G-PROTEIN SPECIFICITY

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DOCTOR OF PHILOSOPHY

by

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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
App(NH)p	adenylyl 5' imidodiphosphate
ATP	adenosine 5' triphosphate
BSA	Bovine serum albumin
cAMP	adenosine 3', 5'-cyclic monophosphate
DADLE	[D-ala$_2$, leu$_5$] enkephalin
DALAMID	[D-ala$_2$, met$_5$] enkephalinamide
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethylsulphoxide
EDTA	Ethylenediaminetetraacetic acid
FCS	foetal calf serum
GDPβS	guanosine 5'-0-(2-thiodiphosphate)
GDP	guanosine 5' diphosphate
Gpp(NH)p	guanylyl 5' imidodiphosphate
G-protein	Guanine nucleotide binding protein
GTPγS	guanosine 5'-0-(3-thiotriphosphate)
GTP	guanosine 5' triphosphate
NAD	nicotinamide adenine dinucleotide
NRS	normal rabbit serum
PBS	phosphate buffered saline
TBS	tris buffered saline
TEMED	N,N,N',N' tetramethylethylenediamine
TCA	tricholroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
TTBS	Tris buffered saline with Tween 20
Tween 20	polyoxyethylenesorbitan monolaurate

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Summary

G-proteins are central mediators of the signal transduction process, conveying information from agonist activated receptor to intracellular effector. However, it is unclear whether G-proteins function in a specific manner, or are promiscuous and able to interact with a wide variety of transmembrane receptors and effectors.

In an attempt to analyse G-protein specificity, the interaction of receptors with G-proteins in the neuroblastoma x glioma NG108-15 cell line was examined. As a means of identifying individual members of the G-protein family, a series of antipeptide antisera were generated against synthetic peptides corresponding to the C-terminal region of the various G-protein α-subunits. These antisera were demonstrated to be specific in their ability to recognise individual members of the G-protein family and thus allowed the identification of the pertussis toxin sensitive G-proteins; Gi2, Gi3, and Go in the NG108-15 cell line. In addition, two forms of the cholera toxin sensitive G-protein, Gs were expressed. However it was not possible to detect expression of Gi1. NG108-15 cells express δ-opioid receptors which function to inhibit adenylyl cyclase in a manner attenuated by prior treatment with pertussis toxin, and a poorly defined growth factor receptor which does not functionally interact with adenylyl cyclase. In membranes derived from NG108-15 cells, agonist activation of the both the δ-opioid receptors and growth factor receptors by DADLE and foetal calf serum respectively, stimulated GTPase activity through activation of separate species of pertussis toxin sensitive G-protein, as assessed by additivity experiments. This demonstrated that tight regulation of receptor-G-protein coupling occurs in membrane systems.

In an attempt to determine which pertussis toxin sensitive G-protein(s) is activated by the δ-opioid receptor, and thus ascertain which G-protein functions to inhibit adenylyl cyclase, a variety of approaches were adopted.

Firstly, although 'Gi' is classically a substrate for pertussis toxin, it is possible to catalyse the ADP-ribosylation of 'Gi' with cholera toxin, when suitable conditions are employed, namely the absence of added guanine nucleotide. In NG108-15 cell membranes, cholera toxin catalysed ADP-ribosylation of 'Gi' was stimulated in a dose-dependent manner by the addition of the δ-opioid agonist, DADLE, thus providing a means of identifying the G-protein which interacts with the δ-opioid receptor.

Secondly, the C-terminal region of the G-protein α-subunit is postulated to be the domain of interaction with receptor. In an attempt to block interaction of the δ-opioid receptor with the G-protein with which the receptor interacts, NG108-15 cell membranes were preincubated with IgG fractions isolated from the various G-protein specific antisera. Functional uncoupling of the δ-opioid receptor from both GTPase stimulation and inhibition of adenylyl cyclase was achieved by preincubation with an IgG fraction isolated from antiserum AS7, an antiserum which specifically recognizes Gi2 in this cell line. Preincubation with IgG fractions isolated from either Gi3, Go or Gs specific antisera were ineffective. Preincubation with an IgG fraction isolated from preimmune serum was also ineffective.

