Agonist regulation of cellular Gsα levels in wild-type and transfected NG108-15 cells

A thesis presented for
the degree of
DOCTOR OF PHILOSOPHY

by

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May 1994

Summary

The neuroblastoma x glioma hybrid cell line, NG108-15, expresses an apparent 1 - 1.5 pmoles of a high affinity IP prostanoid receptor. This receptor is coupled to Gs and activates adenylyl cyclase. Chronic exposure of these cells to the prostanoid agonist, iloprost, results in a desensitisation of prostanoid stimulated adenylyl cyclase activity. In agreement with earlier work (Kelly *et al*, 1990; McKenzie and Milligan, 1990), studies presented in this thesis demonstrate a 50-70% decrease in cellular Gsα levels on prostanoid treatment of NG108-15 cells. Time courses and dose responses of prostanoid treatment showed the down-regulation of Gsα to be concurrent with a down-regulation (50-70%) of the IP prostanoid receptor. Quantitative analysis of the polypeptide levels, on iloprost treatment, revealed that Gsα and the IP prostanoid receptor were down-regulated in a ratio of approximately 8:1 at various degrees of receptor occupancy by agonist.

Although G-protein α-subunit down-regulation is now a well-documented phenomenon during long-term agonist exposure, it does not occur in all cases of chronic agonist treatment. In NG108-15 cells chronic agonist treatment of the A2 adenosine and secretin receptors, which also couple to adenylyl cyclase, does not result in a detectable down-regulation of Gsα (McKenzie *et al*, 1990). These receptors are thought to be expressed at much lower levels than the IP prostanoid receptor (Kelly *et al*, 1990; Gossen *et al*, 1990) potentially activating less G-protein α-subunit in NG108-15 cells. NG108-15 expressing 4000 fmoles/mg membrane protein β2-adrenergic receptor (βN22 cells) demonstrate an approximate 50% down-regulation of Gsα on treatment

with isoprenaline (10μM; 16 hours), whereas NG108-15 cells expressing 300 fmoles/mg membrane protein β2-adrenergic receptor (βN17 cells) show no apparent down-regulation of Gsα on prolonged isoprenaline treatment.

These results show that isoprenaline mediated down-regulation of Gsα in transfected NG108-15 cells is dependent on the level of β2-adrenergic receptor expression, indicating a role for the receptor in regulating Gα levels. This could potentially prove to be a general phenomenon for other receptors and their related G-proteins.

Acknowledgements

I would like to thank several people for their help during the course of the work and without whom it would not have been possible. Professor M. D. Houslay for making the facilities of the Biochemistry Department available. Dr Graeme Milligan for his supervision, advice and suggestions. Dr Gwyn Gould for the use of his laboratory facilities and also his helpful advice during the generation of the transfectants. Fiona and Stevie for their continued patience, advice and support especially during the early stages of the project. Ian for raising the antibodies used in this thesis. Dr Christine Brown for help with some of the data analysis and everyone else in the department who helped in any way with this work. I would finally like to thank Iain for his love and patience and for putting up with me throughout the course of the thesis.

Abbreviations

The abbreviations used in this thesis are as set out in "Instructions to Authors" Biochemical Journal (1985), 225,1-26, with the following additions:

ADP	adenosine 5' diphosphate
βARK	β-adrenergic receptor kinase
ARF	ADP ribosylating factor
ATP	adenosine 5' triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CHO	Chinese hamster ovary
DAG	diacylglycerol
DHA	dihydroalprenolol
DMEM	Dulbeccos' modification of Eagles' medium
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
E.Coli	*Escherichia Coli*
FCS	foetal calf serum
GAP	GTPase activating protein
GDP	guanosine 5' diphosphate
Gpp(NH)p	guanylyl 5' imidodiphosphate
G-protein	guanine nucleotide binding protein
GTPγS	guanosine 5'-O-(3-thio)triphosphate
GTP	guanosine 5' triphosphate
IAP	islet activating protein

NAD	nicotinamide adenine dinucleotide
NaF	sodium fluoride
NaOAc	sodium acetate
NECA	5'-(N-ethyl)carboxamidoadenosine
PBS	phosphate buffered saline
PGE1	prostaglandin E1
PGE2	prostaglandin E2
PGI2	prostaglandin I2
PGF2	prostaglandin F2
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
SV40	simian virus 40
TEMED	N,N,N',N' tetramethylethylenediamine
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane

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CHAPTER 1 - INTRODUCTION

1.1 Historical perspectives

The control of cellular activity is dependent upon the action of a variety of hormones, neurotransmitters and growth factors which bind extracellularly to specific plasma membrane spanning receptors. The mechanism by which the binding of a suitable ligand to such a receptor exerts an intracellular effect has been the subject of much investigation. Pioneering work by Sutherland and Rall, using liver homogenates, demonstrated that, in the presence of glucagon or adrenaline, a heat stable factor was produced which was able to activate phosphorylase in the supernatant fraction (Rall *et al*, 1957). The heat stable factor was later identified as a cyclic monophosphate termed 3'5'-cyclic AMP (cAMP). Glucagon and adrenaline did not directly activate phosphorylase which suggested that adenylyl cyclase, the enzyme catalysing the production of cAMP from ATP, could be hormonally regulated (Rall *et al*, 1957). This data formed the basis for the "second messenger hypothesis", whereby binding of a hormone extracellularly to specific cell surface receptors resulted in the generation of a secondary messenger e.g. cAMP which then altered intracellular metabolism.

The discovery that hormonal regulation of adenylyl cyclase in hepatocytes required GTP (Rodbell *et al*, 1971a, b) suggested the involvement of a guanine nucleotide binding site (Schramm and Rodbell, 1975). GTP resulted in a transient activation of adenylyl cyclase, whereas non-hydrolysable analogues of GTP e.g. guanylyl 5'-[$\beta\gamma$-imido]diphosphate (Gpp[NH]p) were shown to persistently activate adenylyl cyclase (Londos *et al*, 1974). This allowed speculation that the binding site involved in the regulation of adenylyl cyclase activity was capable of binding and hydrolysing GTP. Cassel and Selinger (1976)

corroborated this theory, when they demonstrated that catecholamine stimulation of adenylyl cyclase in the turkey erythrocyte membrane system produced a concomitant increase in the rate of high affinity GTPase activity. GTP hydrolysis appeared to terminate the activation of adenylyl cyclase. This suggested two states, an active GTP bound state and an inactive GDP bound state. Receptor activation was believed to cause the release of bound GDP and its exchange for GTP. This has indeed proved to be the case and will be discussed in greater detail later.

As well as receptor mediated stimulation of adenylyl cyclase, the possibility of hormonal inhibition of adenylyl cyclase, which had been suggested by earlier studies (Murad *et al*, 1962), was clarified by Rodbell and colleagues. They demonstrated in adipocyte preparations that GTP exerts a biphasic effect on adenylyl cyclase activity (Rodbell, 1975). In the absence of stimulatory hormone, guanine nucleotides such as GTP, elicited an inhibition of adenylyl cyclase. It was therefore suggested that adenylyl cyclase activity may be regulated by both stimulatory and inhibitory G-proteins (Rodbell, 1980).

1.2 The identification of the stimulatory and inhibitory G-proteins; Gs and Gi

The isolation of certain exotoxins from cultures of both *Vibrio cholerae* and *Bordetella pertussis*. aided the identification of the stimulatory and inhibitory G-proteins, termed Gs and Gi respectively. Gill and Meren demonstrated that the cholera toxin activation of adenylyl cyclase in pigeon erythrocytes correlated with the radiolabelling of a 42kDa polypeptide, in the presence of [^{32}P]-NAD^{+} (Gill and

Meren, 1978). The presumed reaction of cholera toxin was an ADP-ribosylation, similar to that already detailed for the action of diphtheria toxin on a protein component required for protein synthesis (Collier, 1975). The effect of cholera toxin was similar to that obtained by non-hydrolysable analogues of GTP, thus allowing the identification of Gs as both a substrate for cholera toxin catalysed ADP-ribosylation and a protein capable of binding and hydrolysing guanine nucleotides.

Pertussis toxin, which is also termed islet activating protein (IAP), was similarly isolated from *Bordetella pertussis*. It was shown to produce alterations in receptor mediated control of cyclic AMP production (Katada and Ui, 1981). Initial experiments in rat C6 glioma cells treated with pertussis toxin showed an increase in GTP activation of adenylyl cyclase, concomitant with the transfer of [^{32}P]-ADP-ribose from [^{32}P]NAD$^+$ to a 41kDa membrane associated polypeptide (Katada and Ui, 1982). The release of tonic inhibition of adenylyl cyclase activity paralleled by the modification of the 41kDa protein thus identified this protein as the previously unidentified inhibitory G-protein of the adenylyl cyclase cascade (Gi). IAP functions in a similar manner to cholera toxin in that it is an ADP-ribosyl transferase, catalysing the transfer of ADP-ribose from NAD$^+$ onto the G-protein α-subunit.

In contrast to pertussis toxin, cholera toxin requires the presence of a protein co-factor termed ADP-ribosylation factor (ARF) which is itself able to bind GTP (Kahn and Gilman 1984a), in order to catalyse the ADP-ribosylation of Gs. The functional effect of cholera toxin catalysed ADP-ribosylation of Gsα is to attenuate the ability of Gsα to hydrolyse GTP, thus producing an irreversibly activated Gs α-subunit (Cassel and Selinger, 1977; Birnbaumer *et al*, 1980). After treatment with cholera

toxin, adenylyl cyclase becomes maximally activated and is no longer responsive to hormonal stimulation. In contrast, the functional effects of pertussis toxin catalysed ADP-ribosylation of Giα is to prevent productive coupling between the receptor and the G-protein, thus receptor mediated inhibition of adenylyl cyclase is attenuated after pretreatment with pertussis toxin (Katada and Ui, 1979; Burns *et al*, 1983). This led to the hypothesis that any event which is attenuated by prior treatment with pertussis toxin was indicative of a role for Gi in mediating the response. This has been demonstrated to be too simplistic as will be described later.

1.3 G-protein purification

Initial attempts to purify Gs using GTP affinity chromatography were designed to resolve Gs from adenylyl cyclase, thus proving Gs to be a separate moiety from adenylyl cyclase (Pfeuffer and Helmreich 1975; Spiegel *et al*, 1979). Studies of mutants of the S49 murine lymphoma cell line provided further evidence that receptor, Gs and adenylyl cyclase were separate entities. This cell line was particularly useful as a model to dissect the adenylyl cyclase system since an increase in intracellular cAMP is cytocidal, a characteristic enabling the isolation of mutants which were defective in their ability to produce cAMP. Tomkins and co-workers isolated a clone of S49 which lacked the ability to synthesise cAMP (Bourne *et al*, 1975). Initially designated AC-, later *cyc-*, the clone was shown to be deficient in Gs, and thus provide a powerful functional assay for the purification of Gs. This was exploited by Ross and Gilman who were able to reconstitute adenylyl cyclase activity into

cyc- membranes using a detergent extract of wild-type membranes in which adenylyl cyclase has been inactivated (Ross and Gilman, 1977).

Purified Gs obtained from rabbit liver was shown initially to be composed of α-subunits of either 52 or 45kDa and a 35 kDa β-subunit (Northup *et al*, 1980). Gs was further purified from turkey (Hanski *et al*, 1981) and human erythrocytes (Hanski and Gilman, 1982), using similar methods, although only the 45kDa form of the α-subunit was obtained. The α-subunits corresponded to the guanine nucleotide binding site, as well as the substrate for cholera toxin catalysed ADP-ribosylation (Cassel and Pfeuffer, 1978). Subsequently a low molecular weight γ-subunit (8kDa) was found to be associated with Gs and other G-proteins. Its presence had been missed before due to its poor ability to be stained with conventional protein stains (Hildebrandt *et al*, 1984). Demonstrations of the distinct activities of each subunit came from the activation of purified Gs by AMF ($AlCl_3$, $MgCl_2$ and NaF) or guanosine 5' [3-o-thio] triphosphate (GTPγS - a non-hydrolysable analogue of GTP). Such treatments resolved the α-subunit which activated adenylyl cyclase (Northup *et al*, 1983a) and the β-subunit which stimulated the rate of Gs deactivation (Northup *et al*, 1983b).

The pertussis toxin sensitive "Giα" was initially purified from rabbit liver (Bokoch *et al*, 1983) and human erythrocytes (Codina *et al*, 1983), although the polypeptides isolated from each tissue were of dissimilar molecular weight (41kDa and 39kDa respectively). Attempts to purify Gi from bovine and rat brain led to the conclusion that brain contained two (Sternweis and Robishaw, 1984; Milligan and Klee, 1985) or three (Neer *et al*, 1984) pertussis toxin sensitive polypeptides in the 39-41kDa range, along with the β- and γ-subunits.

1.4 G-protein heterogeneity

Further purification studies and the advent of molecular cloning technology has now revealed a whole family of G-proteins. Indeed, Simon and co-workers (1991) examined the primary amino acid sequences of the distinct α-subunits and arbitrarily classified them into subfamilies which showed a high degree of amino acid homology.

1.4.1 Gs family

At least four forms of Gsα exist, which are obtained from alternative splicing of a transcript produced from a single gene (Bray *et al*, 1986; Kozasa *et al*, 1988). These have been grouped into two subtypes which are termed long and short Gsα. The cDNAs that encode two of the forms of Gsα have been isolated from a bovine adrenal cDNA library and are identical except for a sequence of 46 nucleotides in which the shorter form of Gsα contains alterations in 4 nucleotides and a deletion of 42 others (Robishaw *et al*, 1986). Whereas at least two forms of Gs are expressed in most tissues, their relative amounts usually vary (Mumby *et al*, 1986). Although Gs was originally identified as the G-protein required for receptor mediated stimulation of adenylyl cyclase, the protein has also been shown to couple to the activation of the dihydropyridine-sensitive Ca^{2+} channels (Yatani *et al*, 1987b), the inhibition of the calcium pump in liver membranes (Jouneauz *et al*, 1993) and the inhibition of Na^{+} channels in cardiac myocytes (Schubert *et al*, 1989). Gsα has also been shown to mediate epidermal growth factor-elicited stimulation of rat cardiac adenylyl cyclase (Nair *et al*, 1990).

A protein with 88% amino acid homology to Gsα has been cloned from olfactory epithelia and termed Golf . Its sole location is in the olfactory neuroepithelia where it is responsible for olfactant regulation of adenylyl cyclase (Jones and Reed 1987; Bruch, 1989).

1.4.2 Gi family

Although previously identified by Neer and co-workers (1984) and Gierschik and co-workers (1986a), the pertussis toxin sensitive Gα 40kDa was first purified from bovine brain. It was termed Gi2α, and has since been shown to couple to the inhibition of adenylyl cyclase (McKenzie *et al*, 1988), whereas the previously identified Gα 41kDa was termed Gi1α. HL-60 cells have also been a source of a Gα (41kDa) identified as Gi3α using antipeptide antisera directed against the predicted sequence from cDNA information (Goldsmith *et al*, 1988). Its role in the activation of potassium channels has been demonstrated (Codina *et al*, 1988). In addition to the three forms of Giα identified (Gi1α, Gi2α and Gi3α) other G-protein α-subunits have been termed "Gi-like" due to their high degree of amino acid homology with these proteins.

At least two forms of transducin occur, transducin 1 (Gt1) and transducin 2 (Gt2), which are encoded by separate genes, and are found in rod and cone cells respectively (Lerea *et al*, 1986). These serve to couple rod and cone opsins to cGMP phosphodiesterases. The transducins show an approximate 80% homology with the "Gi-like" proteins. A novel transducin-like G-protein termed gustducin (Gg) has recently been identified from taste tissue (McLaughlin *et al*, 1992) and it

is thought a phosphodiesterase may be involved in its means of signal transduction, due to sequence homology in those regions of the G-protein α-subunit known to interact with receptors and effectors.

Studies using purified preparations of pertussis toxin substrates from bovine brain (Sternweis and Robishaw, 1984; Milligan and Klee, 1985; Neer *et al*, 1984) in conjunction with the purification of rat brain substrates (Katada et al, 1986) led to the identification of the 39kDa G-protein α-subunit termed Go for 'other' (Nukada *et al*, 1986; Itoh *et al*, 1986). cDNA clones of Goα have been isolated from bovine retina (Van Meurs *et al* 1987), rat C6BU1 glioma cells (Itoh *et al* 1986, 1988) and rat olfactory epithelium (Jones and Reed 1987). Additional Goα isoforms have also been identified (Goldsmith *et al*, 1988; Kobayashi *et al*, 1989) immunologically (Goldsmith et al, 1988) and biochemically (Inanobe *et al*, 1989; Hsu *et al*, 1990). The function of Go remains unresolved, early evidence exists to suggest that the G-protein may be involved in the regulation of receptor mediated inhibition of voltage operated Ca^{2+} channels (Hescheler *et al*, 1987; Harris-Warrick *et al*, 1988; Ewald *et al*, 1988). More recent studies demonstrate a role for Go in coupling to somatostatin receptors (Law *et al*, 1991, 1993) and the α_{1B}-adrenergic receptor (Blitzer *et al*, 1993) which serve to inhibit adenylyl cyclase and stimulate phospholipase C activity respectively.

The subclass of "Gi-like" proteins also includes Gxα. Gxα cDNA was isolated from rat brain and was identical to one isolated from human retina and designated Gzα (Fong *et al*, 1988). This protein is pertussis toxin insensitive, however it bears more homology to the "Gi-like" proteins than to members of the Gq class. Antisera specific to Gzα did not identify the Gq proteins (Taylor *et al*, 1990). Gzα exhibits a

remarkably slow rate of guanine nucleotide exchange and a very slow intrinsic GTPase activity when compared to Giα. The biochemical properties of Gzα appear, therefore, to differ from those of Giα, although as yet the physiological significance of this remains undetermined.

1.4.3 Gq/11 family

Numerous studies have implicated a role for G-proteins in coupling receptors to the phospholipid hydrolysing enzyme, phospholipase C (PLC). Recently unique α-subunits that are not substrates for pertussis toxin have been purified and their role in activating PLC is becoming increasingly evident (Exton *et al*, 1990; Pang and Sternweis, 1990).

Simon and colleagues have recently used the polymerase chain reaction (PCR) to detect additional G-protein α-subunit gene products in mouse brain. Five sequences termed Gα10-Gα14 were detected. Four of these sequences were novel, as Gα10 was found to be equivalent to Gαolf. The sequence of the Gα11 clone is closely related to that of the Gα14 (Strathmann *et al*, 1989). Strathmann and Simon more recently obtained a murine cDNA clone termed Gqα which is 88% homologous to G11α and have proposed that these clones represent a third class of α-subunits - the Gq class. Also within this newly designated class of pertussis toxin insensitive G-proteins, three other isotypes have been discovered, which exhibit tissue specific expression. Gα14 is found in stromal and epithelial cells, whilst Gα15 and Gα16 are found in cells derived from the haematopoetic lineage (Simon *et al*, 1991; Amatruda *et al*, 1991).

1.4.4 G12/13 family

Simon and co-workers have also suggested that the less well characterised murine cDNA clones Gα12 and Gα13 represent another class of pertussis toxin resistant α-subunits. The translation products are predicted to share less than 45% identity with other α-subunits and transcripts have been detected in every tissue examined (Strathmann and Simon, 1991).

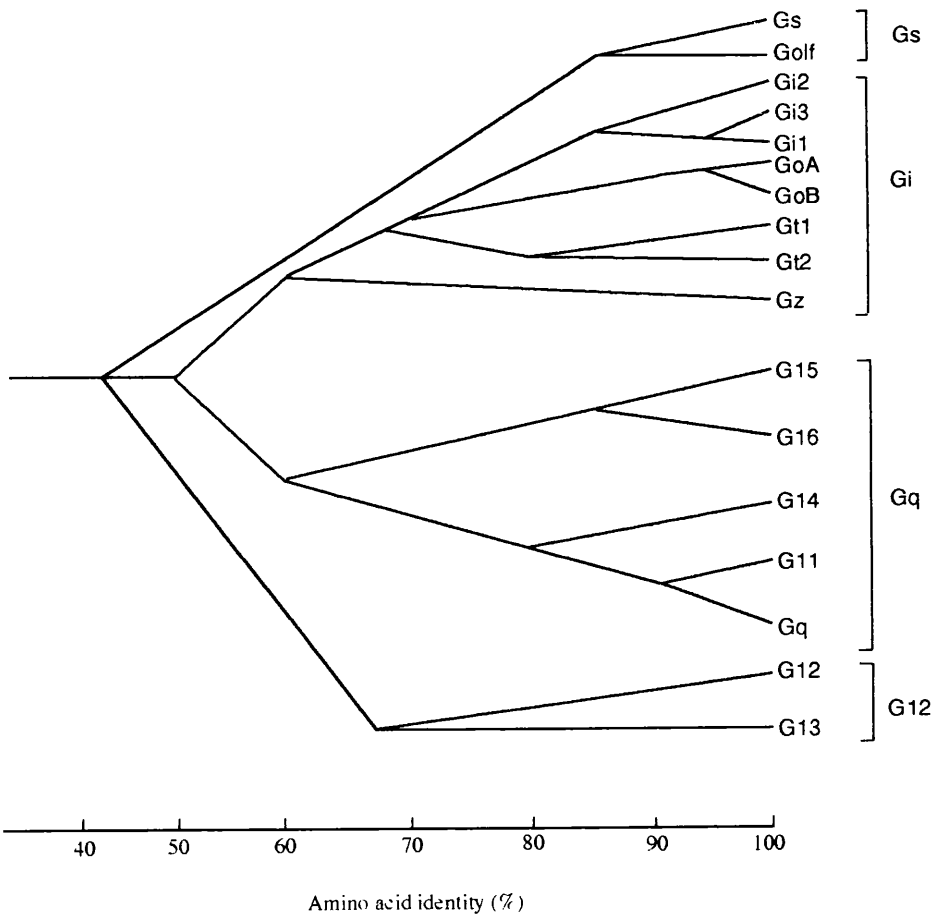
When the predicted amino acid sequences of the G-protein α-subunits are aligned they exhibit impressive sequence homolgy, as represented schematically in figure 1.1. Over 98% identity of amino acid sequence is maintained for Gi2α, Gi3α, Gzα and Goα among evolutionary distinct mammalian species, presumably reflecting an evolutionary pressure to preserve the specific physiological function of each G-protein. The presence of heterotrimeric G-proteins has also been demonstrated in yeast *Saccharomyces cerevisiae* (Nakafuku *et al*, 1987, 1988), *Drosophila melanogaster* (Provost *et al*, 1988; Quan *et al*, 1989; Yoon *et al*, 1989; de Sousa *et al*, 1989) and *Dictyostelium discoideum* (Pitt *et al*, 1992).

1.4.5 Cloning of β and γ subunits

To date four β-subunits have been identified by a combination of purification and molecular cloning; β1 (Sugimoto *et al*, 1985, Fong *et al*, 1986); β2 (Fong *et al*, 1987; Gao *et al*, 1987); β3 (Levine *et al*, 1990) and β4 (von Weizsacker *et al*, 1992). There is an overall homology of approximately 80% between β-subunit isoforms and their molecular

Figure 1.1 G-protein α-subunit amino acid homology.

The G-protein α-subunits were grouped by their amino acid identity and the relationship between the different G-protein α-subunits was displayed. The diagram is adapted from Simon *et al*, 1991 (Science 252: 802-808).



weight is in the range of 35-37kDa. Interestingly the β-subunit amino acid sequence comprises of seven repeating units each about 43 amino acids long. Similar repeating units termed WD40 repeats occur in several other proteins whose functions seem unrelated to signal transduction (van der Voorn and Ploegh, 1992). It is not known, as yet, what features of the repeating unit necessitate its conservation among so many different proteins.

A cDNA corresponding to the γ subunit (γ1), of transducin was initially cloned by Hurley and co-workers (1984). The predicted amino acid sequence from this cDNA suggests a hydrophilic protein of 8.4kDa. Subsequently at least six other γ-subunits have been identified from a variety of tissues and cDNA libraries γ2 from brain; γ3 and γ7 from a bovine brain cDNA library, γ4 from mouse kidney and retina, γ5 from the bovine and rat liver cDNA libraries and γ6 from rat brain (Robishaw *et al*, 1989; Gautam *et al*, 1990; Fisher and Aronson, 1992; Cali *et al*, 1992). There is a much greater diversity in amino acid composition between γ-subunits (27-75% homology) than in the more highly conserved β-subunit. This suggests that the γ-subunit may play a more dominant role in determining the function and specificity of the G-protein than previously anticipated.

1.5 The mechanism of action of G-proteins

1.5.1 The GTPase cycle

Using the available data on the effects of cholera toxin and non-hydrolysable GTP analogues on Gs, together with the ability to measure hormonal stimulation of high affinity GTPase activity, Cassel and Selinger were able to propose a cyclical model to account for G-protein function which is still applicable.

Binding of a ligand to its receptor causes the α-subunit to lose its bound GDP and bind GTP in a Mg^{2+} dependent manner. Agonists act to reduce the concentration of Mg^{2+} required for activation, for example glucagon lowers the magnesium concentration required for GTPγS activation of Gsα from 25mM to 10μM. As the intracellular Mg^{2+} is estimated to be of the order of 2mM it can then be utilised (Iyengar and Birnbaumer, 1982). The binding of GTP reduces the affinity of the receptor for the agonist, resulting in a dissociation of the receptor-G-protein complex and a magnesium dependent dissociation of the α-subunit from the $\beta\gamma$ (Maguire *et al*, 1976). The Gα-GTP is now in an "active" conformation suitable for specific interaction with an effector protein. On completion of effector activation, GTP is hydrolysed to GDP by the intrinsic GTPase of the α-subunit (Rodbell, 1980). The now inactive Gα-GDP can reassociate with its $\beta\gamma$ subunits. This system is cyclical and dependent on ligand binding, GTP and Mg^{2+} (Ransnas *et al*, 1992).

Necessity for agonist occupancy of the receptor can be circumvented by use of non-hydrolysable analogues of GTP, such as

Gpp[NH]p and GTPγS, although the reaction still requires magnesium (Codina *et al*, 1983). G-proteins can also be activated by aluminium fluoride (AlF^{4-}) which mimics the terminal phosphate of GTP when GDP is in the guanine nucleotide binding site (Bigay *et al*, 1985).

1.5.2 Regulation of the GTPase cycle

Several groups have shown that agonist-liganded receptors increase the dissociation rate of bound GDP, therefore vacating the guanine nucleotide binding site and subsequently allowing the binding of GTP (Rodbell, 1980; Schramm and Selinger, 1984). The receptor binds the $\alpha\beta\gamma$ complex much more tightly than it binds to α alone (Fung, 1983; Fung and Nash, 1983; Florio and Sternweis, 1989). Enhanced receptor association with α could, therefore, be a consequence of $\beta\gamma$ interaction with the receptor and indeed, there is evidence to suggest an interaction of $\beta\gamma$ with the β-adrenergic receptor (Im *et al*, 1988). $\beta\gamma$ exhibits a preference for binding α-GDP and a receptor preferentially binds $G\alpha\beta\gamma$ as opposed to $G\alpha$, thus $\beta\gamma$ may serve to ensure the reduction of spontaneous, receptor-independent release of GDP. Additionally $\beta\gamma$ also inhibits the GTPase of the protein (Bourne *et al*, 1991) and the combination of these properties ensure exchange of GTP for GDP and not *vice versa*.

GTPase activating proteins (GAPs) have been identified as being necessary protein co-factors for the efficient GTPase activity in several GTP binding proteins. Compared to the GAP-triggered GTPase activity of p21ras and ribosome triggered GTPase of elongation factor (Ef-Tu), the G-proteins hydrolyse GTP much more slowly, $k_{cat}.GTP=2.4min^{-1}$

(Freissmuth *et al*, 1989; Graziano and Gilman, 1989; Landis *et al*, 1989). However this GTP hydrolytic rate appears to be slow enough to enable signal transduction yet rapid enough to control a turn-off of signal transmission (Bourne *et al*, 1990, 1991). A recent finding has revealed that the effectors phospholipase Cβ-1 (Berstein *et al*, 1992) and cGMP phosphodiesterase (Arshavsky and Bownds, 1992) increase the intrinsic GTPase activity of their activating G-protein α-subunit. It may be that all effectors can deactivate their specific G-protein α-subunits through such a negative feedback mechanism, although this awaits formal demonstration.

1.6 Structural and functional characteristics of G-proteins

1.6.1 The domains in Gα for GTP binding and hydrolysis

Recently the crystalline structure of GTPγS-liganded transducin was elucidated (Noel *et al*, 1993), although the crystal structures of the other Gα have not been determined. The three dimensional folds of its GTP binding site are almost superimposable on those of the GTP binding proteins p21ras (La Cour, 1985; Pai *et al*, 1989; Milburn *et al*, 1990; Pai *et al*, 1990; Bourne *et al*,1991) and the protein elongation factor EF-Tu, although the overall sequence homology between the proteins is only 17% (Valencia *et al*, 1991). The structure of the GTP binding domain in both these proteins and transducin comprises of a hydrophobic core of alternating β-sheets and hydrophilic α-helices. However a stretch of 113 amino acids in transducin (conserved in Gα chains, but missing in the other GTPases) composed of α-helices has been identified near the N-terminal of the protein. This structure has been heavily implicated in

regulating both guanine nucleotide release (GDP) and the binding and hydrolysis of GTP (GAP activity). The helical domain is postulated to restrict movement out of and into the guanine nucleotide binding pocket of the core domain. It has been proposed that to promote the replacement of GDP by GTP the activated receptor potentially "moves" the helical domain, via a conformational change, allowing the exchange of guanine nucleotides. The helical insert is therefore thought to act as an intrinsic activator of GDP exchange for GTP. Indeed for some *ras*-like GTPases a class of separate proteins, guanine nucleotide dissociation inhibitors (GDI), serve to inhibit the release of bound guanine nucleotide. Therefore as well as regulating GTPase activity (acting as an intrinsic GAP protein), the Gα helical domain may also act as an intrinsic GDI that is subject to regulation by hormone receptors (Markby *et al*, 1993; Bourne, 1993) (figure 1.2).

The amino acid residues involved in GTP binding and hydrolysis have been extensively studied in p21*ras* and corresponding residues in transducin have also been shown to bind GTPγS. This is discussed in detail in the following references: Bourne *et al*, 1990; Bourne *et al*, 1991; Markby *et al*, 1993; Noel *et al*, 1993; Bourne, 1993.

1.6.2 Interaction of Gα with other signalling components

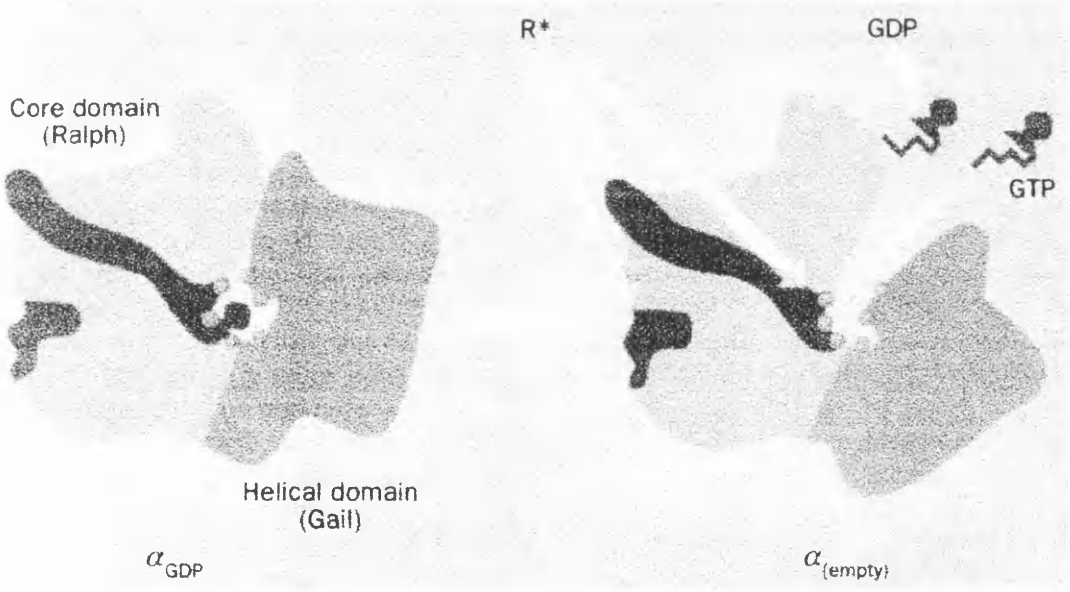
Mutational, biochemical and structural analysis of the G-protein α-subunit has elucidated the regions of the protein involved in its interaction with other polypeptides of the signalling cascade. The extreme C-terminal portion of Gα is primarily responsible for its interaction with receptor (West *et al*, 1985; Sullivan *et al*, 1987; Rall and Harris, 1987; Simonds *et al*, 1989; Gutowski *et al*, 1991; Shenker *et al*, 1991). More

1991). More recent work from Conklin and co-workers showed that in addition to contacting receptors, the extreme C-terminal of Gα also made a major contribution to determining the specificity of the receptor-G-protein interaction (Conklin and Bourne, 1993a). The extreme N-terminus of Gα has been shown to be important for the binding of βγ dimers to the protein (Fung and Nash, 1983; Neer *et al*, 1988; Mazzoni and Hamm 1989; Mazzoni *et al*, 1991; Denker *et al*, 1992; Graf *et al*, 1992; Journot *et al*, 1992), although the GTP binding region has been demonstrated to play some part in this interaction (Lee *et al*, 1992). Mutagenesis studies of Gsα revealed that the key adenylyl cyclase interacting residues were located within a 121 amino acid sequence close to the C-terminal of the protein (Itoh and Gilman, 1991; Berlot and Bourne, 1992;), although other elements which determined the three dimensional structure of the G-protein α-subunit were involved in the regulation of the effector by the G-protein.

Taken together, the emerging picture of the overall three dimensional structure of the G-protein α-subunit, is with the GTP-binding pocket located at the front of the protein and facing the cytoplasm. The back of the protein is oriented towards the plasma membrane and provides binding surfaces for receptor, effector and G-protein βγ subunits, as well as attachment sites for fatty acids that enhance avidity of α-subunits for membranes and βγ (Conklin and Bourne, 1993b).

Figure 1.2 The proposed mechanism of Gα activation by its receptor.

The figure showing the mechanism of Gα activation by a receptor has been adapted from Bourne, 1993 (Nature 366: 628-629). It shows how an activated receptor (R*) may turn on a G-protein, by inducing the helical domain of Gα to pivot away from the Gα core GTPase domain. By binding to surface features the receptor loosens a co-operative web of hydrogen bonds (circles), causing the helical domain to pivot away from the guanine-nucleotide binding site allowing free exchange of GDP for GTP.



1.7 Covalent modifications of G-proteins

G-proteins can be covalently modified in several ways. These modifications are potentially important in regulating the function and cellular targetting of the G-protein and will be discussed below.

1.7.1 ADP-ribosylation

Pertussis toxin and cholera toxin catalysed mono ADP-ribosylation of G-protein α-subunits has been well characterised (section 1.2), but it is still uncertain if ADP-ribosylation of G-protein α-subunits occurs under normal cellular physiological conditions. Both arginine (Moss and Vaughan, 1988; Inageda and Tanuma, 1991) and cysteine specific (Tanuma *et al*, 1987; Tanuma *et al*, 1988) mono (ADP-ribosyl) transferases have been identified in several cell types. Enzymes that catalyse the removal of ADP-ribose from ADP-ribosylated arginine residues have also been identified (Moss *et al*, 1992), suggesting that ADP-ribosylation may be a reversible physiological event (Moss *et al*, 1985). Certainly several groups have produced evidence in support of the ADP ribosylation of G-protein α-subunits by both arginine (Donnelly *et al*, 1992) and cysteine specific mono (ADP ribosyl) transferases (Tanuma and Endo, 1989), although the identification of an endogenously ADP-ribosylated G-protein α-subunit has not yet been reported *in vivo*. The function of this ADP-ribosylation remains unclear, but targetting the protein for an enhanced proteolysis, after agonist activation, has been suggested.

1.7.2 Prenylation

Transfer of the prenyl moiety, farnesyl, to a polypeptide was first described for the yeast mating factor (Kamiya *et al*, 1978; Ishibashi *et al*, 1984; Anderegg *et al*, 1988). Schmidt and co-workers (1984) presented the first evidence that proteins in mammalian cells were also modified by prenyl groups.

Cysteine residues in the C-terminal of the protein are a common characteristic of prenylated proteins. These are organised in a Cys-Xaa-Xaa-Xaa, Cys-Cys or Cys-Xaa-Cys motif. Prenylation of proteins occurs post-translationally and involves the attachment of an isoprenoid moiety to the sulphydryl group of a carboxy terminal cysteine residue via a thioester linkage. Three residues downstream of the prenylated cysteine are then proteolytically removed and the newly exposed C-terminal cysteine residue is methylated (Clarke, 1992).

Parallel studies from two groups (Yamane *et al*, 1990; Mumby *et al*, 1990b) first showed that G-protein γ-subunits purified from bovine brain and the neural cell line PC12 were modified by geranylgeranylation and carboxymethylation on a C-terminal cysteine residue. Furthermore, the γ-subunit of transducin was found to be modified by a farnesyl group (Fukada *et al*, 1990; Lai *et al*, 1990).

The α-carboxy-methylation of mammalian proteins was first demonstrated in *ras* proteins overexpressed in a fibroblast cell line, which incorporated methyl groups with a stability consistent with α-carboxyl methyl esters (Clarke *et al*, 1988). On reconstitution in detergent stripped bovine brain membranes with a methyl transferase the γ-subunit

of G-proteins was first shown to be α-carboxymethylated (Fung *et al*, 1990). The function of the α-carboxymethylation is unknown, however the absence of an α-carboxyl methyl ester on yeast mating factors results in the nearly complete loss of activity (Ishibashi *et al*, 1984; Anderegg *et al*, 1988; Hryeyna *et al*, 1991).

Several lines of evidence, mainly obtained from small molecular weight G-proteins, indicate that the prenyl modification is for the attachment of the protein to the plasma membrane (Hancock *et al*, 1989; Jackson *et al* ,1990; Simonds *et al*, 1991; Hiroyoshi *et al*, 1991; Kato *et al*, 1992). $\beta\gamma$ dimers of heterotrimeric G-proteins containing C-terminal mutants of the γ-subunit demonstrated a low affinity for both α-subunits and adenylyl cyclase, both of which are associated with the plasma membrane. Recent evidence also suggests that the role of the prenyl moiety on the γ-subunit is for the membrane attachment of the $\beta\gamma$ dimer (Iniguez-Lluhi *et al*, 1992). Removal of the C-terminal prenylation signal and replacement with an amino terminal myristoylation site (myristoyl moieties will also bind the protein to the membrane), restores transforming activity to the *ras* protein (Buss *et al*, 1989). This suggests the sole function of prenylation is membrane binding and not interaction of the prenyl group with the receptor or effector.

1.7.3 Myristoylation

The enzyme, N-myristoyltransferase, catalyses the transfer of a myristate moiety to a glycine residue via an amide bond, at the N-terminal of a protein. The consensus sequence for N-terminal myristoylation has now been established to be an amino terminal glycine

with a small uncharged amino acid such as alanine, serine or a hydroxyamine residue commonly found 4 amino acids downstream. Myristoylation is a co-translational rather than a post-translational modification. N-myristoyltransferase activity has been demonstrated in nearly every eukaryotic cell type (Deichaite *et al*, 1988).

The endogenous myristoylation of G-protein α-subunits was first found in isolated proteins from bovine brain (Schultż *et al*, 1987) and human astrocytoma cells (Buss *et al*, 1987). Metabolic labelling of human astrocytoma cells (Buss *et al*, 1987) with [^{3}H]-myristate revealed that Giα and Goα, but not Gsα proteins were myristoylated. On examination of the amino acid sequence of these proteins it was discovered that Giα and Goα had an amino terminal glycine with a serine residue four amino acids downstream. Although Gsα has an amino terminal glycine it does not possess the serine residue, indicating that this residue is essential for myristoylation of Gα. The myristoylation of specific G-protein α-subunits was later studied in depth by their transient expression and subsequent metabolic labelling in COS cells (Jones *et al*, 1990; Mumby *et al*, 1990a). These studies verified that the α-subunits of Gi1, Gi2, Gi3, Go and Gz were substrates for myristoylation and these α-subunits as well as Gsα overexpressed in COS cells were localised to membrane fractions.

Mutagenesis of the N-terminal glycine to alanine in Gi1α and Goα followed by expression of the mutant cDNAs in COS cells resulted in non-myristoylated α-subunits whose localisation was shifted from the plasma membrane to the cytosol (Jones *et al*, 1990; Mumby *et al*, 1990a), indicating a role for myristoylation in membrane attachment. This was further corroborated by more recent studies of *gip2*, an

oncogenic α-subunit of Gi with transforming activity (Lyons *et al*, 1992). Removal of the site of myristoylation by site directed mutagenesis resulted in non-myristoylated *gip2* which lacked transforming activity (Gallego *et al*, 1992).

Co-expression of Go with N-myristoyl transferase in *E. Coli* resulted in a mixture of myristoylated and non-myristoylated Goα (Linder *et al*, 1991). These were purified to homogeneity and through reconstitution studies used to investigate the functional role of myristate. The most striking difference was the inability of the non-myristoylated α-subunit to interact effectively with βγ complexes. In contrast the myristoylated Goα behaved identically to Goα purified from brain. These results are consistent with reports that the amino terminus of Gtα and indeed other Gα subunits is required for the protein's interaction with the βγ complex (Navon *et al*, 1987; Neer *et al*, 1988; Osawa *et al*, 1990).

The relative importance of myristoylation in βγ interaction and membrane localisation appears to vary among the α-subunits ie Gsα is not myristoylated, yet it is still attached to the membrane and interacts with βγ.

1.7.4 Palmitoylation

Palmitoylation of proteins occurs via a thioester linkage on a cysteine residue and is a post-translational modification. As yet no palmitoyltransferases have been purified or cloned and palmitoylation

appears at least in some cases to be non-enzymatic (Yamane and Fung, 1993).

Until recently only members of the *ras* family of small molecular weight G-proteins had been shown to be palmitoylated (Hancock *et al*, 1989). Early studies reported that the α-subunit of the heterotrimeric G-protein Gs, would not undergo palmitoylation, but more recent work by two groups (Degtyarev *et al*, 1993a; Linder *et al*, 1993) demonstrated that Gsα will incorporate [^{3}H]-palmitate. It is thought that the early work missed the palmitoylation due to the low abundance of Gsα in most cells and the resulting low level of incorporation of palmitate. Certainly Linder and co-workers (1993) used high performance liquid chromatography to demonstrate that palmitate and not some other acyl group (for example myristoyl) was incorporated. Furthermore, mutation of a cysteine residue at position 3 of the N-terminal of Gsα (Degtyarev *et al*, 1993a) and Goα (Parenti *et al*, 1993) prevents palmitate incorporation, indicating that this cysteine residue is the site of palmitoylation .

Studies on the palmitoylation of heterotrimeric G-proteins are very much in their preliminary stages and the role of palmitoylation in the functioning of these proteins is still unclear. It seems however that palmitate may play a role in mediating interactions of the G-protein α-subunit with the plasma membrane. Certainly non-palmitoylated Goα shows reduced binding to the membrane (Parenti *et al*, 1993).

The palmitoylation of Gsα may be particularly important because this protein is not myristoylated and the means of membrane attachment outwith its interaction with $\beta\gamma$ remains elusive. Controversy exists over the role of palmitate in Gsα membrane attachment. Degtyarev and co-

workers (1993a) demonstrated that removal of the palmitate moiety from Gsα still produced a protein which is localised to the plasma membrane, whilst Wedegaertner and co-workers (1993) showed by mutational analysis that palmitoylation of Gsα was important for membrane attachment. Audigier and co-workers (1990) by mutational and proteolytic analysis of Gsα demonstrated a role for the C-terminus in membrane attachment, indicating that perhaps palmitoylation of Gsα has a function other than membrane attachment and some other residue and/or covalent modification is responsible for membrane attachment in Gsα. Recent work by Degtyarev and co-workers (1993b) shows an increased palmitoylation of Gsα after activation by both the β-adrenergic receptor and cholera toxin. An explanation for this remains to be found, however, an increased hydrophobicity of the G-protein results and this may function to increase the membrane attachment of the GTP bound form of Gsα, resulting in effector activation.

More investigation is needed to determine unequivocally the role of palmitoylation in membrane attachment of heterotrimeric G-proteins. Palmitoylation has been shown to be a reversible process and it may be that deacylation and acylation of G-proteins controls its function and regulation. Certainly an increased turnover rate of palmitoylation of the β2-adrenergic receptor is observed on treatment with an agonist (Mouilliac *et al*, 1992) although the function of this palmitate turnover remains undetermined (O'Dowd *et al*, 1989; Moffett *et al*, 1993).

1.7.5 Phosphorylation

Phosphorylation of G-proteins is the least well defined of the covalent modifications. The first evidence that G-proteins may be regulated by phosphorylation came with the observation that treatment of platelet membranes with partially purified PKC resulted in phosphorylation of a 41kDa protein, later demonstrated to be Giα, in a stoichiometry of 1 mole Pi per mole of substrate (Katada *et al*, 1985). More recent studies with recombinant G-proteins have shown that the α-subunit of Gz is stoichiometrically phosphorylated by partially purified PKC on a serine residue near the N-terminal, whereas recombinant Gi1α, Gi2α and Gi3α are not labelled under the same conditions (Lounsbury *et al*, 1991). Phosphorylation of G-protein α-subunits has also been reported to occur on both serine and tyrosine residues in a reconstituted system containing purified insulin protein kinase (Krupinski *et al*, 1989) and PKC (Pyne *et al* 1992) .

Although these results demonstrate that the α-subunit of G-proteins can be phosphorylated 'in vitro', a link with physiological events has yet to be conclusively demonstrated. Clearly phosphorylation of G-proteins has strong implications in "cross-talk" between signalling systems.

1.8 The mechanism of G-protein interaction with effector proteins

1.8.1 The effector protein

Among the growing number of effector proteins identified which are regulated by G-proteins are a number of ion channels, the retinal cGMP phosphodiesterase and the expanding adenylyl cyclase and phospholipase C families. The regulation of the latter two families will be detailed here; for information on the regulation of the retinal cGMP phosphodiesterase (Pfister *et al*, 1993) and ion channels (Sternweis and Pang, 1990) the mentioned reviews will prove to be useful.

1.8.1.1 Phospholipase C

Phospholipase C (PLC) catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce two important intracellular second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate. Molecular cloning has revealed at least three classes of phospholipase C; phospholipase C-β, -γ and -δ. A number of isoforms of each class of phospholipase C have been identified (Rhee and Choi, 1992; Rhee, 1991). To date there are four isoforms of PLC-β, and only isoforms from the PLC-β class have been shown to be regulated by heterotrimeric G-proteins (Rhee and Choi, 1992; Kritz *et al*, 1990).

1.8.1.2 Adenylyl cyclase

Adenylyl cyclase catalyses the conversion of intracellular ATP to cAMP, increasing intracellular levels of cAMP. It was only recently that

detailed molecular information about the adenylyl cyclase enzyme has been acquired, following the cloning of several isoforms of the enzyme. Most adenylyl cyclases are associated with the plasma membrane, although certain bacterial enzymes (and perhaps one form in mammalian sperm [Rojas *et al*, 1993]) are cytosolic.

These membrane associated adenylyl cyclases have short cytoplasmic N- and C-terminal regions and two 40kDa cytoplasmic domains (C1 and C2) punctuated by two intensely hydrophobic stretches (M1 and M2), each of which are hypothesised to contain six transmembrane helices (figure 1.3). cDNAs encoding numerous adenylyl cyclases of this type have now been cloned, 6 of these are from mammalian sources: type 1 from brain (Krupinski *et al*, 1989), type11 from brain and lung (Feinstein *et al*, 1991), type 111 from olfactory specific neurons (Bakalyar and Reed, 1990), type 1V from peripheral tissue and the CNS (Gao and Gilman, 1991) and types V (Ishikawa *et al*, 1992) and V1 (Premont *et al*, 1992; Katsushika *et al*, 1992) from S49 lymphoma cells; one is the product of the *rutabaga* gene from *Drosophila melanogaster* (Levin *et al*, 1992), two are from *Dictyostelium discoideum* (Pitt *et al*, 1992) and one is from *Saccharomyces cerevisiae* (Kataoka *et al*, 1985).

Portions of the C1 and C2 domains (C1a and C2a) are well conserved (50-92% homologous). These are very similar to each other and also to the catalytic domains of a series of membrane bound guanylyl cyclases (Chinkers and Garbers, 1991). Catalytic activity of adenylyl cyclase requires both C1a and C2a, suggesting that these two domains may interact to produce the catalytic activity of the enzyme (Tang *et al*, 1992). Similarly the N-terminal and the C-terminal are both required for

the catalytic activity of the protein (Tang *et al*, 1991). The exact site on the adenylyl cyclase polypeptide required for catalytic activity and G-protein interaction is not yet known. The amino acid sequence homology is limited beyond the C1a and C2a domains, but the overall structure is well conserved, suggesting that the two sets of 6 transmembrane helices may play an as yet undiscovered role in the functioning of the enzyme.

Potential for "cross-talk" between signalling pathways exists at the level of the adenylyl cyclase protein. The isoforms of mammalian adenylyl cyclase display differential regulation by $\beta\gamma$ dimers and calcium ions which are detailed in table 1.1. Additionally, several isoforms (types 1, 11 and 111) of adenylyl cyclase have recently been shown to be regulated by protein kinase C (Choi *et al*, 1993; Jacobiwitz *et al*, 1993; Yoshimura and Cooper, 1993).

1.8.2 Gα interaction with effector

1.8.2.1 Gα interaction with adenylyl cyclase

All the mammalian adenylyl cyclase enzymes are activated by Gsα, the Gα originally identified because of this action. For a long time it was thought that inhibition of adenylyl cyclase could not be obtained with activated α-subunits of the putative inhibitory G-proteins (Gi). $\beta\gamma$ dimers were shown to have an inhibitory effect on adenylyl cyclase activity. A mechanism of hormonal inhibition based on this phenomenon was proposed, whereby $\beta\gamma$ dimers that were generated by the inhibitory receptor quenched the activity of the stimulatory G-protein, Gsα.

Inhibition was thus proposed to come about by removal of the stimulatory signal. However, the finding that *cyc-* S49 lymphoma cells which lacked a functional Gsα still exhibited normal inhibitory regulation of adenylyl cyclase argued against this as the sole mechanism of inhibition (Katada *et al*, 1984a). Later McKenzie and co-workers (1988) demonstrated the ability of antibodies specific to Gi2α to suppress receptor mediated inhibition of adenylyl cyclase in NG108-15 cells. Recently cDNAs encoding persistently activated Giα were expressed in several cell systems. Inhibition of, at least some, subtypes of adenylyl cyclase by all three Giα subunits was shown to occur (Wong *et al*, 1991; Lowndes *et al*, 1991; Hermout *et al*, 1991; Wong *et al*, 1992; Taussig *et al*, 1993), indicating a direct role for Giα in the inhibition of adenylyl cyclase.

1.8.2.2 Gα activation of phospholipase C

The G-protein mediated activation of the PLC-β subclass of phospholipase C is specific for the Gq family of heterotrimeric G-proteins (Simon *et al*, 1991). The members of the other G-protein classes have not been found to directly activate PLC. The Gq class only activates PLC-β and not the members of the δ or γ class of phospholipase C (Taylor *et al*, 1990; Wu *et al*, 1992a, 1992b). Both Gα14 and Gα16 activate PLC-β1 (Wu *et al*, 1992b), whereas only Gα16 activates the PLC-β2 isozyme (Lee *et al*, 1992b) to a significant degree.

Figure 1.3 Structure of adenylyl cyclase isoforms.

Figure 1.3 represents the structure of the eukaryotic adenylyl cyclases (adapted from Tang and Gilman, 1992 [Cell 70: 869-872]).

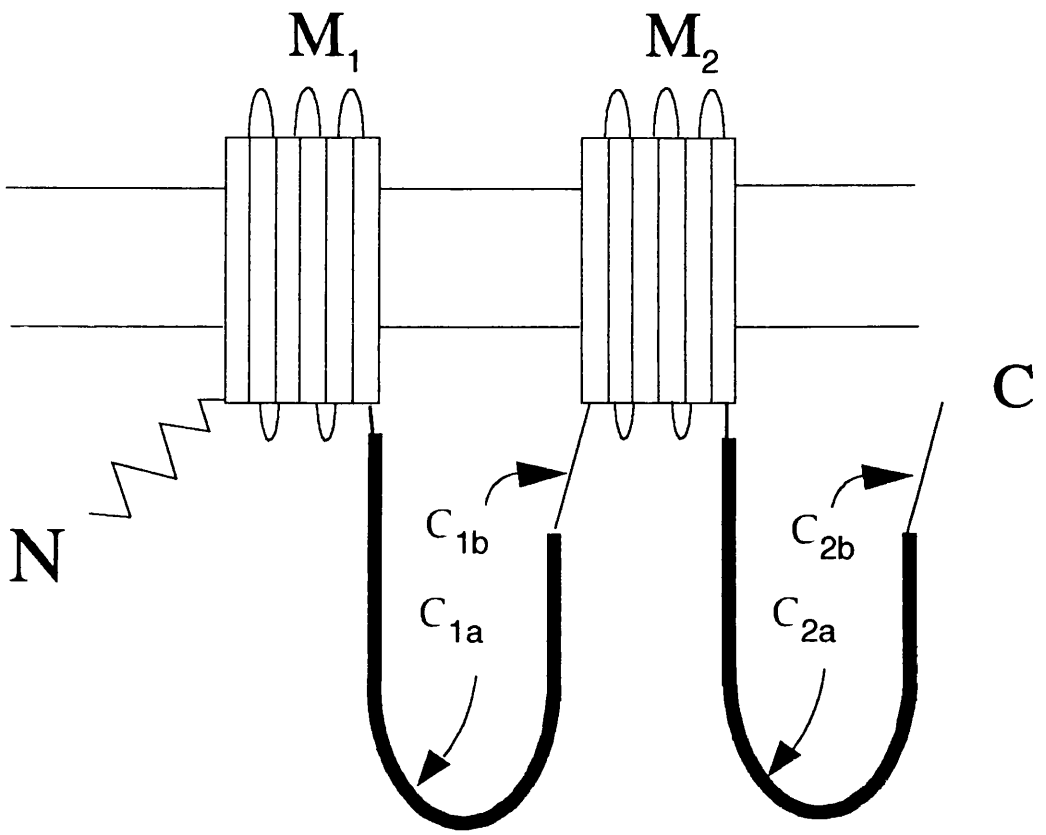


Table 1.1 Properties of eukaryotic adenylyl cyclase isoforms
(adapted from Tang and Gilman, 1992 [Cell 70: 869-872]).

