1983), however Codina *et al.* purified it from human erythrocytes, and found it to be a 39kDa protein (Codina *et al.* 1983). This was the first suggestion of G-protein heterogeneity; that there might be multiple forms of "Gi".

Actions of cholera and pertussis toxin

The study of the identity and function of G-proteins has been greatly aided by the use of bacterial exotoxins. In particular, the roles of cholera and pertussis toxin have already been mentioned. Both toxins are oligomeric proteins with an A-B structure (Gill, 1977) , in which a B-

oligomer binds to particular cell-surface receptors, enabling penetration of the cell by the A-oligomer, wherein it acts as an ADP-ribosyltransferase, catalysing the transfer of ADP-ribose from NAD^+ to the membrane G-proteins which are its substrates. Cholera toxin action requires the presence of a cofactor, ARF (ADP-Ribosylation Factor), which itself binds GTP (Kahn and Gilman, 1984).

To early investigators into the action of adenylyl cyclase their effects appeared to be similar in that both toxins resulted in elevated cAMP levels, and both toxins rendered receptors insensitive to hormones (stimulatory hormones, in the case of cholera toxin, and inhibitory hormones, in the case of pertussis toxin). We now know that these effects are due to their actions on "Gi" and G_s, which work in opposition on the activation of adenylyl cyclase. Exposure of cells to cholera toxin results in ADP-ribosylation of an arginine (Arg ^{201}) residue near the GTP binding site of G_s (Van Dop *et al.* 1984). This modification prevents the cleavage of the terminal phosphate of GTP to GDP, thus G_s becomes constitutively activated, and adenylyl cyclase activity in cholera toxin treated cells is elevated (Cassel and Pfeuffer, 1978).

In contrast, treatment of cells with pertussis toxin resulted in loss of the ability to inhibit adenylyl cyclase: it was shown that pertussis toxin ADP-ribosylated a cysteine residue four amino acids from the C-terminus of the "Gi"- α-subunit and that this prevented functional interaction between the G-protein and its receptor (Ui *et al.* 1984; West *et al.* 1985). This is often referred to as "uncoupling" the G-protein from its receptor. Unlike cholera toxin catalysed ADP-ribosylation of G_s, where $G_{s\alpha}$ is the substrate, pertussis toxin will only ADP-ribosylate the GDP-liganded $\alpha\beta\gamma$ heterotrimer of "Gi" (Mattera *et al.* 1987).

Thus it became possible to distinguish and identify Gs and Gi by use of cholera and pertussis toxin, and for a number of years this was the only

method of detection of G-proteins. However, the realisation that G-proteins were more heterogenous than had at first been suspected, and also the finding that some receptors, particularly those coupled to phospholipase C (PLC), were immune to the effects of both toxins, led to the development of new and more specific methods of G-protein detection (see below).

G-PROTEIN STRUCTURE

1. Alpha Subunit

a: sites for guanine nucleotide binding and GTPase activity

The elucidation of the structure of G-protein alpha subunits has been greatly aided by the X-ray crystallographic analysis of two GTP binding-proteins related to the heterotrimeric G-protein family, namely the bacterial elongation factor involved in ribosomal protein synthesis, EF-Tu (Jurnak, 1985; La Cour *et al.* 1985) , and the product of the Ras gene, $p21^{ras}$ (de Vos *et al.* 1988). Similarities in the 3-dimensional structures of both of these proteins has allowed extrapolation of structural features, particularly with regard to GTP-binding domains and GTPase activity to members of the heterotrimeric G-protein family.

The primary sequences of EF-Tu and $p21^{ras}$ were first compared by Halliday, who identified four conserved stretches (designated A,C,E, and G) which, she predicted, would form part of the guanine-nucleotide binding domain (Halliday, 1984). Comparison of the primary amino-acid sequences of seven G-protein α-subunits aligned with a hypothetical α_{avg} by Masters *et al.* (Masters *et al.* 1986) also revealed a remarkable degree of conservation of sequence, confirming that these same regions were present

in the heterotrimeric G-protein α-subunits. From their comparisons of α-subunits, and using the 3-dimensional structure of EF-Tu as a model, Masters *et al.* were able to predict the 3D structure of their α_{avg} (Figure 1.1). Halliday region A lies in a region important for the regulation or hydrolysis of bound GTP. Mutation of this region in ras proteins results in a reduced GTPase activity (Barbacid, 1987). Region C also appears to be important in the hydrolysis of GTP, lying as it does near the terminal phosphate of the guanine nucleotide and in close proximity to the Mg^{2+} ion. Regions E and G contain conserved hydrophobic amino-acids, suggesting that they form a hydrophobic pocket to facilitate binding of the guanine ring. Again, mutations in this region of $p21^{ras}$ results in lowered binding affinity for guanine nucleotides (Sigal *et al.* 1986).

The crystal structures for EF-Tu and $p21^{ras}$ were obtained for the GDP bound proteins. Of major interest, however, is the conformational change brought about by the exchange of GTP for GDP, since it is this event which activates the G-protein α-subunit. Recently the structure of the Gpp(NH)p-activated Ha^{ras} p21 protein has been analysed (Pai *et al.* 1989). There appeared to be two major conformational changes upon activation. Firstly, a putative effector loop between Ile^{21}-Gly^{48} (in Halliday region A) appeared to have shifted, consistent with an interaction with the terminal phosphate of GTP (Jurnak *et al.* 1990). The second, and most dramatic, conformational change occurred in a polypeptide sequence of 20 amino acids in Halliday region C, beginning with Asp^{57}, the side chain of which is thought to be important in coordination of the Mg^{2+} ion. This segment appears to turn through 120° on activation, bringing the amido- group of Gly^{60} into close proximity to the γ-phosphate of GTP (Jurnak *et al.* 1990).

Figure 1.1

The predicted GDP-binding domain of α_{avg}

Adapted from Iyengar R and Birnbaumer L, Eds. "G-proteins", page 26, Academic Press 1990.

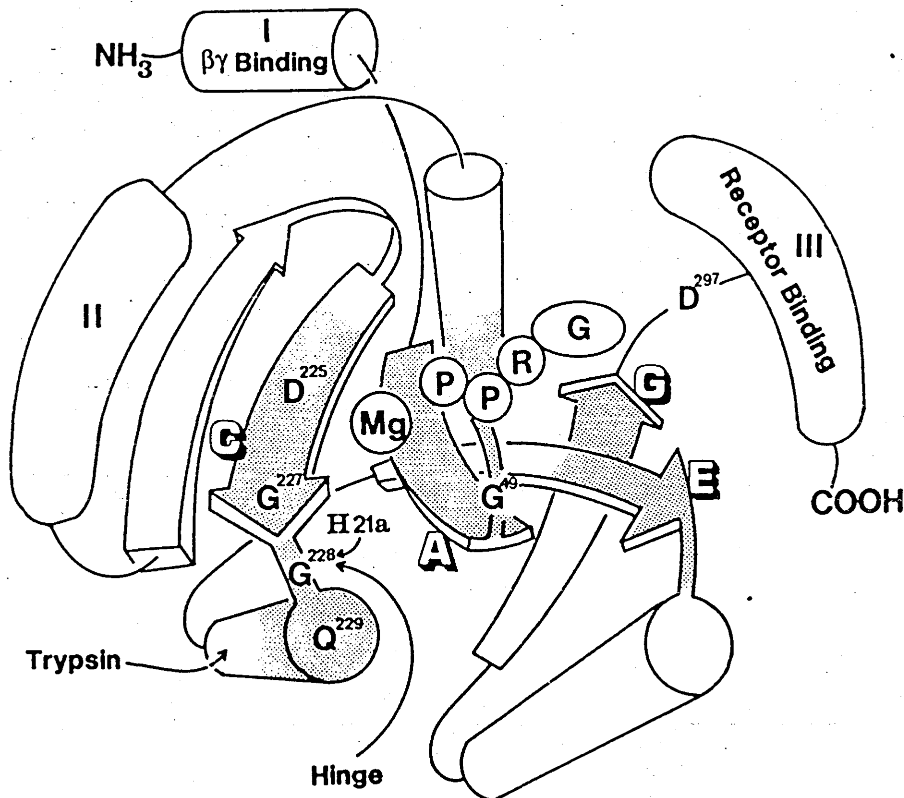


Diagram of the predicted GDP-binding domain of α_{avg} (Masters *et al.*, 1986), based on the crystal structure of the corresponding domain of EF-Tu (Jurnak, 1985). The GDP molecule (G, guanine ring; R, ribose; P, phosphoryl) nestles into a binding site bounded by turns between β strands and α helices; these turns are located in the stippled regions, designated A, C, E, and G, which correspond to the four stretches of similar amino-acid sequence first noted by Halliday (1984)

Key amino -acid residues, numbered in accord with α_{avg}, are indicated by single letters (D, Asp; G, Gly; Q, Gln). The predicted hinge region where flexion is thought to be blocked by the H21a mutation at Gly[228] is indicated by an arrow, as is the site where tryptic cleavage is prevented by activation of the α chain.

While these structural observations have been obtained for p21ras and EF-Tu, the conservation of sequence across the range of guanine-nucleotide binding proteins makes it likely that similar conformational changes will be found to occur in the heterotrimeric G-proteins.

b: Sites for interaction with receptor, effector and βγ

Comparison of the primary sequences of G-protein α-chains reveals three major regions of sequence divergence (Regions I, II, and III in Figure 1.1). As the specificity of α-subunits depends upon their ability to interact with distinct effector and receptor combinations, one would expect that these regions of dissimilarity might encode the receptor- and effector- coupling regions, while the regions of sequence similarity would represent regions common to the function of all G-proteins, ie. guanine nucleotide binding and cleavage.

There is now substantial evidence that the carboxy-terminal region of the α-subunit is the site for receptor coupling (Region III, Figure 1.1). Firstly, pertussis toxin catalysed ADP-ribosylation of a cysteine residue (which is conserved in the various forms of Gi, Go, and transducin) four amino acids from the C-terminus blocks receptor interaction with G-proteins (Ui *et al.* 1984), and prevents binding of transducin to rhodopsin (Van Dop *et al.* 1984). A mutant of the S49 murine lymphoma cell line, called *unc* , which does not show hormonal stimulation of adenylyl cyclase (Haga *et al.* 1977) was shown to contain a mutated form of Gs, in which a single amino acid substitution six amino acids from the C-terminus (Arg→Pro) accounted for the lack of interaction with receptor (Sullivan *et al.* 1987).

Bourne's group also showed that construction of a chimæric α-subunit composed of the N-terminal 60% of murine Gi2 and the C-terminal 40% of murine Gs when expressed in *cyc*$^-$ cells resulted in β-adrenergic stimulation of adenylyl cyclase (mediated by Gs), but not somatostatin inhibition of cyclase (mediated by Gi) (Masters *et al.* 1988). The implication of this result was that the C-terminal portion of Gs conferred on the α-subunit its ability to interact with the β-adrenoceptor, however the chimæric α-subunit would not couple to the somatostatin receptor because it contained only the N-terminal part of Gi, and not the C-terminal portion important for receptor coupling.

Finally, it has been shown by two groups that α-subunit specific antisera raised against the C-terminal decapeptide sequence are capable of uncoupling G-proteins from their receptors. McKenzie and Milligan used antiserum AS7, raised against the C-terminal sequence of Gi2, to block δ-opioid receptor mediated inhibition of forskolin stimulated adenylyl cyclase (McKenzie and Milligan, 1990) and Simonds and Spiegel showed that the adrenergic receptor in human platelets was coupled to adenylyl cyclase via Gi2 by using the same antiserum, AS7, to prevent norepinephrine inhibition of PGE_1 stimulated cyclase (Simonds *et al.* 1989a).

By contrast, the region which couples the α-subunit to its effector is not known. Bourne suggested that Region II (Figure 1.1) might be responsible (Masters *et al.* 1986), however the later results from Bourne's group, discussed above (Masters *et al.* 1988) tend to refute this idea, since the Gi2/Gs chimæra they constructed contained Region II from Gi2, yet this α-subunit did not behave like "Gi", being unable to mediate somatostatin induced inhibition of cyclase, and instead behaved like Gs. Osawa *et al.* have recently constructed their own Gs/Gi2 chimæras and expressed them in COS cells to examine the site for effector-G-protein contact (Osawa *et al.* 1990). They varied the proportions of Gs and Gi and

were able to identify a region of 122 amino acids (Ile^{235}-Arg^{336}) which was involved in interaction of Gs with adenylyl cyclase.

The region responsible for α-subunit interaction with βγ subunits appears to be the N-terminus. Tryptic cleavage of the 21 N-terminal amino-acids from rod cell transducin destroyed its ability to bind to βγ (Navon and Fung, 1987). The Gi2/Gs chimæras constructed by Osawa also threw some light on the βγ interaction site. They found that mutation of the N-terminus prevented regulation of the rate of guanine nucleotide release - one of the functions ascribed to the βγ subunit. They also suggested that the C-terminus and N-terminus of Gα must be spatially close together, since one of their chimæras with a mutated N-terminus inhibited the pertussis toxin catalysed ADP-ribosylation of the C-terminal cysteine of Gi2 (Osawa *et al.* 1990).

C: Membrane Attachment

The hydrophilic nature of much of the α-subunit would suggest that it can readily be extracted from the membrane. In fact, most G-proteins require the use of a detergent before they can be removed from the membrane. This begs the question: are the α-subunits attached in some way to the membrane? It has been well documented that the protein products of ras genes have C-terminus-linked palmitate groups (Barbacid, 1987), and the src oncogene product is N-myristylated (Schultz *et al.* 1985). Both of these lipids have hydrophobic properties which would allow the formation of a membrane anchor. It appears that G-protein α-subunits may be similarly acylated. Schultz *et al.* used reverse-phase HPLC to detect myristate in Goα (Schultz *et al.* 1987). At the same time, Gilman's group demonstrated the incorporation of [^{3}H]-myristic acid into Go, $G\alpha_{40}$ and $G\alpha_{41}$ α-subunits -

but not into Gs or the β-subunit (Buss *et al.* 1987). A variety of N-myristylated proteins share a consensus sequence (Met-Gly-X-X-X-Ser) which also appears in the sequence of Go, "Gi",and transducin α-subunits, but is lacking in Gsα, β_{36} and β_{35} and transducin γ (Schultz *et al.*, 1987). One form of post-translational modification which seems to be lacking in α-subunits is C-terminal isoprenylation. Despite possessing the "CAAX box" sequence required for cysteine-linked isoprenylation (see below, in discussion of βγ-subunit structure) it appears that none of the Gi or Go subunits are modified in this way (Sanford *et al.* 1991). It has also been shown that Gi1 can only become a substrate for such a modification if the carboxy terminus is mutated (Jones and Spiegel, 1990).

2. βγ subunit

Although the β and γ subunits are not covalently linked, they clearly form a single functional unit, as they cannot be dissociated without denaturing the proteins. Also, it is not known whether the functions of the βγ subunit are due to one or other of the proteins alone.

Their are two major forms of β-subunit, with molecular weights of 35- and 36-kDa, (Sternweis and Robishaw, 1984) with the 36kDa form predominating in most tissues (Woolkalis and Manning, 1987). These two forms, sometimes denoted β_1 and β_2 appear to be the products of two separate genes located on chromosomes 1 and 7 respectively (Lochrie and Simon, 1988). The cDNAs for the two forms share 90% homology (Fong *et al.* 1987). Neither sequence suggests membrane spanning domains, special hydrophobic regions or signal sequences for glycosylation, etc, although the sequences for β_1 and β_2 are strictly conserved across a

number of mammalian species, suggesting that the two forms may have distinct functions (Neer and Clapham, 1990). Both forms of β subunit show similarities to the C-terminal region of the yeast cell division cycle gene CDC4, although the significance of this fact is not yet clear (Fong *et al.* 1986).

If less is known about the structure of the β-subunit than the α-, then the γ-subunit is even more mysterious. There do appear to be different forms of γ-subunit, and there is evidence that the γ-subunit from transducin is different to that in brain or erythrocyte G-proteins (Hildebrandt *et al.* 1985). The cDNA for transducin γ-subunit has been cloned and shown to encode a hydrophilic protein (Van Dop *et al.* 1984; Yatsunami *et al.* 1985). It has recently been shown that the γ-subunit is capable of being isoprenylated. The carboxy terminus of brain γ-subunit was shown to contain the Cys-A-A-X sequence (Cysteine, A=aliphatic, X= any amino acid) which occurs as the signal for cysteine thioester isoprenylation in p21^{ras}. Mutant forms of p21^{ras} which lack this "CAAX box" have been shown not to associate with the membrane, and hence are not transforming, suggesting that this modification is important in locating the protein at the membrane (Willumsen *et al.* 1984). The heterotrimeric G-protein γ-subunit, translated in reticulocyte lysate was shown to be labelled with the isoprenoid precursor [^{3}H]-mevalonate (Maltese and Robishaw, 1990). A number of other groups have also demonstrated C-terminal isoprenylation of γ-subunits (see Mumby *et al.* 1990; Lai *et al.* 1990; Sanford *et al.* 1991).

The hydrophilic character of the α-subunits, together with the relative hydrophobicity of the βγ subunit has led to the suggestion that the βγ subunit forms a membrane anchor for the α-subunit (Sternweis, 1986). Sternweis observed that purified α-subunits did not associate with phospholipid vesicles unless in the presence of βγ-subunits. It is clear however, that many α-subunits are myristoylated, or at least are capable of

myristoylation, which makes it a less than compelling argument that this is the only role of the $\beta\gamma$ subunit (see below).

G-PROTEIN HETEROGENEITY

It became clear that there were more than two G-proteins, Gs and "Gi", in 1984, when the search for "Gi" led to the finding of multiple pertussis toxin substrates in bovine brain, where a 39kDa protein was found in addition to the familiar 41kDa pertussis toxin substrate (Sternweis and Robishaw, 1984). This was simply the beginning of a flood of new G-protein discoveries. Initially, G-proteins were detected by virtue of their susceptibility to modification by pertussis or cholera toxin, and because of their differential positioning on SDS-polyacrylamide gels, but nowadays they appear as the result of molecular biology studies. This has allowed the detection of G-protein α-subunits for which there are, as yet, no functions (eg. Strathmann *et al.* 1989: Libert *et al.* 1989). Table 1.1 shows the variety of G-protein forms and functions. The diversity of G-protein subunits has recently been reviewed (Simon, *et al.* 1991).

It was noted earlier that there are at least two forms of Gs_{α} (52 and 45kDa) responsible for the stimulation of adenylyl cyclase. To date four very similar cDNAs for $Gs\alpha$ have been detected in human brain (Bray *et al.* 1986). It seems likely that all four cDNAs are produced from a single gene by differential splicing (Kozasa *et al.* 1988). In addition to stimulation of adenylyl cyclase, Gs has also been shown to activate dihydropyridine-sensitive Ca^{2+} channels (Yatani *et al.* 1987). A form of Gs, known as

"G_{olf}" is expressed only in olfactory tissues, and presumably mediates stimulation of adenylyl cyclase in response to smell (Jones and Reed,1989; Lancet and Pace, 1987).

The nature of "Gi" , the pertussis toxin sensitive inhibitory G-protein for adenylyl cyclase is less clear. Three closely related forms of Gi were cloned from rat C6 glioma cells and a human genomic library (Itoh *et al.* 1988). These forms, classified as Gi1, Gi2, and Gi3, appeared from Itoh's study to be the products of different genes, and have since been detected in a variety of species and tissues (see Lochrie and Simon, 1988 for review). The three forms of "Gi" show a remarkable degree of sequence homology, the overall level being ~90%, with Gi1 and Gi3 sharing 95% homology. Their molecular masses are ~40kDa (Gi2) and ~41kDa (Gi1 and Gi3). Their functions, despite this homology, seem to vary. All three have been shown, in the purified, recombinant form, to open atrial K^+ channels (Yatani *et al.* 1988). Gi2 has been identified as the inhibitor of adenylyl cyclase in a number of systems (Simonds *et al.* 1989a; McKenzie and Milligan, 1990; McClue and Milligan,1990), and in the case of neutrophilic cell lines, Gi2 and Gi3 have been implicated in coupling of chemoattractant receptors to phospholipase C (Gierschik *et al.* 1989).

Go is the major pertussis toxin substrate in brain, accounting for a remarkable 1% of total brain protein. It was first discovered in 1984 (Sternweis and Robishaw, 1984; Neer *et al.* 1984) and has a Mr of 39kDa. Goα has 82% homology with Gi1/Gi3α, and only 50% homology with Gsα (Van Meurs *et al.* 1987). It now seems likely that there are different forms of Go, variously called Go_1, Go_2, and Go*. Studies on brain initially demonstrated the occurrence of two forms of Go, called Go and Go* (Goldsmith *et al.* 1988). More recently, it has been shown that at least two more forms exist in bovine brain (Kobayashi *et al.* 1989; Padrell *et al.* 1991). Go is poorly expressed in peripheral tissues (Homburger *et al.*

TABLE 1.1: G-PROTEIN HETEROGENEITY

G-protein	α-subunit Size	Function	Reference
Gs	45, 52kDa	Adenylyl cyclase stimulation	Bray *et al.* 1986
G_{olf}	45, 52kDa	Adenylyl cyclase stimulation	Jones and Reed 1987
Gi1	41kDa	K^+ channel	Itoh *et al.* 1988
Gi2	40kDa	inhibition of adenylyl cyclase	Itoh *et al.* 1988
Gi3	41kDa	K^+ channel	Itoh *et al.* 1988
Go	39kDa	Ca^{2+} channel	Sternweis *et al.* 1984
Gz	41kDa	Phospholipase C stimulation?	Fong *et al.* 1988
Gp (Gq)	42kDa	Phospholipase C stimulation	Pang *et al.* 1989
$G_{12\text{-}16}$	?	unknown	Simon *et al.* 1991
Td_1	40kDa	cGMP phosphodiesterase	Lerea *et al.* 1986
Td_2	40kDa	cGMP phosphodiesterase	Lerea *et al.* 1986
$\beta_{1\text{-}3}$	35-37kDa	unclear	Levine *et al.* 1990
$\gamma_{1\text{-}4}$	8-9kDa	unclear	Gautam *et al.* 1990

1987). This makes it likely that the function of Go is one exclusive to nervous tissues, and indeed there is now considerable evidence that Go is responsible for coupling neurotransmitter receptors to voltage operated Ca^{2+} channels (VOCCs). Hescheler *et al.* demonstrated that Go was responsible for coupling opioid receptors to Ca^{2+} channels in NG108-15 neuroblastoma x glioma cells (Hescheler *et al.* 1987), and Harris-Warrick *et al.* have shown that antibodies raised against Goα blocked dopamine-induced reductions in VOCCs in snail neurons (Harris-Warrick *et al.* 1988).

While it is clear that a G-protein is involved in coupling receptors to phospholipase C (PLC), the identity of the G-protein(s) involved is unknown, and is the subject of much study (for review, see Boyer *et al.* 1989). One of the problems facing researchers into the G-protein involved, known as Gp, is that in many cases, receptors coupled to PLC are unaffected by either pertussis or cholera toxin. In addition, it seems almost certain that there will be different forms of Gp, if only for the reason that there are a number of isozymes of PLC (see also Boyer *et al.* 1989).

Strathmann and Simon in 1989 used the polymerase chain reaction on mouse brain and sperm and, in addition to the well known G-protein α-subunits, they also detected five unknown α-subunits (Strathmann *et al.* 1989). Analysis of the sequences of some of these α-subunits led Simon and Strathmann to suggest that a separate class of toxin-insensitive α-subunits existed, and that they might be involved in coupling receptors to PLC (Strathmann and Simon, 1990). One member of this class is Gq, isolated by Pang and Sternweis by using an affinity chromatography column of immobilised $\beta\gamma$-subunits (Pang and Sternweis, 1989) and which appeared to be a 42kDa protein, insensitive to pertussis toxin, and not recognised by antibodies against any known G-protein (Pang and Sternweis, 1990). Gq_{α} has only 50% homology with members of the "Gi_{α}" group, or with Gs_{α} and

lacks the cysteine residue at the C-terminus which is the site for pertussis-toxin ADP-ribosylation. It also lacks the sequence for N-terminal myristoylation (Strathmann and Simon, 1990). Gq has now been shown to be capable of regulating the β1 isozyme of PLC (Smrcka *et al.* 1991; Taylor *et al.* 1991).

Another potential member of the "Gp" family is Gz, which was cloned from a human retinal cDNA library, and, again, lacks the site for pertussis toxin catalysed ADP-ribosylation (Fong *et al.* 1988). The mRNA for Gz appears to be localised to nervous tissue but the function of Gz remains speculative. At the same time as Fong *et al.* isolated Gz, Matsuoka *et al.* isolated the cDNA for another pertussis toxin -insensitive G-protein α-subunit, which they called Gx, from rat brain (Matsuoka *et al.* 1988), but it seems likely that Gx and Gz are identical. Gz/x_{α} cDNA encodes a protein of 355 amino acids with a Mr of ~ 41kDa, the mRNA for which appears mostly in brain tissues (Matsuoka *et al.* 1990).

The G-proteins involved in visual transduction, the transducins, occur in two forms. Td_1 is localised to rod cell outer segments, while Td_2 occurs only in cone cells (Lerea *et al.* 1986). They have been extensively studied, due to their abundance in visual tissues and due to the fact that they can be easily extracted without the use of detergents (for review see Stryer, 1986). The sequences of the two transducins differ in ~20% of the amino acids, but both encode proteins of ~40kDa. Their function is clear. They couple their light-activated receptors to cGMP phosphodiesterase, resulting in a rapid drop in cGMP levels which ultimately causes hyperpolarisation of the rod or cone cell, leading to transmission of a nerve impulse (Stryer, 1986).

Just as there are multiple forms of the G-protein α-subunits, it is now becoming clear that there are also many forms of the β- and γ-subunits. There are, to date, three forms of the β-subunit. β1 and β2 have

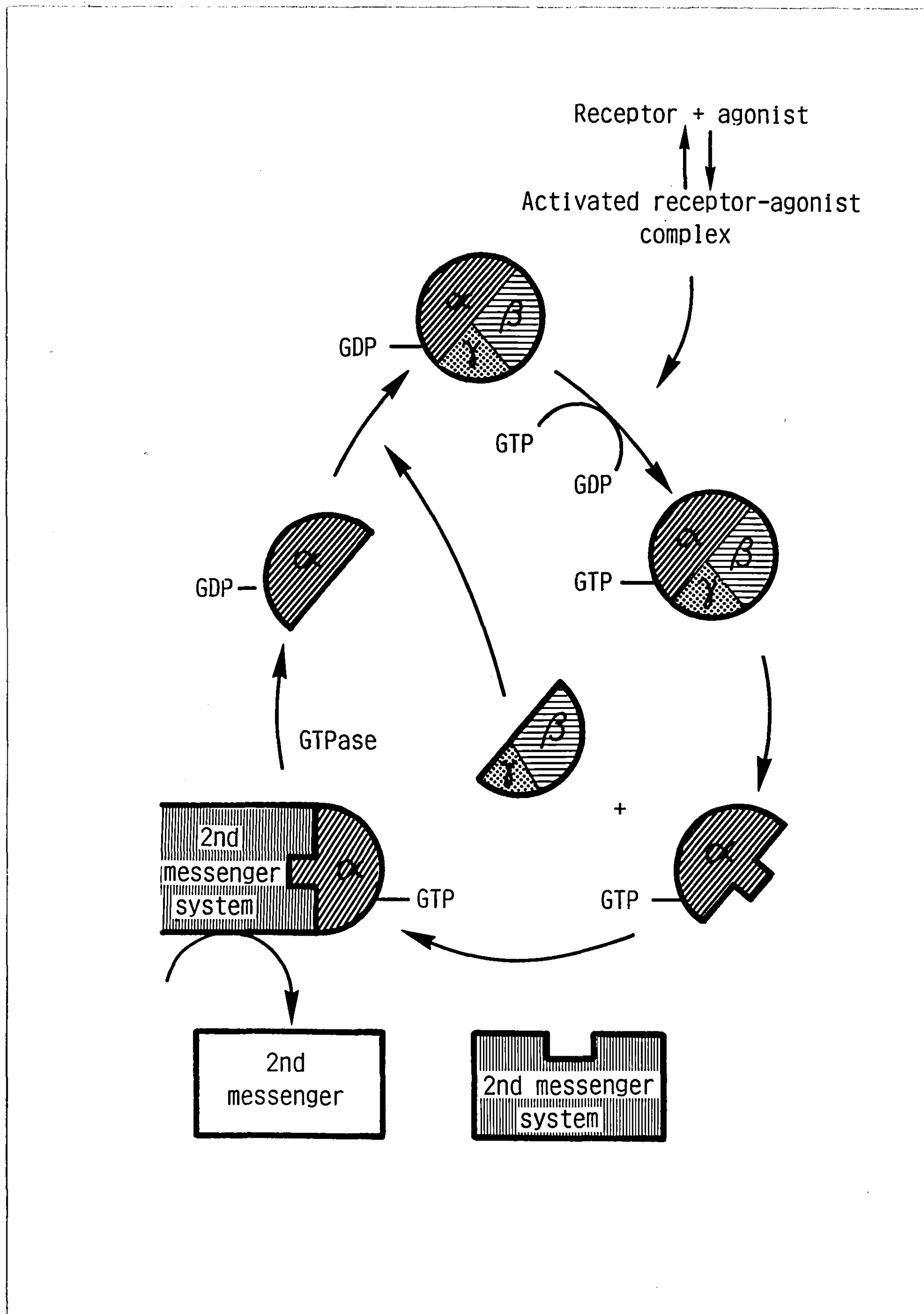
molecular masses of 36 and 35kDa respectively, which are the products of different genes, and which differ in 10% of their amino-acid sequence (Gao *et al.* 1987; Amatruda *et al.* 1988). Recently, β3 has been cloned from human and bovine retinal cDNA libraries (Levine *et al.* 1990). Apparently ubiquitously expressed, β3 has an Mr of~37kDa, and shares 83 and 81% homology with β1 and β2, respectively (Levine *et al.* 1990).

There are at least 4 forms of the γ-subunit. The first γ-subunit cloned (γ_1) was that from bovine retinal transducin (Hurley *et al.* 1984). This cDNA encodes a protein of Mr 8400, which was shown to be both hydrophilic and acidic. Furthermore, the C-terminus contained a CAAX box (see discussion of βγ structure, above), the putative signal for carboxy-terminal isoprenylation in ras oncogene products (Hurley *et al.* 1984). Since then, three other γ-subunits have been cloned: γ_2 from brain and liver (Gautam *et al.* 1989), and recently γ_3 and γ_4 from mouse and bovine brain (Gautam *et al.* 1990). Immunoblot analysis of the tissue distribution of these γ-subunits suggested that γ_1 was expressed solely in retina, γ_3 only in brain, and that γ_2 was ubiquitously expressed (Gautam *et al.* 1990). This suggests that, as with the α-subunits, some forms of γ-subunits are tissue specific.

Figure 1.2

The cycle of G-protein activation and inactivation.

Reproduced from Milligan, 1988.



G-PROTEIN FUNCTION

A. α-subunit function

The model of G-protein function proposed by Cassel and Selinger (1977) has, with some modification, been found to be generally applicable to the mechanism of action of G-proteins. Figure 1.2 illustrates the general principle. Binding of a ligand to its receptor causes a conformational change in the receptor (see adrenergic receptors, below). This conformational change promotes the dissociation of GDP from the G-protein α-subunit, and the rate of GTP association with the α-subunit increases. In the GTP-bound state, the α-subunit dissociates both from its receptor, and also from its βγ subunits. It is now said to be in an "activated" state, in that it can now interact with its intracellular effector. This activation of the G-protein α-subunit, and subsequent dissociation from its receptor also affects the receptor, which now shows a lowered affinity for its ligand (thereby increasing the "off-rate" of the ligand from the receptor, ie. promoting the release of the ligand from the receptor). The activated G-protein α-subunit now interacts with its effector until the intrinsic GTPase activity cleaves the terminal phosphate from GTP, returning the α-subunit to its inactive state, in which it can now re-associate with both its βγ-subunits, and its receptor. The various sequences of this model will now be discussed. A review of the kinetics of guanine nucleotide binding and hydrolysis is provided by Casey and Gilman (Casey and Gilman, 1988).

1. Guanine-nucleotide exchange

Hormone-receptor induced dissociation of GDP from the α-subunit is a Mg^{2+} dependant event (Brandt and Ross, 1986). The presence of

hormone lowers the concentration of magnesium required for GTP binding - eg. glucagon lowers the Mg^{2+} concentration required for GTPγS activation of Gs from 25mM to 10μM (Iyengar and Birnbaumer, 1982). As the intracellular concentration of Mg^{2+} is around 2mM, this ensures rapid activation of Gs on hormone stimulation. The Mg^{2+} ion is also vital for GTP-induced activation of the α-subunit, and for its GTPase activity. The importance of Mg^{2+} in the conformational change has been assessed by measuring the intrinsic tryptophan fluorescence of Gi and Go α-subunits, (Higashijima *et al.* 1987a). In the absence of Mg^{2+}, binding of GTP produces little change in fluorescence, but fluorescence is greatly enhanced in the presence of Mg^{2+}, and then decays in parallel with the hydrolysis of GTP→GDP (Higashijima *et al.* 1987b).

The rate of spontaneous GDP dissociation from the α-subunit is slow (<0.1/min) (Ferguson *et al.* 1986). This helps to stabilise the αβγ heterotrimer until receptor occupation, which increases the rate of GDP dissociation (Brandt and Ross,1986). The high concentration of GTP in the cell then ensures that the nucleotide binding site is rapidly occupied by GTP. Recent studies on the guanine nucleotide binding properties of purified Gi1, Gi2, and Gi3 have suggested that the rate of GDP release and GTP binding may be different for each α-subunit, eg. Gi2α binds GTPγS and releases GDP more rapidly than Gi1α and Gi3α (Carty *et al.* 1990). The importance of these differences in activation kinetics remains to be assessed.

2. Dissociation of α-subunits

Little is known about the mechanism of GTP-induced dissociation of α-subunit from receptor and βγ subunits. The first observation of subunit dissociation was provided by Howlett and Gilman (Howlett and Gilman, 1980) who noticed that persistent activation of Gs with Gpp(NH)p caused a

Wreggett and De Lean (1984) model some systems (eg. receptors which inhibit adenylyl cyclase) where antagonist binding <u>is</u> affected by the presence of guanine nucleotides. Their analysis suggested that in the presence of guanine nucleotides, antagonist binding may be increased, although they note that this property may be related to the potency of the ligand rather than to intrinsic activity (see De Lean *et al.* 1980).

decrease in the size of Gs. In 1981, Stryer *et al.* showed that this was due to dissociation of the heterotrimer into α- and $\beta\gamma$-subunits, one of which (the α-subunit) still had Gpp(NH)p associated with it (Fung *et al.* 1981). It has been shown that persistent guanine nucleotide activation results in release of the α-subunit from the membrane, implying that physical dissociation can occur, (Milligan *et al.* 1988) and Rodbell has suggested that this released α-subunit could form another intracellular second messenger (Rodbell, 1985), however receptor-generated release of α-subunits has yet to be demonstrated.

3. G-protein modulation of receptor-ligand affinity

One of the first observations of the effects of G-proteins was the demonstration of the ability of guanine nucleotides to modulate receptor affinities for ligands. In particular, it was repeatedly observed that GTP and its analogues caused a reduction in radiolabelled agonist binding (eg. Rodbell *et al.* 1971; Maguire *et al.* 1976; Berrie *et al.* 1979). It became clear that this effect was confined to agonist binding, and not to antagonist binding, as the result of studies on the displacement by agonists of β-adrenoceptor antagonist binding (Maguire *et al.* 1976). De Lean suggested the "ternary complex" model, in which there are two affinity states for the β-adrenoceptor, high and low, which are recognised by agonists, and which can be interconverted by GTP and its analogues (De Lean *et al.* 1980). The presence of GTP or Gpp(NH)p causes the receptor to adopt a configuration of low affinity for its agonist, while the absence of GTP results in a high affinity state, antagonist binding being unaffected by the presence of guanine nucleotides (Wregget and De Lean, 1984). It is now clear that agents such as GTP, Gpp(NH)p, and (in the case of "Gi") pertussis toxin, which cause uncoupling of G-proteins from their receptors (ie. the dissociation of the activated α-subunit) all produce an apparent lowering of

the affinity of the receptor for its agonist, presumably as the result of a conformational change in the receptor caused by α-subunit uncoupling. This lowering of receptor affinity for its agonist would promote dissociation of that agonist, and coupling to a fresh molecule of GDP-liganded G-protein.

Receptor affinity changes occur rapidly enough to allow one receptor to interact with a number of molecules of G-protein, this being one of the main methods of amplification of hormone-generated signals. It has been estimated that one β-adrenergic receptor can activate over six molecules of Gs (Brandt and Ross, 1986) within the timespan from the activation of the first Gs until its de-activation by GTP hydrolysis.

This change in receptor affinity has been used by a number of groups to infer the action of G-proteins on their receptor systems (eg. Rodbell 1980; Kurose *et al.* 1983). One group has observed that G-protein-specific antisera can also cause changes in receptor affinity by uncoupling α-subunits from receptors, and used this as a method of identifying Gi2 as the G-protein coupling the δ-opioid receptor to adenylyl cyclase inhibition (McKenzie and Milligan, 1990).

4. Interaction with effector

This is one of the areas of ignorance in G-protein research. The domain for G-protein-effector interaction is unclear, although the work of Osawa suggests that this may lie in the α-subunit C-terminal region (Osawa *et al.* 1990). Exactly how the α-subunit interacts with its effector is not known. Levitzki has argued that Gs and its effector, adenylyl cyclase are coupled *in vivo* (Arad *et al.* 1984), but this idea has not been widely accepted (see Gilman, 1987).

5. GTP hydrolysis

Bourne and colleagues have frequently suggested that the GTPase activity of G-proteins (and not only the heterotrimeric G-proteins) is a kind of molecular switch, with the "on" position being generated upon binding of GTP, and the "off" position by its hydrolysis (see Bourne *et al.* 1990, for example). GTP hydrolysis, depending on its rate, acts as a "timer", allowing one activated G-protein α-subunit to interact with a number of effector molecules until the timer is switched off by the GTPase activity.

The rate of GTP hydrolysis is comparatively slow. The Km for GTP in the GTPase reaction is 0.3μM (Brandt and Ross, 1985), and the catalytic rate, k_{cat} for GTP is around 2-4/min, (Graziano and Gilman, 1989; Brandt and Ross, 1986) so the lifetime of Gα-GTP is many seconds. This allows the activated α-subunit to interact with a number of effector molecules, providing amplification of receptor-generated signals. It has been estimated that activated Gs can interact with adenylyl cyclase to generate over 500 molecules of cAMP, and as a single receptor can activate a number of molecules of Gs, the true amplification is likely to be greater than this (Higashijima *et al.* 1987b).

As has already been noted, the rate of GDP dissociation from the α-subunit is slow, and the catalytic rate for GTP hydrolysis exceeds this by an order of magnitude, hence the rate limiting step in GTP hydrolysis is GDP release (Ferguson *et al.* 1986). Agonist presence does not affect steady state GTP hydrolysis, and by contrast with the comparatively high concentration of Mg^{2+} (10μM) required for GTP binding, hydrolysis of GTP requires only 20nM Mg^{2+} (Brandt and Ross, 1986). The turnover time for Gs to traverse a complete catalytic cycle is of the order of 0.8 min^{-1} (Brandt and Ross 1986).

There are two general models of how $\beta\gamma$ subunits might inhibit adenylyl cyclase. Firstly, as discussed in the text, they could inhibit adenylyl cyclase directly. Secondly, dissociation of "Gi" after receptor activation could lead to generation of free $\beta\gamma$ subunits which then might associate with the α-subunit of Gs, causing it to return to a resting state in which it is not activating adenylyl cyclase. In this model, "Gi" acts as a reservoir of $\beta\gamma$, which, on receptor stimulation, can buffer the release of Gsα. For a review of this "mass action" theory of adenylyl cyclase inhibition , see Gilman, 1987.

B. βγ-subunit function

The idea that the βγ subunit has a function other than the simple physical one of a membrane anchor for the α-subunit is somewhat contentious. There is evidence that purified βγ-subunits can activate muscarinic-gated K^+ channels in chick embryo heart (Logothetis *et al.* 1988). βγ subunits have also been suggested to stimulate phospholipase A_2 (PLA_2) in rod-outer segments, (Jelsema and Axelrod, 1987) and indeed the effect on K^+ channels may be mediated by PLA_2 (Kim *et al.* 1989). It is the proposed effect of βγ on adenylyl cyclase inhibition that generates the greatest controversy, however.

βγ subunits and inhibition of adenylyl cyclase

The question of the identity of the moiety which is responsible for the inhibition of adenylyl cyclase has become confusing and somewhat vexatious. The group led by Neer and Clapham have consistently challenged the orthodox view that it must be the α-subunit of "Gi" which inhibits adenylyl cyclase. In a recent review (Neer and Clapham, 1990) they muster their evidence. They point out that it has been difficult directly to demonstrate inhibition of adenylyl cyclase by G-protein α-subunits. They also note the results of Katada (Katada *et al.* 1987) who inhibited the catalytic subunit of adenylyl cyclase from bovine brain by addition of purified βγ subunits. This result was attributed to the association of βγ subunits with calmodulin, the Ca^{2+} chelator which can activate bovine brain adenylyl cyclase (Salter *et al.* 1980) and the subsequent removal of this calmodulin activation of cyclase. More recently the cDNAs for the α-subunits of Gi1, 2 ,3 and Go were expressed in *E. coli*, purified, and used

in reconstitution experiments, (Linder *et al.* 1990) where it was shown that, despite activation of Gsα with GTPγS or forskolin, and even at high concentrations of α-subunits, none of the expressed "Giαs" were capable of inhibiting adenylyl cyclase purified from bovine brain, even though they were found to be active in other signalling systems.Linder *et al.* do, however, address the possibility that post-translational modification of "Gi" may be required for its action *in vivo*.

Levitzki has recently proposed an alternative hypothesis to account for the role of βγ subunits in adenylyl cyclase inhibition (Marbach *et al.* 1990) . Much of the work on inhibition of adenylyl cyclase by βγ subunits has required the purification of βγ and α subunits. He points out that in the absence of high salt concentrations the β subunit copurifies with the catalytic subunit of Gpp(NH)p activated adenylyl cyclase, implying that the α and βγ subunits of Gs do not dissociate *in vivo* on activation of the enzyme. He suggests that different G-proteins have different tendencies to dissociate in the presence of GTP or its analogues, and that, in the case of adenylyl cyclase, Gsα and βγ subunits do not dissociate, and the active moiety for stimulation of cyclase is GTP-bound Gsαβγ, while the active agent for inhibition of cyclase is GTP-bound Giαβγ.