		Effect of G-proteins		
Type	**Expression**	**Gsα**	**βγ**	**Ca2+**
I	brain	+	-	+
II	brain, lung	+	+	0
III	olfactory	+	0	+
IV	brain, others	+	+	0
V	heart,brain,others	+	0	0
VI	heart,brain,others	+	0	0
rutabaga	mushroom body	+	0	+
AC-A	during aggregation	?	?	?
AC-G	fruiting body	?	?	?
CYR1	constitutive	0	0	0

Adenylyl cyclase types I-VI are mammalian; rutabaga is from *Drosophila melanogaster*, AC-A and AC-G are from *Dictyostelium discoideum*; CYR1 is from *Saccaromyces cerevisiae.* References: type I (Krupinski *et al*, 1989), type II (Feinstein *et al*, 1991), type III (Bakalayer and Reed, 1990), type IV (Gao and Gilman, 1991), type V (Ishikawa *et al*, 1992), type VI (Premont *et al*, 1992; Katsushika *et al*, 1992), rutabaga (Levin *et al*, 1992), AC-A and AC-G (Pitt *et al*, 1992) and CYR1 (Kataoka *et al*, 1985).

1.8.3 $\beta\gamma$ regulation of effector molecules

1.8.3.1 The $\beta\gamma$ dimer

β and γ subunits copurify and although they are not covalently linked, denaturing conditions are still required for their separation. To circumvent the difficulty in purifying individual β and γ subunits, the expression of recombinant β and γ subunits in baculovirus-infected Sf9 insect cells has allowed the purification of dimers composed of different subtypes of β and γ (Robishaw *et al*, 1992; Graber *et al*, 1992). The functional interaction of the different dimers of $\beta\gamma$ has thus been investigated.

Using the Sf9 baculovirus expression system recent studies have revealed that not all of the β-subunits can form dimers with all of the γ-subunits. $\gamma 1$ will associate with $\beta 1$ but not $\beta 2$ (Pronin and Gautam, 1992; Iniguez-Lluhi *et al*, 1992; Clapham and Neer, 1993). However $\gamma 1$ and $\beta 2$ are not likely to encounter each other in a physiological situation since expression of $\gamma 1$ appears restricted to photoreceptors, whereas these cells have been shown not to express $\beta 2$.

Studies using chimeric $\beta 1/\beta 2$ subunits have demonstrated the involvement of the N-terminal of the β-subunit in its interaction with the γ-subunit (Pronin and Gautam, 1992). Cysteine 25 has been postulated to participate in the interaction, however the amino acid sequence surrounding this residue is very similar throughout the β-subunit family. It may be that there are very subtle differences in primary sequence or post-translational modifications of the N-terminal of the distinct β-subunits, thus determining the specificity of their interactions with γ.

1.8.3.2 Effector regulation by $\beta\gamma$

For a long time it was thought that the G-protein α-subunit determined the protein's interaction with receptor and its activation of the effector. The first indication that G-protein $\beta\gamma$ subunits could directly control effector molecules came from the observation that $\beta\gamma$ purified from bovine brain activated the cardiac K^+ channel normally regulated by the muscarinic acetylcholine receptor (Logothetis *et al*, 1987; Codina *et al*, 1987). More recent evidence has demonstrated that both Gα and $\beta\gamma$ can activate the K^+ channel separately (Yamada *et al*, 1993), although it is still uncertain if $\beta\gamma$ subunits activate the channel directly or indirectly through activation of an arachidonic acid metabolite produced by phospholipase A2 activity (Jelsema and Axelrod, 1987; Kim *et al*, 1989; Kurachi *et al*, 1989).

Until recently $\beta\gamma$ dimers had been assigned an indirect role in the regulation of adenylyl cyclase, as described in section 1.8.2.1. The cloning of β and γ subtypes and also of adenylyl cyclase isoforms, has allowed their co-expression in cells and subsequent functional analysis. Using this procedure it has been shown that $\beta\gamma$ regulates adenylyl cyclase activity in a subtype specific manner. Whereas $\beta\gamma$ inhibits type 1 adenylyl cyclase when it is stimulated by either Gsα or Ca^{2+}/calmodullin; $\beta\gamma$ greatly potentiates the stimulatory effect of Gsα on either type 11 or type 1V adenylyl cyclase (Tang and Gilman, 1991). Inhibition of adenylyl cyclase by $\beta\gamma$ is independent of Gsα activation, whereas stimulation by $\beta\gamma$ has an underlying prerequisite for the

stimulation of adenylyl cyclase by Gsα. Other adenylyl cyclases cloned to date have been shown to be insensitive to βγ.

The PLC-β isoforms 1-3 have also been shown to be activated by βγ subunits (Rhee, 1991; Sternweis and Smrcka, 1992, Camps *et al*, 1992 a,b), PLCβ4 however is not activated by βγ (Clapham and Neer, 1993). The sensitivity of the different isoforms of PLC-β to both βγ and Gα varies between isoforms (Camps *et al*, 1992a; Katz *et al*, 1992; Boyer *et al*,1992; Smrcka and Sternweis, 1993). Unlike some of the adenylyl cyclase isoforms the effect of Gα and βγ on the activation of PLCβ isoforms are not dependent on each other suggesting separate regulatory sites for α and βγ on the PLCβ polypeptide. Expression of mutated PLC-β1 in COS-7 cells allowed the identification of the C-terminal of PLC-β1 as the site required for activation by Gqα (Wu *et al*, 1993). Preliminary evidence suggests that βγ binds to the N-terminal of the PLC polypeptide (Clapham and Neer, 1993).

The emerging pattern for G-protein regulation of both adenylyl cyclases and phospholipase C (Clapham and Neer, 1993) seems to be that all forms of the enzyme which are regulated by the heterotrimeric G-proteins are regulated by the α-subunits, though the potency with which and the extent to which the α-subunits regulate the effector may vary. Certainly, the discovery that βγ can regulate some isoforms of the effector proteins alongside Gα indicates that one receptor may regulate two effectors by merely activating one G-protein, thus giving the potential for "cross-talk" between signalling pathways.

1.9 The structure of G-protein coupled receptors

The first step in the transfer of information from the outside to the inside of the cell is the binding of a hormone, neurotransmitter or growth factor to a transmembrane receptor which is present on the plasma membrane of the cell. Cell surface receptors may be functionally classified into several categories, according to the specific signal transduction pathways that they stimulate. These classes include

a) growth factor receptors which have intrinsic tyrosine kinase activity (for example receptors for insulin, PDGF and EGF)(Hunter and Cooper, 1985)

b) multi-subunit receptors which are ion channels (the nicotinic acetylcholine, γ-amino butyric acid and the glycine receptors) (Changeax *et al*, 1987)

c) receptors which function as carrier proteins (transferrin receptor)(Goldstein *et al*, 1985)

d) receptors which activate G-proteins (Dohlman *et al*, 1987; Strader *et al*, 1989a)

1.9.1 The identification of a G-protein coupled receptor

The effect of guanine nucleotides on ligand binding to receptor was first noted by Rodbell and co-workers (1971a), with the demonstration that the affinity of glucagon binding to the plasma membrane of rat liver, was decreased by the presence of guanine nucleotides. Maguire and co-

workers (1976) were later able to demonstrate that in rat C6G1A glioma cell membranes, the binding of β-adrenergic agonists but not antagonists were reduced by the presence of the GTP analogue, Gpp[NH]p. Further studies on β-adrenergic receptors revealed that whilst competition curves for antagonist versus radiolabelled antagonist had pseudo Hill coefficients close to 1, competition curves for agonist versus radiolabelled antagonist are shallower with pseudo Hill coefficients less than 1 (De Lean *et al*, 1980). This data suggested the existence of two affinity states of the receptor for agonist, but only one affinity state for antagonist. In the presence of guanine nucleotides a low affinity state of receptor for agonist was adopted, whereas in the absence of guanine nucleotides the receptor demonstrated a higher affinity for agonist.

In an attempt to account for the body of data emerging on guanine nucleotide effects on ligand binding, De Lean and co-workers (1980) proposed a model involving a ternary complex. In the unstimulated state the inactive G-protein α-subunit (G-inact), coupled to $\beta\gamma$, may interact with the receptor (R), leading to the agonist (H) promoted formation of a high affinity ternary complex (H>R>G-inact). In the presence of guanine nucleotides which activate Gα, the ternary complex is destabilised and both agonist and G-protein can dissociate from the receptor.

Although the ternary complex was originally proposed to account for the effect of guanine nucleotides on agonist binding to receptors linked to stimulation of adenylyl cyclase, ligand binding experiments performed on receptor signalling systems linked to the inhibition of adenylyl cyclase yielded similar results (U'Prichard *et al*, 1978; Koski and Klee, 1981) and led to the general conclusion that if a receptor's

affinity is altered by the presence of guanine nucleotides, then that receptor functions through the activation of a G-protein.

A large number of individual G-protein coupled receptors have now been cloned and sequenced (table 1.2), and the structure and function of these receptors has been a subject of much research in recent years. G-protein coupled receptors have been subdivided into several classes including those for dopaminergic receptors (Vallar and Meldolesi, 1989), muscarinic cholinergic (Birdsall and Hulme, 1983), serotonergic (Schmidt and Peroutka, 1989), adrenergic (Lands, 1967; Gilman, 1987) and olfactory (Jones and Reed, 1987) receptors which each couple to a variety of effectors and ion channels.

When the amino acid sequences of the various cloned G-protein coupled receptors were compared the greatest homology was found in seven hydrophobic regions each consisting of between 20-25 amino acids. Each of these hydrophobic regions is thought to span the plasma membrane once, hence the receptor is thought to be composed of seven transmembrane helices. The regions of greatest diversity are at the receptor's extracellular amino terminus and at the intracellular carboxy terminus and cytoplasmic domains (figure 1.4).

Both the ligand bound and the G-protein activated by the receptor determines the specific cellular response mediated by it. These two structural features of the G-protein coupled receptors have been studied in great depth and will be detailed below.

Table 1.2 The G-protein coupled receptors identified to date

(adapted from Savarese and Fraser, 1992 [Biochem J 283:1-19]).

Peptide receptors	**Neurotransmitter receptors**
Angiotensin	Adenosine*
Adrenocorticotrophin (ACTH)	α-adrenergic*
Bombesin*	β-adrenergic*
Cholecystokinin (CCK)	dopamine*
Choriogonadotrpin*	$GABA_B$
Corticotropin-releasing hormone	Histamine*
Follicle-stimulating hormone	Muscarinic acetylcholine*
Glucagon	Octopamine*
Gonadotrpin-releasing hormone (GnRH)	5-hydroxy-tryptamine (serotonin)*
Growth-hormone-releasing hormone (GRF)	
Kinins (bradykinin, substance P, substance K)*	**Sensory systems**
Luteinizing hormone (LH)*	Rhodopsins (visual)*
Melanocyte-stimulating hormone (MSH)	Olfaction*
Neurotensin*	Taste
Opiates	
Oxytocin	**Other agents**
Parathyroid hormone*	C5a anaphylatoxin*
Somatostatin	Cannabinoids*
Thyrotropin (TSH)*	*mas* oncogene*
Thyrotropin-releasing hormone (TRH)*	fMet-Leu-Phe*
Vasoactive intestinal polypeptide (VIP)*	Prostanoids (PGEs, leukotrienes)*
Vasopressin	Thrombin*
*	IgE*

* Denotes the existence of subtypes of the receptor.

1.9.2 The ligand binding domain of G-protein coupled receptors

A large number of mutagenesis studies have been undertaken to elucidate the domains and specific amino acid residues essential for agonist and antagonist binding to G-protein coupled receptors. Particularly well studied is the β2-adrenergic receptor, and the emerging bank of data suggests that there are structural similarities in the ligand binding domains of all G-protein linked receptors.

β-agonists and β-antagonists exhibit distinct structural similarity. The substituents on the aromatic ring determine whether the compound activates or blocks the receptor (Andrews *et al*, 1986) for example the catechol-hydroxyl groups of isoprenaline, a full agonist appear to be necessary for maximal stimulation of the β2-adrenergic receptor (Strader *et al*, 1989b). Most competitive antagonists, like propranolol, lack these essential hydroxyl moieties and therefore do not activate the receptor (figure 1.5).

Disulphide bridges formed by cysteine 106 and 184 (Dohlman *et al*, 1990); and cysteine 190 and 191 (Fraser *et al*, 1989), in the extracellular domain of the β2-adrenergic receptor are necessary for the high affinity binding of agonists to the receptor. Incubation, and thus activation, of the β2-adrenergic receptor with agonists results in disulphide bond reduction (Rashidbaigi *et al*, 1983), and similarly incubation with disulphide reducing agents activates the β2-adrenergic receptor (Malbon *et al*, 1987; Pederson and Ross, 1985). It has been hypothesised that reversible sulphydryl rearrangements in the extracellular domain of the receptor may stabilise the receptor and Gsα as a high affinity ternary complex and hold the receptor in the active

Figure 1.4 General model for the structure of the β-adrenergic receptor (adapted from a similar figure in Jasper and Insel, 1992 [Biochemical Pharmacology 43:119-130]).

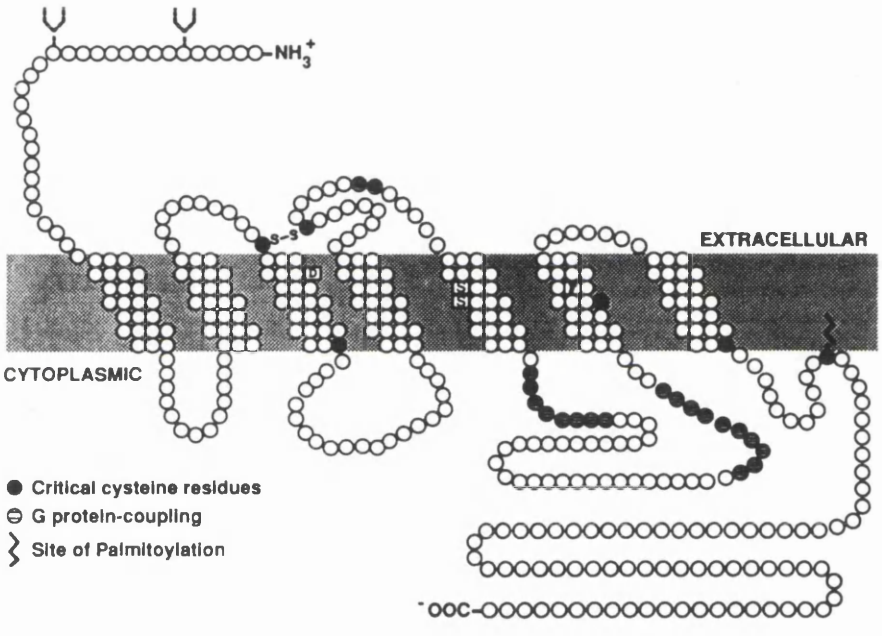
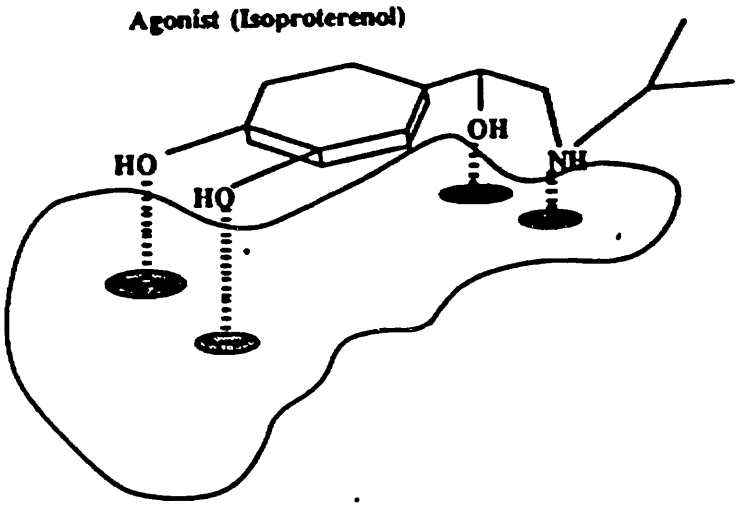
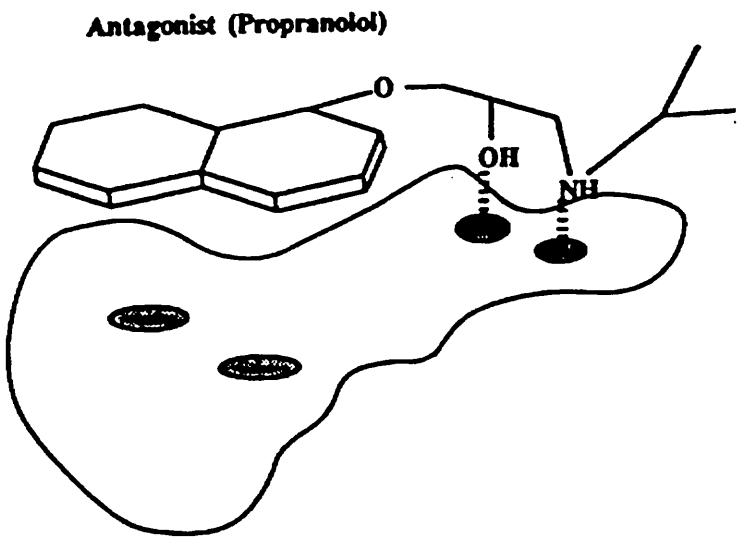


Figure 1.5 β-adrenergic receptor-ligand interaction sites.

Each interaction of a catechol ligand with the β2-adrenergic receptor requires the aliphatic hydroxyl and amino groups. The catechol-hydroxyl groups of isoprenaline interact with sites on the β-adrenergic receptor (serine 204 and 207) to elicit the conformational changes in the receptor necessary for activation. Propranolol, on the contrary, lacks two of these hydroxyl groups not permitting receptor activation through a conformational change. The diagram is adapted from a similar figure in Jasper and Insel, 1992 (Biochemical Pharmacology 43: 119-130).

Figure 1.5


Agonist (Isoproterenol)
HO
HO
OH
NH


Antagonist (Propranolol)
O
OH
NH

state, after agonist dissociation, thus promoting the activation of multiple Gs molecules.

Photoaffinity labelling studies have demonstrated that the hydrophobic domains of the turkey β-receptors (Wong *et al*, 1988), the human platelet α2-adrenergic receptor (Matsui *et al*, 1989) and the D2 dopamine receptor (Pollock *et al*, 1992) were involved in ligand binding. Biophysical analysis of the β2-adrenergic receptor (Tota and Strader, 1990), bacteriorhodopsin (Bayley *et al*, 1981; Mullen *et al*, 1981) and bovine rhodopsin (Findlay *et al*, 1981) also indicated that the ligand binding site was present in the hydrophobic core of the receptor. It has been proposed that the seven transmembrane spanning regions present in G-protein linked receptors form a hydrophobic pocket for ligand binding. Residues on the "inside" of this pocket may therefore confer ligand binding specificity.

Aspartate 113 (which is located in the transmembrane portion of the receptor) is highly conserved among all G-protein coupled receptors that bind agonists or antagonists containing a protonated amine group, like isoprenaline, propranolol and alprenolol. Mutation of aspartate 113 to an asparagine in the β2-adrenergic receptor abolished the binding of β-antagonists and β-agonists (Strader *et al*, 1987a). It has been postulated that the carboxyl group on residue 113 of the β2-adrenergic receptor forms a salt bridge with the amine group present on the aryloxyalkylamines, agonist or antagonist. Similar conclusions on the role of a similarly positioned aspartate residue in ligand binding have been drawn from studies on the α2 C10 receptor and muscarinic receptors (Fraser *et al*, 1988). Using a mutant β2-adrenergic receptor and chemically engineered agonists, serine 204 and serine 207 (in the

fifth transmembrane spanning domain of the receptor) were found to form a hydroxyl bond with the para- and meta- hydroxyl of isoprenaline respectively (Strader *et al*, 1989b). This gives a model of isoprenaline binding to the β2-adrenergic receptor through a carboxy-amine salt bridge at aspartate 113 and hydrogen bonds at serines 204 and 207 (figure 1.5).

More recent work using chimeric receptors points to residues in the seventh membrane spanning domain conferring antagonist/agonist specificity in β1- and β2- adrenergic receptors (Frielle *et al*, 1988; Suryanarayana and Kobilka, 1993a), α2-adrenergic receptors (Kobilka *et al*, 1988) and the 5HT1A receptor (Guan *et al*, 1992). Additional findings from studies of the ligand binding domain of β-adrenergic receptors, and also from parallel studies on α2-adrenergic receptors (Wang *et al*, 1991), m1 muscarinic receptors (Fraser *et al*, 1989) and D2 dopamine receptors (Neve *et al*, 1991) suggest that antagonists and agonists bind to distinct sites, and although these sites are not identical there is likely to be some overlap. The emerging data suggests that although G-protein coupled receptors share common structural domains, the molecular interactions between agonists and antagonists and their corresponding receptors are specified by a very small change in amino acid sequence. Although studies have begun on determining the folding and spatial relationships within the β2-adrenergic receptor (Suryanarayama *et al*, 1992) more investigation is needed to fully elucidate the structure of the ligand binding domain.

1.9.3 Identification of the G-protein coupling domains of receptors

The interaction of a receptor with its G-protein is assumed to occur on the intracellular face of the plasma membrane, on one of the receptor's intracellular loops or its hydrophilic C-terminal tail. The regions of the first and second cytoplasmic loops are highly conserved among G-protein coupled receptors and this led to the suggestion that the more diverse third cytoplasmic loop may be involved in the coupling of the receptor to its specific G-protein(s).

Initial studies demonstrated that deletion of two thirds of the third intracellular loop resulted in a β2-adrenergic receptor which was incapable of activating adenylyl cyclase in mouse L cells (Dixon *et al*, 1987). More specific deletion mutations in the C-terminal and N-terminal of the β2-adrenergic receptor's third intracellular loop again gave a marked decrease in receptor activated adenylyl cyclase (Strader *et al*, 1987b; O'Dowd *et al*, 1988; Hausdorff *et al*, 1990). Similar studies in rhodopsin (Franke *et al*, 1988, 1990) and muscarinic cholinergic receptors (Wess *et al*, 1989) have also demonstrated major roles for the N-and C-terminal of the third intracellular loop in G-protein coupling. Although the third intracellular loop appears to be essential for G-protein coupling, the first and second intracellular loops may also play a minor role (O'Dowd *et al*, 1988; Liggett *et al*, 1991; Cottechia *et al*, 1992), possibly through allosteric interactions with the third intracellular loop and other components of the receptor. Indeed the fifth transmembrane spanning domain in both the D2 dopamine receptor and the α2-adrenergic receptor has been demonstrated to be essential for ligand binding and receptor activation (Pollock *et al*, 1992).

The C-terminal of the third intracellular loop has also been implicated as an important site involved in G-protein interaction (Kjelsberg *et al*, 1992; Ren *et al*, 1993; Samama *et al*, 1993). All 19 possible mutations of alanine 293 and threonine 348 in the C-terminal of the third intracellular loop of the α1B-adrenergic (Kjelsberg *et al*, 1992) and α2C10 (Ren *et al*, 1993) receptors respectively, confer constitutive activity of phospholipase C and an increased affinity for agonists. The fact that all the mutations at a single amino acid site in both receptors results in an increased activity of the receptor suggests that this region may normally function to constrain the G-protein coupling of the receptor, a constraint which is normally relieved by agonist occupancy, although this awaits formal demonstration.

The C-terminal tail of the G-protein coupled receptors may also be involved in determining the receptor's G-protein specificity. Cysteine 341 in the C-terminal tail of the β2-adrenergic receptor is palmitoylated (O'Dowd *et al*, 1989). Mutant receptors lacking this residue and hence lacking palmitoylation are uncoupled from Gs and the receptor is highly phosphorylated (O'Dowd *et al*, 1988), suggesting that this mutation promotes the accessibility of specific receptor sites to kinases and leads to receptor uncoupling in the absence of receptor activation. Certainly acylation of proteins has been to be shown necessary for the translocation of proteins to the plasma membrane (James and Olsen, 1990) and it is possible that the palmitoylated cysteine 341 in the β2-adrenergic receptor forms a fourth intracellular loop by this means. Agonist activation of the β2-adrenergic receptor has been shown to result in an increased turnover of palmitate on this protein (Mouilliac *et al*, 1992). The release of palmitate is most likely to result in a conformation change, perhaps the release of the membrane association of this fourth intracellular loop, and

the exposure of potential phosphorylation sites. Interestingly, direct activation of protein kinase A, by dibutyryl cAMP results in the phosphorylation of the consensus PKA sequences located in the third intracellular loop but not of the site located near cysteine 341 (Bouvier *et al*, 1989). Phosphorylation of this site may only occur on removal of palmitate and thus result in an uncoupling of the receptor from its G-protein.

The role of palmitoylation in receptor-G-protein coupling still remains controversial. Removal of palmitate from a cysteine residue on the C-terminus of the α2A-adrenergic receptor resulted in no decrease in receptor-G-protein coupling (Kennedy and Limbird, 1993). Chemical removal of palmitate from a cysteine residue at the C terminus of rhodopsin resulted in an enhanced receptor-Gt coupling (Morrison *et al*, 1991) whereas mutational analysis showed no perturbed rhodopsin-Gt coupling (Karnik *et al*, 1988). The differing findings observed for palmitoylation at this site suggest that receptor acylation may play distinct functional roles at different receptor-G-protein interfaces.

1.10 Desensitisation of the β2-adrenergic receptor on exposure to agonist

For most receptors agonist stimulation is followed by desensitisation. Desensitisation is characterised by a reduction in the response mediated by the receptor over time, despite the continuous presence of the stimulus. Of the G-protein coupled receptors the mechanism of desensitisation of the β2-adrenergic receptor to agonists

has been the most widely investigated (Hausdorff *et al*, 1990; Collins *et al*, 1992).

The mechanism of the agonist mediated desensitisation of the β2-adrenergic receptor varies depending on the length of time of agonist exposure and the concentration of both agonist and magnesium ions used in the assay. Most studies to date have detailed the mechanism of β2-adrenergic receptor desensitisation at relatively high concentrations of both agonist (μM) and Mg^{2+} (mM) and these will be discussed below.

1.10.1 Desensitisation of the β2-adrenergic receptor on short-term exposure to agonist

Exposure of the β2-adrenergic receptor to agonist is followed by a rapid uncoupling of the receptor from Gs and homologous desensitisation of the β2-adrenergic receptor. Initially on agonist binding, the cytosolic kinase, β-adrenergic receptor kinase (βARK), is translocated to the plasma membrane where it phosphorylates the β2-adrenergic receptor partially uncoupling it from Gs. The interaction of βARK with the receptor allows its interaction with a second protein, β-arrestin, which serves to completely uncouple Gs from the receptor by binding to the receptor (Benovic *et al*, 1987b; Lohse *et al*, 1990). The receptor is then sequestered from the plasma membrane into distinct vesicles within the cell (Hertel *et al*, 1983). A similar mechanism for regulating rhodopsin function was found in the visual system (Wilden *et al*, 1986; Palczewski and Benovic, 1991a; Hargrave and McDowell, 1992)

1.10.1.1 β-adrenergic receptor kinase

The 80kDa protein β-adrenergic receptor kinase (βARK) was originally identified as enzyme activity present in a crude cytosolic fraction of S49 lymphoma cells which specifically phosphorylated the agonist occupied form of the receptor (Benovic *et al*, 1986). Now several isoforms of βARK have been cloned namely βARK1 (Benovic *et al*, 1989), βARK2 (Benovic *et al*, 1991), rhodopsin kinase (Lorenz *et al*, 1991) and *Drosophila* GPRK1 and GPRK2 (Cassill *et al*, 1991). Recent evidence points to the additional existence of a muscarinic acetylcholine receptor kinase (Haga and Haga 1989, 1990). βARK is present in a number of tissues (Benovic *et al*, 1987b; Benovic *et al*, 1989; Arriza *et al*, 1992) and its specificity is poorly defined. It will phosphorylate the agonist occupied forms of the human platelet α2-adrenergic receptor (Benovic *et al*, 1987a), the chicken heart muscarinic receptor (Kwatra *et al*, 1989), rhodopsin (Palczewski *et al*, 1988, Lorenz *et al*, 1991), a PGE1 receptor (Strasser *et al*, 1986), the somatostatin receptor (Mayor *et al*, 1987) and the human muscarinic cholinergic receptors (Richardson *et al*, 1993). It was initially proposed that βARK may be a general adenylyl cyclase linked kinase, but recently both βARK1 and 2 have been shown to phosphorylate the substance P receptor, which couples to Gq/11 and phospholipase C (Kwatra *et al*, 1993). Unlike βARK, rhodopsin kinase exhibits high specificity for rhodopsin and a poor affinity for the β2-adrenergic receptor (Palczewski *et al*, 1991a). βARK may be a general receptor kinase which functions only on the agonist occupied conformation of any G-protein coupled receptor but this remains to be established.

The increased association of βARK with the plasma membrane on agonist occupancy of the receptor has been shown to be promoted by the βγ subunits of the heterotrimeric G-proteins (Kameyama *et al*, 1993). βγ attaches to βARK at its C-terminal 130 amino acids (Koch *et al*, 1993). The geranylgeranyl isoprenoid residue on the γ-subunit of the G-protein (Mumby *et al*, 1990; Simonds *et al*, 1991) serves as a membrane anchor for the kinase (Pitcher *et al*, 1992; Inglese *et al*, 1992), allowing it to associate with the membrane and phosphorylate the β2-adrenergic receptor. Indeed peptide inhibition studies have demonstrated that the first and third intracellular loops of the receptor, appear to be essential for its phosphorylation by βARK, although phosphorylation appears to be predominantly on the C-terminal tail (Benovic *et al*, 1987a; Bouvier *et al*, 1988; Clark *et al*, 1989).

βγ has also been shown to facilitate the activation of the muscarinic acetylcholine receptor kinase (Haga and Haga 1990, 1992; Kameyama *et al*, 1993), although phosphorylation of rhodopsin by rhodopsin kinase is not stimulated by βγ (Kelleher and Johnson, 1988; Haga and Haga, 1992). The interaction of βARK and indeed the muscarinic acetylcholine receptor kinase with different βγ dimers could potentially be a way for refining receptor substrate specificity. Indeed, preliminary studies have shown specific βγ dimers to be more potent than others in enhancing βARK activity (Muller *et al*, 1993).

1.10.1.2 β-arrestin

The 48kDa protein arrestin was originally identified through its enhanced binding to phosphorylated and not to unphosphorylated

rhodopsin (Wilden *et al*, 1986). Now at least three isoforms of mammalian arrestin have been isolated and cloned; arrestin from the retina and β-arrestin 1 (Lohse *et al*, 1990) and 2 (Attramada *et al*, 1992) from bovine brain. These isoforms exhibit an overall homology of 60%. Southern blot analysis suggests there may be as many as four distinct genes which hybridise with a β-arrestin cDNA probe in bovine brain (Attramada *et al*, 1992), indicating the potential for additional isoforms of mammalian arrestin to exist.

Unlike βARK the arrestins appear to be highly specific in their interaction with a receptor, arrestin with rhodopsin and β1- and β2-arrestin with the β2-adrenergic receptor. Arrestins bind to their specific receptor with a 1:1 stoichiometry (Lohse *et al*, 1990; Palczewski *et al*, 1991b). The most divergent part of the arrestin polypeptide is its C-terminal tail. It is tempting to speculate that this region determines receptor specificity. Some sequence similarity has been noted between the C-terminal tail of arrestin and the C-terminal tail of transducin (Shinohara *et al*, 1987). The C-terminal portion of the G-protein α-subunit also interacts with the receptor (Masters *et al*, 1986). This suggests that arrestin/β-arrestin may mimic the G-protein α-subunits and bind to receptors at an identical site resulting in the receptor G-protein uncoupling.

It has recently been shown that arrestins can bind nucleotides (Glitcher and Ruppel, 1989), inositol phosphates (Palczewski *et al*, 1991a), Ca^{2+} ions (Huppertz *et al*, 1990) and be phosphorylated (Matsumoto and Yamada, 1991; Weyland and Kuhn, 1990; Levine *et al*, 1991). Binding of these small intracellular signalling molecules may contribute to the regulation of arrestin function. The existence of

different subtypes of arrestin, as well as the possibility of modifying the proteins via binding of certain ligands or via phosphorylation, may allow arrestin to play different roles in cell signalling, and also gives it the potential to be involved in "cross-talk" between signalling pathways.

1.10.1.3 Receptor sequestration

Receptor sequestration is the process whereby the plasma membrane bound receptor is removed from the plasma membrane but remains in the cell, usually in distinct membrane vesicles. On removal of agonist the receptor quickly returns to the plasma membrane and is fully functional (Hertel *et al*, 1983). Receptor down-regulation, on the contrary, is the means by which a plasma membrane bound receptor is internalised and lost completely from the cell.

On exposure to agonist there is a rapid uncoupling of the β2-adrenergic receptor from Gs and then a sequestration of the receptor away from the cell surface. β2-adrenergic ligand binding has been detected in membrane vesicles distinct from the plasma membrane (and devoid of Gs and the adenylyl cyclase catalytic subunit) using a combination of hydrophobic and hydrophilic radiolabelled antagonist binding (Hertel *et al*, 1983; Stadel *et al*, 1983).

Early studies expressing mutant β2-adrenergic receptors in mouse L cells suggested a role for Gs coupling in β2-adrenergic receptor sequestration (Strader *et al*, 1987c). However, further studies using mutants of the S49 lymphoma cell line have indicated that the presence of Gs in the plasma membrane was not a necessity for receptor

sequestration, but was required for receptor down-regulation (Rich *et al*, 1989; Gonzales *et al*, 1992) (see section 1.10.2.1). There is no strong evidence for receptor sequestration being mediated by cAMP, βARK or PKC (Perkins *et al*, 1991) and only agonist occupation is clearly required for receptor sequestration.

1.10.2 Desensitisation of G-protein coupled receptors on long term exposure to agonist

Long term exposure to hormones or agonists is unlikely to occur in a physiological situation although it may be important in certain clinical conditions (for example in alcoholism where cells are exposed to high concentrations of ethanol for long periods of time [Mochly-Rosen *et al*, 1988]). On long term exposure to agonist the β2-adrenergic receptor undergoes down-regulation and there is a resulting homologous desensitisation of adenylyl cyclase. Existing evidence suggests that several discrete processes contribute to the regulation of membrane associated β2-adrenergic receptor levels on prolonged (hours) exposure to agonist. These include, firstly, a proteolytic degradation of the receptor protein, and secondly, transcriptional regulation and changes in mRNA stability to maintain the new lower levels of receptor expression.

1.10.2.1 Receptor down-regulation

Receptor down-regulation is the process whereby the total cellular levels of a particular receptor is decreased on agonist exposure. Down-regulation can be demonstrated by the very slow return of the receptor to

the plasma membrane on removal of agonist, indicating the necessity for *de novo* protein synthesis.

Mutant cell lines with lesions in adenylyl cyclase can still undergo receptor down-regulation indicating the cAMP independent nature of the effect (Mahan *et al*, 1985; Gonzales *et al*, 1989; Hadcock *et al*, 1989b). Additionally, mutant receptors with impaired phosphorylation sites for protein kinases can still be both down-regulated and sequestered (Strader *et al*, 1987c; Campbell *et al*, 1991). Studies using mutant cell lines, indicated the necessity for unimpaired receptor-G-protein coupling for down-regulation to occur (Gonzales *et al*, 1989; Rich and Iyengar, 1990).

Several lines of evidence suggest that endocytosis by clathrin coated vesicles is the mechanism whereby β2-adrenergic receptors are sequestered (Hertel *et al*, 1985; Hertel *et al*, 1986; Heuser *et al*, 1989; Liao *et al*, 1990). Tyrosine residues in the C-terminal tail of several G-protein coupled receptors have been implicated in agonist induced endocytosis (Davis *et al*, 1986; Vega and Strominger, 1989; Lobel *et al*, 1989). Certainly Valinquette and co-workers (1990) have demonstrated the involvement of two tyrosine residues in the C-terminal tail of the β2-adrenergic receptor in receptor down-regulation. Mutations of these tyrosine residues also affect G-protein coupling again suggesting that G-protein coupling is a necessity for down-regulation. Although there have been findings to the contrary (Hertel *et al*, 1990; Hausdorff *et al*, 1991) the majority of the evidence to date indicates a role for the C-terminal of G-protein coupled receptors in receptor down-regulation (Liggett *et al*, 1993; Nussenzveig *et al*, 1993)

Receptor-G-protein coupling and hence the C-terminal of the β2-adrenergic receptor appear to both be involved in determining receptor down-regulation. However conflicting evidence exists for the involvement of G-protein coupling and the receptor's C-terminal in receptor sequestration. Indeed it has yet to be established unequivocally whether receptor sequestration and down-regulation are distinct processes acting through different pathways, or if the two processes are sequential. Agonist regulated internalisation of the β2-adrenergic receptor shares common cellular components used by the constitutively recycling receptor for transferrin (von Zastrow *et al*, 1992). Having shown that β2-adrenergic receptors are internalised into endosomes von Zastrow and co-workers (1992) have suggested that endosomes may be a common branchpoint in recycling and protein sorting to lysosomes. This hypothesis is however difficult to reconcile with the fact that the avian β2-adrenergic receptor does not undergo sequestration (Hertel *et al*, 1990) and that mutant receptors and cell lines with impaired receptor-G-protein coupling will undergo down-regulation but not sequestration. Indeed, more investigation is needed to fully elucidate the fate of the receptor after internalisation.

1.10.2.2 Regulation of gene expression

The marked increase in intracellular cAMP observed following agonist binding to the β2-adrenergic receptor leads to the activation of many cellular substrates, including members of the CREB family of transcription factors (Karpinski *et al*, 1992 and references therein). Activation of CREB, through a cascade of events, results in its binding to the cAMP response element (CRE) in the 5' promoter region of the β2-

adrenergic receptor gene (Collins *et al*, 1990), which in turn increases the rate of transcription, leading to a transient increase in receptor expression.

A sustained decrease in β2-adrenergic receptor mRNA levels on prolonged agonist exposure has been observed (Hadcock and Malbon, 1988), in both DDT_1-MF_2 hamster vas deferens cells and S49 lymphoma cells, and is a result of an increased destabilisation of the receptor mRNA (Hadcock *et al*, 1989a). It is partially dependent on cAMP (Hadcock *et al*, 1989b; Hough and Chuang, 1990). The actual sequences necessary for destabilisation of the receptor mRNA remain to be determined, but the β2-adrenergic receptor mRNA contains the consensus destabilisation sequences (AUUUA) in its 3' non-coding region. Recently proteins have been identified that bind to AUUUA sequence in RNA coding for lymphokines and proto-oncogenes (Gillis and Malter, 1991; Bohjanen *et al*, 1991). The complexing of RNA with such adenosine-uridine binding factors has been suggested to regulate RNA degradation. The mRNA poly A tract length has also been implicated in the regulation of mRNA stability (Bernstein and Ross, 1989).

1.10.3 Heterologous desensitisation of the β2- adrenergic receptor

A brief synopsis of the heterologous desensitisation of the β2-adrenergic receptor will be detailed below. Heterologous desensitisation and "cross-talk" between signalling pathways in a variety of systems will be discussed in greater depth in the forthcoming chapters. Heterologous desensitisation is the process whereby a response mediated through one

receptor is desensitised through activation of a distinct receptor. It has been demonstrated with receptors that activate the same signalling pathway or distinct pathways, and is usually mediated through some component of the signal transduction cascade downstream of the receptor, for example the G-protein, the effector or the second messenger. The most widely characterised mechanism of heterologous desensitisation of the β2-adrenergic receptor is its phosphorylation by the protein kinases A and C, which are activated by the second messengers cAMP and DAG (diacylglycerol) respectively.

Early studies using mutant S49 cell lines that demonstrated that heterologous desensitisation did not require either cAMP or Gsα, led to the conclusion that cAMP-dependent protein kinase played little or no role in this desensitisation (Green and Clark, 1981). The finding that concentrations of free Mg^{2+} in cell free assays of adenylyl cyclase exceeding 1-2mM obscured the cAMP-dependent and PKC-mediated desensitisations, whereas assays in the physiological range of free Mg^{2+} for S49 cells (0.2-0.6mM) allowed the observation of the cAMP- and PKC-induced heterologous desensitisations (Clark *et al* 1987, 1988; Johnson *et al*, 1986, 1990), led workers to reconsider.

The site of phosphorylation on the β2-adrenergic receptor by both PKA (Clark *et al*, 1989; Hausdorff *et al*, 1989) and PKC (Johnson *et al*, 1990) was found to be in the third intracellular loop of the receptor. Similarly PKA consensus sequences in the third intracellular loop of the M1 muscarinic receptor (which activates PLC) have been implicated in mediating its heterologous desensitisation (Lameh *et al*, 1992; Lee and Fraser, 1993). This same region is involved in receptor-G-protein coupling suggesting that phosphorylation of the receptor by PKA (Kunkel

et al, 1989) or PKC during desensitisation uncouples it from its G-protein. Recently, studies on reconstituted liposomes have demonstrated that the cAMP-mediated effect requires only the receptor and the receptor-Gs interaction and PKA mediated phosphorylation appears to disrupt the receptor-G-protein coupling (Bouvier *et al*, 1987; Pitcher *et al*, 1992).

The demonstration that the β2-adrenergic receptor can be phosphorylated by PKC, and that the M1 muscarinic receptor can be phosphorylated by PKA, clearly suggests the existence of "cross-talk" between signalling pathways, although the exact role of these modifications remain to be determined. There is obviously the potential for other proteins to contribute to heterologous desensitisation. Modification of G-protein α-subunit levels during prolonged exposure to agonist has become an increasingly documented phenomenon, and recently much work has been focused on determining its role in heterologous desensitisation.

1.11 Regulation of cellular G-protein levels

Prolonged exposure of the HM1 receptor in CHO cells (Mitchell *et al*, 1993), the GnRH receptor in αT3 cells (Shah and Milligan, unpublished observations) and the TRH receptor long form in EMBK cells (Kim and Milligan, unpublished observations) to agonist results in a non-selective down-regulation of Gq/11α. In rat adipocytes agonist treatment of the A1 adenosine receptor results in a 90% decrease in membrane associated levels of Gi1α and Gi3α and a 50% decrease in Gi2α and β-subunit (Green *et al*, 1990). Treatment of NG108-15 cells

with PGE1 (an agonist at the IP prostanoid receptor) results in an approximate 50% loss of membrane associated Gsα (McKenzie and Milligan, 1990). The mechanism behind the down-regulation of the G-protein α-subunit remains to be unequivocally determined, however evidence exists for control at the level of transcription (Hadcock *et al*, 1990) and protein degradation (Mitchell *et al*, 1993).

Enhanced proteolysis of the G-protein α-subunit has been shown to follow prolonged agonist treatment of the receptor (Green *et al*, 1992; Mitchell *et al*, 1993). The mechanism behind the proposed proteolysis has been the subject of much investigation. Treatment of cells with cholera toxin permanently activates the Gsα subunit. Cholera toxin catalyses the transfer of an ADP-ribose moiety to Gsα in the presence of NAD^+ and a 20kDa GTP binding protein, ARF (ADP-ribosylating factor), which is necessary for cholera toxin function (Schleiffer *et al*, 1982). Cholera toxin treatment of NG108-15 cells and rat liver membranes resulted in a cAMP-independent decrease in Gsα present at the plasma membrane (MacLeod *et al*, 1989; Lynch *et al*, 1990) with no change in Gsα mRNA levels. In GH3 cells Chang and Bourne (1989) demonstrated that activation of Gsα by cholera toxin enhanced its rate of proteolytic degradation. It is uncertain if the increased proteolytic degradation is a result of the dissociation of Gsα from βγ, or of the ADP-ribosylation of the protein, or indeed of some other less well-characterised post-translational modification (see section 1.7).

1.11.1 The involvement of the ADP-ribosylation of Gsα in its down-regulation

The hypothesis that the adenylyl cyclase cascade is regulated through endogenously, reversible (mono) ADP-ribosylation of the Gsα protein by mono (ADP-ribosyl) transferases and ADP-ribose glycohydrolase has been investigated (Tanuma *et al*, 1987, 1988; Tanuma, 1990; Tanuma and Endo 1989, 1990). Certainly, endogenous ADP-ribosylation of Gsα has been demonstrated in chicken spleen (Obara *et al*, 1991), human platelets (Inageda *et al*, 1991) and rat adipocytes (Jacquemin *et al*, 1986). Recent studies from Donnelly and co-workers (1992) demonstrated that inhibition of the mono (ADP-ribosyl) transferases increased the membrane associated levels of Gsα in NG108-15 cells, indicating a potential role for ADP-ribose in releasing Gsα from the plasma membrane.

The discovery that ADP-ribosylation of Gsα was capable of regulating its membrane associated levels suggested that agonist treatment of cells may result in the activation of a mono (ADP-ribosyl) transferase, ADP-ribosylating Gsα and thus activating it and releasing it from the plasma membrane. This was shown not to be the case for the IP prostanoid receptor which couples to Gsα in NG108-15 cells (Donnelly *et al*, 1992). It remains to be unequivocally determined whether agonist induced ADP-ribosylation is the mechanism whereby Gsα is lost from the plasma membrane of other cells by distinct receptors. Treatment of mouse mastocytoma cells with both a prostanoid (activating adenylyl cyclase via Gsα) and cholera toxin, resulted in an additive activation of adenylyl cyclase and Gsα release, indicating that in

this cell line the two processes appear to involve different mechanisms (Negishi *et al*, 1992).

1.11.2 The involvement of G-protein activation in Gα down-regulation

Ransnas and co-workers (1992) demonstrated that the release of Gsα from the plasma membrane is likely to accompany Gs subunit dissociation. Certainly, Gsα is less hydrophobic than the holomeric Gs and is thus more soluble and attracted to the cytosol (Stryer and Bourne, 1986; Sternweis, 1986; Neer and Clapham, 1988). Levis and Bourne (1992) showed an increased degradation rate of Gsα on release from the plasma membrane after agonist activation of S49 lymphoma cells. This enhanced proteolysis was not a direct result of the loss of membrane attachment, and the authors suggest that the conformational change in Gsα induces the loss of membrane attachment.

Whether receptor activation results in a promotion of the rate of degradation of the G-protein by a conformational change, which allows greater access to a proteolytic enzyme, or from a covalent modification (or loss of one) of the G-protein α-subunit, or a combination of these will remain a subject of much research in forthcoming years.

1.12 Research aims

The role the receptor plays in the agonist mediated control of G-protein α-subunit levels has still to be fully determined. In some cells both the receptor and G-protein α-subunit are down-regulated on prolonged agonist treatment, and in others only the Gα or receptor is down-regulated. Chapter 3 aims to further examine the role of the receptor in Gsα down-regulation. The effect of varying the levels of receptor expression, by cellular transfection, on the agonist mediated down-regulation of the G-protein α-subunit will be examined and discussed in later chapters.

CHAPTER 2 - MATERIALS AND METHODS

Materials and Methods

Materials

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

2.1.1 General reagents

BRL, Paisley, Scotland

Prestained molecular weight markers, Lipofectin reagent, restriction enzymes, restriction enzyme react buffers, agarose.

Bio 101 Incorporated, Stratech Scientific Ltd., Luton, UK.

Geneclean kit.

Boehringer (UK) Ltd, Lewes, East Sussex, UK.

GTPγS, GppNHp, GDP, GDPβS, AppNHp, dithiothreitol, creatine phosphate, creatine phosphokinase, triethanolamine hydrochloride, tris, thymidine.

DIFCO Laboratories, Detroit, Michigan, USA.

Bacto-agar.

Lab. Supplies, Loughborough, UK.

Acrylamide, N,N'-methylenebisacrylamide, hydrogen peroxide.

Koch-Light Lab.Ltd, Haverhill, Suffolk, UK.

DMSO, sodium potassium tartrate.

M and B, Dagenham, UK.

Ammonium persulphate.

National Diagnostics, Aylesbury, Buckinghamshire, UK.

"Ecoscint" scintillation fluid.

New England Biolaboratories, Bishop's Stortford, Herts., UK

DNA molecular weight standards.

Promega, Chilworth Research Centre, Southampton, UK.

T4 DNA ligase.

Schering Health Care, Burgess Hill, Sussex, UK.

Iloprost.

Sigma Chemical Company, Poole, Dorset.

Cholera toxin, bovine serum albumin, NAD, TEMED, trypsin, arginine hydrochloride, N-ethylmaleimide, o-diansidine hydrochloride, ATP disodium salt, cAMP sodium salt, Coomassie blue R-250, Bromophenol blue, thimerosal, Protein A Sepharose, Dowex AG50W-X4 (200-400 mesh), Alumina (neutral), forskolin, (-) propranolol, (-) isoproterenol [(+) bicarbonate salt], PGE1.

Whatman International Ltd. Maidstone, UK.

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

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

2.1.2 Tissue culture plasticware

A. and J. Beveridge Ltd., Edinburgh, Scotland.

Falcon tissue culture 100mm petri dishes.

Bibby Science Products Ltd, Stone, Staffordshire, UK.

75cm^2 tissue culture flasks.

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

Biofreeze vials.

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

50ml centrifuge tubes.

2.1.3 Cell culture media

GIBCO Life Technologies, Paisley, U.K.

Dulbeccos' modification of Eagles' 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., UK.

Foetal calf serum, normal calf serum.

Sigma Chemical Company, Poole, Dorset, UK.

CPSR-3.

2.1.4 Radiochemicals

Amersham PLC, Amersham, Buckinghamshire.

[8-^{3}H] Adenosine 3',5'-cyclic phosphate, ammonium salt (26Ci/mmol)

Adenosine 5'-[α-^{32}P] triphosphate, triethylammonium salt (400Ci/mmol)

[^{3}H]-Prostaglandin E1 (54Ci/mmol)

[^{3}H]-Dihydroalprenolol (56Ci/mmol)

2.1.5 Standard buffers

Phosphate buffered saline (10x PBS)

2g potassium chloride
80g sodium chloride
2g potassium dihydrogen orthophosphate
11.4g disodium hydrogen orthophosphate, heptahydrate
to a final volume of 1 litre, pH 7.4.

2.1.6 Molecular biology tools

The cDNA for the β2-adrenergic receptor was a kind gift from Dr S. Griffiths, Department of Biochemistry, University of East Anglia, UK.

The plasmid pJM16 (LK444) was a kind gift from Dr John Miles, Department of Biochemistry, University of Glasgow, UK.

The plamid pSVL was a gift from Dr Yasmin Shakur, Department of Biochemistry, University of Glasgow, UK.

Methods

2.2 Cell Culture

2.2.1 Cell growth

NG108-15 neuroblastoma x glioma hybrid cells were a kind gift from Dr W. Klee (N.I.H., Bethesda, M.D. USA). NG108-15 cells, βN22 and βN17 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) CPSR-3 which had been heat inactivated at 56^{o}C for 2 hours. The medium was supplemented with glutamine (2mM), hypoxanthine (0.1mM), aminopterin (1μM) and thymidine (16μM). Both penicillin (100 units/ml) and streptomycin (100mg/ml) were routinely included. This medium will henceforth be termed DMEM/10% (v/v) CPSR. βN clones were selected for by the addition of 0.8mg/ml geneticin sulphate to the growth medium.

S49 lymphoma and *cyc-* cells were a kind gift from Dr K. P. Ray (Glaxo Group Research Ltd, Greenford, Middlesex, U.K.). They were grown in 25cm^2 tissue culture flasks in 0.0375% (w/v) sodium bicarbonate buffered Dulbeccos' modification of Eagles' Medium (DMEM), containing 10% (v/v) foetal calf serum which had been heat inactivated at 56^{o}C for 2 hours. This medium was supplemented with glutamine (2mM), penicillin (100 units/ml) and streptomycin (100mg/ml). These cells were maintained in cell culture at a density of between 5 x 10^5 - 2 x 10^6 cells/ml medium.

COS-1 cells (a kind gift from Dr Yasmin Shakur; Biochemistry Department, University of Glasgow) were grown in DMEM/10% (v/v) FCS supplemented with glutamine (2mM), penicillin (100 units/ml) and streptomycin (100mg/ml) as described for S49 lymphoma cells. COS-1 cells were subcultured in a manner identical to NG108-15 cells.