The identity of the G-protein moiety responsible for mediating inhibition of adenylyl cyclase is, therefore, not as clear as had first been thought. Nevertheless, it is documented that antibodies directed against the α-subunits of Gi2 are capable of alleviating the receptor-driven inhibition of adenylyl cyclase. Simonds *et al.* (1989a) have achieved this in the human platelet with the adrenergic inhibition of cyclase, and McKenzie and Milligan (1990) have also shown this using the NG108-15 cell line, where cyclase is inhibited by the δ-opioid receptor. It might be argued that in these cases, the lack of inhibition is due to the prevention of βγ subunit release from the uncoupled α-subunit, however recently further evidence

has supported the role of the α-subunit as the mediator of adenylyl cyclase inhibition. Wong *et al.* reported the expression of a mutant form of Gi2α in human embryo kidney cell line 293. This mutant Gi2α was shown to constitutively inhibit adenylyl cyclase (Wong *et al.* 1991), even when expressed at levels where the α-subunit clearly exceeded βγ levels.

It may be that different approaches used to address this question of the identity of the true inhibitor of adenylyl cyclase, and the possible involvement of βγ subunits, are producing results which may not be applicable *in vivo*. It is possible that addition of sufficient βγ will indeed cause inhibition of cyclase, however the relevant question would then be, does the enzyme *in vivo* ever see such levels of βγ ? It has been shown in the S49 murine lymphoma cell, where cyclase is inhibited by somatostatin, that added βγ subunits were much less effective than somatostatin in inhibiting isoproterenol stimulation of the β-adrenergic receptor, although they found that βγ was a more potent inhibitor of basal cyclase activity than somatostatin (Hildebrandt and Kohnken, 1990). It was proposed that hormonal and βγ inhibition of cyclase occur by different mechanisms, and that α_i is the primary mediator of hormone inhibition of the enzyme.

It is worth noting that there is a strong theoretical argument against the βγ subunit being solely responsible for cyclase inhibition. If one believes that it is the diversity of the α-subunits that accounts for the fidelity of coupling of G-proteins to their receptors, then it seems unlikely that the βγ subunits, which are even more homologous in sequence than the α-subunits, could confer the specificity of coupling which is apparent *in vivo*. Stimulation of any receptor would then result in release of βγ subunits which could inhibit cyclase.

Use of Anti-Peptide Antisera to detect and modify G-protein function

For many years, the only methods of G-protein detection were indirect. Hormonal simulation or inhibition of adenylyl cyclase activity could be shown to be dependant on the presence of GTP or its analogues. Alteration in receptor affinity by guanine nucleotides could be detected by radioligand binding studies. But these methods could not identify the G-proteins responsible. The use of the bacterial toxins cholera and pertussis toxin could only divide G-proteins into two broad classes, both of which expand with the discovery of ever more cDNAs encoding G-protein α-subunits. To date, the most effective and specific method for detection of G-proteins is the use of immunological probes. These antibodies, raised against various parts of various G-protein components have allowed assesment of G-protein distribution, detection of similarities and differences between members of the G-protein family and, finally, they have been used recently to probe specificity of G-protein function. For a recent review, see Spiegel (Spiegel, 1990).

In 1984 Hamm and Bownds raised an antibody against frog rod outer segments, which bound to transducin. This antibody blocked light-activation of GDP/GTP exchange and cGMP phosphodiesterase activity (Hamm and Bownds, 1984). They were later able to show, by use of tryptic cleavage, that the antibody recognised an epitope near the C-terminus of Tdα, (Deretic and Hamm, 1987) and thus provided evidence that it was the C-terminus of the α-subunit which interacted with receptor.

The antibody raised by Hamm and Bownds was fortuitous in its functional effect. Nowadays "designer antibodies" can be generated, allowing the targeting of specific G-proteins. Determination of the peptide sequences of Gs and Gi allowed the generation of synthetic peptides

corresponding to parts of G-protein α,β, or γ-subunits, and this provided the means for the immunochemical distinction of new forms of the various subunits. For example, the two distinct forms of transducin were shown to be tissue specific by the use of such antibodies (Lerea *et al.* 1986). In a similar fashion, Mumby *et al.* were able to identify different forms of the β-subunit (Mumby *et al.* 1986). Antipeptide antisera raised against the C-terminus of Tdα detected various forms of Gi (Gi1 and Gi2) in neutrophils and brain (Falloon *et al.* 1986; Goldsmith *et al.* 1987).

The use of specific antipeptide antisera unambiguously to identify the G-protein coupling to specific receptors has already been mentioned. McKenzie and Milligan used antiserum AS7, raised against Tdα, but which also recognises Gi1 and Gi2α, to identify Gi2 as the G-protein coupling the δ-opioid receptor in the neuroblastoma x glioma cell line, NG108-15, to inhibition of adenylyl cyclase. They were aided in this task by the fact that the NG108-15 cell was shown to express only Gi2, and not Gi1, making their antibody specific for this G-protein alone in this cell line (McKenzie and Milligan, 1990). Similarly, Simonds *et al.* used AS7 to show that the adrenergic receptor in the human platelet was coupled to inhibition of adenylyl cyclase via Gi2 (Simonds *et al.* 1989a).

The functions of Go and Gs have also been probed using C-terminal specific antisera. Antibodies raised against the Gsα carboxy terminus were shown to block receptor-mediated activation of Gs and adenylyl cyclase in membranes from S49 cells (Simonds *et al.* 1989b). A number of groups have used antisera against Go to show its interaction with calcium channels. As has been mentioned above, (see G-Protein Heterogeneity) Harris-Warrick *et al.* raised an antibody against Go and injected it into snail neurons, where they found that it prevented dopamine-induced reductions in voltage-dependant Ca^{2+} currents (Harris-Warrick *et al.* 1988). McFadzean *et al.* showed that injection of NG108-15 cells with the antibody

OC1, raised against the C-terminus of Go, prevented noradrenaline-induced reductions in calcium currents (McFadzean *et al.* 1989).

It is clear, therefore, that G-protein-specific antisera provide a powerful tool for investigation of identity, prevalence, and function of G-proteins.

ADRENERGIC RECEPTORS

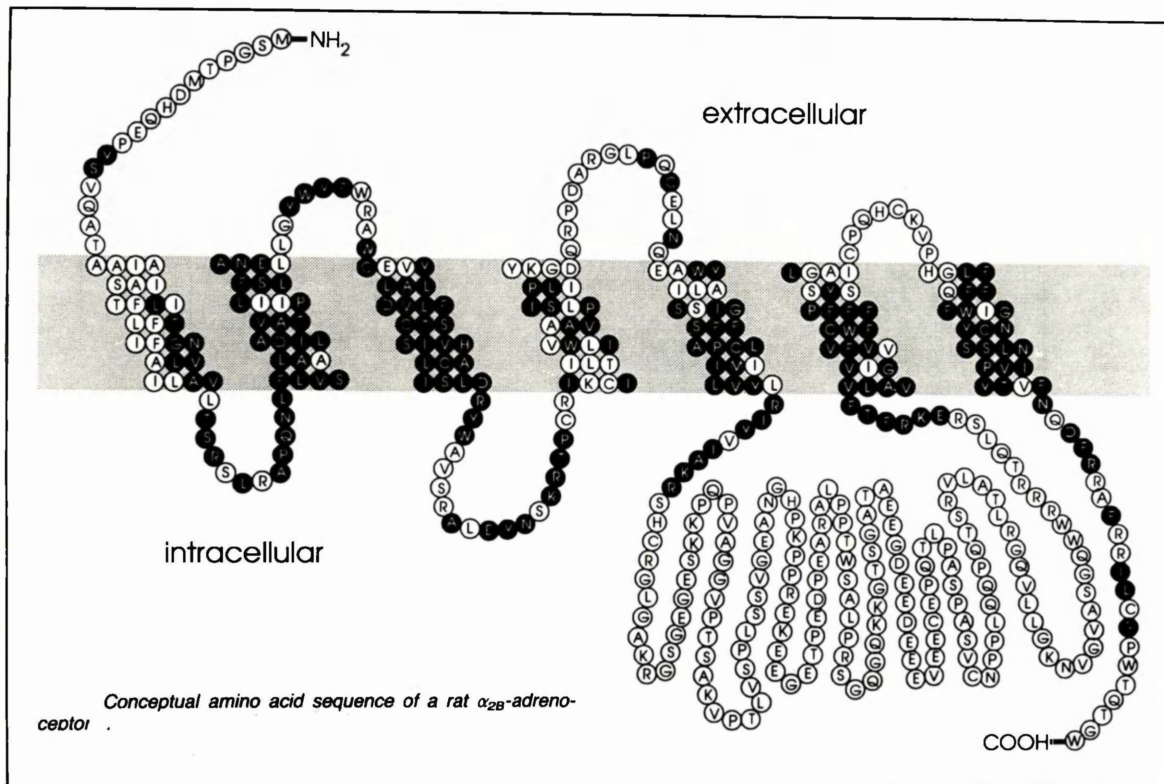
Adrenergic receptors mediate the effects of the catecholamines adrenaline and noradrenaline in the body. As a group they are well characterised, with many of the receptor subtypes purified to homogeneity, and the genes or cDNAs isolated and sequenced. They will be discussed here as archetypes of receptors which interact with heterotrimeric G-proteins.

The structure of the adrenergic receptors seems to fall into a pattern common to a family of receptors which all couple to heterotrimeric G-proteins (for review see O'Dowd *et al.* 1989). The adrenoceptors have molecular weights between 64 and 80 kDa. Each appears to be an integral membrane glycoprotein consisting of a single polypeptide chain, ranging in size from 477 amino acids (for the human β_1 receptor) to 413 amino acids (human β_2). Elucidation of the primary sequences has shown similarities between the adrenergic receptors and bacteriorhodopsin, (the principal membrane protein of the purple bacterium *Halobacterium halobium*) for which structural information has been obtained by electron diffraction and hydropathicity analysis. The striking common feature of the adrenergic receptors, and indeed of all the members of the family of G-protein linked receptors, is the appearance in the primary sequence of seven stretches of ≈20-28 hydrophobic amino acids which seem to represent membrane spanning α-helical regions. The amino acid sequences around these domains presumably represent intra- and extra-cellular loops. Figure 1.3 shows the organisation of G-protein coupled receptors within the plasma membrane.

Figure 1.3

The organisation of G-protein coupled receptors within the plasma membrane

Adapted from Harrison *et al.* 1991.



Conceptual amino acid sequence of a rat α_{2B}-adrenoceptor.

The putative membrane spanning sequences are highly conserved: the α_2-adrenergic receptors so far cloned share ≈75% amino-acid homology in this region, with the least homology being found in those regions thought to lie close to the extracellular surface. This may indicate that the amino acids in this region of the receptors account for their unique pharmacological properties, ie. this region encompasses the ligand-binding pocket. Some evidence for this comes from site-directed mutagenesis of the hamster β_2-adrenergic receptor, converting $Asp^{113} \rightarrow Asn$ on the third transmembrane helix, which resulted in a receptor with markedly attenuated affinities for both adrenergic agonists and antagonists, and which was unable to mediate isoproterenol stimulation of adenylyl cyclase activity (Strader *et al.* 1988). It has also been shown that ligand binding in the rat α_{2B} receptor depends upon a cysteine residue (Cys^{169}) in the second extracellular loop. This cysteine is conserved among all members of the seven-transmembrane-region family and mutation of this cysteine to phenylalanine resulted in a receptor which failed to bind the adrenergic antagonist rauwolscine (Zeng *et al.* 1990). Furthermore, it has been reported that the alkylating agent $[^{125}I]$pBABC, a probe which covalently binds to the hamster β_2-adrenoceptor, binds to either the His^{93} or Ser^{92} on the extracellular face of the second transmembrane helix (Dohlman *et al.* 1988).

The N-terminus lies outside the cell, and has consensus sequences for glycosylation. The β_1, β_2, and α_{2A} adrenergic receptors have been shown to contain oligosaccharides, although the α_{2B} receptor is distinctive amongst adrenergic receptors in that it lacks the N-terminal glycosylation sequence (Zeng *et al.* 1990). The role of N-terminal glycosylation is unclear. It has been shown recently that removal of the glycosylation sites of the hamster β_2-adrenergic receptor had no effects on ligand binding or coupling to G-proteins, however only half of the total number of non-

glycosylated receptors appeared at the cell surface, which suggests that glycosylation may be important in localising the receptor to the membrane surface (Rands *et al.* 1990).

The C-terminus lies within the cytosol, and it has been shown that certain serine and threonine residues in the carboxy tail are substrates for phosphorylation. The β-adrenergic receptor is phosphorylated by a specific kinase (βARK) when occupied by agonist (Benovic *et al.* 1986b). This phosphorylation, which is independant of cAMP levels, results in uncoupling of the β-adrenergic receptor from G_S (Benovic *et al.* 1986a). It has been shown that mutant β-adrenergic receptors truncated at the C-terminus which are exposed to the agonist isoproterenol do not show the rapid (2 minutes) decrease in adenylyl cyclase activity observed in the wild type receptor (Bouvier *et al.* 1988). Thus, the C-terminal region of the adrenergic receptors may be important as part of the mechanism for desensitization or down-regulation of the receptors, the first step of which involves phosphorylation of the agonist-occupied receptor, and subsequent uncoupling of the G-protein linked to that receptor.

The intracellular (cytoplasmic) loops of the receptors have been shown to be vital in coupling of the receptor to its G-protein. Various groups have used mutant receptors to demonstrate that the third cytoplasmic loop, in particular, is critical for receptor-G-protein coupling. Strader *et al.* have constructed deletion mutants of the hamster β_2-adrenergic receptor, removing most of the loop (residues 239-272), or alternatively only the residues at the amino- (222-229) and carboxy- (258-270) terminal portions of the loop. In each case, the mutant receptor showed impaired ability to mediate stimulation of adenylyl cyclase (Strader *et al.* 1987). It is noteworthy that the amino acid sequences in these regions are highly conserved among different members of the adrenergic receptor subfamily. Further evidence that the third cytoplasmic loop couples the

receptor to its G-protein comes from the construction of chimeric receptors where the third loop from the human platelet (α2-C10) receptor was replaced with the corresponding loop from the β_2-adrenergic receptor, and expressed in *Xenopus* oocytes. In this way, a receptor was created which stimulated adenylyl cyclase in response to α_2-adrenergic agonists, which would normally inhibit cyclase (Kobilka *et al.* 1988).

Taken together, a model of receptor function and activation can be suggested from these data. The glycosylation of sites on the N-terminus ensures correct placement of the receptor at the cell surface. A ligand binding pocket is formed from the extracellular amino acid residues of the transmembrane helices in the receptor. The negatively charged residues in helices II and III may form a charge network which comprises both high- and low- affinity states of the receptor and which, in the absence of bound ligand, may be complexed with water, or mono- or di-valent cations. Displacement of these residues upon binding of ligand may result in the movement of one transmembrane helix relative to the others, and this movement is then transmitted to the third cytoplasmic loop, allowing activation of the G-protein coupled to the receptor. Persistent activation of the receptor causes phosphorylation of the C-terminal serine and threonine residues, resulting in receptor uncoupling from G-protein, and ultimately, receptor downregulation.

α ADRENERGIC RECEPTOR PHARMACOLOGY

Subdivision of adrenergic receptors into α and β was first suggested by Ahlquist (for review, see Ahlquist, 1967) in order to account for the differential effects of some sympathomimetic amines, and this was

validated in 1958 when selective blockade of β-adrenoceptors was demonstrated (Moran and Perkins, 1958). The further subdivision of β receptors into β_1 and β_2 types was originally proposed by Furchgott (Furchgott, 1967). Subdivision of the α–adrenergic receptor came about when it was found that the α receptor, which had previously been thought of as a "post-synaptic" receptor, stimulation of which caused a pharmacological effect, could also occur pre-synaptically, this time in the form of an inhibitory receptor, stimulation of which resulted in negative feedback on adrenaline release from the synapse. Thus the postsynaptic receptor became the α_2 receptor, while the presynaptic receptor was called the α_1 receptor. Nowadays the concept of "pre-" or "post-" synaptic forms of the α adrenergic receptor has broken down, and the terms α_1 and α_2 simply denote receptors with different pharmacological characteristics. In terms of function, however, the distinction holds good. The α_1 receptors
phospholipase
couple to stimulation of the membrane associated enzyme phospholipase C, which cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate into the two second messenger molecules inositol(1,4,5,) trisphosphate and diacylglycerol. The α_2 receptors in contrast, couple to inhibition of adenylyl cyclase.

The pharmacology of the different α_2 subtypes has been reviewed by Bylund (Bylund, 1988) and the relationship between pharmacological and genetic subtypes by Harrison *et al.* (1991). All the α_2 receptors have a high affinity (i.e.K_D <20nM) for the antagonists rauwolscine and its diastereoisomer, yohimbine. It is now accepted that the two well characterised subtypes of the α_2 receptor, α_{2A} and α_{2B}, can be distinguished by their differential affinities for the adrenergic agonist oxymetazoline, and for the antagonist prazosin. The α_{2A} subtype has a high affinity for oxymetazoline and a low affinity for prazosin, while the α_{2B} subtype has a high affinity for both oxymetazoline and prazosin (Bylund *et*

al. 1988). A third subtype was also reported by Bylund, this time on OK cells, derived from opossum kidney. This α_{2C} subtype is similar to the α_{2B} in that it has a high affinity for prazosin, however the K_D for yohimbine is similar to that expected for the α_{2A} receptor (Murphy and Bylund, 1988).

To date, several different α adrenoceptors have been cloned (see Table 1.2). The first clone was from the human genome chromosome 10 (α_2-C10) and was isolated using a partial amino-acid sequence from the purified human platelet α_2 adrenoceptor (Kobilka *et al.* 1987). The gene for this receptor was subsequently expressed permanently in CHO cells and bound adrenergic ligands with the expected profile of an α_{2A} receptor (Fraser *et al.* 1989). Identification of the α_{2B} receptor has been less straightforward. Southern blots of human genomic DNA probed with a radiolabelled fragment of the α_2-C10 clone resulted in the appearance of three bands, which suggested that there were at least two other α_2 adrenoceptors in the human genome , probably located on chromosomes 2 and 4 (Kobilka *et al.* 1987). Two more clones, α_2-C4 and RNGα_2, a Rat Non Glycosylated clone, were subsequently isolated by screening human and rat kidney cDNA libraries with cloned DNA or oligonucleotides under conditions of reduced stringency (Regan *et al.* 1988, Zeng *et al.* 1990). The human genomic form of the RNGα_2 receptor was localised to chromosome 2 (α_2-C2) by two groups (Lomasney *et al.* 1990; Weinshank *et al.* 1990). The α_2-C4 and α_2-C2 clones both encoded receptors with the expected pharmacological characteristics of the α_{2B} receptor, however it has been shown that the α_2-C2/RNGα_2 clones do not have the consensus sequence for N-terminal glycosylation, and that mRNA for the RNGα_2 receptor is present in extracts of neonatal rat lung, as well as in rat kidney (Zeng *et al.* 1990). This makes it likely that the α_2-C2/RNGα_2 receptor is the true α_{2B} receptor. It is possible that the α_2-C4 receptor is the α_{2C} subtype suggested by Bylund's studies on the opossum kidney OK cell line.

TABLE 1.2: α-ADRENERGIC RECEPTORS

Subtype	Clone	Reference
α_{1A}	-	Harrison *et al.* 1991
α_{1B}	-	Cotecchia *et al.* 1988
α_{1C}	-	Schwinn *et al.* 1990
α_{2A}	C10	Kobilka *et al.* 1987
α_{2B}	C2	Weinshank *et al.* 1990
α_{2B}	RNG	Zeng *et al.* 1990
α_{2C} (?)	C4	Regan *et al.* 1988
α_{2B} (?)	-	Flordellis *et al.* 1991
α_{2D}	RG20	Lanier *et al.* 1991

Within the last few months a glycosylated receptor with the pharmacological characteristics of an α_{2B} type has been cloned from rat brain. This shows 87% sequence homology with the α2-C4 receptor, but has two potential *N*-glycosylation sites (Flordellis *et al.* 1991). Finally, the existence of a fourth α_2 subtype has been suggested by the isolation of a clone (RG20) from a rat genomic library which has a lower affinity for rauwolscine than the other α_2-receptors, and which has 89% sequence homology to the α2-C10 receptor (Lanier *et al.* 1991). This has been suggested as the presynaptic α_{2D} receptor.

RESEARCH AIMS

Although the adrenergic receptors are very well characterised in terms of their structure and pharmacology, very little is known about their interactions with G-proteins. The aim of the work presented in this thesis was to examine the coupling of adrenergic receptors to G-proteins and, in particular, to address the question of specificity of interaction of the receptor and G-protein.

Two model systems were used. Firstly, the neuroblastoma x glioma cell line, NG108-15, which is a popular model system for the study of signal transduction processes. This cell line, which is discussed in detail in Chapter 3, expresses an adrenergic receptor which is known to mediate inhibition of adenylyl cyclase (Sabol and Nirenberg, 1979). The identity of the G-protein mediating this effect was unknown, and it was decided to attempt this identification.

The second model system utilised in these studies was the Rat 1 fibroblast cell line. These cells do not normally express an adrenergic receptor, but the DNA for the human platelet adrenergic receptor (α2-C10) was transfected into this cell, in order to examine the specificity of coupling of this receptor, in an "unnatural" surrounding, to G-proteins. This work is discussed in Chapter 4.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

All reagents employed were of the highest quality available and were obtained from the following suppliers;

GENERAL REAGENTS

B.R.L., Paisley, Scotland.

Prestained molecular weight markers.

Boehringer (U.K.) Ltd, Lewes, East Sussex

GTPγS, Gpp(NH)P, GDP, GDPβS, App(NH)p, Dithiothreitol, Creatine phosphate, Creatine phosphokinase, Triethanolamine hydrochloride, Tris, Thymidine

F.S.A. Lab. Supplies, Loughborough.

Acrylamide, N,N'-methylenebisacrylamide, Hydrogen peroxide

Koch-Light Lab.Ltd, Haverhill, Suffolk.

DMSO, sodium potassium tartrate

M & B, Dagenham.

Ammonium persulphate

National Diagnostics, Aylesbury, Buckinghamshire

'Ecoscint" scintillation fluid

Pfizer Central Research, Sandwich, Kent.

UK14,304 tartrate (bromoxidine).

Porton Products, Porton Down, Salisbury, Wiltshire.

Pertussis toxin

Reckitt and Coleman, Dansom Lane, Kingston-upon-Hull.

Idazoxan

Sigma Chemical Co., Poole, Dorset.

Cholera toxin, Bovine Serum Albumin, NAD, TEMED, Trypsin, Norit-A charcoal, Ouabain, Theophylline, Arginine hydrochloride, N-ethylmaleimide, o-dianisidine hydrochloride, ATP disodium salt, cAMP sodium salt, Keyhole limpet Haemocyanin, Freund's complete adjuvant, Freund's incomplete adjuvant, Coomassie blue R-250, Bromophenol Blue, Thimerosal, Protein-A Sepharose 4B, Tween 20, Dowex AG50W-X4 (200-400 mesh), Alumina (neutral), Imidazole, (-) Adrenaline bitartrate, (-) Noradrenaline bitartrate, Yohimbine hydrochloride, Oxymetazoline hydrochloride, Prazosin hydrochloride, Forskolin.

Whatman International Ltd. Maidstone.

GF/C Glassfibre filters, 3mm Chromatography paper, No. 1 Filter paper.

All other reagents used were obtained from BDH (Dorset, Poole, England).

TISSUE CULTURE PLASTICWARE

Bibby Science Products Ltd, Stone, Staffordshire.

$75cm^2$ tissue culture flasks.

Costar, 205 Broadway, Cambridge M.A., U.S.A.

Biofreez vials

Elkay Products, Shrewsbury, M.A., U.S.A.

50ml centrifuge tubes

CELL CULTURE MEDIA

Gibco Life Technologies, Paisley, U.K.

Dulbecco's modification of Eagle's medium (10x), Donor Calf Serum, Glutamine (200mM), Sodium Bicarbonate, HAT(50x): Hypoxanthine (0.1mM), Aminopterin (1mM),Thymidine (16mM), Penicillin (100 I.U./ml) and Streptomycin (100mg/ml) (100x).

Imperial Labs, West Portway, Andover, Hants.

Foetal Calf Serum, Normal Calf Serum

RADIOCHEMICALS

Amersham plc, Amersham, Buckinghamshire

Guanosine 5'-[γ-^{32}P] triphosphate, triethylammonium salt (product no. PB144) 10Ci/mmol

[8-^{3}H] Adenosine 3',5'-cyclic phosphate, ammonium salt (product no. TRK304) 24Ci/mmol

Adenosine 5'-[α-^{32}P] triphosphate, triethylammonium salt

(product no. PB10160) 10mCi/ml

New England Nuclear, Boston, Mass., U.S.A

[Adenylate-^{32}P]-Nicotinamide adenine dinucleotide,
di(triethylammonium) salt.
(product no. NEG-023X) 800Ci/mmol
[methyl-^{3}H] Yohimbine
(product no. NET-659) 79.1Ci/mmol

STANDARD BUFFERS

Phosphate Buffered Saline (PBS)

2g potassium chloride
80g sodium chloride
2g potassium dihydrogen orthophosphate
21.6g disodium hydrogen
orthophosphate, heptahydrate
to a final volume of 1 litre, pH7.4.

Tris Buffered Saline (TBS)

500mM sodium chloride,
20mM Tris-HCl , pH 7.5.

Tris Buffered Saline with Tween 20 (TTBS)

500mM sodium chloride
20mM Tris-HCl , pH 7.5
0.05% (v/v) Tween 20.

METHODS

1. CELL CULTURE

Cell growth.

NG108-15 neuroblastoma x glioma hybrid cells were a kind gift from Dr. W. Klee (N.I.H., Bethesda, M.D. USA) and were grown in 75cm^2 tissue culture flasks in 0.0375% (w/v) sodium bicarbonate buffered Dulbecco's modification of Eagle's Medium (DMEM), containing 10% (v/v) foetal calf serum (FCS) which had been heat inactivated at 56°C for 90 minutes. The medium was supplemented with glutamine (2mM), hypoxanthine (0.1mM), aminopterin (1mM) and thymidine (16mM). Both penicillin (100 units/ml) and streptomycin (100mg/ml) were routinely included. This growth medium will henceforth be termed DMEM/10% (v/v) FCS.

Rat 1 fibroblasts were obtained from Dr P. White (Beatson Institute for Cancer Research, Glasgow). Rat 1 fibroblasts and clone 1C cells were grown in 75cm^2 tissue culture flasks in 0.0375% (w/v) sodium bicarbonate buffered Dulbecco's modification of Eagle's medium (DMEM), containing 10%(v/v) newborn calf serum (NCS) which had been heat inactivated at 56°C for 90 minutes. This medium was supplemented with glutamine (2mM), and penicillin (100 units/ml) and streptomycin (100mg/ml) were routinely included. Selection of transfected clones was maintained by addition of geneticin (800μg/ml) to the growth medium.

All cells were grown in a humidified atmosphere of 5% CO_2/ 95% air.

Cell subculture.

Confluent cells (typically 10^7 cells per $75cm^2$ flask) were passaged using a trypsin solution containing 0.1% (w/v) trypsin, 0.67mM EDTA and 10mM glucose in PBS. Growth medium was removed from the cells and 2mls of trypsin solution added. When the cells had been removed from the surface of the flask, trypsinisation was stopped by the addition of two volumes of growth medium. This cell suspension was centrifuged at 800 x g in an MSE Centaur centrifuge for two minutes to pellet the cells. The cell pellet was resuspended in growth medium and plated out as required.

Cell maintenance.

Confluent cells were removed from the surface of the flask by trypsinisation as described above and the cells resuspended in freezing medium, which consisted of 8% (v/v) DMSO in FCS (for NG108-15 cells) or 8% (v/v) DMSO in NCS (for Rat 1 fibroblasts). The suspension was aliquoted into 0.5 ml volumes into Biofreez vials, frozen overnight packed in cotton wool at -80°C, and then transferred to liquid nitrogen for storage.

Cells to be brought up from liquid nitrogen storage were thawed immediately at 37°C, resuspended in 10 mls of appropriate growth medium, and centrifuged at 800 x g in an MSE Centaur centrifuge for two minutes to pellet the cells. The cell pellet was resuspended in growth medium and plated out in a final volume of 10mls in a 75 cm^2 flask.

Toxin treatment of cells.

Confluent cells to be treated with toxin were given a change of medium into medium supplemented with either pertussis toxin (to a final concentration of 25ng/ml) or cholera toxin (to a final concentration of 100ng/ml). A parallel set of control flasks were treated with an equal

volume of vehicle. After 16 hours treatment, cells were harvested as described below.

Cell Harvesting.

When confluent, growth medium was removed from the cell culture flask and 10mls of ice -cold PBS added. Cells were gently washed from the surface of the flask, collected in a 50ml conical tube and centrifuged at 800 x g in a Beckman TJ6 centrifuge for 10 minutes. The resulting cell pellet was washed with ice-cold PBS and re-centrifuged. The final pellet was stored at -80°C until use. Pellets which had been stored for up to one year were found to retain full activity.

2. PRODUCTION OF PLASMA MEMBRANES

Membranes were produced according to Koski and Klee (1981). Frozen cell pellets were thawed and suspended in 5 volumes of ice-cold 10mM Tris-HCI., 0.1mM EDTA pH 7.5 and homogenised with 15 strokes of a Potter homogeniser.

The homogenate was centrifuged at 500 x g for 10 minutes in a Beckman L5-50B centrifuge with a Ti50 rotor, to remove unbroken cells and nuclei. Plasma membranes were collected by centrifugation of the supernatant at 48,000 x g for 10 minutes, washed in 10 volumes of the same buffer and after a second centrifugation, were resuspended in the same buffer to a final protein concentration of between 1-4 mg/ml, aliquoted, and stored at -80°C until required.

Protein Determination.

The method used is based on that of Lowry (Lowry *et al*, 1951).

Stock Solutions;

A) 2% (w/v) sodium carbonate in 0.1M sodium hydroxide.

B) 1% (w/v) copper sulphate.

C) 2% (w/v) sodium potassium tartrate.

Just prior to use, the stock solutions were mixed in the following ratio, A:B:C, 100:1:1, to produce solution D.

Protein standards were prepared using a 1mg/ml bovine serum albumin fraction 5, and a standard curve constructed for a maximum of 30μg of protein per sample. Unknowns were assayed in 2, 4 and 8μl volumes in duplicate. 1ml of solution D was added to each sample, mixed and left to stand for 10 minutes. 100ml of Folin and Ciocalteau reagent diluted 1:1 with H_2O was added to each sample, mixed and allowed to stand for a further 20 minutes. The absorbance of light by each sample was assessed spectrophotometrically at 750nm in an LKB Ultrospec 2 spectrophotometer.

3. ANTIBODY PRODUCTION.

All antisera used were generated against synthetic peptides, essentially as described by Goldsmith and colleagues, (Goldsmith *et al.* 1987). Synthetic peptides were obtained from Dr. C.G. Unson, the Rockerfeller University, New York, U.S.A. with the exception of the peptide NLKLEDGISAAKDVK, which was synthesised by Dr. A. I. Magee, N.I.M.R., Mill Hill, London, and the peptide KNNLKECGLY which was obtained from Biomac Ltd., Glasgow, U.K.

3mg of the appropriate peptide and 10mg of keyhole limpet haemocyanin were dissolved slowly in 1ml of 0.1M phosphate buffer pH 7·0. 0·5ml of 21mM glutaraldehyde (also in 0·1M phosphate buffer, pH 7·0) was then added dropwise with stirring and the combined 1·5ml incubated overnight at room temperature.

The 1·5ml solution was mixed with an equal volume of complete Freund's adjuvant and briefly sonicated with a Branson 'soniprobe' (Type 7532B). 1ml aliquots of the resulting emulsion were injected in multiple subcutaneous sites in New Zealand white rabbits. Four weeks later each animal received a booster immunization with material identically prepared, except one half as much keyhole limpet haemocyanin and peptide were injected in incomplete Freund's adjuvant.

Bleeds were performed monthly with approximately 15ml taken from the ear vein and collected into a glass container. Blood was left to clot overnight at 4°C and the plasma removed and centrifuged at 1000 x g in a Beckman TJ 6 centrifuge for 10 minutes to pellet any remnants of the clot. The supernatants thus produced were aliquoted into 250ml volumes and stored at -80°C until use.

A range of different antisera were produced in the manner described, as summarised in Table 2.1 ;

Table 2.1: Anti G-protein antisera

Antiserum	Peptide Used	G-protein sequence	Antiserum Identifies
AS7	KENLKDCGLF	Tdα 341-350	Td, Gi1, Gi2
OC1	ANNLRYCGLY	Goα 345-354	Go
IM1	NLKEDGISAAKDVK	Goα 22-35	Go
I3B	KNNLKECGLY	Gi3α 345-354	Gi3
CS1	RMHLRQYELL	Gsα 385-394	Gs
I1C	LDRIAQPNYI	Gi1α 159-168	Gi1
BN1	MSELDQLRQE	Gβ 1-10	β1, β2

ANTIBODY PURIFICATION

Crude antisera were chromatographed on a 1·5cm x 2cm column of protein-A-sepharose 4B. A 5ml volume of antiserum in glycine buffer (1.5M Glycine, 3M NaCl, pH 8.9) was added to the column and allowed to equilibrate. The column was washed with glycine buffer until the eluate had an A_{280} of zero and was then eluted with 100mM citric acid, pH 4.0 into 2M Tris/HCl , pH 7.5. The eluted IgG fractions were dialysed overnight against 1000 volumes of 10mM Tris-HCl and 0.1mM EDTA pH 7.5, and lyophilised. Just prior to use, samples were reconstituted to the required dilution with the same buffer.

4. GEL ELECTROPHORESIS

Gel electrophoresis was carried out according to the discontinuous system described by Laemmli (1970).

A. RESOLVING GEL PREPARATION

Stock Solutions; (stored at 4°C and filtered through Whatmans No.1 filter paper prior to use)

Solution A	1.5M Tris,0.4% (w/v) SDS, pH 8.8 with HCI .
Solution B	0.5M Tris,0.4% (w/v) SDS, pH 6.8 with HCI..
Solution C	30% (w/v) Acrylamide,0.8% (w/v) N,N'-methylene bisacrylamide.
Solution D	30% (w/v) Acrylamide,0.15% (w/v) N,N'-methylene bisacrylamide.
Solution E	50% Glycerol.
Solution F	10% (w/v) Ammonium persulphate (made daily)
Solution G	TEMED.

Reservoir buffer; 0.025M Tris, 0.192M Glycine, 0.1% (w/v) SDS, pH 7.5.

10% (w/v) Acrylamide/0.25% (w/v) N,N'-methylene -bisacrylamide gels were prepared from stock solutions as follows;-

Solution	Volume (ml)
A	6
C	8
E	1.6
F	0.09
G	0.008

To a final volume of 24ml with H_2O.

The solution was immediately mixed and poured into a LKB gel casting apparatus, which consisted of 180 x 160 mm glass plates with 1.5mm spacers. The gel was layered with 0.1% (w/v) SDS to exclude air, and left to set at room temperature for approximately 2 hours.

12.5% (w/v) Acrylamide/0.0625 % (w/v) N,N'-methylene bisacrylamide gels were prepared from the stock solution as follows;

Solution	Volume (ml)
A	12
D	20
E	4
F	0.160
G	0.015

To a final volume of 36ml with H_2O

The solution was immediately mixed and poured into a Bio-Rad Protean II gel casting apparatus, which consisted of 200 x 200 mm glass plates with 1.5mm spacers. The gel was layered with 0.1% (w/v) SDS to exclude air, and left to set at room temperature for approximately 2 hours.

B. STACKING GEL PREPARATION

Stacking gels were prepared from the stock solutions as follows;-

Solution	Volume (ml)
B	3.75
C	1.5
F	0.150
G	0.008

To a final volume of 15 ml with H_2O.

The solution was mixed, layered on top of the resolving gel and allowed to polymerize around a 15 well teflon plate.

Electrophoresis was performed overnight at either 50V (Bio-Rad system) or 100V (Protean II system).

C. SAMPLE PREPARATION

PROTEIN PRECIPITATION

Samples were prepared for gel electrophoresis by sodium deoxycholate/ trichloroacetic acid precipitation; 6.25µl of 2% (w/v) sodium deoxycholate was added to each sample, followed by 750µl of H_2O, and then 250µl of 24% (w/v) trichloroacetic acid. Samples were centrifuged in a Hettich Mikro Rapid/K centrifuge at 12000 x g for 20 minutes, after which time the supernatants were removed and the pellets dissolved in 20µl of 1M Tris base followed by 20µl of Laemmli buffer, which consisted of 5M Urea, 0.17M SDS, 0.4M DTT, 50mM Tris-HCl pH 8.0, 0.01% Bromophenol Blue.

PROTEIN ALKYLATION

As a means of obtaining better resolution of the pertussis toxin sensitive G-proteins, samples to be run on 12.5% (w/v) Acrylamide 0.0625% (w/v) N,N'-methylenebisacrylamide gels were firstly alkylated by treatment with N-ethylmaleimide (NEM) (Sternweis and Robishaw, 1984).

Samples were centrifuged in a Hettich Mikro Rapid/K at 12000 x g, the supernatants discarded and the pellets resuspended in 20µl of 10mM Tris-HCl , 1mM EDTA pH 7.5. 10µl of 5% (w/v) SDS, 50mM DTT was added to each sample, mixed, and placed in a boiling water bath for 15 minutes. Samples were then cooled to hand temperature and 10µl of 100mM NEM added. After 15 minutes at room temperature 20µl of Laemmli buffer was added to each sample, leaving the samples ready for gel electrophoresis.

D. GEL PROTEIN STAINING

After electrophoresis, the gel was placed in a tray on a stirring table and covered in stain solution which consisted of 0.1% (w/v) Coomassie blue in 50% (v/v) H_2O, 40 % (v/v) Methanol, 10% (v/v) Glacial Acetic acid for 1 hour. The stain solution was discarded and the gel soaked in destain solution (identical to stain solution, but lacking coomassie blue) until excess stain had been removed to leave a clear background, and proteins were apparent on the gel as discrete bands.

E. AUTORADIOGRAPHY

Gels to be autoradiographed were firstly stained for protein with coomassie blue as described above, and dried down onto Whatman 3mm chromatography paper at 80°C for 1 hour under vacuum. The dried gel was

placed next to KODAK X-OMAT X-ray film in a cassette with intensification screens for up to 1 week at -80 °C. Films were developed by hand using Kodak LX 24 developer and FX 40 fixer.

5. WESTERN BLOTTING

Proteins were separated under appropriate resolving conditions on SDS/polyacrylamide gels overnight at 50V. The proteins were transferred to a nitrocellulose sheet (Schleicher and Schuell) for 2 hours at 1.5mA in an LKB transblot apparatus (Towbin *et al.* 1979) with blotting buffer which consisted of 0.192M Glycine, 25mM Tris, 20% (v/v) methanol. The sheet was then "blocked" for 2 hours in 5% (w/v) gelatin in TBS, after which time gelatin was washed off with distilled H_2O, and the nitrocellulose sheet incubated overnight at 30 °C with the appropriate dilution of antiserum in 1% (w/v) gelatin in TBS. Next day, the antiserum was removed and the sheet subjected to a series of washes in distilled H_2O, TTBS, and TBS for 10 minutes each, after which the blot was incubated with a second antibody (peroxidase conjugated goat anti-rabbit IgG) for 2 hours at room temperature. The second antibody was then removed and the sheet subjected to the series of washes in distilled H_2O, TTBS and TBS as before. The blot was then developed in 40ml of 10mM Tris pH 7.5 with 0.025% (w/v) 0-dianisidine as substrate. Development of the blot was begun by addition of 10μl of hydrogen peroxide, and stopped by immersion of the blot in sodium azide (1% w/v).

Both first and second antibodies could be reused up to four times, and were stored at 4 °C using 0.004% (w/v) thimerosal as an anti-bacterial agent.

6. ADP-RIBOSYLATION

Membranes to be ADP-ribosylated were diluted in 10mM Tris-HCI , 0.1mM EDTA pH 7.5, to a protein concentration of between 1 to 3 mg/ml. 20µl aliquots were assayed in a final volume of 50µl containing the following;-

250mM potassium phosphate buffer, pH 7.0
3mM [^{32}P] NAD (~4x10^6 c.p.m.)
20mM Thymidine
1mM ATP pH 7.5
100mM GTP pH 7.5
20mM Arginine/HCI

The appropriate toxin was added at a final concentration of 10mg/ml (pertussis toxin) or 50mg/ml(cholera toxin). Both toxins were activated prior to use by preincubating with an equal volume of 100mM DTT for 1 hour at room temperature.

The ribosylation assay was initiated by addition of membranes and transferral of tubes to a 37°C water bath. Assays proceeded for 1- 2 hours and were terminated by removal to ice followed by sodium deoxycholate/ trichloroacetic acid precipitation as detailed above. Samples were then resolved under appropriate SDS/polyacrylamide gel electrophoresis.

7. GTPASE ASSAY

The assay monitors the release of ^{32}Pi from γ[^{32}P]GTP and is essentially performed as by Koski and Klee (Koski and Klee, 1981), which is a modification of the method described by Cassel and Selinger except that the concentration of 5' adenylyl-imidodiphosphate (App(NH)p) was lowered to 0.1mM (Cassel and Selinger, 1976).

The assay system contained 0.5mM γ[^{32}P]GTP (approx. 50,000 c.p.m.), .1mM App(NH)p, 1mM ATP, 1mM ouabain, 10mM creatine phosphate, 5 units creatine phosphokinase, 100mM sodium chloride, 5mM magnesium chloride, 2mM dithiothreitol, 0.1 mM EDTA, 12.5 mM Tris.HCl and 3 to 10µg membrane protein in a final volume of 100µl at pH 7.5.

Aliquots of the reaction mixture (50µl) were added to tubes on ice containing membrane protein and the appropriate drug. Low affinity hydrolysis of γ[^{32}P]GTP was assessed in the presence of 100mM GTP, blank values were determined by the replacement of membrane protein with buffer. Hydrolysis of γ[^{32}P]GTP at 0°C was negligible.

The reaction was initiated by transferring the tubes to a 37°C water bath. After 20 minutes, the tubes were immersed in an ice bath and 900µl aliquots of 20mM phosphoric acid (pH 2.3) containing 5% (w/v) activated charcoal were then added. After centrifugation for 20 minutes at 12000 x g in an MSE microcentaur centrifuge, radioactivity was measured in 500µl aliquots of the supernatant fluids by Cerenkov counting in a Rackbeta scintillation counter set to the tritium counting channel.

In some experiments membrane protein was preincubated with antibodies which had been purified on a column of protein-A sepharose and reconstituted to the required concentration. Preincubation was at 37°C for 1 hour in the presence of the GTPase assay components excluding γ[^{32}P]GTP

and receptor ligands. After preincubation, $\gamma[^{32}P]$GTP and ligands were added and the assay proceeded as normal.

8. RADIOLIGAND BINDING ASSAY

Binding assays were performed by the rapid filtration method as described by Pert and Snyder, (Pert and Snyder, 1973) in 10mM Tris-HCl, 50mM sucrose, 20mM magnesium chloride pH 7.5, containing 20-150μg of membrane protein and radiolabelled ligand in a final volume of 250μl. Non-specific binding was assessed in parallel tubes containing an appropriate drug. Blank values were determined by replacement of membrane protein with buffer.