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

2.2.2 Cell subculture

Confluent cells (approximately 10^7 cells per $75cm^2$ flask) were passaged using 0.1% (w/v) trypsin, 0.67mM EDTA and 10mM glucose in PBS (x1). Growth media was removed from the cells and 3ml trypsin solution added. When the cells had been removed from the surface of the flask, trypsinisation was stopped by the addition of three 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.

2.2.3 Cell maintenance

Confluent cells were removed from the surface of the flask by trypsinisation and the cells resuspended in freezing medium, which consisted of 8% (v/v) DMSO in CPSR (for NG108-15 cells and βN clones) or 8% (v/v) DMSO in FCS (for S49 lymphoma and COS-1 cells). This suspension was aliquoted into 1.0ml volumes into Biofreeze 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 10ml appropriate growth medium, and centrifuged at 800 x g in an MSE centaur centrifuge for two minutes to pellet the cells. In the case of NG108-15 cells, βN clones and COS-1 cells the cell pellet was resuspended in growth medium and plated out in a final volume of 10ml in a $75cm^2$ flask. For S49 lymphoma cells the pellet was resuspended in 1ml growth medium, the cell number was counted using a haemocytometer, and the pellet resuspended in an appropriate volume of growth medium to ensure the cells were maintained between $5x10^5$ - $2x10^6$ cells/ml medium.

2.2.4 Cell harvesting

When confluent, growth medium was removed from the cell culture flask and 10 ml ice cold PBS (x1) added. Cells were gently washed from the surface of the flask, collected in a 50ml centrifuge tube and centrifuged at 800 x g in a Beckman TJ6 centrifuge for 5 minutes. The resulting cell pellet was washed with ice cold PBS (x1) 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.

2.3 Production of a crude membrane preparation

Membranes were prepared according to Koski and Klee (1981). Frozen cell pellets were thawed and suspended in approximately 4ml ice cold 10mM Tris-HCl, 0.1mM EDTA pH 7.5 and homogenised with 25 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 5ml 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^{o}C until required.

2.4 Protein determination

The method used was based on that of Lowry *et al*, 1951.

Stock solutions

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

1% (w/v) copper sulphate

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 V, and a standard curve constructed for a maximum of 25µg protein per sample. Unknowns were assayed in 2 and 4µl volumes in duplicate. 1ml solution D was added to each sample, mixed and left to stand for 10 minutes. 100µl Folin's 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.

2.5 Antibody production

All antisera 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 Rockefeller 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.

A range of antisera were produced in a similar manner as summarised in table 2.1

Table 2.1 The generation of G-protein subunit-specific antisera

Antiserum	Peptide used	G protein sequence	Antiserum identifies
IMI	NLKEDGISAAKDVK	Goα22-35	Goα
I3B	KNNLKECGLY	Gi3α345-354	Gi3α
CS1	RMHLRQYELL	Gsα385-39	Gsα
BN1	MSELDQLRQE	Gβ1-10	β1,β2
CQ2	QLNLKEYNLV	Gqα351-360 G11α 350-359	Gq/11α
SG1	KENLKDCGLF	Tdα341-350	Gi2α, Gi1α Tdα

2.6 Gel electrophoresis

Gel electrophoresis was carried out according to the method of Laemmli (1970).

2.6.1 Resolving gel preparation

Stock solutions (stored at 4°C)

Solution A	1.5M Tris, 0.4% (w/v) SDS, pH 8.8 with HCl.
Solution B	0.5M Tris, 0.4% (w/v) SDS, pH 6.8 with HCl
Solution C	30% (w/v) Acrylamide, 0.8% (w/v) N,N'-methylene bisacrylamide
Solution D	50% (v/v) glycerol
Solution E	10% (w/v) Ammonium persulphate (made daily)
Solution F	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 the stock solutions as follows:-

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

To a final volume of 24ml 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.

2.6.2 Stacking gel preparation

Solution	Volume (ml)
B	3.75
C	1.5
E	0.15
F	0.008

To a final volume of 15ml with H_2O.

The solution was mixed, layered on top of the resolving gel and allowed to polymerise around a 15 well Teflon plate. Electrophoresis was performed overnight at 60V.

2.6.3 Sample preparation by 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 microfuge at 12000 x g for 10 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% (w/v) bromophenol blue.

2.6.4 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 approximately 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 the proteins were apparent on the gel in discrete bands.

2.6.5 Western blotting

Proteins were separated under appropriate resolving conditions on SDS polyacrylamide gels overnight at 60V. The proteins were transferred to a nitrocellulose sheet 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 PBS (1x), after which time the gelatin was washed off with distilled water and the nitrocellulose sheet incubated for at least 2 hours at 30°C with the appropriate dilution of antiserum in 1% (w/v) gelatin in PBS/0.2% (v/v) NP40. The antiserum was then removed and the nitrocellulose sheet given two ten minute washes with PBS/0.2% (v/v) NP40, after which the blot was incubated with a second antibody (peroxidase conjugated goat anti-rabbit IgG) for at least 2 hours at 30°C. The second antibody was then removed and the sheet subjected to two ten minute washes in PBS (x1). The blot was then developed in 40ml PBS (x1) pH 7.4 with 0.025% (w/v) o-diansidine as substrate. Development of the blot was initiated by the addition of up to 20µl of hydrogen peroxide, and then stopped by immersion of the blot into sodium azide (1% w/v).

First antibodies could be reused up to three times and second antibodies could be reused twice. Both antibodies were stored at 4°C using 0.004% (w/v) thimerosal as an anti-bacterial reagent.

2.6.6 Quantitation of immunoblots

Initially immunoblots were quantified by overlaying the colorimetrically developed blot with [^{125}I]-labelled goat anti-rabbit IgG (0.1μCi/ml) in 1% (w/v) gelatin / PBS (x1) / 0.2% (v/v) NP40 for 2 hours. After extensive washing firstly with PBS / 0.2% (v/v) NP40 and then with PBS (x1), the blot was air-dried, the coloured bands were excised and the radioactivity was assessed in a gamma radiation counter.

The acquisition of a Shimadzu Flying Spot Scanning machine allowed the colorimetrically developed blots to be densitometrically scanned and the results were expressed as a percentage of control values.

2.7 Radioligand binding assay

Binding assays were performed by the rapid filtration method as described by Pert and Snyder (1973), in 10mM Tris-HCl, 50mM sucrose, 20mM magnesium chloride pH 7.3, containing 5-100μg 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 the transferral of tubes to a 30^{o}C water bath for 30 minutes, after which time the tubes were removed to ice. The samples were then rapidly filtered through Whatman GF/C glassfibre filters which had been presoaked in assay buffer, followed with three washes of the filter with 5ml ice cold assay buffer using a Brandell Cell

Harvester. Initially, before the acquisition of the Brandell Cell Harvester, a ten well binding manifold was used, samples were spotted on to Whatman GF/C filters and the filters washed three times with 5ml ice cold buffer. Filters were soaked overnight in Ecoscint scintillation fluid prior to counting in a Rackbeta scintillation counter.

In some instances, the binding data was manipulated according to the equations derived by Scatchard (1949) or DeBlasi *et al*, 1989.

2.8 Adenylyl cyclase assay

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

2.8.1 Sample preparation

Reaction mixtures of 50µl containing; 4mM creatine phosphate, 100mM NaCl, 100U/ml creatine phosphokinase, 200mM Tris.HCl pH 7.5, 10mM magnesium chloride, 0.4mM ATP pH 7.5, 2.0mM cAMP, 2.0µM GTP pH 7.5, [α-^{32}P]-ATP (1x10^6 cpm), were added to tubes containing between 5-20µg membrane protein and the appropriate ligands to a final volume of 100µl. Reaction tubes were kept on ice at all times and the reaction started by removal to a 30^{o}C water bath. After 15 minutes the reaction was terminated by removal to ice and the addition of

100µl stopper solution which comprised 2% (w/v) SDS, 45mM ATP, 1.3mM cAMP. 50µl of [8-^{3}H]-3'5'cAMP (approximately 10000 cpm) was added to each tube prior to boiling for 10 minutes. 750µl H_2O was then added to each sample, and the [^{32}P]-cAMP content of each tube determined.

2.8.2 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 (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 volume of firstly, 1M hydrochloric acid, then 1M NaOH and finally 1M Hydrochloric acid. The Dowex was mixed with water to a slurry (1:1 v/v) and then 2ml removed and added to glass wool stoppered columns. The water was allowed to drain out and the columns washed with 2ml 1M hydrochloric acid and stored at room temperature. Prior to use, the columns were washed with 4ml 1M NaOH and then 4ml 1M hydrochloric acid followed by 20ml water.

The alumina columns were prepared by the addition of 1.0g dry neutral alumina to glass wool stoppered columns and the columns washed with 12ml 1M imidazole buffer pH 7.3 followed by 15ml 0.1M imidazole pH 7.3 and then stored at room temperature. On the day of use, each column was washed with 8ml 0.1M imidazole pH 7.3.

2.8.3 Separation of cAMP on Dowex columns

Prior to sample chromatography, the nucleotide elution profiles for each column were determined. This was performed by applying [^{3}H]-cAMP to the columns and determining the elution volume.

Stock [^{3}H]-cAMP was diluted in water to give approximately 10000 cpm in 50µl. 50µl cAMP solution were added to 950µl of water and applied to a Dowex column. The cAMP was eluted from the column by successive washes of the column with 0.5ml water. Fractions were collected in a vial with 5ml 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. Recovery from the Dowex columns was always greater than 70%.

The elution volume required to elute the cAMP from the alumina columns was determined as for the Dowex columns except the eluting buffer was 0.1M imidazole pH 7.3. Recoveries were similar to that obtained for the Dowex columns.

2.8.4 Determination of cAMP produced by membrane fractions

Samples (total volume 950µl) were added to prepared Dowex columns and the ATP eluted with 0.5ml water. 6.0ml 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 14 ml Ecoscint, with 6ml 0.1M imidazole pH 7.3. 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.

2.9 Reconstitution of Gsα into S49 lymphoma *cyc-* membranes

Reconstitution assays were performed according to the method of Milligan and Klee (1985). Sodium cholate (1%, w/v) was used (1 hour, 4^{o}C) to extract protein from membrane preparations of NG108-15 cells. Samples were centrifuged at 25 p.s.i. (150000 x g) in an airfuge (Beckman instruments) and the supernatant fraction was taken as the soluble extract. Up to 5µl extract (containing the equivalent of up to 5µg NG108-15 membrane protein) was added to a final volume of 100µl containing 10mM NaF or 10mM NaCl, 50mM HEPES pH 8.0, 10µg bovine serum albumin, 10 units of creatine phosphokinase, 6mM $MgCl_2$, 0.2mM EGTA, 2mM 2-mercaptoethanol, [^{3}H]-cAMP (15000 cpm), 0.2mM [α-^{32}P]ATP (2 x 10^6 cpm) and 10 µg S49 lymphoma *cyc-* cell membranes. Samples were incubated at 30^{o}C for 60 minutes and the

[^{32}P]-cAMP generated was measured using the Salomon cyclase assay (section 2.8).

Up to 5µg solubilised NG108-15 cell membranes gave a linear increase in reconstituted adenylyl cyclase activity in 10µg *cyc-* cell membranes (McKenzie and Milligan, 1990).

Cloning technology (Sambrook *et al*, 1989)

Glassware, eppendorfs, pipette tips and buffer solutions, were autoclaved for 45 minutes at 15 p.s.i. before being used in contact with bacteria or DNA.

2.10 General solutions used during cloning

50x TAE: 242g Tris base
57.1ml glacial acetic acid
100ml 0.5M EDTA (pH 8.0)
made up to 1 litre

DNA loading buffer (sample incubation buffer):

0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
15% (w/v) Ficoll (Type 400, Pharmacia)
in water

2x YT medium: 16g/l Bactotryptone
(pH 7.4) 10g/l yeast extract
5g/l NaCl

Agar plates: 15g Bacto-agar made up to 1 litre with 2x YT medium and the appropriate concentration of antibiotic.

2.11 Agarose gel electrophoresis

The DNA sample was mixed with approximately 10µl sample incubation buffer and loaded onto a 1% (w/v) agarose gel. Ethidium bromide was added to the electrophoresis buffer (1x TAE) to a final concentration of 0.5µg/ml. The gel was run at 100mA for approximately 30 minutes. It was then examined under UV light and photographed.

2.12 Phenol:chloroform extraction of DNA

The sample containing the DNA was diluted 1:1 with phenol:chloroform:isoamyl alcohol (25:24:1) and subsequently vortexed and pulsed in a microfuge. The DNA present in the upper aqueous phase was removed and diluted 1:1 with chloroform:isoamyl alcohol (24:1), vortexed, pulsed and the chloroform:isoamyl alcohol extraction repeated on the supernatant.

The supernatant from the second extraction was collected and 0.3 volumes 3M sodium acetate (NaOAc) pH 5.0 and 2.5 volumes ethanol were added. The sample was frozen at -20°C for 60 minutes and then

spun in a microfuge at 4°C for 30 minutes to collect the precipitated DNA. The supernatant was discarded and the eppendorf containing the sample was half-filled with ice cold 70% ethanol. The sample was vortexed and spun at 4°C for 15 minutes. The supernatant was removed and the sample dried under vacuum in an Aquavac. The DNA sample was resuspended in an appropriate volume of sterile water.

2.13 RNAase treatment of DNA

1µl RNase (100µg/ml) was added to the DNA sample (approximate volume 30µl) and incubated at 37°C for 1 hour. The sample was then purified by phenol:chloroform extraction of the DNA (section 2.12). (The RNase was prepared in 10mM Tris-HCl , 15mM NaCl pH 7.5 and boiled for 15 minutes to remove any DNase activity before use.)

2.14 Restriction enzyme reactions.

Up to 10µg DNA was incubated with 1µl restriction enzyme and 1x react buffer specific for that restriction enzyme, at 37°C for 90 minutes. The sample was then RNAase treated (section 2.13) and phenol:chloroform treated in order to extract the digested DNA (section 2.12).

2.15 DNA ligation reactions

The predigested DNA vector and insert (in a ratio of vector:insert 1:10) were heated to 65°C for 5 minutes in the same sterile eppendorf tube. The DNA was then cooled on ice. The reaction reagents were added to the tube as follows : 0.5mM ATP, 1x T4 DNA buffer and 1µl T4 DNA ligase to a final volume of between 10-20 µl. The reaction was kept at 4°C overnight.

T4 DNA ligase buffer (10x):

0.5M Tris.HCl (pH 7.6)
100mM $MgCl_2$
100mM dithiothreitol
500µg/ml BSA (Fraction V, Sigma)

2.16 Preparation of competent *E.Coli*

2ml of an overnight culture of *E.Coli* (either NM522 or HB109) was incubated with 40ml 2xYT medium for 3 hours. The *E. Coli* suspension was spun at 3000 rpm for 5 minutes in a TJ6 Beckman centrifuge. The supernatant was discarded and 20ml ice cold 50mM $CaCl_2$ added. The sample was left for 20 minutes on ice and then spun at 3000 rpm for 5 minutes. The supernatant was discarded and 4ml 50mM $CaCl_2$ added. The competent *E. Coli* could be left at 4°C for up to 48 hours. Maximum transforming efficiency occurred between 12-24 hours of $CaCl_2$ treatment.

2.17 Transformation of competent *E.Coli*

200µl competent *E.Coli* was added to the plasmid DNA (ligation reaction) and gently mixed. The reaction was left on ice for 90 minutes and then heat shocked at 37°C for 90 seconds. The sample was returned to ice, 400µl 2xYT medium was added and it was then shaken at 37°C for 1-2 hours. The samples were plated out on the appropriate antibiotic bactoagar plates to select for the *E.Coli* which had taken up the plasmid. The bactoagar plates were incubated at 37°C overnight and inspected the next day for colonies. Colonies were picked and grown in 3ml 2xYT medium overnight.

2.18 DNA minipreps from transformed *E.Coli*

From 3ml overnight cultures of *E.Coli* a 1.5ml suspension was taken and spun for 30s in a microfuge. The supernatant was removed and the pellet redissolved in 100µl glucose buffer (50mM glucose, 25mM Tris.Cl pH 8.0, 10mM EDTA pH 8.0). The sample was left for 5 minutes on ice for the cells to lyse. 200µl freshly prepared 1% (w/v) SDS/0.2M NaOH was added, the sample shaken and left on ice for 20 minutes to allow solubilisation of the membranes. 300µl 3M K/5M OAc was added and the sample left on ice for 5 minutes. The sample was spun for 5 minutes in a microfuge and the supernatant removed. Phenol:chloroform was used to extract the DNA from the sample (section 2.12).

2.19 "Geneclean" using the Geneclean kit

The appropriate fragment of DNA was cut out of the agarose gel. 500µl sodium iodide solution, from the Geneclean kit, was added to the fragment of gel in an eppendorf tube and incubated at 47°C-55°C for several minutes, until the agarose was completely melted. 5µl heavily vortexed glassmilk was added, and the sample was left on ice for 5 minutes. It was pulsed for 5 seconds in a microfuge, the supernatant was discarded and 500µl Geneclean solution added to the sample. It was then washed three times in Geneclean and the pellet resuspended in e.g. 10µl sterile water and incubated at 50-55°C for 3 minutes. The sample was spun for 30 seconds in a microfuge, the supernatant removed and this procedure repeated on the supernatant. The resulting supernatant contained the DNA from the gel fragment.

2.20 $CsCl_2$ purification of DNA from a large scale culture of transformed *E.Coli*

500ml of an overnight culture of transformed *E.Coli* was spun in a JA14 rotor in a Beckman J2.21 centrifuge at 2000 x g for 5 minutes at 4°C. The pellet was resuspended in 9ml glucose buffer (50mM glucose, 25mM Tris.HCl pH 8.0, 10mM EDTA pH 8.0) and left for 5 minutes on ice. To each tube was added 0.5ml lysozyme (50mg/ml) and the tubes were left to stand at room temperature for 5 minutes. 10ml 0.2M NaOH/1% (w/v) SDS was added to each tube and left for 5 minutes at room temperature. 7.5ml 3MK/5M OAc buffer was then added to each

tube which were shaken vigorously. The samples were left for at least 10 minutes on ice, and then spun at 30000 x g for 20 minutes at 4°C.

The supernatant was transferred to a new tube by passing it through 2 layers of muslin. It was mixed with an equal volume of propan-2-ol and left at room temperature for at least 30 minutes. The samples were spun at 12000 x g for 30 minutes at room temperature and the supernatant discarded. The pellet was washed with 20ml 70% ethanol, again at room temperature. The ethanol was discarded, the pellet dried, resuspended in 30ml 10mM Tris/0.1mM EDTA pH 7.4 and then transferred to sterile tubes containing 30.0g caesium chloride. The tubes were mixed gently until all the caesium chloride had dissolved and 2.4ml of 10mg/ml ethidium bromide solution was added and the tube mixed gently.

The samples were transferred to Beckman VTi50 tubes and spun at 242000 x g for 20 hours at 20°C, in the VTi50 vertical rotor, in the Beckman L8 centrifuge. The linear DNA, present half-way up the tube, was extracted out. Any ethidium bromide present was removed by washing repeatedly with an equal volume of water saturated butan-1-ol. The aqueous phase was then dialysed against 2 litres 10mM Tris/0.1mM EDTA pH 7.4 for 4 hours at 4°C.

The DNA solution was removed from the tubing and precipitated with 2.5 volumes ethanol at -20°C overnight. The samples were spun at 6500 x g for 30 minutes at 4°C in a Beckman J2.21 centrifuge, then washed with 70% ethanol and the pellet dried using an Aquavac. The pellet was redissolved in a total of 500µl 10mM Tris/0.1mM EDTA pH 7.4.

2.21 Quantitation of DNA

5µl and 10µl DNA sample were made up to 1ml with water and the absorbance at 260 nm read. An absorbance of 1.0 at this wavelength indicated the presence of 50µg plasmid DNA, thus allowing the calculation of the amount of DNA in the sample.

The ratio $OD_{(260nm)}/OD_{(280nm)}$ allows the purity of the nucleic acid to be calculated. Pure preparations of DNA and RNA have $OD_{(260nm)}/OD_{(280nm)}$ values of 1.8 and 2.0, respectively. If there is contamination with protein or phenol, the $OD_{(260nm)}/OD_{(280nm)}$ is significantly less than the values given above and accurate quantitation of the amount of nucleic acid present was not possible.

2.22 Transfection of plasmid DNA into mammalian cells

Cells were transfected using the Lipofectin reagent according to the manufacturer's instructions (GIBCO BRL).

NG108-15 or COS-1 cells were plated out to 30-50% confluency for stable transfections, and 80% confluency for transient transfections, on 100mm petri dishes in DMEM/10% (v/v) CPSR or DMEM/10% (v/v) FCS respectively. 10µg DNA was diluted with sterile water to 50µl. 50µg Lipofectin Reagent was diluted to 50µl with sterile water. 50µl each of the DNA and Lipofectin were mixed in a polystyrene tube and left for 15 minutes at room temperature. The cells were washed twice with 5ml serum-free media and 5ml serum-free media then added to the

cells. The 100µl DNA/Lipofectin complex was added to the cells dropwise and then left for 5-24 hours at 37°C in 5% CO_2. After the appropriate time 5ml 20% serum was added to the cells and they were left for 2-3 days. For transient transfections the cells were then harvested. For stable transfections the cells were plated out in the appropriate selection medium, in this case 0.8mg/ml geneticin sulphate. Cell colonies grew which were resistant to 0.8 mg/ml geneticin sulphate and these were selected and expanded.

0.8mg/ml geneticin was determined to be the minimum concentration of geneticin that would kill every wild-type NG108-15 cell within 7 days. This was demonstrated by treating NG108-15 cells with increasing concentrations of geneticin and assessing their viability by eye daily. When NG108-15 cells die they become non-adherent to the flask surface. After 7 days incubation in 0.8mg/ml geneticin all the NG108-15 cells had become non-adherent and expired.

2.23 Data analysis

Where appropriate, data were analysed for statistical significance using the Student's two-tailed t-test (paired). Dose response and displacement curves were analysed using the Kaleidograph curve fit programme, courtesy of Syntex Research Centre, Edinburgh.
The IC50 (corrected for receptor occupancy) was calculated by dividing the measured IC50 by (1 + L/Kd) where L is the concentration of radioligand used.

CHAPTER 3

Prolonged agonist exposure of the IP prostanoid receptor in NG108-15 cells, results in a concurrent down-regulation of receptor and Gsα.

Introduction

Human platelets aggregate on damaged epithelial cells that line blood vessel walls during vascular injury. Numerous factors initiate this aggregation process, and these include exposed collagen on the subendothelial matrix (Wilner *et al* ,1968), and ADP released from damaged cells (Born, 1962). The developing platelet plug is further increased in size by the pro-aggregatory factors ADP (Born 1962, Born and Kratzer 1965), thromboxane A2 and the prostaglandin endoperoxides released from platelets during aggregation (Hamberg *et al* 1975). The increase in the size of the platelet aggregate is finally attenuated by several anti-aggregatory factors including prostaglandin I2 (PGI_2) (Moncada *et al*, 1976) and adenosine (Born *et al* , 1965) (a product of ATP hydrolysis) released from the adjacent endothelial cells during injury. Both PGI2 (Gorman *et al*, 1977, Tateson *et al*, 1977) and adenosine (Haslam and Rosson, 1975, Huttemann *et al*, 1984) mediate inhibition of platelet aggregation through activation of adenylyl cyclase. Both adenosine and PGI_2 analogues, therefore, have therapeutic potential as 'anti-platelet' aggregation agents, but this has proved problematic with the discovery that exposure of human platelets to PGI_2 and to adenosine eventually results in a desensitisation of adenylyl cyclase, which serves to relieve the inhibition of platelet aggregation (Sinzinger *et al*, 1981). The ability to control the desensitisation of adenylyl cyclase to adenosine and PGI_2 is therefore important in controlling platelet aggregation .

The neuronal-like hybrid cell line, NG108-15, was formed by the cell fusion of the 6-thioguanine-resistant clonal mouse neuroblastoma cell line N18TG2 and the bromodeoxyuridine-resistant rat glioma cell

line C6-BU1, followed by selection with hypoxanthine, aminopterin and thymidine (HAT) medium (Hamprecht *et al*, 1985) and these cells are readily grown in cell culture. Like human platelets, NG108-15 cells express receptors for adenosine (of the A2 subtype) and prostaglandins (of the IP prostanoid subtype), both of which couple to the activation of adenylyl cyclase. Because of this, the NG108-15 cell line has been used as a readily available system to study both types of receptor and their respective mechanisms of desensitisation on exposure to agonist or hormone.

Several groups have attempted to determine the mechanism of both homologous and heterologous desensitisation of adenylyl cyclase activation in NG108-15 cells, through exposure of these cells to hormones and agonists acting at the A2 adenosine receptor and the IP prostanoid receptor. Kenimer and Nirenberg (1981) showed that on prolonged exposure (hours) of NG108-15 cells to prostaglandin E1 (PGE1), there was a reduction in the capacity for adenylyl cyclase activation by each of PGE1, the A2 adenosine receptor agonist, 2-chloroadenosine and sodium fluoride (NaF). Prostanoid treatment of NG108-15 cells therefore resulted in both homologous and heterologous desensitisation of adenylyl cyclase. This was accompanied by a 30-50% decrease in membrane associated levels of Gsα, measured both immunologically (McKenzie and Milligan, 1990) and by cholera toxin catalysed [^{32}P]-ADP-ribosylation (Kelly *et al*, 1990).

Heterologous desensitisation of an effector is postulated to be mediated through some component downstream of the receptor in the signal transduction cascade. Protein kinases A (Clark *et al*, 1989; Hausdorff *et al*, 1989) and C (Johnson *et al*, 1990), activated by the

second messengers cAMP and diacylglycerol (DAG) respectively, will phosphorylate the β2-adrenergic receptor and result in a desensitisation of receptor-mediated adenylyl cyclase activity through the uncoupling of the G-protein from its receptor. The role of the guanine nucleotide binding protein in heterologous desensitisation has also been implicated in several systems (Kassis and Fishman, 1982; Garrity *et al*, 1984; Rich *et al* ,1984). Studies, initially using toxin-catalysed [^{32}P]-ADP-ribosylation of G-protein α-subunits (Parson and Stiles 1987; Kelly *et al*, 1990) and, subsequently, using antisera specific to G-protein α-subunits (Green *et al*, 1990; McKenzie and Milligan, 1990; Mullaney *et al*, 1993), have demonstrated that prolonged agonist stimulation of many G-protein coupled receptors results in a down-regulation of the activated G-protein α-subunit. In several of these systems, heterologous desensitisation of the effector stimulated by the activated G-protein α-subunit is a coincidental occurrence (Kelly *et al*, 1990; McKenzie and Milligan 1990; Green *et al* 1990, 1992).

The apparent loss of Gsα from the plasma membrane on prostanoid treatment of NG108-15 cells may play an important role in the prostanoid-mediated heterologous desensitisation of adenylyl cyclase in these cells, although this remains to be unequivocally demonstrated. If Gsα down-regulation is to account, even partially, for the heterologous desensitisation, then it must be the limiting factor in the signal transduction cascade activating adenylyl cyclase. This could occur simply through the quantity of Gsα present in the plasma membrane being insufficient to couple to all of the receptor or effector or, alternatively, because of constraints, perhaps cytoskeletal, preventing receptor-G-protein and G-protein-effector interactions. It is, therefore, important to quantify the amount of both IP prostanoid

receptor:Gsα and A2 adenosine receptor:Gsα in the cells and also the ratio of activation/down-regulation to further elucidate if Gsα is stoichiometrically responsible for heterologous desensitisation. Recent estimates of receptor:Gα ratios in several cell lines predict a molar ratio of between 1:10 and 1:200 (Neubig *et al*, 1985; Alousi *et al*, 1991; Ransnas *et al* 1988). G-protein α-subunits appear to be present in excess over their respective receptors. Additionally, the receptor-Gα ratio of activation is in the region of 1:10 for "Gi-like" proteins in both rat adipocytes and human neutrophils (Green *et al*, 1990; Mueller *et al*, 1991). The ratios for Gsα:IP prostanoid and A2 adenosine receptor activation/down-regulation remain to be determined in NG108-15 cells and, until the stoichiometry is resolved, the question of whether Gsα is quantitatively limiting for prostanoid mediated adenylyl cyclase activation will stay unanswered.

In this chapter a brief characterisation of prostanoid binding in NG108-15 cells will be discussed and the levels of IP prostanoid receptor expression in NG018-15 cells determined. The mechanism behind the homologous desensitisation of the IP prostanoid receptor will be explored and discussed. The IP prostanoid receptor:Gsα stoichiometry in the plasma membrane will be determined, as will the ratio of receptor:Gsα down-regulation and the implication of these results discussed.

Figure 3.1 Time course of [^{3}H]-PGE1 binding to NG108-15 cell membranes.

25µg NG108-15 cell membranes were incubated at 30^{o}C with 10nM [^{3}H]-PGE1. At various times the samples were removed from the water bath and placed on ice to terminate the reaction. The samples were then rapidly filtered through Whatman GF/C filters and washed three times with 5ml ice cold buffer, as described in section 2.7. A parallel series of incubations was performed in the presence of 10µM PGE1 for each time point in order to determine non-specific binding. The results were expressed as the specific binding of [^{3}H]-PGE1 to the cell membrane (mean ± SD, n=3), from a single experiment.

Figure 3.1

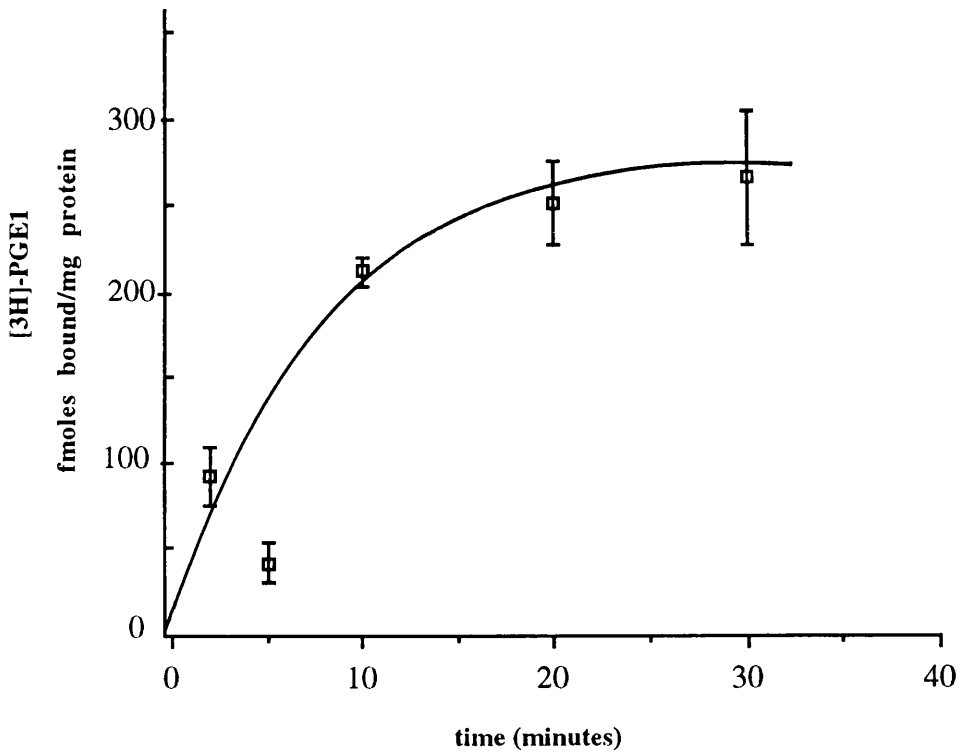

[3H]-PGE1
fmoles bound/mg protein
300
200
100
0
0
10
20
30
40
time (minutes)

Figure 3.2 The incubation of increasing amounts of NG108-15 cell membrane protein with [^{3}H]-PGE1.

Increasing amounts of NG108-15 cell membranes (5 - 100μg) were incubated at 30°C for 30 minutes with 10nM [^{3}H]-PGE1. Samples were then placed on ice, rapidly filtered through Whatman GF/C filters and washed three times with 5ml ice cold buffer, as described in section 2.7. A parallel set of incubations was performed in the presence of 10μM PGE1 to determine non-specific binding. The results were expressed as specific binding in dpm (mean ± SD, n=3) of a single experiment, of which one other produced similar data.

Figure 3.2

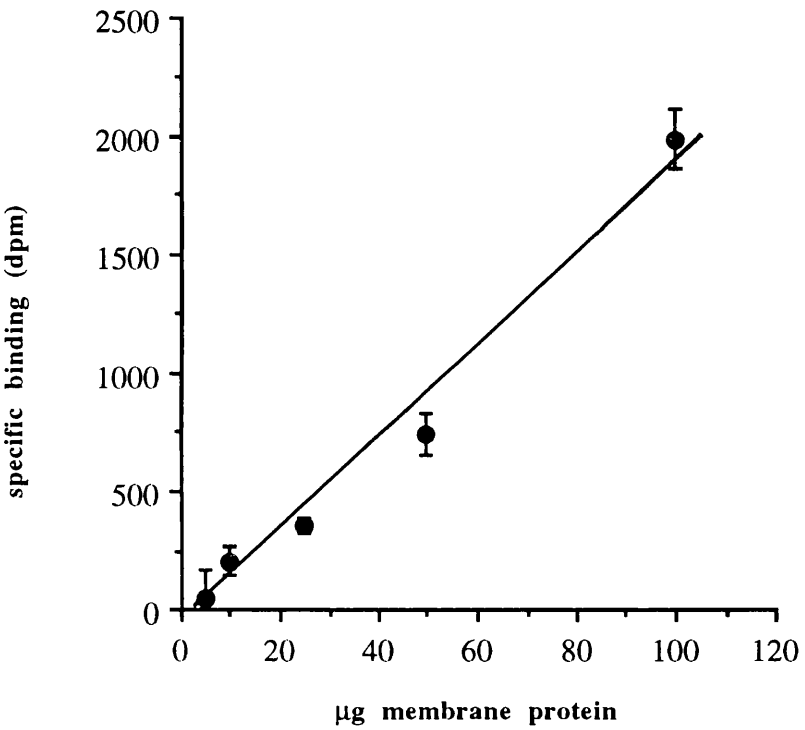

2500
2000
1500
1000
500
0
specific binding (dpm)
0
20
40
60
80
100
120
µg membrane protein

Figure 3.3 Displacement of [^{3}H]-PGE1 binding with increasing concentrations of PGE1 in NG108-15 cell membranes.

NG108-15 cell membranes (25μg) were incubated for 30 minutes at 30°C with 10nM [^{3}H]-PGE1 and increasing concentrations of PGE1 (between 100μM - 0.1nM). Samples were then placed on ice, rapidly filtered through Whatman GF/C filters and washed three times with 5ml ice cold buffer as described in section 2.7. The results were expressed as the specific binding of [^{3}H]-PGE1 (the mean of triplicates), from a single experiment of which two others gave similar results. SD values for the individual points were always less than 15% of the mean value. The IC50 (corrected for receptor occupancy) for the experiment displayed was 26.2nM.

Figure 3.3

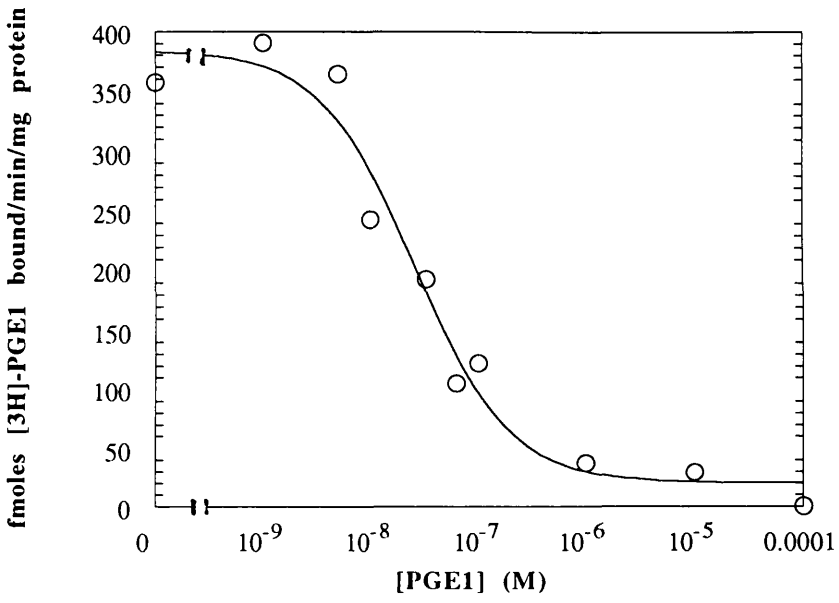

fmoles [3H]-PGE1 bound/min/mg protein
400
350
300
250
200
150
100
50
0
0
10-9
10-8
10-7
10-6
10-5
0.0001
[PGE1] (M)

Figure 3.4 Displacement of [^{3}H]-PGE1 binding with increasing concentrations of iloprost in NG108-15 cell membranes.

25μg NG108-15 cell membranes were incubated for 30 minutes at 30°C with 10nM [^{3}H]-PGE1 and various concentrations of iloprost (between 10μM - 0.1nM). Samples were then placed on ice, filtered through Whatman GF/C filters and washed three times with 5ml ice cold buffer as described in section 2.7. The results shown were expressed as specific binding of [^{3}H]-PGE1 (the mean of triplicates), of a single experiment of which four others gave similar results. SD values for the individual points were always less than 10% of the mean value. The IC50 (corrected for receptor occupancy) for the experiment displayed was 17.4nM.

Figure 3.4

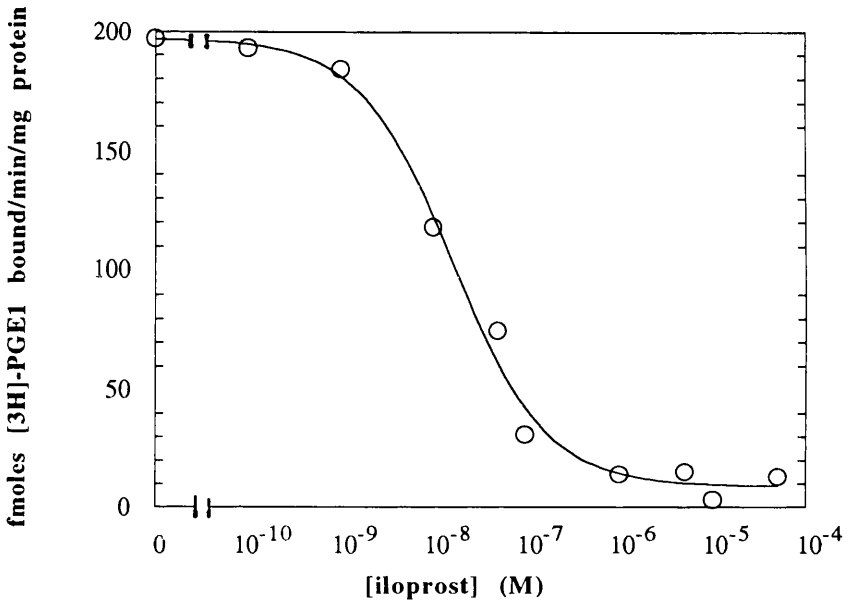

fmoles [3H]-PGE1 bound/min/mg protein
200
150
100
50
0
0
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[iloprost] (M)

Figure 3.5 Saturation binding of [^{3}H]-PGE1 in membranes prepared from NG108-15 cells.

50μg NG108-15 cell membranes were incubated with 5 or 10nM [^{3}H]-PGE1 and increasing concentrations of PGE1 (10-90nM) for 30 minutes at 30^{o}C as described in appendix 1 and section 2.7. The reaction was terminated by returning the samples to ice prior to rapid filtration through Whatman GF/C filters and subsequent washing with ice cold buffer as described in section 2.7. A saturation binding curve was constructed (figure 3.5a) as described in appendix 1. The data was then manipulated according to Scatchard (1949) (figure 3.5b). A Bmax of 1080 fmoles bound/mg membrane protein and a Kd of 29.4 nM was calculated. This was a representative experiment (mean ± SD, n=3), of which four others were performed and gave similar results.

Figure 3.5a

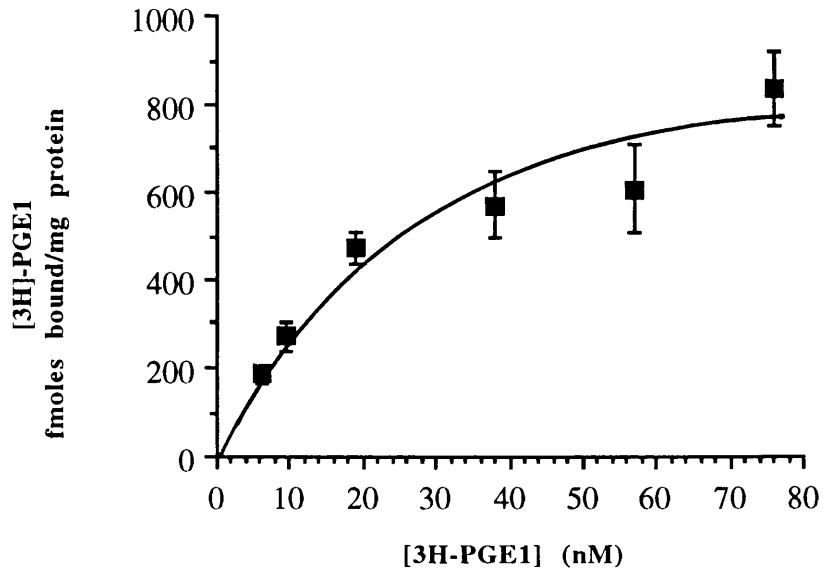


Figure 3.5b

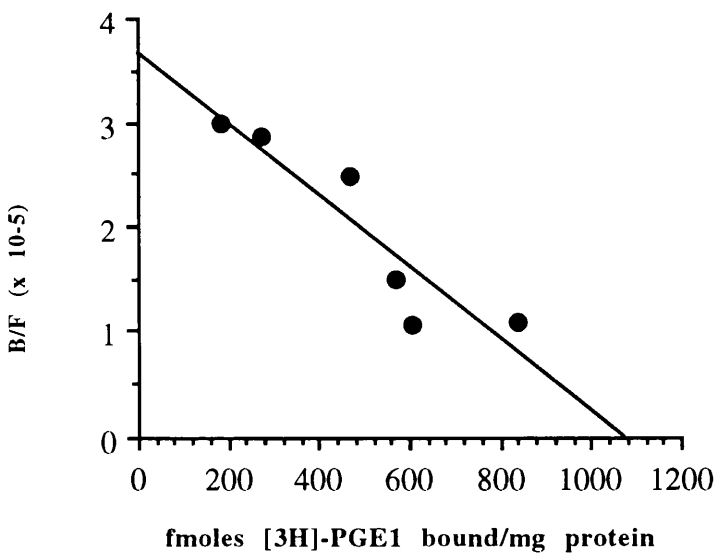


Figure 3.6 Western blotting analysis of membranes prepared from control and iloprost treated NG108-15 cells using antisera CS1 and SG1.

40μg membrane protein prepared from control and iloprost (1μM; 16 hours) treated NG108-15 cells were TCA precipitated, run on a 10% (w/v) acrylamide/0.25% (w/v) N,N'-methylene-bisacrylamide gel and western blotted for Gsα using the antiserum CS1, at a dilution of 1:250, as described in section 2.6 (figure 3.6a). Similarly, 100μg membranes from control and iloprost treated cells were western blotted for the presence of Gi2α using SG1 at a dilution of 1:400 (figure 3.6b). Lanes 1 and 3 in both figures were control membranes and lanes 2 and 4 were membranes from iloprost treated cells. The blots were quantified by overlaying them with an [^{125}I]-labelled goat anti-rabbit IgG as described in section 2.6. There was a 66±6% (mean ± SD) decrease of membrane associated Gsα levels in iloprost treated NG108-15 cells. There was no change in Gi2α levels (95±6%, mean ± SD).

Figure 3.6a

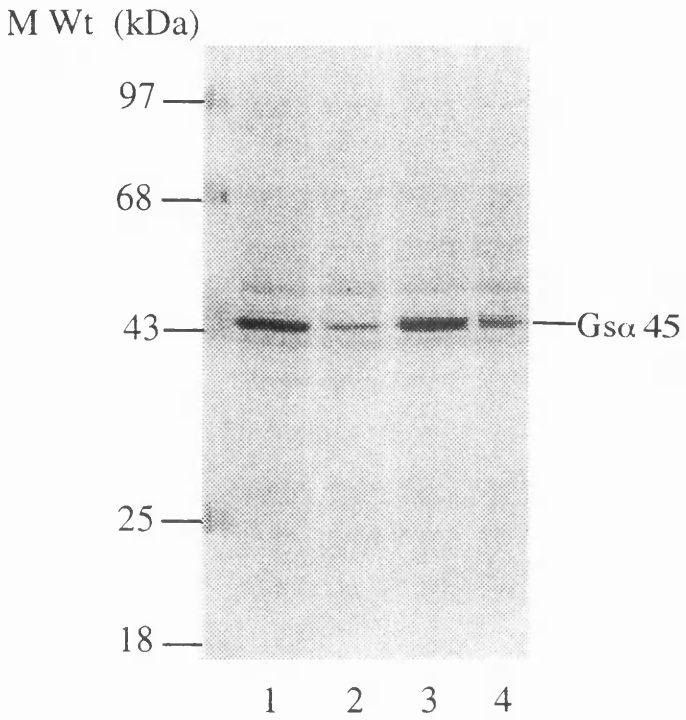


Figure 3.6b

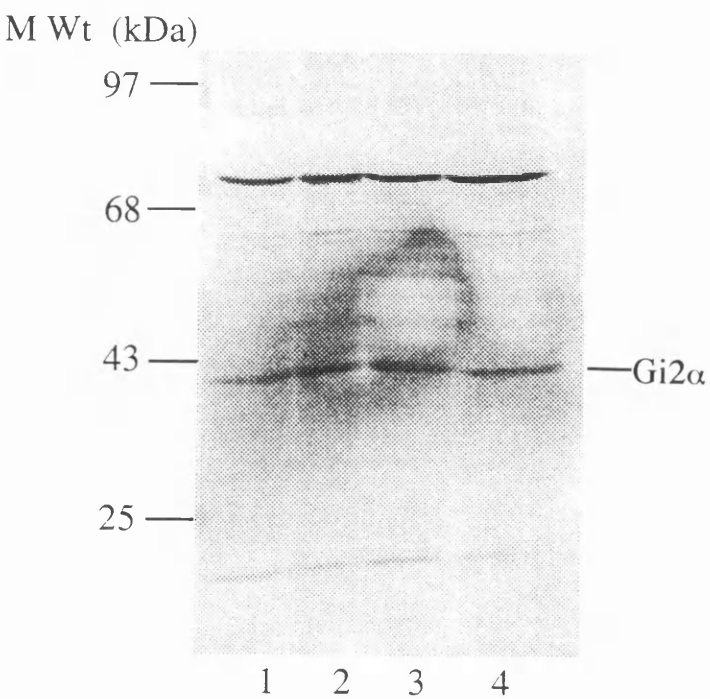


Figure 3.6c Coomassie blue stained SDS gel from figures 3.6a and 3.6b

After western blotting the gel from figures 3.6a and b was stained in Coomassie blue stain and then destained to assess if equal amounts of protein had been loaded on the different lanes. Lanes 1-4 correspond to lanes 1-4 in figure 3.6a and lanes 5-8 correspond to lanes 1-4 in figure 3.6b.

Figure 3.6c

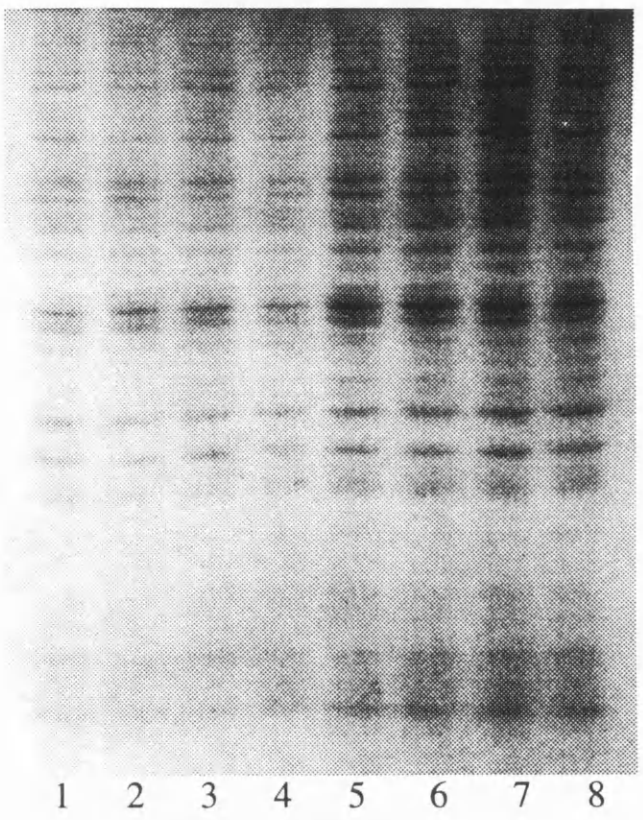


Figure 3.7 Western blotting analysis of increasing amounts of control and iloprost treated NG108-15 cell membranes, with an antiserum generated against the C-terminal decapeptide of Gsα, CS1.

5 - 100 µg membrane protein from control and iloprost treated (1µM; 16 hours) NG108-15 cells were TCA precipitated, run on a 10% (w/v) acrylamide / 0.25% (w/v) N,N'-methylene bisacrylamide gel and western blotted using the Gsα specific antiserum CS1 at a dilution of 1:250 as described in section 2.6. Lanes 1-6 were control membranes; lanes 7-12 were iloprost treated membranes. Lanes 1 and 7 were loaded with 5µg membrane protein, lanes 2 and 8, 10µg; lanes 3 and 9, 25µg; lanes 4 and 10, 50µg; lanes 5 and 11, 75µg; lanes 6 and 12, 100µg. The blots were quantitated using densitometric scan analysis as described in section 2.6. Membrane associated Gsα levels from iloprost treated cells were calculated as a percentage of control, for each amount of membrane protein loaded as follows: 5µg, 24%; 10µg, 46%; 25µg, 49%; 50µg, 59%; 75µg, 60%; 100µg, 54%.

Figure 3.7

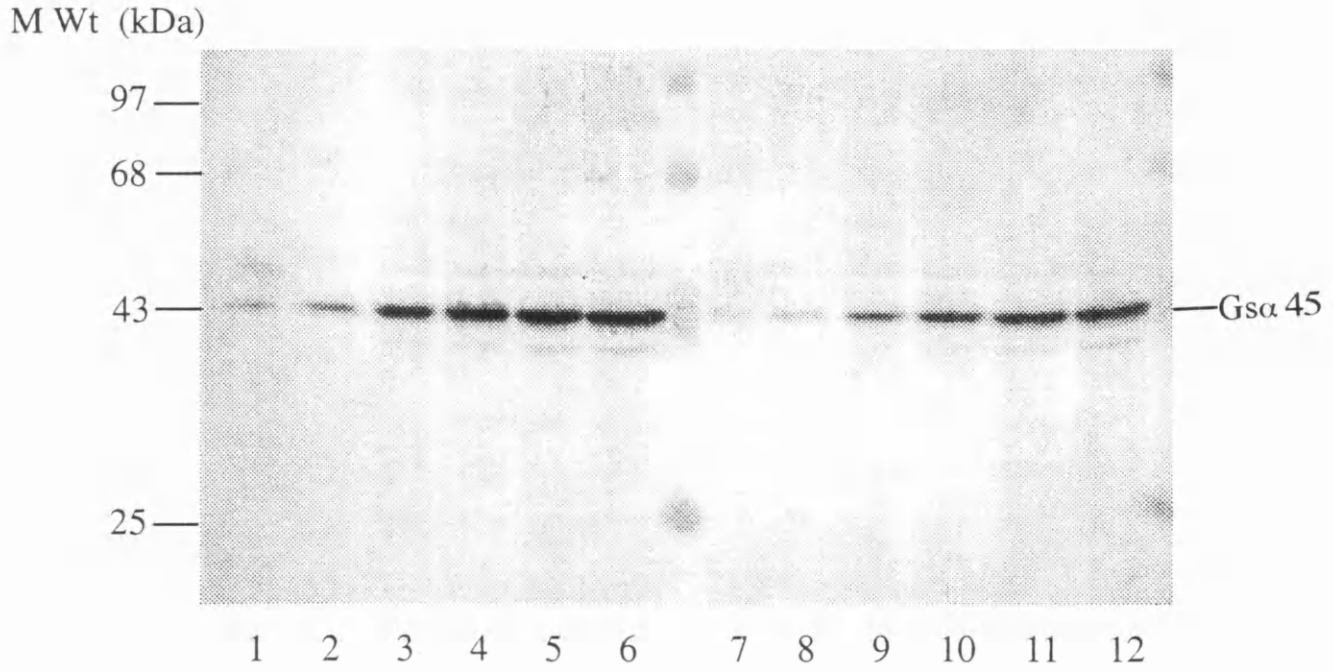


Figure 3.8 Saturation binding of [^{3}H]-PGE1 in membranes prepared from control and iloprost treated NG108-15 cells.