The assay was initiated by transferral of tubes to a 30°C water bath for 30 minutes, after which time the tubes were removed to ice and a 200μl volume rapidly filtered through Whatman GF/C glassfibre filters which had been presoaked in assay buffer, followed with three 5ml washes of the filter with ice-cold buffer. Filters were soaked overnight in Ecoscint scintillation fluid prior to counting in a Rackbeta scintillation counter. In some assays, membranes were pre-incubated with an IgG fraction purified from either normal rabbit serum or an anti-G-protein antiserum (as detailed above) for 60 minutes at 30°C prior to addition of radiolabelled ligand. After the pre-incubation period the assay continued as normal. In binding assays designed to determine the rate at which equilibrium binding was achieved, the assay volume was increased to 1000μl, with protein and ligand concentrations altered accordingly. After transferral to a 30°C water bath, aliquots (100μl) were removed and filtered at varying time intervals, filters being then treated as described.

In some instances, data was manipulated according to the method of Scatchard (Scatchard, 1949).

9. ADENYLYL CYCLASE ASSAY

This was essentially the method of Salomon, (Salomon 1979) except that the amount of $\alpha[^{32}P]$ATP was reduced to 1μCi per sample. This assay monitors the production of $[^{32}P]$ cAMP from the substrate $\alpha[^{32}P]$ATP. The cAMP thus produced is separated from unreacted $\alpha[^{32}P]$ATP by a two-step column method.

SAMPLE PREPARATION

Briefly, reaction mixtures of 50μl containing; 5mM creatine phosphate, 100mM NaCl, 100U/ml creatine phosphokinase, 25mM Tris acetate pH7.0, 5mM Mg acetate, 0.5mM ATP pH 7.0, 0.05mM cAMP, 1mM DTT, 0.1mg/ml BSA, 10mM GTP pH 7.0, $\alpha[^{32}P]$ATP ($1x10^6$ c.p.m.), between 5 to 20μg of membrane protein., together with the ligand(s) of interest. Reaction tubes were kept on ice at all times and the reaction started by removal to a 30°C water bath. After 15 mins, the reaction was terminated by removal to ice and the addition of 100μl of stopper solution which was 2% (w/v) SDS, 45mM ATP, 1.3mM 3'5'cAMP. 50μl of [8-^{3}H]3'-5'cAMP (approx. 10,000 c.p.m) was added to each tube prior to boiling for 15 mins. 750μl of water was then added to each sample, and the ^{32}P cAMP content of each tube determined.

PREPARATION OF DOWEX AND ALUMINA COLUMNS

The method used to quantitate the amount of cAMP produced by each sample was identical to that of Salomon (Salomon, 1979) and involves the

separation of cyclic AMP from other nucleotides by Dowex and then alumina chromatography. Dowex H^+ 50x4(200-400) was washed in twice its packed volume with 1M Hydrochloric acid and then with the same volume of water four times. The Dowex was mixed with water to a slurry (1:1 v/v ratio) and then 3mls removed and added to glass wool stoppered columns. The water was allowed to drain out and the columns washed with 2mls of 1M hydrochloric acid and stored at room temperature. Prior to use, the columns were washed with 1ml of 1M hydrochloric acid followed by 20mls of water.

The alumina columns were prepared by the addition of 0.6g of dry neutral alumina to glass wool stoppered columns and the columns washed with 12mls of 1M Imidazole buffer pH 7.3 followed by 15mls of 0.1M Imidazole buffer pH 7.3 and then stored at room temperature. On the day of use, each column was washed with 8mls of 0.1M Imidazole pH 7.3.

SEPARATION OF CAMP ON DOWEX COLUMNS

Prior to sample chromatography, the nucleotide elution profiles for each column were determined. This was performed by applying a mixture of [^{3}H] cAMP and [^{32}P]ATP to the column and determining the elution volume.

Stock [^{3}H]cAMP was diluted with water to give approximately 10,000 c.p.m. in 50µl. [^{32}P]ATP was diluted from stock of 1mCi/ml to give approximately 2000 c.p.m. in 50µl. 50µl of each of the cAMP and ATP solutions were added to 900µl of water and the mixture applied to a dowex column. The ATP and cAMP were eluted from the column by successive washes of the column with 0.5mls of water. Fractions were collected in a vial with 5mls of Ecoscint and radioactivity determined by scintillation counting using a dual label programme. The elution volumes required to elute the cAMP from the Dowex columns were then determined

graphically. Typical recovery from the Dowex columns was always greater than 80%.

The elution volume required to elute the cAMP from the alumina columns was determined as for the Dowex columns except that only [^{3}H]cAMP was used, and the eluting buffer was 0.1M Imidazole. Recoveries were similar to that obtained for the Dowex columns.

DETERMINATION OF CAMP PRODUCED BY MEMBRANE FRACTIONS

Samples (total volume 950µl) were added to prepared Dowex columns and the ATP eluted with 1.8mls of water. 3.5mls of water was then added to the Dowex columns and this eluate allowed to run directly onto the alumina columns. The cAMP fraction was eluted into vials containing 12mls of Ecoscint, with 6mls of 0.1M Imidazole pH7.3. The recovery of cAMP from the columns was routinely greater than 75%, when recovery fell below 60% the columns were discarded and fresh columns prepared. This gave a column life of approximately 6 months.

The cAMP fractions obtained were counted on a dual label scintillation counting programme which automatically corrected for 'spillover' from each channel. The amount of cAMP produced by each sample was calculated by taking into account the recovery from each column, based on the recovery of the [^{3}H]cAMP internal standard. Data was thus calculable in pmoles of cAMP produced per minute per milligram of membrane protein, and the assay was sensitive to approximately 5 pmoles/min/mg.

10. STATISTICAL ANALYSIS

Where appropriate, data were analysed for statistical significance using Student's two-tailed t-test (paired).

CHAPTER 3

COUPLING OF THE ADRENERGIC RECEPTOR IN NG108-15 CELLS TO G-PROTEINS

INTRODUCTION

The neuroblastoma x glioma cell line NG108-15 (also called 108CC15) is a popular model system for the study of signal transduction in neuronal type cells. This cell line was generated by fusion of the 6-thioguanine-resistant mouse neuroblastoma cell line N18TG2 and the bromodeoxyuridine-resistant rat glioma cell line C6BU1 followed by selection with hypoxanthine, aminopterin, thymidine (HAT) medium and cloning (Hamprecht, 1977). These cells exhibit many of the properties usually ascribed to neurons, including excitable membranes, formation of functional synapses, and neurotransmitter synthesis and release (see Hamprecht *et al.* 1985, for review).

In addition, NG108-15 cells express a wide range of receptors, including adrenergic, muscarinic and opioid types, and a number of different second messenger generating systems, including stimulation and inhibition of adenylyl cyclase, and stimulation of phospholipase C. These intracellular effectors are coupled to their receptors by various heterotrimeric G-proteins (Milligan *et al.* 1990), but the designation of which G-protein couples to which receptor is not clear. Neither is the specificity of this coupling well defined; it is not known whether one class of receptor couples to only one G-protein type, or whether there is "promiscuity" of coupling, wherein a receptor couples to whichever G-protein is available.

This chapter describes the results obtained from a study of the coupling of the adrenergic receptor expressed in NG108-15 cells to its G-protein. It has been known since 1975 that noradrenaline caused inhibition of PGE_1 stimulated adenylyl cyclase activity in NG108-15 cells (Traber *et al.* 1975). Sabol and Nirenberg showed that this inhibition could be blocked by the α-adrenergic antagonists phentolamine and

dihydroergotamine, but not by β-adrenergic antagonists such as propranolol, (Sabol and Nirenberg, 1979) suggesting that the receptor involved was of the α-subtype. In addition, they found that inhibition of adenylyl cyclase was GTP dependant. Radioligand binding studies showed that the radiolabelled α_2-adrenergic antagonist [^{3}H]-yohimbine bound with high affinity to a site on membranes from NG108-15 cells, while the α_1 antagonist radioligand, [^{3}H]-prazosin did not (Kahn *et al.* 1982). It was further shown that [^{3}H]-adrenaline binding to NG108-15 membranes was reduced by high concentrations of GTP and Gpp(NH)p, while these agents had no effect on [^{3}H]-yohimbine binding. Kahn *et al.* suggested that NG108-15 cells possessed a receptor of the α_2-adrenergic subtype which was coupled to adenylyl cyclase in a GTP-dependant, inhibitory manner. Their data supported the idea that the receptor occured in two affinity states, high and low, both labelled with the same affinity by [^{3}H]-yohimbine, while [^{3}H]-adrenaline labelled only the high affinity state.

It is clear, therefore, that NG108-15 cells possess an adrenergic receptor of the α_2-subtype which is coupled to inhibition of adenylyl cyclase. The regulation of receptor affinity by GTP and Gpp(NH)p suggests the involvement of a G-protein in this coupling (see Introduction). Klee *et al.* showed that pretreatment of NG108-15 cells with pertussis toxin abolished the inhibition of adenylyl cyclase by adrenaline, hence the G-protein involved is a pertussis toxin substrate (Klee *et al.* 1985). However, it has been demonstrated that there are at least three pertussis toxin substrates expressed in NG108-15 cells (McKenzie and Milligan, 1990), hence the identity of the G-protein responsible for coupling the adrenergic receptor in NG108-15 cells to inhibition of adenylyl cyclase is unclear. It was decided to make use of specific anti-G-protein antibodies to attempt to identify the G-protein which functions as "Gi", the inhibitory G-protein of adenylyl cyclase in NG108-15 cells.

This chapter shows how this identification was achieved. Firstly, a study of the G-protein complement of NG108-15 cells was made, using antibodies against various G-protein α-subunits, and pertussis and cholera toxin catalysed ADP-ribosylation of these subunits to define the G-proteins present. These results confirmed the presence of Gs, Gi2, Gi3, and Go in NG108-15 cells. Secondly, the adrenergic receptor in NG108-15 cells was characterised by radioligand binding, suggesting that the receptor was of the α_{2B}-subtype. It was further shown that agonist displacement of antagonist binding to the receptor was reduced by Gpp(NH)p, a GTP analogue known to uncouple G-proteins from their receptors. Finally, it was shown that pre-incubation of membranes from NG108-15 cells with antibodies raised against the C-terminal sequence of Gi2 had a similar effect to Gpp(NH)p on ligand binding, and that this effect was specific to the antiserum against Gi2, antisera against equivalent regions of Gi3, Go, and Gs being ineffective. These results suggest that the adrenergic receptor in NG108-15 cells interacts directly, and specifically, with Gi2.

RESULTS

1. CHARACTERISATION OF THE G-PROTEIN COMPLEMENT OF NG108-15 CELLS

Immunoblots of membranes from NG108-15 cells using the antisera described in Table 2.1 (Chapter 2) demonstrated the presence of a range of G-protein α-subunits (Figure 3.1). Antiserum OC1, raised against a decapeptide of the C-terminal sequence of Goα, recognised a 39kDa protein in NG108-15 membranes which comigrated with Go from rat brain. Antiserum AS7 recognised a 40 kDa protein in membranes from NG108-15 which comigrated with Gi2 from rat brain and C6BU1 cells. AS7 also identified a 41kDa protein in rat brain which is Gi1, however this peptide was not recognised in NG108-15 membranes or from membranes of C6BU1 cells. Antiserum AS7 was raised against the C-terminal 10 amino-acids of the α-subunit of transducin, and as this sequence is (with a single amino acid difference) common to transducin, Gi1, and Gi2, AS7 recognises all of these α-subunits (Spiegel, 1990). Antiserum I3B, which was raised against the Gi3α C-terminus recognised a single protein in membranes from NG108-15, which had an apparent molecular weight of 41kDa, and which comigrated with Gi3α from C6BU1 cells and rat adipocytes. Antiserum CS1 recognised two proteins in membranes from NG108-15, which comigrated with the two forms of Gs from rat adipocyte membranes. The identities of the G-protein α-subunits from the membranes used as standards in these immunoblots had previously been established in a study by Mitchell *et al.* (Mitchell *et al.* 1989). These immunoblots, therefore, showed that membranes from NG108-15 cells contained the α-subunits of Gs, Go, Gi2, and Gi3. Figure

3.2 demonstrates the expression of the G-protein β-subunit in membranes from NG108-15 cells.

Thiol-activated pertussis toxin-catalysed ADP-ribosylation of membranes from NG108-15 cells in the presence of [^{32}P] NAD$^+$ followed by SDS-PAGE under conditions which allowed resolution of proteins in the 35-45 kDa range, and subsequent autoradiography, showed that pertussis toxin incorporated radiolabel into 3 proteins (Figure 3.3). These proteins had apparent molecular weights of 39, 40 and 41kDa, and were found to comigrate with proteins identified on immunoblots as Goα, Gi2α, and Gi3α, respectively (Figure 3.3). Hence the pertussis toxin substrates from membranes of NG108-15 cells are the α-subunits of Go, Gi2, and Gi3. Treatment of NG108-15 cells with pertussis toxin (50ng/ml for 16 hours) resulted in complete ADP-ribosylation of pertussis toxin substrates, as was confirmed by challenging membranes from pertussis toxin-treated NG108-15 cells with fresh thiol-activated pertussis toxin in the presence of [^{32}P]NAD$^+$ as substrate (Figure 3.4). Membranes from control cells were shown to be [^{32}P]ADP-ribosylated, but membranes from pertussis toxin pretreated cells were not (Figure 3.4).

Thiol-activated cholera toxin-catalysed ADP-ribosylation of membranes from NG108-15 cells in the presence of [^{32}P] NAD$^+$ followed by SDS-PAGE under resolving conditions, and subsequent autoradiography showed that cholera toxin incorporated radioactivity into two proteins of apparent molecular weight 42 and 44 kDa (Figure 3.5). These proteins comigrated with those recognised on immunoblots by the antiserum CS1. Hence the cholera toxin substrates of membranes from NG108-15 cells are two forms of Gsα.

The experimental design of "uncoupling experiments" to investigate which G-protein was coupled to a particular receptor required that membranes from NG108-15 cells be incubated with protein-A Sepharose-

purified anti-G-protein antisera. Protein-A purification of the plasma fraction of rabbit blood results in an antibody fraction free from other plasma proteins which may interfere with antibody binding. It was therefore necessary to establish that protein-A purified antisera recognised the same G-protein α-subunits as crude antiserum. Figures 3.6 and 3.7 show that the antisera OC1, CS1, I3B, and AS7, when purified on Protein-A Sepharose (as detailed in Chapter 2) show the same specificity as the crude antisera, indeed there is some improvement in the signal, as background bands recognised by the crude antisera are not detected by the protein-purified antisera (Figure 3.6).

2. CHARACTERISATION OF THE ADRENERGIC RECEPTOR IN NG108-15 CELLS.

The adrenergic receptor in NG108-15 cells was characterised by use of the specific α_2-adrenergic antagonist radioligand, [^{3}H]-yohimbine (Daiguji *et al.* 1980). Radioligand binding experiments established that membranes from NG108-15 cells bound [^{3}H]-yohimbine rapidly at 30°C, with equilibrium being attained after 5-10 minutes (Figure 3.8). Non-specific binding, as assessed by co-incubation of [^{3}H]-yohimbine with 100μM(-) noradrenaline, reached equilibrium within 1 minute (results not shown). This binding was linear over a range of NG108-15 membrane concentrations from 25-200μg protein (Figure 3.9). All subsequent assays, unless otherwise stated, were performed at 30°C, for 30 minutes, using 100μg of membrane protein.

It should be noted that the slopes of the displacement curves in Figure 3.11 are not simple, with Hill slopes that are not equal to 1. This might suggest that prazosin and oxymetazoline are binding to two forms of a single receptor, or that they are binding to a number of receptors which have similar affinities for yohimbine, but which have different affinities for oxymetazoline and prazosin. This has some implications which are mentioned in the Discussion (page 103)

Saturation binding isotherms indicated that, in the presence of 20mM Mg^{2+}, [^{3}H]-yohimbine bound to a single high affinity site (Figure 3.10a). Scatchard transformation of the results suggested a Kd of 2.3 (± 0.5) nM (mean ± SD, n=4), and a mean B_{max} of 93.6 (± 9.4) fmoles/mg, (Figure 3.10b). Non-specific binding was typically 40-50% of total binding (results not shown).

Bylund *et al.* have shown that the subtypes of the α_2-adrenoceptor can be discriminated pharmacologically on the basis of their affinities for the adrenergic agonist oxymetazoline, and the antagonist, prazosin (Bylund *et al.* 1988). [^{3}H]-yohimbine binding to membranes of NG108-15 cells was displaced by oxymetazoline with a Ki of 24.0 (± 6.9) nM (mean ± SEM, n=3), and by prazosin with a Ki of 33.1 (± 2.5) nM (n=4) (Figure 3.11). Ki was estimated from IC_{50} values corrected for receptor occupancy by the method of Cheng and Prussoff (Cheng and Prussoff, 1973). This high affinity for both prazosin and oxymetazoline is typical of an α_{2B} adrenergic receptor (Bylund 1988).

The effect of the poorly-hydrolysed GTP analogue, Gpp(NH)p, on the α_{2B}-receptor in membranes from NG108-15 cells was examined by displacing [^{3}H]-yohimbine with noradrenaline (Figure 3.12). The presence of the GTP analogue (100μM) caused a shift in the IC_{50} for noradrenaline displacement of [^{3}H]-yohimbine from 0.35 (±0.3)μM to 4.8(±3.2)μM (means ± SEM, n=4), suggesting that the receptor possesses multiple affinity states, which can be modulated by guanine nucleotides (see Chapter 1). Gpp(NH)p altered the apparent Hill slopes (n_H) from 0.52 to 1.03, indicating a decrease in cooperativity, or a shift from a multiple affinity state receptor to a receptor with only one affinity state. This effect of Gpp(NH)p is common to agents which interfere with receptor-G-protein coupling, and was used in the following experiments to examine receptor-G-protein coupling.

Displacement of [^{3}H]-yohimbine binding with a single concentration of noradrenaline in the presence of agents which interfere with receptor-G-protein coupling results in an measured increase in specific [^{3}H]-yohimbine binding - this being due to the reduction in receptor affinity for agonist, while antagonist binding remains unaffected (see Chapter 1 for a discussion of G-protein modulation of receptor affinities). This apparent increase in [^{3}H]-yohimbine binding in the presence of noradrenaline therefore represents a measure of the receptor-G-protein coupling. Uncoupling of the adrenergic receptor from NG108-15 cells by preincubation of membranes from NG108-15 cells with specific anti-G-protein antisera should thus be detected as an increase in [^{3}H]-yohimbine binding.

3. UNCOUPLING OF THE ADRENERGIC RECEPTOR IN NG108-15 CELLS

The rationale of uncoupling experiments using specific anti-G-protein antisera required that membranes from NG108-15 cells be incubated with specific anti-G-protein antisera followed by radioligand binding of [^{3}H]-yohimbine in the presence of a displacing concentration of noradrenaline. It has previously been established that a 60 minute preincubation period at 30°C was sufficient to elicit binding of antisera to G-proteins under these assay conditions (McKenzie and Milligan, 1990, and FR McKenzie PhD thesis, University of Glasgow 1989). Table 3.1 shows that [^{3}H]-yohimbine binding to membranes from NG108-15 cells

was unaffected by preincubation of membranes for up to 60 minutes at 30°C, and Figure 3.13 shows that preincubation of membranes from NG108-15 cells with a protein-A purified IgG fraction from normal rabbit serum or from antiserum AS7, or with 100μM Gpp(NH)p, did not affect total specific [^{3}H]-yohimbine binding.

Figure 3.14 shows the effect of incubation of membranes from NG108-15 cells with antisera raised against specific G-protein α-subunits followed by assessment of specific [^{3}H]-yohimbine binding in the presence of 1μM (-) noradrenaline, a concentration which displaces ≈70% of specific [^{3}H]-yohimbine binding (see Figure 3.12). Incubation with protein-A purified IgG from normal rabbit serum, or from antisera OC1, CS1 or I3B does not affect (-) noradrenaline displacement of [^{3}H]-yohimbine binding. However, incubation with Gpp(NH)p (100μM), or with protein-A purified IgG from antiserum AS7 caused a statistically significant increase in apparent [^{3}H]-yohimbine binding ($P<0.001$, Student's t-test). This effect is likely to be due to the alteration in the affinity state of the receptor which results from uncoupling of the G-protein from the receptor. Antiserum AS7 was raised against a synthetic decapeptide representing the C-terminus of transducin$_\alpha$, which sequence is, with a single amino acid difference, shared by Gi1$_\alpha$ and Gi2$_\alpha$. In NG108-15 cells, AS7 recognises the α-subunit of Gi2 only (Figure 3.1), therefore this uncoupling effect of AS7 must be due to interaction of the antibody with Gi2.

Coincubation of membranes from NG108-15 with protein-A purified AS7 and 100μM Gpp(NH)p (Figure 3.15) produced no greater increase in [^{3}H]-yohimbine binding in the presence of 1μM noradrenaline than incubation with protein-A purified AS7 or Gpp(NH)p alone, suggesting that antiserum AS7 had completely uncoupled the α_{2B} receptor in NG108-15 cells, and that no other G-protein was involved. This idea

was supported by experiments using membranes from pertussis toxin pretreated NG108-15 cells, where incubation with protein-A purified AS7 or 100μM Gpp(NH)p caused no further increase in [^{3}H]-yohimbine binding in the presence of 1μM noradrenaline (Figure 3.16).

Figure 3.1

IMMUNOBLOT ANALYSIS OF MEMBRANES FROM NG108-15 CELLS

Membranes from NG108-15 cells were separated on SDS-PAGE as described in Chapter 2, and western blotted onto nitrocellulose sheets. Western blots were incubated with anti-G-protein antisera as described in Chapter 2. Samples were incubated with 1:200 dilutions of:

A: antiserum OC1 (1; 100 μg NG108-15 membranes, 2; 10 μg rat cerebral cortex membranes)

B: antiserum CS1 (1; 100 μg NG108-15 membranes, 2; 50 μg rat white adipocyte membranes)

C: antiserum I3B (1; 100 μg NG108-15 membranes, 2; 50 μg rat white adipocyte membranes, 3; 100 μg rat glioma C6 BU1 membranes)

D: antiserum AS7 (1; 100 μg NG108-15 membranes, 2; 50 μg rat cerebral cortex membranes, 3; 50 μg rat glioma C6 BU1 membranes).

Primary antisera were detected by incubation with a donkey-anti-rabbit horseradish peroxidase-linked second antiserum, as detailed in Chapter 2. The substrate for the enzyme was o-dianisidine.

Figure 3.1

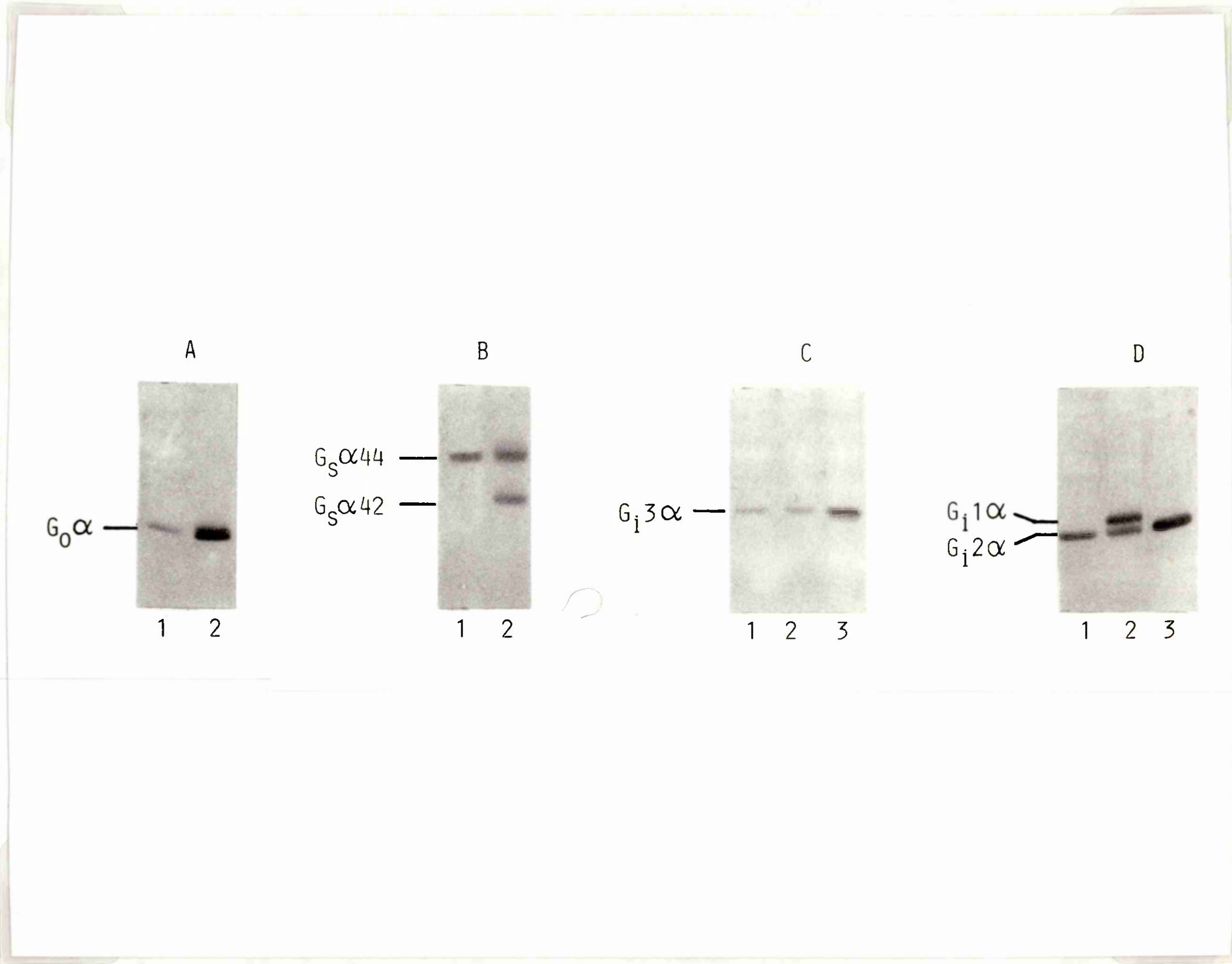


Figure 3.2

IMMUNOBLOT DETECTION OF β-SUBUNIT IN MEMBRANES FROM NG108-15 CELLS.

Membranes from NG108-15 cells (100μg) were separated on SDS-PAGE and western blotted onto nitrocellulose as described in Chapter 2. Western blots were then immunoblotted with antiserum BN1, which detects the G-protein β-subunit. Lanes are membranes from:

A. NG108-15 (100μg)

B. Rat brain (100μg)

Figure 3.2

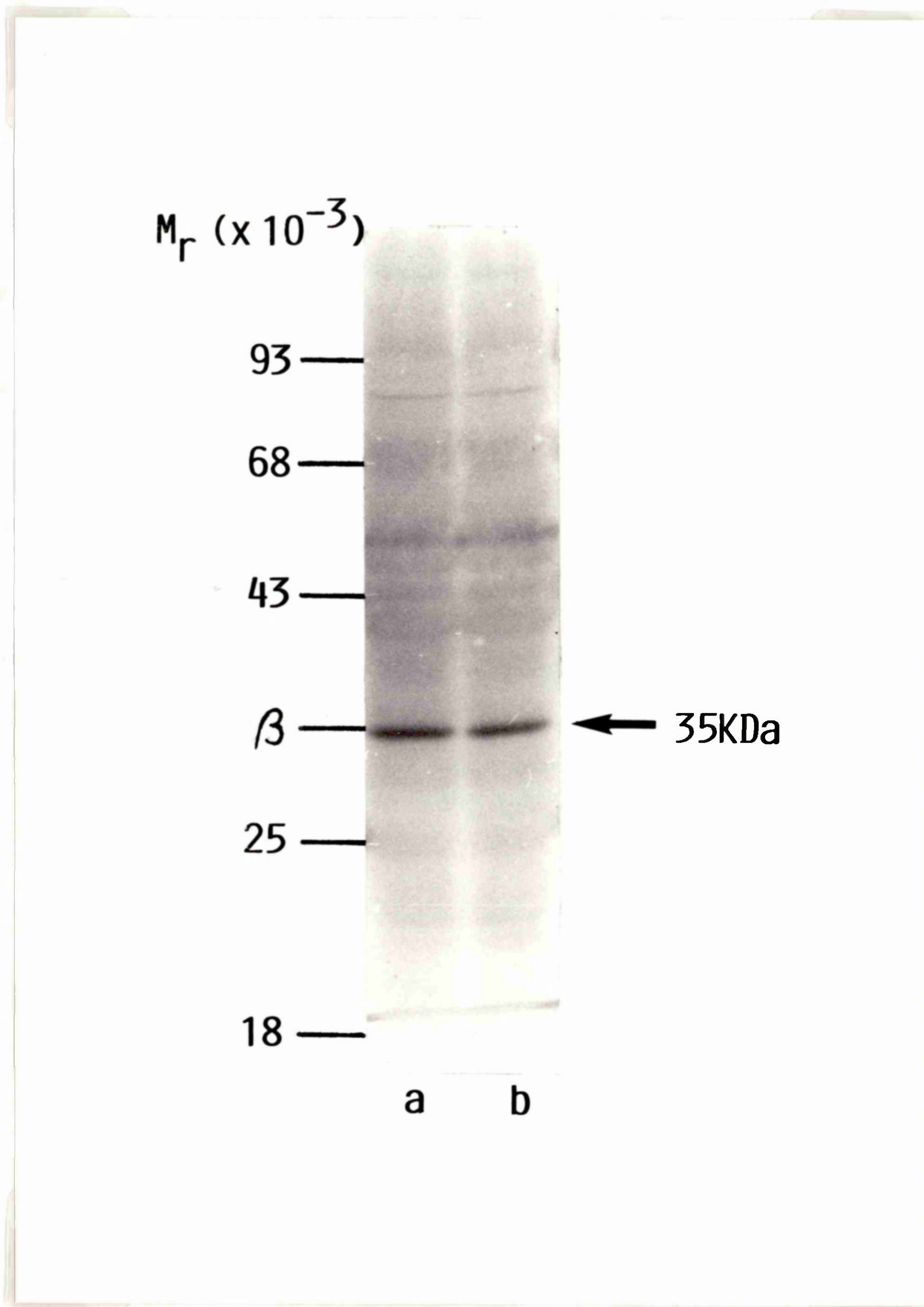


Figure 3.3

PERTUSSIS TOXIN SUBSTRATES IN MEMBRANES FROM NG108-15 CELLS

Membranes from NG108-15 cells (100μg) were ADP-ribosylated by thiol-activated pertussis toxin for 90 minutes at 37°C,using [^{32}P]NAD^{+} as substrate, as detailed in Chapter 2. ADP-ribosylated membranes were separated on SDS-PAGE, and western blotted onto nitrocellulose. The nitrocellulose was immunoblotted with antisera, as indicated below. The immunoblot was developed, dried and subsequently autoradiographed, as described in Chapter 2.

Lane A shows an autoradiograph of the region of the immunoblot encompassing proteins of the 35-45kDa range.

Lanes B-D show the region of the immunoblots encompassing proteins of the 35-45kDa range.

Lane B was immunoblotted with antiserum I3B, which recognises the α-subunit of Gi3.

Lane C was immunoblotted with antiserum AS7, which recognises the α-subunits of Gi2 and Gi1.

Lane D was immunoblotted with antiserum IM1, which recognises the α-subunit of Go.

None of the proteins identified incorporated radioactivity in the absence of activated pertussis toxin.

Figure 3.3

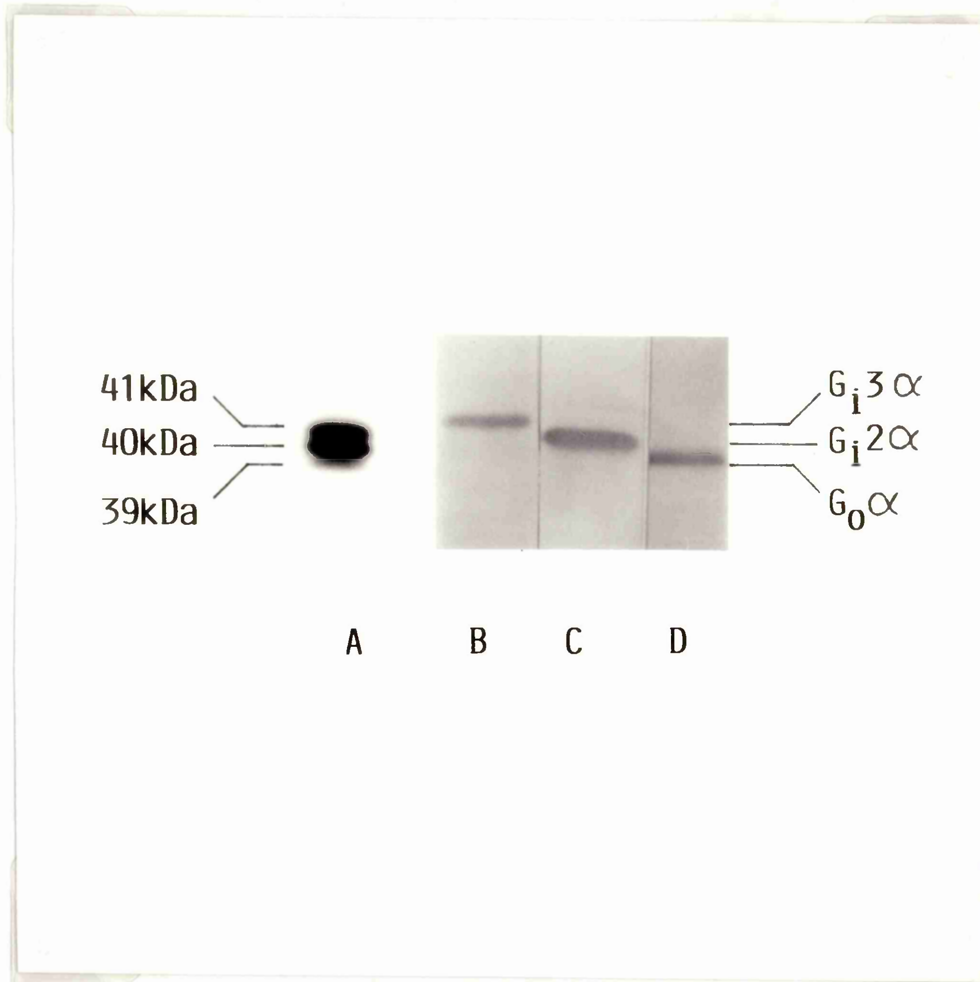


Figure 3.4

PERTUSSIS TOXIN SUBSTRATES IN MEMBRANES FROM PERTUSSIS TOXIN PRETREATED NG108-15 CELLS

NG108-15 cells were grown in the presence of pertussis toxin (50ng/ml) for 16 hours, and membranes prepared as described in Chapter 2. Membranes from control and pertussis toxin-pretreated NG108-15 cells (100μg) were ADP-ribosylated by thiol-activated pertussis toxin for 90 minutes at 37°C, using [^{32}P]NAD^{+} as substrate, as detailed in Chapter 2. Ribosylated membranes were separated on 10% SDS-PAGE, followed by drying of the gel, and subsequent autoradiography. This figure shows an autoradiograph of the region of the gel encompassing proteins of the 35-45kDa range.

Lane 1: Membranes (100μg) from untreated NG108-15 cells

Lane 2: Membranes (100μg) from pertussis toxin-treated NG108-15 cells

None of the proteins identified incorporated radioactivity in the absence of activated pertussis toxin.

Figure 3.4

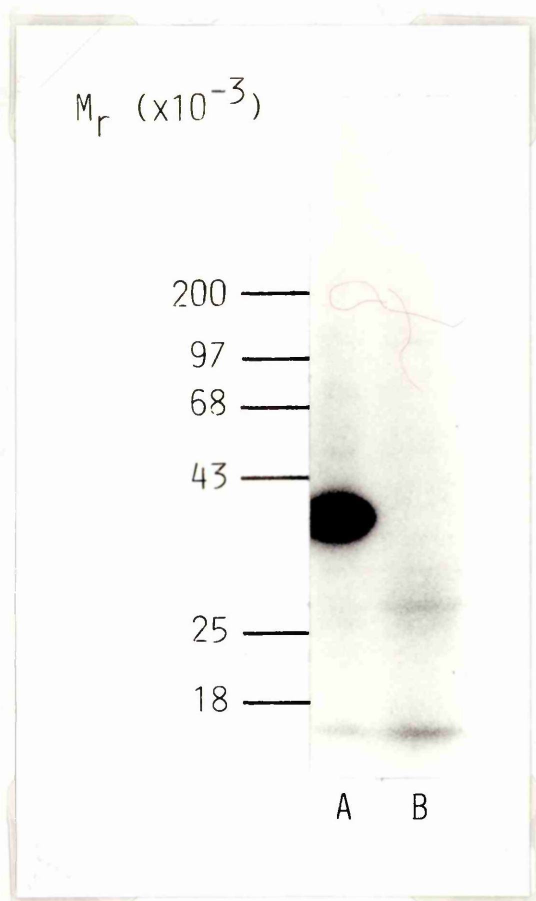


Figure 3.5

CHOLERA TOXIN SUBSTRATES IN MEMBRANES FROM NG108-15 CELLS.

Membranes from NG108-15 cells (100µg) were ADP-ribosylated by thiol-activated cholera toxin for 90 minutes at 37°C, using [^{32}P]NAD^{+} as substrate, as detailed in Chapter 2. ADP-ribosylated membranes were separated on SDS-PAGE, and western blotted onto nitrocellulose. The nitrocellulose was immunoblotted with antiserum CS1, which detects the α-subunit of Gs, as described in Chapter 2. The immunoblot was developed, dried and subsequently autoradiographed. This figure shows:

Lane A: an autoradiograph of the region of the immunoblot encompassing proteins of the 35-55kDa range.

Lane B: the region of the immunoblot encompassing proteins of the 35-55kDa range.

None of the proteins identified incorporated radioactivity in the absence of activated cholera toxin.

Figure 3.5

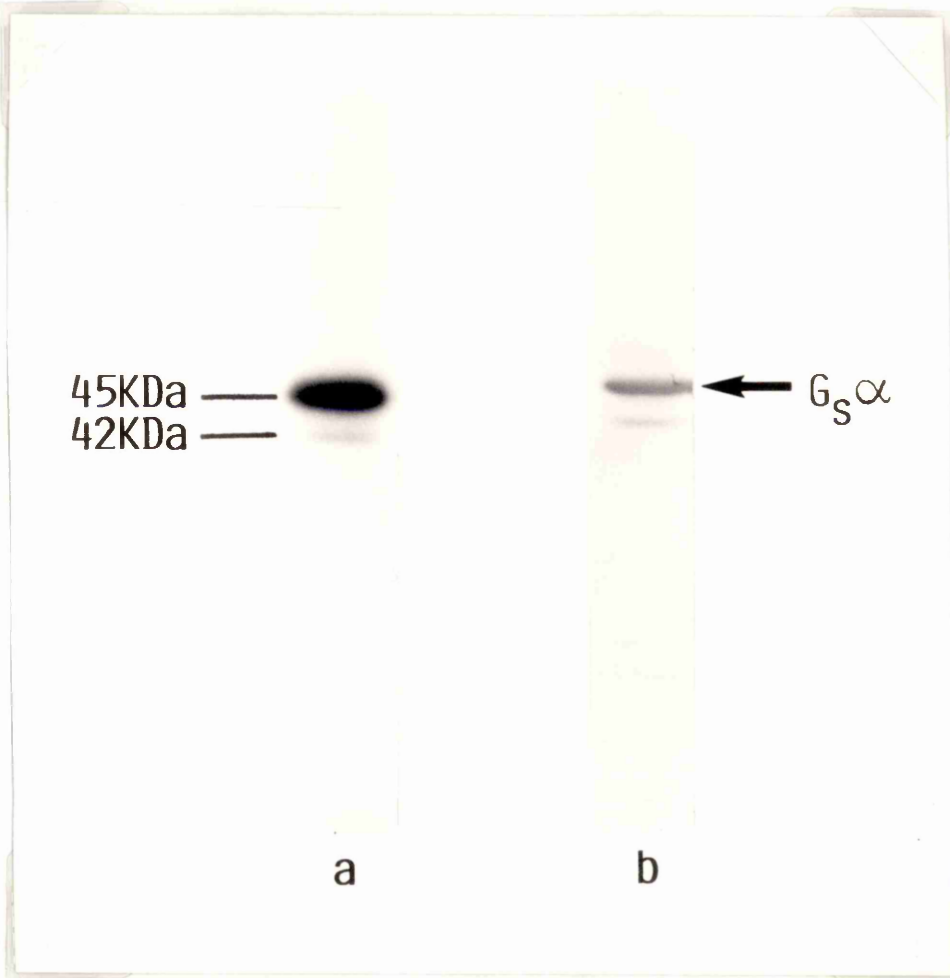


Figure 3.6

SPECIFICITY OF PROTEIN-A PURIFIED ANTISERA

Protein-A purified antisera were prepared as detailed in Chapter 2, and used at a dilution of 1:200 in 1% gelatin in TBS. Crude antisera were used as described in the legend to Figure 3.1. Membranes from NG108-15 cells (10, 50, and 100μg) were separated on SDS-PAGE and western blotted as described in Chapter 2. Western blots were incubated with crude and protein-A purified antisera as illustrated.