50µg NG108-15 cell membranes were incubated with 5 or 10nM [^{3}H]-PGE1 and increasing [PGE1] (0-90nM), for 30 minutes at 30^{o}C as described in appendix 1. Non-specific binding was determined in the presence of 10µM PGE1. The reaction was terminated by returning the samples to ice prior to rapid filtration through Whatman GF/C filters and subsequent washing with ice cold buffer as described in section 2.7. A saturation binding curve was constructed as described in appendix 1. The data was then manipulated according to Scatchard (1949). A Bmax of 1530 fmoles bound/mg membrane protein and a Kd of 38.5 nM was calculated for control membranes (□). Membranes from iloprost treated cells gave a Bmax of 550 fmoles bound/mg membrane protein and a Kd of 30.4 nM (♦). This was a representative experiment (mean ± SD, n=3), of which one other was performed which gave a similar result.

Figure 3.8

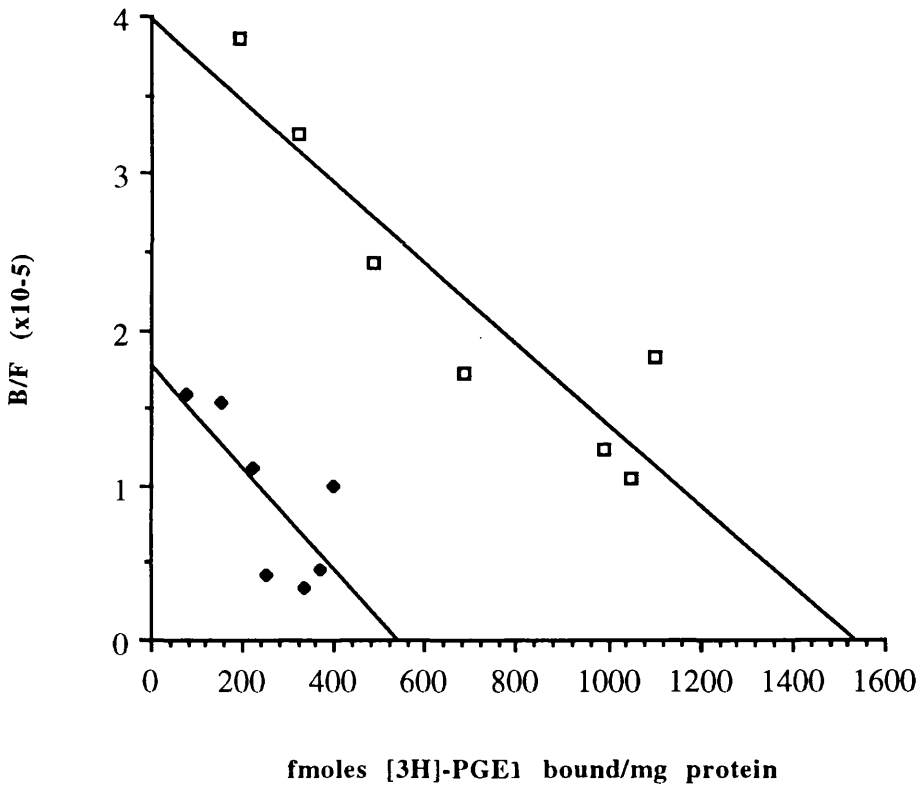

4
3
2
1
0
B/F (x10-5)
0
200
400
600
800
1000
1200
1400
1600
fmoles [3H]-PGE1 bound/mg protein

Figure 3.9 **Measurement of adenylyl cyclase activity in response to increasing concentrations of iloprost in membranes prepared from control and iloprost treated NG108-15 cells.**

Adenylyl cyclase activity was assessed in 10μg each of control (o) and iloprost treated (1μM; 16 hours) (□) NG108-15 cell membranes as described in section 2.8. Adenylyl cyclase activity was amplified in the presence of increasing concentrations (0 - 10μM) of iloprost. EC50 values for adenylyl cyclase stimulation in control (15.3nM) and iloprost treated (37.0nM) cells were calculated. The results shown were the mean of triplicates, from a single experiment which was representative of three experiments performed. SD values for the individual points were always less than 15% of the mean value.

Figure 3.9

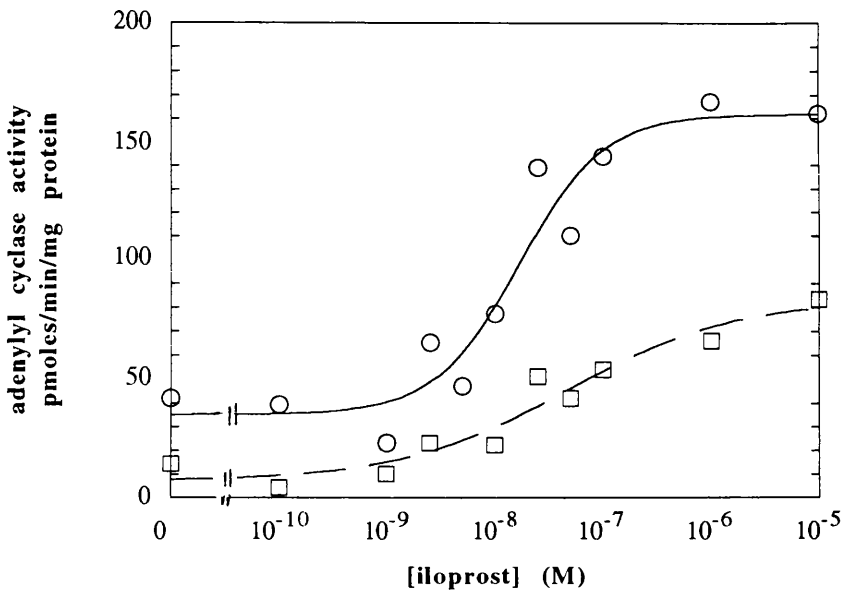

adenylyl cyclase activity
pmoles/min/mg protein
200
150
100
50
0
0
10-10
10-9
10-8
10-7
10-6
10-5
[iloprost] (M)

Figure 3.10 Analysis of Gsα levels, by western blotting, in membranes prepared from NG108-15 cells which were treated separately with iloprost and forskolin.

40μg NG108-15 cell membranes prepared from control, forskolin treated (10μM; 16 hours) and iloprost treated (1μM; 16 hours) cells were TCA precipitated, loaded onto a 10% (w/v) acrylamide / 0.25% (w/v) N,N'-methylene bisacrylamide gel and western blotted for Gsα using a 1:250 dilution of antiserum CS1, as described in sections 2.6. Lane 1 represented control membranes, lane 2 forskolin treated and lane 3 iloprost treated. Densitometric scan analysis was performed as described in section 2.6 and the data taken as a percentage of control levels. For this single experiment forskolin treatment gave 108% and iloprost 36% of control levels of Gsα. This data was from a single experiment but four others performed gave similar results.

Figure 3.10

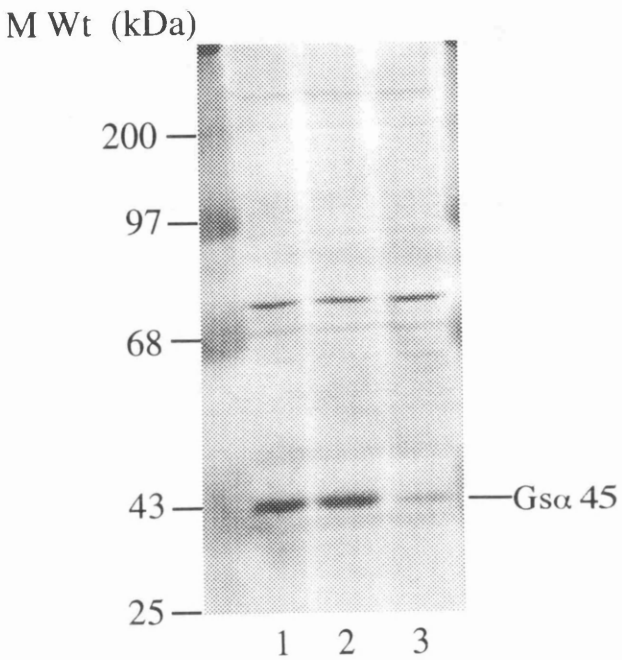


Figure 3.11 Measurement of Gsα levels and [^{3}H]-PGE1 binding in membranes prepared from NG108-15 cells which were treated for increasing lengths of time with iloprost.

NG108-15 cells were treated for various times (0 - 12 hours) with 1μM iloprost. Membranes prepared from each of the time points were TCA precipitated, run on a 10% (w/v) acrylamide / 0.25% (w/v) methylene bisacrylamide gel and western blotted for Gsα, using a 1:250 dilution of CS1 antisera. Immunoblots of Gsα (▲) were quantitatively analysed using an [^{125}I]-labelled anti-rabbit IgG second antibody overlay as described in section 2.6. Results were expressed as a percentage of control Gsα levels. The same membranes from the time course were solubilised (2μg) and analysed for NaF stimulated adenylyl cyclase activity when reconstituted into 10μg *cyc-* cell membranes (o) as described in more detail in section 2.9

25μg membranes prepared from cells from the various time incubations with iloprost were assayed for [^{3}H]-PGE1 binding (□) as described in section 2.7 and appendix 1. Non-specific binding was determined in the presence of 10μM PGE1. Results were expressed as a percentage of control levels.

Results for cyc- reconstitution experiments were expressed as the mean from three individual experiments. Results for both Gsα levels and [^{3}H]-PGE1 binding were expressed as the mean ± SD, from three individual experiments, performed on separate membrane preparations.

Figure 3.11

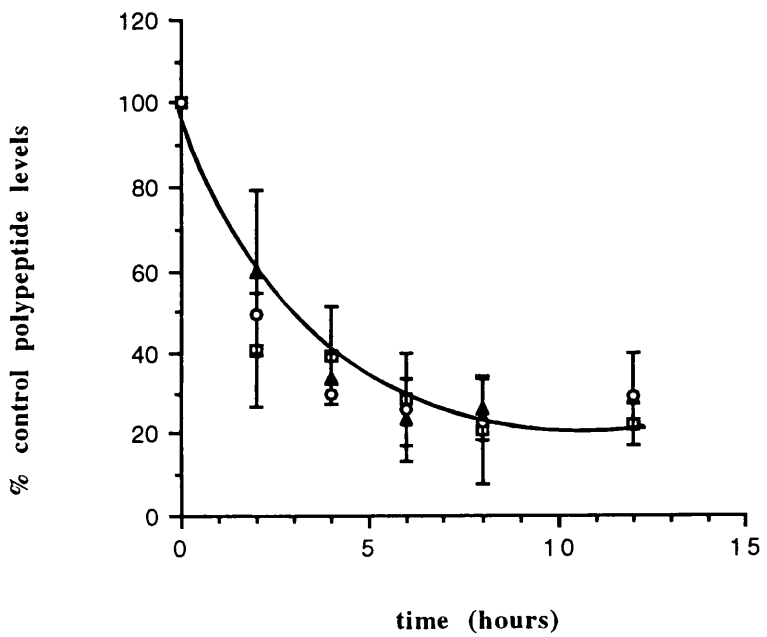

120
100
80
60
40
20
0
0
5
10
15
% control polypeptide levels
time (hours)

Figure 3:12 Analysis of Gi2α levels, by western blotting, in membranes prepared from NG108-15 cells treated with iloprost for increasing lengths of time.

100µg membranes prepared from NG108-15 cells from the time course of 1 µM iloprost treatment in figure 3.12 were TCA precipitated, run on a 10% (w/v) acrylamide / 0.25% (w/v) N,N'-methylene bisacrylamide gel and western blotted for Gi2α using the antiserum SG1 (1:400) as described in section 2.6 (figure 3.12a). Lane 1 - 0 hours; lane 2 - 2 hours; lane 3 - 4 hours; lane 4 - 6 hours; lane 5 - 8 hours; lane 6 - 12 hours. The immunoblot was quantified by overlaying it with an [^{125}I]-labelled goat anti-rabbit IgG as described in section 2.6 and expressing the results as a percentage of control Gi2α levels (figure 3.12b).

Figure 3.12a

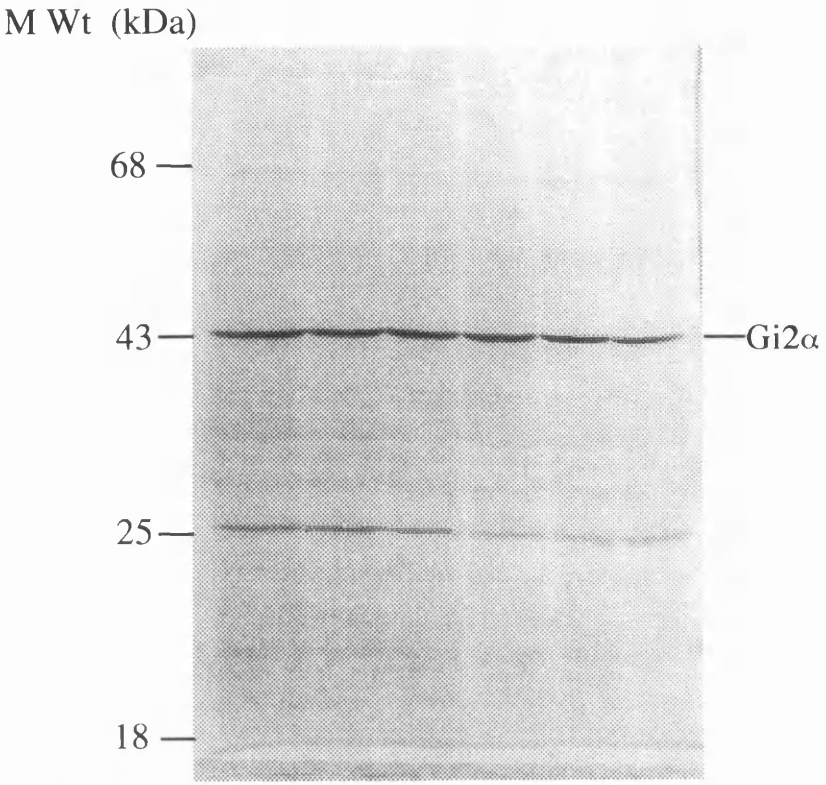


Figure 3.12b

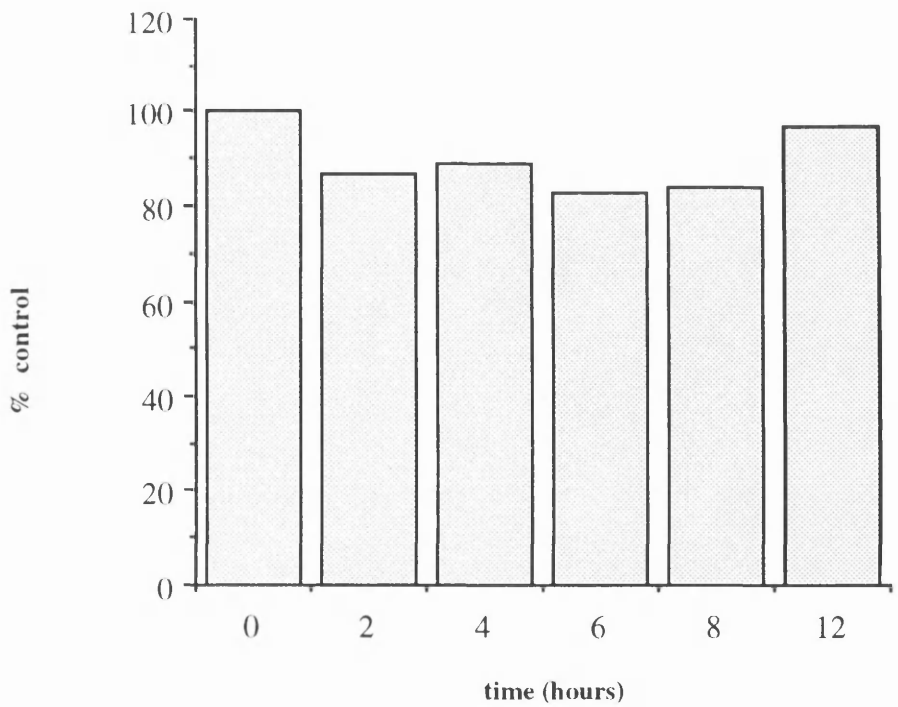


Figure 3.13 Analysis of Gsα levels, by western blotting, in membranes and cytosol prepared from control and iloprost treated NG108-15 cells.

NG108-15 cells were treated with 1μM iloprost for 3 hours. The cells were harvested, washed twice with PBS (x1) and homogenised as described in section 2.3. The homogenate was spun at 150 000 x g for 30 minutes. The supernatant was taken as "cytosol" and the pellet as "membranes". 40μg membranes (lanes 1 and 3) and cytosol (lanes 2 and 4) from control (lanes 1 and 2) and iloprost (lanes 3 and 4) treated cells were TCA precipitated, run on a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα, using a 1:250 dilution of CS1 antiserum, as described in section 2.6.

The immunoblot was densitometrically scanned and the iloprost treated membrane fraction taken as a percentage of the control (57%). The cytosolic fractions were too low in Gsα levels to detect accurately on the densitometric scanner. The results were taken from a single experiment that was representative of three performed.

Figure 3.13

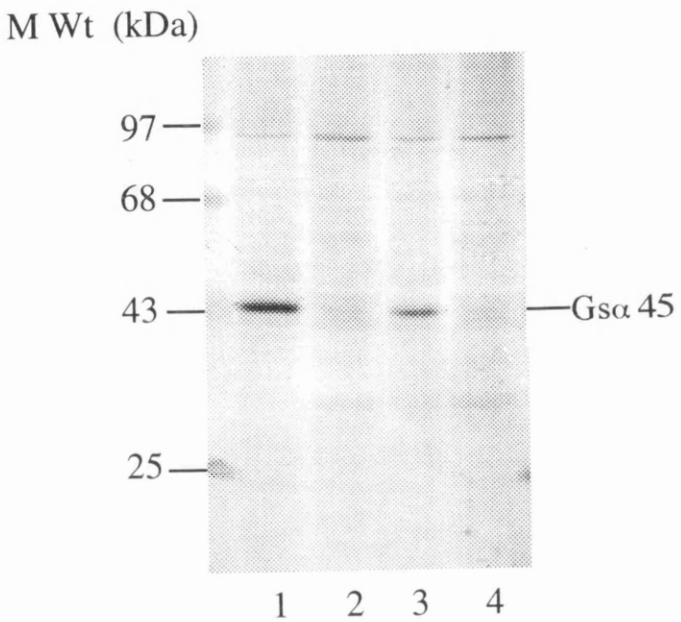


Figure 3.14 Molar quantitation of membrane associated Gsα levels in NG108-15 cell membranes.

40μg control (lane4) and iloprost treated (1μM; 16 hours) (lane 3) NG108-15 cell membranes were TCA precipitated, run on a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα using CS1, at a dilution of 1:250, as described in section 2.6. Alongside *E. Coli* expressed Gsα long (45KDa) and Gsα short (42KDa) were run. Lanes 1 and 2 were loaded with Gsα long 6ng and 18ng respectively and lanes 5 and 6 were loaded with Gsα short 6ng and 18 ng. The blot was quantitated with [^{125}I]-labelled goat anti-rabbit IgG. In the experiment displayed, Gsα45 in untreated membranes bound 836 cpm, whereas that in iloprost-treated membranes bound 494 cpm as measured in a gamma counter, a decrease of some 41% (figure 3.14a).

40μg membranes from control and iloprost treated NG108-15 cells were TCA precipitated and loaded on to a SDS polyacrylamide gel alongside various amounts (0-24ng) of *E. Coli*- expressed Gsα long. The gel was immunoblotted using the antiserum CS1 at a 1:250 dilution as described in section 2.6. The immunoblot was quantitated using an [^{125}I]-labelled goat anti-rabbit IgG overlay as described in section 2.6, and counted on a gamma counter. The amount of Gsα long form loaded onto the gel was plotted against the corresponding number of [^{125}I] counts bound on the blot (figure 3.14b). The amount of Gsα long form in the membranes prepared from control and iloprost treated NG108-15 cells was then calculated from the standard curve. Control NG108-15 cell membranes expressed 9.6±1.5 pmoles Gsα/mg membrane protein and membranes from iloprost treated cells expressed 3.0±0.3 pmoles Gsα/mg membrane protein. The results were expressed as the mean ± SD (n=3) taken from analysis of three individual membrane preparations.

Figure 3.14a

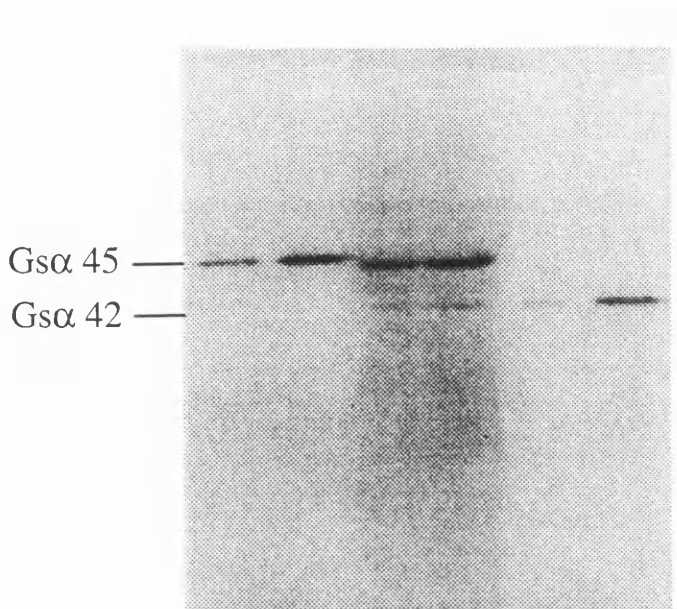


Figure 3.14b

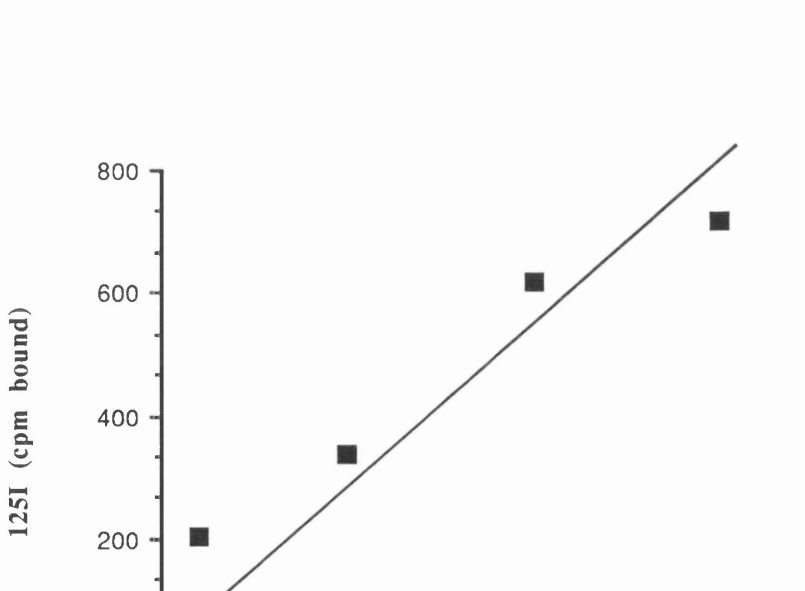


Figure 3.15 Measurement of membrane associated Gsα levels and [^{3}H]-PGE1 binding in membranes prepared from NG108-15 cells which have been treated with increasing concentrations of iloprost.

NG108-15 cells were treated with increasing concentrations of iloprost (0 - 1μM) for 8 hours. Membranes were prepared, and 40μg membrane protein was TCA precipitated, resolved on a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα, using a 1:250 dilution of the antiserum CS1, as described in section 2.6. The blot was quantitated using [^{125}I]-labelled goat anti-rabbit IgG and the results expressed as a percentage of control Gsα levels (□).

The cell membranes from the treatments with iloprost were also analysed for [^{3}H]-PGE1 binding using 10nM [^{3}H]-PGE1, and 10μM PGE1 to define non-specific binding as described in section 2.7 and appendix 1. Results were taken as a percentage of the control [^{3}H]-PGE1 binding (■).

The results were expressed as the mean ± SD (n=3), for a single experiment which was performed three times with similar results.

Figure 3.15

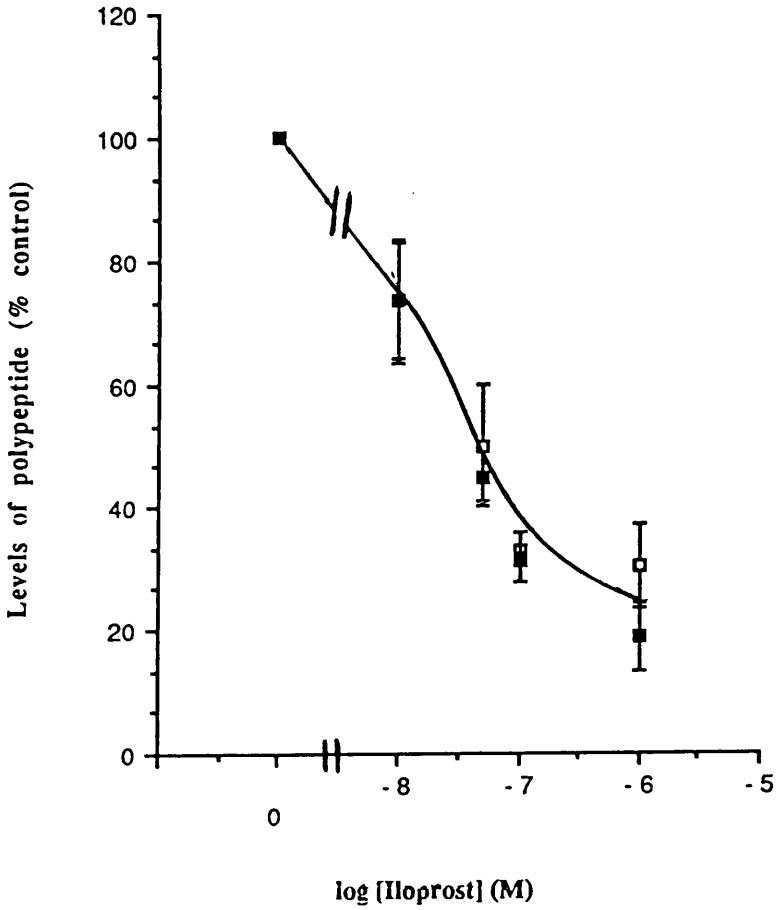

120
100
80
60
40
20
0
Levels of polypeptide (% control)
0
-8
-7
-6
-5
log [Iloprost] (M)

Figure 3.16 The effect of receptor occupancy on the stoichiometry of Gsα:receptor down-regulation.

The fractional occupancy of the IP prostanoid receptor during treatment with various concentrations of iloprost was calculated based on an estimated IC50 (corrected for receptor occupancy) of 14.8nM for iloprost, calculated from the membrane preparations analysed. Molar ratios of the Gsα and IP prostanoid receptor that is eliminated from the plasma membrane remained essentially constant with the fractional occupancy of the receptor. Fractional occupancy varied between 0.2 and 0.96. Ratios of the stoichiometry of loss of Gsα : receptor in the experiment displayed were between 8.5 and 10.1. In two further experiments the ratios of G-protein α-subunit to receptor loss were between 6.9 and 9.4.

Figure 3.16

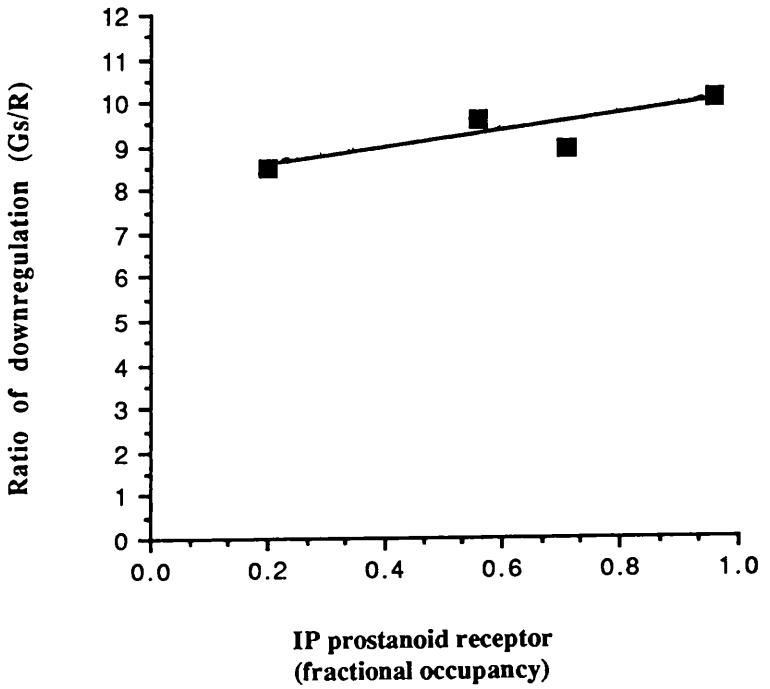

Ratio of downregulation (Gs/R)
12
11
10
9
8
7
6
5
4
3
2
1
0
0.0
0.2
0.4
0.6
0.8
1.0
IP prostanoid receptor
(fractional occupancy)

Table 3.1 The effect of 100μM GTPγS on [^{3}H]-PGE1 binding to control and iloprost treated NG108-15 cell membranes.

50μg control and iloprost treated (1μM, 16 hours) NG108-15 cell membranes were incubated for 30 minutes at 30°C with 40nM [^{3}H]-PGE1, in the presence of 100μM GTPγS. Non-specific binding was defined in the presence of 10μM PGE1. The reaction was terminated by returning the samples to ice and the [^{3}H]-PGE1 binding measured as described in section 2.7. The results were expressed as the mean ± SD (n=3), from a single representative experiment of which two others produced similar results.

Table 3.1

Treatment	[^{3}H]-PGE1 (fmoles bound/ mg membrane protein)	% control
control	912±88	100
+ GTPγS	496±44	54
iloprost (1μM)	276±20	30
iloprost + GTPγS	140±40	15

Table 3.2 Measurement of adenylyl cyclase activity in membranes prepared from control and iloprost treated NG108-15 cells. The effect of 20mM $MnCl_2$.

Adenylyl cyclase activity was assessed in 10μg of both control and iloprost treated (1μM, 16 hours) NG108-15 cell membranes as described in section 2.8. Additionally, when the adenylyl cyclase assay was performed in the presence of 20mM $MnCl_2$ the $MgCl_2$ routinely included in the assay mix was excluded. The results were expressed as mean ± SD (n=3), for a single experiment which was representative of three performed.

Table 3.2

Treatment	Adenylyl cyclase activity (pmoles/min/mg protein)
control	45.8 ± 6.2
control + Mn	14.3 ± 2.9
iloprost	11.0 ± 2.4
iloprost + Mn	14.2 ± 2.5

Results

Equilibrium binding of [^{3}H]-PGE1 (10nM) to NG108-15 cell membranes was achieved after 10 - 20 minutes incubation at 30°C (figure 3.1). An incubation time of 30 minutes at 30°C was subsequently used to ensure equilibrium binding. Increasing the amount of membrane protein in each assay increased the specific binding of [^{3}H]-PGE1 linearly up to 100μg membrane protein (figure 3.2). Routinely up to 50μg membrane protein was used per assay.

Both PGE1 (figure 3.3) and iloprost (figure 3.4) competed the binding of 10nM [^{3}H]-PGE1 with high affinity. The IC50 (corrected for receptor occupancy) for PGE1 displacement of [^{3}H]-PGE1 was 25.6±6.0 nM (mean ± SD, n=3) and the IC50 (corrected for receptor occupancy) for iloprost displacement was 16.9±5.4 nM (mean ± SD, n=5). The IC50 values (corrected for receptor occupancy) were calculated using the measured Kd=30nM (figure 3.5) for [^{3}H]-PGE1 binding. No further displacement of [^{3}H]-PGE1 was obtained above 10μM PGE1 and 1μM iloprost. 10μM PGE1 was subsequently used to define non-specific binding of [^{3}H]-PGE1. Routinely non-specific binding was approximately 20% of the total binding.

Saturation binding of [^{3}H]-PGE1 was performed on membranes prepared from NG108-15 cells to determine the levels of IP prostanoid receptor (figure 3.5) as described in appendix 1. Manipulating the data according to Scatchard (1949) (figure 3.5) gave an apparent Bmax of 1208±397 fmoles/mg membrane protein (mean ± SD, n=5) and a Kd of 31.1±4.3 nM (mean ± SD, n=5) was calculated. Manipulating the data produced from [^{3}H]-PGE1 self-displacement curves (figure 3.3)

according to the equations derived by DeBlasi *et al* (1989) (described in appendix 1), gave a calculated Bmax of 994 ± 410 fmoles/mg membrane protein (mean ± SD, n=3) and a Kd of 35.7±19.2 nM (mean ± SD, n=3,), not statisitically different to the results obtained by Scatchard analysis of the saturation binding data (figure 3.5) (p=0.80 for Bmax values and p=0.82 for the Kd values). The calculated Bmax and Kd, using the equations derived by DeBlasi *et al* (1989) are, however, based on the assumption that the high [Mg^{2+}] used in the PGE1 binding assay will force the entire population of the IP prostanoid receptor into a single high affinity state (Costa *et al*, 1990) as discussed more fully in appendix 1.

Saturation binding of [^{3}H]-PGE1 was performed on both control and iloprost treated NG108-15 cell membranes as described in appendix 1 (figure 3.8), and the data manipulated according to Scatchard (1949). A Bmax of 1530 fmoles/mg membrane protein and a Kd of 38.5 nM was calculated for the IP prostanoid receptor population in control membranes in these experiments, whilst in membranes prepared from iloprost treated cells the Bmax was 550 fmoles/mg membrane protein and the Kd was 30.4 nM. No significant change in the Kd of the IP prostanoid receptor for [^{3}H]-PGE1 was observed on iloprost treatment (p=0.73). Using a single concentration of both [^{3}H]-PGE1 (10nM) and PGE1 (10μM), binding analysis was performed on membranes prepared from control and iloprost treated NG108-15 cells. The estimated percentage decrease in [^{3}H]-PGE1 binding on iloprost treatment of NG108-15 cells was 69.2±9.6 % (mean ± SD, n=7). This indicated an apparent loss of IP prostanoid receptor from the plasma membrane. The pseudo Hill coefficients for [^{3}H]-PGE1 binding in control NG108-15 cell membranes was 1.05±0.25 and in iloprost treated cell membranes was 1.11±0.17, indicating an apparent single affinity state of the IP prostanoid receptor in

both control and iloprost treated NG108-15 cells indicating that the high [Mg^{2+}] used in the binding assay appeared to have been effective in coupling all of the IP prostanoid receptor to a G-protein (appendix 1).

However it was further necessary to examine the IP prostanoid receptor and its coupling to G-proteins in both control and iloprost treated NG108-15 cells. Both control membranes and membranes prepared from iloprost treated cells were incubated with 100μM GTPγS, and analysed for [^{3}H]-PGE1 binding using a single concentration of both radioligand (10nM) and PGE1 (10μM) as described in appendix 1 (table 3.1). Incubation with 100μM GTPγS resulted in a 54% decrease in specific [^{3}H]-PGE1 binding in membranes prepared from untreated NG108-15 cells. Following treatment of NG108-15 cells with iloprost there was a 51% decrease in specific [^{3}H]-PGE1 binding to the membranes when incubated with GTPγS. The IP prostanoid receptor appears still to be coupled to a G-protein after iloprost treatment.

Treatment of NG108-15 cells with 1μM iloprost for 16 hours resulted in a 56±8% (mean ± SD, n=8) decrease in the amount of membrane associated Gsα detectable by the C-terminal decapeptide antibody CS1 (figure 3.6a). The antiserum SG1 will only recognise Gi2α in NG108-15 cells because these cells do not express detectable levels of Gi1α (McKenzie and Milligan, 1990). Using SG1 no detectable changes in membrane associated Gi2α levels were observed on iloprost treatment (figure 3.6b). When examining the levels of G-protein α-subunits immunologically by western blotting, the gel was subsequently stained in Coomassie blue for every experiment. The protein loadings in each lane, for different cell treatments, were examined and determined to be very similar, indicating that any changes observed in

immunologically detectable Gα were not a result of varying amounts of protein in each lane. Additionally iloprost treatment of NG108-15 cells did not produce any detectable gross alteration in membrane protein profile (figure 3.6c).

High concentrations of ethanol (mM) have been shown to reduce membrane associated levels of Gsα in NG108-15 cells (Mochley-Rosen *et al*, 1988). The vehicle for the agonist iloprost contains approximately 0.1% ethanol and treatment of NG108-15 cells with vehicle only, resulted in no significant down-regulation of Gsα (McKenzie and Milligan, 1990), indicating that the vehicle itself plays no role in iloprost induced Gsα down-regulation.

Increasing amounts of membrane protein (5-100μg) from both control and iloprost treated NG108-15 cells were analysed for Gsα levels by western blotting. The percentage of Gsα lost from the plasma membrane on treatment with iloprost was calculated for each amount of membrane protein: 5μg 24%; 10μg 46%, 25μg 49%; 50μg 59%, 75μg 60%, 100μg 54% (figure 3.7).

A robust stimulation of adenylyl cyclase by iloprost was observed in untreated NG108-15 cells (figure 3.9) with an EC50 of 25.8±9.1 nM (mean ± SD, n=3). The EC50 for adenylyl cyclase activation by iloprost in membranes from iloprost treated cells [EC50 = 57.7±18.6 nM (mean ± SD, n=3)] was approximately two-fold higher than in control cells (p=0.035). Iloprost treatment of NG108-15 cells (16 hours, 1μM) resulted in a 59±13% (mean ± SD, n=6) decrease in the basal levels of adenylyl cyclase activity and a 61±14% (mean ± SD, n=6) decrease in maximal (1μM) iloprost stimulated adenylyl cyclase.

Basal adenylyl cyclase activity was measured in the presence of 20mM $MnCl_2$. This is believed to be a condition which attenuates the interaction of Gsα and the adenylyl cyclase catalytic subunit (Limbird *et al*, 1979). The basal level of adenylyl cyclase activity, without the contribution of tonic Gsα activation of its catalytic moiety, can thus be measured. No significant difference (p=0.977) was observed between basal adenylyl cyclase activity in both control membranes and iloprost treated cell membranes in the presence of $MnCl_2$ (table 3.2). Iloprost treatment of NG108-15 cells therefore did not modify the levels of activity of the adenylyl cyclase polypeptide.

NG108-15 cells were treated for 16 hours with 10μM forskolin to determine if the loss of Gsα from the plasma membrane was dependent on elevated intracellular cAMP levels. Forskolin activates a combination of the catalytic moiety of adenylyl cyclase and Gsα, independently of receptor, increasing intracellular levels of cAMP (Darfler *et al*, 1982). On prolonged exposure of NG108-15 cells to 10μM forskolin and hence to high levels of cAMP there was no significant change in membrane associated levels of Gsα. Expressed as a percentage of control values, densitometric scan analysis revealed levels of Gsα in forskolin treated cells to be 100±7% (mean ± SD, n=3) (figure 3.10), indicating the cAMP-independent nature of Gsα down-regulation.

A half-maximal time of 2-3 hours for both IP prostanoid receptor and Gsα down-regulation was observed in membranes prepared from NG108-15 cells which had been treated with 1μM iloprost over a range of times (0-12 hours) (figure 3.11). To determine if Gsα remaining in the plasma membrane after iloprost treatment was fully functional, and

could activate adenylyl cyclase, membranes prepared from iloprost treated NG108-15 cells over a time course were solubilised with 1% (w/v) sodium cholate (1 hour, 4°C). Solubilised extracts were reconstituted into *cyc-* S49 lymphoma cell membranes and adenylyl cyclase activity measured, in the reconstituted system, as described in section 2.9. The level of Gsα activity (as assessed by NaF stimulated adenylyl cyclase activity) remaining in the NG108-15 plasma membrane after iloprost treatment, was expressed as a percentage of control membranes. This was very similar to the amount of immunologically detectable Gsα lost from the plasma membrane on iloprost treatment at every time point, indicating the temporal loss of both Gsα protein and activity. There was no corresponding change in membrane associated Gi2α levels over the time course of iloprost treatment (figure 3.12).

Cytosolic preparations from control and iloprost treated cells were examined for the presence of Gsα to determine if the G-protein α-subunit was released into the cellular cytosol on iloprost treatment of NG108-15 cells (figure 3.13). A very small proportion (not capable of being accurately quantitated by densitometric scanning) of the total cellular Gsα was present in the cytosol of control NG108-15 cells. No increased levels of Gsα were observed in cytosol derived from cells which had been treated with iloprost for 3 hours. [3 hours was the time determined to be half-maximal for iloprost mediated Gsα down-regulation (figure 3.11)].

Membranes prepared from control and iloprost treated NG108-15 cells were resolved by SDS polyacrylamide gel electrophoresis alongside recombinant *E. Coli.* expressed Gsα45 and Gsα42, and western blotted for Gsα using an antiserum raised to the C-terminal decapeptide of Gsα

(table 2.1). Gsα45, was the most abundant form of Gsα expressed in NG108-15 cells. Less than 10% of the total membrane associated Gsα was Gsα42, and although it also appears to be down-regulated, because of its low level of expression this effect was difficult to characterise (figure 3.14a). All further reference to Gsα in these cells will refer to the 45kDa form unless otherwise stated.

To quantify the molar amount of Gsα present within control NG108-15 membranes and the amount of Gsα down-regulated on iloprost treatment of cells, membranes prepared from control and iloprost treated NG108-15 cells were resolved by SDS polyacrylamide gel electrophoresis alongside a range of concentrations of the recombinant Gsα45 (6-24ng). The samples were then quantified by assessing the binding of a [^{125}I]-labelled goat anti-rabbit IgG to the colorimetrically developed immunoblots. Standard curves of the amount of Gsα45 and the corresponding number of [^{125}I]-labelled goat anti-rabbit IgG counts bound were constructed (figure 3.14b) and the amount of Gsα present in control and iloprost treated cell membranes calculated from these curves. This quantitation indicated that Gsα45 was present in NG108-15 cell membranes at some 9.6±1.5 pmoles/mg membrane protein (mean ± SD, n=4). Treatment with iloprost (1μM, 16 hours) decreased the membrane associated levels of this polypeptide to 3.0±0.3 pmoles/mg membrane protein (mean ± SD, n=3).

Treatment of NG108-15 cells with a range of concentrations of iloprost gave an EC50 of approximately 30nM iloprost for both Gsα and IP prostanoid receptor down-regulation. (figure 3.15). The fractional occupancy of the IP prostanoid receptor was calculated for each concentration of iloprost used to treat the cells, using an estimated IC50

(corrected for receptor occupancy) for iloprost displacement of PGE1 of 14.8nM. At all concentrations of iloprost the molar ratio of Gsα and IP prostanoid receptor down-regulation was calculated (figure 3.16). The ratio was 1:7-10 receptor:Gsα and did not substantially vary with the fractional occupancy of the IP prostanoid receptor.

Discussion

Prostaglandins produce a broad range of biological effects in diverse tissues and cells through their binding to specific receptors on the plasma membrane. The interaction of prostaglandin and prostacyclin receptors with G-proteins has been identified by both radioligand binding and biochemical studies (Haluska *et al*, 1989). A classification of different subtypes of prostaglandin receptor has been proposed by Coleman and co-workers (1985, 1987), based on functional criteria, using the rank order of potencies of natural prostaglandins and a few relatively selective agonists and antagonists. The five main types of prostaglandin receptor are TP, DP, IP, FP and EP recognising with high affinity thromboxane A2, PGD2, PGI2, PGF2α and PGE2 respectively. The diverse and sometimes opposite effects of the prostaglandins on cell function have been attributed to their interaction with both adenylyl cyclase and phospholipase C (Samuelsson *et al*, 1978; Black and Wakelam, 1990), depending on the cell type or tissue assayed.

Scatchard manipulation of data obtained from saturation binding of [^{3}H]-PGE1 revealed that NG108-15 cells express an apparent 1 - 1.5 pmoles / mg membrane protein of an IP prostanoid receptor, which has a Kd of approximately 30nM for [^{3}H]-PGE1 (figure 3.5). Alternatively, manipulation of the competition binding data (figure 3.3), using the equations derived by DeBlasi *et al* (1989), produced similar values for the IP prostanoid receptor levels and Kd estimates (figure 3.3 and appendix 1). Other measurements of the IP prostanoid receptor levels in NG108-15 cells have revealed much lower estimates (Kelly *et al*, 1990), but more recent studies have produced evidence of IP prostanoid receptor levels similar to those observed in this thesis (Donnelly *et al*, 1992). The

pharmacological and molecular characteristics of this receptor have not been well-studied. However, on the basis of the relative potencies of PGE1 and iloprost to displace the specific binding of [^{3}H]-PGE1 and [^{3}H]-iloprost, and the lack of specific binding of [^{3}H]-PGE2 to membranes of these cells, it has been argued that the receptor in NG108-15 cells is an IP prostanoid receptor rather than a receptor for E-series prostaglandins (Carroll and Shaw, 1989).

Until recently, very few receptors for metabolites of arachidonic acid had been defined at the molecular level. Recently, a receptor on the human platelet for thromboxane A2 (Hirata *et al*, 1991), a murine PGF receptor (Sugimoto *et al*, 1994) and several murine receptors for prostaglandin E2 have been cloned. Examination of the primary amino acid sequences of these receptors revealed that they contained the seven hydrophobic stretches of amino acids which are indicative of G-protein-coupled receptors. Indeed, the thromboxane A2 receptor activates phospholipase C through the pertussis toxin insensitive protein, Gq/11 (Shenker *et al*, 1991). Three subtypes of the receptors for PGE_2 were identified; EP1 (Watabe *et al*, 1993), EP2 (Sugimoto *et al*, 1992) and EP3 (Honda *et al*, 1993), which are coupled to calcium mobilisation, and the stimulation and inhibition of adenylyl cyclase respectively, through the corresponding G protein(s). This characterisation has proven to be too simplistic. Recent studies from Namba and co-workers (1993) demonstrated that alternative splicing of the gene for the EP3 receptor generates four isoforms of the receptor differing only in their C-terminal tail. The C-terminal of a G-protein coupled receptor is known to be a site for determining G-protein interaction and specificity (Conklin and Bourne 1993a). These isoforms of the receptor differentially coupled to various second messenger systems, through their individual interaction

with different G-proteins (Negishi *et al*, 1989; Goureau *et al*, 1992). More receptors for metabolites of arachidonic acid are likely to be cloned in the near future, and it will be interesting to examine if these receptors are also alternatively spliced in the C-terminal tail.

A high affinity, G-protein coupled, IP prostanoid receptor was observed in the plasma membrane of NG108-15 cells after iloprost treatment (figure 3.8, table 3.1). Although apparently coupled to a G-protein it must be noted that the remaining IP prostanoid receptor may not all be coupled to Gs. Recent evidence from Negishi and co-workers (1993) showed that the EP_{3C} receptor can couple to both Gs and Go, so it cannot be definitively stated that all of the IP prostanoid receptor couples only to Gs. Desensitisation of the β2-adrenergic receptor on treatment with agonist results in a rapid uncoupling of the receptor from Gs, due to phosphorylation of the receptor by the protein kinase βARK, and subsequent binding of arrestin (Hausdorff *et al*, 1990). From the results presented here it cannot be determined if a rapid uncoupling of the IP prostanoid receptor from Gs occurs in the early stages of agonist exposure. Certainly on long term prostanoid exposure there appears to be no, or very little, uncoupling of the receptor from Gs. 50-70% of both the IP prostanoid receptor (figure 3.8) and Gsα are down-regulated (figure 3.6) on iloprost treatment, and it is uncertain if IP prostanoid receptor-G-protein uncoupling occurs, either by a previously undefined "IP prostanoid receptor kinase" or protein kinase A (PKA), before the receptor and G-protein are internalised. Previous studies by Keen and co-workers (1992) are in agreement with these results. This group demonstrated that after iloprost, and also forskolin, treatment of NG108-15 cells, the IP prostanoid receptor is still coupled to a G-protein in the cell membrane (Krane and Keen, 1992).

The similarity of the half-maximal concentrations for adenylyl cyclase activation (figure 3.9) and Gsα down-regulation (figure 3.15), by iloprost (approximately 30nM), suggested that the Gsα down-regulation may be mediated via cAMP-dependent events. Treating NG108-15 cells with the diterpene forskolin, which activates a combination of the adenylyl cyclase catalytic subunit and Gsα, elevating intracellular cAMP levels (Darfler *et al*, 1982), had no effect on the levels of membrane associated Gsα (figure 3.10). Additionally iloprost treatment had no effect on the membrane associated levels of Gi2α (figure 3.6 and 3.12). These results were in direct contrast to studies in S49 lymphoma cells, which demonstrated that agonist activation of the β2-adrenergic receptor resulted in a cAMP-dependent decrease in Gsα and an increase in Gi2α (Hadcock *et al*, 1990). The changes in membrane associated Gsα and Gi2α were reflected in the corresponding mRNA levels. On the contrary, prostanoid treatment of NG108-15 cells resulted in no change in Gsα mRNA levels (McKenzie and Milligan, 1990).

The cAMP dependent nature of the desensitisation in S49 lymphoma cells has been attributed to an enhanced transcription rate of the gene for Gi2α. One of the cAMP-dependent transcription factors binds to the cAMP response element in the 5' promoter region of the Gi2α gene (Brann *et al*, 1987) and activates its transcription. No cAMP response element is present in the gene encoding Gsα and the observed decrease in Gsα, in the S49 lymphoma cell line, is most likely to be the result of a cAMP-dependent decreased stability of the Gsα mRNA. Gsα mRNA contains two AUUUA rich consensus sequences in its 3' untranslated region which are thought to target the mRNA for enhanced degradation (Loganzo and Fletcher, 1993).

On the contrary, Gsα down-regulation by prostanoids in NG108-15 cells is a cAMP-independent event (figure 3.10; Krane and Keen, 1992; Keen *et al*, 1992), independent of the regulation of Gα mRNA levels and also independent of regulation at the level of protein synthesis (McKenzie and Milligan, 1990). This suggests that the regulation of Gsα protein levels is conducted through an enhanced proteolysis of the G-protein α-subunit, targetted either through the physical dissociation of the G-protein or some reversible covalent modification of the protein (as discussed in section 1.11). Certainly the mechanism of desensitisation of the IP prostanoid receptor appears to be different from that of the β2-adrenergic receptor, although this could also be attributed to differences in receptor desensitisation between cell types.

On short term exposure to agonist the β2-adrenergic receptor is rapidly sequestered from the plasma membrane (Hertel *et al*, 1983). Long term agonist exposure results in a cellular down-regulation of the β2-adrenergic receptor (Collins *et al*, 1992). Although there are suggestions of PKA phosphorylation mediating an enhanced proteolysis of the β2-adrenergic receptor (Campbell *et al*, 1991; Bouvier *et al*, 1989), most evidence points to a down-regulation of the receptor through internalisation and transfer into the endosomal pathway where degradation subsequently occurs (von Zastrow *et al*, 1992). The sustained decreased cellular levels of β2-adrenergic receptor are due to a cAMP-dependent decreased stability of the β2-adrenergic receptor mRNA, which contains an AUUUA rich sequence in its 3' non-coding region (Hadcock *et al*, 1989a). Several other G-protein coupled adenylyl cyclase-linked receptors contain this sequence in their mRNA, suggesting that perhaps they undergo a similar process in long term desensitisation (McFarland *et al*, 1989; Dearry *et al*, 1990; Sprengel *et*

al, 1990). The IP prostanoid receptor down-regulation has been shown, at least in part, to be dependent on cAMP (Wiltshire *et al*, 1990; Keen *et al*, 1992). This indicates either the presence of the AUUUA consensus sequences in the 3' untranslated region of its mRNA allowing a decrease in its stability, or a CRE in the 5' region of the gene allowing the binding of the transcription factor CREM which, unlike CREB, serves to down-regulate the transcription of the gene (Foulkes *et al*, 1991). However, the gene for the IP prostanoid receptor, in NG108-15 cells, has not yet been cloned and the presence of these, and potentially other, sequences which are capable of regulating the mRNA levels, will, until then, remain speculative.

Accumulating evidence suggests that an additional pathway exists to regulate β2-adrenergic receptor mRNA levels that is independent of cAMP. In S49 lymphoma cells isoprenaline gave a much greater decrease in receptor mRNA levels than forskolin (Hadcock *et al*, 1989b). Additional studies with S49 lymphoma cells which have mutants in the cAMP-producing part of the pathway suggested that there is an additional pathway which exists for regulating receptor mRNA that does not require stimulated levels of cAMP but nonetheless does require unstimulated cAMP-dependent protein kinase activity (Hadcock *et al*, 1989b). Forskolin stimulation of NG108-15 cells resulted in a 30% down-regulation of IP prostanoid receptor (Keen *et al*, 1992), whereas maximal down-regulation by iloprost was 50-70% (figure 3.8). Another unidentified pathway involved in IP prostanoid receptor down-regulation may exist in NG108-15 cells, perhaps identical to the cAMP-independent one discovered by Hadcock and co-workers (1988, 1989b).

Time courses and dose responses for the down-regulation of the IP prostanoid receptor and Gsα are very similar suggesting that both polypeptides may be co-internalised (figure 3.11). Green and co-workers have similarly observed a concurrent down-regulation of the A1 adenosine receptor and "Gi-like" proteins, in a ratio of receptor:Gi of 1:10, in rat adipocytes on prolonged agonist exposure (Green *et al*, 1990). It will be interesting to determine if both polypeptides are sequestered and degraded in the same membrane vesicle, or if distinct processes control the down-regulation of each polypeptide. The emerging body of evidence indicates that down-regulation of the β2-adrenergic receptor requires its coupling to Gs (chapter 1) suggesting that the receptor-G-protein complex may be the target for down-regulation. The reason for this remains unclear because in the majority of studies Gsα is not down-regulated along with the β2-adrenergic receptor (Gonzales *et al*, 1989; Rich and Iyengar, 1990). It may be that G-protein coupling serves to expose certain residues of the receptor, which act as substrates for, as yet, undefined cytoskeletal elements, potentially allowing the internalisation and subsequent down-regulation of the receptor, however this remains to be unequivocally determined. Certainly the δ-opioid receptor in NG108-15 cells is down-regulated on agonist exposure without coupling to Gi2α (Law *et al*, 1985), indicating the possible existence of distinct receptor specific mechanisms of receptor down-regulation.

Several isoforms of adenylyl cyclase have recently been cloned (as described in section 1.8.1). These have been shown to be differentially regulated by PKC, G-protein βγ dimers and Ca^{2+} (table 1.1). The exact nature of the adenylyl cyclase(s) expressed in NG108-15 cells is not known, however, there was no change in the functional capacity of the

catalytic component of adenylyl cyclase on iloprost treatment, indicating no alteration in the levels of activity of the effector protein (table 3.2). The decrease in basal adenylyl cyclase activity in membranes from iloprost treated NG108-15 cells appears to be due to the loss of tonic activation of adenylyl cyclase by the decreased levels of membrane associated Gsα. The diminished levels of IP prostanoid receptor may also contribute to the decreased basal adenylyl cyclase activity through decreasing the 'empty' receptor stimulation of adenylyl cyclase (more fully discussed in chapter 4).

There was no apparent release of the Gsα subunit to the cytosol after 2-3 hours of iloprost treatment of NG108-15 cells (figure 3.13), unlike the β2-adrenergic receptor in S49 lymphoma cells which gave an agonist stimulated release of Gsα to the cytosol (Ransnas *et al*, 1989). A P1 pellet (which comprises of all the cellular material minus the cytosol - see figure legend 3.13) was examined immunologically for Gsα levels in the above experiment. An approximate 50% decrease in Gsα was observed upon iloprost treatment, with no increase in cytosolic levels, indicating a cellular down-regulation of Gsα, suggesting an internalisation of the polypeptide into endosomal compartments and subsequent protein degradation Two recent studies have demonstrated that iloprost treatment of mouse mastocytoma cells (Negishi *et al*, 1992) and human platelets (Mollner *et al*, 1992) resulted in a complexing of the Gsα with an unknown protein in the cytosol and plasma membrane respectively. This is in agreement with Takahashi and co-workers (1991) who showed that factors present in the cytosol promoted the release of Gi2α from mouse mastocytoma cells, on receptor stimulation, and the released Gi2α was identified as a high molecular weight soluble complex with unidentified components. This unknown protein may

serve to target the G-protein α-subunit for enhanced proteolysis, before internalisation.

Heterologous desensitisation and the down-regulation of Gsα or the impairment of Gsα function were shown to be coincidental occurrences in several systems upon prolonged agonist exposure (Rich *et al*, 1984; Garrity *et al*, 1983; Kassis and Fishman 1982; Green *et al*, 1990, 1992). Kelly and co-workers (1990) demonstrated the iloprost mediated heterologous desensitisation of adenylyl cyclase in NG108-15 cells and a coincidental loss of cholera toxin catalysed [^{32}P]-ADP-ribosylation. Prostanoid mediated Gsα down-regulation was first detected immunologically by McKenzie and Milligan (1990) in the NG108-15 cell line and studies performed here (figure 3.6) further confirm that data. Approximately one pmole of IP prostanoid receptor was down-regulated for every 7-10 pmoles Gsα at various degrees of receptor occupancy by agonist (figure 3.16). This was in agreement with similar studies on the A1 adenosine receptor in rat adipocytes which showed a down-regulation of receptor:Gi1α and receptor:Gi3α ratios of 1:10 (Green *et al*, 1990).

Approximately 3 pmoles fully functional Gsα were left in the plasma membrane after iloprost treatment of NG108-15 cells (figure 3.11, 3.15). Kim and co-workers (unpublished observations) recently showed, using high affinity [^{3}H]-forskolin binding to measure Gsα-adenylyl cyclase complexes in NG108-15 cells, that Gsα is in a 70-fold excess over the adenylyl cyclase catalytic moiety. If heterologous desensitisation of adenylyl cyclase in NG108-15 cells by iloprost is mediated through Gsα down-regulation several points must be considered. Firstly, the quantity of Gsα remaining in the plasma

membrane after iloprost treatment must be examined to determine if it is sufficient to couple to all the A2 adenosine receptor (or secretin receptor) and then maximally activate adenylyl cyclase; and secondly the capacity of the remaining Gsα to interact with each of the receptor and effector proteins of the signalling cascade must be explored. These points will be discussed below.

0.5 pmoles IP prostanoid receptor (approximately the amount remaining in NG108-15 cell membranes after iloprost treatment) will down-regulate approximately 3-5 pmoles Gsα on agonist treatment, thus it appears that there is a sufficient amount of Gsα left in the plasma membrane (approximately 3 pmoles) to be activated by a large proportion, if not by all, of the remaining IP prostanoid receptor. (This is only the case if the ratio of receptor:Gsα activation is similar to that of down-regulation. The ratio of receptor:Gsα activated is not necessarily identical to the stoichiometry of down-regulation. Rapid recycling of activated Gsα by βγ dimers may make the ratio of Gsα activation higher than that of its down-regulation.)

The levels of A2 adenosine receptor remain undetermined in NG108-15 cells due to the lack of availability, until recently, of a suitable radioligand (Keen *et al*, 1989) allowing no determination of the stoichiometry of A2 adenosine receptor:Gsα activation on agonist exposure. It is therefore uncertain if the A2 adenosine receptor requires more than 3 pmoles Gsα to maximally activate adenylyl cyclase. The elucidation of the ratio of A2 adenosine receptor:Gsα activation will be important in determining if the quantity of Gsα remaining in the NG108-15 cells after iloprost treatment is limiting for A2 adenosine receptor

activation of adenylyl cyclase and thus responsible for the resultant heterologous desensitisation.

After iloprost treatment all of the remaining membrane associated Gsα is fully functional (figure 3.11). It may not, however, be capable of interacting with the IP prostanoid and A2 adenosine receptors and, indeed, the other components of the signalling cascade for a number of reasons. It has been shown that the lateral mobility of G-protein coupled receptors is greater than for those of the tyrosine kinase family (Jans, 1992). Decreased lateral mobility of the V2 vasopressin receptor in LLC-PK1 renal epithelial cells, using pretreatments with either low temperature or NH_4Cl, resulted in a decreased receptor mediated production of cAMP, with no effect on non-receptor stimulated adenylyl cyclase activity. This suggests a role for the lateral mobility of receptors in regulating receptor coupling to G-proteins and hence the effector (the "mobile receptor hypothesis"). Although, as yet, poorly investigated the lateral mobility of G-proteins may also be important in determining its coupling to the receptor and effector. Indeed, preliminary studies in NG108-15 cells using fluorescently labelled (tetramethylrhodamine) Goα and βγ demonstrated their restricted mobility within the plane of the plasma membrane (Kwon *et al*, 1992). Constraints imposed on the lateral mobility of both receptor and G-protein, may therefore serve to control receptor-G and G-effector interactions, indicating the potential for another point of producing diversity in signalling pathways, whereby selective release of these constraints could result in either, one receptor activating only one or several G-proteins or several G-proteins activating only one or several effectors.

Interestingly, temperature and NH_4Cl pretreatments which affect the lateral mobility of the V2 vasopressin receptor and subsequently decrease cAMP production, also affect the actin cytoskeleton (Jans *et al* 1992), suggesting that cytoskeletal elements may regulate the lateral mobility of the signalling elements in the plasma membrane. Evidence is accumulating from several groups that G-protein α-subunits can associate with cytoskeletal structures. Octyl glucoside, a detergent which preserves the membrane-cytoskeletal interaction, was used to extract these elements from rat brain synaptosomes. This yielded large guanine nucleotide sensitive polydisperse structures (Nakamura and Rodbell, 1990). It is uncertain if these polydisperse structures of Gα subunits are distinct elements from single heterotrimeric G-proteins or if they represented the native form of the protein. It appears that the α-subunits of heterotrimeric G-proteins can self-associate along with cytoskeletal elements. Wang and co-workers (1990) have also shown that Gsα and Gi1α bind to tubulin with high affinity whereas Gi2α and Gi3α do not. Tubulin is a GTP-binding protein with structural homology to heterotrimeric G-proteins (Mandelkow *et al*, 1985; Rasenick *et al*, 1989;). It can transfer its guanine nucleotides to both Gsα and Gi1α thereby activating the G-protein. Because tubulin only transfers its GTP to Gsα and Gi1α it has been suggested that tubulin is an adenylyl cyclase regulatory protein. Several 'in vivo' studies have been performed in S49 lymphoma cells, which lack Gi1α, using the inhibitors of microtubule polymerisation, colchicine and vinblastine (Insel and Kennedy, 1978; Kennedy and Insel, 1979; Leiber *et al*, 1993). An enhancement of β-adrenergic receptor stimulated adenylyl cyclase was observed in cells treated with these inhibitors. More recent studies on S49 lymphoma cells treated with colchicine demonstrated an increase in high affinity [^{3}H]-forskolin binding, which measures the formation of Gsα-adenylyl

cyclase complexes, when compared to untreated cells (Leiber *et al*, 1993). Inhibition of tubulin polymerisation appeared to enhance the interaction of Gs with adenylyl cyclase, suggesting that tubulin ordinarily places constraints on Gsα adenylyl cyclase interactions and compartmentalisation of Gs exists, whereby not all of Gsα ordinarily has access to adenylyl cyclase. Constraints on the interaction of Gsα and adenylyl cyclase may, therefore, be involved in the heterologous desensitisation of adenylyl cyclase in NG108-15 cells.

In NG108-15 cells there is a 70-fold excess of Gsα over adenylyl cyclase (Kim *et al*, 1994, unpublished observations). Unless the stoichiometry of Gsα-adenylyl cyclase interaction is very much greater than one, the adenylyl cyclase catalytic component is quantitatively the limiting factor in the signalling cascade. If Gsα is to account for iloprost mediated heterologous desensitisation of adenylyl cyclase in NG108-15 cells, either constraints on the interaction of Gsα and adenylyl cyclase must occur as suggested above, or all of the Gsα activated does not interact with adenylyl cyclase. Indeed, Gsα has been shown to couple to the inhibition of calcium channels (Khac *et al*, 1991), to allow opening of voltage dependent calcium channels (Kameyama *et al*, 1985; Yatani *et al*, 1987), to inhibit the activity of voltage-dependent Na^+ channels (Schubert *et al*, 1989) and to inhibit Mg^{2+} flux (Maguire and Erdos, 1980). The interaction of Gsα with these effectors must, therefore, be examined in NG108-15 cells.

Recent findings from Kleuss and co-workers (1992, 1993) have shown that preferential α-subunit coupling with subtypes of β and γ G-protein subunits demonstrate differential coupling to receptors. All of the remaining Gsα in the plasma membrane after iloprost treatment may

not be capable of interacting with the IP prostanoid or A2 adenosine receptors through preferential coupling to specific subtypes of $\beta\gamma$ which signals Gsα coupling to another receptor/effector. The EP_{3C} receptor, recognising PGE2, has been shown to couple to both Gs and Go (Negishi *et al*, 1993). Some of the IP prostanoid receptor remaining after iloprost treatment may already be coupled to a G-protein distinct from Gs, and therefore be incapable of interacting it, and so stimulating adenylyl cyclase.

In rat adipocytes the involvement of the "Gi-like" protein down-regulation in agonist mediated heterologous desensitisation of adenylyl cyclase inhibition has been shown (Green *et al*, 1992), and it seems likely that Gsα plays a pivotal role in heterologous desensitisation of adenylyl cyclase stimulation in NG108-15 cells. It must, however, be considered that the down-regulation of Gsα and the development of heterologous desensitisation are merely coincidental occurrences and another mechanism is involved in mediating the iloprost induced heterologous desensitisation of adenylyl cyclase in NG108-15 cells. The most likely alternative is via a cAMP-dependent protein kinase, and, indeed, elevating intracellular cAMP with forskolin results in a 30% decrease in NECA stimulated adenylyl cyclase activity in NG108-15 cells (Wiltshire *et al*, 1990; Keen *et al*, 1992). However, inhibitors of protein kinase A (H7) and βARK (heparin) do not affect the desensitisation of the A2 adenosine receptor (Malkhandi and Keen 1992) and it has been proposed by Keen and co-workers (1992) that cAMP plays only a minor role in the desensitisation because the loss of receptor responsiveness is much smaller with forskolin than with either iloprost or NECA. Alternatively heterologous desensitisation of adenylyl cyclase could be the result of increased phosphodiesterase activity breaking down cAMP produced by

the adenylyl cyclase enzyme. In rat Sertoli cells the level of mRNA transcripts encoding a high affinity cAMP phosphodiesterase is increased by more than 100-fold when these cells are treated with dibutyryl cAMP (Swinnen *et al*, 1989). cAMP can therefore regulate the expression of one of its own degrading enzymes by an intracellular feedback mechanism and it must not be discounted that this is a possibility for iloprost mediated heterologous desensitisation in NG108-15 cells .

In summary, one pmole of IP prostanoid receptor is down-regulated for every 8-10 pmoles Gsα in a cAMP-independent manner. Down-regulation of the IP prostanoid receptor and Gsα demonstrate identical time courses and dose responses following iloprost treatment, indicating the temporally concurrent down-regulation of receptor and Gsα. The involvement of the down-regulation of Gsα in iloprost-induced heterologous desensitisation of adenylyl cyclase in NG108-15 cells remains to be unequivocally demonstrated, although emerging evidence strongly suggests that cytoskeletal elements serve to constrain Gsα in specific "pools" within the plasma membrane of S49 lymphoma cells. This requires to be investigated more fully in NG108-15 cells. Potentially these "pools" could differentially interact with receptor and/or effector. Similarly the role of $\beta\gamma$ dimers in determining G-protein-receptor coupling, and perhaps Gsα-adenylyl cyclase coupling requires further investigation in NG108-15 cells.

CHAPTER 4

Characterisation of the elements of the adenylyl cyclase signalling cascade in NG108-15 cells transfected with the β2-adrenergic receptor.

Introduction

The NCB20 cell line is a half-sister of the NG108-15 cell line and was formed by the fusion of the N18TG2 mouse neuroblastoma with cells of embryonic hamster brain (Minna *et al*, 1972). NCB20 cells express approximately 100 fmoles/mg membrane protein of a high affinity IP prostanoid receptor (Kelly *et al*, 1990) . This receptor is likely to be of the same molecular species as the IP prostanoid receptor in the NG108-15 cell because of their joint parentage, the N18TG2 mouse neuroblastoma. The IP prostanoid receptor in NCB20 cells couples to adenylyl cyclase (Blair *et al*, 1980, 1981) through Gsα activating it 10-15 fold. Interestingly, there is no apparent down-regulation of Gsα on iloprost treatment of NCB20 cells (Leigh *et al*, 1985; Mullaney and Milligan, unpublished observations). The down-regulation of Gsα on iloprost treatment appears therefore not to be a unique feature of the IP prostanoid receptor, but it may be dependent either on the cell line expressing it or on its level of expression. The IP prostanoid receptor is expressed at high levels, 1.0 - 1.5 pmoles/mg membrane protein (figure 3.5), in NG108-15 cells where it is capable of down-regulating Gsα on prolonged agonist treatment, whereas in NCB20 cells the levels of IP prostanoid receptor expression are much lower. Additionally, Gsα down-regulation, by the activation of the IP prostanoid receptor, has been shown to be dependent on receptor occupancy (figure 3.16). The agonist mediated down-regulation of Gsα may, therefore, be dependent on the level of receptor expression.

The A2 adenosine and secretin receptors also activate adenylyl cyclase in NG108-15 cells, and prolonged agonist activation of these receptors does not result in a down-regulation of Gsα (McKenzie *et al*,

1990). These receptors may also be expressed at low levels unable to produce an apparent agonist mediated Gsα down-regulation. Until recently measuring the levels of A2 adenosine receptor expression in the NG108-15 cell has proved problematic due to the lack of a specific radioligand (Keen *et al*, 1989). It is therefore uncertain as to the level of A2 adenosine receptor expression in these cells. Certainly, the secretin receptor is expressed at very low levels, between 25-30 fmoles/mg membrane protein, in NG108-15 cells (Gossen *et al*, 1990).

The expression of another Gsα-coupled receptor at similar levels to the IP prostanoid receptor in NG108-15 cells, and the examination of the effects of agonist at this receptor, would potentially determine if Gsα down-regulation was unique to the IP prostanoid receptor in NG108-15 cells. Furthermore, isolation of NG108-15 cells expressing differing levels of a distinct Gsα-coupled receptor would enable the further elucidation of the involvement of receptor levels in Gsα down-regulation.

It has been well-established that the β2-adrenergic receptor activates adenylyl cyclase through Gsα. Several groups (Ransnas *et al*, 1989; Hadcock *et al*, 1990) have detected a loss of Gsα from the plasma membrane on prolonged isoprenaline treatment of S49 lymphoma cells. Transfection of NG108-15 cells with a cDNA for the β2-adrenergic receptor was undertaken, with the objective of selecting clones with differing levels of receptor expression and subsequently examining the effect of isoprenaline treatment on membrane associated Gsα levels in these clones.

In this chapter the method of transfection of NG108-15 cells with a cDNA for the human β2-adrenergic receptor will be briefly described.

The β2-adrenergic receptor expressed in the NG108-15 cells will be characterised, as will the elements proximal to cAMP production in the adenylyl cyclase signalling cascade, to determine whether the transfection process resulted in any changes in IP prostanoid receptor, G-protein or adenylyl cyclase protein expression.

Figure 4.1 Full length nucleotide and deduced amino acid sequence of a cDNA for the human β2-adrenergic receptor (Kobilka *et al*, 1987).

The ATG and the termination codon, TGA, in the 5' untranslated region are underlined. Within the coding sequence the sites of N-linked glycosylation are indicated by *. The consensus cAMP-dependent protein kinase A phosphorylation sites are boxed. In the 3' untranslated region three potential polyadenylation sequences are underlined.

Figure 4.1

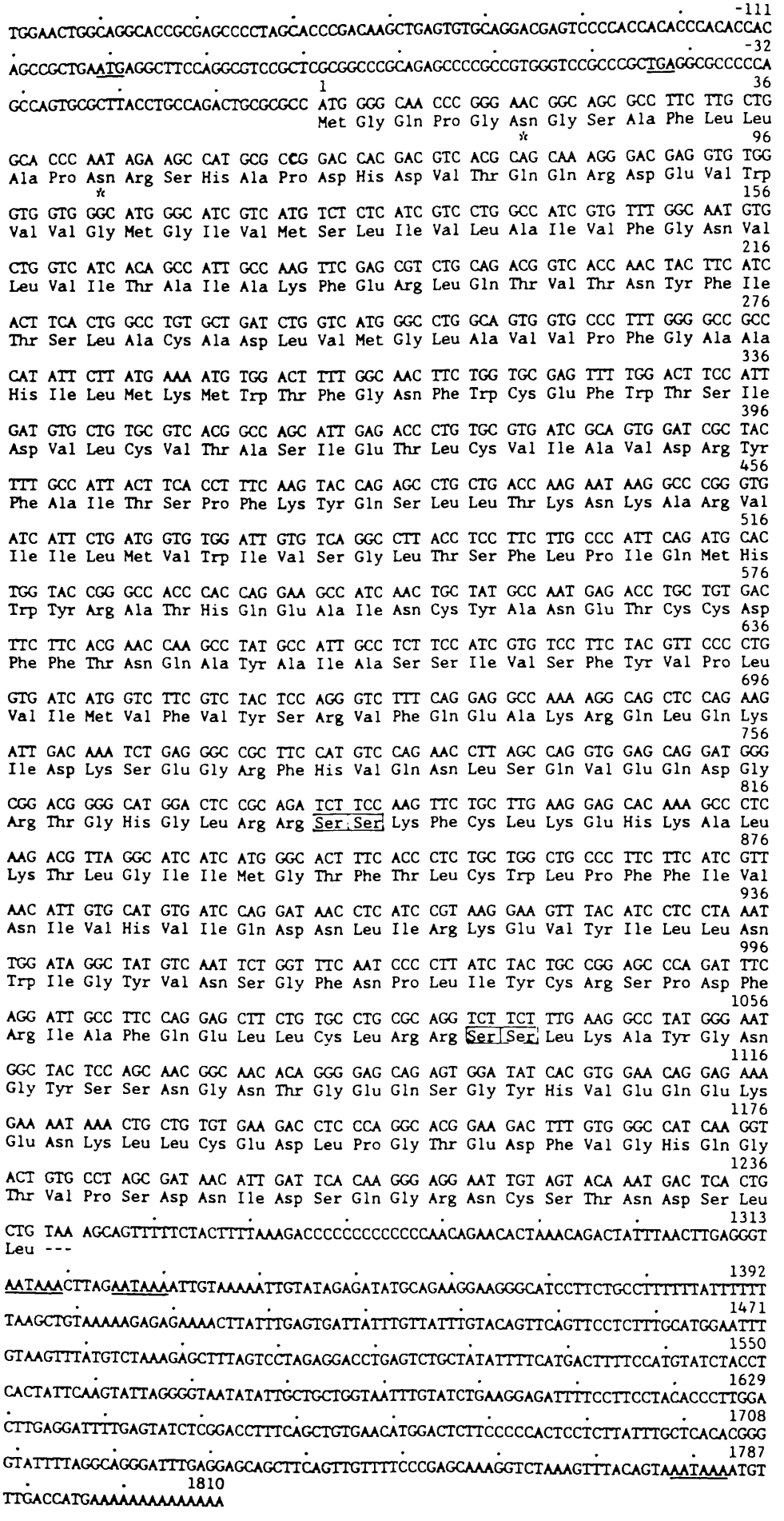

-111
TGGAACTGGCAGGCACCGCGAGCCCCTAGCACCCGACAAGCTGAGTGTGCAGGACGAGTCCCCACCACACCCACACCAC
-32
AGCCGCTGAATGAGGCTTCCAGGCGTCCGCTCGCGGCCCGCAGAGCCCCGCCGTGGGTCCGCCCGCTGAGGCGCCCCCA
1
36
GCCAGTGCGCTTACCTGCCAGACTGCGCGCC ATG GGG CAA CCC GGG AAC GGC AGC GCC TTC TTG CTG
Met Gly Gln Pro Gly Asn Gly Ser Ala Phe Leu Leu
96
GCA CCC AAT AGA AGC CAT GCG CCG GAC CAC GAC GTC ACG CAG CAA AGG GAC GAG GTG TGG
Ala Pro Asn Arg Ser His Ala Pro Asp His Asp Val Thr Gln Gln Arg Asp Glu Val Trp
156
GTG GTG GGC ATG GGC ATC GTC ATG TCT CTC ATC GTC CTG GCC ATC GTG TTT GGC AAT GTG
Val Val Gly Met Gly Ile Val Met Ser Leu Ile Val Leu Ala Ile Val Phe Gly Asn Val
216
CTG GTC ATC ACA GCC ATT GCC AAG TTC GAG CGT CTG CAG ACG GTC ACC AAC TAC TTC ATC
Leu Val Ile Thr Ala Ile Ala Lys Phe Glu Arg Leu Gln Thr Val Thr Asn Tyr Phe Ile
276
ACT TCA CTG GCC TGT GCT GAT CTG GTC ATG GGC CTG GCA GTG GTG CCC TTT GGG GCC GCC
Thr Ser Leu Ala Cys Ala Asp Leu Val Met Gly Leu Ala Val Val Pro Phe Gly Ala Ala
336
CAT ATT CTT ATG AAA ATG TGG ACT TTT GGC AAC TTC TGG TGC GAG TTT TGG ACT TCC ATT
His Ile Leu Met Lys Met Trp Thr Phe Gly Asn Phe Trp Cys Glu Phe Trp Thr Ser Ile
396
GAT GTG CTG TGC GTC ACG GCC AGC ATT GAG ACC CTG TGC GTG ATC GCA GTG GAT CGC TAC
Asp Val Leu Cys Val Thr Ala Ser Ile Glu Thr Leu Cys Val Ile Ala Val Asp Arg Tyr
456
TTT GCC ATT ACT TCA CCT TTC AAG TAC CAG AGC CTG CTG ACC AAG AAT AAG GCC CGG GTG
Phe Ala Ile Thr Ser Pro Phe Lys Tyr Gln Ser Leu Leu Thr Lys Asn Lys Ala Arg Val
516
ATC ATT CTG ATG GTG TGG ATT GTG TCA GGC CTT ACC TCC TTC TTG CCC ATT CAG ATG CAC
Ile Ile Leu Met Val Trp Ile Val Ser Gly Leu Thr Ser Phe Leu Pro Ile Gln Met His
576
TGG TAC CGG GCC ACC CAC CAG GAA GCC ATC AAC TGC TAT GCC AAT GAG ACC TGC TGT GAC
Trp Tyr Arg Ala Thr His Gln Glu Ala Ile Asn Cys Tyr Ala Asn Glu Thr Cys Cys Asp
636
TTC TTC ACG AAC CAA GCC TAT GCC ATT GCC TCT TCC ATC GTG TCC TTC TAC GTT CCC CTG
Phe Phe Thr Asn Gln Ala Tyr Ala Ile Ala Ser Ser Ile Val Ser Phe Tyr Val Pro Leu
696
GTG ATC ATG GTC TTC GTC TAC TCC AGG GTC TTT CAG GAG GCC AAA AGG CAG CTC CAG AAG
Val Ile Met Val Phe Val Tyr Ser Arg Val Phe Gln Glu Ala Lys Arg Gln Leu Gln Lys
756
ATT GAC AAA TCT GAG GGC CGC TTC CAT GTC CAG AAC CTT AGC CAG GTG GAG CAG GAT GGG
Ile Asp Lys Ser Glu Gly Arg Phe His Val Gln Asn Leu Ser Gln Val Glu Gln Asp Gly
816
CGG ACG GGG CAT GGA CTC CGC AGA TCT TCC AAG TTC TGC TTG AAG GAG CAC AAA GCC CTC
Arg Thr Gly His Gly Leu Arg Arg Ser Ser Lys Phe Cys Leu Lys Glu His Lys Ala Leu
876
AAG ACG TTA GGC ATC ATC ATG GGC ACT TTC ACC CTC TGC TGG CTG CCC TTC TTC ATC GTT
Lys Thr Leu Gly Ile Ile Met Gly Thr Phe Thr Leu Cys Trp Leu Pro Phe Phe Ile Val
936
AAC ATT GTG CAT GTG ATC CAG GAT AAC CTC ATC CGT AAG GAA GTT TAC ATC CTC CTA AAT
Asn Ile Val His Val Ile Gln Asp Asn Leu Ile Arg Lys Glu Val Tyr Ile Leu Leu Asn
996
TGG ATA GGC TAT GTC AAT TCT GGT TTC AAT CCC CTT ATC TAC TGC CGG AGC CCA GAT TTC
Trp Ile Gly Tyr Val Asn Ser Gly Phe Asn Pro Leu Ile Tyr Cys Arg Ser Pro Asp Phe
1056
AGG ATT GCC TTC CAG GAG CTT CTG TGC CTG CGC AGG TCT TCT TTG AAG GCC TAT GGG AAT
Arg Ile Ala Phe Gln Glu Leu Leu Cys Leu Arg Arg Ser Ser Leu Lys Ala Tyr Gly Asn
1116
GGC TAC TCC AGC AAC GGC AAC ACA GGG GAG CAG AGT GGA TAT CAC GTG GAA CAG GAG AAA
Gly Tyr Ser Ser Asn Gly Asn Thr Gly Glu Gln Ser Gly Tyr His Val Glu Gln Glu Lys
1176
GAA AAT AAA CTG CTG TGT GAA GAC CTC CCA GGC ACG GAA GAC TTT GTG GGC CAT CAA GGT
Glu Asn Lys Leu Leu Cys Glu Asp Leu Pro Gly Thr Glu Asp Phe Val Gly His Gln Gly
1236
ACT GTG CCT AGC GAT AAC ATT GAT TCA CAA GGG AGG AAT TGT AGT ACA AAT GAC TCA CTG
Thr Val Pro Ser Asp Asn Ile Asp Ser Gln Gly Arg Asn Cys Ser Thr Asn Asp Ser Leu
1313
CTG TAA AGCAGTTTTTCTACTTTTAAAGACCCCCCCCCCCCCCAACAGAACACTAAACAGACTATTTAACTTGAGGGT
Leu ---
1392
AATAAACTTAGAATAAAATTGTAAAAATTGTATAGAGATATGCAGAAGGAAGGGCATCCTTCTGCCTTTTTTATTTTTT
1471
TAAGCTGTAAAAAGAGAGAAAACTTATTTGAGTGATTATTTGTTATTTGTACAGTTCAGTTCCTCTTTGCATGGAATTT
1550
GTAAGTTTATGTCTAAAGAGCTTTAGTCCTAGAGGACCTGAGTCTGCTATATTTTCATGACTTTTCCATGTATCTACCT
1629
CACTATTCAAGTATTAGGGGTAATATATTGCTGCTGGTAATTTGTATCTGAAGGAGATTTTCCTTCCTACACCCTTGGA
1708
CTTGAGGATTTTGAGTATCTCGGACCTTTCAGCTGTGAACATGGACTCTTCCCCCACTCCTCTTATTTGCTCACACGGG
1787
GTATTTTAGGCAGGGATTTGAGGAGCAGCTTCAGTTGTTTTCCCGAGCAAAGGTCTAAAGTTTACAGTAAATAAAATGT
1810
TTGACCATGAAAAAAAAAAAAAA

Figure 4.2 Plasmid map of the expression vector pSVL

Figure 4.2

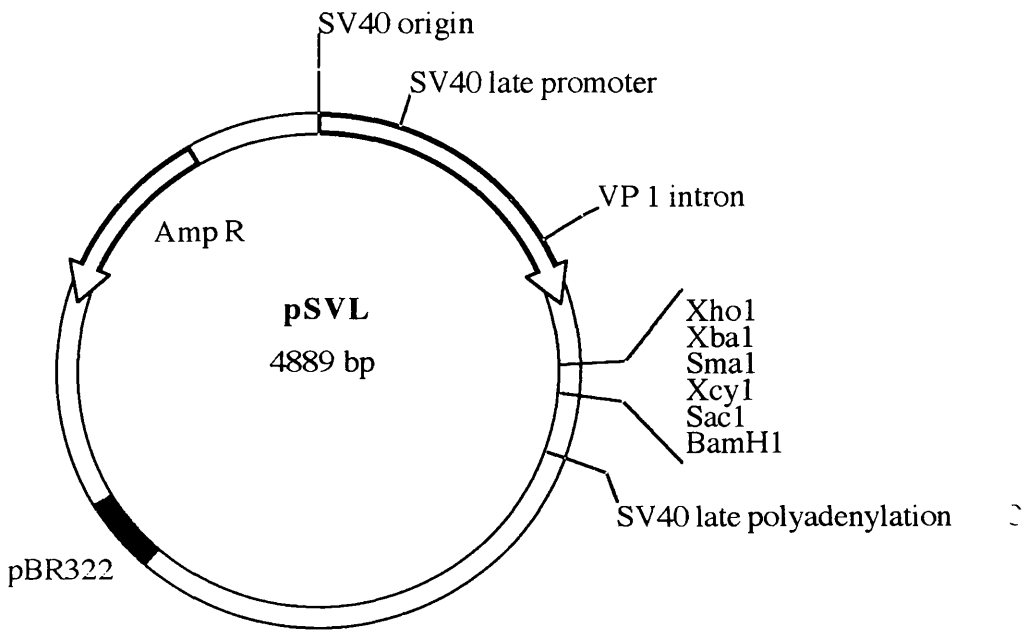

SV40 origin
SV40 late promoter
VP 1 intron
Amp R
pSVL
4889 bp
Xho1
Xba1
Sma1
Xcy1
Sac1
BamH1
SV40 late polyadenylation
pBR322

Figure 4.3 **[^{3}H]-DHA binding analysis in membranes prepared from control COS-1 cells and COS-1 cells which had been transfected with a cDNA for the human β2-adrenergic receptor.**

COS-1 cells were transfected with 10μg plasmid DNA (β2-adrenergic receptor cloned into pSVL) as described in appendix II(ii). Control and transfected cells were harvested, washed twice in PBS (x1) and membranes were prepared as described in section 2.3. The membranes were analysed for specific [^{3}H]-DHA binding. 25μg membranes from both control and transfected COS-1 cells were incubated with 10nM [^{3}H]-DHA for 30 minutes at 30^{o}C. Samples were then placed on ice, filtered through Whatman GF/C filters using a Brandell cell harvester and washed three times with 5ml ice cold buffer as described in section 2.7. Non-specific binding was determined by incubating a parallel set of membranes with 10nM [^{3}H]-DHA and 10μM (-) propranolol. Untransfected COS-1 cell membranes specifically bound 120±12 fmoles/mg membrane protein (1) and transfected COS-1 membranes bound 523±85 fmoles/mg membrane protein (2), a 4.4-fold increase in [^{3}H]-DHA binding.

Figure 4.3

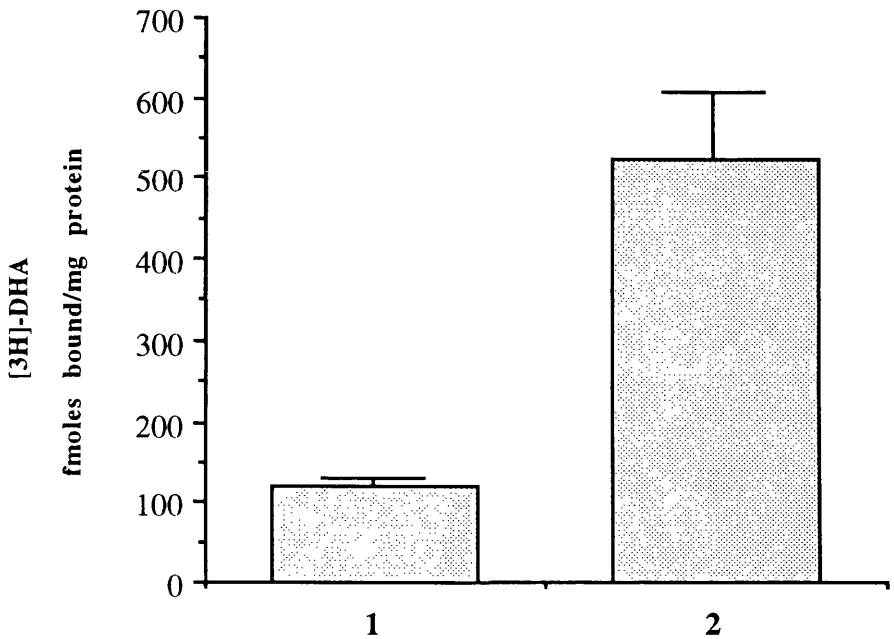


Figure 4.4 **Plasmid map of the expression vector pJM16 or LK444 (Gunning *et al*, 1987).**

Figure 4.4

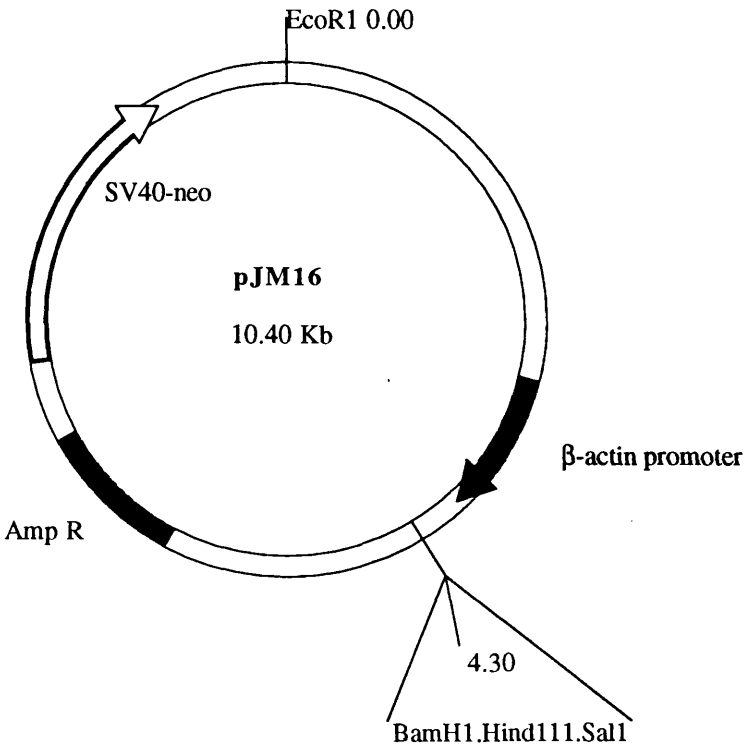

EcoR1 0.00
SV40-neo
pJM16
10.40 Kb
β-actin promoter
Amp R
4.30
BamH1.Hind111.Sal1

Figure 4.5 Increasing the incubation time of membranes, prepared from βN22 and βN17 cells, with [^{3}H]- DHA.

Membranes from βN22 (5μg) and βN17 (25μg) cells were incubated with 0.5 nM [^{3}H]-DHA at 30^{o}C for increasing lengths of time (0 - 45 minutes). Samples were then returned to ice, rapidly filtered through Whatman GF/C filters and washed three times with ice cold buffer as described in section 2.7. A parallel series of incubations were performed using 10μM (-) propranolol to determine non-specific binding. Figure 4.5a represents [^{3}H]-DHA binding in membranes of βN22 cells and figure 4.5b represents binding in βN17 membranes. The results were expressed as the mean ± SD (n=3), of which two other identical experiment in βN22 gave similar results.

Figure 4.5a

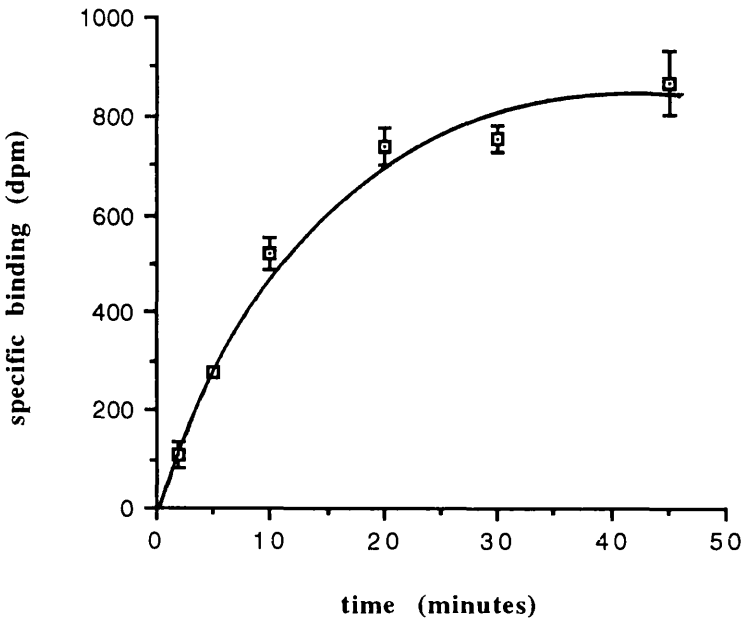


Figure 4.5b

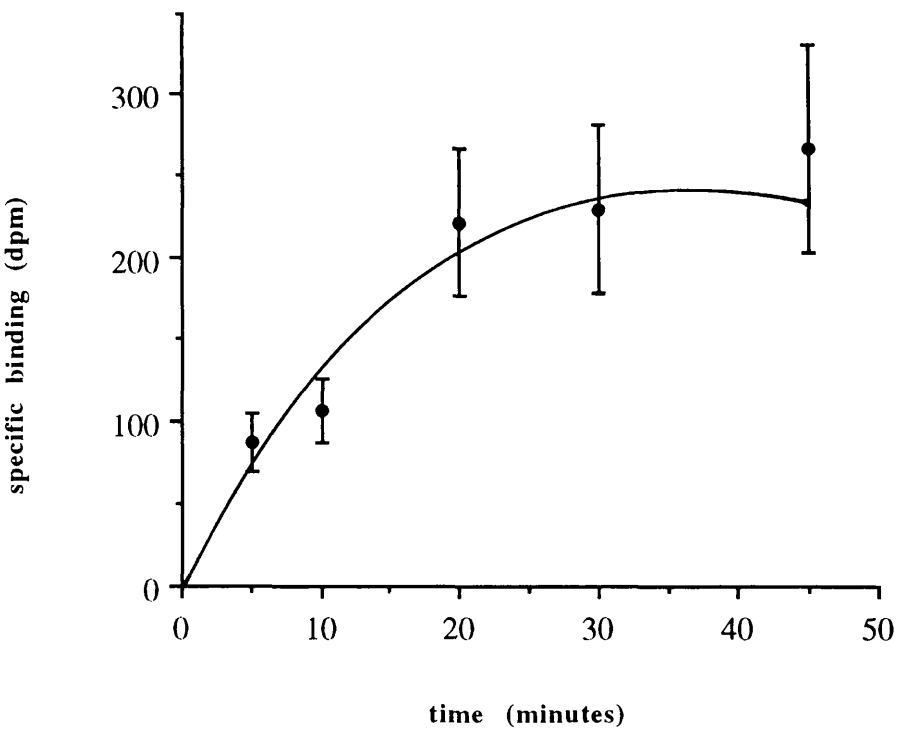


Figure 4.6 [^{3}H]-DHA binding analysis on increasing amounts of βN22 and βN17 cell membranes.

βN22 (0 - 45μg) and βN17 (0 - 50μg) membranes were incubated for 30 minutes at 30°C with [^{3}H]-DHA. Samples were then returned to ice, rapidly filtered through Whatman GF/C filters and washed three times with ice cold buffer by a Brandell cell harvester as described in section 2.7. Non-specific binding was determined for each protein amount using 10μM (-) propranolol. Figure 4.6a represents [^{3}H]-DHA binding in βN22 cell membranes and figure 4.6b represents its binding in βN17. The results were expressed as mean ± SD (n=3), from a single experiment of which one other produced similar results in βN22.

Figure 4.6a

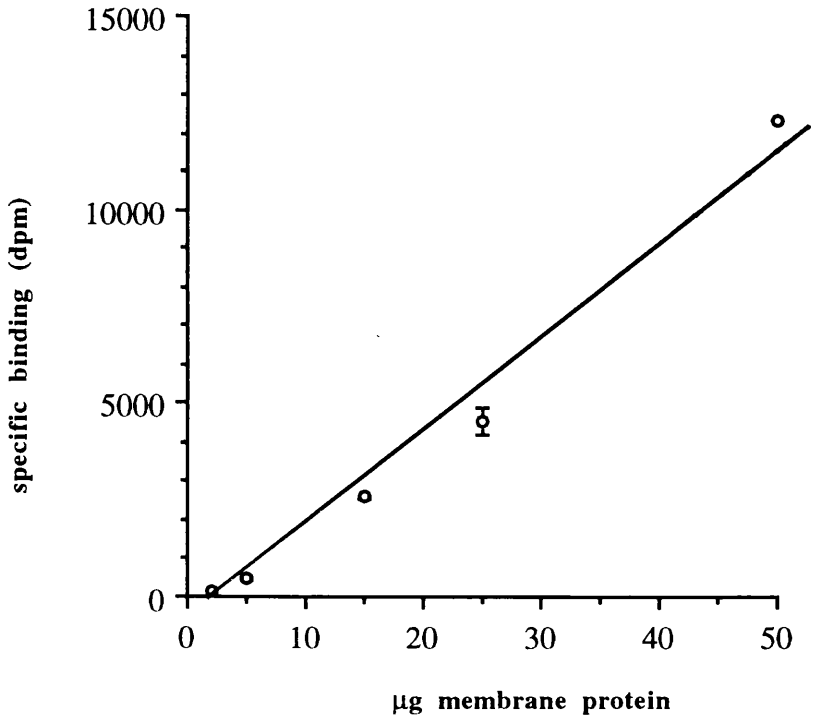


Figure 4.6b

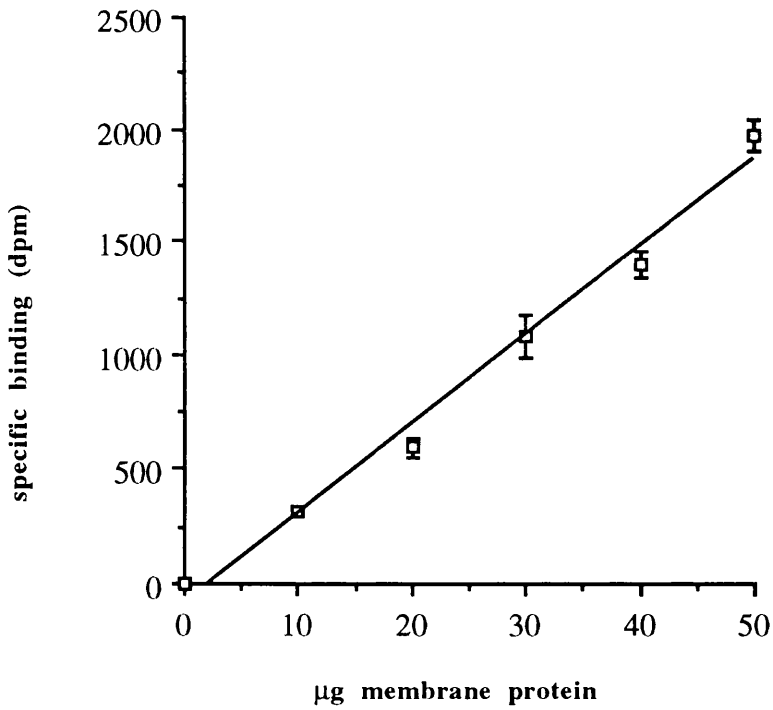


Figure 4.7 [^{3}H]-DHA saturation binding analysis in membranes prepared from clone βN22.

10μg βN22 cell membranes were incubated with various concentrations of [^{3}H]-DHA (0 - 5.4 nM) for 30 minutes at 30°C. Samples were then returned to ice, rapidly filtered through Whatman GF/C filters using a Brandell cell harvester and washed three times with ice cold buffer as described in section 2.7. Non-specific binding was determined at each concentration of [^{3}H]-DHA using 10μM (-) propranolol. A saturation binding curve was constructed (figure 4.7a). Specific fmoles bound/mg membrane protein were plotted (□) and non-specific binding (●) was also plotted expressed in dpm. The specific binding was manipulated according to Scatchard (1949) (figure 4.7b). A Bmax of 4300 fmoles/mg membrane protein with a Kd of 0.37 nM was calculated. Two other experiments were performed which essentially produced similar results.

Figure 4.7a

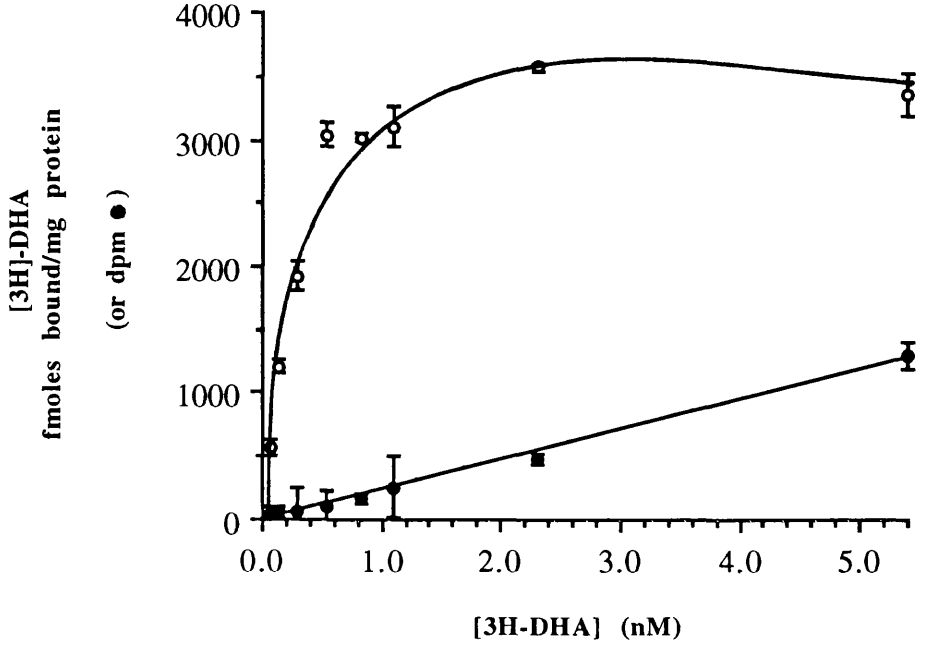


Figure 4.7b

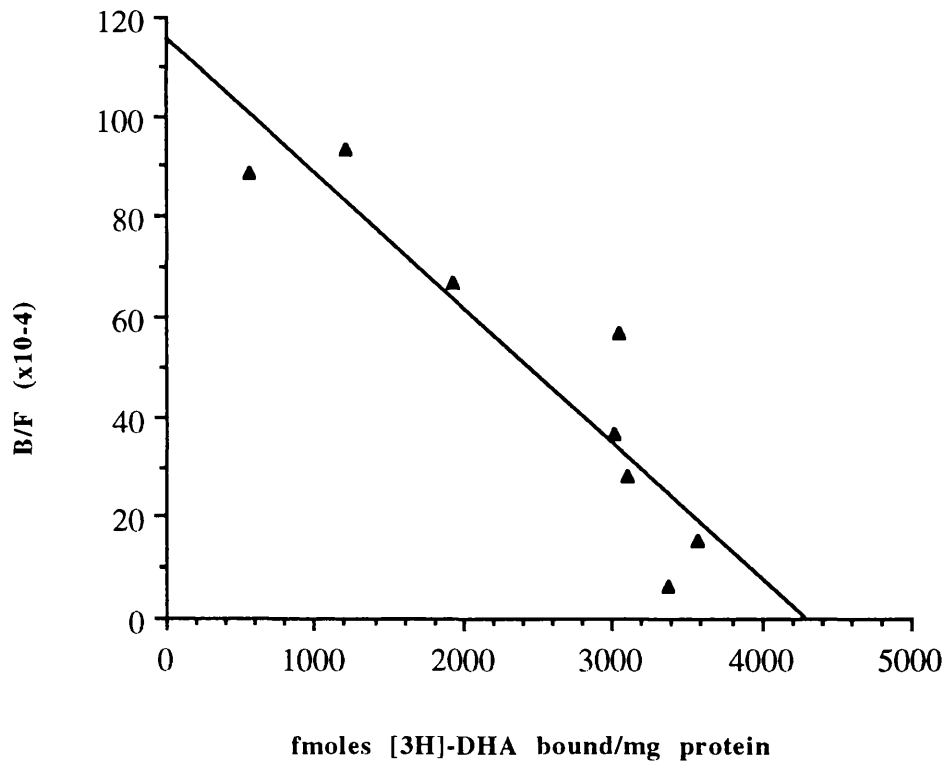


Figure 4.8 [^{3}H]-DHA saturation binding analysis in membranes prepared from clone βN17.

25µg βN17 membranes were incubated with various concentrations of [^{3}H]-DHA (0 - 5.5 nM) for 30 minutes at 30^{o}C. Samples were placed on ice, rapidly filtered through Whatman GF/C filters and washed three times with ice cold buffer using a Brandell cell harvester according to section 2.7. Non-specific binding was determined at each concentration by the binding of [^{3}H]-DHA in the presence of 10µM (-) propranolol. A saturation binding curve was constructed of the specific binding of [^{3}H]-DHA in βN17 cell membranes (figure 4.8a). Non-specific binding increased linearly with [(^{3}H)-DHA] (results not shown). The data was manipulated according to Scatchard (1949) (figure 4.8b). A Bmax of 380 fmoles bound/mg membrane protein and a Kd of 0.26 nM was calculated. This was a representative experiment of three performed.