Figure 3.6

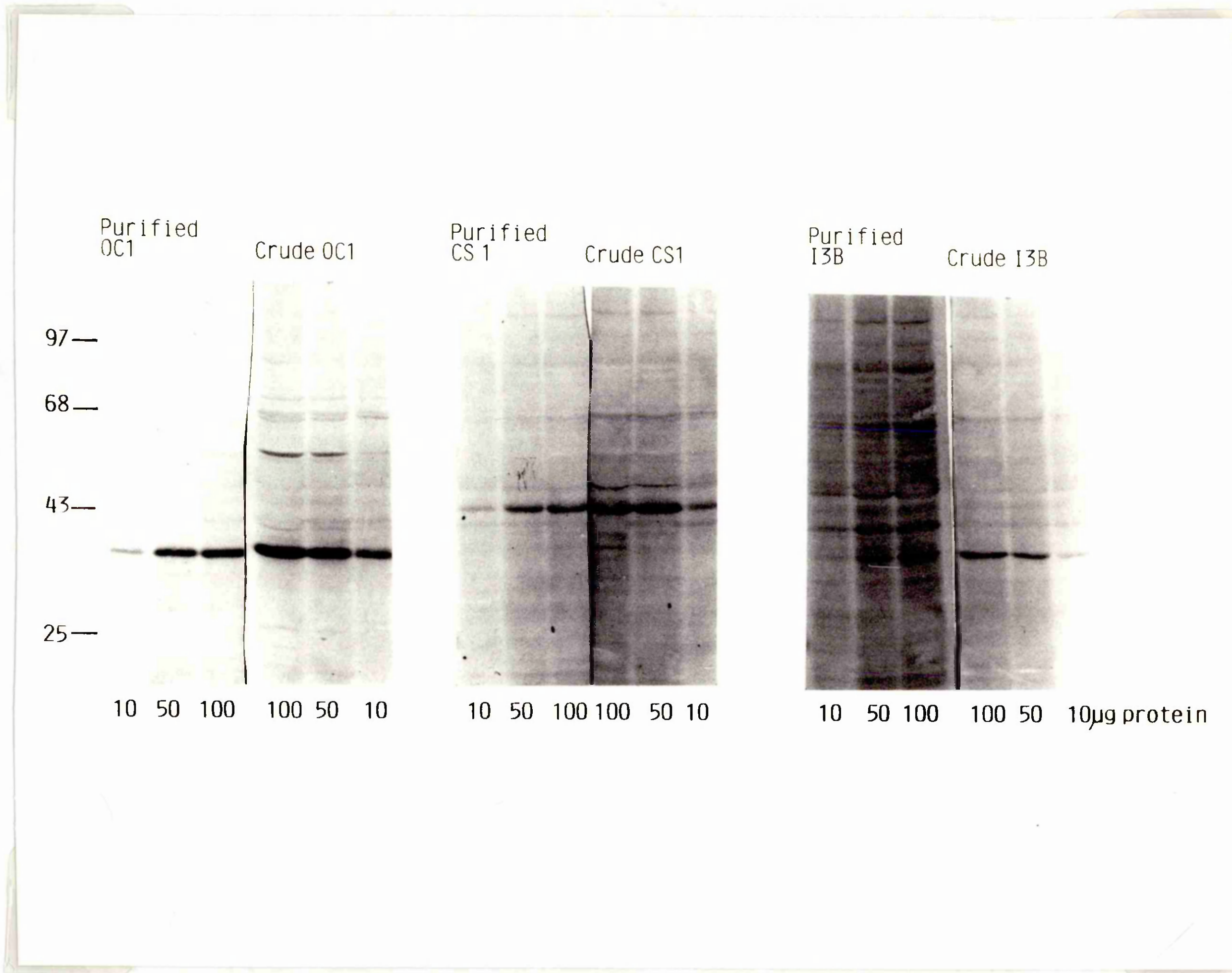


Figure 3.7

SPECIFICITY OF PROTEIN-A PURIFIED ANTISERUM AS7

Protein-A purified antiserum AS7 was prepared as detailed in Chapter 2, and used at a dilution of 1:200 in 1% gelatin in TBS. Crude antiserum was used as described in the legend to Figure 3.1. Membranes from NG108-15 cells (10, 50, and 100μg) were separated on SDS-PAGE and western blotted as described in Chapter 2. Western blots were incubated with crude and protein-A purified antiserum AS7 as illustrated.

Figure 3.7

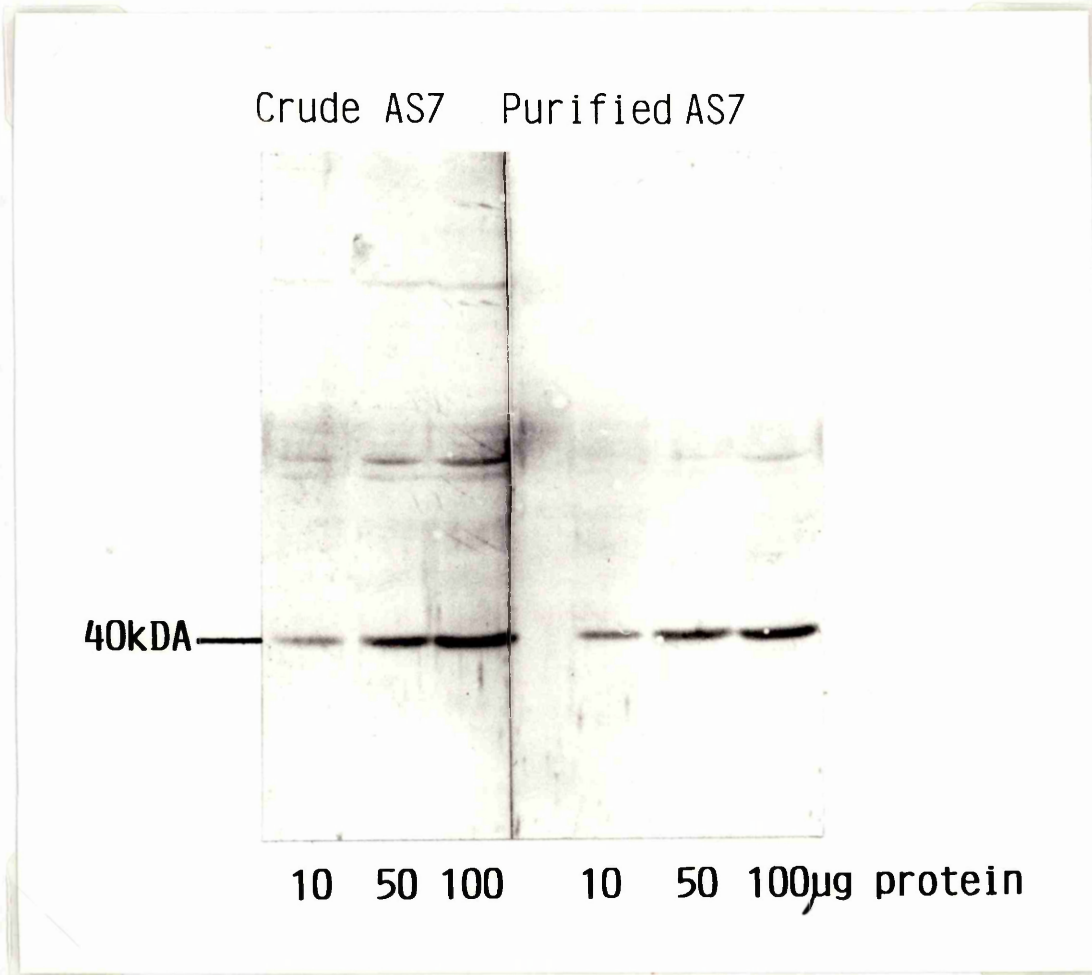


Figure 3.8

TIME COURSE OF ASSOCIATION OF [3H]-YOHIMBINE SPECIFIC BINDING TO MEMBRANES FROM NG108-15 CELLS

Specific [^{3}H]-yohimbine (10nM) binding to membranes (100µg) of NG108-15 cells at 30°C was assessed at the time points shown. Non-specific binding was defined by parallel incubation with (-) noradrenaline (100µM). Points shown are mean (± SD) of triplicate determinations from a single experiment, which was repeated twice using different NG108-15 membrane preparations, with similar results being obtained.

Figure 3.8

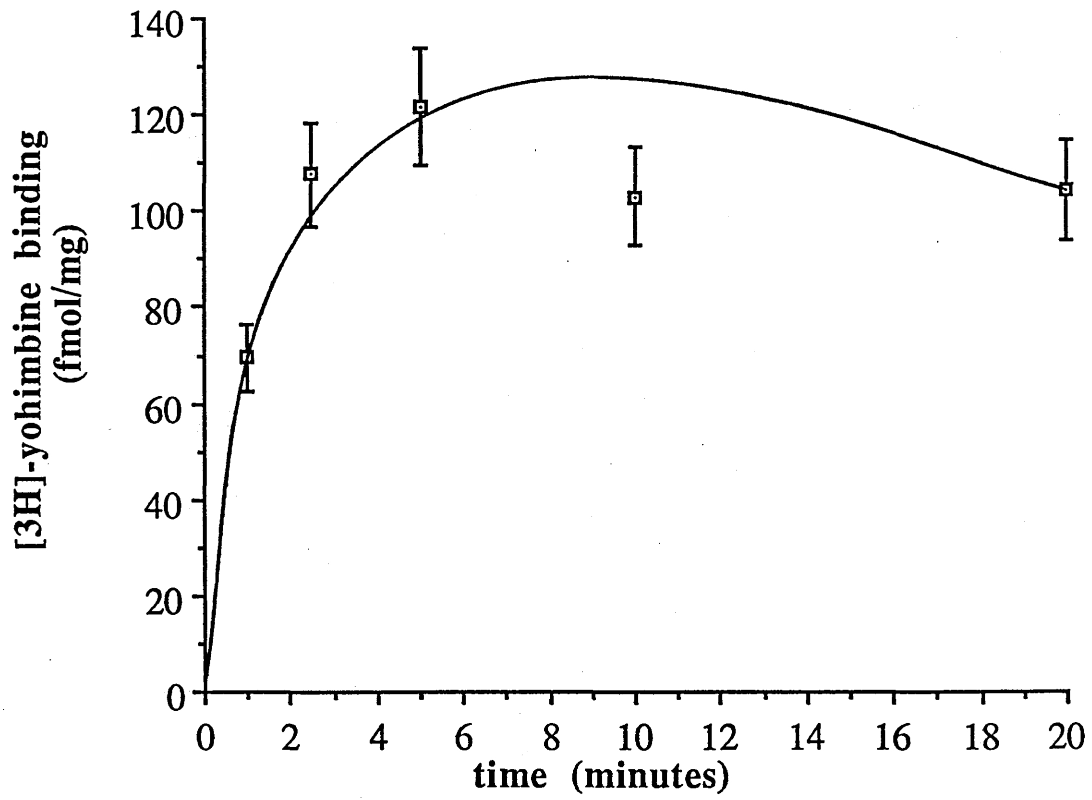


Figure 3.9

SPECIFIC BINDING OF [3H]-YOHIMBINE TO INCREASING AMOUNTS OF MEMBRANES FROM NG108-15 CELLS

Specific [^{3}H]-yohimbine (10nM) binding to increasing amounts of membranes of NG108-15 cells at 30°C was assessed as shown. Non-specific binding was defined by parallel incubation with (-) noradrenaline (100µM). Points shown are mean (± SD) of triplicate determinations from a single experiment, which was repeated twice using different NG108-15 membrane preparations, with similar results being obtained.

Figure 3.9

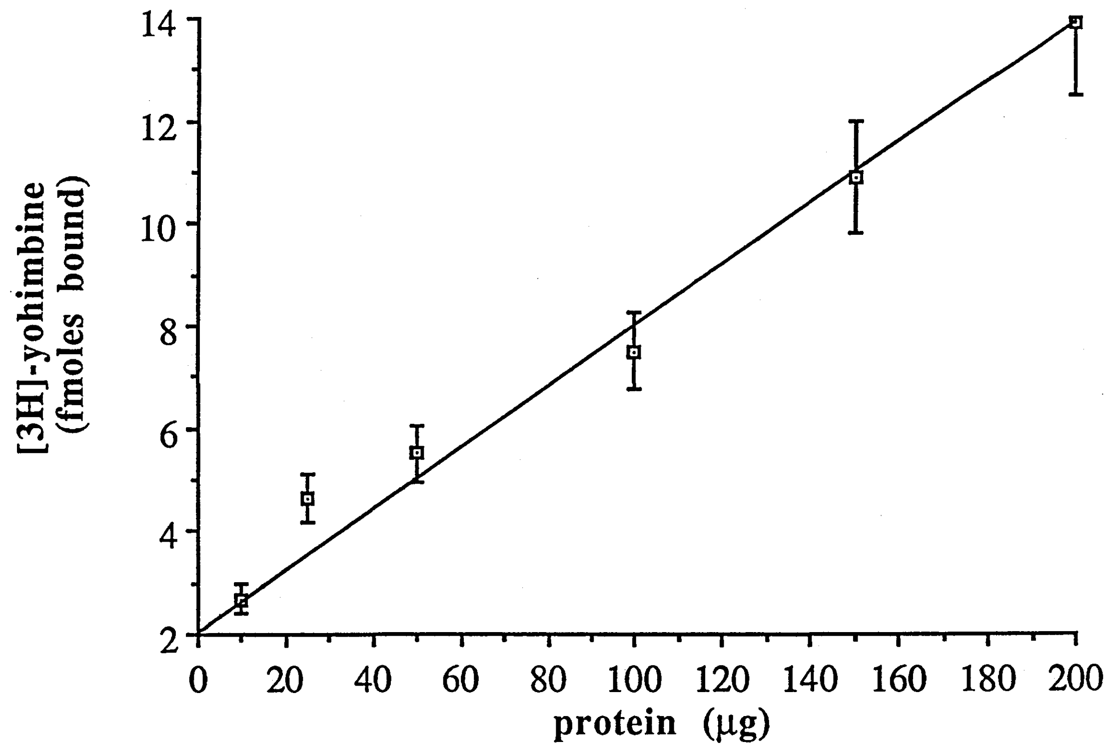


Figure 3.10a

SPECIFIC BINDING OF INCREASING CONCENTRATIONS OF [3H]-YOHIMBINE TO MEMBRANES FROM NG108-15 CELLS

Membranes (100µg) of NG108-15 cells were incubated for 30 minutes at 30°C with various concentrations of [^{3}H]-yohimbine as shown. Non-specific binding was determined in the presence of 100µM (-) noradrenaline, and increased in a linear fashion over the range of [^{3}H]-yohimbine concentrations, being 35-50% of total binding (not shown). Points shown are means (± SEM) from 4 separate experiments using different membrane preparations.

Figure 3.10b

SCATCHARD TRANSFORMATION

Data from Figure 3.10a have been transformed by the method of Scatchard (Scatchard, 1949). Results suggested a Kd of 2.3 (±0.5) nM (mean ± SEM, n=4), and a mean B_{max} of 93.6 (±9.4) fmoles/mg.

Figure 3.10a

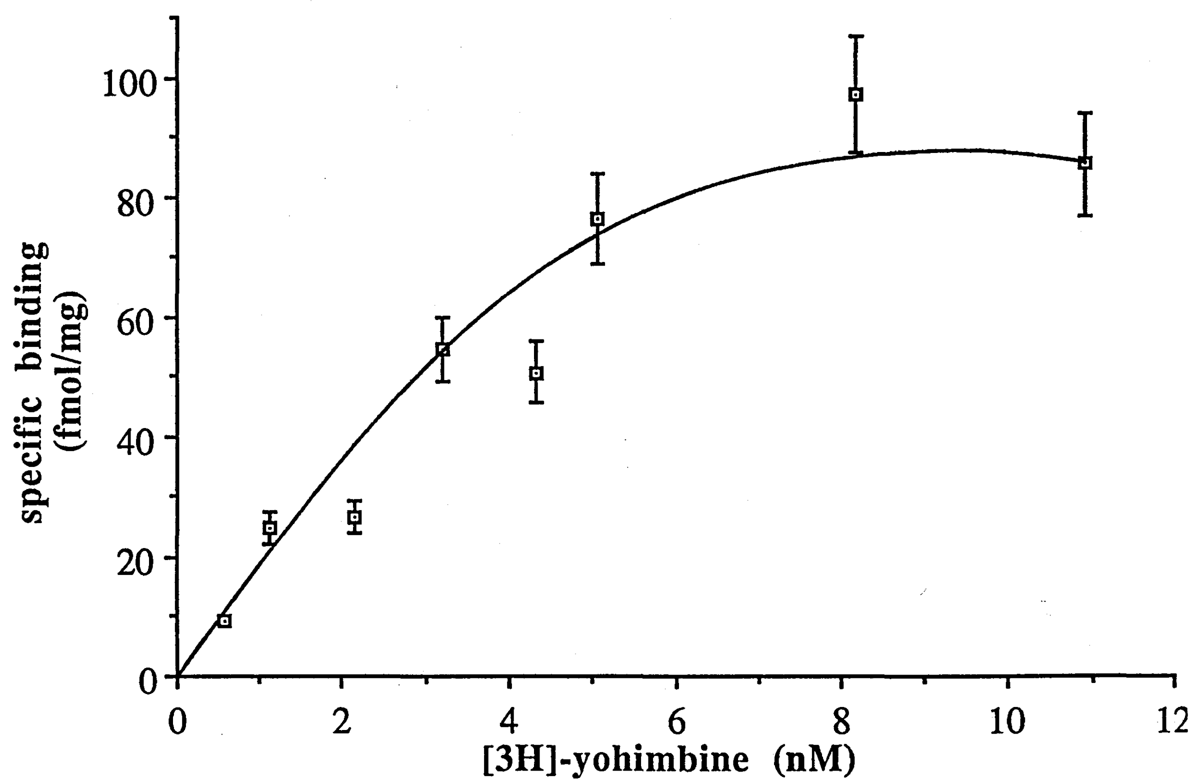


Figure 3.10b

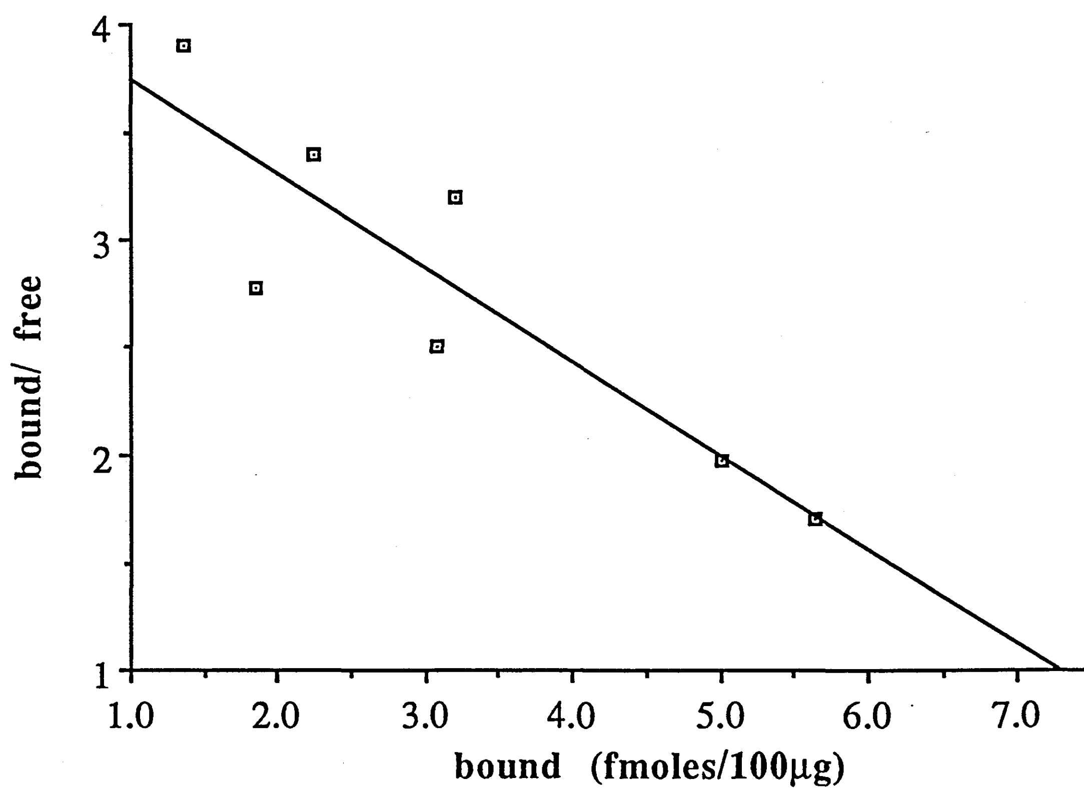


FIGURE 3.11

DISPLACEMENT OF [3H]-YOHIMBINE BINDING TO MEMBRANES FROM NG108-15 CELLS BY OXYMETAZOLINE AND PRAZOSIN

Displacement of [^{3}H]-yohimbine (10nM) to membranes (100µg) of NG108-15 cells by prazosin (◆) and oxymetazoline (□) was assessed at 30°C for 30 minutes. Points shown are means of triplicate determinations (SD has been omitted for clarity, but was less than 10% of the mean in each case) from a single experiment which was repeated three times using different membrane preparations. For this experiment, IC_{50} for oxymetazoline was 0.12µM, and for prazosin, 0.2µM. 100% specific [^{3}H]-yohimbine binding was 88.4 (±4.3) fmoles/mg for prazosin, and 95.4 (±9.4) fmoles/mg for oxymetazoline.

Figure 3.11

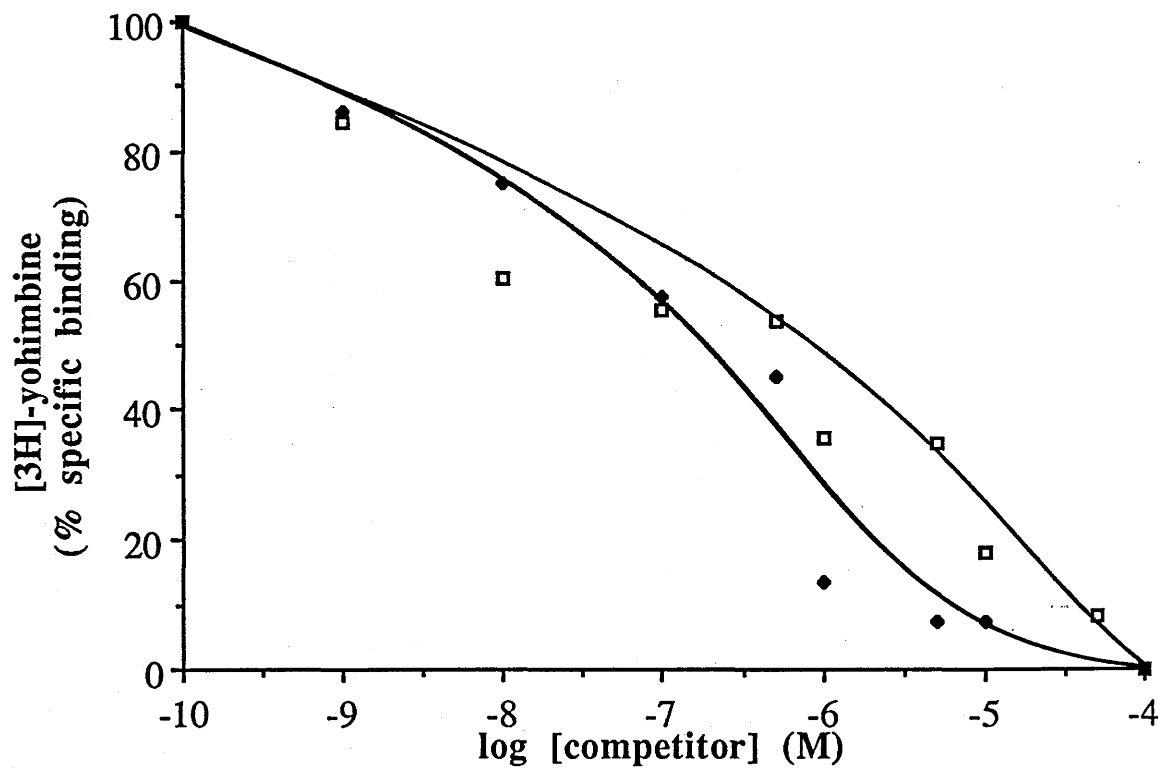


Figure 3.12

EFFECT OF GPP(NH)P ON DISPLACEMENT OF [3H]-YOHIMBINE BINDING BY NORADRENALINE IN MEMBRANES FROM NG108-15 CELLS

Displacement of [^{3}H]-yohimbine (10nM) binding by (-) noradrenaline in membranes from NG108-15 cells (100μg) was measured in the presence (◆) and absence (□) of 100μM Gpp(NH)p at 30°C for 30 minutes. Points shown are means from four experiments using different membrane preparations. SEM has been omitted for clarity, but was less than 10% of the mean in all cases. 100% specific binding was 90.3 (±4.5) fmoles/mg for control, and 95.5 (±7.3) fmoles/mg for gpp(NH)p samples.

Figure 3.12

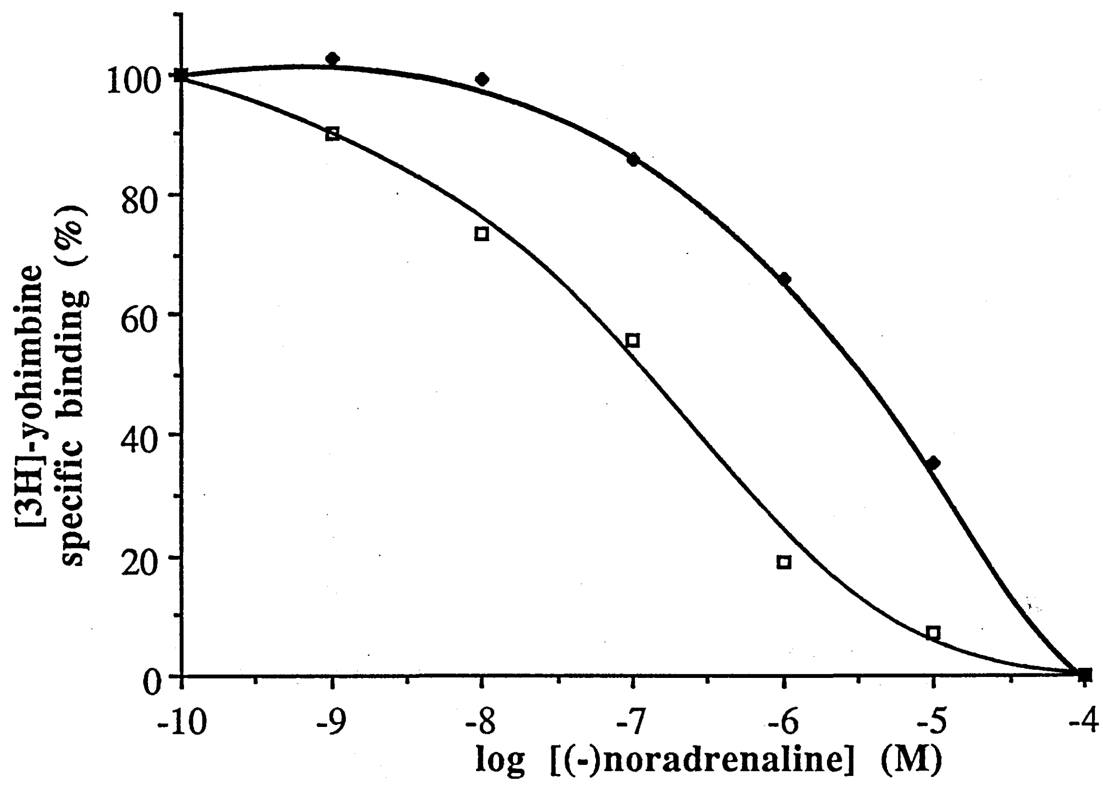


Table 3.1

EFFECT OF 60 MINUTE PREINCUBATION OF MEMBRANES FROM NG108-15 CELLS AT 30°C ON [3H]-YOHIMBINE BINDING

Membranes from NG108-15 cells (100µg) were incubated for 60 minutes at 30°C, or at 0°C, or not preincubated before addition of (-) noradrenaline (100µM) or buffer, and [^{3}H]-yohimbine (10nM). Incubation continued for a further 30 minutes before assessing specific binding by rapid filtration, as described in Chapter 2. Results are mean ± SEM, n=3.

Condition	[^{3}H]-yohimbine binding (fmoles/mg)
Not preincubated	73.4 (±7.4)
Preincubated 60 minutes, 30°C	74.3 (±6.5)
Preincubated 60 minutes, 0°C	76.1(±7.5)

Figure 3.13

EFFECT OF PREINCUBATION WITH ANTISERA OR GPP(NH)P ON TOTAL [3H]-YOHIMBINE BINDING TO MEMBRANES FROM NG108-15 CELLS

Membranes from NG108-15 cells (100μg) were incubated for 60 minutes at 30°C with protein-A purified antiserum (10μg/sample) or 100μM Gpp(NH)p as shown, before addition of buffer or (-) noradrenaline (100μM), and [^{3}H]-yohimbine (2nM). Incubation continued for a further 30 minutes before specific binding was assessed by rapid filtration, as described in Chapter 2. Points represent the means ± SEM of four experiments performed on different membrane preparations. 100% specific binding was 23.9 (±0.7) fmoles/mg.

Figure 3.13

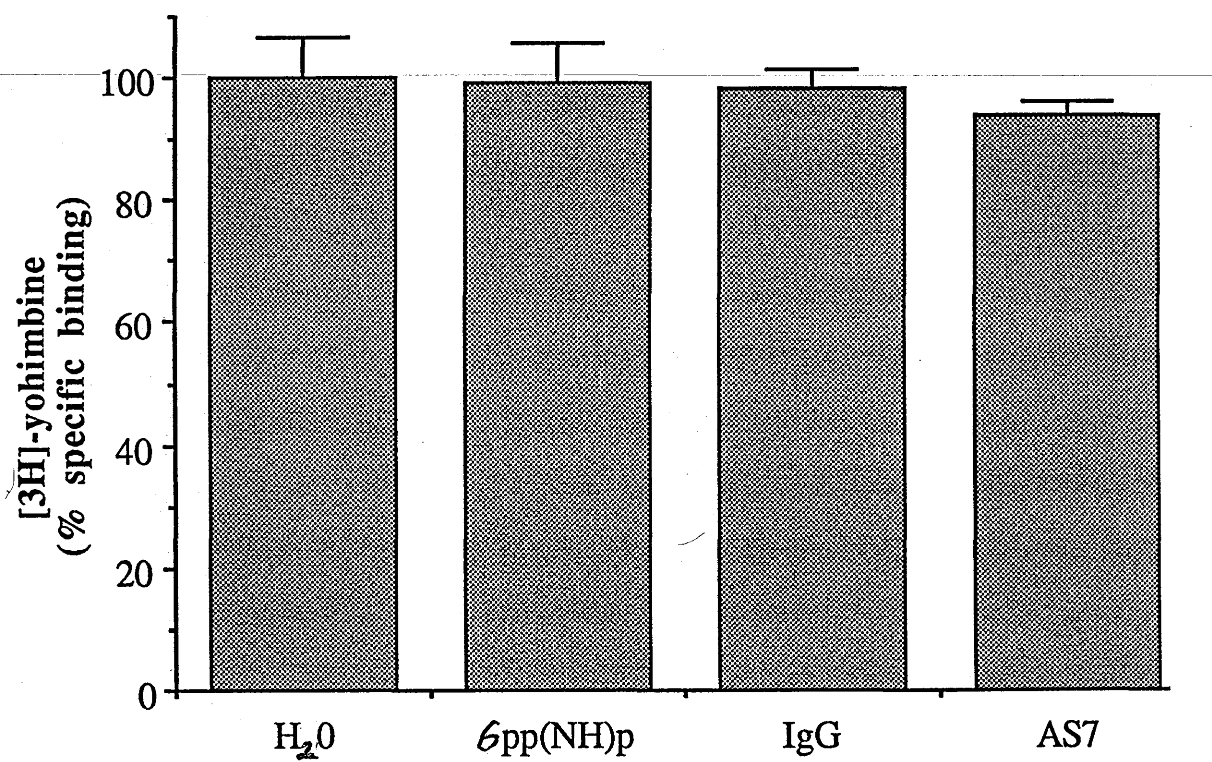


Figure 3.14

EFFECT OF PREINCUBATION WITH ANTISERA OR GPP(NH)P ON SPECIFIC [3H]-YOHIMBINE BINDING TO MEMBRANES FROM NG108-15 CELLS

Membranes from NG108-15 cells (100μg) were incubated for 60 minutes at 30°C with protein-A purified antiserum (10μg/sample) or 100μM Gpp(NH)p as shown, before addition of buffer or (-) noradrenaline (1μM), and [^{3}H]-yohimbine (2nM). Incubation continued for a further 30 minutes before specific binding was assessed by rapid filtration, as described in Chapter 2. Points represent the means ± SEM of four experiments performed on different membrane preparations. 100% specific binding was 24.6 (±1.4) fmoles/mg. *P<0.001 (Student's t-test).

Figure 3.14

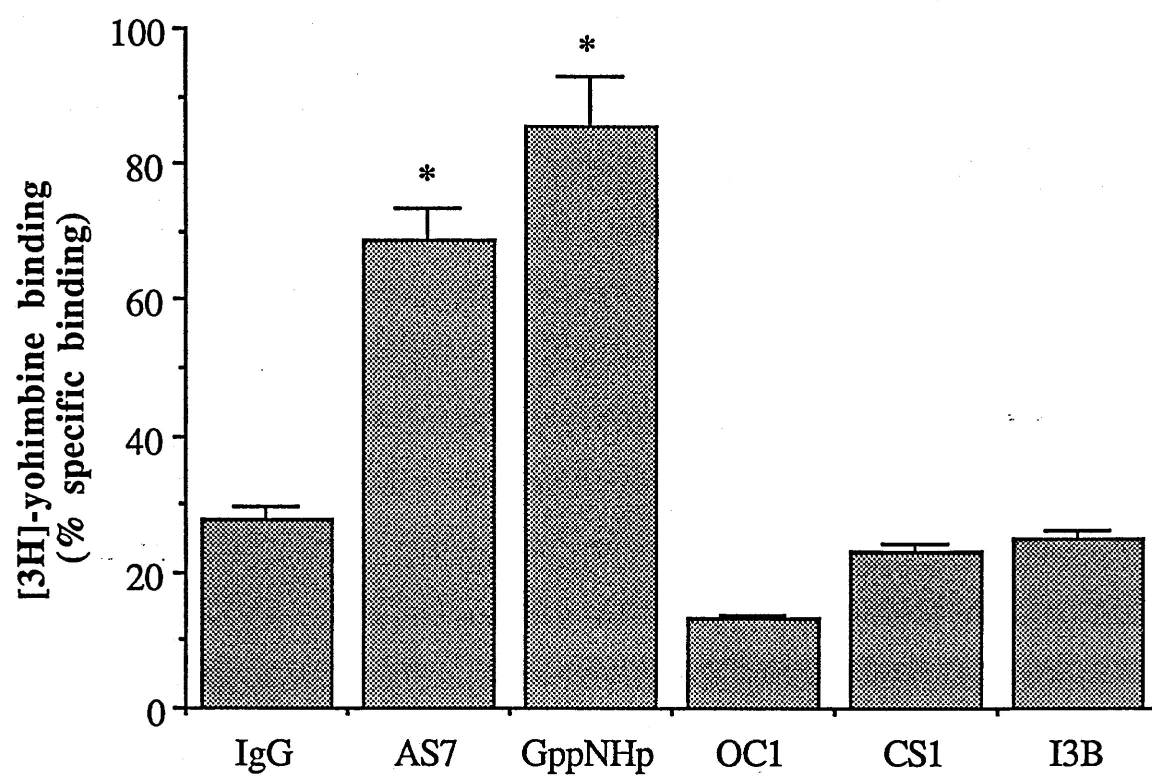


Figure 3.15

EFFECT OF COINCUBATION WITH ANTISERUM AS7 AND GPP(NH)P ON SPECIFIC BINDING OF [3H]-YOHIMBINE TO MEMBRANES FROM NG108-15 CELLS

Membranes from NG108-15 cells (100µg) were incubated for 60 minutes at 30°C with protein-A purified AS7 (10µg/sample) and/or Gpp(NH)p (100µM) as shown, before addition of buffer or (-) noradrenaline (1µM), and [^{3}H]-yohimbine (2nM). Incubation continued for a further 30 minutes before specific binding was assessed by rapid filtration, as described in Chapter 2. Points represent the means ± SEM of four experiments performed on different membrane preparations. 100% specific binding was 25.0 (±2.4) fmoles/mg.

Figure 3.15

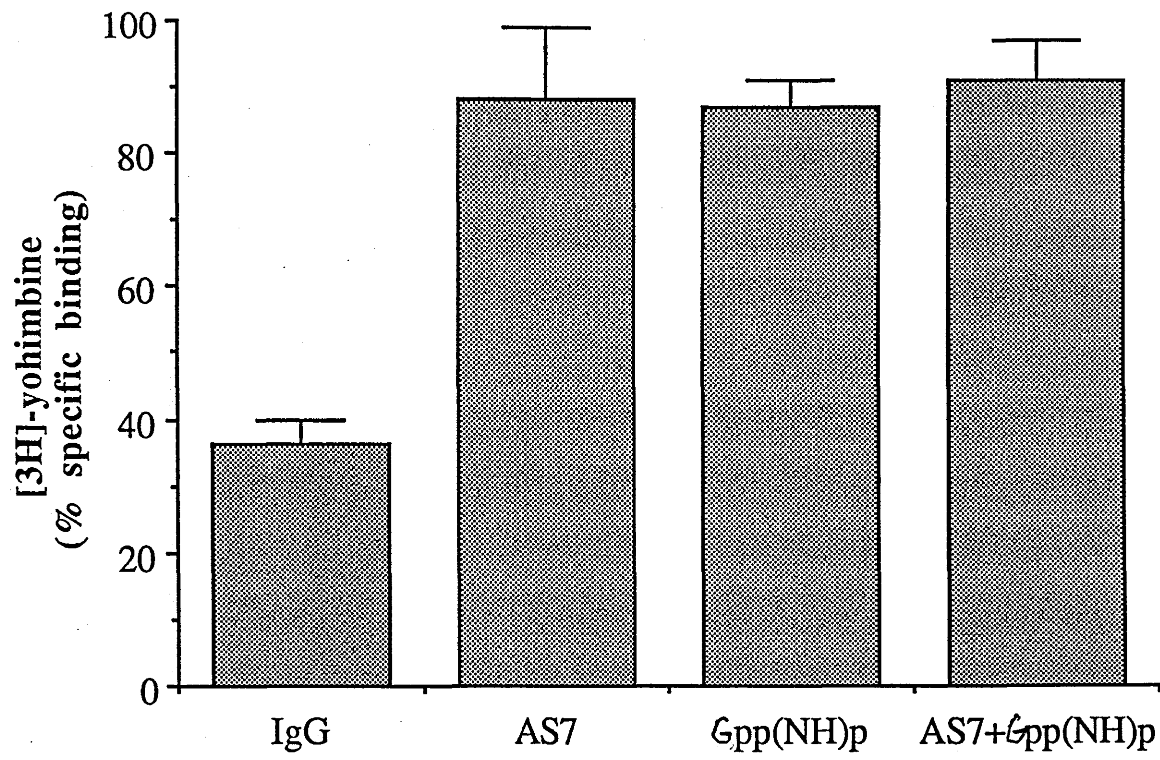
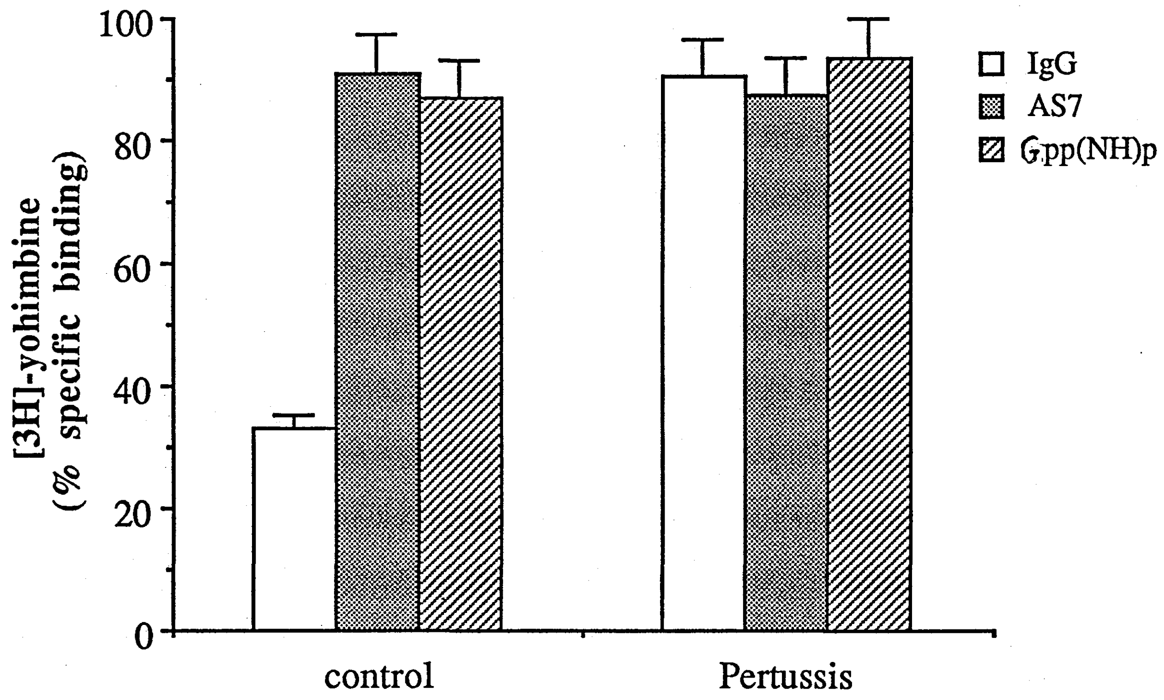


Figure 3.16

EFFECT OF AS7 OR GPP(NH)P PREINCUBATION ON SPECIFIC BINDING OF [3H]-YOHIMBINE TO MEMBRANES OF PERTUSSIS TOXIN PRETREATED NG108-15 CELLS

Membranes from control or pertussis toxin pretreated (50ng/ml, 16 hours) NG108-15 cells (100µg) were incubated for 60 minutes at 30°C with protein-A purified AS7 (10µg/sample) or Gpp(NH)p (100µM) as shown, before addition of buffer or (-) noradrenaline (1µM), and [^{3}H]-yohimbine (2nM). Incubation continued for a further 30 minutes before specific binding was assessed by rapid filtration, as described in Chapter 2. Points represent the means ± SEM of three experiments performed on different membrane preparations. 100% specific binding was 25.1 (±1.7) fmoles/mg (control membranes) and 23.4 (±0.9) fmoles/mg (pertussis toxin treated membranes).

Figure 3.16



DISCUSSION

NG108-15 is a commonly used cell line for the study of signal transduction in a "neuronal" type cell (Milligan *et al*, 1990, Hamprecht *et al.* 1985). It has proved useful for the study of δ-opioid receptor coupling to G-proteins (McKenzie and Milligan, 1990), differentiation (Mullaney and Milligan, 1989) and calcium channel regulation by G-proteins (McFadzean *et al.* 1989). These studies have all taken advantage of the presence in NG108-15 cells of a range of receptor types and intracellular effectors. Most of these studies have been concerned with identifying the G-protein(s) responsible for mediating the intracellular effects of receptor activation.

One of the first receptors identified on the NG108-15 cell was an adrenergic receptor (Traber *et al.* 1975), which was shown to be coupled to inhibition of adenylyl cyclase (Sabol and Nirenberg, 1979). This inhibition was later shown to be pertussis toxin sensitive (Klee *et al.* 1985), indicating that a G-protein which was modified by this toxin was involved in the coupling of the adrenergic receptor to adenylyl cyclase inhibition. The generation of specific G-protein antipeptide antibodies (see Chapter 1) capable of both identifying specific G-proteins and interfering with receptor-G-protein coupling (McKenzie and Milligan, 1990; Simonds *et al.* 1989a, 1989b) suggested that it might be possible to use such antisera to identify the G-protein(s) responsible for coupling the adrenergic receptor to adenylyl cyclase inhibition in NG108-15 cells.

The method used in this study involved measuring changes in receptor affinity on uncoupling of receptor from G-protein. It is worth addressing the question of why this particular method was chosen. There are a number of consequences of receptor activation, as

outlined in Chapter 1. Firstly, agonist occupation of receptor causes an increase in guanine-nucleotide exchange at the G-protein, resulting in a GTP-bound heterotrimer, which then dissociates into α- and βγ-subunits (Fung *et al.* 1981). The α-subunit is now activated, and can interact with its intracellular second-message generator -in the case of the adrenergic receptor in NG108-15 cells, this is an inhibitory action on adenylyl cyclase. After a period of time, an intrinsic GTPase activity of the α-subunit cleaves the terminal phosphate of GTP, restoring the GDP-bound form of the α-subunit (Cassel and Selinger, 1977), which then reassociates with both the βγ subunits and the receptor to return the cycle to the resting state. There are a number of points of this cycle which could be used directly to assess G-protein activation, notably the stimulation of GTPase activity on receptor activation, and also the direct measurement of adenylyl cyclase inhibition (Milligan, 1988). Both of these techniques have been used, in conjunction with specific anti-G-protein antisera, to identify G-protein coupling to receptors (Simonds *et al.* 1989a, 1989b; McKenzie and Milligan, 1990). Why were these, arguably more direct, measurements of G-protein activation not used here?