Figure 4.8a

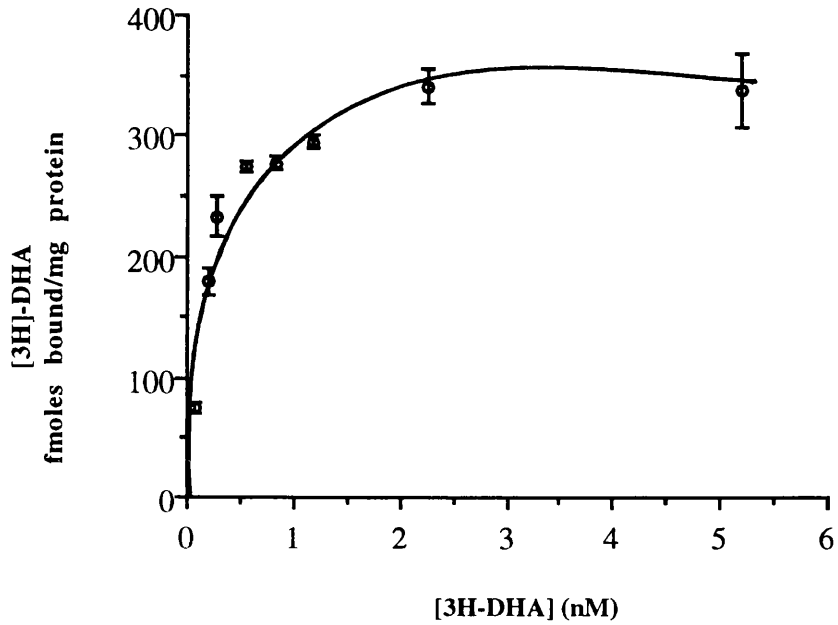


Figure 4.8b

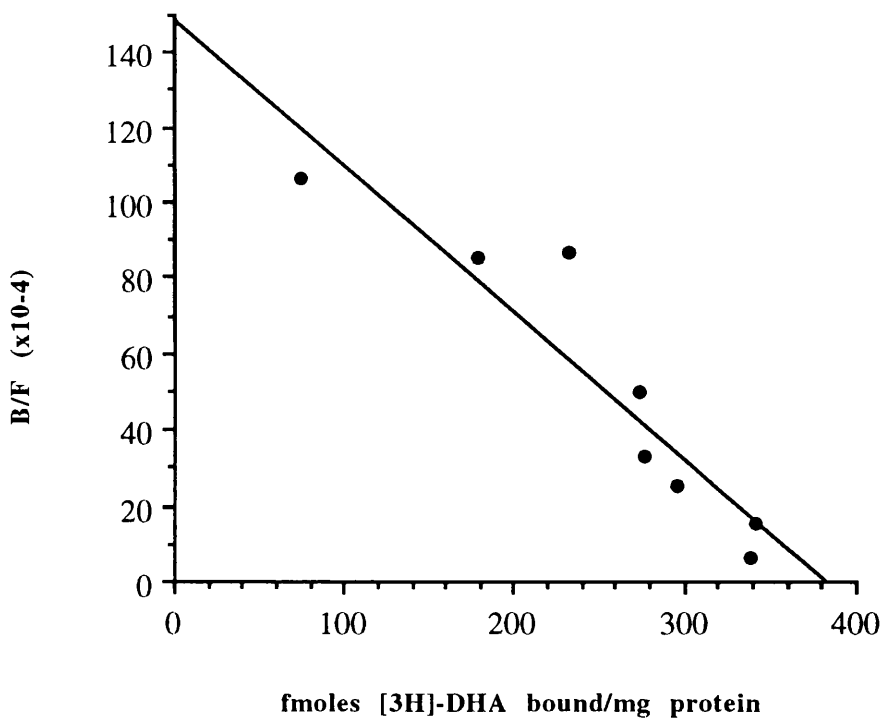


Figure 4.9 Saturation binding of [^{3}H]-PGE1 in membranes from clones βN22 and βN17.

25μg membranes prepared from βN22 and βN17 cells were incubated with 5 or 10nM [^{3}H]-PGE1 and increasing [PGE1] (0-90nM), for 30 minutes at 30^{o}C, as described in appendix 1. Samples were then placed on ice, filtered through Whatman GF/C filters and washed three times with ice cold buffer by a Brandell cell harvester, as described in section 2.7. The data was manipulated according to Scatchard (1949) for βN22 (figure 4.9a) and βN17 (figure 4.9b). The Bmax was 2100 fmoles/mg membrane protein and the Kd was 26.0 nM for βN22 cell membranes. For βN17 the Bmax was 1120 fmoles/mg membrane protein and the Kd was 21.1 nM. Two other identical experiments produced essentially the same results in βN22 membranes and one other experiment was performed in βN17 membranes with a similar result.

Figure 4.9a

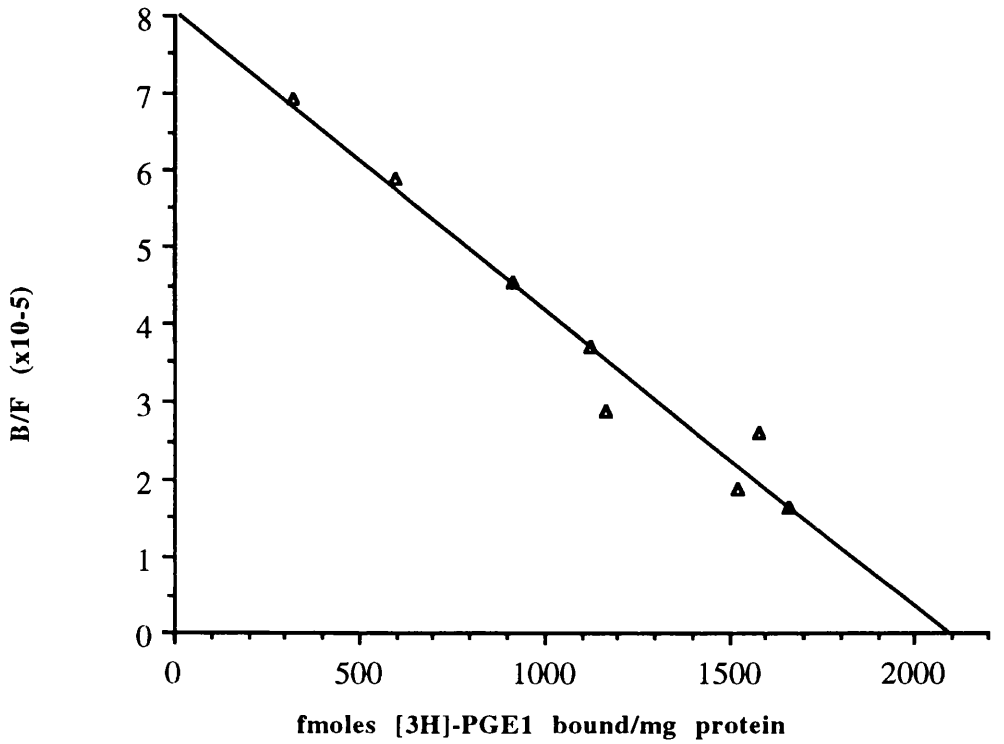


Figure 4.9b

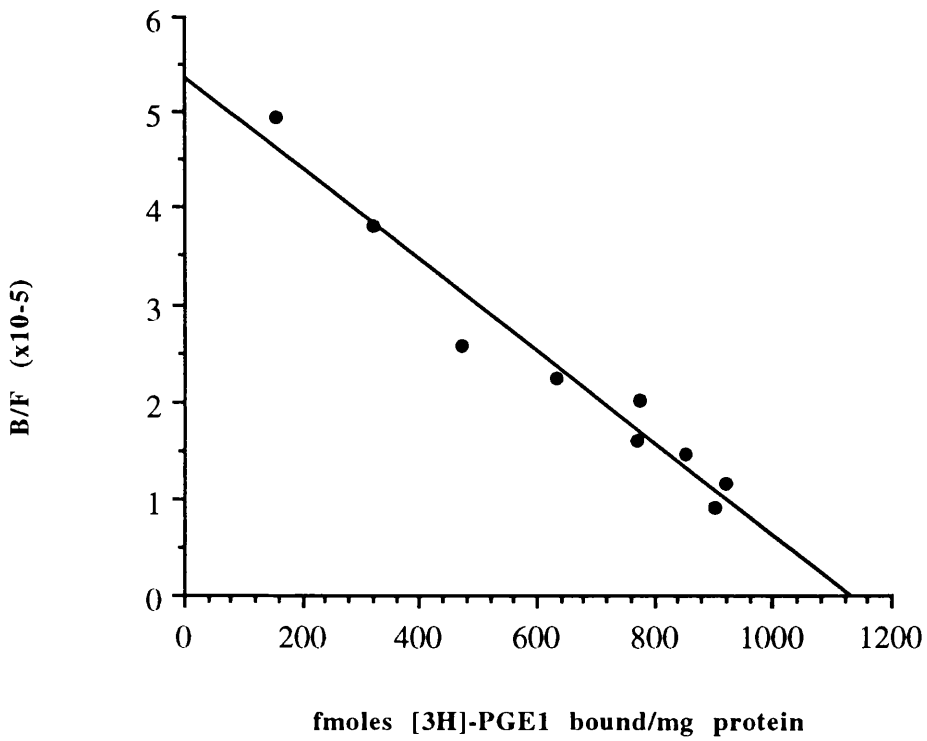


Figure 4.10 Western blotting analysis of Gα in membranes prepared from βN22 and NG108-15 cells.

Membranes prepared from wild-type NG108-15 cells and βN22 cells were TCA precipitated, loaded onto a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for the various Gα, as described in section 2.6. Lane 1 was loaded with NG108-15 membranes and lane 2 with βN22 membranes. Panel A shows 40μg membrane protein western blotted with CS1 (1:250); panel B shows 50μg protein blotted with SG1 (1:400); panel C shows 100μg protein blotted for I3B (1:1000); panel D shows 50μg protein blotted with IM1 (1.250) and panel E shows 50μg protein blotted with CQ2 (1:1000) as described in table 2.1. Colorimetrically developed blots were densitometrically scanned and βN22 levels taken as a percentage of NG108-15 levels as follows: Gsα = 98±6%; Gi2α = 93±7%; Gi3α = 99±6%; Goα = 103±8% and Gq/11α = 100±10% (mean ± SD, n=3). The results were from three immunoblots using individual membrane preparations of cells.

Figure 4.10

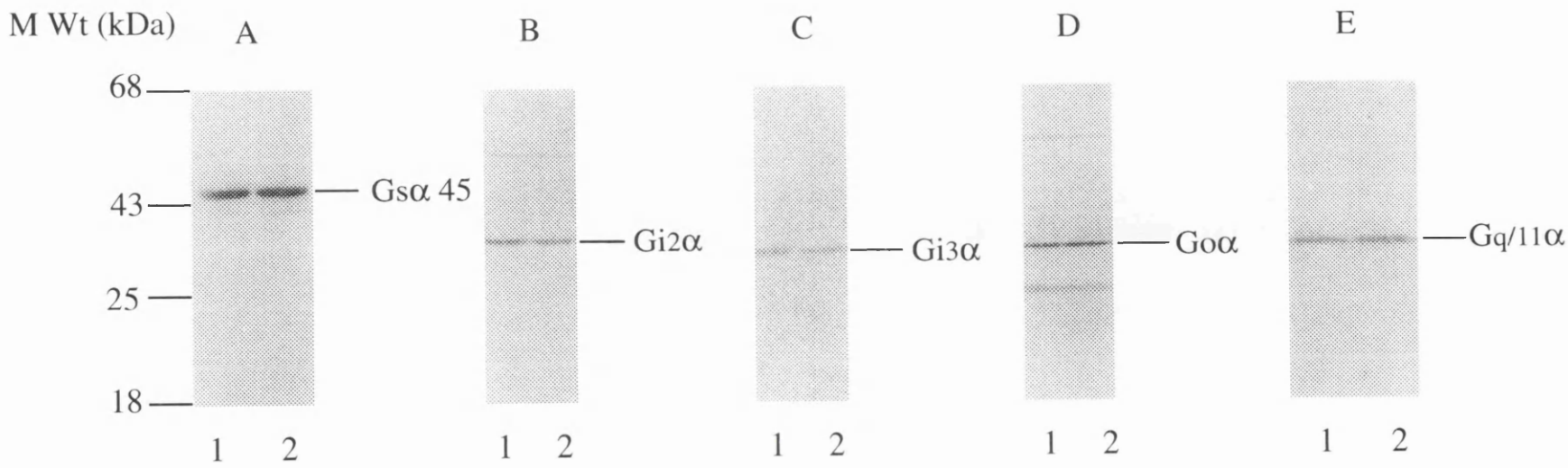

M Wt (kDa)
A
B
C
D
E
68
43
25
18
Gsα 45
Gi2α
Gi3α
Goα
Gq/11α
1 2
1 2
1 2
1 2
1 2

Figure 4.11 Western blotting analysis of Gα in membranes from βN17 and NG108-15 cells.

Membranes prepared from wild-type NG108-15 cells and βN17 cells were TCA precipitated, loaded onto a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for the various Gα, as described in section 2.6. Lane 1 was loaded with NG108-15 membranes and lane 2 with βN17 membranes. Panel A shows 50μg membrane protein western blotted with SG1 (1:400); panel B shows 100μg protein blotted for I3B (1:1000); panel C shows 50μg protein blotted with CQ2 (1:1000); panel D shows 40μg membrane protein western blotted with CS1 (1:250) and panel E shows 50μg protein blotted with IM1 (1.250) and as described in table 2.1. Colorimetrically developed blots were densitometrically scanned and βN17 levels taken as a percentage of NG108-15 levels as follows: Gsα = 100±11%; Gi2α = 94±1%; Gi3α = 100±6%; Goα = 145±6% and Gq/11α = 105±13% (mean ± SD, n=3). The results were from three immunoblots using individual membrane preparations of cells.

Figure 4.11

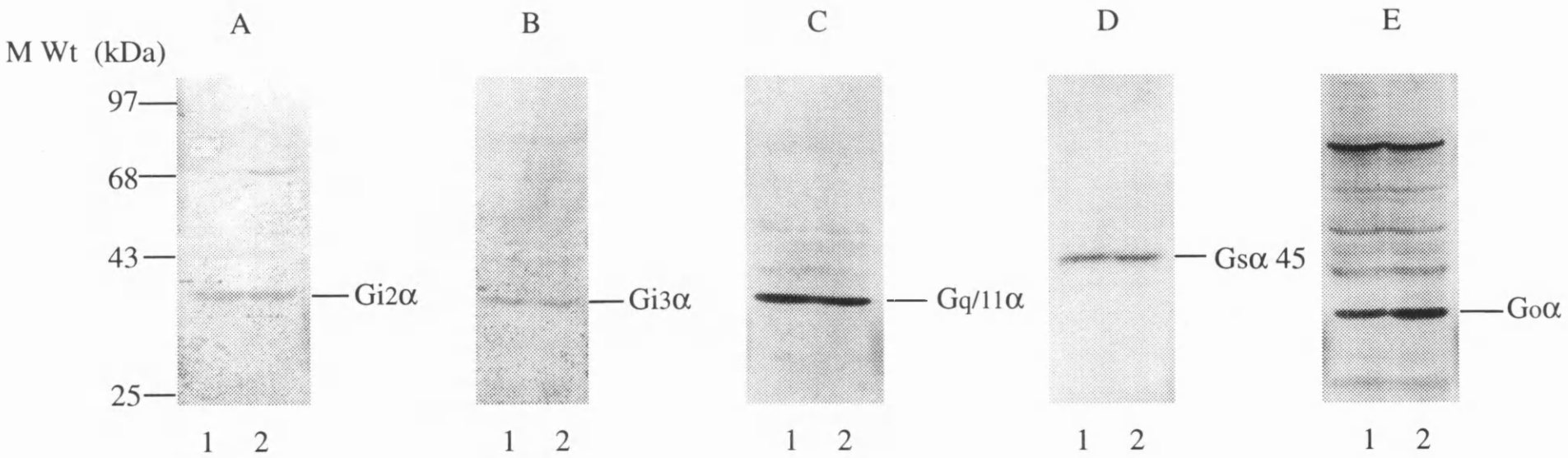

A
B
C
D
E
M Wt (kDa)
97
68
43
25
Gi2α
Gi3α
Gq/11α
Gsα 45
Goα
1 2
1 2
1 2
1 2
1 2

Figure 4.12 **Examination of the cellular morphology of NG108-15 (a) and βN17 (b) cells.**

Figure 4.12a

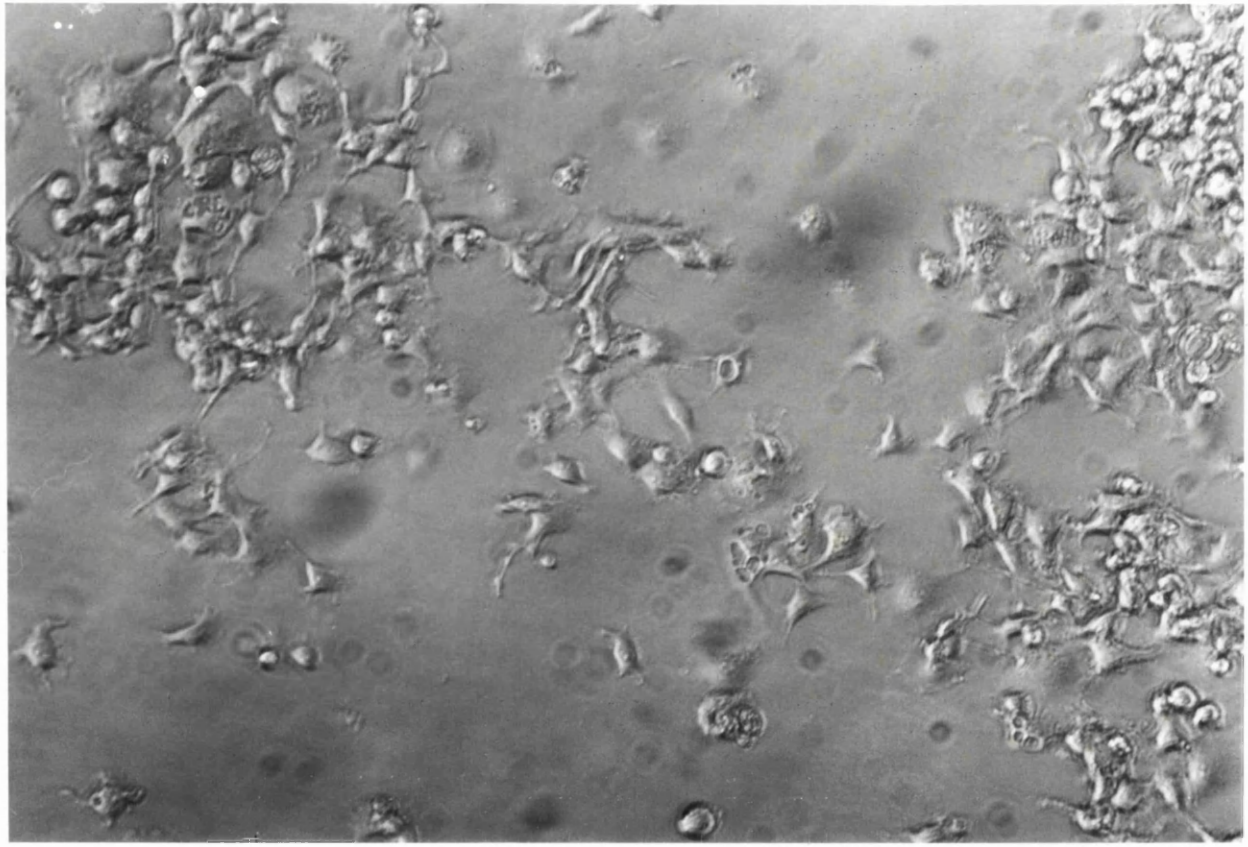


Figure 4.12b

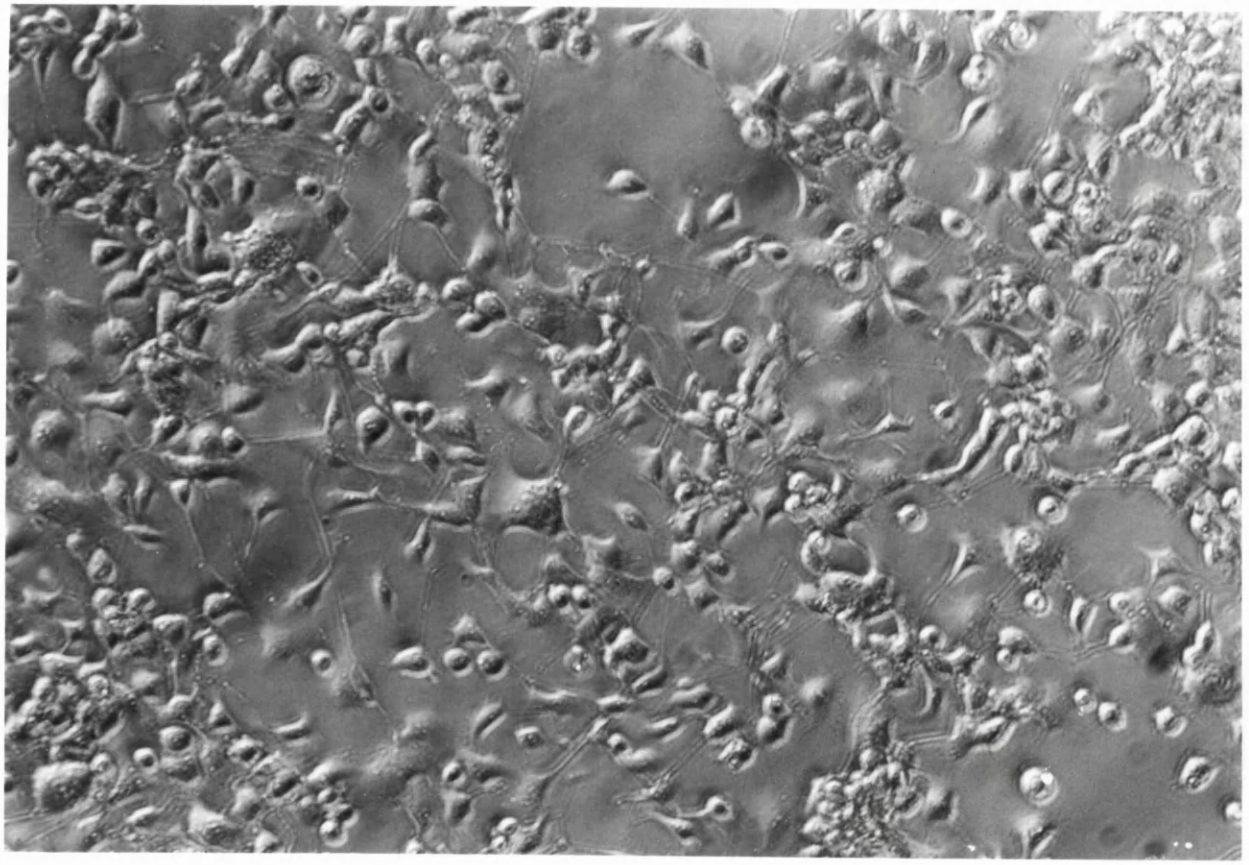


Figure 4.13 Displacement of 0.5nM [^{3}H]-DHA by increasing concentrations of isoprenaline ± 100μM GTPγS, in membranes prepared from βN22 cells.

10μg βN22 membranes were incubated with 0.5nM [^{3}H]-DHA and increasing concentrations of isoprenaline (0 - 100μM), in the presence and absence of 100μM GTPγS, for 30 minutes at 30°C. Samples were returned to ice, rapidly filtered through Whatman GF/C filters using a Brandell cell harvester and washed as described in section 2.7. The IC50 (corrected for receptor occupancy) (-GTPγS) was 86nM (o) and the IC50 (corrected for receptor occupancy) (+ GTPγS) was 207nM (□). In the experiment shown there was an approximate two-fold increase in the IC50 (corrected for receptor occupancy) on incubation with 100μM GTPγS. However, another three experiments were performed and on pooling the data this increase did not achieve statistical significance : IC50 (-GTPγS) = 141±55nM and IC50 (+ GTPγS) = 216±30nM (mean ± SD, n=4), p=0.11.

Figure 4.13

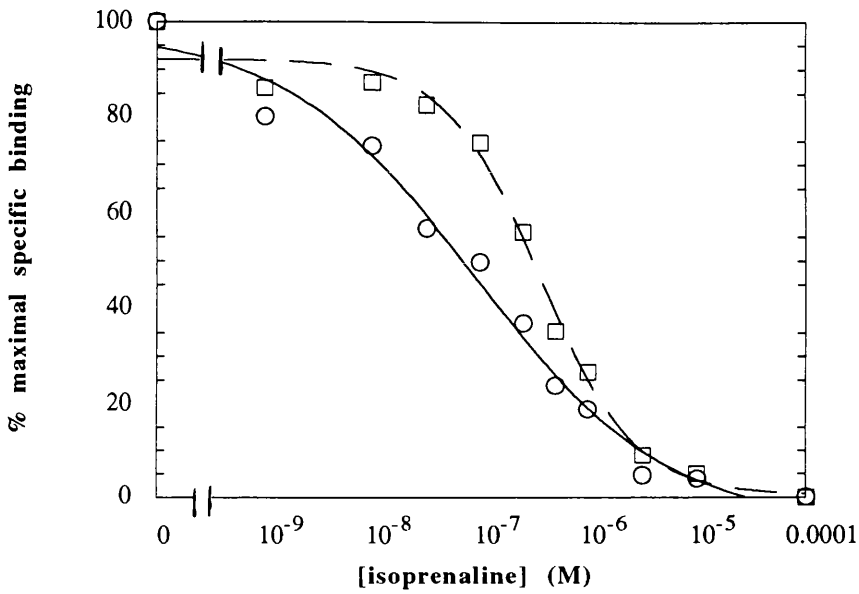

% maximal specific binding
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
0.0001
[isoprenaline] (M)

Figure 4.14 Displacement of 0.5nM [^{3}H]-DHA with increasing concentrations of isoprenaline ± 100μM GTPγS, in membranes prepared from βN17 cells.

25μg βN17 membranes were incubated with 0.5nM [3H]-DHA and increasing concentrations of isoprenaline (0 - 100μM), in the presence and absence of 100μM GTPγS, for 30 minutes at 30°C. Samples were placed on ice and rapidly filtered through Whatman GF/C filters using a Brandell cell harvester and washed three times with ice cold buffer as described in section 2.7. The IC50 (corrected for receptor occupancy) (-GTPγS) was 41nM (o) and the IC50 (corrected for receptor occupancy) (+ GTPγS) was 263nM (□). There was an approximate ten-fold increase in the IC50 (corrected for receptor occupancy) on incubation with 100μM GTPγS. The results were expressed as the mean of triplicate assay points. One other identical experiment produced a similarl result.

Figure 4.14

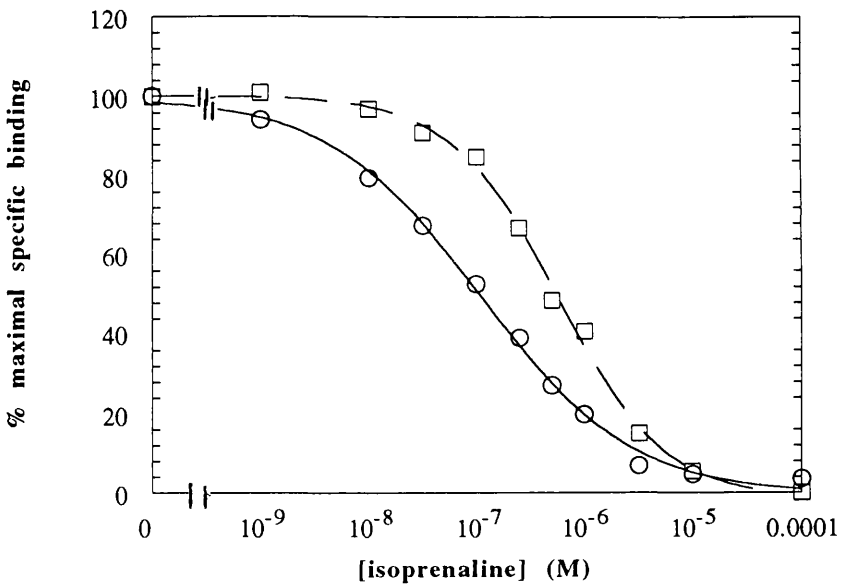

% maximal specific binding
120
100
80
60
40
20
0
0
10-9
10-8
10-7
10-6
10-5
0.0001
[isoprenaline] (M)

Figure 4.15 Analysis of the ability of sodium cholate extracts of membranes, prepared from NG108-15 and βN22 cell, to activate adenylyl cyclase when reconstituted into S49 lymphoma *cyc-* cell membranes.

2μg each of βN22 and NG108-15 cell membranes were solubilised using 1% (w/v) sodium cholate (1 hour, 4°C), extracts were reconstituted into S49 lymphoma *cyc-* cell membranes and adenylyl cyclase activity measured as described in section 2.9. The adenylyl cyclase activity was amplified in the presence of 10mM NaF. NG108-15 cells (1) gave a 20.5±1.4 pmoles cAMP formed/min/mg membrane protein stimulation over basal with NaF, and βN22 cells (2) gave a 23.0±1.8 pmoles cAMP formed/ min/mg membrane protein stimulation. The results were expressed as the mean ± SD (n=3), of a single experiment. Two others produced similar results.

Figure 4.15

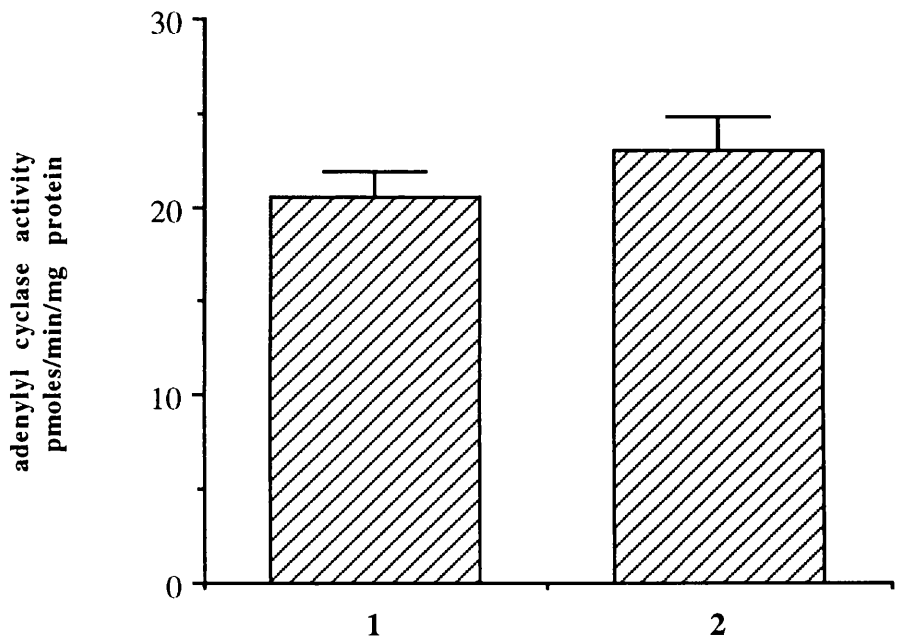

adenylyl cyclase activity
pmoles/min/mg protein
30
20
10
0
1
2

Figure 4.16 Measurement of adenylyl cyclase activity in membranes prepared from βN22 and βN17 cells in response to increasing concentrations of iloprost.

10μg membranes prepared from either βN22 (o) or βN17 (□) cells were analysed for adenylyl cyclase activity as described in section 2.8. Adenylyl cyclase activity was amplified in the presence of increasing concentrations of iloprost (0-10μM). The basal enzyme activity was subtracted from the adenylyl cyclase activity measured at each concentration of iloprost. Each value was then calculated as a percentage of the maximal adenylyl cyclase response. The results were expressed as the mean of tripicate assay points, from a single experiment. Two others produced similar results in both βN22 and βN17 cell membranes.

Figure 4.16

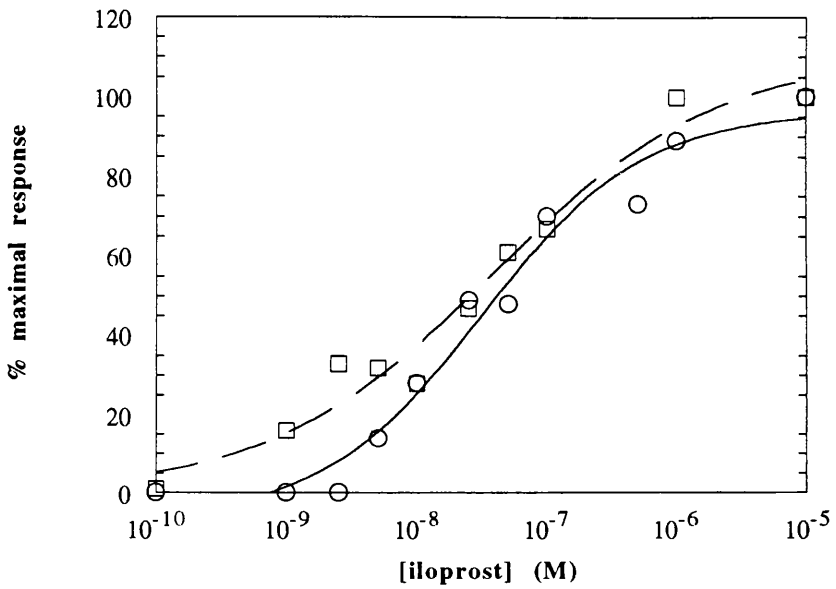

% maximal response
120
100
80
60
40
20
0
10-10
10-9
10-8
10-7
10-6
10-5
[iloprost] (M)

Figure 4.17 Measurement of adenylyl cyclase activity in membranes prepared from βN22 and βN17 cells in response to increasing concentrations of isoprenaline.

10μg membranes prepared from either βN22 (□) or βN17 (o) cells were assayed for adenylyl cyclase activity as described in section 2.8, in the presence of increasing concentrations of isoprenaline (0 - 100μM). The basal enzyme activity was subtracted from the adenylyl cyclase activity measured at each of the concentrations of isoprenaline. Each value was then taken as a percentage of the maximal adenylyl cyclase response and plotted graphically. The results were taken as the mean of triplicate assay points, from a single experiment of which two others (βN17) and four others (βN22) produced similar results.

Figure 4.17

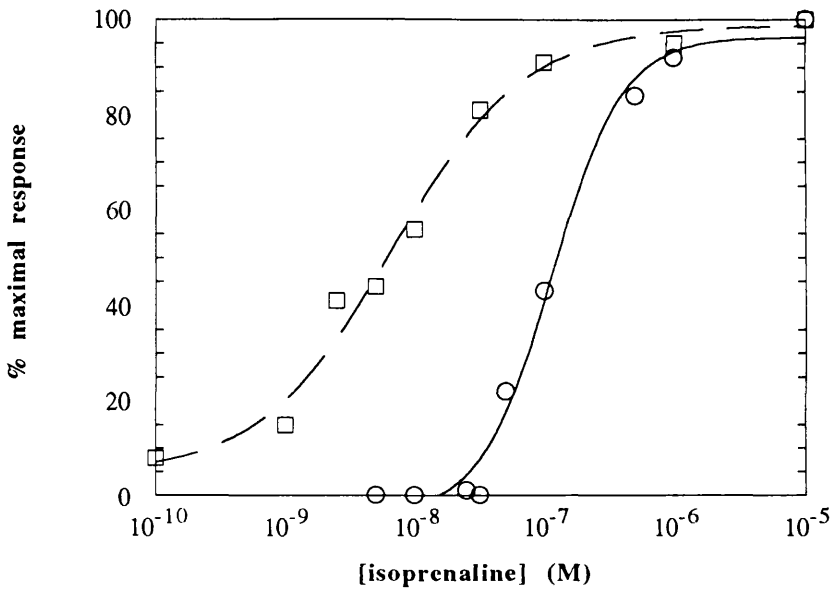

100
80
60
40
20
0
% maximal response
10-10
10-9
10-8
10-7
10-6
10-5
[isoprenaline] (M)

Table 4.1 Measurement of [^{3}H]-DHA binding in membranes prepared from wild-type NG108-15 cells.

25μg membranes prepared from NG108-15 cells were incubated with either 5.4nM or 1.45 nM [^{3}H]-DHA for 30 minutes at 30°C. The reaction was placed on ice, filtered through Whatman GF/C filters using a Brandell cell harvester and washed three times with ice cold buffer as described in section 2.7. Non-specific binding was determined in the presence of 10μM (-) propranolol. The results were expressed as the mean ± SD (n=3), from a single experiment of which three others gave similar results.

Table 4.1

	dpm	
[^{3}H-DHA] (nM)	**non-specific binding**	**total binding**
5.4	3109±71	2969±130
1.45	1018±48	1128±33

Table 4.2 Incubation of membranes prepared from βN22 and βN17 with [^{3}H]-PGE1 in the presence of 100μM GTPγS.

25μg membranes prepared from βN22 and βN17 cells were incubated with 10nM [^{3}H]-PGE1 in the presence or absence of 100μM GTPγS for 30 minutes at 30°C and analysed for [^{3}H]-PGE1 binding as described in section 2.7. Non-specific binding was determined using 10μM PGE1. The results were expressed as the mean ± SD (n=3), from a single experiment. Three others produced similar results.

Table 4.2

Treatment	[^{3}H]-PGE1 (fmoles bound/mg protein)	% control
βN22 - GTPγS	147±15.4	100
βN22 + GTPγS	39.7±12.1	27
βN17 - GTPγS	163±23	100
βN17 + GTPγS	87.7±4.0	54

Table 4.3 Measurement of agonist stimulated adenylyl cyclase activity in βN22, βN17 and NG108-15 cell membranes.

10μg βN22 and NG108-15 cell membranes were analysed for adenylyl cyclase activity as described in section 2.8, in the presence of 10μM isoprenaline and 1μM iloprost (table 4.3a). The results were the mean ± SD (n=3), from a single experiment. Two others produced similar results.

Similarly, in a separate experiment, 10μg βN17 and NG108-15 cell membranes were analysed for adenylyl cyclase activity as described in section 2.8, in the presence of 10μM isoprenaline and 1μM iloprost (table 4.3b). The results were the mean ± SD (n=3) of a single experiment. Two others produced similar results.

Table 4.3

a)

	Adenylyl cyclase activity (pmoles/min/mg membrane protein)	
	NG108-15	**βN22**
basal	25.4±5.0	81.5±7.0
isoprenaline (10μM)	29.0±3.0	193±5.8
iloprost (1μM)	180±4.2	192±5.2

b)

	Adenylyl cyclase activity (pmoles/min/mg membrane protein)	
	NG108-15	**βN17**
basal	25.4±10.9	21.0±5.9
isoprenaline (10μM)	16.8±8.7	138.1±1.0
iloprost (1μM)	248.0±5.8	183.0±13.2

Table 4.4 Measurement of basal adenylyl cyclase activity in the presence of 20mM $MnCl_2$ in membranes prepared from clones βN22 and βN17.

Basal adenylyl cyclase was measured in 10μg membranes prepared from βN22, βN17 and NG108-15 cells as described in section 2.8. Basal adenylyl cyclase activity was also measured in the presence of 20mM $MnCl_2$. When $MnCl_2$ was included in the assay, the $MgCl_2$ routinely added was excluded. The results were the mean ± SD (n=3), from a single experiment. Two others gave similar data.

Table 4.4

	Adenylyl cyclase activity (pmoles/min/mg membrane protein)		
	NG108-15	**βN22**	**βN17**
Basal	31.6±1.3	84.3±1.1	31.0±1.3
+ Mn	11.2±1.2	12.7±1.4	11.0±1.6

Table 4.5 Measurement of basal adenylyl cyclase activity in βN22 and NG108-15 cell membranes on incubation with 10μM propranolol.

10μg membranes prepared from βN22 and NG108-15 cells were analysed for adenylyl cyclase activity as described in section 2.8, in the presence of 10μM propranolol. The results were the mean ± SD (n=3), from a single experiment. Two others produced similar results.

Table 4.5

	Adenylyl cyclase activity (pmoles/min/mg membrane protein)	
	NG108-15	**βN22**
Basal	33.0±2.3	89.1±4.3
Propranolol (10μM)	33.3±2.3	60.4±0.7

Results

Before commencing the longer, more laborious task of producing stable transfects of NG108-15 cells with a cDNA for the β2-adrenergic receptor, it was first necessary to determine that the cDNA available (figure 4.1) would express the β2-adrenergic receptor, by use of a transient transfection system. A cDNA for the human β2-adrenergic receptor was cloned into the plasmid pSVL (figure 4.2) downstream of the late SV40 promoter as described in appendix ll(ii). 10μg plasmid DNA was used to transiently transfect simian COS-1 cells using the Lipofectin reagent as explained in section 2.22. Transfected COS-1 cells were harvested 51 hours after the addition of 20% foetal calf serum and membranes were prepared from the cells as described in section 2.3. The β-adrenergic receptor antagonist [^{3}H]-dihydroalprenolol ([^{3}H]-DHA)(10nM) was used to assess β2-adrenergic receptor binding. Samples were incubated for 30 minutes at 30^{o}C and non-specific binding was determined in the presence of 10μM (-) propranolol. Transiently transfected COS-1 cell membranes demonstrated a 4.4-fold increase in [^{3}H]-DHA binding over control COS-1 cell membranes (figure 4.3), indicating that the cDNA for the human β2-adrenergic receptor encoded for the receptor in COS-1 cells.

NG108-15 cell membranes were assayed for specific [^{3}H]-DHA binding to determine if the cells endogenously expressed any β2-adrenergic receptor. This was found not to be the case (table 4.1). Having established that the cDNA would encode a β2-adrenergic receptor, NG108-15 cells were stably transfected with this cDNA as described in appendix ll(iii). The cDNA for the human β2-adrenergic receptor was cloned into the plasmid pJM16 (figure 4.4) downstream of

the β-actin promoter. NG108-15 cells were stably transfected with 10μg plasmid DNA as explained in section 2.22. Geneticin resistant (0.8mg/ml) cell colonies were picked and expanded. Membranes were prepared from the clones as described in section 2.3 and analysed for the β2-adrenergic receptor using [^{3}H]-DHA binding as described for the transient transfection (figure 4.3).

Two geneticin resistant clones βN22 and βN17 were selected which expressed 4000 and 300 fmoles β2-adrenergic receptor/mg membrane protein respectively. Time courses of 0.5nM [^{3}H]-DHA binding on both clones showed that binding equilibrium was achieved after approximately 20 minutes incubation at 30^{o}C (figure 4.5). Samples were incubated at 30^{o}C for 30 minutes to ensure equilibrium binding. The amount of membrane protein in the binding assay was increased and the corresponding number of specifically bound [^{3}H]-DHA counts were measured in both clones (figure 4.6). A linear relationship between the amount of membrane protein and the number of specifically bound counts was observed in βN22 cell membranes with up to 45μg membrane protein. Subsequently up to 25μg membrane protein was used in βN22 [^{3}H]-DHA binding assays. In βN17 cells the number of specifically bound counts increased linearly with the amount of membrane protein up to 50μg. Routinely up to 40μg membrane protein was used in βN17 [^{3}H]-DHA binding assays.

Saturation binding using [^{3}H]-DHA was performed on both of the positive clones selected. Scatchard analysis of the data revealed that βN22 expressed 4317±375 fmoles receptor/mg membrane protein (mean ± SD, n=3) and this bound with high affinity (Kd = 0.32±0.20nM) (figure 4.7). Similarly, βN17 expressed 292±76 fmoles receptor/mg

membrane protein (mean ± SD, n=3) which also bound with high affinity (Kd = 0.52±0.20 nM) (figure 4.8). Non-specific binding increased linearly with [^{3}H-DHA] in both cell lines.

The level of IP prostanoid receptor expression was measured in both clonal cell lines by saturation binding of [^{3}H]-PGE1 as described in appendix 1. βN22 expressed 1210±731 fmoles receptor/mg membrane protein (mean ± SD, n=3) with a Kd for [^{3}H]-PGE1 of 21.7±7.9 nM (mean ± SD, n=3) (figure 4.9a), and βN17 expressed 1048±103 fmoles/mg membrane protein with a Kd for [^{3}H]-PGE1 of 18.7±3.5 nM (mean ± SD, n=2) (figure 4.9b). In both clones, βN22 (p=0.86) and βN17 (p=0.82) the levels of IP prostanoid receptor were not significantly different to that measured by saturation binding in the wild-type NG108-15 cell line (1208±397 fmoles bound/mg protein). The pseudo Hill coefficients were calculated for [^{3}H]-PGE1 binding in βN22 (1.03±0.1) and in βN17 (1.03±0.07). There was at least a 50% decrease in specific [^{3}H]-PGE1 binding, in membranes prepared from both clones, when measured in the presence of 100μM GTPγS (table 4.2).

Equivalent amounts of NG108-15 and βN22 cell membranes were western blotted for various Gα subunits using antisera raised against the C-terminal decapeptide of Gα as described in table 2.1. Expressed as a percentage of Gα levels in NG108-15 membranes there were no changes in membrane associated Gsα (98±6%); Gi2α (93±7%); Gi3α (99±6%); Goα (103±8%) and Gq/11α (100±10) in βN22 membranes (mean ± SD, n=3) (figure 4.10). There were also no changes in levels of Gsα (100±11), Gi2α (94±1%), Gi3α (100±6%) and Gq/11α (105±13%) in βN17 cell membranes, expressed as a percentage of NG108-15 Gα levels (mean ± SD, n=3)(figure 4.11). Surprisingly, Goα levels were

substantially higher in βN17 cells (145±6%, mean ± SD, n=3, $p<0.005$). Wild-type NG108-15 cells exhibit a 250-300% increase in membrane associated Goα on cellular differentiation, which can be induced by treatment of the cells with various cAMP-elevating agents (Mullaney and Milligan, 1989). Differentiation of NG108-15 cells results in cells with very long neuronal-like processes which adhere less well to the flask surface (Hamprecht *et al*, 1985). Examination of βN17 cells revealed no detectable difference in the cellular morphology when compared to wild-type NG108-15 cells, indicating that the βN17 cells were not differentiated (figure 4.12).

Membranes prepared from βN22 cells were incubated with 0.5nM [^{3}H]-DHA and the radioligand was displaced with increasing concentrations of isoprenaline (0-100μM). The displacement was additionally performed in the presence of 100μM GTPγS. The IC50 (corrected for receptor occupancy) was 141±55 nM (mean ± SD, n=4) in βN22 membranes, and on incubation with GTPγS the IC50 (corrected for receptor occupancy) was 216±30 nM (mean ± SD, n=4). There was no significant difference in the IC50 (corrected for receptor occupancy) on incubation of βN22 cell membranes with GTPγS, p=0.11 (figure 4.13). The pseudo Hill coefficient of βN22 in the absence of GTPγS was 0.72±0.05 and in the presence of 100μM GTPγS was 0.78±0.11.

Isoprenaline (0-100μM) displacement of 0.5nM [^{3}H]-DHA in membranes from βN17 cell membranes was also examined. βN17 membranes gave an IC50 (corrected for receptor occupancy) of 41 ± 11 nM (mean ± SD, n=3). On incubation with GTPγS the IC50 (corrected for receptor occupancy) was 280 ± 25 nM (mean ± SD, n=2). In the presence of GTPγS there was an approximate 7-fold increase in the

IC50 (corrected for receptor occupancy) in βN17 cell membranes. This was statistically significant p=0.007 (figure 4.14). The pseudo Hill coefficient for isoprenaline displacement of [^{3}H]-DHA in βN17 in the absence of GTPγS was 0.54 ± 0.1 while in the presence of GTPγS it increased to 1.02 ± 0.13.

Both iloprost and isoprenaline gave a robust stimulation of adenylyl cyclase activity in membranes prepared from βN22 and βN17 cells (table 4.3). However, basal adenylyl cyclase activity in βN22 membranes was 2-3 fold higher than that observed in wild-type NG108-15 and βN17 membranes (table 4.3). Wild-type NG108-15 cell membranes and membranes prepared from both clones showed no significant difference in basal adenylyl cyclase levels when assayed in the presence of 20mM $MnCl_2$ (table 4.4), indicating the expression of similar amounts of adenylyl cyclase in all three cell lines.

To determine if some of the membrane associated Gsα had been constitutively activated during the transfection process, membranes from both βN22 and NG108-15 cells were solubilised with 1% (w/v) sodium cholate, reconstituted into S49 lymphoma *cyc-* membranes and adenylyl cyclase activity measured as described in section 2.9. Extracts from both NG108-15 and βN22 gave identical basal adenylyl cyclase activities in reconstituted *cyc-* membranes and 10mM NaF was necessary for adenylyl cyclase stimulation over basal levels in both cell lines (figure 4.15).

Basal adenylyl cyclase activity was measured in both βN22 and NG108-15 membranes in the presence of 10μM (-) propranolol (a β-adrenergic antagonist) (table 4.5). There was a 28.0±3.6% (mean ±

SD, n=3) decrease in βN22 basal adenylyl cyclase activity on incubation with propranolol, with no reduction in basal activity in NG108-15 membranes.

Iloprost stimulation of adenylyl cyclase activity in membranes from both βN22 and βN17 cells gave EC50 values which did not significantly differ from that of wild-type NG108-15 cells (figure 4.16). The EC50 for iloprost stimulation of adenylyl cyclase in βN22 was 21.8±16.4 nM (mean ± SD, n=3, p=0.813), in βN17 it was 29.7±5.3 nM (mean ± SD, n=3, p=0.681) and in NG108-15 cells it was 25.8±9.1 nM (mean ± SD, n=3).

Incubation of wild-type NG108-15 cell membranes with 10μM isoprenaline gave no significant stimulation of adenylyl cyclase activity over basal levels (table 4.3). Membranes prepared from both clones βN22 and βN17 were incubated with increasing concentrations of isoprenaline and adenylyl cyclase activity measured (figure 4.17). EC50 values for isoprenaline stimulated adenylyl cyclase activity were calculated for both clones. The EC50 was 57.7±28.9 nM (mean ± SD, n=3) for adenylyl cyclase activation by isoprenaline in βN17 membranes and the EC50 was 8.1±4.7 nM (mean ± SD, n=5) for βN22 membranes. There was an approximate 7-fold decrease in the EC50 value for adenylyl cyclase activation by isoprenaline in βN22 membranes when compared to βN17, p=0.007 (figure 4.17).

Discussion

Prolonged treatment of NG108-15 cells with a prostanoid agonist results in a sustained down-regulation of cellular levels of Gsα (Kelly *et al*, 1990; McKenzie and Milligan, 1990; figure 3.6) whereas agonist treatment of other less highly expressed Gsα coupled receptors in the same cell do not affect membrane associated Gsα levels (McKenzie *et al*, 1990). To examine the effect of an agonist on a Gsα-linked receptor, distinct to the IP prostanoid receptor, in NG108-15 cells, but expressed at similar levels, the cells were transfected with a cDNA for the human β2-adrenergic receptor. The transfection process produced two cell lines which were examined in detail, namely βN22 and βN17, which expressed some 4000 (figure 4.7) and 300 (figure 4.8) fmoles receptor/mg membrane protein respectively. This was assessed by the specific binding of the β-adrenergic antagonist [^{3}H]-DHA ([^{3}H]-dihydroalprenolol).

Expression of foreign DNA in cells may perturb the metabolism and gene expression of proteins ordinarily expressed in the cell, because the DNA can insert into the host chromosome at a non-specified site. Indeed the high basal adenylyl cyclase activity in βN22 cells (table 4.3) which serves to elevate cAMP within the cell, may itself result in the altered gene and/or protein expression of one or more of the components of the adenylyl cyclase cascade for several reasons more fully detailed in chapter 1. cAMP can induce the destabilisation of mRNA if the correct consensus sequences are present (Hadcock *et al*, 1989); it can also enhance or inhibit gene transcription if the gene contains a cAMP response element and cAMP can also activate PKA which phosphorylates numerous cellular proteins with a variety of resultant

effects (Collins *et al*, 1992). However, the high basal production of cAMP in βN22 cells did not affect the polypeptide levels of G-protein α-subunits (figure 4.10) in this cell line. The levels of IP prostanoid receptor expression in βN22 cells (figure 4.9a) were not significantly different to those in wild-type NG108-15 cells despite the down-regulation of this receptor in NG108-15 cells being partially dependent on cAMP (Wiltshire *et al*, 1990).

βN17 cells showed no increased basal adenylyl cyclase activity when compared to wild-type NG108-15 cells (table 4.3) and there was no change in the levels of expression of the IP prostanoid receptor (figure 4.9b) or G-protein α-subunits except Goα (figure 4.11). There was an unexpected 45% increase in Goα expression in βN17 cells. Wild-type NG108-15 cells will differentiate when treated with agents which elevate intracellular cAMP and there is a resultant 250-300% increase in membrane associated Goα. The morphology of the NG108-15 cells also changes, exhibiting slow growth and extending long neuronal-like processes (Hamprecht *et al*, 1985; Mullaney and Milligan, 1989). Examination of the morphology of βN17 (figure 4.12), revealed no change from wild-type NG108-15 cells indicating no cellular differentiation. It is uncertain of the reason for this increase in membrane associated Goα. βN17 cells do not have a higher basal adenylyl cyclase activity than wild-type NG108-15 cells, so it is unlikely to be a cAMP dependent process. It may simply be that the transfection process has disrupted the genome in some manner affecting the expression of Goα or even perhaps decreasing its rate of turnover.

Receptor theory predicts that after the addition of GTPγS, a condition that should uncouple all the receptor from Gsα giving a pseudo

Hill coefficient of 1.0, agonist binding will decrease as the affinity of the receptor for agonist and not antagonist decreases. This should produce a rightward shift in the agonist displacement curve (Ross *et al*, 1977). This was not the case in βN22 (figure 4.14), although in some experiments there was a small (2-fold) effect this did not achieve statistical significance when the data from several experiments was pooled. Because the pseudo Hill coefficient did not increase to 1.0 in the presence of excess guanine nucleotides, this indicates two affinity states of the receptor (G-protein-coupled and uncoupled). The lack of "shift" is therefore not simply a result of contaminating guanine nucleotides in the membrane preparation which would uncouple the β2-adrenergic receptor from Gs before it was assayed in the binding experiment. Indeed, at least a 50% decrease in [^{3}H]-PGE1 binding was observed in βN22 cell membranes on incubation with 100μM GTPγS (table 4.2), essentially identical to that observed in NG108-15 cells. It appears that even in the presence of an excess of a guanine nucleotide there might still exist a small pool of G-protein coupled β2-adrenergic receptor. The observation of a guanine nucleotide resistant population of receptors has been made in several systems, including the pituitary D2 dopamine receptor (Wregget and DeLean, 1984), the brain dopamine receptor (Wregget and Seeman, 1984) and the cardiac muscarinic cholinergic receptor (Burgisser *et al*, 1982). Grigoriadis and Seeman (1983) postulated that complete conversion with guanine nucleotide in these systems would only be observed in the presence of millimolar concentrations of sodium ions, but this was not the case for the pituitary D2 dopamine receptor (Wregget and DeLean, 1984). Sodium ions at this concentration have been shown to uncouple a variety of receptors from their G-protein (Gierschik *et al*, 1989), and it may be useful to perform the GTPγS 'shift' in βN22 cell membranes in the presence of

millimolar concentrations of sodium ions. It was, however, evident that at least some of the β2-adrenergic receptor in βN22 cells was capable of coupling to Gsα because of the robust receptor mediated adenylyl cyclase stimulation (table 4.3).