Preliminary experiments using NG108-15 membranes established that (-) noradrenaline and (-) adrenaline generated a small, but significant, increase in GTPase activity , of the order of 5-10% above basal (results not shown). However this GTPase activity was lost on the preincubation of the membranes for 60 minutes at 30°C required for antibody binding. Similarly, experiments using (-) noradenaline to inhibit adenylyl cyclase activity showed ≈30% inhibition of cyclase activity in membranes from NG108-15 cells (results not shown, and Sabol and Nirenberg 1979), but again, this inhibition was abolished on preincubation of membranes for 60

minutes. The method using the change in receptor affinity as an assay for G-protein coupling to receptor was the only one robust enough to survive the preincubation period. To make a virtue of necessity, it should also be observed that, for a number of systems, the change of receptor affinity on uncoupling from a G-protein is the only method of detection of G-protein coupling, because either the stimulation of GTPase activity is undetectable, or (as in the case of receptors liked to phospholipase C) effector activity cannot usually be measured in membrane fractions, and we wished to demonstrate the applicability of the technique.

It is clear that, where a sufficiently large GTPase activity is obtained, this is the method of choice for assessing receptor-G-protein coupling. The reasons why GTPase stimulation is not always measurable are not clear. In some cases, this may be due to the relatively low numbers of activated G-proteins present, such that the signal-to-noise ratio is too low to allow assessment of stimulated activity over basal. If, as has been reported by Carty *et al.*, (1990) different G-proteins have different activation rates, lack of detectable GTPase activity may simply be due to the kinetics of activation of the G-protein concerned. In other cases, there seems to be a relationship between the number of receptors interacting with a population of G-proteins, and the detectability of stimulated GTPase response. It is notable that McKenzie and Milligan (1990) were able to detect δ-opioid stimulation of GTPase activity in NG108-15 cells of around 50% above basal, where they reported a Bmax for [^{3}H]-DADLE binding of 400 fmoles/mg, while the results reported here suggest a Bmax for [^{3}H]-yohimbine binding of 93 fmoles/mg, this number of receptors producing a poorly detectable GTPase response of only ~10% above basal.

The reasons for the loss of GTPase activity and adenylyl cyclase inhibition after preincubation are more obvious. Although it was clear that preincubation caused no loss of [^{3}H]-yohimbine binding to membranes from NG108-15 cells (Table 3.1), preliminary experiments using the tritiated adrenergic agonist, [^{3}H]-UK14,304 suggested a loss of some 20% of [^{3}H]-UK14,304 binding sites after 60 minutes preincubation at 30°C (Control specific [^{3}H]-UK14,304 binding 76.6 (±3.5) fmoles/mg, incubated membranes specific [^{3}H]-UK14,304 binding 62.6 (±1.8) fmoles/mg, results are mean ± SEM, n=2). The implication of these data is that the receptors were being gradually uncoupled by the process of preincubation *per se*, adopting a low affinity state for their agonist which is detected as a reduction in [^{3}H]-UK14,304 binding. Despite this, there remained sufficient coupled receptors for the antibody incubation experiments to be successfully performed.

It has previously been reported that NG108-15 cells express the α-subunits of Gi2, Gi3, two forms of Gs, and Go (McKenzie *et al.* 1988). The results obtained here confirm this finding. The antiserum AS7, which was raised against a peptide sequence representing the C-terminal 10 amino-acids of bovine transducin (Falloon *et al.* 1986), detects not only transducin, but also Gi1 and Gi2, which share identical C-terminal sequences, differing in only a single amino acid to that of transducin (Itoh *et al.* 1986). In membranes from NG108-15 cells, AS7 recognises only a single band on western blots, which appears to be Gi2 (Figure 3.1). Gi1 appears not to be expressed in NG108-15 cells, as it is not detectable immunologically (Figure 3.1), and the mRNA for Gi1 is not detected in NG108-15 cells (McKenzie and Milligan, 1990).

The pertussis toxin substrates of NG108-15 cells are shown in Figure 3.3 to be Gi2, Gi3, and Go. As adrenergic inhibition of adenylyl cyclase in NG108-15 cells is inhibited by pertussis toxin pretreatment (Klee *et al.* 1985), one or more of these G-proteins is responsible for coupling the receptor to adenylyl cyclase. Mullaney and Milligan (1990) have shown that isoforms of Go exist in membranes from NG108-15, however the separation technique used here does not allow detection of these two forms. Pertussis toxin pretreatment of NG108-15 cells for 16 hours (50ng/ml) completely ADP-ribosylates the group of pertussis toxin substrates, preventing subsequent ADP-ribosylation with $[^{32}P]NAD^{+}$ as substrate (Figure 3.4). The cholera toxin substrates of NG108-15 membranes are shown in Figure 3.5. Two forms of Gs are identified, with apparent Mr 42 and 44kDa, with the 44kDa form predominating in both immunoblot and ADP-ribosylation study (Figure 3.5). Although 4 cDNA clones of Gs have been reported in human brain (Bray *et al.* 1986), it is not known which of these clones is expressed in NG108-15.

The α_2-adrenergic receptor in NG108-15 cells was characterised by radioligand binding using the tritiated antagonist, $[^{3}H]$-yohimbine (Figure 3.10). Previous studies of $[^{3}H]$-yohimbine binding to NG108-15 membranes have generated different Kd values. Kahn *et al.* reported a Kd of 9.1nM (Kahn *et al.* 1982), while Bylund *et al.* reported a Kd of 1.76nM (Bylund *et al.* 1988). The results obtained here suggested a Kd of 2.3nM, in close agreement with Bylund. The Bmax obtained in this study was, at 93.6fmoles/mg, significantly lower than that reported by both Kahn (258.4 fmoles/mg) and Bylund (153 fmoles/mg). The reason for this is likely to be that different clones of the cells , with different receptor numbers, have been used.

The characterisation of the α_2 receptor subtype was on the basis of affinity of the receptor for oxymetazoline and prazosin, as suggested by Bylund (Bylund *et al.* 1988; Bylund, 1988). The α_{2A} adrenergic receptor, the prototype of which occurs on the human platelet, has been shown to possess a high affinity for oxymetazoline, and a low affinity for prazosin. The α_{2B} adrenergic receptor, the prototype for which occurs in neonatal rat lung tissue, has a high affinity for both prazosin and oxymetazoline. Displacement of [^{3}H]-yohimbine binding by oxymetazoline and prazosin in membranes from NG108-15 cells produced Ki values of 24.0nM and 33.1nM, respectively (Figure 3.11). These data suggest that the α_2-adrenergic receptor on NG108-15 cells is of the α_{2B} subtype, which result is in agreement with that obtained by Bylund (Bylund *et al.* 1988).

The effect of guanine nucleotides and their analogues on agonist affinities of receptors was one of the first observations which led to the development of the idea that a guanine-nucleotide binding protein was required for the full functioning of many signal transduction systems (Rodbell *et al.* 1971; Rodbell, 1980). In membranes from NG108-15 cells, addition of 100μM Gpp(NH)p, a poorly hydrolysed analogue of GTP, caused a reduction in the ability of (-) noradrenaline to compete for [^{3}H]-yohimbine binding sites (Figure 3.12). IC_{50} values for (-) noradrenaline displacement of [^{3}H]-yohimbine increased from 0.35μM to 4.8μM in the presence of Gpp(NH)p. Similarly, pseudo-Hill slopes changed from 0.52 to 1.03, indicating a decrease in receptor cooperativity in the presence of the GTP analogue. These changes are

consistent with the theory that the receptor affinity for agonist is governed by the coupling state of its G-protein. When the GDP-liganded G-protein is coupled to its receptor, the receptor has a high affinity for its agonist, however if the G-protein becomes activated by binding of GTP, or an analogue such as Gpp(NH)p, then the receptor adopts a low affinity state for its agonist (Wregget and De Lean, 1984). Similarly, agents which cause the uncoupling of the G-protein from its receptor, such as pertussis toxin-catalysed ADP-ribosylation of the C-terminus of the G-protein α-subunit, or preincubation with antisera which bind to the C-terminus, will cause the receptor to adopt a low affinity state for its agonist, resulting in a shift in apparent Kd for the agonist (Milligan *et al.* 1990; McKenzie and Milligan, 1990). A single concentration of 1μM(-) noradrenaline was chosen for use in subsequent "uncoupling" experiments, this concentration displacing ≈80% of specific [^{3}H]-yohimbine binding in the absence of Gpp(NH)p, but only displacing ≈30% of specific [^{3}H]-yohimbine binding in the presence of Gpp(NH)p (Figure 3.12). Hence, it would be anticipated that agents which caused uncoupling of the receptor from its G-protein would cause a similar apparent increase in [^{3}H]-yohimbine binding.

The first evidence that the C-terminus of the G-protein α-subunit was involved in coupling to receptors came from the observation that pertussis toxin-catalysed ADP-ribosylation of a cysteine residue 4 amino-acids from the C-terminus of the α-subunits of Td, Gi1, Gi2, Gi3 and Go prevented functional interaction between receptor and G-protein (Katada and Ui, 1979, 1982, West *et al.* 1985). This idea was supported by the observation that the lesion responsible for the *unc* mutant of S49 murine lymphoma cells, in which Gs is uncoupled from receptors which stimulate adenylyl cyclase, is the replacement of an Arg by a Pro residue near the extreme C-terminus

of Gs (Sullivan *et al.* 1987). Furthermore, antisera which recognise the C-terminus of transducin prevented rhodopsin activation of transducin (Hamm and Bownds, 1984). Subsequent to this observation, (as has been detailed in Chapter 1) a number of groups have used anti-C-terminal antibodies to interfere with receptor-G-protein coupling in systems as diverse as human platelet, snail neurons, and S49 murine lymphoma cells. Hence it seemed likely that use of anti-C-terminal antisera would allow the identification of the G-protein which interacts with the α_{2B} receptor in NG108-15 cells.

It was established that preincubation with an IgG fraction from normal rabbit serum, or with 100µM Gpp(NH)p did not affect total [^{3}H]-yohimbine binding in the absence of (-) noradrenaline (Figure 3.13). However, in the presence of 1µM (-) noradrenaline, preincubation of membranes from NG108-15 cells with antisera raised against the C-teminal sequences of a range of G-protein α-subunits showed that the antiserum AS7 caused a statistically significant increase ($P<0.001$, Student's t-test) in measured [^{3}H]-yohimbine binding (Figure 3.14). Preincubation with 100µM Gpp(NH)p also caused this increase in [^{3}H]-yohimbine binding, however preincubation with an IgG fraction from normal rabbit serum, or with antisera raised against the C-terminal sequences of Go (OC1), Gs (CS1), and Gi3 (I3B) did not have this effect. The implication of this apparent increase in [^{3}H]-yohimbine binding is that antiserum AS7 and 100µM Gpp(NH)p are both able to uncouple the G-protein that interacts with the α_{2B}-adrenergic receptor in NG108-15. In the case of Gpp(NH)p, this is achieved by activation of the G-protein α-subunit, and in the case of antiserum AS7, by binding to the C-terminus of the α-subunit, preventing functional interaction between G-protein and receptor. As it has been established that, in membranes from NG108-15, antiserum

AS7 recognises Gi2 only (Figure 3.1, and McKenzie *et al.* 1988), we can conclude that the α_{2B}-adrenergic receptor in NG108-15 cells interacts specifically with Gi2.

Two further experiments suggested that the α_{2B} receptor interacts with Gi2 alone, and that no other G-protein is involved. Co-incubation of antiserum AS7 and 100μM Gpp(NH)p (Figure 3.15) produced no greater increase in [^{3}H]-yohimbine binding than either AS7 or Gpp(NH)p alone. This indicated that antiserum AS7, at the concentration used (10μg AS7 per 100μg NG108-15 membranes), was able fully to uncouple the receptor from Gi2, producing the same degree of effect as 100μM Gpp(NH)p, which would at this concentration completely uncouple all the G-proteins present. Finally, it was shown that no further increase in [^{3}H]-yohimbine binding was obtained when AS7 or Gpp(NH)p were preincubated with membranes from NG108-15 cells which had been pretreated with pertussis toxin (Figure 3.16). This would be expected if pertussis toxin had already completely ADP-ribosylated Gi2 *in vivo*, making it impossible for further uncoupling to occur as a result of antibody preincubation. Hence these experiments indicated that Gi2 alone was coupled to the α_{2B}-adrenergic receptor in NG108-15 cells, and that antiserum AS7 had completely uncoupled the G-protein from its receptor.

These results provide evidence that Gi2 acts as "Gi", the inhibitory G-protein of adenylyl cyclase, for the adrenergic receptor of NG108-15. It has now been shown for a number of receptor systems that Gi2 acts as "Gi", namely the δ-opioid receptor in NG108-15 (McKenzie and Milligan, 1990), and the α_{2A}-receptor in human platelets (Simonds *et al.* 1989a), so this may indicate that, where it is expressed, and where there is an inhibitory receptor for adenylyl cyclase, Gi2 has a general role as "Gi".

McFadzean *et al.* have shown that injection of NG108-15 cells with protein-purified antisera raised against the C-terminus of Go attenuated the adrenaline-induced reduction of calcium current (McFadzean *et al.* 1989), while injection of AS7 was without effect. These studies arose from the findings of Hescheler *et al.* , who reported δ-opioid receptor-mediated inhibition of Ca^{2+} currents in NG108-15 which was pertussis toxin sensitive. The effect of pertussis toxin could be reversed, however, by subsequent injection of purified Gi and Go, Go being up to 10 times more potent than Gi (Hescheler *et al.* 1987). The results of McFadzean *et al.* suggest that the adrenergic receptor in NG108-15 is coupled to inhibition of Ca^{2+} currents via Go, and this supports the results of Hescheler in suggesting that Go is capable of the control of Ca^{2+} currents in NG108-15. The results reported herein have shown that AS7, which recognises Gi2 only, completely uncouples the α_{2B} receptor in NG108-15, and the coincubation experiment (Figure 3.15) and pertussis toxin experiment (Figure 3.16) suggested that no other G-protein was involved in coupling to this receptor. Hence there is an apparent contradiction between the results of McFadzean *et al.*, and the findings reported here.

One possible explanation for this apparent contradiction is that there are separate, but pharmacologically similar adrenergic receptors in NG108-15 cells, each of which interacts with a different G-protein, and each of which is coupled to a different intracellular second-message generating system. Two results reported here argue against this interpretation. Firstly, the saturation binding isotherms of [^{3}H]-yohimbine binding suggested a single-site binding affinity (Figure 3.10). To date, only 3 well defined subtypes of the α_2-adrenoceptor have been reported (Harrison *et al.* 1991), all of which can be

The data presented in Figure 3.11 suggest that the Ki values for oxymetazoline and prazosin are similar to those observed previously for a receptor of the α_{2B} subtype (Bylund *et al.* 1988). However, the slopes of the displacement curves suggested that oxymetazoline and prazosin were binding to more than one receptor (see note to page 72). If this were the case, then it is possible that two receptors with similar affinities for yohimbine (ie. subtypes of the α_2-adrenergic receptor) exist on NG108-15 cells, and the corrollary of this would be that one receptor might couple exclusively to Go, while the other couples exclusively to Gi2.

discriminated pharmacologically by their affinities for oxymetazoline and prazosin (Bylund, 1988). The assessment of these affinities in NG108-15 (Figure 3.11) clearly suggested an α_{2B} subtype adrenoceptor, so it seems unlikely that another receptor subtype is involved. The second piece of evidence against the "two receptors" hypothesis is the finding that preincubation of NG108-15 membranes with the antiserum OC1, raised against Go did not cause any apparent increase in [^{3}H]-yohimbine binding, as would be expected if the "other" α_2-receptor were coupled to Go. If there were two receptors present with virtually identical affinities for [^{3}H]-yohimbine, (and for prazosin and oxymetazoline) it should have been possible to detect the increase in apparent [^{3}H]-yohimbine binding which accompanies uncoupling of the receptor. However, this was not detected.

It seems much more likely that the reason for the present failure to detect any coupling of the adrenergic receptor in NG108-15 to Go lies with the G-proteins themselves. It is customary, when measuring Ca^{2+} currents by electrophysiological methods, to first differentiate the cells in question, in order to maximise the currents (Hamprecht *et al.* 1985). Differentiation is usually achieved by preincubation of the cells with agents which raise levels of intracellular cAMP, such as dibutyryl cAMP, forskolin, and PGE_1 (Nirenberg *et al.* 1983). Accompanying this elevation in intracellular cAMP levels is a morphological change in the cells, resulting in the extrusion of neurite-like processes. It has also been shown by Mullaney that differentiation of NG108-15 cells results in changes in the levels of Gi and Go (Mullaney *et al.* 1988). Mullaney and Milligan noticed that levels of Go were low in comparison to those of Gi in undifferentiated cells, but that treatment with dibutyryl cAMP or forskolin resulted in an approximate doubling of the levels of Go (Mullaney and Milligan,

1989). Concurrent with this increase in the level of Go, there was also a decrease in levels of the G-protein detected by the antiserum AS7 (Mullaney *et al.* 1988).

From these data, it seems clear that the process of chemical differentiation results in an alteration in the levels of Gi2 and Go. The results obtained by McFadzean *et al.* showing coupling of the adrenergic receptor in NG108-15 to Go, and the results reported here, showing coupling of the same adrenergic receptor exclusively to Gi2, can therefore be compatible. In the undifferentiated state, NG108-15 cells express higher levels of Gi2 than Go, and the α_{2B} adrenergic receptor couples to inhibition of adenylyl cyclase via Gi2. However, on differentiation, NG108-15 cells express higher levels of Go, and the levels of Gi2 fall. Under these conditions, the α_{2B} receptor can be shown to couple to inhibition of voltage sensitive Ca^{2+} channels via Go. The corrollary of this hypothesis is that the α_{2B} adrenergic receptor in NG108-15 cells is physically capable of interacting with two different G-proteins, although it is not clear that these interactions ever occur simultaneously.

With reference to this point, it has been shown by Ashkenazi that the M2 muscarinic receptor can interact simultaneously with adenylyl cyclase and phospholipase C, ie. that a single receptor can interact at one time with different G-proteins to generate different intracellular effects (Ashkenazi *et al.* 1987). The results obtained here suggest that the adrenergic receptor in NG108-15 interacts specifically with only one G-protein at a time, the identity of which is dependant on the differentiation state of the cell. Hence the degree of specificity of coupling between G-protein and the adrenergic receptor in NG108-15 is a property not of the sequence of the G-protein α-subunit, but of

the prevalence of that G-protein, in terms of absolute amounts available for coupling to the receptor.

Mullaney and Milligan have shown recently that NG108-15 cells express two isoforms of Go, which they call Go1 and Go2 (Mullaney and Milligan, 1990). They show that Go1 is equivalent to one form of Go that is expressed in rat brain, but that Go2 is not equivalent to Go*,the other form of Go expressed in rat brain (Goldsmith *et al.* 1988). Furthermore, chemical differentiation of NG108-15 cells results in an increase in the levels of Go1 only, levels of Go2 being unaffected (Mullaney and Milligan 1990). These data again raise the question of why interaction of the α_{2B} receptor with Go in undifferentiated NG108-15 cells was not detected. If control of voltage-operated calcium channels is mediated by only one form of Go (presumably Go1), then it is possible that the levels of Go1 in undifferentiated NG108-15 cells are so low that interaction with the α_{2B} receptor does not occur, or occurs to such a small extent that it cannot be detected using the methods described here.

Despite the substantial degree of sequence homology between G-protein α-subunits, it is clear that, *in vivo*, a high degree of specificity is obtained. Gi2 and Gi3 share 90% sequence homology, yet on the basis of the results obtained here, the α_{2B}-adrenergic receptor in NG108-15 cells is capable of discriminating between the two. This is also the case for the α_{2A} receptor in the human platelet, where again, the receptor can discriminate between Gi2 and Gi3 (Simonds *et al.* 1989a). The interaction of the α_{2B} receptor in NG108-15 with Gi2 and Go under different conditions is therefore clearly a matter of interest. Since the receptor is capable of discriminating between two such similar α-subunits as those of Gi2 and Gi3 (90% sequence homology),

and yet will interact under different conditions with Gi2 and Go, which share some 80% sequence homology, this raises the question: what is the determining factor of G-protein specificity ? The studies in Chapter 4 attempt to address this question by transfecting the α_{2A} adrenergic receptor into a cell line which does not express an adrenergic receptor, in order to examine the specificity of coupling of G-proteins to this receptor in an "alien" environment.

CHAPTER 4

INTERACTION OF THE α_2-C10 ADRENERGIC RECEPTOR IN RAT 1 FIBROBLASTS WITH G-PROTEINS

INTRODUCTION

The coupling of adrenergic receptors to their G-proteins has not been widely studied. The α_1 adrenergic receptor has been shown in a number of systems to couple to stimulation of phospholipase C, although the identity of the G-protein responsible for this coupling is unclear, and its identification is made more difficult by its insensitivity to pertussis or cholera toxin (Berridge and Irvine, 1984; Burch *et al.* 1986). Both the α_{2A} and α_{2B} adrenergic receptors have been shown to couple to inhibition of adenylyl cyclase via a pertussis toxin sensitive G-protein (Jakobs *et al.* 1978; Klee *et al.* 1985), but there are several pertussis toxin substrates among the family of G-proteins, (Milligan, 1988) and the precise identity of these G-proteins remains to be established.

The α_{2A}-adrenergic receptor was the first α_2-adrenoceptor to be cloned. The clone identified was found to be localised to human chromosome 10 (Kobilka *et al.* 1987). This clone, designated α_2-C10, when expressed in COS cells showed identical characteristics to the pharmacologically defined α_{2A} adrenergic receptor, the prototype of which is found on human platelets, (Harrison *et al.* 1991) and which possesses a high affinity for the adrenergic agonist oxymetazoline and the antagonist yohimbine, but a low affinity for the antagonist prazosin (Murphy and Bylund 1988). Thus the pharmacological and molecular genetic definitions of the α_{2A} receptor have converged.

The α_{2A} receptor in the human platelet has been shown to couple to inhibition of adenylyl cyclase via a pertussis toxin sensitive G-protein (Jacobs *et al.* 1978). Simonds *et al.* have used antisera raised against the C-terminal sequences of various G-protein α-subunits to study the specificity of coupling of the α_{2A} receptor to G-proteins (Simonds *et al.* 1989a). They showed that preincubation of platelet membranes with the antiserum AS7,

which in the platelet recognises Gi2 only, resulted in abolition of the adrenaline-induced inhibition of PGE_1-stimulated adenylyl cyclase activity. Antisera raised against Go, Gi3, Gs or Gz did not have this effect. They concluded that Gi2 was the principal transducer of the α_{2A} receptor-mediated inhibition of adenylyl cyclase in human platelets. Clearly a high degree of specificity of coupling obtains here, as the receptor can discriminate between Gi2 and Gi3, which share some 90% sequence homology (Itoh *et al.* 1988).

The previous chapter has shown that the α_{2B}-adrenergic receptor in NG108-15 cells is also specifically coupled to Gi2, but that the receptor also has the potential to couple specifically to Go. The specificity of this coupling is shown by the fact that, like the α_{2A} receptor in human platelets, the receptor does not couple to Gi3. Go, on the other hand, has only ~80% sequence homology to Gi2 (Van Meurs *et al.* 1987), and yet the α_{2B} receptor can, under the right circumstances, also couple to this G-protein. The receptor therefore exhibits a high degree of discrimination between G-proteins, apparently regardless of their sequence similarity or dissimilarity. What, then is the factor which determines the specificity of G-protein coupling to receptors?

This chapter describes an attempt to examine this question by studying the specificity of coupling to G-proteins of the α_{2A} receptor in an "unnatural" environment. Milligan *et al.* have recently reported the transfection of the α_{2A} receptor clone (α_2-C10) into the Rat 1 fibroblast cell line (Milligan *et al.* 1991). Briefly, they constructed a transfection vector which contained the entire α_{2A} genomic DNA sequence together with a neomycin resistance gene, and transfected Rat 1 fibroblasts by calcium phosphate precipitation. They were then able to select clones which had been successfully transfected by growing them in G418 (geneticin sulphate)-supplemented medium. One clone, called 1C, was shown to

express high levels of the α_{2A} receptor, and this clone was used here to study the coupling of this receptor to its G-proteins.

The identity of the adrenergic receptor transfected into the 1C clone was first confirmed by radioligand binding. It was found that the receptor bound [^{3}H]-yohimbine with high affinity, and displacement of yohimbine binding by oxymetazoline and prazosin suggested that the receptor was, indeed, of the α_{2A} subtype. The G-protein complement of clone 1C was assessed by immunoblotting membranes with antisera specific for various G-protein α-subunits, and by pertussis and cholera toxin catalysed ADP-ribosylation. These data indicated that the 1C clone expressed Gi1, Gi2, Gi3, and Gs. Coupling of the α_{2A} receptor to G-proteins in membranes from clone 1C was assessed by agonist-stimulated GTPase measurement. The α_2-adrenergic agonist UK14,304 was shown to stimulate a high affinity GTPase activity in membranes from clone 1C which was sensitive to both yohimbine and pertussis toxin. It was shown that preincubation of membranes from clone 1C with antisera raised against the C-terminal sequences of Gi1, Gi2, and Gi3 caused a reduction of the UK14,304-stimulated GTPase activity. Finally it was shown that preincubation with antiserum AS7, which recognises Gi1 and Gi2, completely abolished the UK14,304 inhibition of forskolin stimulated adenylyl cyclase activity of membranes from clone 1C. The implications of these results will be discussed.

RESULTS

1. THE ADRENERGIC RECEPTOR EXPRESSED IN CLONE 1C CELLS

The generation of the 1C clone has been described by Milligan and coworkers (Milligan *et al.* 1991). Radioligand binding with the α_2-adrenergic ligand [^{3}H]-yohimbine (Daiguji *et al.* 1980) established that membranes from 1C cells bound [^{3}H]-yohimbine rapidly at 30°C, with equilibrium being attained after 10 minutes (Figure 4.1). Non-specific binding was assessed by co-incubation with the α_2-adrenergic antagonist idazoxan (100µM). This reached equilibrium within 1 minute (results not shown). Specific binding was linear over a range of 1C membrane concentrations from 10-150µg protein (Figure 4.2). Subsequent radioligand binding experiments were performed at 30°C for 30 minutes using 20-50µg protein.

Saturation binding isotherms indicated that, in the presence of 20mM Mg^{2+}, [^{3}H]-yohimbine bound to a single site with high affinity (Figure 4.3a). Scatchard transformation of these results (Figure 4.3b) suggested a Kd of 1.0(± 0.2) nM, and a B_{max} of 3152 (± 310) fmoles/mg membrane protein (means ± SEM, n=4). Non-specific binding was typically 8-15% of total binding (results not shown).

Characterisation of the adrenergic receptor subtype was on the basis of differential affinities for the agonist oxymetazoline, and the antagonist prazosin, according to the scheme of Bylund (1988). Displacement of specific [^{3}H]-yohimbine binding to membranes of clone 1C cells (Figure 4.4) produced IC_{50} values of 13.8 (± 5.6) nM and 4.8 (± 1.5) µM for oxymetazoline and prazosin respectively (means ± SEM, n=3 for both drugs). Estimation of Ki by the method of Cheng and Prussoff (Cheng and

Prussoff, 1973) gave values of 1.4nM and 0.9μM, respectively. The high affinity for oxymetazoline and low affinity for prazosin is typical of the α_{2A}-adrenergic subtype (Murphy and Bylund, 1988). These data confirm that the receptor expressed in the 1C clone is equivalent to an α_{2A}-receptor (Milligan *et al.* 1991).

2. THE G-PROTEIN COMPLEMENT OF CLONE 1C CELLS

Immunoblots of membranes from clone 1C cells with the antisera described in Table 1 of Chapter 2 demonstrated the presence of a range of G-protein α-subunits (Figure 4.5). Antiserum I1C, raised against a synthetic decapeptide representing amino acids 159-168 of Gi1α recognised a single protein in membranes from clone 1C which comigrated with Gi1α from rat cerebral cortex. Antiserum SG1, which was raised against the C-terminal 10 amino acids from the α-subunits of Gi1 and Gi2, demonstrated the presence of a single band on a 10% SDS-polyacrylamide gel (Figure 4.5) which comigrated with Gi2α from C6BU1 cells. Separation of membranes from clone 1C on 12% SDS-PAGE followed by immunoblotting with the antiserum AS7, which also recognises the C-terminal sequences of Gi1 and Gi2α (Figure 4.7a) demonstrated that the single band recognised by antiserum SG1 in Figure 4.5 is indeed a composite of the α-subunits of Gi1 and Gi2. Antiserum I3B, which was raised against a synthetic peptide corresponding to the C-terminal decapeptide of Gi3α, recognised a single protein on immunoblots of clone 1C membranes, which comigrated with Gi3α from rat C6BU1 cells (Figure 4.5). Antiserum IM1, which was raised against a synthetic peptide representing amino-acids 22-35 of Goα, recognised Goα from rat brain, but failed to detect any such protein in

membranes from clone 1C. Antiserum CS1 recognised two proteins in membranes from clone 1C which comigrated with the two forms of Gsα from rat glioma C6BU1 membranes. These immunoblots suggested that membranes from clone 1C cells contained the α-subunits of Gi1, Gi2, Gi3, and Gs, but that they lack Goα. Figure 4.6 demonstrates the expression of the G-protein β-subunit in membranes from clone 1C cells.

Thiol-activated pertussis toxin-catalysed ADP-ribosylation of membranes from clone 1C cells in the presence of [^{32}P]NAD$^+$ followed by SDS-PAGE under conditions which allowed resolution of proteins in the 35-45kDa range, and subsequent autoradiography, showed that pertussis toxin incorporated radiolabel into 2 bands (Figure 4.7a). These bands had apparent molecular weights of 40 and 41kDa, and were found to comigrate with proteins identified on immunoblots as Gi1, Gi2, and Gi3, the upper of the two ADP-ribosylated bands being a composite of Gi1 and Gi3 (Figure 4.7a). Pertussis toxin catalysed ADP-ribosylation of membranes from NG108-15 and clone 1C cells (Figure 4.7b) showed that pertussis toxin failed to incorporate radiolabel in membranes from clone 1C cells into a band with apparent molecular weight of 39kDa, which has been shown to comigrate with Goα (Chapter 3, Figure 3.3). Hence the pertussis toxin substrates in membranes of clone 1C cells appear to be the α-subunits of Gi1, Gi2, and Gi3, while Goα appears to be undetectable by antibodies raised against Go, or by pertussis toxin-catalysed ribosylation. Pretreatment of clone 1C cells with pertussis toxin *in vivo* (50ng/ml, 16 hours) completely prevented subsequent *in vitro* pertussis toxin-catalysed incorporation of radiolabelled [^{32}P]ADP-ribose (Figure 4.8).

Thiol-activated cholera toxin-catalysed ADP-ribosylation of membranes from clone 1C cells in the presence of [^{32}P]NAD$^+$ followed by SDS-PAGE under resolving conditions and subsequent autoradiography revealed that cholera toxin incorporated radioactivity into two bands of

apparent molecular weight 42 and 44kDa. These bands comigrated with two proteins recognised on immunoblots by the antiserum CS1, raised against Gsα (Figure 4.9). Hence the cholera toxin substrates of membranes from clone 1C cells are two forms of Gsα.

As was described in Chapter 3, uncoupling experiments designed to investigate the identity of G-proteins coupling to the adrenergic receptor in clone 1C cells required the use of anti-G-protein antisera which had been protein-A purified to remove plasma proteins which might otherwise interfere with the action of the antisera. It was therefore necessary to demonstrate that protein-A purified antisera showed the same G-protein specificity as crude antisera. Figure 4.10 shows that the antisera AS7, CS1, and I3B when purified on protein-A sepharose, recognised the same G-protein α-subunits as crude antisera.

3. UNCOUPLING OF THE GTPASE ACTIVITY IN CLONE 1C CELLS

The α_2-adrenergic agonist UK14,304 stimulated a high affinity GTPase activity at 37°C in membranes from clone 1C cells, with an EC_{50} of 250.0 ±10.0nM (mean ±SEM, n=3) (Figure 4.11). This GTPase response was sensitive to the α_2-adrenergic antagonist yohimbine (Figure 4.12), which inhibited the response with Ki=1.2 ±0.4nM (mean ±SEM, n=3). This high affinity GTPase response was also completely abolished by pretreatment of clone 1C cells with pertussis toxin (50ng/ml for 16 hours) (see Figure 4.19). Basal and UK14,304 (10µM) stimulated GTPase

responses were found to be essentially linear over a range of clone 1C membrane concentrations from 2-10μg membrane protein (Figure 4.13).

In order to examine the identity of the G-protein(s) responsible for transducing this GTPase effect, a method similar to that outlined in Chapter 3 was utilised, whereby membranes from clone 1C cells were incubated with anti-G-protein antisera for 60 minutes at 37°C, and then assayed for 10μM UK14,304 stimulated GTPase activity. The period of 60 minutes preincubation time has previously been shown to maximise the effects of added antisera (McKenzie and Milligan, 1990). It was first necessary to establish the effects of a 60 minute preincubation period at 37°C on the UK14,304 stimulated GTPase activity *per se*. Figure 4.14 shows that preincubation of membranes from clone 1C cells for various times at 37°C resulted in a reduction of 10μM UK14,304 stimulated GTPase activity. By 60 minutes, there was a reduction in UK14,304 stimulated GTPase activity of ~50%, with a similar reduction in basal GTPase activity also being observed (Figure 4.14).

Preincubation of membranes from clone 1C cells (5μg) for 60 minutes at 37°C with 10μg of protein-A purified antisera raised against the α-subunits of Gs (antiserum CS1), Go (antiserum OC1), Gi1+Gi2 (antiserum AS7) and Gi3 (antiserum I3B), resulted in a statistically significant reduction of 10μM UK14,304 stimulated GTPase activity in the cases of AS7 and I3B ($P<0.001$, Student's t-test). There was no such reduction in GTPase activity in the cases of antisera OC1 or CS1, and preincubation of clone 1C membranes with an IgG fraction from normal rabbit serum showed that antibody incubation *per se* did not affect basal or agonist stimulated GTPase activity (Figure 4.15). These results implied that antiserum AS7, which recognises Gi1 and Gi2,and antiserum I3B, which recognises Gi3, were inhibiting the the α_{2A} receptor-mediated UK14,304 GTPase stimulation in clone 1C membranes by preventing the interaction of

the C-termini of these G-proteins with the receptor, and therefore that Gi1, Gi2, and Gi3 are all involved in coupling to this receptor.

Incubation of 5μg of clone 1C membranes with increasing concentrations of protein-A purified AS7 (Figure 4.16) or I3B (Figure 4.17) showed that maximum inhibition of 10μM UK14,304 stimulated GTPase occurred at around 10μg of antiserum per sample. This maximum inhibition was 35-40% for both antibodies, and did not increase at antibody concentrations up to 100μg per sample (results not shown). Co-incubation of membranes from clone 1C cells with antisera AS7 and I3B caused a statistically significant greater reduction than incubation with antiserum AS7 or I3B alone, up to a maximum of 70-75% inhibition (Figure 4.18; $P<0.001$, Student's t-test, comparing AS7+I3B with AS7 or I3B alone). In no case was complete abolition of the 10μM UK14,304 stimulated GTPase observed, the stimulated GTPase response in the presence of antisera AS7 and I3B together always being significantly greater than basal values ($P<0.001$, Student's t-test, comparing AS7+I3B to basal). These results suggest that, while antisera AS7 and I3B are capable of substantially attenuating the α_{2A}-receptor mediated UK14,304 GTPase response in clone 1C membranes, they are not capable of completely attenuating this response, and that some other G-protein may be involved in transducing a part of this GTPase response.

Pretreatment of clone 1C cells with pertussis toxin (50ng/ml for 16 hours) resulted in complete abolition of the 10μM UK14,304 stimulated GTPase response in membranes from pertussis toxin treated cells (Figure 4.19). Hence any G-protein transducing this GTPase response other than Gi1, Gi2 or Gi3, must also be a substrate for pertussis toxin-catalysed ADP-ribosylation.

4. UNCOUPLING OF ADENYLYL CYCLASE INHIBITION IN CLONE 1C CELLS

It has been shown that the α_{2A} receptor in the human platelet is coupled to inhibition of adenylyl cyclase, and that preincubation of platelet membranes with antiserum AS7, which recognises Gi2 only in the platelet, completely abolishes this inhibition (Simonds *et al.* 1989a). It was clearly of interest to examine the α_{2A} receptor expressed in clone 1C cells for any similar inhibition, and to determine whether Gi2 was also responsible for coupling this receptor to adenylyl cyclase. Figure 4.20 shows that forskolin (1nM-100μM) stimulated an adenylyl cyclase activity in membranes from clone 1C cells. This cyclase activity was inhibited by coincubation with the α_2-adrenergic agonist UK14,304 (Figure 4.21), with maximal inhibition being 40% at a UK14,304 concentration of 10μM. Pretreatment of clone 1C cells with pertussis toxin (50ng/ml for 16 hours) completely abolished the 10μM UK14,304 inhibition of 10μM forskolin stimulated adenylyl cyclase activity (Figure 4.22).Thus, the α_{2A} receptor in clone 1C cells, like that in human platelets, couples to inhibition of forskolin stimulated adenylyl cyclase in a pertussis toxin-sensitive manner.

In order to determine which G-protein(s) were responsible for transducing this inhibition of stimulated adenylyl cyclase, the method of preincubation with anti-G-protein antisera was used, in a manner similar to that used earlier to examine the UK14,304 stimulated GTPase effect. Again, it was necessary first to establish the effect of a 60 minute preincubation period on adenylyl cyclase activity in membranes from clone 1C cells (Figure 4.23). Preincubation of clone 1C membranes at 37°C for periods up to 60 minutes resulted in reductions of basal, 10μM forskolin stimulated adenylyl cyclase activity, and 10μM UK14,304 inhibition of forskolin

stimulated activity (Figure 4.23). Mean percent inhibition remained constant throughout the preincubation, at 37.3 (±5.3)% (mean ±SEM, n=3).

Preincubation of membranes from clone 1C cells with protein-A purified IgG from normal rabbit serum, or with antisera against the C-terminal sequences of Gs (antiserum CS1), or Gi3 (antiserum I3B) did not cause any reduction in 10μM UK14,304 inhibition of 10μM forskolin stimulated adenylyl cyclase activity (Figure 4.24). However preincubation with protein-A purified antiserum AS7, which in clone 1C cells recognises Gi1 and Gi2, caused complete abolition of the UK14,304 inhibition of forskolin stimulated adenylyl cyclase activity (Figure 4.24). These results suggest that the α_{2A} receptor expressed in clone 1C cells , like that expressed in the human platelet, is coupled to inhibition of adenylyl cyclase activity via Gi2 and/or Gi1.

Figure 4.1

TIME COURSE OF ASSOCIATION OF [3H]-YOHIMBINE SPECIFIC BINDING TO MEMBRANES FROM CLONE 1C CELLS

Specific [^{3}H]-yohimbine (10nM) binding to membranes (50µg) of clone 1C cells at 30°C was assessed at the time points shown. Non-specific binding was defined by parallel incubation with idazoxan (100µM). Points shown are mean (± SD) of triplicate determinations from a single experiment, which was repeated twice using different clone 1C membrane preparations, with similar results being obtained.

Figure 4.1

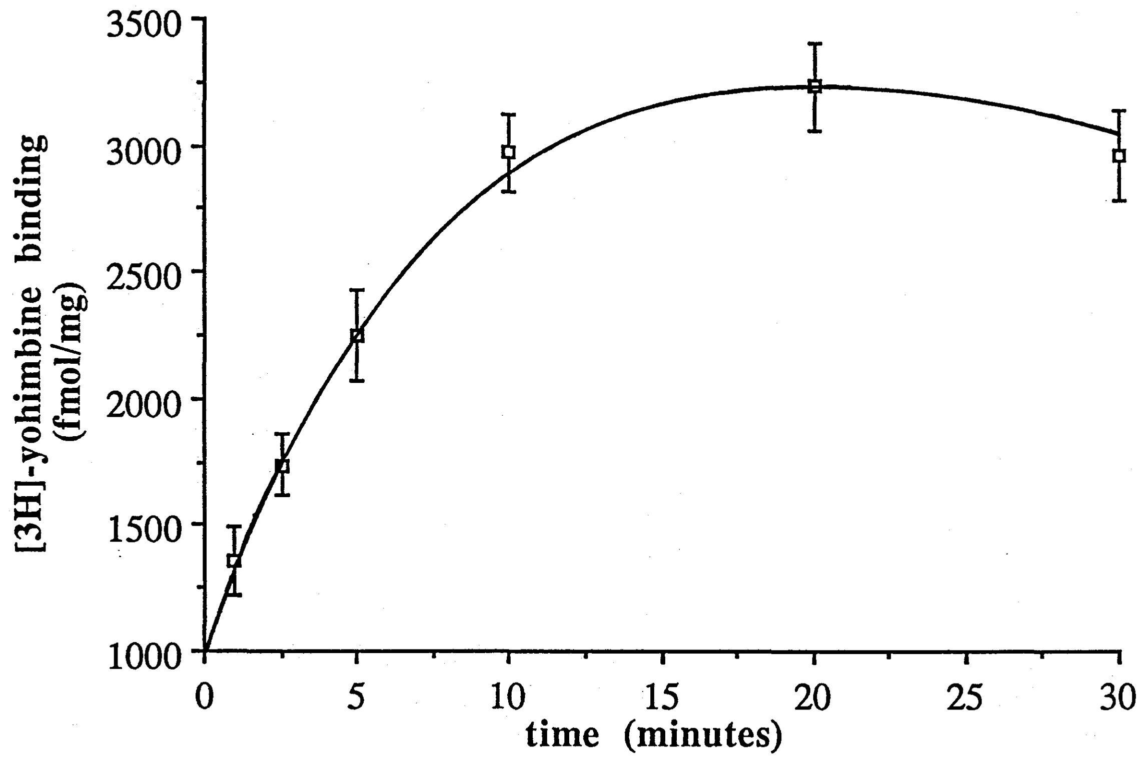


Figure 4.2

SPECIFIC BINDING OF [3H]-YOHIMBINE TO INCREASING AMOUNTS OF MEMBRANES FROM CLONE 1C CELLS

Specific [^{3}H]-yohimbine (10nM) binding to increasing amounts of membranes of clone 1C cells at 30°C was assessed as shown. Non-specific binding was defined by parallel incubation with idazoxan (100µM). Points shown are mean (± SD) of triplicate determinations from a single experiment, which was repeated twice using different clone 1C membrane preparations, with similar results being obtained.

Figure 4.2

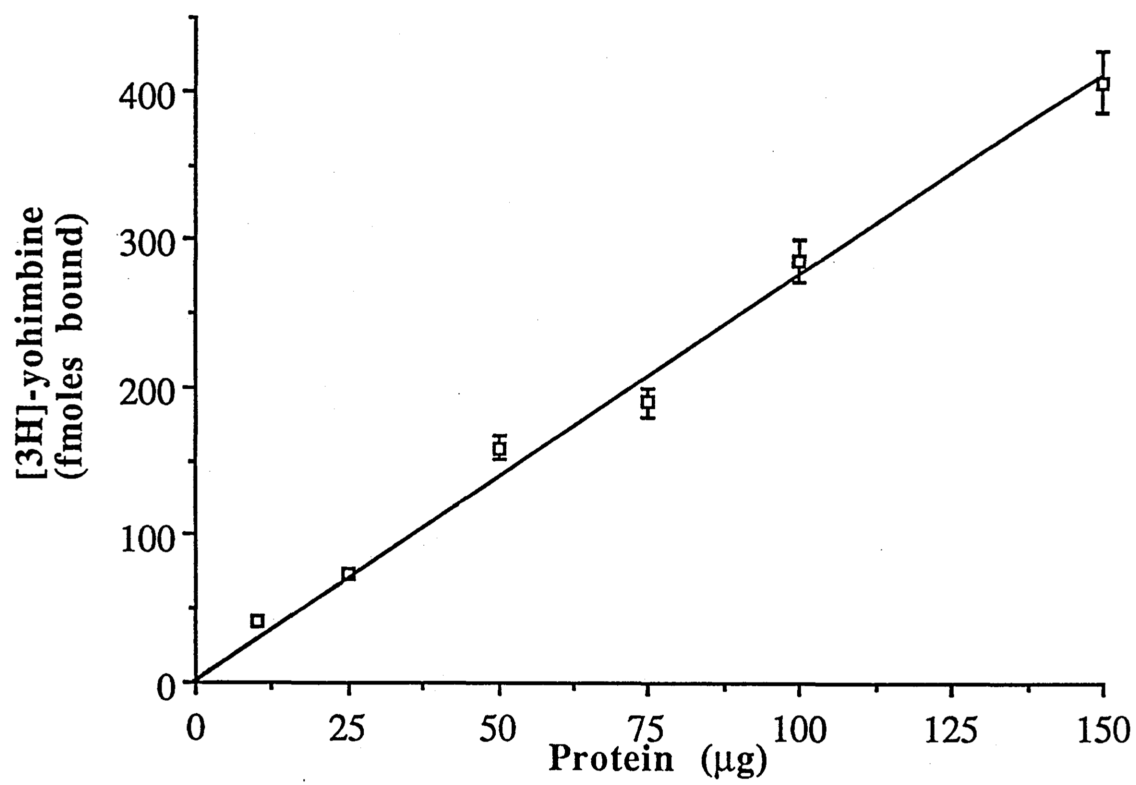


Figure 4.3a

SPECIFIC BINDING OF INCREASING CONCENTRATIONS OF [3H]-YOHIMBINE TO MEMBRANES FROM CLONE 1C CELLS

Membranes (17μg) of clone 1C cells were incubated for 30 minutes at 30°C with various concentrations of [^{3}H]-yohimbine as shown. Non-specific binding was determined in the presence of 100μM idazoxan, and increased in a linear fashion over the range of [^{3}H]-yohimbine concentrations, being 8-15% of total binding (not shown). Points shown are means (± SEM) from 4 separate experiments using different membrane preparations.

Figure 4.3b

SCATCHARD TRANSFORMATION

Data from Figure 4.3a have been transformed by the method of Scatchard (Scatchard, 1949). Results suggested a Kd of 1.0 (±0.2) nM (mean ± SEM, n=4), and a mean B_{max} of 3152 (±310) fmoles/mg.

Figure 4.3a

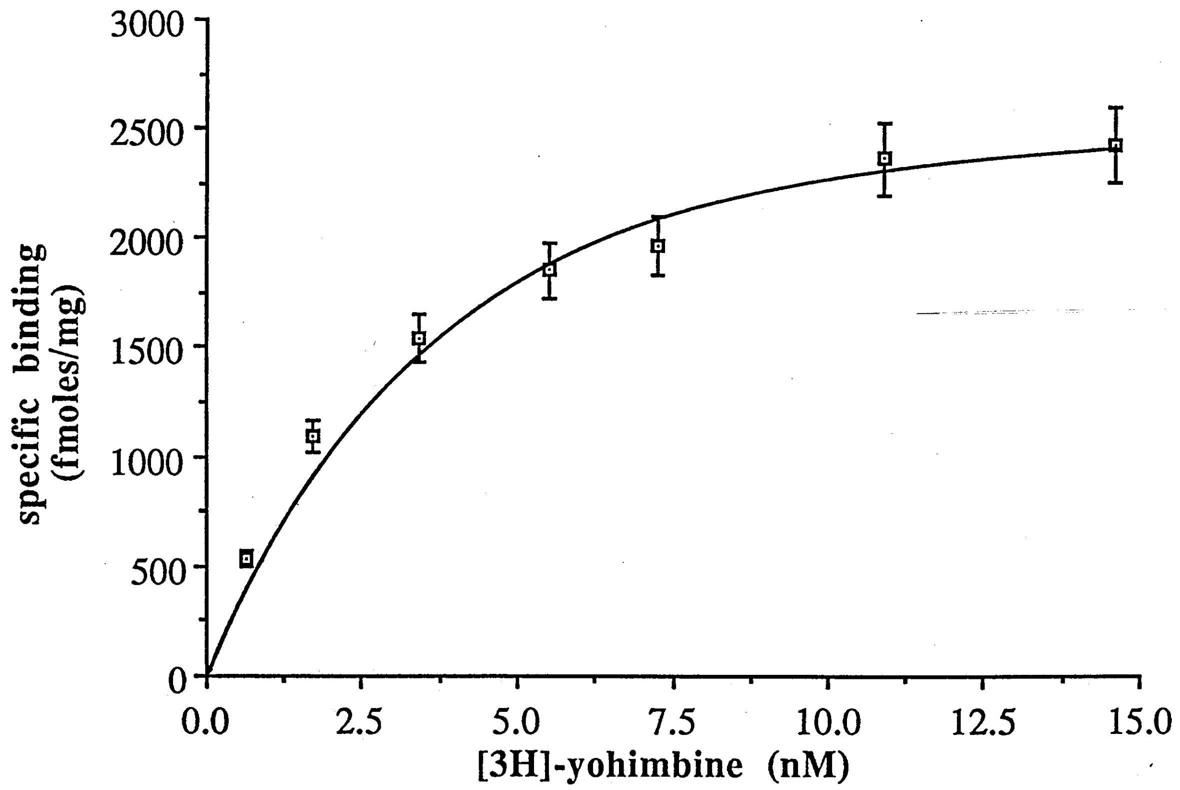


Figure 4.3b

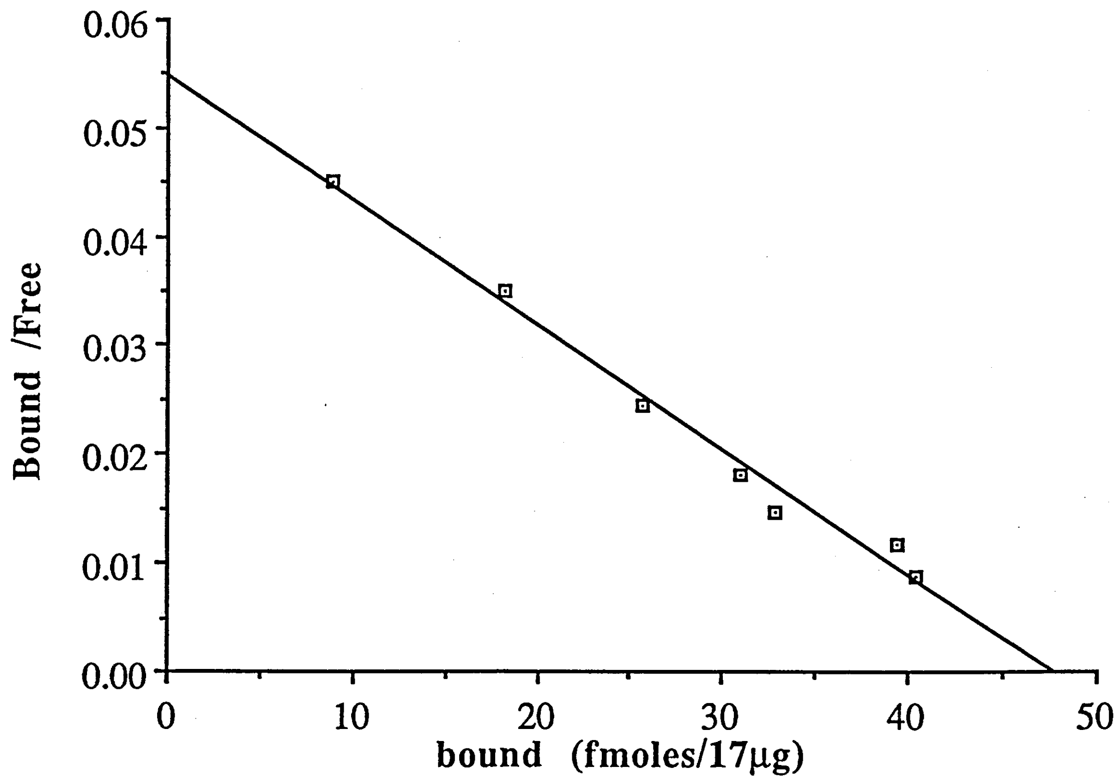


FIGURE 4.4

DISPLACEMENT OF [3H]-YOHIMBINE BINDING TO MEMBRANES FROM CLONE 1C CELLS BY OXYMETAZOLINE AND PRAZOSIN

Displacement of [^{3}H]-yohimbine (5nM) to membranes (50μg) of clone 1C cells by prazosin (❑) and oxymetazoline (◆) was assessed at 30°C for 30 minutes. Points shown are means of triplicate determinations (SD has been omitted for clarity, but was less than 10% of the mean in each case) from a single experiment which was repeated three times using different membrane preparations. For this experiment, IC_{50} for oxymetazoline was 31.0 nM, and for prazosin, 5.0 μM. 100% specific [^{3}H]-yohimbine binding was 2830 (±211) fmoles/mg for prazosin, and 2894 (±187) fmoles/mg for oxymetazoline.

Figure 4.4

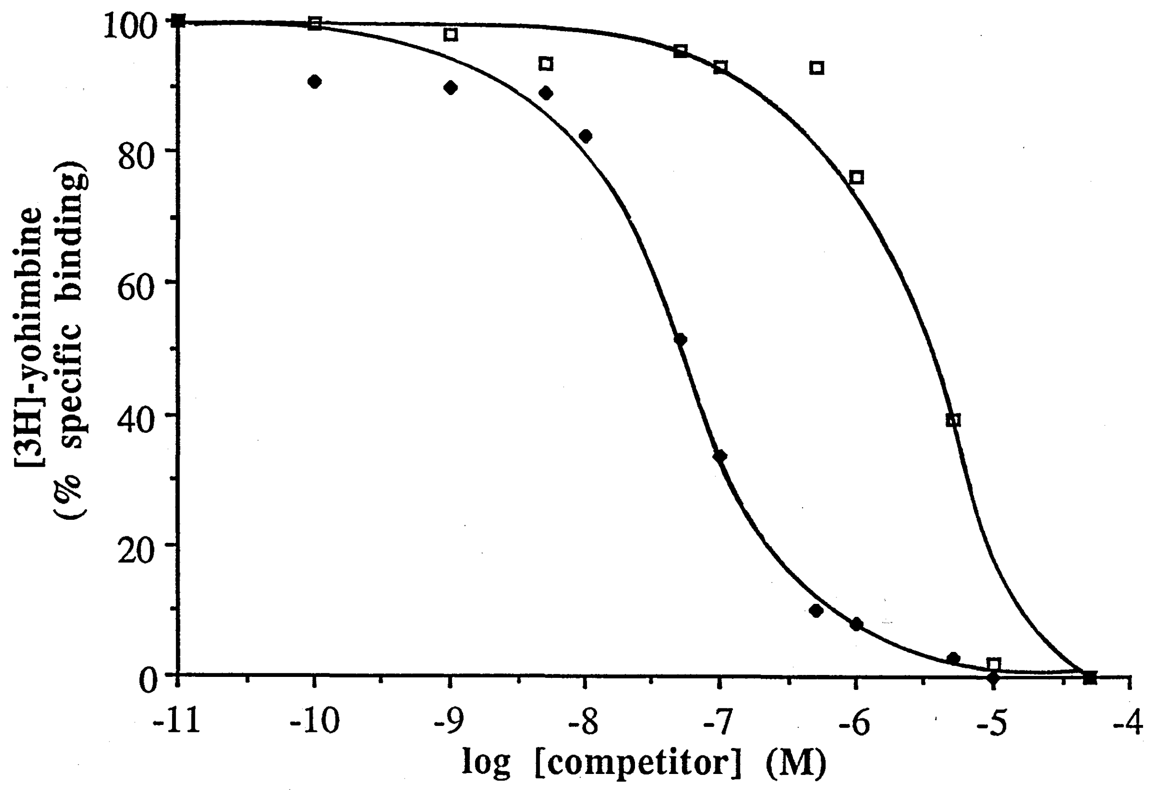


Figure 4.5

IMMUNOBLOT ANALYSIS OF MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells were separated on SDS-PAGE as described in Chapter 2, and western blotted onto nitrocellulose sheets. Western blots were incubated with anti-G-protein antisera as described in Chapter 2. Samples were incubated with 1:200 dilutions of:

A: antiserum I1C (1; 25 μg rat cerebral cortex membranes, 2; 50 μg clone 1C membranes)

B: antiserum SG1 (1; 25μg rat glioma C6BU1 membranes, 2; 50 μg clone 1C membranes)

C: antiserum I3B (1; 25μg rat glioma C6BU1 membranes, 2; 50 μg clone 1C membranes)

D: antiserum IM1 (1; 10 μg rat cerebral cortex membranes, 2; 50 μg clone 1C membranes).

E: antiserum CS1 (1; 25μg rat glioma C6BU1 membranes, 2; 50 μg clone 1C membranes)

Primary antisera were detected by incubation with a donkey-anti-rabbit horseradish peroxidase-linked second antiserum, as detailed in Chapter 2. The substrate for the enzyme was o-dianisidine.

Figure 4.5

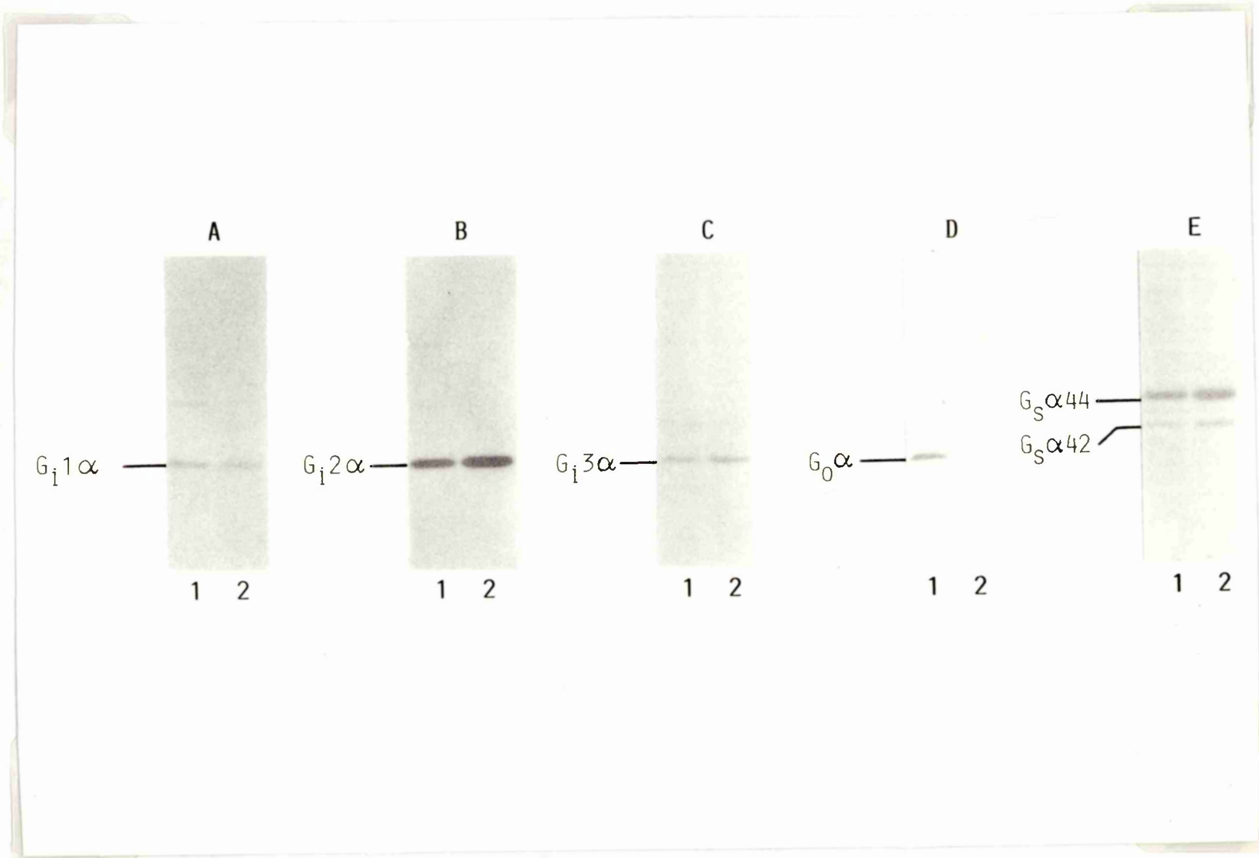


Figure 4.6

IMMUNOBLOT DETECTION OF β-SUBUNIT IN MEMBRANES FROM CLONE 1C CELLS.

Membranes from clone 1C cells (100μg) were separated on SDS-PAGE and western blotted onto nitrocellulose as described in Chapter 2. Western blots were then incubated with a 1:200 dilution of antiserum BN1, which detects the G-protein β-subunit. Blots were developed as described in the legend to Figure 4.5. Lanes are:

A: molecular weight standards

B. clone 1C membranes (100μg)

C. rat brain membranes (100μg)

Figure 4.6

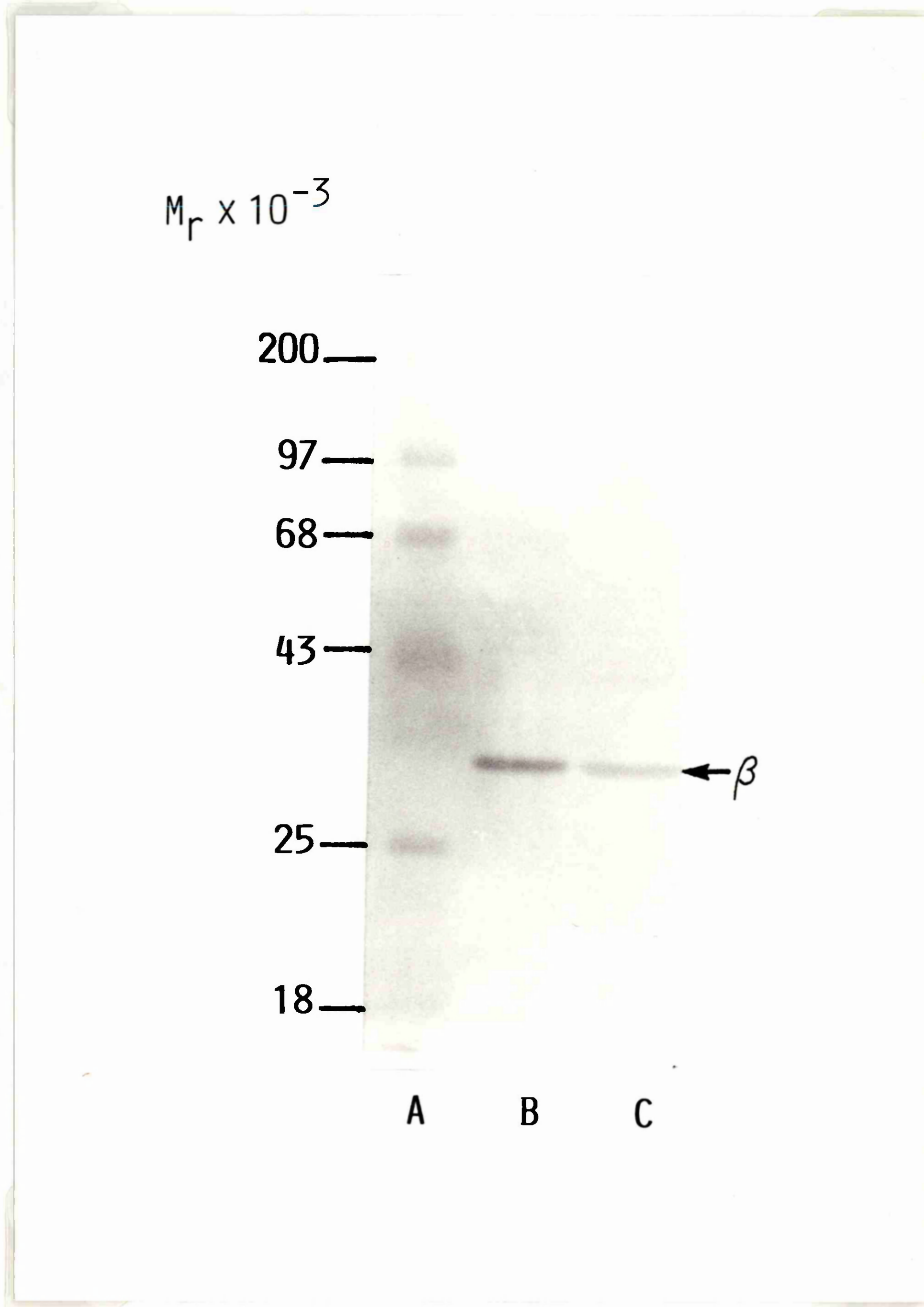


Figure 4.7a

PERTUSSIS TOXIN SUBSTRATES IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (50μg) were ADP-ribosylated by thiol-activated pertussis toxin for 90 minutes at 37°C,using [^{32}P]NAD^{+} as substrate, as detailed in Chapter 2. ADP-ribosylated membranes were separated on SDS-PAGE, and western blotted onto nitrocellulose. The nitrocellulose was immunoblotted with a 1:200 dilution of antisera, as indicated below. The immunoblot was developed, dried and subsequently autoradiographed, as described in Chapter 2. Lanes A-C show the region of the immunoblots encompassing proteins of the 35-45kDa range.

Lane A was immunoblotted with antiserum I3B, which recognises the α-subunit of Gi3.

Lane B was immunoblotted with antiserum AS7, which recognises the α-subunits of Gi2 and Gi1.

Lane C was immunoblotted with antiserum I1C, which recognises the α-subunit of Gi1.

Lane D shows an autoradiograph of the region of the immunoblot encompassing proteins of the 35-45kDa range.

None of the proteins identified incorporated radioactivity in the absence of activated pertussis toxin.

Figure 4.7a

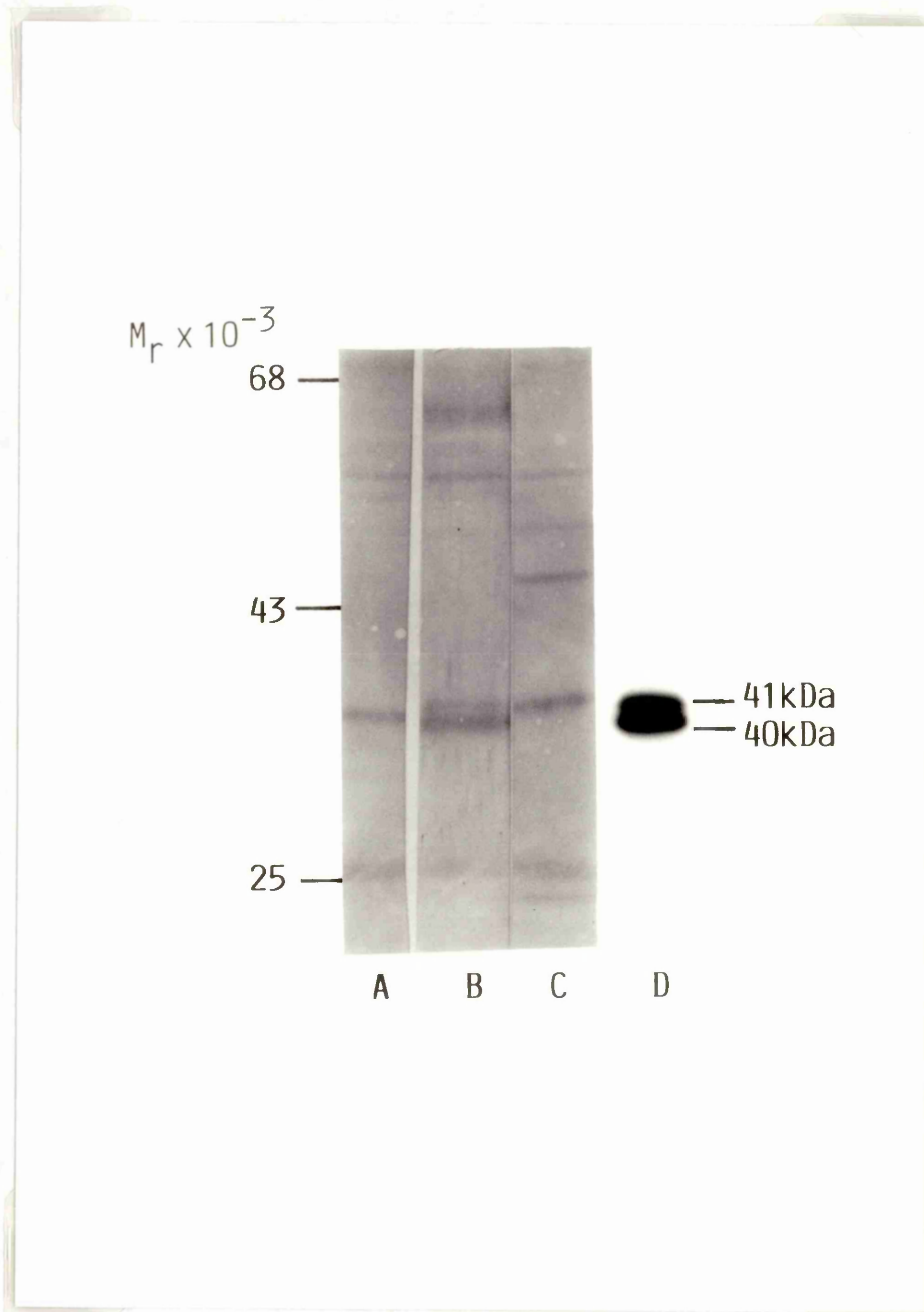


Figure 4.7b

PERTUSSIS TOXIN SUBSTRATES IN MEMBRANES FROM CLONE 1C CELLS AND NG108-15 CELLS

Membranes from clone 1C cells (50μg) and NG108-15 cells (50μg) were ADP-ribosylated by thiol-activated pertussis toxin for 90 minutes at 37°C, using [^{32}P]NAD^{+} as substrate, as detailed in Chapter 2. Ribosylated membranes were separated on SDS-PAGE, followed by drying of the gel, and subsequent autoradiography. This figure shows an autoradiograph of the region of the gel encompassing proteins of the 35-45kDa range.

Lane A: NG108-15 membranes (50μg)

Lane B: Clone 1C membranes (50μg)

None of the proteins identified incorporated radioactivity in the absence of activated pertussis toxin.

Figure 4.7b

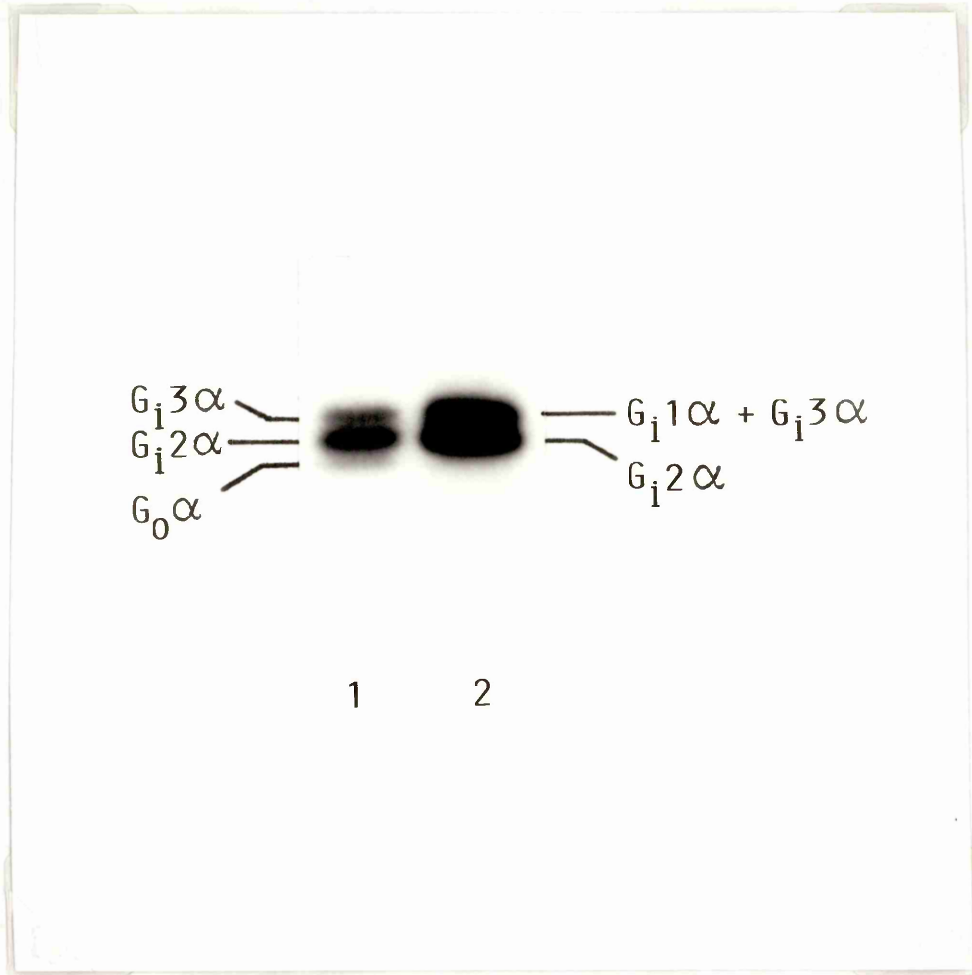


Figure 4.8

PERTUSSIS TOXIN SUBSTRATES IN MEMBRANES FROM PERTUSSIS TOXIN PRETREATED CLONE 1C CELLS

Clone 1C cells were grown in the presence of pertussis toxin (50ng/ml) for 16 hours, and membranes prepared as described in Chapter 2. Membranes from control and perussis toxin-pretreated clone 1C cells (50µg) were ADP-ribosylated by thiol-activated pertussis toxin for 90 minutes at 37°C, using [^{32}P]NAD^{+} as substrate, as detailed in Chapter 2. Ribosylated membranes were separated on SDS-PAGE, followed by drying of the gel, and subsequent autoradiography. This figure shows an autoradiograph of the region of the gel encompassing proteins of the 35-45kDa range.

Lane 1: Membranes (50µg) from untreated clone 1C cells

Lane 2: Membranes (50µg) from pertussis toxin-treated clone 1C cells

None of the proteins identified incorporated radioactivity in the absence of activated pertussis toxin.

Figure 4.8

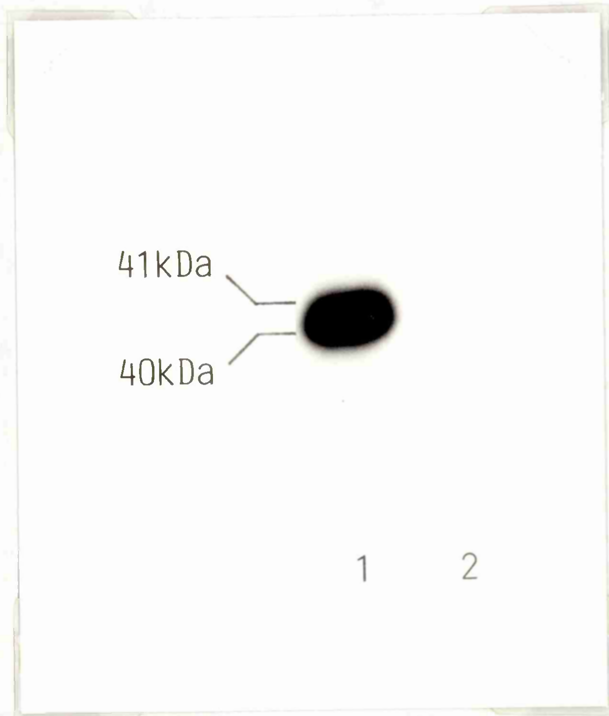


Figure 4.9

CHOLERA TOXIN SUBSTRATES IN MEMBRANES FROM CLONE 1C CELLS.

Membranes from clone 1C cells (50µg) were ADP-ribosylated by thiol-activated cholera toxin for 90 minutes at 37^oC, using [^{32}P]NAD^+ as substrate, as detailed in Chapter 2. ADP-ribosylated membranes were separated on SDS-PAGE, and western blotted onto nitrocellulose. The nitrocellulose was incubated with a 1:200 dilution of antiserum CS1, which detects the α-subunit of Gs, as described in Chapter 2. The immunoblot was developed, as described in the legend to Figure 4.5, dried and subsequently autoradiographed. This figure shows:

Lane A: an autoradiograph of the region of the immunoblot encompassing proteins of the 35-55kDa range.

Lane B: the region of the immunoblot encompassing proteins of the 35-55kDa range.

None of the proteins identified incorporated radioactivity in the absence of activated cholera toxin.

Figure 4.9

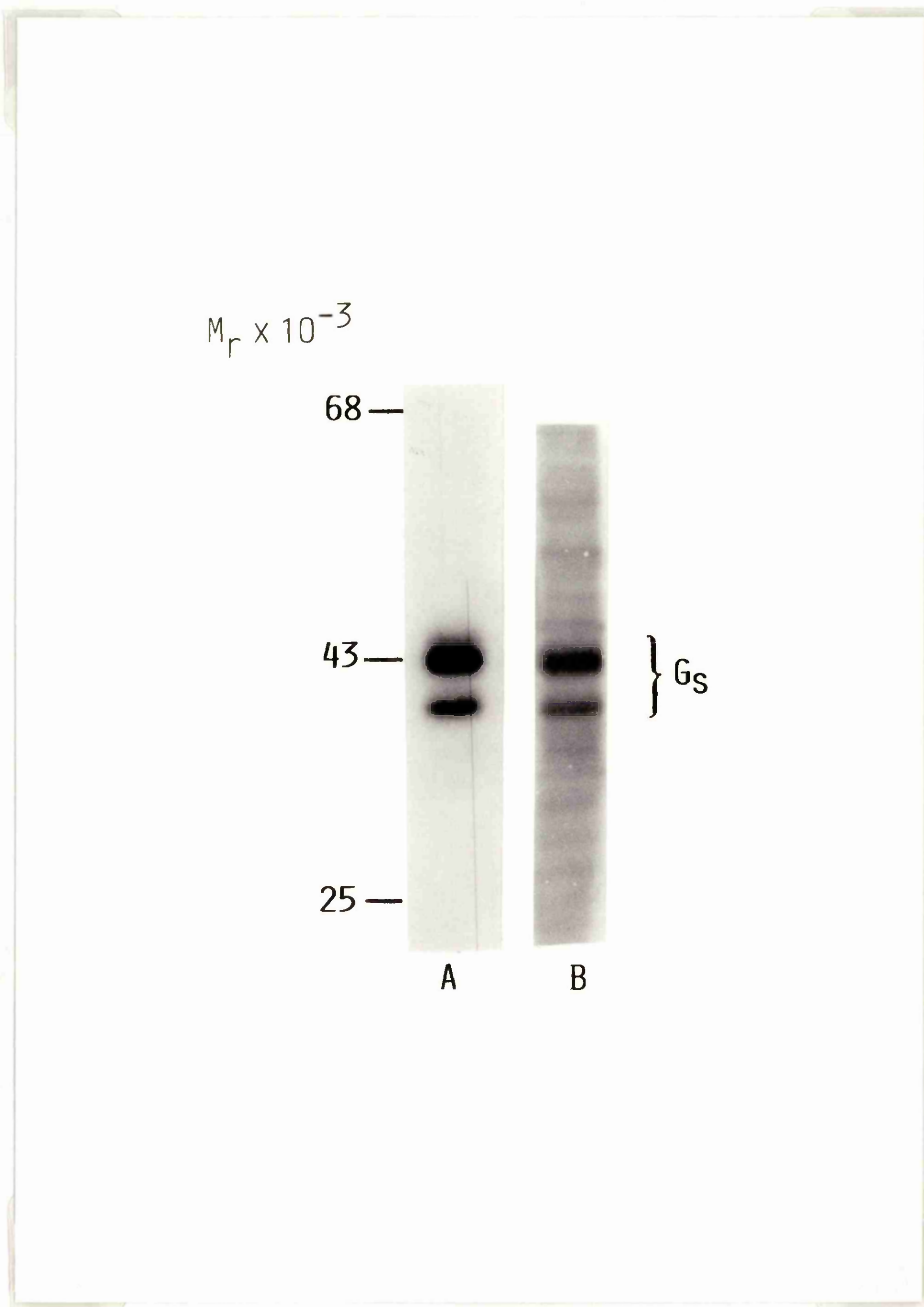


Figure 4.10

SPECIFICITY OF PROTEIN-A PURIFIED ANTISERA

Protein-A purified antisera were prepared as detailed in Chapter 2, and used at a dilution of 1:200 in 1% gelatin in TBS. Crude antisera were used as described in the legend to Figure 1. Membranes from clone 1C cells (50, and 100μg) were separated on SDS-PAGE and western blotted as described in Chapter 2. Western blots were incubated with crude and protein-A purified antisera as illustrated. Blots were developed as described in the legend to Figure 4.5.

Figure 4.10

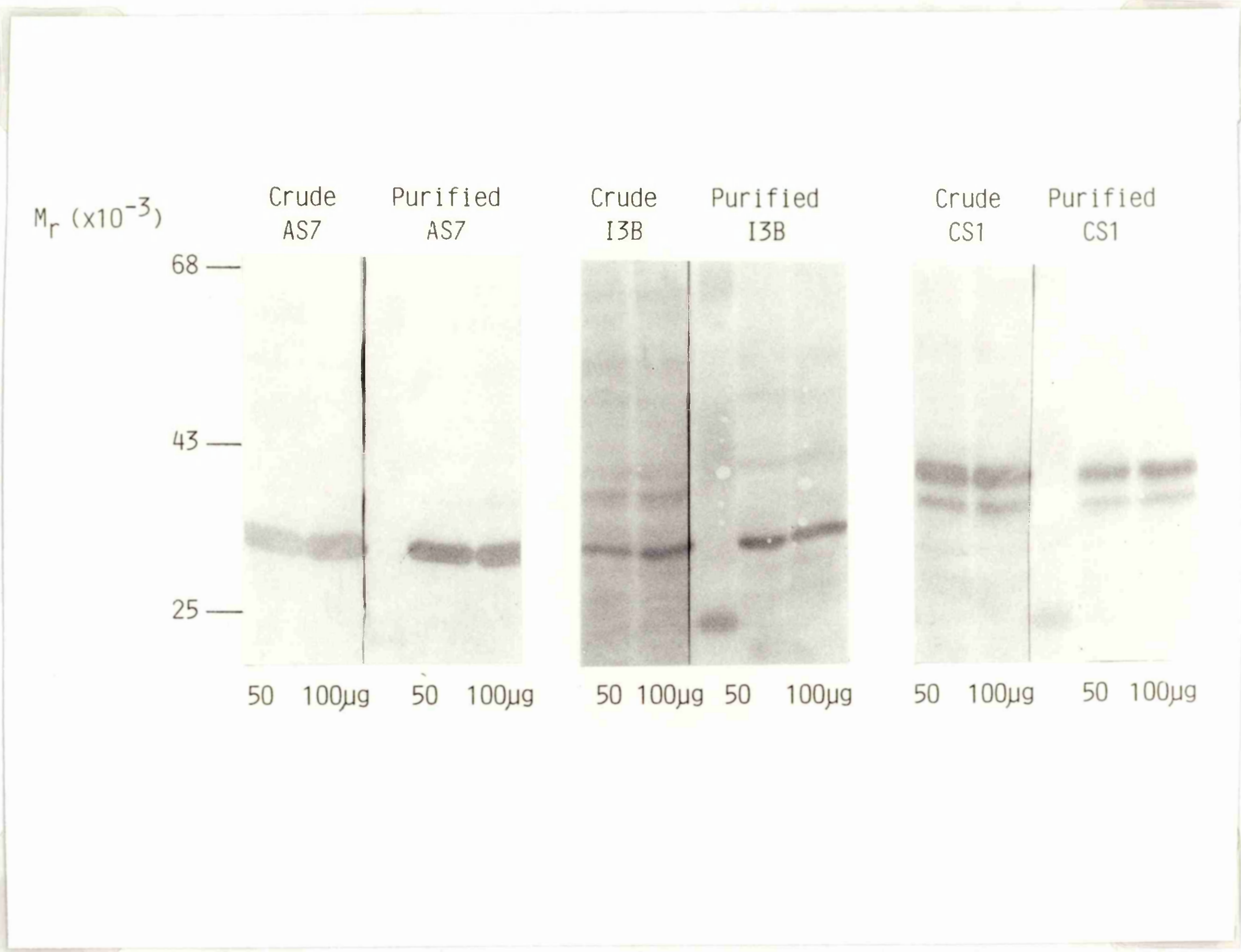


Figure 4.11

UK14,304 STIMULATED HIGH AFFINITY GTPASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C (10µg/sample) were assessed for high affinity GTPase activity for 20 minutes at 37°C as described in Chapter 2. Points are mean (±SEM) of three separate experiments using different clone 1C membrane preparations. EC_{50}=250.0 (±10.0) nM.