There was a 7-fold increase in the IC50 (corrected for receptor occupancy) of the agonist displacement curve in the presence of excess guanine nucleotide in βN17 cell membranes (figure 4.14) indicating that at least some of the 300 fmoles β2- adrenergic receptor coupled to Gsα. Unlike the β2-adrenergic receptor in βN22 cells, the pseudo Hill coefficient increased to 1.0 in the presence of GTPγS demonstrating that all the receptor had been uncoupled from Gsα in βN17 cell membranes.

Isoprenaline was able to stimulate adenylyl cyclase activity in membranes isolated from both clone βN22 and βN17 but not wild-type NG108-15 cells (table 4.3). The measured EC50 for isoprenaline stimulated adenylyl cyclase activity in βN22 cells (8nM) was some 7-fold to the left of the dose response curve in membranes from βN17 cells (figure 4.17), whereas dose responses measuring iloprost stimulated adenylyl cyclase activity were identical in both clones (figure 4.16). IP prostanoid receptor levels expressed in βN17 and βN22 cells were similar (figure 4.9) whereas the level of expression of the β2-adrenergic receptor in the two clones differs by some 13-fold (figure 4.7, 4.8). In chapter 3, a stoichiometry of down-regulation of Gsα and the IP prostanoid receptor in NG108-15 cells was calculated of some 8 pmoles Gsα:1 pmole receptor at all levels of receptor occupancy. If this represents the ratio of activation of G-protein by receptor, then as the IP prostanoid receptor is present at approximately 1 pmole/mg membrane protein and Gsα at some 10 pmoles/mg membrane protein there is likely

to be a slight molar excess of Gsα even when the entire pool of Gsα is activated by high concentrations of prostanoid agonist. If the β2-adrenergic receptor were to show similar G-protein interaction stoichiometry it might be anticipated that the entire pool of available Gs would be activated by agonist occupation of only a fraction of the available β2-adrenergic receptor in βN22 and as such the dose response curve for G-protein and hence adenylyl cyclase activation might be anticipated to be to the left of the receptor occupancy curve, as indeed is the case. There appears, therefore, to be "spare" β2-adrenergic receptors in clone βN22, whereby a fractional occcupancy can produce a maximal response, unlike βN17 where maximal occupancy is necessary to elicit a maximal response.

Basal adenylyl cyclase levels in βN22 membranes were approximately 2-fold higher than in both NG108-15 and βN17 cell membranes (table 4.3). This was not due to either increased levels of expression (figure 4.10) or an increased intrinsic activity of Gsα (figure 4.15) in βN22 cells, produced by some artefact of the transfection process.

The basal level of an effector enzyme represents its activity in the absence of agonist activation of the receptor. This is not necessarily the same as being independent of receptor regulation. "Empty" receptor regulation of G-proteins has been recorded for the δ-opioid receptor in NG108-15 cells (Costa and Herz, 1989; Costa *et al*, 1990). Evidence in favour of such a model comes from the ability of agents with "negative intrinsic" activity to reduce basal high affinity GTPase activity (Costa and Herz, 1989; Costa *et al*, 1990). The observation that antagonists with "negative intrinsic" activity can decrease the basal GTPase activity

tonically activated by "empty" receptor indicates that the "empty" receptor can stimulate the G-protein, although it is not possible to determine whether this apparent activation results from a truly spontaneous interaction between "empty" receptors and G-proteins or from some other mechanism. Certainly it was not possible to ascertain whether a β-receptor antagonist would be able to reduce either basal or agonist stimulation of GTPase activity in membranes of βN22 cells because of the well-established technical difficulties in recording receptor-mediated stimulation of Gs GTPase activity within the background of the basal activity of other high affinity GTPases (Milligan, 1988). However, addition of the β-adrenergic receptor antagonist propranolol resulted in a substantial reduction in basal adenylyl cyclase in membranes of βN22 cells without altering the basal adenylyl cyclase in membranes from wild-type NG108-15 cells (table 4.5). Addition of an agonist stabilises or promotes the probability of a receptor adopting an active conformation which can then stimulate a signalling cascade. An antagonist by contrast must limit or deny the receptor such a conformation. As such, in cells expressing high levels of a receptor it may be that the number of receptors anticipated to be in the 'agonist' conformation at any instant may be sufficient to significantly stimulate the signal pathway.

Clearly a simple explanation for the elevated basal adenylyl cyclase activity in βN22 membranes might relate to an enhanced expression of the adenylyl cyclase catalytic moiety in the cells, associated either with the transfection process or simply within the clone isolated. The basal adenylyl cyclase activities in membranes of wild-type NG108-15 cells and from both clone βN22 and βN17 were not different when the assay was performed in the presence of Mn^{2+} rather than in the presence

of Mg^{2+} (table 4.4). This condition is believed to be the best indication of the basal activity of the catalytic moiety of adenylyl cyclase without regulation of this activity by guanine nucleotide binding proteins (Limbird *et al*, 1979). Such results further indicate that the enhanced basal activity of adenylyl cyclase in βN22 membranes results from tonic G-protein stimulation of the effector rather than elevated levels of the adenylyl cyclase catalytic moiety resulting artefactually from the transfection process.

In summary, clones βN22 and βN17 express 4000 and 300 fmoles β2-adrenergic receptor / mg membrane protein respectively. Both clones expressed identical levels of IP prostanoid receptor, adenylyl cyclase catalytic subunit and the α-subunits from Gi2, Gi3, Gs and Gq/11, as did the wild-type NG108-15 cell. There was a 45% increase in Goα expression in βN17 with no accompanying change in cell morphology. Both the IP prostanoid receptor and the β2-adrenergic receptor were coupled to Gsα in βN17 and βN22. A robust isoprenaline stimulation of adenylyl cyclase in βN22 was observed but there was no shift to the right in the agonist displacement curve of the β2-adrenergic receptor on addition of excess guanine nucleotides. Both clones gave a robust stimulation of adenylyl cyclase activity by iloprost and isoprenaline. "Spare" β2-adrenergic receptors exist for adenylyl cyclase activation in βN22 but not in βN17. The high basal adenylyl cyclase activity measured in βN22 cell membranes appears to be, at least in part, a result of "empty" receptor regulation of adenylyl cyclase.

CHAPTER 5

Examination of the effects of prolonged agonist treatment on membrane associated G-protein α-subunit levels in transfected NG108-15 cells.

Introduction

Agonist regulation of G-protein α-subunit levels has been demonstrated in several cellular systems. As described in detail in chapter 3 prolonged prostanoid treatment of NG108-15 cells gave a down-regulation of cellular Gsα levels (Kelly *et al*, 1990; Milligan and McKenzie, 1990). In rat adipocytes PIA treatment resulted in a 90% decrease in membrane associated levels of Gi1α and Gi3α, and a 50% decrease in Gi2α and β-subunit (Green *et al*, 1990). Chronic exposure of each of the HM1 receptor in transfected CHO cells (Mullaney *et al*, 1993), the GnRH receptor in αT3 cells (Shah and Milligan, unpublished observations) and the TRH receptor long form in EMBK cells (Kim *et al*, unpublished observations) to agonists resulted in a non-selective down-regulation of Gq/11α. In the aforementioned systems the activated G-protein α-subunit was subsequently down-regulated. Additionally, in other cases the levels of G-protein α-subunit which had not been activated were also regulated. Treatment of S49 lymphoma cells with isoprenaline which activates the β2-adrenergic receptor gave a 25% decrease in membrane associated levels of Gsα and a 3-fold increase in Gi2α (Hadcock and Malbon, 1990).

The emerging body of evidence suggests that the down-regulation of the activated G-protein α-subunit on prolonged agonist exposure is a general phenomenon (Milligan and Green, 1991). There are, however, several exceptions. Treatment of the δ-opioid receptor in NG108-15 cells with agonist results in a down-regulation of the δ-opioid receptor but not of Gi2α, (Law *et al*, 1985) and prolonged agonist stimulation of the β2-adrenergic receptor in several cell lines does not give a coincidental down-regulation of Gsα (Rich and Iyengar, 1989; Gonzales

et al, 1992). As discussed in chapter 4 agonist treatment of the A2 adenosine and secretin receptors in NG108-15 cells (McKenzie *et al*, 1990b) and the IP prostanoid receptor in NCB20 cells demonstrated no down-regulation of Gsα (Kelly *et al*, 1990, Mullaney and Milligan, unpublished observations). The reason for the discrepancy in agonist mediated down-regulation of Gα between systems remains uncertain. Several possibilities exist; the genetic make-up of the cell in which the receptor is expressed may differentially control the agonist-mediated down-regulation of Gα. Alternatively, the amount of Gsα down-regulated may be dependent on the amount of each receptor expressed in every individual cell. Indeed, in chapter 3 it was shown that the IP prostanoid receptor will down-regulate Gsα in a molar ratio of approximately 1:8, at various levels of receptor occupancy on prostanoid treatment.

In chapter 4 the generation and characterisation of two clones of NG108-15 cells, βN22 and βN17, expressing very high (4000 fmoles/mg membrane protein) and moderate (300 fmoles/mg membrane protein) levels of β2-adrenergic receptor respectively was described. Examination of the agonist regulation of Gsα levels in these transfected NG108-15 cells, where treatment with a prostanoid agonist results in a down-regulation of Gsα and agonist treatment of the A2 adenosine and secretin receptors do not, should allow the further elucidation of the role of receptor levels in G-protein α-subunit down-regulation.

Figure 5.1 Analysis of Gsα levels in membranes prepared from control and isoprenaline treated NG108-15 cells by western blotting.

NG108-15 cells were treated with 10μM isoprenaline for 16 hours. Control and treated cells were harvested and cell membranes made as described in section 2.3. 20μg (lanes 1, 2), 40μg (lanes 3, 4) and 60μg (lanes 5, 6) membranes were TCA precipitated, loaded onto a 10% (w/v) acrylamide / 0.25% (w/v) N, N'-methylene bisacrylamide gel and western blotted for Gsα using the antiserum CS3 (1:250 dilution), as described in section 2.6. Lanes 1,3 and 5 were loaded with control membranes and lanes 2,4 and 6 were loaded with membranes prepared from isoprenaline treated cells. The developed immunoblot was densitometrically scanned and the results taken as a percentage of control Gsα levels; 20μg protein: 110%; 40μg protein: 104% and 60μg protein: 112%. Results obtained from one other identical experiment performed on a separate membrane preparation of cells gave similar results.

Figure 5.1

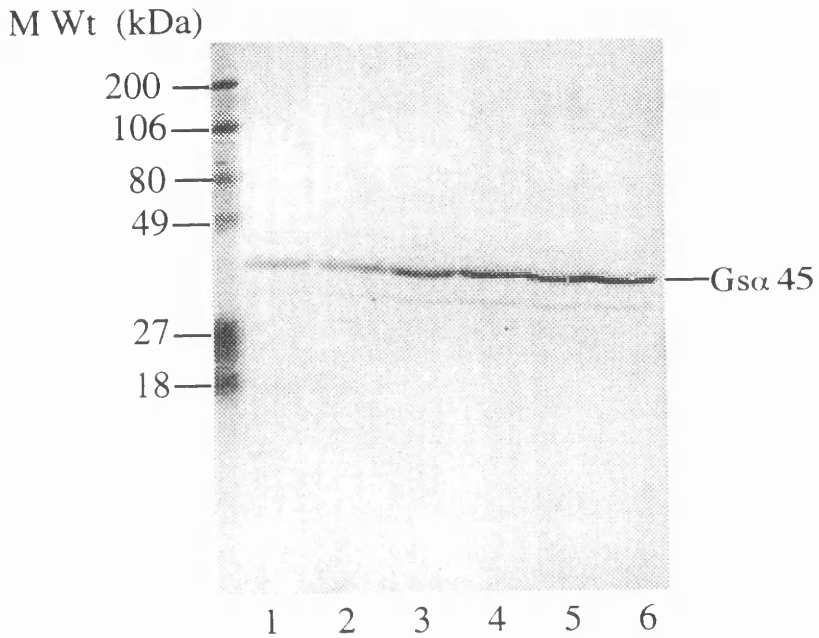


Figure 5.2 Analysis of Gα levels in membranes prepared from control and isoprenaline treated βN22 membranes, by western blotting.

Cell membranes were prepared from control and isoprenaline (10μM, 16 hours) treated βN22 cells according to section 2.3. Membranes were loaded onto a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted as described in section 2.6. Lanes 1 and 2 in (A) were loaded with 40μg membrane protein, lanes in panel D were loaded with 100μg membrane protein and all other lanes were loaded with 50μg protein. Lane 1 was loaded with control βN22 membranes, lane 2 membranes prepared from isoprenaline treated βN22 cells. Panel A was western blotted with CS3 (1:250) recognising Gsα, panel B with SG1 (1:400) recognising Gi2α, panel C with CQ2 (1:1000) for Gq/11α, panel D with IM1 (1:250) for Goα and panel E with I3B (1.1000) for Gi3α as described in table 2.1.

Colorimetrically developed blots were densitometrically scanned and the isoprenaline treated membrane Gα levels taken as a percentage of control levels for that particular Gα. Isoprenaline treatment resulted in the following membrane associated levels of Gα: Gsα = 48±10%, Gi2α = 105±5%, Gi3α = 104±17%, Goα = 110±5%, Gq/11α = 96±19%. The results were expressed as mean ± SD, from the analysis of separate membrane preparations, n=14 for Gsα and n=3 for the other Gα.

Figure 5.2

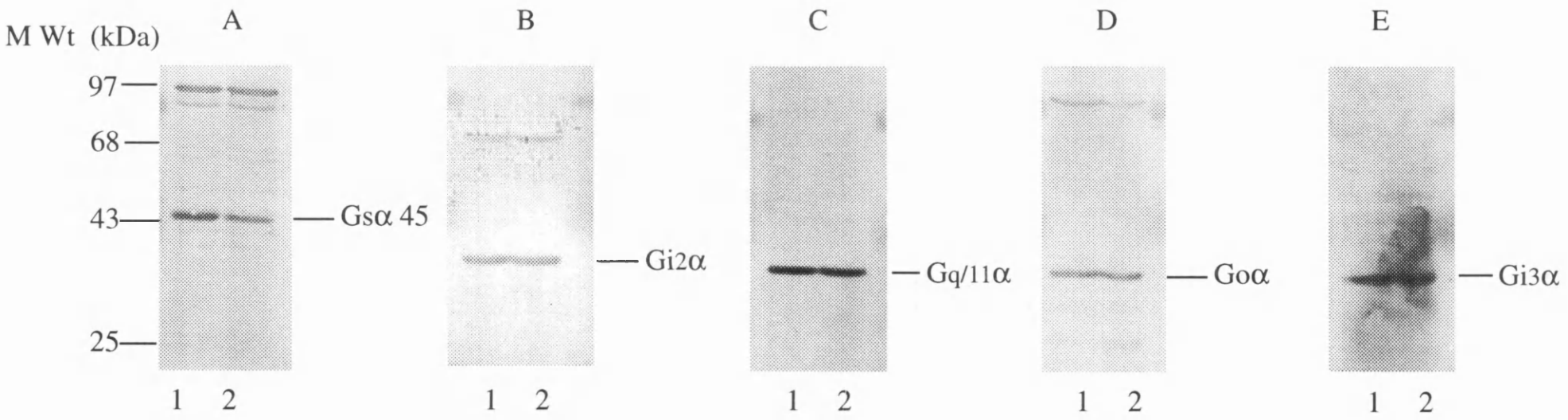

M Wt (kDa)
97
68
43
25
A
Gsα 45
B
Gi2α
C
Gq/11α
D
Goα
E
Gi3α
1 2
1 2
1 2
1 2
1 2

Figure 5.3 Molar quantitation of Gsα levels in membranes prepared from control and isoprenaline (10μM, 16 hours) treated βN22 cells.

30μg βN22 cell membranes prepared from control (lanes 1) and isoprenaline (10μM, 16 hours) treated (lanes 2) cells were run on a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα with antiserum CS3 (1:250) as described in section 2.6 (figure 5.3a). Increasing amounts of recombinant Gsα long form (2-25μg) were loaded alongside the samples (lane 3, 2μg; lane 4, 4μg; lane 5, 6μg; lane 6, 8μg; lane 7, 10μg; lane 8, 15μg; lane 9, 20μg and lane 10, 25μg). The colorimetrically developed blot was densitometrically scanned and the amount of Gsα long form plotted graphically against the corresponding value from the densitometric scan analysis (figure 5.3b).

The amount of Gsα 45 present in control and isoprenaline βN22 membranes was calculated from the standard curve, and the results from all three experiments were pooled and expressed as mean ± SD: control = 8.4±1.1 pmoles and isoprenaline treated = 4.9±0.9 pmoles.

Figure 5.3a

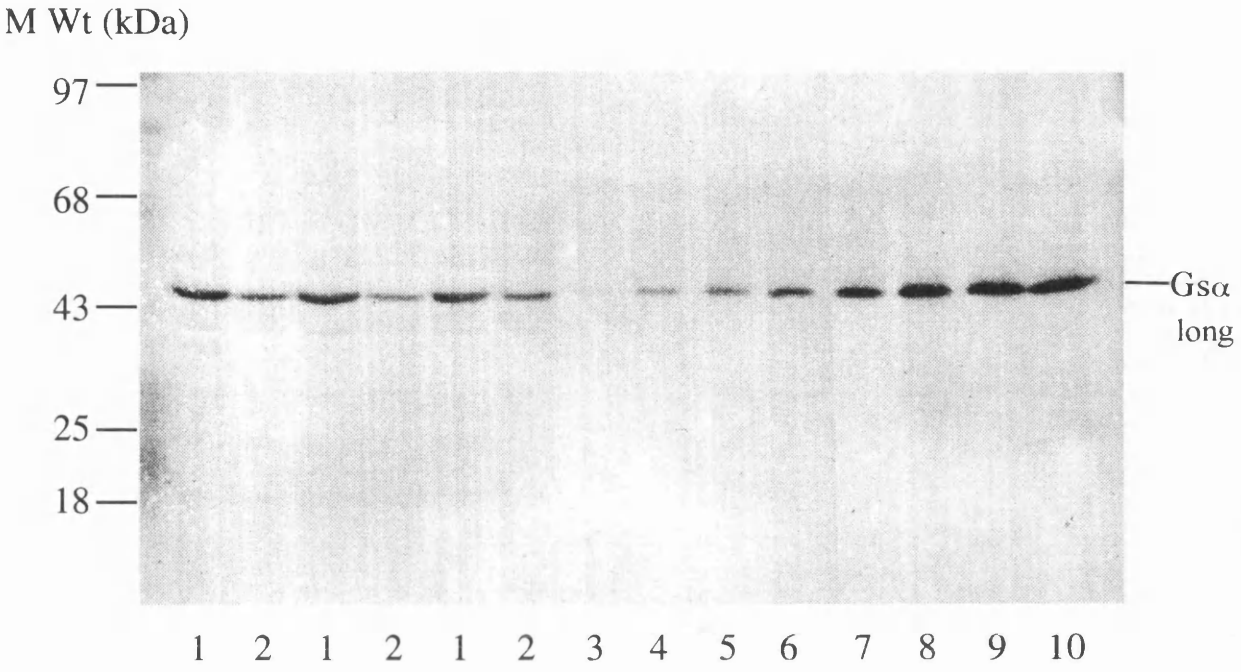


Figure 5.3b

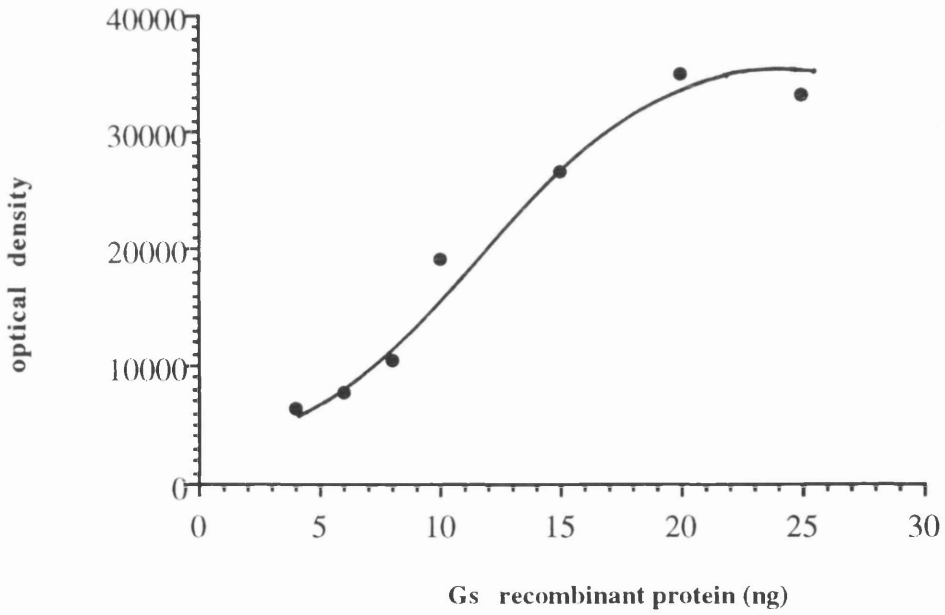


Figure 5.4 Analysis of G-protein β-subunit levels, by western blotting, in membranes prepared from control and isoprenaline (10μM, 16 hours) treated βN22 cells.

50μg membrane protein prepared from control (lane 1) and isoprenaline treated (lane 2) βN22 cells were run on a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for the G-protein β-subunit using the antipeptide antiserum BN3 (1:200 dilution), as described in section 2.6. The developed immunoblot was densitometrically scanned and the β-subunit levels in isoprenaline treated membranes expressed as a percentage of control levels. The results from scans of three identical immunoblots, from separate membrane preparations of cells were pooled and the treated value taken as a percentage of the control (90±9% [mean ± SD, n=3]).

Figure 5.4

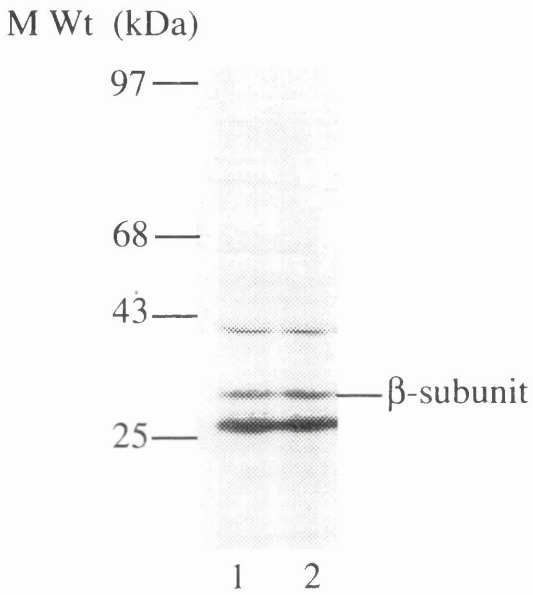


Figure 5.5 Analysis of Gsα levels, by western blotting, in membranes prepared from βN22 cells exposed to increasing concentrations of isoprenaline for 16 hours.

βN22 cells were treated with increasing concentrations (0-100μM) of isoprenaline for 16 hours. Membranes were made from these cells as described in section 2.3. 30μg membrane protein from cells treated with each concentration of isoprenaline was loaded on to a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα using the antipeptide antiserum CS3 (1:250), as described in section 2.6. Lane (1) was loaded with control βN22 membranes, (2) 0.1nM, (3) 1nM, (4) 10nM, (5) 100nM, (6) 1μM, (7) 10μM, (8) 100μM isoprenaline treated cell membranes (figure 5.5a). The developed immunoblots were densitometrically scanned and Gsα levels expressed as a percentage of control levels (figure 5.5b). The results were expressed as mean ± SD from data pooled from analysis of three individual membrane preparations.

Figure 5.5a

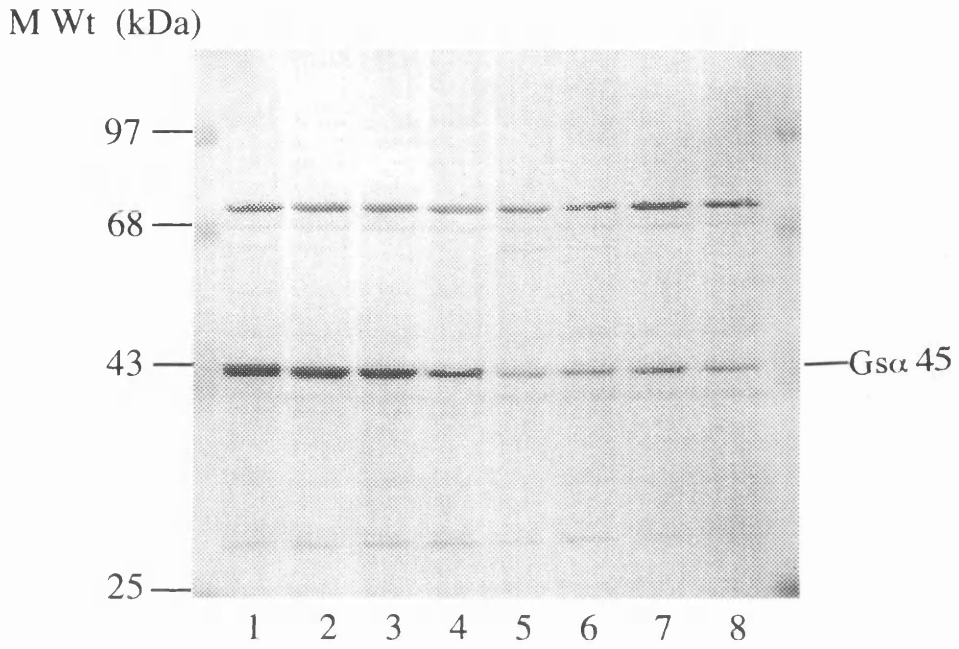


Figure 5.5b

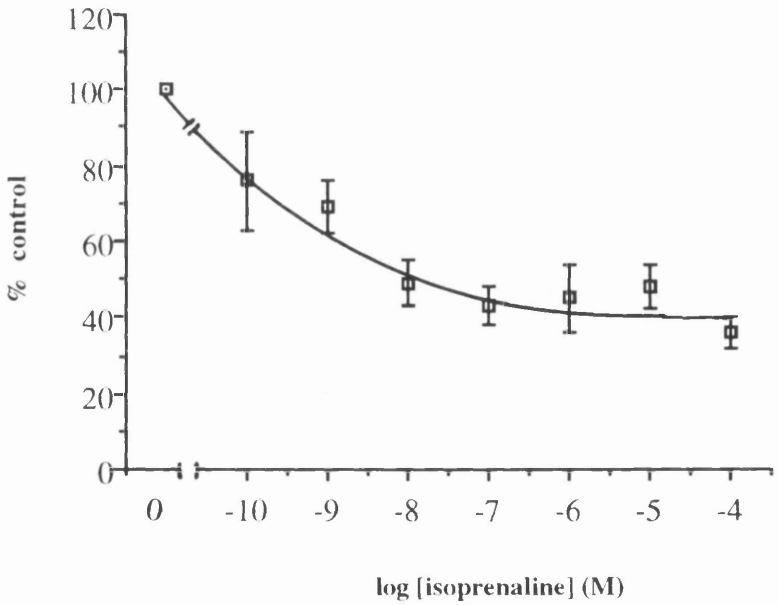


Figure 5.6 Analysis of Gsα levels, by western blotting, in membranes prepared from βN22 cells which were treated with 10μM isoprenaline for increasing lengths of time.

βN22 cells were treated with 10μM isoprenaline for varying lengths of time. Cell membranes were made as described in section 2.3, and 30μg membrane protein from each time point was loaded on to a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα using CS3 (1:250) as described in section 2.6. Lane (1) was loaded with control membranes; lane (2) 1 hour isoprenaline treatment; (3) 2 hours; (4) 4 hours; (5) 8 hours; (6) 12 hours; (7) 16 hours (figure 5.6a). Developed blots were densitometrically scanned and the results obtained from three individual membrane preparations were pooled and expressed as a percentage of control values, mean ± SD (n=3) (figure 5.6b).

Figure 5.6a

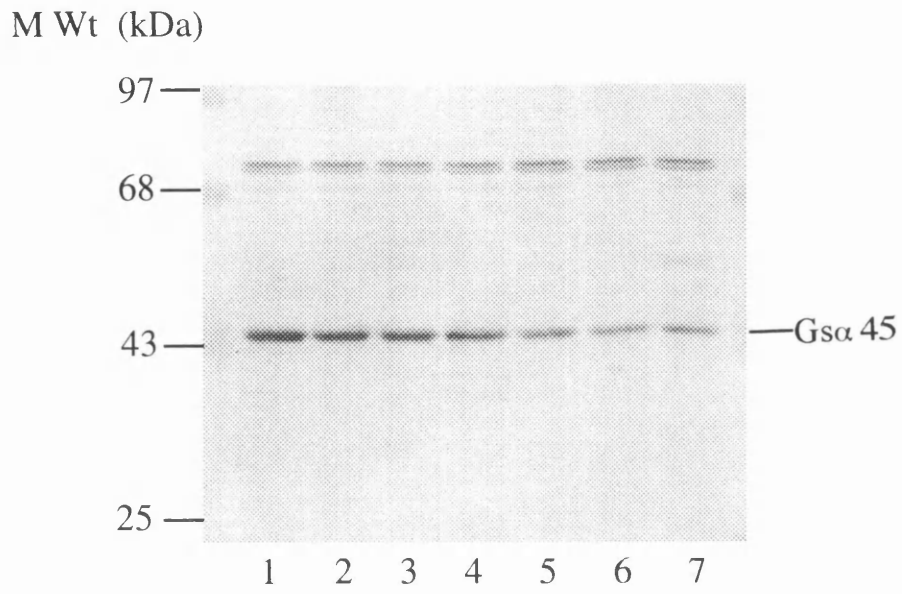


Figure 5.6b

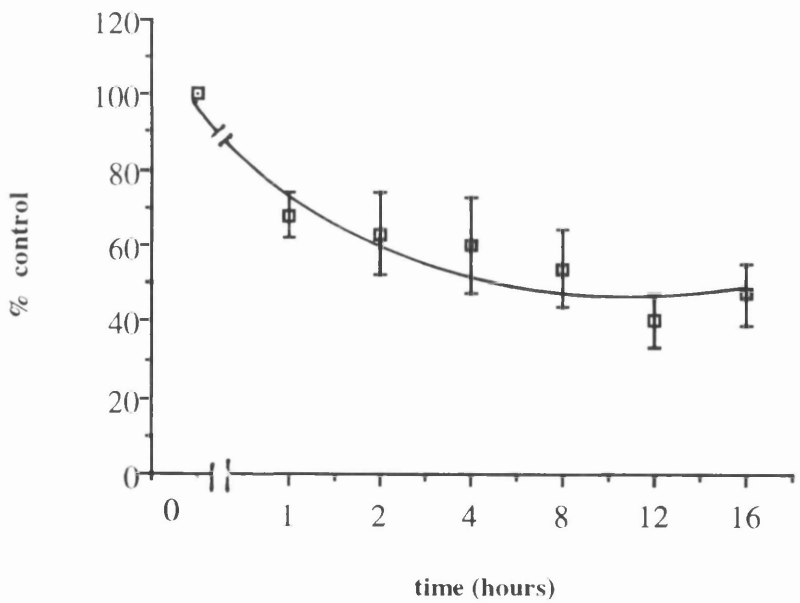


Figure 5.7 Analysis of Gsα levels, by western blotting, in membrane and cytosol prepared from βN22 cells, treated individually with 10μM isoprenaline and 1μM iloprost.

βN22 cells were treated individually with 10μM isoprenaline and 1μM iloprost for 3 hours. The cells were harvested, washed twice in PBS (x1) and homogenised as described in section 2.3. The homogenate was spun at 150 000 x g for 30 minutes in a Beckman airfuge. The supernatant was taken as "cytosol" and the pellet as "membranes". 30μg membrane protein (lanes 1,3 and 5) and cytosol protein (lanes 2,4 and 6) from control (lanes 1,2), isoprenaline treated (lanes 3,4) and iloprost treated (lanes 5,6) βN22 cells were TCA precipitated, resolved on a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα using CS3 antisera (1:250), as described in section 2.6 (figure 5.7). No detectable increase in cytosolic Gsα was observed in iloprost and isoprenaline treated cells. The membrane protein bands were densitometrically scanned and expressed as a percentage of control: isoprenaline treated was 22% and iloprost was 47% (for the immunoblot displayed). The results were from a single experiment representative of three performed.

Figure 5.7

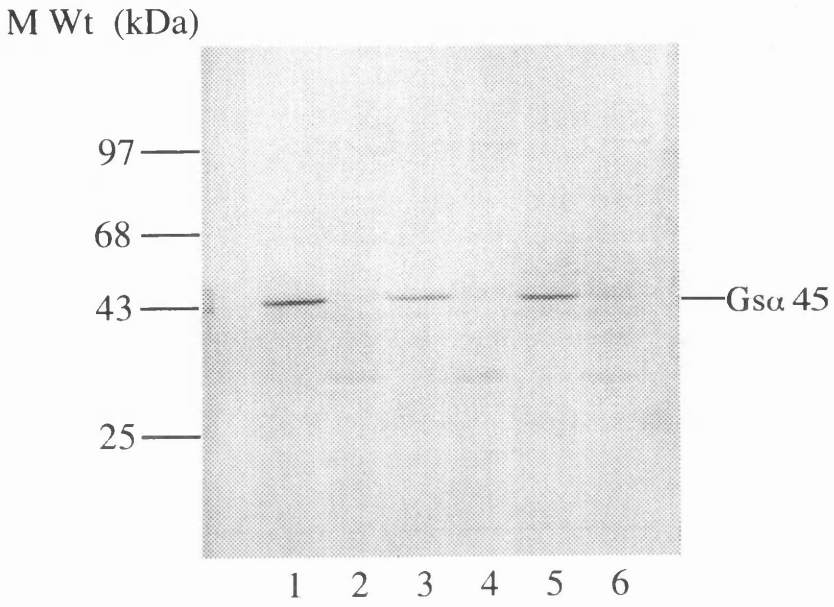


Figure 5.8 **Analysis of the ability of sodium cholate extracts of membranes, prepared from control and 10μM isoprenaline treated βN22 cells, to activate adenylyl cyclase when reconstituted into S49 lymphoma *cyc-* cell membranes.**

2μg each of control and isoprenaline treated (10μM, 16 hours) βN22 cell membranes were solubilised in 1% (w/v) sodium cholate on ice for 1 hour, reconstituted into 10μg *cyc-* membranes and adenylyl cyclase activity measured as described in section 2.9. 10mM NaF gave a stimulation of adenylyl cyclase activity over basal levels of 10.1±1.7 pmoles cAMP formed/minute/mg membrane protein in control cell membranes (1) and 5.2±2.0 pmoles cAMP formed/minute/mg membrane protein in membranes from isoprenaline treated cells (2), a decrease of 51.5% ($p<0.05$). The results were expressed as mean ± SD (n=3), of a single experiment which was representative of two others performed.

Figure 5.8

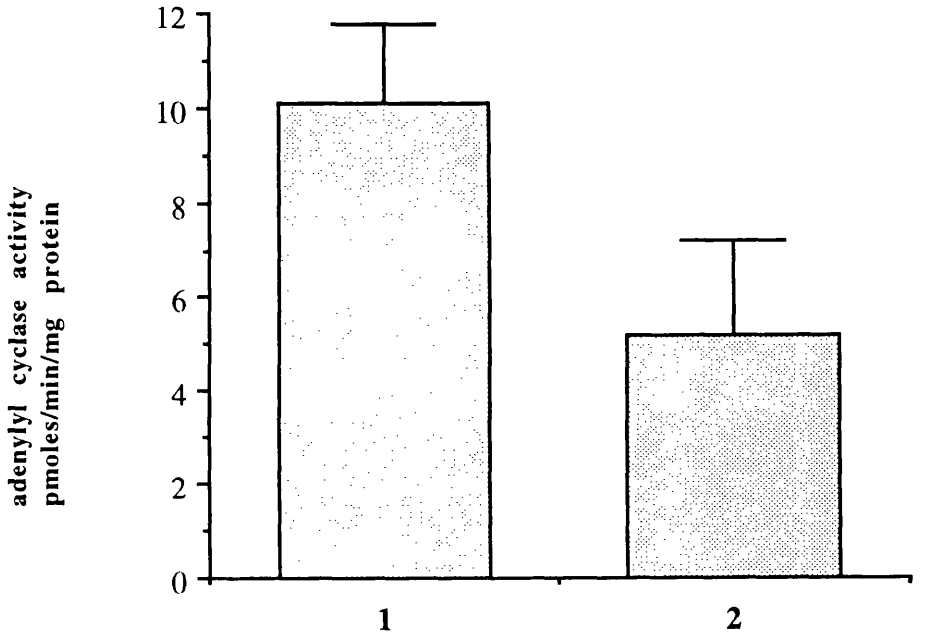

adenylyl cyclase activity
pmoles/min/mg protein
12
10
8
6
4
2
0
1
2

Figure 5.9 Analysis of Gα levels, by western blotting, in membranes prepared from control and isoprenaline treated βN17 cells.

Membranes were prepared from control and isoprenaline (10μM, 16 hours) treated βN17 cells according to section 2.3. Membranes were loaded on to a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted as described in section 2.6. Lanes 1 and 2 in (A) were loaded with 40μg and 20μg membrane protein (left to right) and all other lanes were loaded with 50μg membrane protein. Lane 1 was loaded with control βN17 membranes and lane 2 with membranes from isoprenaline treated βN17 cells. Panel A was western blotted with CS3 (1:250) recognising Gsα, panel B with SG1 (1:400) recognising Gi2α, panel C with I3B (1.1000) for Gi3α, panel D with IM1 (1:250) for Goα and panel E with CQ2 (1:1000) for Gq/11α as described in table 2.1.

Colorimetrically developed blots were densitometrically scanned and the isoprenaline treated membrane Gα levels taken as a percentage of control levels. Isoprenaline treatment gave the following levels of Gα: Gsα = 97±9%, Gi2α = 102±9%, Gi3α = 98±24%, Goα = 102±11%, Gq/11α = 104±6%. The results were expressed as mean ± SD from analysis of separate membrane preparations, n=6 for Gsα, n=2 for Gq/11α and n=3 for the other Gα.

Figure 5.9

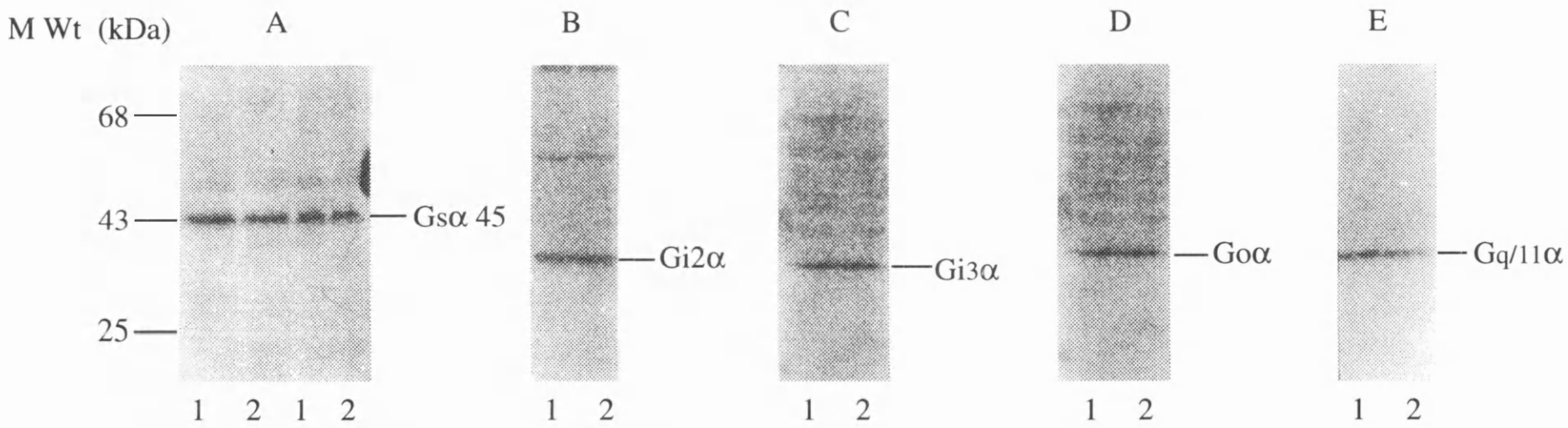

M Wt (kDa)
A
B
C
D
E
68
43
25
Gsα 45
Gi2α
Gi3α
Goα
Gq/11α
1 2 1 2
1 2
1 2
1 2
1 2

Figure 5.10 Analysis of Gα levels, by western blotting, in membranes prepared from control and iloprost treated βN22 cells.

Membranes were prepared from control and iloprost (1μM, 16 hours) treated βN22 cells as described in section 2.3. Membranes were loaded onto a 10% (w/v) acrylamide / 0.25% (w/v) N,N'-methylene bisacrylamide gel and western blotted as described in section 2.6. Lanes 1 and 2 in (A) were loaded with 40μg membrane protein and all other lanes were loaded with 50μg membrane protein. Lane 1 was loaded with control βN22 and lane 2 with membranes from iloprost treated (1μM; 16 hours) βN22 cells. Panel A was western blotted with CS3 (1:250) recognising Gsα, panel B with SG1 (1:400) recognising Gi2α, panel C with 13B (1:1000) recognising Gi3α, panel D with IM1 (1:250) for Goα, panel E with CQ2 (1:1000) for Gq/11α as described in table 2.1.

Colorimetrically developed blots were densitometrically scanned and the iloprost treated membrane Gα levels taken as a percentage of control for that particular Gα. Iloprost treatment gave the following levels of Gα: Gsα = 50±15%, Gi2α = 107±12%, Goα = 106±11%, Gq/11α = 97±1%. The results were expressed as mean ± SD from analysis of separate membrane preparations, n=7 for Gsα and n=3 for the other Gα.

Figure 5.10

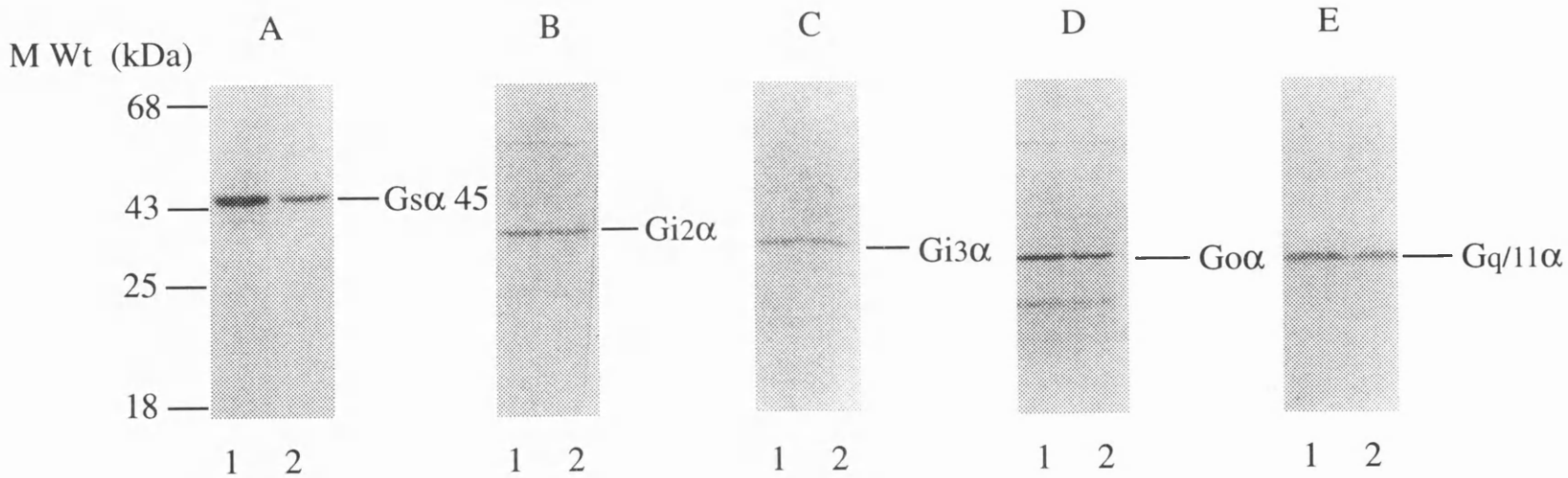

M Wt (kDa)
68
43
25
18
A
B
C
D
E
Gsα 45
Gi2α
Gi3α
Goα
Gq/11α
1 2
1 2
1 2
1 2
1 2

Figure 5.11 Analysis of Gα levels, by western blotting, in membranes prepared from control and iloprost treated βN17 cells.

Membranes were prepared from control and iloprost (1μM, 16 hours) treated βN17 cells as described in section 2.3. Membranes were loaded onto a 10% (w/v) acrylamide / 0.25% (w/v) N,N'-methylene bisacrylamide gel and western blotted as described in section 2.6. Lanes 1 and 2 in (A) were loaded with 40μg membrane protein and all other lanes were loaded with 50μg membrane protein. Lane 1 was loaded with control βN17 membranes and lane 2 with membranes prepared from iloprost treated βN17 cells (figure 5.11). Panel A was western blotted with CS3 (1:250) recognising Gsα, panel B with SG1 (1:400) recognising Gi2α, panel C IM1 (1:250) for Goα, panel D with CQ2 (1:1000) for Gq/11α as described in table 2.1.

Colorimetrically developed blots were densitometrically scanned and the iloprost treated membrane Gα levels taken as a percentage of control for that particular Gα. Iloprost treatment gave the following levels of Gα: Gsα = 49±14%, Gi2α = 89±11%, Goα = 96±10%, Gq/11α = 99±10%. The results were expressed as mean ± SD of analysis from separate membrane preparations (n=3).

Figure 5.11

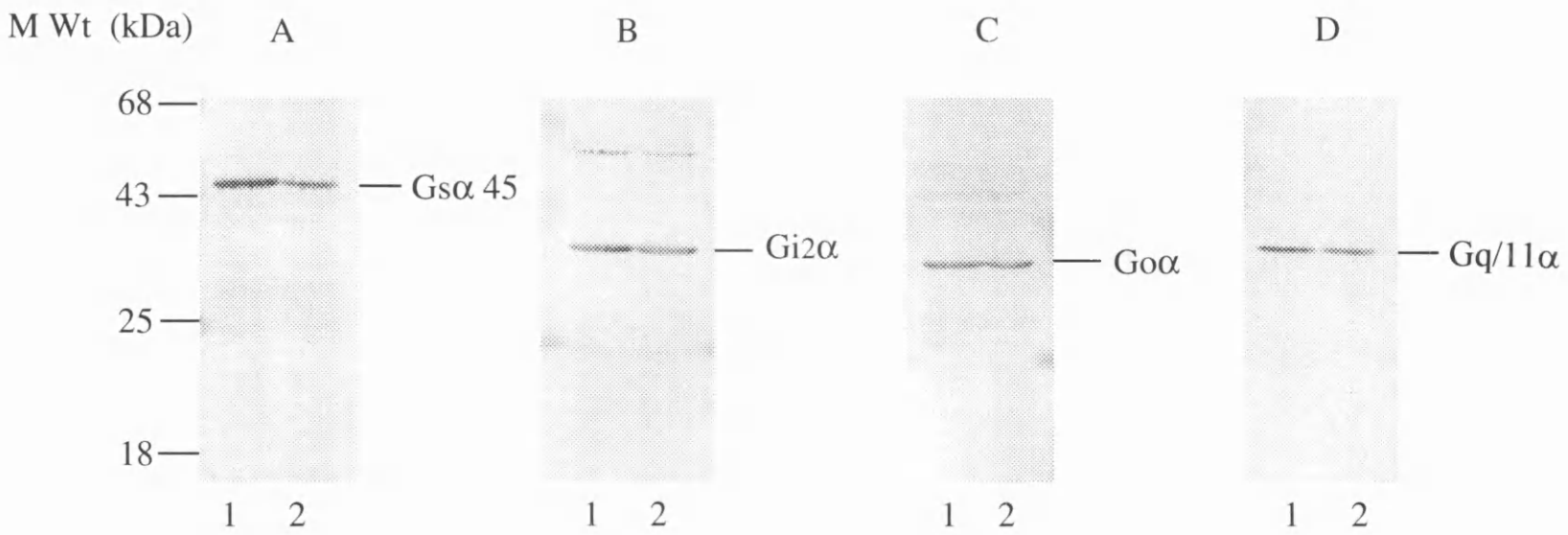


Figure 5.12 Analysis of Gsα levels in membranes prepared from βN22 cells treated with increasing concentrations of iloprost.

βN22 cells were treated for 16 hours with increasing concentrations of iloprost. Membranes were prepared form the treated cells and 30μg loaded onto a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted for Gsα using the antiserum CS3 (1:250) as described in section 2.6. Lane 1 was loaded with control βN22 membranes, lane 2 with 1nM iloprost treated cells, lane 3: 10nM, lane 4: 50nM, lane 5: 100nM, lane 6: 500nM, lane 7: 1μM and lane 8: 10μM (figure 5.12a). The developed blot was densitometrically scanned and the results from three individual experiments pooled (mean ± SD) and expressed graphically (figure 5.12b).

Figure 5.12a

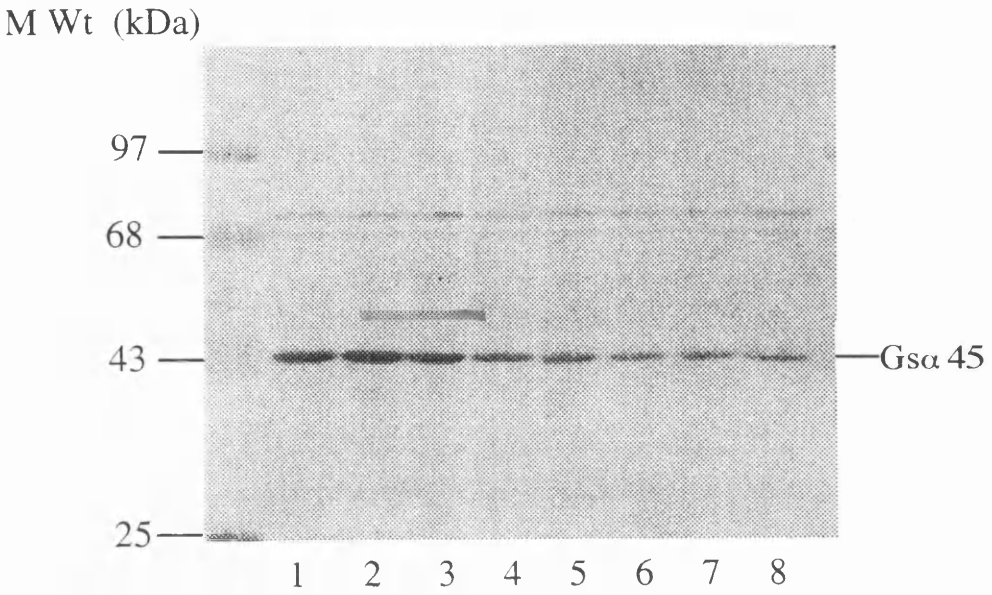


Figure 5.12b

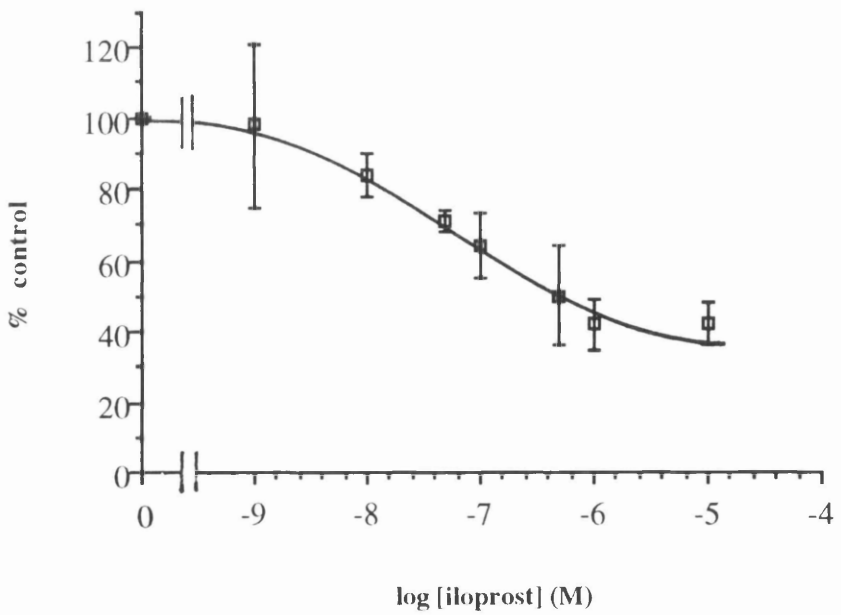


Figure 5.13 Analysis of Gsα levels in membranes prepared from βN22 cells treated with 1μM iloprost for increasing lengths of time.