Figure 4.11

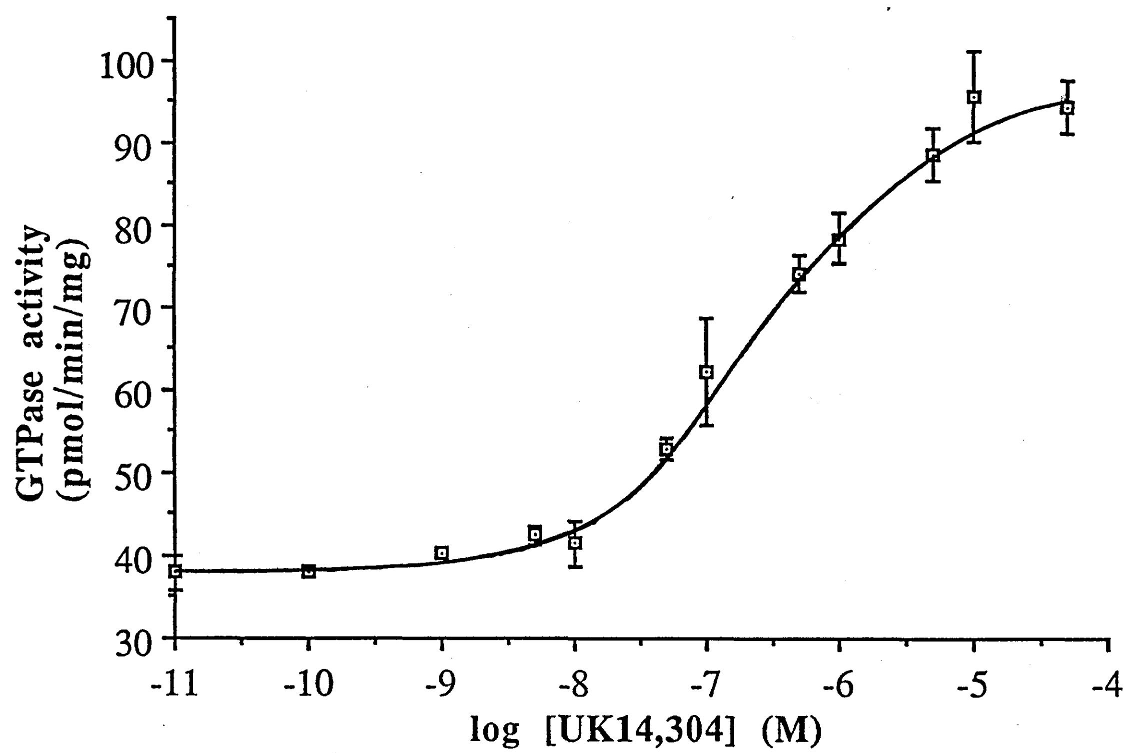


Figure 4.12

YOHIMBINE SENSITIVITY OF UK14,304 STIMULATED GTPASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes (10μg/sample) from clone 1C cells were incubated with yohimbine (0.1-50μM) before assessment of 10μM UK14,304 stimulation of GTPase activity at 37°C for 20 minutes, as described in Chapter 2. Results are mean (±SD) of triplicate determinations from a single experiment, which was repeated twice, using different membrane preparations, with similar results. For this experiment, IC_{50} = 150.0 (±9.3) nM.

Figure 4.12

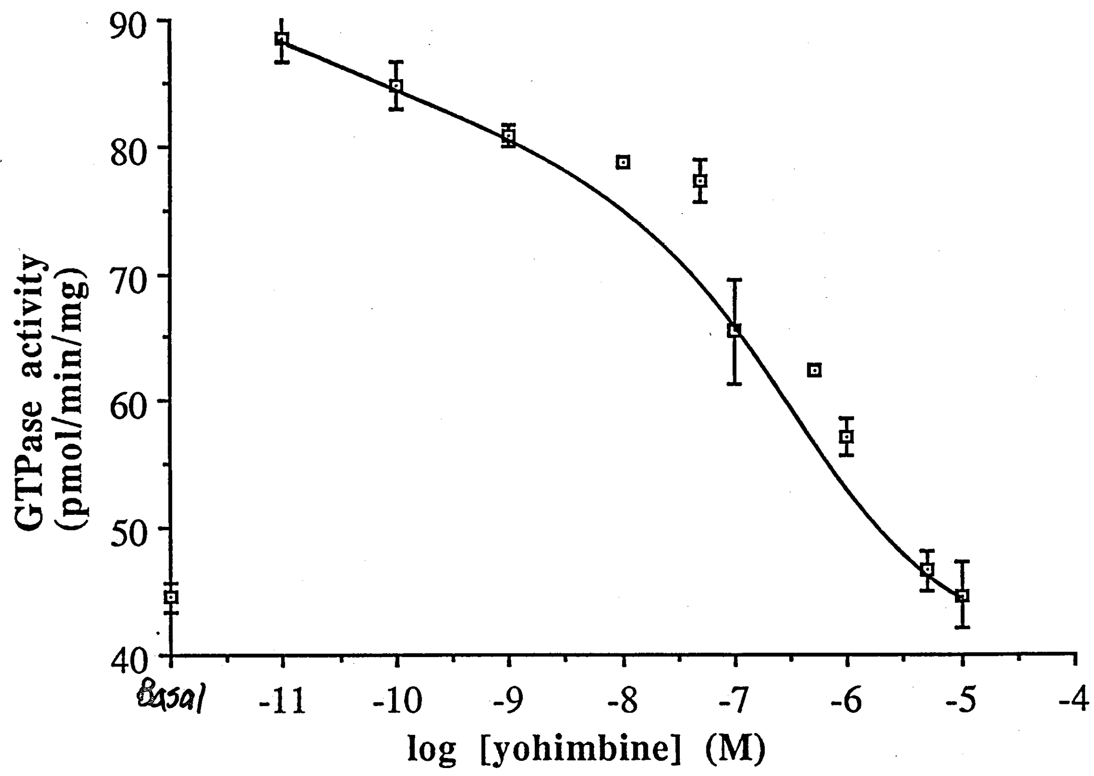


Figure 4.13

UK14,304 STIMULATION OF GTPASE ACTIVITY IN INCREASING AMOUNTS OF CLONE 1C MEMBRANES

Membranes from clone 1C cells, at the concentrations shown, were assessed for 10μM UK14,304 stimulation of GTPase activity for 20 minutes at 37°C, as described in Chapter 2. Results are means of triplicate determinations from a single experiment, which was repeated using a different membrane preparation, with similar results. SD has been omitted for clarity, but was less than 5% of the mean for all points. (□)=basal GTPase, (■)=stimulated GTPase, (O)=GTPase increase/ basal.

Figure 4.13

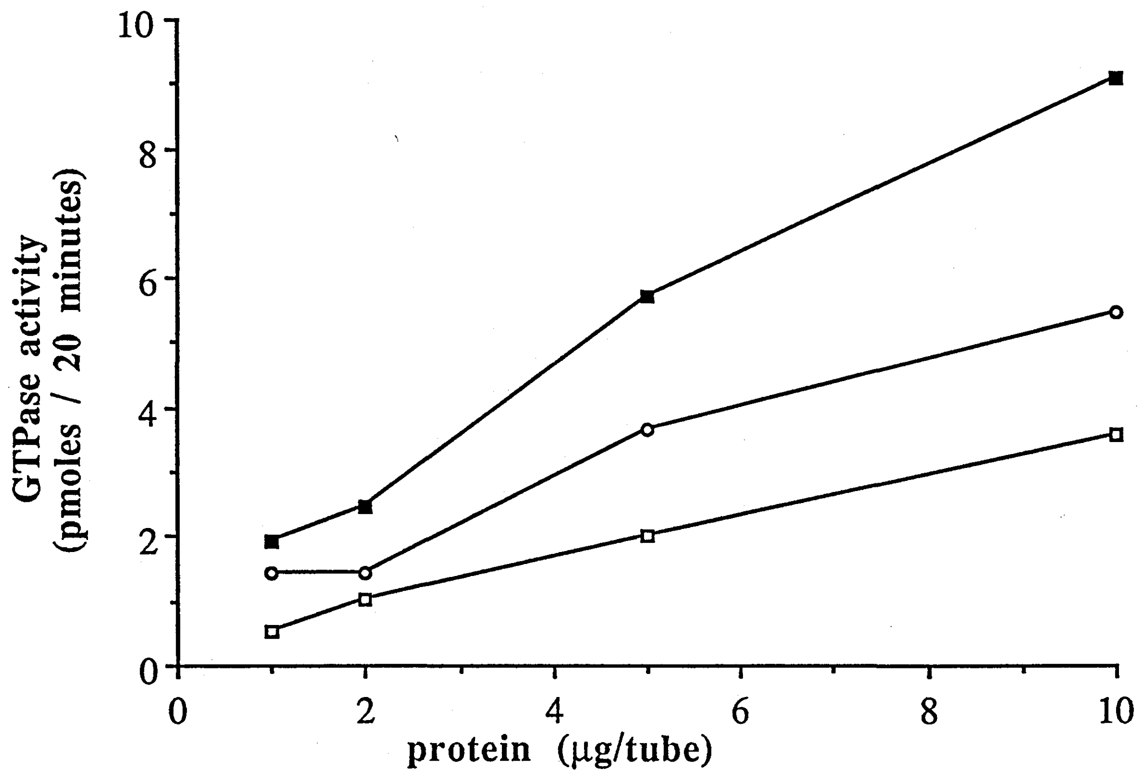


Figure 4.14

EFFECT OF 37°C PREINCUBATION ON UK14,304 STIMULATED GTPASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (10µg) were incubated at 37°C for the times shown before addition of GTPase reaction mixture and assessment of 10µM UK14,304 stimulated GTPase activity for 20 minutes at 37°C, as described in Chapter 2. Results are means (±SEM) of three experiments, performed using different membrane preparations. Data are expressed as % of basal (■) or of total (ie. basal + UK14,304 stimulated) response (□). 100% was 42.3 ± 2.2 pmol/min/mg membrane protein for basal GTPase samples, and 87.3 ± 5.6 pmol/min/mg membrane protein for total 10µM UK14,304 stimulated samples (means ±SEM, n=3).

Figure 4.14

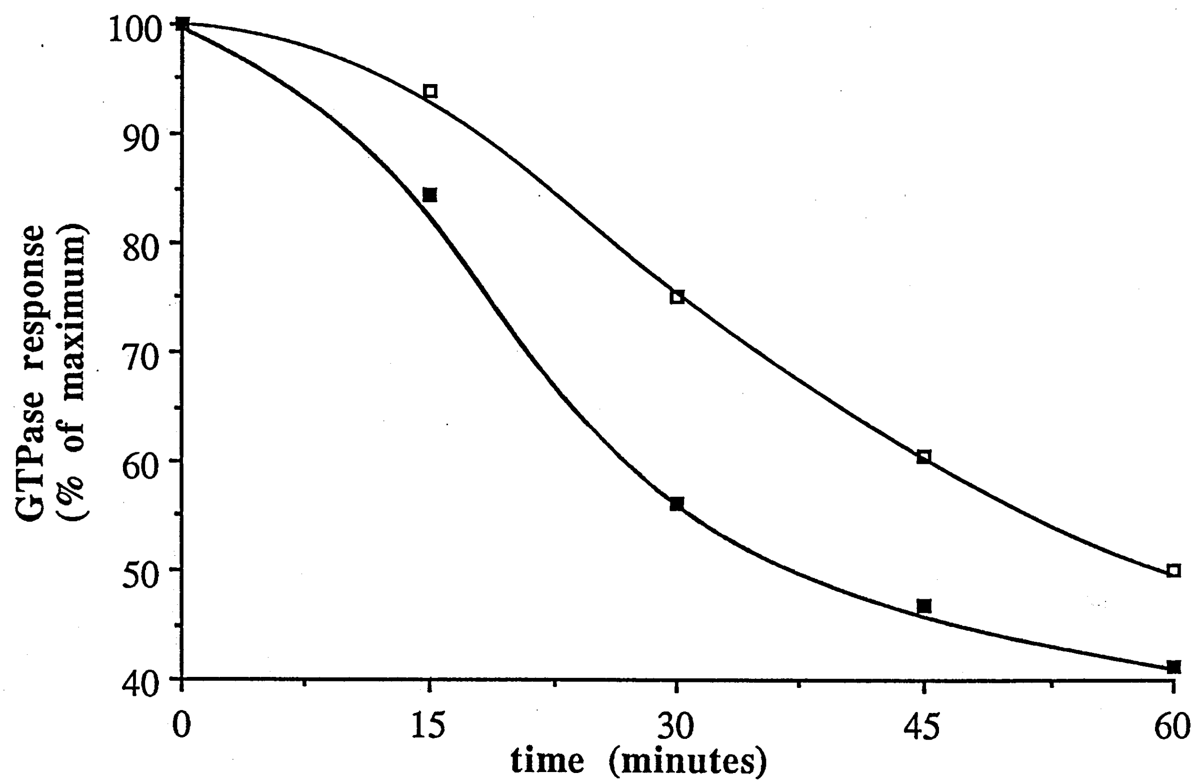


Figure 4.15

EFFECT OF PREINCUBATION WITH ANTI-G-PROTEIN ANTISERA ON UK14,304 STIMULATED GTPASE IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (5μg/sample) were preincubated for 1 hour at 37°C with protein-A purified IgG (10μg) from normal rabbit serum or from anti-G-protein antisera (10μg) as shown, in GTPase assay mix without [γ-^{32}P]GTP. After preincubation, [γ-^{32}P]GTP and 10μM UK14,304 were added and incubation continued for a further 20 minutes. Points are means (± SEM) from four experiments performed using different membrane preparations. Results are expressed as increases over basal GTPase. Basal GTPase activity was 20.8 ±4.0 pmoles/minute/mg membrane protein preincubated with H_2O, and 22.3 ± 1.4 pmoles/minute/mg membrane protein preincubated with IgG (means ±SEM, n=4). *significantly different from IgG samples, $P<0.001$ (Student's t-test).

Figure 4.15

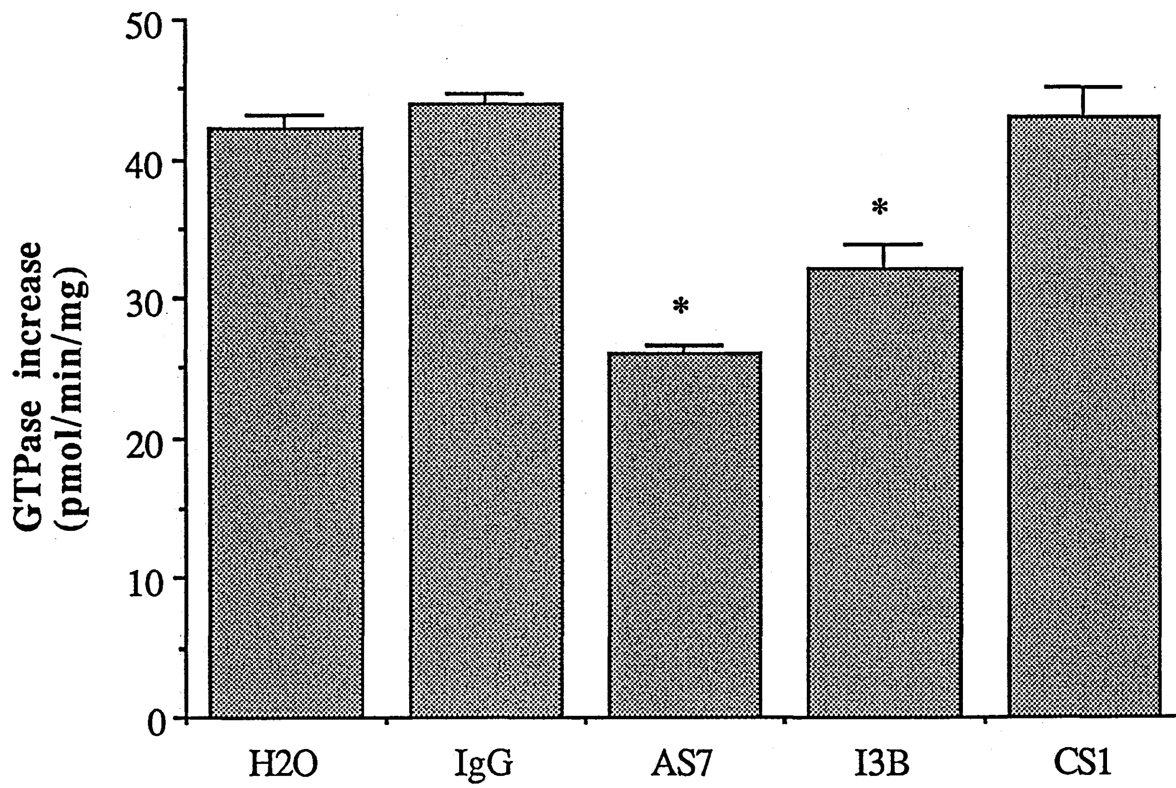


Figure 4.16

EFFECT OF PREINCUBATION WITH INCREASING CONCENTRATIONS OF AS7 ON UK14,304 STIMULATED GTPASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (5μg) were preincubated for 1 hour at 37°C with protein-A purified antiserum AS7 at the concentrations shown, before assessment of 10μM UK14,304 stimulated GTPase activity, as described in the legend to Figure 4.15. Points are means ± SEM from three experiments performed using different membrane preparations. 100% represented an increase over basal GTPase of 41.2 (±1.7) pmoles/min/mg membrane protein.

Figure 4.16

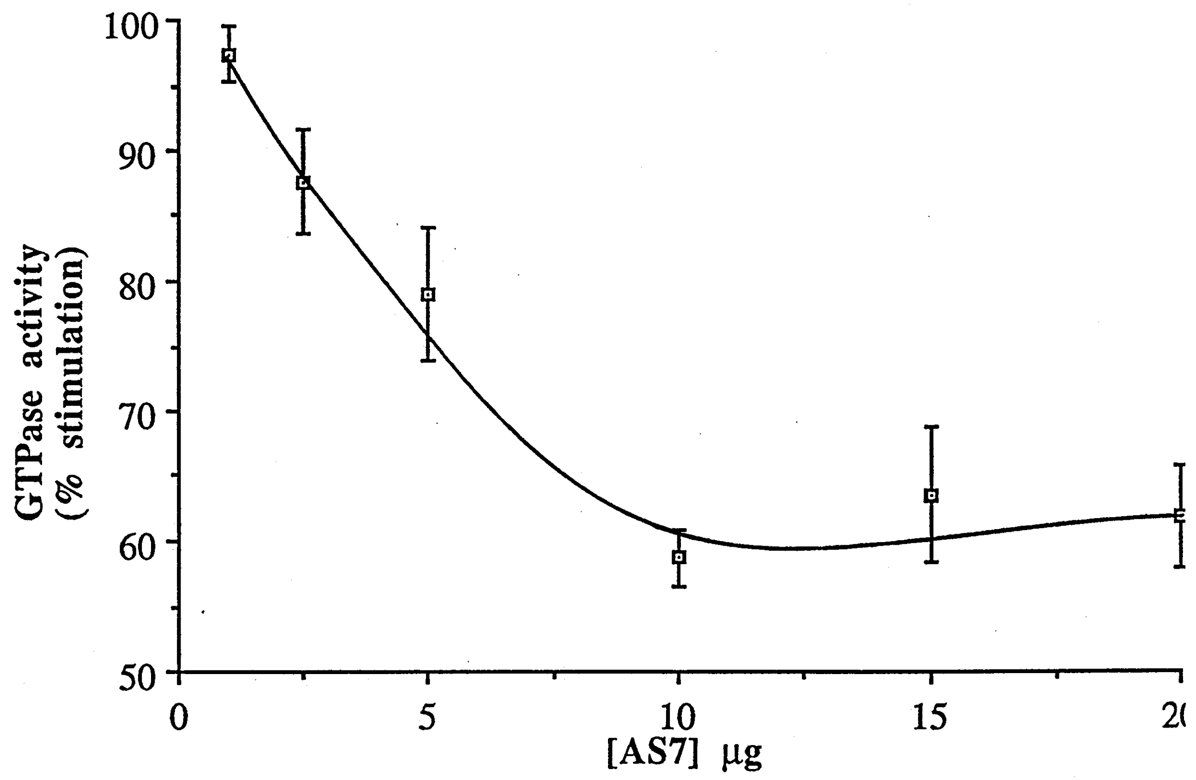


Figure 4.17

EFFECT OF PREINCUBATION WITH INCREASING CONCENTRATIONS OF I3B ON UK14,304 STIMULATED GTPASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (5μg) were preincubated for 1 hour at 37°C with protein-A purified antiserum I3B at the concentrations shown, before assessment of 10μM UK14,304 stimulated GTPase activity, as described in the legend to Figure 4.15. Points are means ± SEM from three experiments performed using different membrane preparations. 100% represented an increase over basal GTPase of 42.0 (±2.0) pmoles/min/mg membrane protein.

Figure 4.17

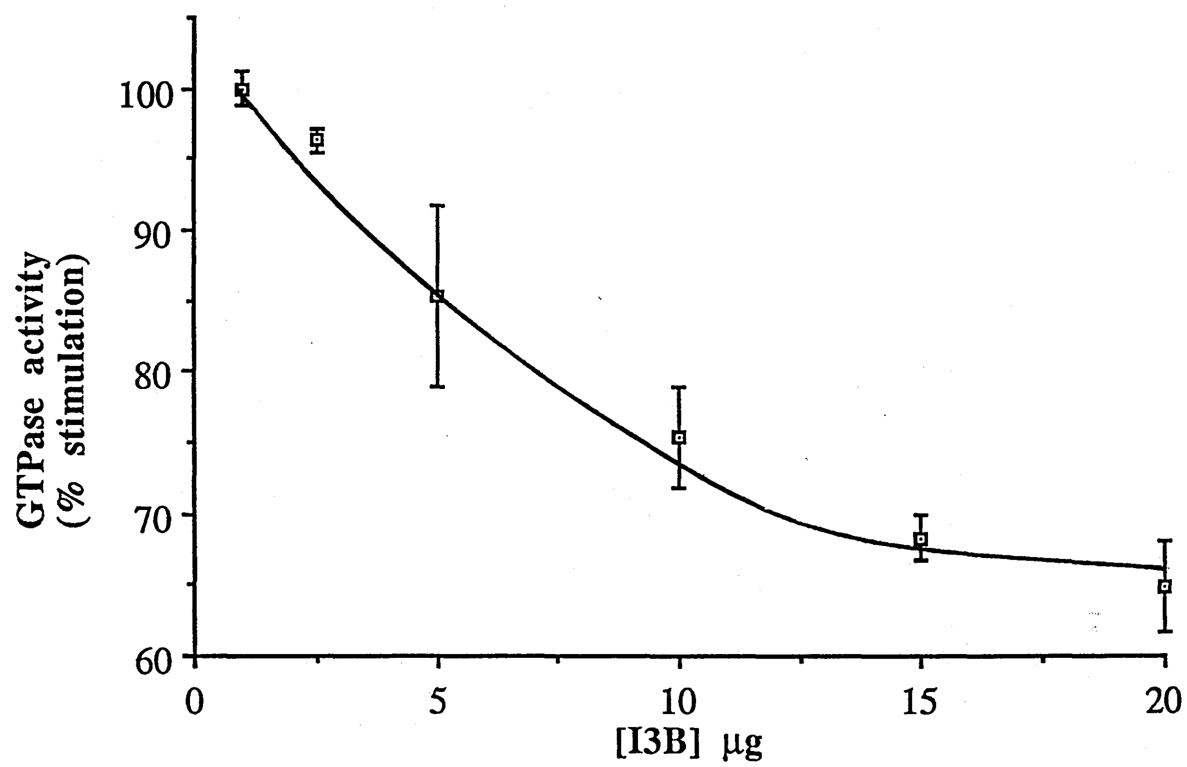


Figure 4.18

EFFECT OF CO-INCUBATION WITH AS7 AND I3B ON UK14,304 STIMULATED GTPASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (5µg) were preincubated with protein-A purified IgG from normal rabbit serum, or from antiserum AS7 and/or antiserum I3B (10µg), as shown, for 1 hour at 37°C, before assessment of 10µM UK14,304 GTPase activity, as described in the legend to Figure 4.15. Points are means ± SEM from six experiments, performed using different membrane preparations. Results are expressed as increases over basal GTPase, which was 24.5 (±3.0) pmoles/minute/mg membrane protein. *significantly different from IgG samples, P<0.001. **significantly different from AS7 or I3B samples alone, P<0.001, Student's t-test.

Figure 4.18

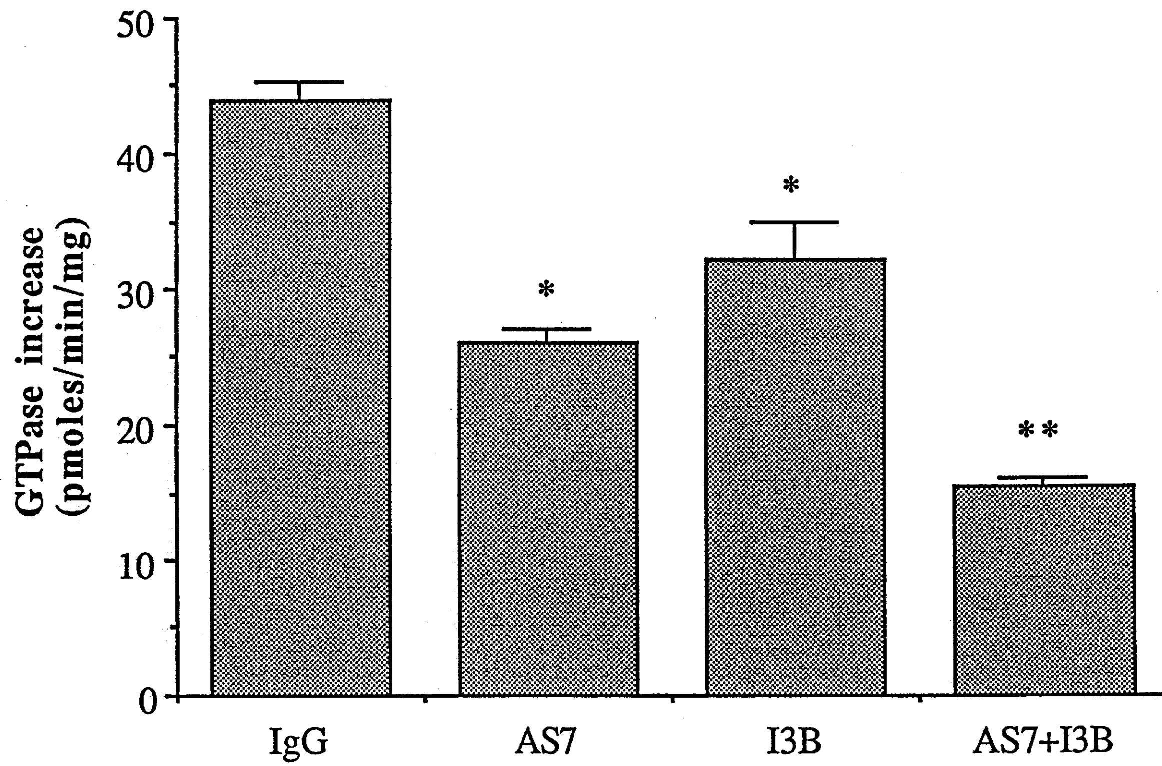


Figure 4.19

UK14,304 STIMULATED GTPASE ACTIVITY IN MEMBRANES FROM PERTUSSIS TOXIN PRETREATED CLONE 1C CELLS

Membranes (5μg) from control and pertussis toxin (50ng/ml) treated clone 1C cells were preincubated for 1 hour at 37°C with protein-A purified IgG from normal rabbit serum (10μg) , or from antiserum AS7 (10μg), before assessment of 10μM UK14,304 stimulatedGTPase activity, as described in the legend to Figure 4.15. Results are means ±SEM from three experiments performed on different membrane preparations. Results are expressed as increases over basal GTPase, which were 25.8 (±2.3) pmol/min/mg membrane protein for control membranes, and 13.7 (±1.1) pmol/min/mg membrane protein for pertussis toxin treated membranes.

Figure 4.19

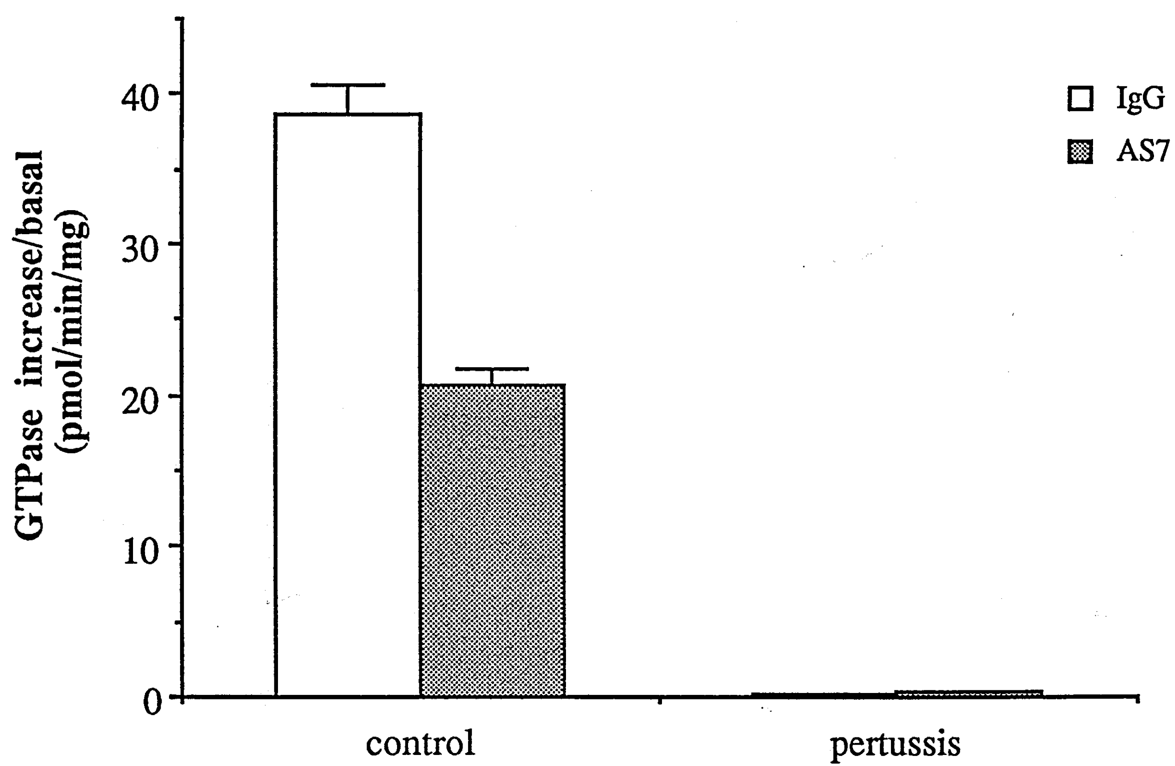


Figure 4.20

FORSKOLIN STIMULATED ADENYLYL CYCLASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Forskolin stimulated adenylyl cyclase activity in membranes from clone 1C cells (5μg) was assayed at 30°C for 15 minutes, as described in Chapter 2. Results are mean (±SEM) of three experiments performed on different membrane preparations. EC_{50}= 59.2 (±2.0) nM.

Figure 4.20

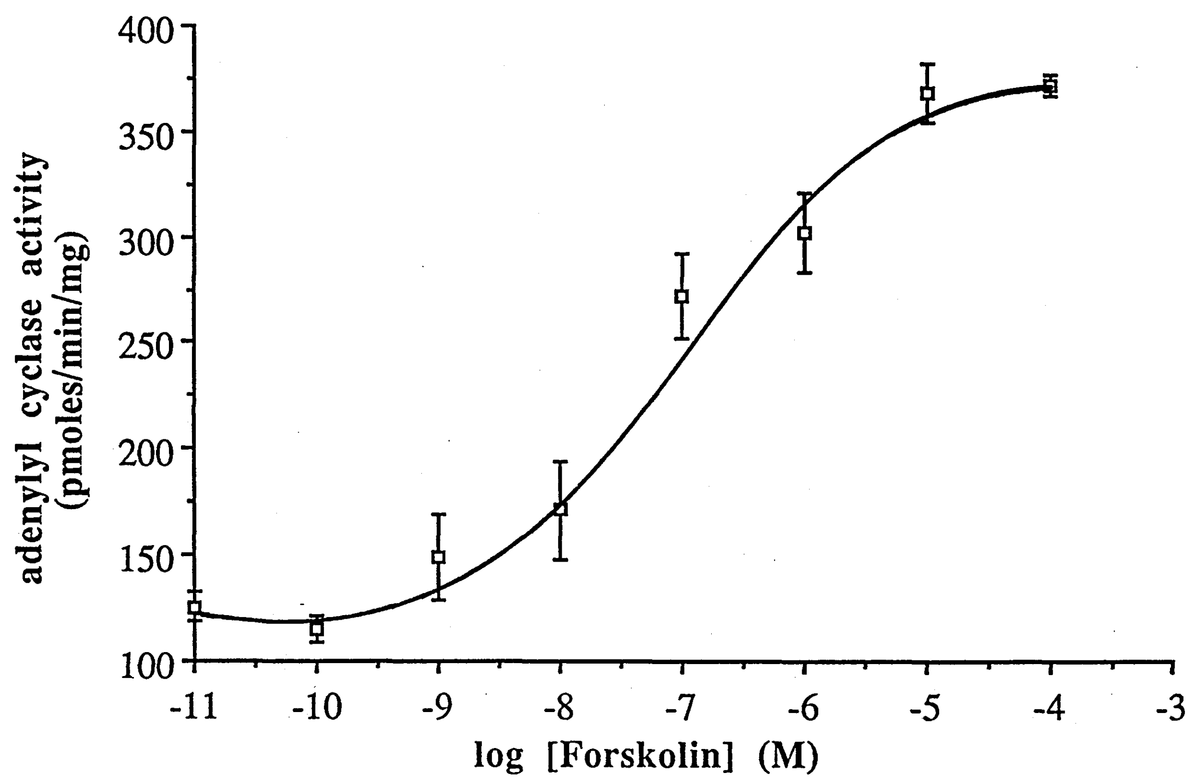


Figure 4.21

UK14,304 INHIBITION OF FORSKOLIN STIMULATED ADENYLYL CYCLASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

UK14,304 inhibition of 10μM forskolin-stimulated adenylyl cyclase activity in membranes (5μg) from clone 1C cells was assayed at 30°C for 15 minutes, as described in Chapter 2. Results are means (±SEM) from three experiments performed using different membrane preparations. IC_{50}=0.12 (±0.1)μM.

Figure 4.21

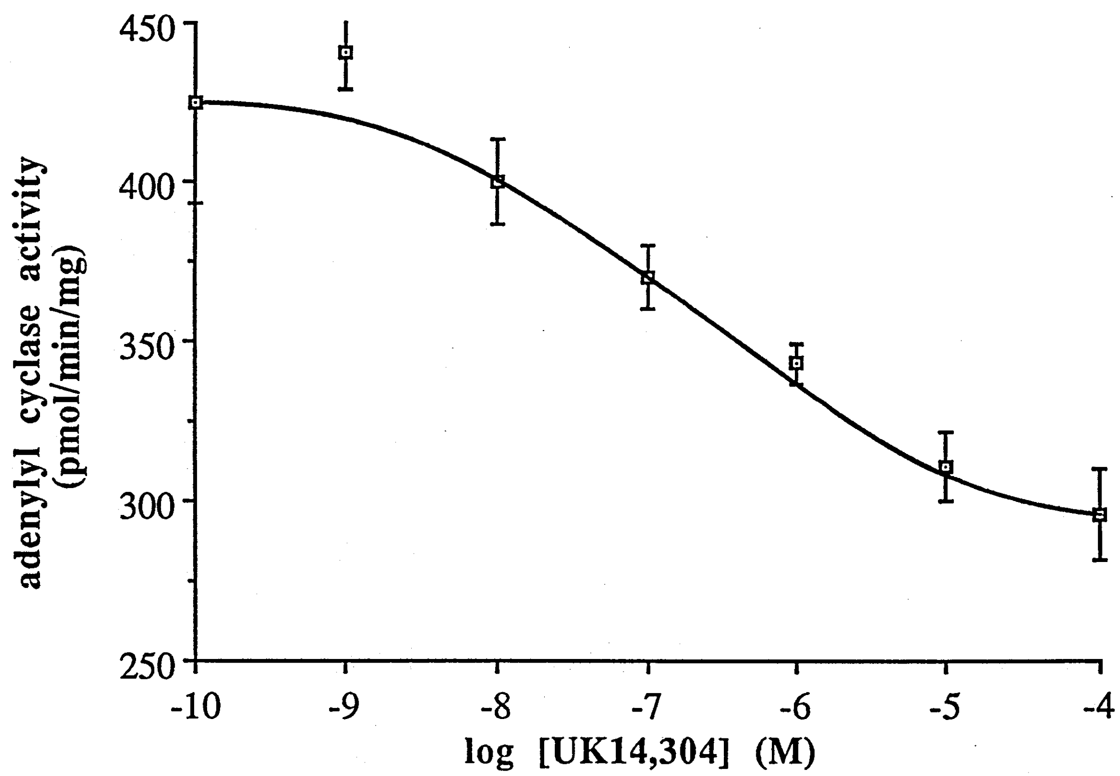


Figure 4.22

UK14,304 INHIBITION OF FORSKOLIN STIMULATED ADENYLYL CYCLASE ACTIVITY IN MEMBRANES FROM PERTUSSIS TOXIN TREATED CLONE 1C CELLS

Membranes (5μg) from clone 1C cells grown in pertussis toxin (50ng/ml), or control membranes were assayed for 10μM UK14,304 inhibition of 10μM forskolin stimulated adenylyl cyclase activity as described in Chapter 2. Results are means ±SEM from three experiments performed on different membrane preparations. 100% adenylyl cyclase activity was 476 (±33) pmoles/minute/mg membrane protein (control membranes) and 520 (±55) pmoles/minute/mg membrane protein (pertussis toxin treated membranes). *significantly reduced compared to control forskolin stimulated samples, Student's t-test.

Figure 4.22

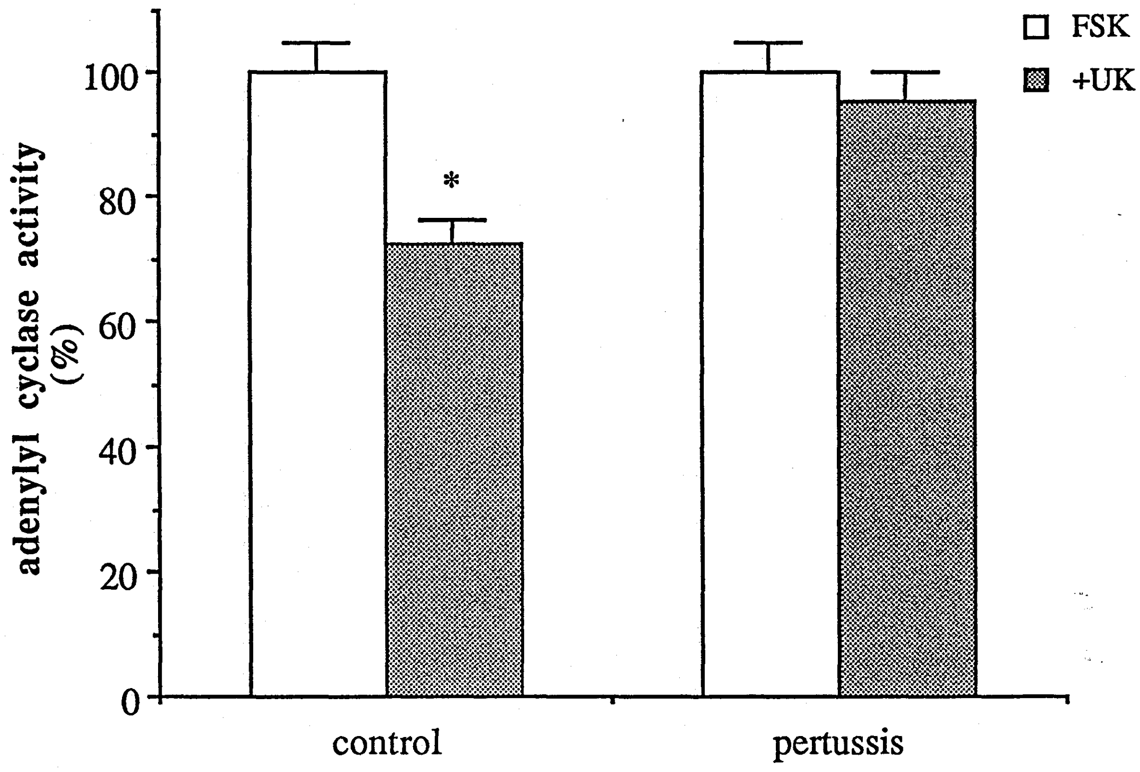


Figure 4.23

EFFECT OF 30ºC PREINCUBATION ON UK14,304 INHIBITION OF FORSKOLIN STIMULATED ADENYLYL CYCLASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (5μg) were incubated at 30ºC for the times shown in adenylyl cyclase mixture without added α[^{32}P]ATP. After the preincubation, α[^{32}P]ATP, 10μM forskolin and 10μM UK14,304 were added, and incubation continued for a further 15 minutes. Assay of adenylyl cyclase activity was as described in Chapter 2. Results are means of triplicate determinations from a single experiment which was repeated twice using different membrane preparations, with similar results. SD has been omitted for clarity, but was less than 10% of the mean in all cases. Symbols are: basal adenylyl cyclase (❑), 10μM forskolin (▲), 10μM UK14,304 (■). Mean UK14,304 inhibition over the preincubation period was 37.3(±5.3) pmoles/minute/mg membrane protein.

Figure 4.23

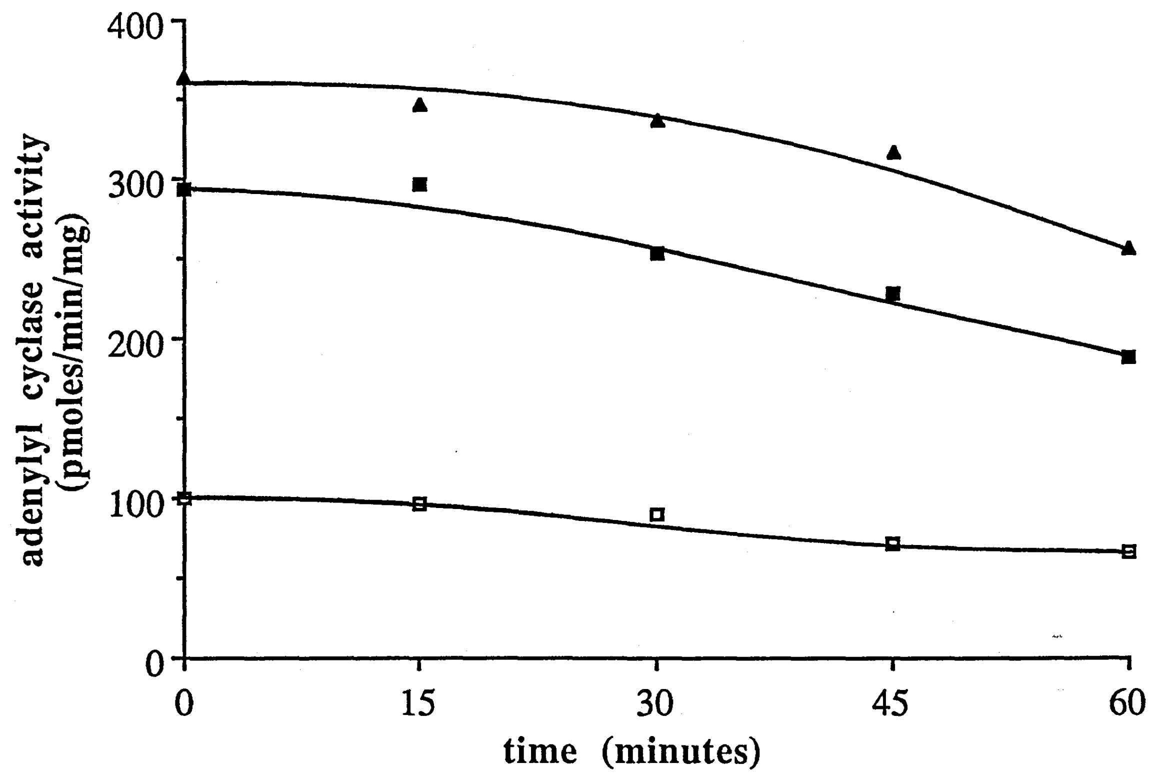
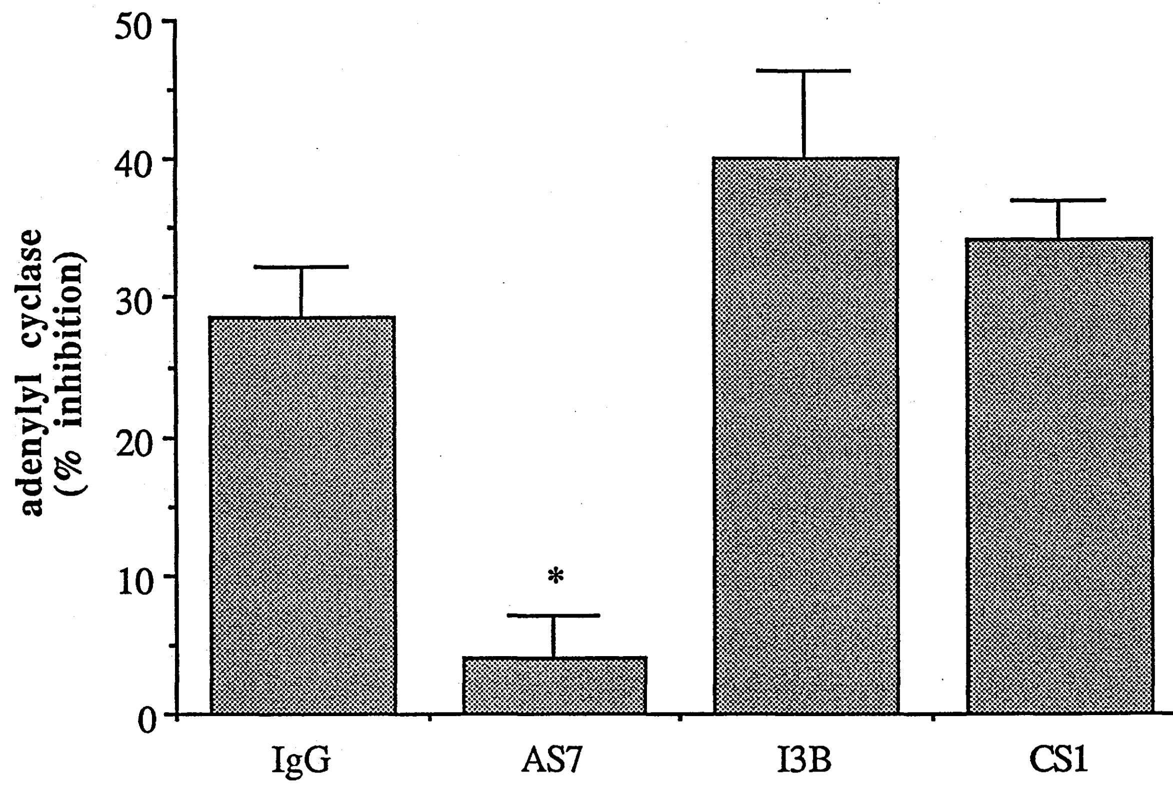


Figure 4.24

EFFECT OF PREINCUBATION WITH ANTI-G-PROTEIN ANTISERA ON UK14,304 INHIBITION OF FORSKOLIN-STIMULATED ADENYLYL CYCLASE ACTIVITY IN MEMBRANES FROM CLONE 1C CELLS

Membranes from clone 1C cells (5µg) were preincubated for 1 hour at 30°C with protein-A purified anti-G-protein antisera (10µg/sample) in adenylyl cyclase mixture without α[^{32}P]ATP. After preincubation, α[^{32}P]ATP, 10µM forskolin and 10µM UK14,304 were added, and incubated for a further 15 minutes. Assay of adenylyl cyclase activity was as described in Chapter 2. Results are means (±SEM) from four experiments performed using different membrane preparations. Basal adenylyl cyclase activity was 67.5 (±3.3) pmoles/minute/mg, forskolin stimulated activity was 257.3 (±25.2) pmoles/minute/mg (mean ±SEM). *significantly different from IgG samples, $P<0.001$ (Student's t-test).

Figure 4.24



DISCUSSION

The Rat 1 fibroblast is a clonal cell line which has been shown to express receptors for endothelin, bradykinin, and platelet-derived growth factor, all coupled to stimulation of phospholipase C (Muldoon *et al.* 1989; Parries *et al.* 1987). Rat 1 fibroblasts do not appear to express an α_2-adrenergic receptor *in vivo* (Milligan *et al.* 1991). The Rat 1 fibroblast clone used in these studies (clone 1C) was transfected with the genomic DNA for the human platelet α_{2A} receptor (clone α2-C10) by Milligan *et al.* in order to examine the specificity of coupling of this receptor to its G-proteins (Milligan *et al.* 1991).

In their initial report on clone 1C, Milligan *et al.* measured agonist activation of the α_{2A} receptor by using a method of $[^{32}P]NAD^+$ dependant $[^{32}P]$ADP-ribosylation of "Gi", wherein G-proteins which are normally pertussis toxin substrates are ADP-ribosylated by cholera toxin. This method arose from the observation that, in the absence of added guanine nucleotides, cholera toxin could catalyse the ADP-ribosylation of a 40kDa protein in a mouse macrophage cell line - ie. that it could ADP-ribosylate substrates other than Gsα (Aksamit *et al.* 1985). All G-protein α-subunits possess an arginine residue equivalent to that which is the substrate for cholera toxin-catalysed ADP-ribosylation, however, in the case of "Gi"-like G-proteins (ie. the class of G-proteins which are pertussis toxin substrates) it is believed that some conformational change, dependant on the presence of guanine nucleotide, hides this residue, making it inaccessible to cholera toxin under conventional ADP-ribosylation conditions (Milligan, 1988). It has been shown that in the absence of guanine nucleotide, and in the presence of agonists which activate the receptors coupled to "Gi"-like G-proteins, there is an enhancement of

cholera toxin-catalysed ADP-ribosylation of these "Gi"-like G-proteins (Giershik and Jacobs, 1987; Milligan and McKenzie 1988). The explanation of this enhancement of ADP-ribosylation in the presence of an agonist was that receptor activation catalysed GDP release in the normal manner (see Chapter 1), but that the lack of added guanine nucleotides resulted in an α-subunit without any bound guanine nucleotide, thus exposing the arginine residue which is the site for cholera toxin action (Milligan, 1988).

Milligan *et al.* used this method to demonstrate that, in the absence of guanine nucleotides, and in the presence of 10μM UK14,304, cholera toxin catalysed the incorporation of radiolabelled [^{32}P]ADP-ribose into a 40kDa protein, as well as the 45 and 42kDa forms of Gsα (Milligan *et al.* 1991). Using the antisera SG1 and I3B, which identify the α-subunits of Gi1+Gi2, and Gi3, respectively, they found that it was possible to immunoprecipitate two radiolabelled proteins in a manner dependant on UK14,304 activation of the α_{2A} receptor; these proteins were identified as Gi2 and Gi3α. It was concluded that the α_{2A} receptor that had been transfected into the Rat 1 fibroblast clone 1C must therefore be coupled to Gi2 and Gi3 (Milligan *et al.* 1991).

The results of Milligan *et al.* have essentially been confirmed here, although the measurement of UK14,304 stimulated high affinity GTPase activity allows a more precise quantification of receptor activation than does the use of UK14,304 stimulation of cholera toxin [^{32}P]ADP-ribosylation of "Gi". Furthermore, the GTPase results reported here suggest that Gi2 and Gi3 alone do not account for the full extent of α_{2A} receptor-stimulated GTPase activity in clone 1C cells, and that another G-protein may therefore be involved in coupling to this receptor.

The identity of the receptor expressed in clone 1C cells was confirmed by radioligand binding. [^{3}H]-yohimbine binding suggested the

presence of a single high affinity binding site in membranes from clone 1C cells (Figure 4.3a, 4.3b) with a Kd of 1nM, and a Bmax of some 3pmoles/mg membrane protein. The α_2-receptor subtype was characterised by displacing [^{3}H]-yohimbine with oxymetazoline and prazosin, which allows discrimination of the α_{2A} and α_{2B} subypes (Bylund 1988). The Ki values obtained (Figure 4.4) indicated a receptor with high affinity for oxymetazoline (Ki=1.4nM) but a substantially lower affinity for prazosin (Ki=900nM). These data are characteristic of a receptor of the α_{2A} subtype, and confirm the identity of the receptor transfected into this cell line (Milligan *et al.* 1991).

The G-protein complement of clone 1C cells was assessed by immunoblotting membranes from cells with antibodies raised against specific G-proteins, as described in Chapter 2 (Figures 4.5, 4.6). The presence of three forms of "Gi" was demonstrated by immunoblotting membranes from clone 1C cells with the antisera raised against Gi1, Gi2, and Gi3 (Figure 4.5). Antiserum AS7, which was raised against the C-terminus of transducin$_\alpha$, and which also recognises the C-termini of Gi1$_\alpha$ and Gi2$_\alpha$, therefore recognises the α-subunits of both Gi1 and Gi2 in membranes from clone 1C cells. This contrasts with the findings for NG108-15 cells (Chapter 3, and McKenzie *et al.* 1988), where antiserum AS7 recognises Gi2α only, as Gi1 appears not to be expressed in NG108-15 cells (McKenzie and Milligan, 1990). The presence of Gi1 and Gi2 in clone 1C cells limits the conclusions that can be drawn from the use of antiserum AS7 in "uncoupling" experiments with membranes from these cells, however the use of antiserum AS7 cannot be avoided or improved upon in these circumstances, because it is the C-terminus of the G-protein α-subunit which is thought to be responsible for coupling to the receptor (Chapter 1), and it is this part of Gi1 and Gi2 which is recognised by antiserum AS7.

The pertussis toxin substrates from membranes of clone 1C cells are shown in Figure 4.7a to be Gi1, Gi2, and Gi3. Go was not detected on immunoblots (Figure 4.7a) and comparison of pertussis toxin-catalysed [^{32}P]ADP-ribosylated membranes from clone 1C cells with those from membranes of NG108-15 cells, which do express Go (Figure 4.7b), also failed to detect the α-subunit of Go. These results suggested that, in clone 1C cells, Go was either expressed at levels not detectable by immunoblotting or radiolabelled ADP-ribosylation, or that Go was not expressed at all.

It was a notable feature of membranes from clone 1C cells that the relative levels of Gi1,Gi2 and Gi3, as assessed by immunoblotting, appeared to be markedly different (Figure 4.5) . Gi2 levels appeared to be much higher than those of either Gi1 or Gi3, and Gi1 levels appeared to be the lowest of the three pertussis toxin substrates. The relative levels of all the pertussis toxin substrates were compared with those from NG108-15 cells both by immunoblotting (results not shown) and by pertussis toxin-catalysed [^{32}P]ADP-ribosylation (Figure 4.7b). These data indicated that levels of Gi2, in particular, were greater in membranes from clone 1C cells than in equivalent amounts of NG108-15 cells. This fact may be of importance in the discussion of the relative abilities of anti-G-protein antibodies to uncouple adrenergic receptors from functional responses in NG108-15 and clone 1C cells (see below).

Immunoblotting with the antiserum CS1, which recognises Gsα, and cholera toxin-catalysed [^{32}P]ADP-ribosylation of membranes from clone 1C cells, both suggested that two forms of Gsα with apparent molecular weights of 44 and 42kDa were expressed in clone 1C cells (Figures 4.5, 4.9). The higher molecular weight form appears to be predominant. As with NG108-15 cells, it is not clear which of the four isoforms of Gsα known to date are expressed in clone 1C cells (Bray *et al.* 1986).

Incubation of membranes from clone 1C cells with the α_2-adrenergic full agonist UK14,304 (bromoxidine) resulted in a substantial stimulation of high affinity GTPase activity (Figure 4.11). The magnitude of this GTPase stimulation (an increase over basal activity of some 60 pmoles/minute/mg of membrane protein) may be compared with that obtained using the δ-opioid receptor agonist, [D-Ala-D-Leu]enkephalin (DADLE), in membranes from NG108-15 cells, where a stimulation over basal of approx. 10 pmoles/minute/mg membrane protein was obtained (McKenzie *et al.* 1988). The stimulated GTPase response in the clone 1C is therefore of an exceptional size. The magnitude of stimulation appears to be related to receptor number; this was discussed in Chapter 3 with reference to the adrenergic receptor in NG108-15, where it was observed that the α_{2A} receptor in human platelets mediated a detectable GTPase response while the α_{2B} receptor in NG108-15 cells did not, and it was speculated that the difference in receptor number was an important factor. Further evidence in favour of this suggestion came from another clone of the Rat 1 fibroblast transfected with the α2-C10 receptor which was isolated at the same time as the 1C clone. This clone, called 1D, showed specific [^{3}H]-yohimbine binding of ~810 fmoles/mg membrane protein and 10μM UK14,304 stimulated a high affinity GTPase activity of some 20 pmoles/minute/mg membrane protein over basal activity (data from C.Carr, personal communication). Hence a clone with a lower receptor number showed a lesser UK14,304 stimulated GTPase response. While it is likely that other factors will play a part in determining the size of a GTPase response, such as the levels of expression of the G-proteins coupling to these receptors, it seems that the level of receptor number is an important factor.

The UK14,304 stimulated GTPase response was sensitive to the α_2-adrenergic antagonist yohimbine (Figure 4.12), and was completely

abolished by prior treatment of 1C cells with pertussis toxin (Figure 4.19). From these results it can be inferred that the UK14,304 GTPase effect was due to activation of an α_2-adrenergic receptor (presumably the α_{2A} receptor), and that this subsequently resulted in a GTPase response via activation of a pertussis toxin substrate or substrates.

The identity of the pertussis toxin substrates coupling to the α_{2A} receptor in clone 1C cells was examined by use of "uncoupling experiments", in which antibodies raised against the C-terminal region of the various G-proteins present in clone 1C cells were incubated with membranes from these cells and then assayed for UK14,304 stimulated GTPase activity. It was reasoned that antibodies which bound to the G-protein which normally couples to the α_{2A} receptor would prevent interaction of receptor and G-protein, thus preventing receptor-mediated stimulation of high affinity GTPase activity. The C-terminus of the α-subunit has been implicated as the likely site of interaction of the G-protein with its receptor (the evidence for this suggestion has been discussed in Chapter 1 and Chapter 3), and the functional use of antibodies raised against synthetic peptides representing the C-terminus of various G-protein α-subunits (as discussed in Chapter 1 and Chapter 3) has been validated in a number of different systems (Hamm and Bownds, 1984; Harris-Warrick *et al.* 1988; Simonds *et al.* 1989a, 1989b; McKenzie and Milligan 1990).

As a prelude to these uncoupling experiments it was necessary to establish the effect of the preincubation phase on UK14,304 stimulated GTPase activity (Figure 4.14). Preincubation of clone 1C membranes for 1 hour at 37°C resulted in the loss of ~50% of both stimulated and basal GTPase activity. This effect has been noted before, (McKenzie *et al.* 1988) and is usually attributed to a gradual uncoupling of the receptor from its G-protein during the preincubation phase. Radioligand binding has shown that antagonist binding to receptors under these conditions is unchanged,

yet agonist binding gradually decreases during the preincubation, this change in receptor affinity for agonist suggesting uncoupling of the G-protein(s) interacting with the receptor (see comments on this phenomenon in NG108-15 cells in the Discussion of Chapter 3). Nevertheless, despite this loss of receptor-stimulated GTPase response, sufficient activity remained to allow assessment of uncoupling in the clone 1C membranes by using GTPase activity as the measure of receptor-G-protein coupling.

Preincubation of 5μg of clone 1C membranes for 1 hour with protein-A purified IgG from normal rabbit serum or from antisera OC1 or CS1 (10μg) did not affect basal or 10μM UK14,304 stimulated high affinity GTPase activity, however preincubation with protein-A purified IgG from antisera AS7 or I3B resulted in a statistically significant reduction of 10μM UK14,304 stimulated GTPase activity (Figure 4.15). Basal GTPase activity was unaffected by preincubation with antibody AS7 or I3B (results not shown). The implication of these results is that the protein-A purified antisera AS7 (which was raised against the α-subunits of Gi1 and Gi2) and I3B (raised against Gi3α) had bound to their target α-subunits, preventing interaction of these α-subunits with the α_{2A} receptor in clone 1C membranes. This interference subsequently prevents the receptor-mediated stimulation of high affinity GTPase activity, and hence it may be concluded that the α_{2A} receptor in membranes from clone 1C cells interacts with Gi1, Gi2, and Gi3. In order to assess the maximum extent of inhibition of UK14,304 stimulated GTPase activity, membranes (5μg) from clone 1C cells were incubated with increasing amounts of the antisera AS7 and I3B (Figures 4.16 and 4.17). It was found that both antisera were maximally effective at concentrations of 10μg per assay. Antiserum AS7 appeared to be more potent, since it showed 90% of its maximum efficacy at a concentration of 5μg per assay, and it has already been suggested that the levels of Gi2 were higher in clone 1C cells than

those of Gi3 (Figure 4.5 and Figure 4.7b, lane 2). The antiserum I3B was less potent than antiserum AS7, becoming maximally effective only at concentrations of 10μg per assay (Figure 4.17). Despite this, both antisera at 10μg inhibited 10μM UK14,304 stimulated GTPase activity in clone 1C cells by a similar proportion, and even at antibody concentrations up to 100μg per sample, inhibition by antiserum AS7 or I3B did not exceed 40%.

These results raise a number of questions of interpretation. Firstly, as was mentioned earlier, the use of antiserum AS7 in this context limits the conclusions that can be drawn, since this antiserum recognises the C-terminal regions of the α-subunits of both Gi1 and Gi2 (Spiegel, 1990), both of which are expressed in clone 1C cells (Figure 4.5). Although both immunoblotting and pertussis toxin-catalysed [^{32}P] ADP-ribosylation (Figure 4.7a, 4.7b) suggest that the levels of Gi1 in clone 1C cells are low (and substantially lower than those of Gi2) it cannot be assumed from these results that Gi1 does not play a role in transducing the α_{2A}-receptor-mediated high affinity GTPase response observed here. It might be supposed that only a G-protein present in high amounts would be capable of generating the substantial UK14,304 GTPase response, and indeed Gi2 is present in high amounts, both relative to Gi1 and Gi3, and also relative to Gi2 levels in NG108-15 cells (Figure 4.7b). Despite this, the possibility exists that a fraction of this GTPase response is due to Gi1. However, the data of Carty *et al.* (1990) showed that different G-protein α-subunits have different activation kinetics, and that Gi2 in particular bound GTPγS and released GDP more rapidly than either Gi1 or Gi3. As it is the release of GDP that is the rate-limiting-step in the GTPase reaction (Ferguson *et al.* 1986), this evidence would lend more weight to suggestions that Gi1, if involved at all, plays only a small role in transducing the UK14,304-stimulated GTPase effect. The initial study by Milligan *et al.* on the 1C

clone showed that agonist-driven cholera toxin [^{32}P]ADP-ribosylation of 1C membranes (see above) followed by immunoprecipitation with antisera AS7 and I3B resulted in precipitation of two bands, which corresponded to Gi2 and Gi3 (Milligan *et al.* 1991). Immunoprecipitation of Gi1 was not observed, however the authors were careful not to conclude that Gi1 was therefore not involved in coupling to the α_{2A} receptor in clone 1C cells, as it was possible that due to the low level of expression of Gi1, its immunoprecipitation might not be detected.

Another problem attendant on the interpretation of the "GTPase uncoupling" results is the question of relative levels of antibody required to account for all the G-proteins in clone 1C membranes, and whether sufficient antibody had been added to abolish completely the 10μM UK14,304 stimulated GTPase effect. The data in Figure 4.18 are relevant to this point. Co-incubation of membranes from clone 1C cells with antisera AS7 and I3B showed that their effects were additive (Figure 4.18), but complete inhibition of 10μM UK14,304 stimulated GTPase activity was never observed. There always remained a statistically significant UK14,304 stimulated GTPase response (in Figure 4.18, Column AS7+I3B was significantly different from basal GTPase, $P<0.001$, Student's t-test). There are a number of possible reasons for this. The simplest is to suggest that insufficient antibody had been added to the reaction to uncouple all the receptors. The 1C clone expresses, as has been observed previously, a high number of α_{2A} receptors, and a high level of Gi2. It is difficult using the methods available here, to quantitate the levels of antibody necessary to uncouple all the G-proteins from all the receptors, except by trial and error, however increasing the antibody concentration up to 100μg per sample, and decreasing the 1C membrane concentration to 2μg per sample (the lowest concentration giving linear stimulated increases over basal, see Figure 4.13) did not produce any

greater inhibition of stimulated GTPase, the results being essentially the same as those in Figure 4.18 (data not shown). This contrasts with the results of uncoupling experiments in membranes from NG108-15 cells, where antiserum AS7, at a concentration of 10µg per sample, was able completely to uncouple the α_{2B} receptor from Gi2 (Chapter 3, Figures 3.12-3.14), however NG108-15 cells express lower levels of both α_{2B} receptor and Gi2 (Chapter 3, and Chapter 4 figure 4.7b). It is clear that the use of antibodies in uncoupling experiments of this kind has limitations, particularly as the antisera used here form only a part of an IgG fraction obtained from rabbit serum, and it is difficult to assess in a manner other than the purely qualitative, what proportion of the total IgG is the anti-G-protein IgG.

The second possible interpretation of the results indicating an incomplete antibody-attenuation of 10µM UK14,304 stimulated GTPase activity is that clone 1C cells express another G-protein besides Gi1, Gi2, and Gi3, which is responsible for transducing a part of the GTPase effect. This G-protein would have to be a pertussis toxin substrate, since the UK14,304 GTPase stimulation is completely pertussis toxin sensitive (Figure 4.19). It would also have to be undetected by any of the antibodies so far raised against G-protein α-subunits. Most of the known pertussis toxin sensitive G-proteins have had antibodies raised against them, these include Td1, Td2, Gi1, Gi2, Gi3 and Go (Spiegel, 1990). If another pertussis toxin substrate exists in clone 1C cells, then it must be completely novel. One thing is clear: this result was not anticipated by the use of UK14,304-driven cholera toxin-catalysed [^{32}P]ADP ribosylation of pertussis toxin substrates (Milligan *et al.* 1991), and if another pertussis toxin substrate is found in clone 1C cells, then this will be as the result of the application of a quantitative assessment of receptor-G-protein coupling, rather than a qualitative one.

The work of Simonds *et al.* on the coupling to G-proteins of the α_{2A} receptor in the human platelet involved using antibodies raised against synthetic decapeptides corresponding to the C-termini of various G-proteins in a similar manner to that reported here (Simonds *et al.* 1989a). Platelet membranes were preincubated with the antisera and then assayed for noradrenaline inhibition of PGE_1-stimulated adenylyl cyclase activity. It was found that preincubation with the antiserum AS7 resulted in attenuation of the noradrenaline-induced inhibition of PGE_1 stimulated adenylyl cyclase activity, and that this effect was not produced by antisera against the other G-proteins detected in platelets. Antiserum AS7 is specific for Gi2 alone in platelet membranes, as Gi1 appears not to be expressed (Simonds *et al.* 1989a). From these results, it was concluded that Gi2 mediates α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes.

Hence it seems clear that the α_{2A} receptor expressed *in vivo* interacts specifically with Gi2 to inhibit adenylyl cyclase. It was therefore of interest to examine the α_{2A} receptor expressed in the "unnatural" environment of the Rat 1 fibroblast clone 1C for a similar effect. Membranes from clone 1C cells were shown to possess a forskolin-stimulated adenylyl cyclase activity (Figure 4.20) which could be inhibited by the α_2-adrenergic agonist UK14,304 (Figure 4.21). This α_2-adrenergic inhibition of adenylyl cyclase activity was pertussis toxin sensitive (Figure 4.22). Preincubation of membranes from clone 1C cells with protein-A purified IgG from normal rabbit serum, or from antisera CS1 or I3B (which recognise the C-termini of Gs and Gi3, respectively), did not affect UK14,304 inhibition of forskolin-stimulated adenylyl cyclase. However preincubation with protein-A purified IgG from antiserum AS7 completely abolished 10μM UK14,304 inhibition of 10μM forskolin stimulated adenylyl cyclase activity (Figure 4.24). Hence it appears that

antiserum AS7, which in clone 1C cells recognises the C-termini of both Gi1 and Gi2, completely uncouples the α_{2A} receptor from inhibition of adenylyl cyclase, presumably by binding to Gi1 and Gi2 and preventing their interaction with the receptor.

The caveats that applied to the interpretation of the GTPase uncoupling results (above) apply here, *ie.* that antiserum AS7 recognises both Gi1 and Gi2, but the arguments against the role of Gi1 also apply, and it is probably safe to infer that these results suggest that Gi2 in clone 1C cells is the primary mediator of the α_{2A}-receptor-induced inhibition of adenylyl cyclase activity. Unlike the data obtained for GTPase stimulation, these data show that Gi3 has no role in the inhibition of adenylyl cyclase activity. In this respect, these results show that the α_{2A} receptor retains its specificity of inhibitory coupling to adenylyl cyclase, since both *in vivo*, in the human platelet (Simonds *et al.* 1989a), and now in the unnatural environment of the rat fibroblast clone 1C, the α_{2A} receptor only couples to inhibition of adenylyl cyclase via Gi2. As was noted of the α_{2B} receptor in NG108-15, which also couples to inhibition of adenylyl cyclase via Gi2 (Chapter 3 Discussion), this is a considerable degree of specificity, since the amino acid sequences of Gi2 and Gi3 are 90% homologous (Itoh et al. 1988).

Yet it has also been shown here that Gi3 <u>is</u> activated by the α_{2A} receptor in clone 1C cells, as an antiserum against the C-terminal region of Gi3 attenuates the UK14,304-stimulated high affinity GTPase activity (Figure 4.15, Figure 4.17). Simonds *et al.* did not assess receptor stimulated GTPase activity, which would have indicated whether the α_{2A} receptor in the human platelet showed some interaction with Gi3, hence it is impossible to state categorically that there has been a loss of specificity of G-protein coupling to the α_{2A} receptor, but their results suggested that, despite the presence of Gi3 in platelet membranes, Gi2 alone mediated the

functional response of the α_{2A} receptor. This would imply that there has indeed been a loss of specificity of G-protein coupling to the α_{2A} receptor when expressed in the 1C clone. If the process of transfection and expression has not modified the receptor structure, how is the α_{2A} receptor now able to interact with a different G-protein? If there has genuinely been a loss of integrity of receptor-G-protein interaction in clone 1C cells, then the results reported here might be construed as evidence that within the membrane there is some degree of compartmentalisation of G-proteins, or that some post-translational modification of the various G-proteins plays a role in determining the specificity of receptor coupling *in vivo*. As to the cause of the loss of specificity in clone 1C cells, it is possible that the high level of receptor expression is important here. It may be speculated that, *in vivo*, receptors are localised to the cell membrane in the presence of a select surrounding of G-protein types, thus ensuring that the receptor only interacts with particular G-proteins. The over-expression of the α_{2A} receptor in clone 1C cells may result in the placing of receptors in "inappropriate" areas, where it can now interact with novel G-proteins.

The function of the activated Gi3 in clone 1C cells is also a matter for speculation. The results of the adenylyl cyclase uncoupling experiments (Figure 4.24) seem quite conclusive: Gi3 does not mediate the interaction of the α_{2A} receptor in clone 1C cells with adenylyl cyclase. However, it has recently been shown that UK14,304 stimulates a pertussis toxin sensitive phospholipase D activity in clone 1C cells, but not in parental Rat 1 fibroblasts (E. MacNulty, personal communication). It is tempting to speculate that this PLD activity might be transduced by Gi3, but this will be difficult to prove until a method of assessing PLD activity in membranes or broken cells is obtained, or until a method of introducing antibodies into whole cells is obtained.

Finally, the results obtained from the clone 1C cells suggest that the α_{2A} receptor interacts simultaneously with multiple pertussis toxin substrates. This is not the first report of multiple interaction between receptor and G-protein. Gierschik *et al.* noted the interaction of the fMet-Leu-Phe receptor in human leukemia HL-60 cells with Gi2 and Gi3 (Gierschik *et al.* 1989). By using the method of agonist-driven cholera toxin-catalysed [^{32}P]ADP-ribosylation of pertussis toxin substrates (Giershik and Jacobs,1987) they were able to demonstrate the incorporation of radiolabelled [^{32}P]ADP-ribose into two protein bands, and on the basis of relative mobilities on SDS-polyacrylamide gels identified them as Gi2 and Gi3. It has already been noted that this method suffers from the drawback that the results can only demonstrate qualitative, and not quantitative interaction. Hence it is likely that the results reported here give the first quantitative assessment of receptor-activation of multiple G-proteins.

CHAPTER 5

CONCLUSIONS

CONCLUSIONS

The initial observations of the effects of guanine-nucleotides on adenylyl cyclase activity were treated in some quarters with skepticism (see Gilman, 1989). Nowadays it is accepted, and indeed expected, that most receptors will be coupled to intracellular second-message-generating systems via the actions of one or more members of the signal-transducing class of guanine-nucleotide binding proteins. The scheme of G-protein activation proposed by Cassel and Selinger wherein G-proteins are activated by binding GTP and inactivated as the result of the intrinsic GTPase activity of the G-protein α-subunit (Cassel and Selinger, 1977) has been found to be widely applicable. Examination by X-ray crystallography of the structures of the $p21^{ras}$ protein and of bacterial elongation factor EF-Tu (de Vos *et al.* 1988; Jurnak, 1985) has shown that certain structural features are common to most guanine-nucleotide binding proteins, particularly with respect to the guanine nucleotide binding domains (Halliday, 1984; Masters *et al.* 1986). The allocation of functions to parts of the G-protein molecule is well advanced, with general acceptance for the idea that the C-terminus of the α-subunit represents the site of receptor interaction (Ui *et al* 1984; Sullivan *et al.* 1987), and that the N-terminus of the α-subunit represents the site of $\beta\gamma$-interaction (Navon and Fung, 1987; Osawa *et al.* 1990). The use of molecular biological techniques has allowed the isolation of novel G-proteins, including recently the long-awaited discovery of a G-protein mediating receptor interaction with phospholipase C (Pang and Sternweis, 1990; Taylor *et al.* 1991). Undoubtedly, more new G-protein subunits will be isolated, adding to the already extensive family of heterotrimeric signal-transducing G-proteins (see Chapter 1, Table 1.1).

Increasingly, the focus of research has shifted from examination of the structural or functional features of G-protein subunits to addressing questions relating to the function of the heterotrimer within the cell membrane, *ie* how it interacts with its receptor, or with its second message-generating effector. The work reported in this thesis has attempted to address the question of specificity of interaction of G-proteins. Although there is a large family of G-proteins, their structures, and in particular the sequences of the α-subunits, are all remarkably similar. The degree of sequence similarity extends from around 50% (for Gi2α and Gsα) to 82% (for Gi2 and Go), to 95% (for Gi1α and Gi3α) (Itoh *et al.* 1988; Van Meurs *et al.* 1987). If the α-subunits are so homologous in sequence, is it reasonable to expect any degree of specificity of interaction between G-protein α-subunits and receptors, and if such specificity exists, how can it be accounted for?

There is a body of conflicting evidence on the question of the existence of specificity of interaction between G-protein and receptor. Yatani *et al.* (1988) used purified, recombinant forms of Gi1, Gi2, and Gi3, and found that they were all capable of opening excised atrial K^+ channels. Moving to the model system under investigation here, namely the α_2-adrenergic receptor system, it was shown that the α_2-adrenergic receptor purified from human platelets, when inserted into phospholipid vesicles, was capable of interacting with both Gi and Go, but was much less effective in interacting with Gs (Cerione *et al.* 1986). One interesting finding reported by this group was that Gi copurified with the α_2-adrenergic receptor through the initial steps of receptor purification (Cerione *et al.* 1986). More recently the α_2-C10 and α_2-C4 adrenergic receptor clones were overexpressed in COS-7 cells, purified and inserted into phospholipid vesicles with recombinant forms of Gs, Gi1, Gi2, Gi3, and Go (Kurose *et al.* 1991). Again, both the α_2-receptors were found to

interact with all the "Gi" like G-proteins (order of specificity Gi3>Gi1≥Gi2>Go), but not with Gs. It seems clear from these observations that a basic level of specificity of interaction between receptors and G-proteins obtains. Receptors may appear to interact with closely related members of the G-protein family (such as the different members of the "Gi" group of pertussis toxin substrates, or the different forms of Gs), but are unlikely also to interact with G-proteins significantly different in sequence, hence a receptor which will interact with Gi will not interact with Gs, which is much less similar in sequence to Gi.

As was observed by Kurose *et al* in their discussion, these reconstitution experiments demonstrate the *potential* for interaction of adrenergic receptors with multiple G-proteins, however they do not exclude the possibility of specific interaction of receptors and G-proteins. Indeed, one reconstitution study using the purified dopamine D_2 receptor from bovine pituitary and resolved G-proteins from bovine brain demonstrated that the D_2 receptor interacted with a 10 fold higher affinity with Gi2 than with Gi1, Gi3, or Go (Senogles *et al.* 1990). However, it is usually studies of receptor-G-protein specificity using native membranes, or whole cells, which tend to report a high degree of specificity of interaction. McKenzie and Milligan showed that the δ-opioid receptor in the NG108-15 cell line was coupled specifically to inhibition of adenylyl cyclase activity via Gi2 (McKenzie and Milligan, 1990). Simonds *et al.* showed that the α_2-adrenergic receptor in human platelets was coupled to inhibition of adenylyl cyclase activity, also via Gi2 alone (Simonds *et al.* 1989a). It was also shown by Simonds *et al.* that Gs alone mediated receptor-activated stimulation of adenylyl cyclase in S49 cells (Simonds *et al.* 1989b). The data reported in Chapter 3 suggested that the α_{2B} adrenergic receptor in the NG108-15 cell line was coupled specifically to Gi2, although it was clear that the specificity of this coupling was

dependent on the differentiation state of the cell, and that the same receptor could, under different circumstances, couple to Go (Chapter 3; McClue and Milligan 1990).

So there is evidence that specificity of interaction of receptors with G-proteins occurs *in vivo*, even though the potential exists for the interaction of some receptors with multiple G-proteins, as demonstrated by reconstitution experiments, and by the work reported in Chapter 3 on the adrenergic receptor in NG108-15 cells. If it is accepted, then, that specificity of interaction does occur, this raises the question of *how* it occurs. This leads us to consider the work reported in Chapter 4. The α_{2A} receptor in the human platelet has been shown in native membranes to couple to inhibition of adenylyl cyclase via Gi2 (Simonds *et al* 1989a). The reconstitution experiments of Cerione *et al* indicated that the receptor has the potential to couple to Gi and Go (Cerione *et al.* 1986), this finding being supported by the results of Kurose *et al.*, which suggested that the α_2-C10 receptor could couple to Gi1, Gi2, Gi3, and Go (Kurose *et al.* 1990). It has been shown in Chapter 4 that transfection of this receptor into the Rat 1 fibroblast cell line results in the expression of an α_{2A} receptor which can interact with Gi2 and Gi3, possibly with Gi1, and possibly with another pertussis toxin substrate (Chapter 4, Results). Once again, this receptor was able to interact only with G-proteins which have a high degree of sequence homology (*ie.* the members of the class of pertussis toxin substrates), but not with G-proteins possessing a substantially different sequence (*ie.* Gs). It was also shown in Chapter 4 that inhibition of adenylyl cyclase activity in the 1C clone was mediated solely via Gi2, and that Gi3, although activated by the α_{2A} receptor, does not participate in inhibition of adenylyl cyclase (Chapter 4, Figure 23).

These data, examining the specificity of coupling of the α_{2A} receptor in the clone 1C cells, indicate a "half-way house" between the results from

reconstitution experiments, which generally report a low level of receptor-G-protein specificity, and the results from native membranes, which tend to show a high degree of receptor-G-protein specificity. It is clear that the α_{2A} receptor, when transfected into an unnatural environment, can interact with multiple G-proteins, however it is also apparent that the specificity of the G-protein coupling to its intracellular effector is tightly maintained. In a similar way, the α_{2B} receptor in NG108-15 cells can, under different circumstances, activate both Gi2 and Go, however the activated Gi2 will only interact with adenylyl cyclase, and activated Go only with Ca^{2+} channels (see Discussion in Chapter 3). Does this imply that receptors interact with G-proteins with a relatively low degree of specificity, but that subsequent G-protein interaction with intracellular second-message generators has a high degree of fidelity?

There are some problems with this hypothesis, notably the assumption that specific G-proteins interact only with specific intracellular effectors. For example, while it has been shown for a number of systems that Gi2 is the G-protein responsible for mediating the inhibition of adenylyl cyclase (McKenzie and Milligan 1990; Simonds *et al.* 1989a; Chapter 3), it has also been shown that Gi2 is capable of transducing signals to other effectors, notably to stimulation of a phospholipase C in the human monoblastic U937 cells (F.M.Mitchell, personal communication) and in the human leukemia HL-60 cell line (Gierschik *et al.* 1989). If this is so, then the reason for the specificity of Gi2 in each situation cannot lie in its primary sequence, *ie* not in the fact that it is Gi2. This suggests that specificity of G-protein interaction has more to do with differential or tissue specific expression, or with membrane compartmentation, and that particular G-proteins interact with specific receptors and intracellular effectors simply because they happen to be the only ones expressed in a particular tissue, or because they happen to be the only G-proteins that a

receptor has access to. This may explain the apparent anomaly of results (from native membranes) which suggest specificity of coupling (eg. Chapter 3), while other results (including reconstitution experiments, and Chapter 4) suggest G-protein promiscuity of interaction.

Evidence for G-protein compartmentalisation is scarce, however one group has shown differential localisation of Gi2 and Gi3 in a kidney epithelial cell line LLC-PK$_1$ (Ercolani *et al.* 1990). The LLC-PK$_1$ cell line is polarised, *ie* the cells have a distinct orientation which is required for proper functioning. By using anti-G-protein antibodies and immunofluorescence it was shown that Gi2 was localised to the basolateral membrane of this polar cell line, while Gi3 was localised to the apical membrane and the Golgi. Transfection of the LLC-PK1 cells with the gene for Gi2 resulted in an increase in Gi2 levels, which was still found to be localised only to the basolateral membrane. While it is difficult to extrapolate these results in a cell line which depends for its function on having a "top" and a "bottom" to other cells which are not thus polarised, these results do set a precedent, and indicate that the concept of G-protein compartmentalisation can be supported by experimental evidence.

Tissue-specific expression of G-proteins has been noticed since the beginning of G-protein research (see Chapter 1), and if compartmentalisation of G-proteins can be shown to be more widely applicable, then it may be possible to explain the specificity of interaction of G-proteins with receptors and intracellular effectors as being due to the availability to receptors of only certain G-proteins, which, in turn, are able to interact only with specific effectors in the locale. The results described in Chapter 4 for the interaction of the α_{2A} adrenergic receptor with G-proteins in the 1C clone could than be interpreted in a similar manner. While in the human platelet the α_{2A} receptor may only interact with Gi2, on transfection into the Rat 1 fibroblast the receptor is now expressed in

areas where it has access to Gi3 as well as Gi2. The similarity of sequence of the two G-proteins allows the receptor now to interact with Gi3 as well as Gi2. In any event, the apparent loss of specificity of G-protein coupling to the α_{2A} receptor in the 1C clone suggests that results arising from the use of recombinant G-proteins and transfected receptors to attribute receptor-G-protein specificity must be interpreted with caution.

The results of the experiments on adenylyl cyclase activity in the 1C clone also deserve some comment (Chapter 4, Figures 4.20-4.24). It is interesting to note that, despite the expression of some 3000 fmoles/mg of α_{2A} receptor in the clone 1C cells (as measured by specific [^{3}H]-yohimbine binding, Chapter 4 Figure 4.3a), the degree of adenylyl cyclase inhibition by UK14,304 is over the range 37-50%, which compares with α_{2B}-receptor mediated inhibition of adenylyl cyclase activity in NG108-15 cells of 35-60% (Sabol and Nirenberg, 1979). These levels of inhibition are not dissimilar, despite the findings that NG108-15 cells express the α_{2B} receptor in the much lower range of 100-250 fmoles/mg (as measured by specific [^{3}H]-yohimbine binding, Chapter 3 Figure 3.10, and Murphy and Bylund, 1988). It may be that the α_{2A} receptor interacts less efficiently with Gi2 to produce inhibition of adenylyl cyclase activity than does the α_{2B} receptor, but this extreme difference in receptor number might still have been anticipated to produce a greater degree of cyclase inhibition. Although GTPase activity appears to be closely linked to receptor number (see Discussions to Chapter 3 and Chapter 4), it would appear that inhibition of adenylyl cyclase activity is not. Also, the presence of substantially higher levels of Gi2 in clone 1C cells by comparison with those in NG108-15 cells (Chapter 4, Figure 4.7b) has not resulted in any greater inhibition of adenylyl cyclase activity. It may be that the important factor here is the balance in the levels of Gs and Gi2, ensuring that

adenylyl cyclase inhibition is not more than ~40%. What is not clear, however, is how a 40% inhibition of stimulated adenylyl cyclase activity can affect the cell, and why 100% inhibition of stimulated adenylyl cyclase activity is not observed.

One further question remains to be addressed with respect to adenylyl cyclase inhibition in the clone 1C cells. Do the results from Chapter 4 shed any light on the question of which G-protein subunit, α- or βγ-, is responsible for mediating inhibition of adenylyl cyclase activity (see Chapter 1 for a discussion of the evidence for and against βγ-subunit mediated inhibition of adenylyl cyclase)? The use of antibodies directed against the α-subunit of Gi1/Gi2 alone has been shown to attenuate receptor-mediated inhibition of adenylyl cyclase activity in the clone 1C cells (Chapter 4, Figure 4.24). Unfortunately, no conclusions can be drawn from these results about the moiety responsible for mediating adenylyl cyclase inhibition, since prevention of receptor-G-protein contact by the antibodies also prevents the subsequent dissociation of the G-protein which might give rise to activated Gα or free βγ, the two species which are thought, variously, to be responsible for inhibition of adenylyl cyclase. Considering Levitzki's hypothesis (see Chapter 1) that it is the heterotrimer which is the active body in cyclase inhibition (Marbach *et al.* 1990), once again no conclusions may be drawn, since the interference of G-protein contact with receptor prevents the stimulation of guanine-nucleotide exchange which results in the GTP-bound, activated form of the G-protein heterotrimer which is proposed as the transducer of cyclase inhibition. However, application of Occam's Razor would lead one to favour the simplest conclusion, *ie.* that it was, in this system, the α-subunit of Gi2 which was responsible for mediating inhibition of adenylyl cyclase.

CHAPTER 6

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