βN22 cells were treated with 1μM iloprost for increasing lengths of time (0-16 hours). Membranes were made from each treatment as described in section 2.3. 30μg membrane was loaded on to a 10% (w/v) acrylamide / 0.25% N,N'-methylene bisacrylamide gel and western blotted using the antiserum CS3 (1:250). Lane 1 was loaded with control βN22 membranes, lane 2 with βN22 cell membranes treated for 1 hour with iloprost, lane 3: 2 hour, lane 4: 4 hour, lane 5: 8 hour, lane 6: 12 hour and lane 7: 16 hour (figure 5.13a). The developed blot was densitometrically scanned and the results from three individual experiments pooled (mean ± SD) and expressed graphically (figure 5.13b).

Figure 5.13a

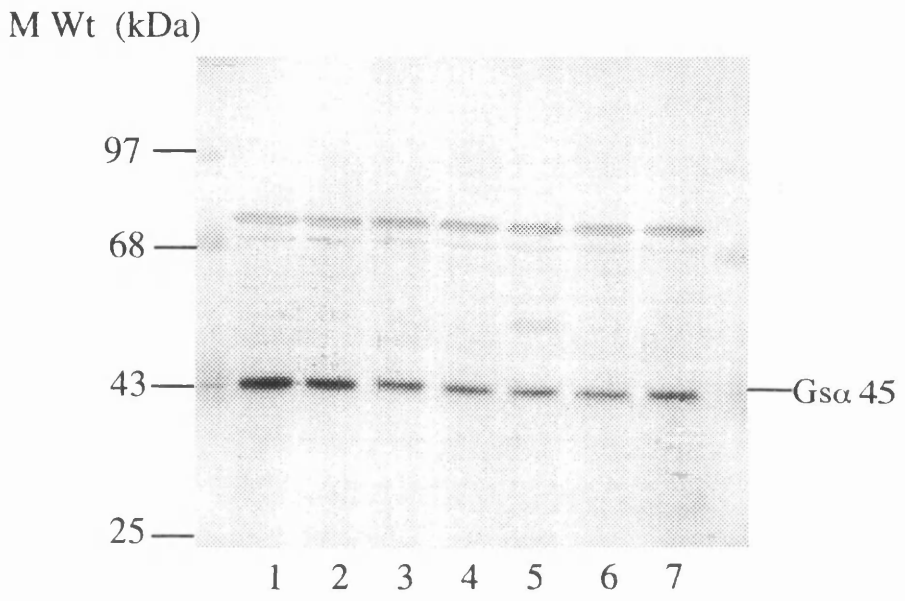


Figure 5.13b

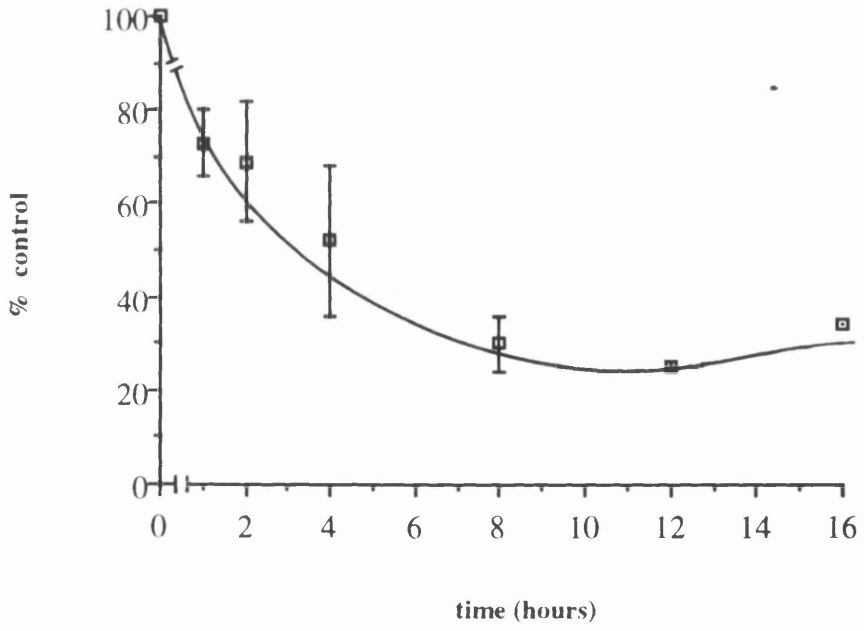


Figure 5.14 Measurement of the adenylyl cyclase activity, in response to increasing concentrations of isoprenaline, in membranes prepared from control and isoprenaline treated βN22 cells.

10μg membranes produced from control (o) and isoprenaline (□) treated (10μM, 16 hours) βN22 cells were incubated with increasing concentrations of isoprenaline (0 - 0.1mM) and the adenylyl cyclase activity measured as described in section 2.8. The EC50 for adenylyl cyclase stimulation by isoprenaline in control membranes was 4.3 nM and in isoprenaline treated membranes was 14.3 nM. The result was from a single experiment representative of three performed.

Figure 5.14

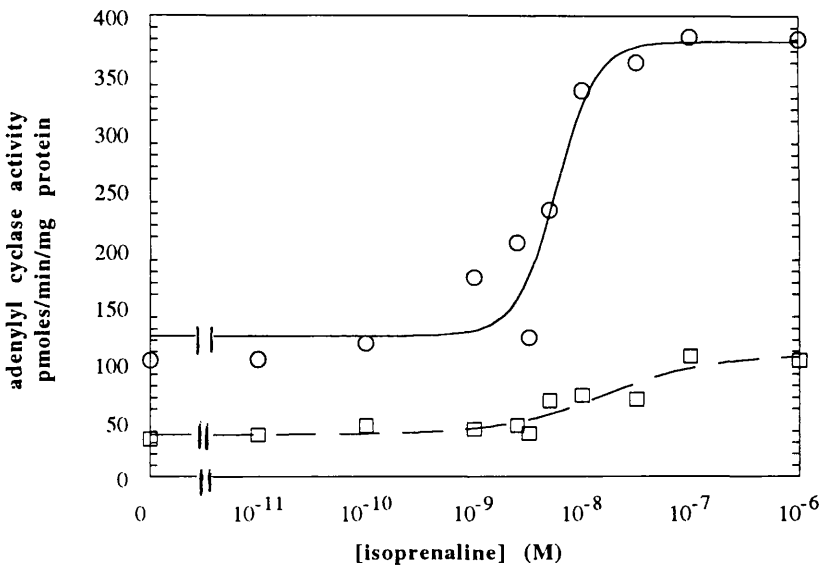

adenylyl cyclase activity
pmoles/min/mg protein
400
350
300
250
200
150
100
50
0
0
10-11
10-10
10-9
10-8
10-7
10-6
[isoprenaline] (M)

Figure 5.15 The effect of isoprenaline, iloprost and a combination of these agonists on membrane associated levels of Gsα in βN22 cells as assessed by western blotting.

βN22 cells were treated with isoprenaline (10μM) or iloprost (1μM) or a combination of these two agonists for 16 hours. Cells were harvested and membranes prepared as described in section 2.3. 40μg membrane protein was loaded on to a 10% (w/v) acrylamide / 0.25% (w/v) N,N'-methylene bisacrylamide gel and western blotted for Gsα using the antiserum CS3 (1:250) as described in section 2.6. Lane (1) was loaded with control membranes, lane (2) with membranes prepared from isoprenaline treated cells, lane (3) with membranes prepared from iloprost treated cells and lane (4) with membranes from cells which had been treated with both isoprenaline and iloprost. The developed immunoblots were densitometrically scanned and values taken as a percentage of control Gsα levels. The following levels of Gsα were observed on treatment with isoprenaline: 53±10% (mean ± SD, n=6), iloprost: 52±16% (mean ± SD, n=6) and both agonists: 36±8% (mean ± SD, n=6).

Figure 5.15

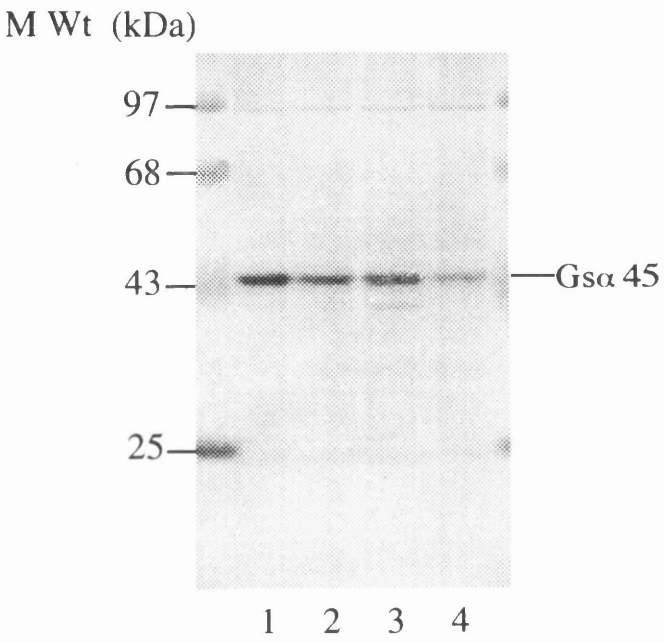


Table 5.1 [^{3}H]-PGE1 binding analysis in membranes prepared from control and iloprost treated βN22 and βN17 cells.

25μg membranes from control and iloprost treated βN22 and βN17 cells were incubated with 10nM [^{3}H]-PGE1 for 30 minutes at 30^{o}C. The reaction was returned to ice and rapidly filtered through Whatman GF/C filters as described in section 2.7. Non-specific binding in membranes was defined as the [^{3}H]-PGE1 bound in the presence of 10μM PGE1. The results were expressed as the mean ± SD (n=3) of a single experiment. Two others produced similar results.

Table 5.1

Cell	[^{3}H]-PGE1 bound (fmoles/ mg membrane protein) control	iloprost	% control
βN22	226±77	136±32	40
βN17	246±5	134±10	54

Table 5.2 Measurement of adenylyl cyclase activity in membranes from control and isoprenaline treated βN22 cells in the presence of 20mM $MnCl_2$.

10μg membranes prepared from control and isoprenaline treated (10μM, 16 hours) βN22 cells were assayed for basal adenylyl cyclase activity as described in section 2.8. In some cases 20mM $MnCl_2$ was added to the incubation, and in these cases $MgCl_2$ was excluded from the reaction mix. The results were expressed as the mean ± SD (n=3) from a single experiment which was representative of three performed.

Table 5.2

Treatment	Adenylyl cyclase activity (pmoles /min/mg membrane protein)
control	86.6±10.3
control + Mn	23.7±10.6
isoprenaline (10μM)	17.0±9.6
isoprenaline + Mn	18.3±9.9

Table 5.3 Measurement of basal and iloprost stimulated adenylyl cyclase activity in membranes from control and iloprost treated βN22 cells.

10μg membranes prepared from control and iloprost treated (1μM, 16 hours) βN22 cells were assayed for adenylyl cyclase activity as described in section 2.8 in the presence of 1μM iloprost. The results were the mean ± SD (n=3) of a single experiment. One other produced a similar result.

Table 5.3

	Adenylyl cyclase activity (pmoles /min/mg membrane protein)	
Treatment	**basal**	**iloprost (1μM)**
control	157±10.3	489±46.1
iloprost (1μM)	52.0±9.8	319±13.8

Table 5.4 Measurement of agonist stimulated adenylyl cyclase activity in membranes from control, isoprenaline treated (10μM) and iloprost treated (1μM) βN17 cells.

Adenylyl cyclase activity was measured according to section 2.8 in membranes (10μg) prepared from control, isoprenaline (10μM, 16 hours) and iloprost (1μM, 16 hours) treated βN17 cells. In several cases adenylyl cyclase activity was amplified in the presence of agonist (either 10μM isoprenaline or 1μM iloprost). Results were taken from a single experiment mean ± SD (n=3). Two others produced similar results.

Table 5.4

	Adenylyl cyclase activity (pmoles/min/mg membrane protein)		
Treatment	**basal**	**isoprenaline**	**iloprost**
control	60.0±5.0	281±16.7	378±10.5
isoprenaline (10μM)	49.7±9.3	74.6±8.2	-
iloprost (1μM)	32.1±5.7	-	195±7.6

Table 5.5 [^{3}H]-DHA binding analysis in membranes prepared from control and isoprenaline treated βN17 and βN22 cells.

25μg membranes prepared from control and isoprenaline treated (10μM, 16 hours) βN17 (a) and βN22 cells (b) were incubated with [^{3}H]-DHA for 30 minutes at 30°C. βN17 membranes were incubated with 0.5nM [^{3}H]-DHA and βN22 membranes were incubated with the concentrations indicated in table 5.5b. The reaction was terminated by returning the samples to ice and filtering through Whatman GF/C filters as described in section 2.7. Non-specific binding was determined by the binding of [^{3}H]-DHA to cell membranes in the presence of 10μM (-) propranolol. Results were expressed as the mean ± SD (n=3), from a single experiment. Two others produced similar results.

Table 5.5a

Treatment	**[^{3}H]-DHA (fmoles bound/mg membrane protein)**
control	281±8.6
isoprenaline (10μM)	65.9±5.4

Table 5.5b

[^{3}H-DHA] (nM)	**fmoles bound/mg protein**		**%**
	control	**isoprenaline**	**control**
1.1	3100±160	2520±73	81
0.13	1202±53	936±29	78

Results

Wild-type NG108-15 cells were treated with 10μM isoprenaline for 16 hours. The membrane-associated levels of Gsα did not vary between control membranes (100%) and membranes prepared from isoprenaline treated cells (104±10%, mean ± SD, n=4) (figure 5.1), indicating that prolonged isoprenaline treatment of wild-type NG108-15 cells does not result in Gsα down-regulation.

βN22 cells, expressing approximately 4000 fmoles β2-adrenergic receptor/mg membrane protein, were treated with 10µM isoprenaline for 16 hours. Membranes prepared from these cells showed a decrease in Gsα levels to 48±11% (mean ± SD, n=14) of control membranes, as measured by the C-terminal decapeptide antiserum CS3 (figure 5.2, panel A). The membrane associated levels of other G-protein α-subunits were determined in both control and isoprenaline treated βN22 cells by western blotting using specific antipeptide antisera as described in table 2.1. The results were expressed as a percentage of control levels (mean ± SD n=3). No changes in levels of membrane associated Gi2α (105±5%), Gi3α (104±17%), Goα (110±5%), Gq/11α (102±13%) and β-subunit (90±9%) were observed after isoprenaline treatment of βN22 cells (figure 5.2, 5.4).

Control and isoprenaline (10µM, 16 hours) treated βN22 cell membranes were resolved by SDS-PAGE alongside various amounts (2-25ng) of the *E. Coli* expressed long form of Gsα (45kDa) (figure 5.3a). The colorimetrically developed blot was densitometrically scanned and the amount of recombinant Gsα 45kDa loaded on to the gel plotted against the corresponding numerical value of the densitometric scan

(figure 5.3b). The amount of Gsα long form in both control and isoprenaline treated βN22 cell membranes was then calculated from the standard curve. Control βN22 had 8.4±1.1 pmoles Gsα/mg membrane protein (mean ± SD, n=3) and membranes from isoprenaline treated βN22 cells had 4.9±0.9 pmoles Gsα/mg membrane protein (mean ± SD, n=3).

The loss of Gsα from the plasma membrane of βN22 cells on exposure to isoprenaline was both dependent on the concentration of isoprenaline used and the length of time the cells were exposed to it. The half-maximal time for isoprenaline mediated Gsα down-regulation was 2-4 hours in βN22 cells (figure 5.6), and the concentration of isoprenaline necessary to produce half-maximal Gsα down-regulation was approximately 0.5 - 1.0 nM (figure 5.5).

βN22 cells were treated separately with 10μM isoprenaline and 1μM iloprost for 3 hours, the time determined to be half-maximal for down-regulation of Gsα for both iloprost and isoprenaline. Equivalent amounts of "membrane" and "cytosol" prepared (as described in figure legend 5.7) from both control and agonist treated βN22 cells were western blotted for Gsα using the antipeptide antiserum CS3 (figure 5.7). There was no detectable increase in cytosolic levels of Gsα after treatment of βN22 cells with either isoprenaline or iloprost.

Sodium cholate extracts prepared from membranes of control and isoprenaline treated βN22 cells were reconstituted into S49 lymphoma *cyc-* cell membranes and adenylyl cyclase activity measured as described in section 2.9. A 45.6±14.9% (mean ± SD, n=3) decrease in 10mM NaF stimulated adenylyl cyclase activity was observed in *cyc-* cell

membranes, reconstituted with solubilised extracts from isoprenaline treated βN22 membranes, when compared to control extracts (figure 5.8). This was similar to the amount of immunologically detectable Gsα lost from the plasma membrane on iloprost treatment (figure 5.2).

βN17 cells were treated with 10μM isoprenaline for 16 hours and membrane associated Gα levels measured by western blotting with antipeptide antisera specific for the individual Gα as described in table 2.1. Expressed as a percentage of control Gα levels, no change in membrane associated Gsα levels on isoprenaline treatment of the clone βN17 was observed (97±9%, mean ± SD, n=6). No apparent change in levels of the other G-protein α-subunits was observed, Gi2α = 102±9% (n=3), Gi3α = 98±24% (n=3), Goα = 102±11% (n=3) and Gq/11α = 104±6% (n=2) (figure 5.9).

Iloprost treatment (1μM, 16 hours) of both βN22 and βN17 cells resulted in a loss of membrane associated Gsα. Expressed as a percentage of control levels, Gsα levels were decreased to 50±15% (mean ± SD, n=7) in βN22 cell membranes (figure 5.10) and to 49±14% (mean ± SD, n=3) in βN17 membranes (figure 5.11). Treatment of βN22 cells with increasing concentrations of iloprost revealed a half-maximal concentration for Gsα down-regulation of approximately 50nM (figure 5.12). Time courses of iloprost treatment of βN22 cells showed a half-maximal time of 1-2 hours for Gsα down-regulation (figure 5.13). In both βN22 and βN17 cell membranes no change in other Gα subunit levels were observed on prostanoid treatment, as assessed by western blotting with specific antipeptide antisera for the various Gα subunits. Densitometric scan analysis, expressed as a percentage of control values (mean ± SD, n=3), revealed quantitative levels of Gi2α (107±12%), Goα

(106±11%) and Gq/11α (97±1%) in βN22, and in βN17 Gi2α (89±11%), Goα (96±10%) and Gq/11α (99±10%). A decrease in 10nM [^{3}H]-PGE1 binding was observed on iloprost treatment: [^{3}H]-PGE1 binding in iloprost treated βN22 cells was 49.0±7.9% (expressed as a percentage control levels) and in iloprost treated βN17 cells was 53.0±5.6% (table 5.1). The percentage loss of Gsα and [^{3}H]-PGE1 binding from the plasma membrane on iloprost treatment of both clones was essentially identical to that in wild-type NG108-15 cells.

Adenylyl cyclase activity, measured in membranes prepared from control and isoprenaline treated (10μM, 16 hours) βN22 cells, showed an EC50 for adenylyl cyclase activation by isoprenaline in control membranes of 7.8±6.6 nM (mean ± SD, n=3) and 28.1±14.3nM (mean ± SD, n=3) in membranes prepared from isoprenaline treated cells (figure 5.14). There was no significant difference in the EC50 on isoprenaline treatment (p=0.060). Basal adenylyl cyclase activity decreased to 41.7±9.0% (mean ± SD, n=3) of control levels on isoprenaline treatment of βN22 cells. Similarly maximal isoprenaline stimulation of adenylyl cyclase decreased to 42.0±18.0% (mean ± SD, n=3) of control. Basal adenylyl cyclase activity in the presence of 20mM $MnCl_2$ was identical in membranes prepared from both control and isoprenaline treated βN22 cells (table 5.2).

Iloprost treatment (1μM, 16 hours) of βN22 cells demonstrated a decrease in basal (30%) and iloprost activated (65%) adenylyl cyclase. The results were expressed as a percentage of adenylyl cyclase activity measured in control membranes (table 5.3).

Isoprenaline treatment of βN17 cells resulted in no change in basal adenylyl cyclase activity, when compared to that of control βN17 cells. Whereas isoprenaline gave a robust stimulation of adenylyl cyclase in control βN17 membranes, little or no stimulation of adenylyl cyclase with isoprenaline was observed in membranes prepared from isoprenaline treated βN17 cells (table 5.4). A decrease in basal (39.5±15.8%, mean ± SD, n=3) and iloprost stimulated (50.3±16.6%, mean ± SD, n=3) adenylyl cyclase was observed in membranes prepared from iloprost treated (1μM, 16 hours) βN17 cells (the results were expressed as a percentage of control levels).

Using a single concentration of [^{3}H]-DHA (table 5.5), membranes prepared from control βN22 and isoprenaline (10μM, 16 hours) treated cells were assayed for specific [^{3}H]-DHA binding. Non-specific binding was determined using 10μM (-) propranolol. Specific [^{3}H]-DHA binding in membranes prepared from isoprenaline treated βN22 cells was 75.8±12% (mean ± SD, n=7, p=0.004) of control levels (table 5.5b). Similarly, membranes prepared from βN17 cells treated with isoprenaline demonstrated a decrease in [^{3}H]-DHA binding to 21.8±3.8% (mean ± SD, n=3, p=0.001) of control levels (table 5.5a).

βN22 cells were treated with 10μM isoprenaline and 1μM iloprost both together and separately for 16 hours and the Gsα levels examined in membranes prepared from these cells using the antipeptide antiserum CS1 as described in table 2.1 (figure 5.15). Expressing the results as a percentage of control Gsα levels, isoprenaline treatment reduced levels of Gsα to 53±10% (mean ± SD, n=6), iloprost treatment reduced levels of Gsα to 52±16% (mean ± SD, n=6) and a combination of both agonists to 36±8% (mean ± SD, n=6). The down-regulation of Gsα levels in cells

which had been treated with both agonists was however less than additive with down-regulation of Gsα in cells which had been treated with each agonist separately.

Discussion

Receptor levels have been implicated in regulating the amount of G-protein α-subunit down-regulated on prolonged agonist treatment of cells (chapter 3). In NG108-15 cells, chronic exposure of the IP prostanoid receptor to agonist results in a down-regulation of Gsα (Kelly *et al*, 1990; McKenzie and Milligan, 1990) whereas agonist activation of the apparently less highly expressed A2 adenosine and secretin receptors do not (McKenzie *et al* 1990).

NG108-15 cells expressing 4000 fmoles β2-adrenergic receptor/mg membrane protein (βN22 cells) were treated for 16 hours with the agonist isoprenaline. A dose-dependent, time-dependent down-regulation of some 50% of the cellular Gsα was observed (figure 5.5, 5.6). The membrane associated levels of other Gα were unchanged (figure 5.2), as were the membrane associated levels of β-subunit (figure 5.4). Gsα down-regulation was therefore not a distinct molecular feature of the IP prostanoid receptor in the NG108-15 cell line.

Moreover, NG108-15 cells expressing 300 fmoles β2-adrenergic receptor (βN17 cells) did not detectably down-regulate Gsα on prolonged isoprenaline exposure (figure 5.10). Although it is virtually impossible to examine the expression and function of every protein in the two clones to determine if the transfection process has disrupted the cellular metabolism, both clones express essentially identical amounts of Gsα, Gi2α, IP prostanoid receptor and adenylyl cyclase (chapter 4). The major difference between the two cell lines appears to be their relative levels of β2-adrenergic receptor expression. Receptor number, therefore, appears to be pivotal to isoprenaline mediated down-regulation

of Gsα in the transfected NG108-15 cell. Clone βN22, which expresses extremely high levels (4000 fmoles/mg membrane protein) of the β2-adrenergic receptor, will down-regulate Gsα on prolonged agonist exposure, whereas βN17, which expresses moderate levels (300 fmoles/mg membrane protein) of the receptor, does not appear to down-regulate Gsα. Indeed, higher levels of receptor occupancy by agonist are capable of down-regulating more Gsα than lower levels of receptor occupancy, as previously described. The IP prostanoid receptor in NG108-15 cells, can down-regulate Gsα, in a molar ratio of approximately 1:8 (receptor:G) at various degrees of receptor occupancy by iloprost (chapter 3). It seems likely that as the level of receptor expression increases (to a maximal) the amount of Gsα down-regulated by agonist treatment also increases and eventually reaches a plateau. If Gsα down-regulation does increase linearly with increasing levels of receptor expression then isoprenaline treatment of the clone βN17 may result in a barely detectable down-regulation in the levels of Gsα which cannot be measured within the sensitivity of the assays used. It would therefore be informative to select more clones of NG108-15 cells expressing β2-adrenergic receptor levels of between 300 and 4000 fmoles and examine the effect of agonist on cellular Gsα levels in these cells.

Having demonstrated that isoprenaline mediated down-regulation of Gsα in transfected NG108-15 cells is dependent on the level of β2-adrenergic receptor expression, it seems likely that the A2 adenosine and secretin receptors also down-regulate Gsα on prolonged agonist exposure but to such a small degree that it is not detectable within the sensitivity of the assays used. Certainly, NG108-15 cells express approximately only 25-30 fmoles/mg membrane protein of the secretin receptor (Gossen *et*

al, 1990), which would, on agonist treatment, potentially down-regulate only 1-2% Gsα (if the stoichiometry of secretin receptor:Gsα down-regulation was similar to that of the IP prostanoid receptor and Gsα) and this would not be detected by western blotting. The measurement of the levels of A2 adenosine receptor expression has proven problematic until recently (Keen *et al*, 1989), but the determination of these levels will help to further elucidate the role of the receptor in Gsα down-regulation. Although it has been shown that agonist mediated Gsα down-regulation is not unique to the IP prostanoid receptor it may not be a general phenomenon and may still be restricted to certain subtypes of G-protein coupled receptors, that is, the IP prostanoid receptor and the β2-adrenergic receptor when each is expressed at a sufficiently high level in a cell.

No apparent down-regulation of Gsα was observed on prostanoid treatment of NCB20 cells (Kelly *et al*, 1990; Mullaney and Milligan, unpublished observations), and this is most likely to be a result of the low levels of IP prostanoid receptor expression (100 fmoles) in these cells (Kelly *et al*, 1990). If the stoichiometry of down-regulation of the IP prostanoid receptor:Gsα in NCB20 cells is similar to that of the IP prostanoid receptor and Gsα in NG108-15 cells, then 100 fmoles IP prostanoid receptor would down-regulate approximately 5% total cellular Gsα if NCB20 cells express similar amounts of Gsα to NG108-15 cells. A 5% loss of Gsα cannot be detected within the sensitivity of our assays.

Recent studies, in transfected NCB20 cells expressing approximately 6000 fmoles β2-adrenergic receptor, demonstrated a down-regulation of 50-70% membrane associated Gsα on prolonged isoprenaline treatment (Mullaney and Milligan, unpublished

observations). Isoprenaline mediated down-regulation of Gsα, unlike iloprost, does not distinguish between NG108-15 and NCB20 cells. Furthermore, the role of receptor levels in Gα down-regulation is additionally validated by this data. From this data it seems likely that the down-regulation of G-protein α-subunits on prolonged agonist exposure, is a general phenomenon and stoichiometrically dependent on the level of receptor. Examination of the levels of receptor expression in cell lines which do not appear to down-regulate their respective G-protein α-subunit on prolonged agonist exposure will also be useful in determining the role of receptor number in the down-regulation of Gα.

In both clones, βN22 (figure 5.10, 5.12, 5.13) and βN17 (figure 5.11), chronic treatment with iloprost produced a down-regulation of both the IP prostanoid receptor (table 5.1) and Gsα essentially identical to that of wild-type NG108-15 cells. The lack of Gsα down-regulation by isoprenaline in βN17 was not an artefact produced by the transfection process preventing Gsα down-regulation in this clone. Co-addition of maximal concentrations of iloprost and isoprenaline to βN22 cells resulted in a less than additive down-regulation of Gsα in βN22 cell membranes (figure 5.15). It appears that the IP prostanoid and β2-adrenergic receptors work through the same pool of Gsα in the cell membrane and only 50-70% Gsα is capable of interacting with both receptors. This leaves at least 30% cellular Gsα which cannot be mobilised by a combination of agonist, and is therefore inaccessible to, or incapable of, being activated by both receptors. 20-30% Gsα in S49 lymphoma cells is present in a low density membrane fraction (Svoboda *et al*, 1992), which may indicate the presence of Gsα in discrete membrane compartments. Similarly β-adrenergic receptor activation in rat adipocytes can modify the cellular distribution of Gs and of Gi, by

promoting a movement of these proteins to a low density endosomal fraction (Haraguchi and Rodbell, 1990), indicating the potential for compartmentalisation of Gs and Gi in rat adipocytes. The crude "membrane" preparation used in the experiments with NG108-15 cells and the two clones (described in section 2.3) will include these low density membrane fractions because only the heavier nuclear components and cytosol are removed during the "membrane" production. If Gs is present in these low density vesicles in NG108-15 cells it will not be immediately accessible to the plasma membrane, and hence the receptor, thereby restricting the agonist mediated down-regulation of Gsα. It will be interesting to examine the effect of co-addition of half-maximal concentrations of agonist to determine if 50% Gsα is down-regulated at these concentrations - 25% by each agonist.

In previous studies (chapter 3) prostanoid induced down-regulation of Gs in NG108-15 cells (figure 3.15) was shown to follow an agonist dose-response curve which mirrored agonist occupancy curves for the receptor (figure 3.4), indicating the lack of "spare" receptors. This was not the same for the β2-adrenergic receptor in βN22 cells where approximately 100-fold less agonist is required to produce half-maximal Gsα down-regulation (figure 5.5) than is required for half-maximal receptor occupancy (figure 4.13) The combination of these observations demonstrates an apparent receptor reserve ("spare" receptors) for the β2-adrenergic receptor, but not the IP prostanoid receptor mediated Gsα down-regulation in the βN22 clone. "Spare" β2-adrenergic receptors were also noted in chapter 4 when the EC50 for adenylyl cyclase activation by isoprenaline in βN22 was 25-fold less than the concentration of isoprenaline required for half-maximal receptor occupancy. This may well be a reflection of the relative levels of the

receptors and Gs. A stoichiometry of down-regulation of Gs by the IP prostanoid receptor of approximately 8 pmoles Gs: 1 pmole receptor was calculated at all levels of receptor occupancy (chapter 3). If the β2-adrenergic receptor were to show similar G-protein activation stoichiometry, it might be anticipated that the entire pool of available Gs would be activated by agonist occupation of only some 25-35% of the available receptors, and as such the dose response curve for G-protein activation and down-regulation might be anticipated to be to the left of the receptor occupancy curve, as was indeed the case.

Preliminary data showed an approximate 20-25% down-regulation of the β2-adrenergic receptor on prolonged isoprenaline treatment of βN22 cells (table 5.5b). This was approximately 1000 fmoles receptor. If the stoichiometry of β2-adrenergic receptor: Gsα down-regulation in clone βN22 was similar to that of the IP prostanoid receptor and Gs in wild-type NG108-15 cells, it would be anticipated that 1000 fmoles β2-adrenergic receptor would down-regulate 50-70% total cellular Gsα, as indeed was the case. In βN17, although there was no apparent down-regulation of Gsα on isoprenaline treatment there was a marked 80% down-regulation of β2-adrenergic receptor (table 5.5a). This represents some 240 fmoles β2-adrenergic receptor and it might be anticipated, that there would be an approximate 10% concurrent down-regulation of Gsα on isoprenaline treatment of βN17 cells which would be very difficult to detect by western blotting and scan analysis. We must, however, be wary of drawing strong conclusions from a single point binding assay using a single concentration of antagonist and competing drug. Although the change in specific antagonist binding is most likely to be the result of a physical loss of the receptor polypeptide from the plasma membrane, this may not necessarily be the case. Antagonist binding has been shown

to vary depending on the coupling state of the receptor with the G-protein (Wregget and DeLean, 1984; Schutz and Freissmuth, 1992 and references therein). Saturation binding analysis must therefore be performed in both control and isoprenaline treated βN22 and βN17 cells to affirm if the apparent loss of β2-adrenergic receptor from the plasma membrane of these cells, on isoprenaline treatment, is a physical loss of the polypeptide or simply the result of some affinity change in the receptor.

No detectable release of Gsα to the cytosol was observed upon iloprost and isoprenaline treatment of βN22 cells (figure 5.9), contrary to observations by Ransnas and co-workers (1989), who demonstrated a 50% increase in cytosolic Gsα in S49 lymphoma cells treated with isoprenaline. However, Haraguchi and Rodbell (1990) showed that isoprenaline treatment of rat adipocytes redistributed Gs into low density membrane fractions, suggesting the internalisation of Gs in discrete membrane vesicles in S49 lymphoma cells. In both wild-type and transfected NG108-15 cells Gsα may be internalised either separately, or with the respective receptor, on agonist exposure and subsequently degraded. Certainly, as discussed in chapter 3, the coupling of the receptor and G-protein has been shown to be necessary for receptor down-regulation to occur in several systems, even with receptors which do not down-regulate their respective G-protein on prolonged agonist exposure (Gonzales *et al*, 1989; Rich and Iyengar, 1990). It may well be that the combination of receptor and G-protein α-subunit in NG108-15 cells is the target for cellular down-regulation.

Homologous desensitisation of prostanoid mediated adenylyl cyclase activity was observed in both clones, βN22 (table 5.3) and βN17

(table 5.4), essentially identical to wild-type NG108-15 cells. Homologous desensitisation of isoprenaline mediated adenylyl cyclase activity occurred in βN22 (figure 5.14) and βN17 cells, although further characterisation, through dose response curves is necessary in βN17 membranes. Inclusion of 20mM manganese chloride in the adenylyl cyclase assay revealed essentially identical levels of basal adenylyl cyclase activity in both control and isoprenaline treated βN22 cells. No functional modification of the catalytic subunit of adenylyl cyclase occurred on isoprenaline treatment, indicating that the decrease in basal adenylyl cyclase activity on isoprenaline stimulation appeared to be a result of either the loss of membrane associated Gsα, or a combination of the loss of Gsα and β2-adrenergic receptor which tonically stimulates adenylyl cyclase (table 5.2).

In summary, receptor number appears to be pivotal in determining Gsα down-regulation on chronic isoprenaline exposure of transfected NG108-15 cells. NG108-15 cells expressing 4000 fmoles β2-adrenergic receptor down-regulate Gsα on prolonged isoprenaline exposure, in a time and concentration dependent manner, whereas NG108-15 cells expressing only 300 fmoles β2-adrenergic receptor do not. Preliminary studies on the β2-adrenergic receptor levels after isoprenaline treatment in βN22 cells suggest a similar stoichiometry of receptor:Gsα down-regulation as does the IP prostanoid receptor and Gsα in wild-type NG108-15 cells. Although homologous desensitisation of adenylyl cyclase, by both iloprost and isoprenaline, has been demonstrated in both clones βN22 and βN17, the role of Gsα in heterologous desensitisation of adenylyl cyclase in both these cell lines can now be examined in these model systems.

CHAPTER 6 - CONCLUSIONS

Chapter 6 - discussion

The mechanism of agonist-mediated, G-protein α-subunit down-regulation has been and is presently being widely studied. As discussed more fully in the previous chapters, control of protein turnover appears to be the major contributing factor to the down-regulation of the α-subunit, however the role that the receptor plays in this process has not been explored in great depth. Studies presented in this thesis demonstrate that the level of receptor expression and, indeed, the degree of receptor occupancy by agonist, determine the amount of G-protein α-subunit down-regulated. Dose response curves of both iloprost and isoprenaline treatment of wild-type and transfected NG108-15 cells respectively show at low receptor occupancy with agonist relatively small amounts of Gsα are down-regulated, whereas at a high degree of receptor occupancy there is a large amount of Gsα down-regulation (chapter 3). Also, NG108-15 cells expressing relatively low levels of the β2-adrenergic receptor (βN17 cells) do not show a down-regulation of Gsα on agonist treatment, whereas a high level of β2-adrenergic receptor expression (βN22 cells) results in a marked (50%) Gsα down-regulation on agonist treatment (chapter 5).

The mechanism whereby the receptor influences the levels of Gsα down-regulation has not been unequivocally determined, however, the stoichiometry of IP prostanoid receptor:Gsα down-regulation has been calculated to be approximately 1:8-10 at various degrees of receptor occupancy, indicating that as receptor occupancy by agonist increases so does the amount of Gsα down-regulated. Although this ratio may not be identical to the ratio of IP prostanoid receptor:Gsα activation, it appears that G-protein activation is a prerequisite for the down-

regulation of Gsα. Levis and co-workers (1992) demonstrated that the activation of Gs resulted in a conformational change which increased the susceptibility of the protein (Gsα) to proteolysis. The enhanced proteolysis of the G-protein α-subunit upon agonist exposure does not appear to be dependent on its activation specifically by the receptor, because activation of Gsα by cholera toxin has also resulted in an increased turnover of the Gsα polypeptide (Chang and Bourne, 1989). The enhanced proteolysis appears, therefore, to rely on the prior activation of the G-protein. Since the amount of G-protein activated will increase with a higher degree of receptor occupancy with agonist, there will be an increased susceptibility to proteolysis and eventually cellular down-regulation.

As well as the activation induced conformational change of Gα, the addition or removal of covalent modifications of the G-protein α-subunit have also been implicated in targetting the protein for proteolysis. These include ADP-ribosylation and palmitoylation (as discussed in chapter 1). In addition, several cases of prolonged activation of the G-protein through receptor stimulation have resulted in the association of Gα with another unidentified polypeptide, which may function to signal the protein for an enhanced proteolysis (Takahashi *et al*, 1991; Mollner *et al*, 1992; Negishi *et al*, 1992).

Prostanoid treatment of NG108-15 cells resulted in a concurrent down-regulation of IP prostanoid receptor and Gsα. There was no apparent loss of functional adenylyl cyclase from the plasma membrane on agonist treatment, thus it appears that the receptor and G-protein α-subunit are the only known elements of the membrane associated signal transduction cascade internalised. It is still unclear if the receptor and

Gsα are taken up in the same endocytic vesicle and degraded together. Alternatively it is possible that they may be taken up separately or there may be a branching point after internalisation of both proteins, whereby receptor and G-protein are "sorted" either for degradation or alternative intracellular functions.

Rodbell's "programmable messenger hypothesis" originally arose from the observation that G-protein α-subunits were not irreversibly associated with the plasma membrane (Rodbell, 1985). It postulates that G-protein α-subunit release from the plasma membrane could potentially result in the activation of non-plasma membrane bound effectors in discrete cellular compartments (Rodbell, 1985). Gs has been identified in discrete vesicles subcellularly (Haraguchi and Rodbell, 1990; Svoboda *et al*, 1992), and functions in addition to the activation of adenylyl cyclase have also been detected (as discussed in section 1.4.1). It is uncertain if these additional functions are connected to the subcellular localisation of Gs, although it may be the case that the internalisation of Gsα on prolonged agonist exposure results in the activation of intracellular effectors before Gsα is degraded.

As well as a potential role for activating subcellular effector proteins, the internalisation and subsequent down-regulation of Gsα has been heavily implicated in heterologous desensitisation of adenylyl cyclase activity (as discussed in depth in chapter 3). NG108-15 cells transfected with the β2-adrenergic receptor (βN22 cells) represent an ideal system in which to study the role of Gsα down-regulation in the heterologous desensitisation of adenylyl cyclase. Preliminary studies, examining the effect of dual agonist (isoprenaline and iloprost) treatment on Gsα levels in βN22 cells, revealed a less than additive loss

of Gsα from the plasma membrane when compared to individual agonist treatment suggesting that the interaction of Gsα with its receptor may be restricted in some way, perhaps through cytoskeletal restraints or differential βγ dimer affinities which serve to arrange the plasma membrane associated Gsα subunit into separate "pools" within the plasma membrane. Recent evidence, from Kim and co-workers (1994), reveals that in NG108-15 cells a 70-fold molar excess of Gsα over Gsα-adenylyl cyclase complexes exists, suggesting that the interaction of Gsα and adenylyl cyclase is also limited, perhaps for similar reasons to the interaction of receptor and Gsα.

Using transfected NG108-15 cell lines, expressing the β2-adrenergic receptor (βN22 and βN17), the role of Gsα down-regulation in heterologous desensitisation of adenylyl cyclase can be examined in greater detail, using agonist treatment of the β2-adrenergic receptor and the IP prostanoid receptor. Although more difficult to explore, the identification of "sub-pools" of Gs which distinguish in their interaction with receptor and effector, can potentially be investigated in the transfected cell lines using both inhibitors of cytoskeletal elements and radiolabelled agonist binding studies in a similar manner to Graeser and co-workers (1993).

APPENDICES

Appendix 1 [^{3}H]-PGE1 binding analysis

Maguire and co-workers (1976) first observed that guanine nucleotides decreased the affinity of the β2-adrenergic receptor for agonist. Furthermore, competition curves whereby radiolabelled antagonist is competed by agonist gives a pseudo Hill coefficient of less than 1.0 indicating the presence of more than one affinity state of the receptor (DeLean *et al*, 1980) An agonist has thus been shown to bind with high affinity to a receptor that is coupled to its G-protein and with low affinity to a receptor that is not coupled to its G-protein. Using a radiolabelled agonist to quantify the amount of receptor expressed in a cell is therefore problematic as the measured amount of receptor detected will depend on the affinity state of the receptor at the time of the assay. The affinity of a receptor for antagonist, on the contrary, has not been shown to decrease in the presence of guanine nucleotides, although small increases in affinity have been observed in some cases (Schutz *et al*, 1992). It is therefore more accurate to use a radiolabelled antagonist, than agonist, to determine receptor number.

There is, however, no commercially available radiolabelled antagonist specific for the IP prostanoid receptor. It has therefore been necessary to use the [^{3}H]-agonist, [^{3}H]-PGE1, to determine the levels of IP prostanoid receptor expression in NG108-15 cells. DeBlasi and co-workers (1989) have derived the equations necessary to estimate the Kd and Bmax of a receptor population using a radiolabelled agonist and competing with the same unlabelled agonist. There are two ways of performing the experiments.

l(i) Competitive binding

Competition binding is performed by taking a single concentration of radioligand and competing it with increasing concentrations of the same unlabelled ligand. A displacement curve can then be plotted, producing three values: the top plateau (total binding of radioligand without competitor); the bottom plateau (non-specific binding of the ligand); and the IC50 (the concentration of unlabelled ligand that displaced half the specific binding of the radioligand). Subtracting the value (cpm) of the bottom plateau from that of the top plateau yields the specific binding (Bo). Derivations from the equation of Cheng and Prussoff (1973) (1) allow the Kd and Bmax to be calculated from these values (Kc is the Kd of the unlabelled ligand, and Kh is the Kd of the radiolabelled ligand).

$$IC50 = Kc(1+L/Kh) \quad (1)$$

If it is assumed that the affinity of the radioligand for the receptor is the same as that of unlabelled ligand (Kc=Kh=Kd) then the Kd can be calculated using equation 2, where L is the concentration of radiolabelled ligand used.

$$Kd = IC50 - L \quad (2)$$

According to the law of mass action:

$$Bmax = Bo(Kd+L)/L \quad (3)$$

By substituting equation 3 with equation 2 the Bmax can be calculated using equation 4.

$$Bmax = Bo\ IC50 / L \qquad (4)$$

To sum up, the equations 1-4 allow the calculation of receptor number and affinity from the measured values Bo and IC50 obtained from the competitive binding data. These equations are based on four assumptions.

1. The labelled and unlabelled ligands must have an identical affinity for the receptor.
2. There must be no cooperativity between ligand binding sites.
3. Only a small fraction of the total ligand is bound to the receptors so that the free [ligand] = total [ligand].
4. Only one class of affinity site exists. Because agonist binding is being examined it is highly likely that the receptor is present in two affinity states, coupled and uncoupled to a G-protein. The agonist binding experiments were therefore performed in the presence of a high concentration of Mg^{2+} (20mM). Under such conditions all the receptors in the untreated cell membranes are converted into a high affinity state for agonists (Costa *et al*, 1990) and hence are all presumably coupled to a G-protein.

The mechanism by which magnesium ions mediate this effect is currently unknown and open to debate. It is well-established that Mg^{2+} is a requirement for G-protein function (Iyengar and Birnbaumer, 1982), however the concentration of Mg^{2+} required for GTPase activity is vastly different from the concentration required for hormonal stimulation of

adenylyl cyclase (5nM and 10μM, respectively), which may indicate that Mg^{2+} can affect G-protein function by two distinct mechanisms (Gilman, 1987). When GTP or GTPγS is bound to the G-protein α-subunit, the interaction of Mg^{2+} at the active site occurs with high affinity, and is presumably sufficient for G-protein activation (Higashijima *et al*, 1987), however the presence of high Mg^{2+} concentrations seems to be a requirement for dissociation of GDP, association of GTP and subunit dissociation (Brandt and Ross, 1986).

Neomycin, an inositol phospholipid binding aminoglycoside antibiotic can exert a similar effect on the binding of agonist to receptor as Mg^{2+} (Herrmann *et al*, 1989). In contrast to magnesium, neomycin did not induce G-protein activation in the presence of GTP, suggesting that the induction of high affinity binding was not a consequence of an ability to promote G-protein dissociation. The effect of neomycin was also noted for two other aminoglycoside antibiotics, gentamycin and streptomycin. These cationic compounds are commonly employed as inhibitors of phospholipid hydrolysis (Cockcroft and Gomperts, 1985) and have been shown to specifically interact with inositol phospholipids, an ability also observed for magnesium (McLaughlin and Whitaker, 1988). It is possible to speculate that the induction of a high affinity state of receptor for agonist is not as a consequence of a direct interaction of Mg^{2+} with either G-protein or receptor, but instead as a result of the binding of magnesium and neomycin to the membrane lipid components, to produce an alteration in the local lipid environment surrounding the receptor and G-protein which facilitates the coupling of G-proteins to agonist bound receptors (Herrmann *et al*, 1989).

l(ii) Saturation binding

Saturation binding analysis can be performed on membranes using a very similar protocol to the competition binding data. A single, non-saturating, concentration of [^{3}H]-PGE1 (10nM) and various concentrations of unlabelled PGE1 (0-90nM) are incubated with membranes and the binding assay performed as described in section 2.7. The results are calculated by dividing the specific binding by the specific radioactivity of the ligand. This yields the number of receptors occupied by both labelled and unlabelled ligand. The data can then be manipulated according to Scatchard (1949) and the Bmax and Kd of the receptor population calculated.

The saturation binding analysis generates values with large errors, as the large number of binding sites (of labelled and unlabelled ligand) at high concentrations of unlabelled ligand is derived by dividing a few specific cpm by a very low specific activity. However, DeBlasi and co-workers (1989) compared the efficiency of both saturation and competitive binding analysis using the same amount of radioligand in each case. The accuracy of either method decreased as the amount of non-specific binding increases, but for any particular value of non-specific and total binding the accuracy of either method was essentially identical.

Appendix 11 Transfection of mammalian cells with plasmid DNA

The introduction of genes into mammalian cells and their subsequent expression requires the insertion of the gene into a mammalian expression vector. One of the most commonly used expression vectors is the plasmid. Plasmids usually contain prokaryotic sequences that facilitate the propagation of the vector in bacteria. A gene encoding antibiotic resistance will permit the selection of bacteria that harbour the recombinant plasmids, allowing their growth and DNA multiplication and extraction. A limited number of unique restriction sites in non-essential regions of the plasmid are also required to be present to allow the insertion of DNA sequences. The most basic eukaryotic expression vector contains a eukaryotic promoter element to mediate transcription of foreign DNA sequences and also the signals necessary for the efficient polyadenylation of the transcript (Sambrook *et al*, 1989).

Unlike the signals for RNA processing which can function efficiently in all mammalian cells, the activities of transcription controlling elements can vary considerably between different cell types. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription. The combination of different recognition sequences and varying levels of expression of transcription factors determines the efficiency with which a given gene is transcribed in a particular cell type. The simian virus 40 (SV40) early gene promoter is active in a variety of tissues and for this reason vectors incorporating this promoter have been widely used (Sambrook *et al*, 1989).

II(i) The simian virus 40 (SV40) promoter

The SV40 origin of replication comprises of a coding region of approximately 300 base pairs. The early and late genes are transcribed in opposite directions, and hence opposite strands, from the origin of replication. The early gene region of SV40 is transcribed immediately after viral infection. This gene encodes for two proteins, through differential RNA splicing, the small T antigen and the large T antigen. The large T antigen represses its own synthesis by binding to the mRNA start site for early gene expression. It also activates late gene transcription by binding to the late SV40 promoter. The large T antigen binds with a weaker affinity to the site of late gene activation than to the early promoter, therefore it first shuts off its own synthesis and then activates late gene transcription (McKnight *et al*, 1981).

Both the SV40 early and late promoters have been constructed into vectors and used to control the expression of genes in a variety of mammalian cell lines. The use of SV40 origin containing transient expression vectors was greatly facilitated by the development of COS-1 cells that were derived by the transformation of simian CV-1 cells with an origin defective SV40 genome. COS-1 cells constitutively express the wild-type SV40 large T antigen and contain all of the cellular factors necessary to drive the replication of SV40 origin containing plasmids (Gluzman *et al*, 1981). High levels of gene expression can result if the cDNA to be expressed is cloned into a plasmid under the control of a SV40 late promoter, and transfected into COS-1 cells. The constitutive production of the large T antigen in COS-1 cells will overexpress the cloned gene by activating the late SV40 promoter.

II(ii) Transient expression of the β2-adrenergic receptor in COS-1 cells

A cDNA for the human β2-adrenergic receptor (figure 4.1) was cloned into the plasmid pSVL (figure 4.2) downstream of the SV40 late promoter and transiently transfected into COS-1 cells as follows.

The cDNA for the human β2-adrenergic receptor was cloned into the unique Eco R1 restriction site (5'-3' with respect to the T7 promoter) of the plasmid pGEM-7Zf (+). To excise out the cDNA for the β2-adrenergic receptor, the pGEM-7Zf (+) plasmid was digested with the restriction enzymes Xho1 and BamH1 (section 2.14). The restriction digest was run on a 1% (w/v) agarose gel (section 2.11) and the band corresponding to the human β2-adrenergic receptor cDNA (2305 base pairs) "Genecleaned" (section 2.19).

The plasmid pSVL was also digested with Xho1 and BamH1. The cut pSVL and "Genecleaned" human β2-adrenergic receptor cDNA were then ligated together (section 2.14), and the ligation reaction transformed into competent *E.Coli* (section 2.16, 2.17), which were subsequently plated out on ampicillin (25μg/ml) bactoagar plates. The DNA was extracted from ampicillin resistant colonies of *E.Coli* (section 2.18) and restriction digests performed on the plasmid DNA with Pst1 and BamH1 (section 2.14) to determine the orientation of the insert in the plasmid. Purified plasmid DNA was produced, using $CsCl_2$ centrifugation (section 2.20), from one of the colonies which contained the plasmid with the receptor cDNA insert present in the correct orientation. 10μg purified plasmid DNA was used to transiently transfect COS-1 cells using the Lipofectin reagent (section 2.22).

II(iii) Stable expression of the human β2-adrenergic receptor in NG108-15 cells

To ensure high levels of expression of a cloned gene it is necessary to use a very strong promoter to drive transcription and so drive high gene expression. Most promoters show a significant variation between different cell types. It is much more convenient to use a promoter that is highly active in all cells. Since β-actin is abundantly expressed in all non-muscle cells it was reasoned that the β-actin promoter may give a high level of gene expression in a wide range of cell lines. Gunning and co-workers (1987) evaluated the ability of the β-actin promoter to drive high level accumulation of antisense transcripts in a variety of human and rodent cell lines and discovered that the β-actin promoter was consistently as strong or stronger than the SV40 promoter. For this reason they constructed a plasmid which contained the β-actin promoter linked to unique restriction sites and a SV40 polyadenylation signal. This plasmid pJM16 (or LK444) (figure 4.4) also contained a neomycin resistant gene, controlled by the early SV40 promoter, suitable for selection of successfully transfected mammalian cells with geneticin sulphate (G418).

Successful transfection of NG108-15 cells with a cDNA for a P400 protein encoding an IP_3 receptor was shown by Furiuchi and co-workers (1989). The cDNA used was cloned into a modified version of the plasmid pbact-CAT9 (Supattapone *et al*, 1988) downstream of the β-actin promoter and the plasmid then transfected into NG108-15 cells. A moderate level of expression of the IP_3 receptor was obtained. NG108-15 cells were therefore able to express the necessary control elements to initiate transcription from the β-actin promoter. It was thus decided to

use this promoter for expressing the human β2-adrenergic receptor in NG108-15 cells. The cDNA for the human β2-adrenergic receptor was cloned into the plasmid pJM16 and transfected into NG108-15 cells as described below.

pJM16, or LK444 (figure 4.4), was digested (section 2.14) with the restriction enzymes BamH1 and Sal1 (which is compatible with a Xho1 site during ligation reactions). "Genecleaned" β2-adrenergic receptor cDNA [as prepared for section ll(ii)], cut with Xho1 and BamH1, was ligated into the digested pJM16 (section 2.15). Competent *E.Coli* were transformed with the ligation reactions (section 2.16, 2.17), spread on ampicillin (25μg/ml) bactoagar plates and ampicillin resistant colonies of *E.Coli* were selected. Plasmid DNA was extracted from the bacterial colonies (section 2.18) and run on a 1% (w/v) agarose gel (section 2.11) to determine the presence of the cDNA insert. The plasmid DNA from a colony with an insert present, and thus in the correct orientation, was purified using $CsCl_2$ centrifugation (section 2.20). 10μg purified plasmid DNA was then stably transfected (section 2.22) into NG108-15 cells using the Lipofectin reagent. Cells were grown in the presence of 0.8mg/ml geneticin, the minimum concentration determined to kill all wild-type NG108-15 cells.

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