Thirdly, pertussis toxin catalysed ADP-ribosylation of Gi attenuates productive coupling between receptor and Gi, leaving the receptor in a state with lowered affinity for agonist. Preincubation of NG108-15 cell membranes with an IgG fraction isolated from antiserum AS7 produced the same functional consequence as pertussis toxin pretreatment. As before, preincubation with IgG fractions derived from antisera specific for Gi3, Go, Gs or preimmune serum were ineffective.

These data demonstrate that in cell membranes, receptors are specific in their ability to activate G-proteins, the δ-opioid receptor functions through activation of Gi2, and that Gi2 must therefore be the G-protein which mediates inhibition of adenylyl cyclase in NG108-15 cell membranes.

Chapter 1

Introduction.

1.1 Historical perspectives

The control of cellular activity is dependent upon the concerted action of a variety of hormones, neurotransmitters and growth factors which bind to specific receptors traversing the plasma membrane. The mechanism by which the binding of a suitable ligand to a transmembrane receptor exerts an intracellular effect is currently unclear and is the subject of much endeavour. Pioneering work by Sutherland and Rall on the effects of glucagon and adrenaline in liver homogenates, demonstrated that in the presence of hormone, a heat stable factor was produced which was able to activate phosphorylase in the supernatant fraction, whilst glucagon and adrenaline were without effect (Rall et al., 1957). The heat stable factor was identified as being an adenine ribonucleotide and was designated 3,5 AMP (Sutherland and Rall, 1957). Further research identified 3,5 AMP as a cyclic monophosphate which is now termed 3',5' cyclic AMP (cAMP). The enzyme catalysing the production of 3,5 AMP from ATP was known as adenylyl cyclase. This data allowed the formulation of the second messenger hypothesis, whereby binding of hormone (primary messenger) to specific receptors on the plasma membrane elicited the production of a secondary messenger, (cAMP) which was functionally active and able to regulate intracellular events.

An insight into the transduction mechanism of hormonal binding to control of adenylyl cyclase came in 1971, when Rodbell and colleagues demonstrated that hormonal regulation of adenylyl cyclase in hepatocytes required guanosine triphosphate (GTP) (Rodbell et al., 1971a). This requirement had not been previously noted, due to heavy contamination of available preparations of ATP with GTP. The difference in activation of adenylyl cyclase produced by the addition of GTP and poorly hydrolysed analogues of GTP such as Gpp(NH)p and GTPγS, suggested the involvement of a guanylnucleotide binding site (Schramm and Rodbell, 1975). Both GTPγS and Gpp(NH)p were able to promote the persistent activation of adenylyl cyclase, which was in contrast to the effect of GTP, which produced a transient activation. This allowed speculation that the guanylnucleotide

binding site involved in the regulation of adenylyl cyclase activity was capable of binding and hydrolysing GTP. In 1976, this theory was corroborated by Cassel and Selinger, who demonstrated that hormonal stimulation of adenylyl cyclase in the turkey erythrocyte membrane system produced a concomitant increase in the rate of high affinity GTPase activity. The elucidation of a technique to measure high-affinity GTPase activity in membrane preparations (Cassel and Selinger, 1976) allowed the proposal of a mechanism for control of adenylyl cyclase whereby the coupling of a stimulatory hormone to receptor caused the exchange of GDP for GTP on a regulatory protein. The hydrolysis of GTP on the regulatory protein resulted in the termination of activation of adenylyl cyclase (Cassel and Selinger, 1977). The isolation of a GTP-binding protein from pigeon erythrocytes by Pfeuffer supported the earlier work and led to the concept that the transduction of information from receptor to effector required a GTP-binding protein (G-protein) (Pfeuffer, 1977). Such proteins are capable of binding and hydrolysing GTP.

In addition to 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, who 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 identification of the stimulatory and inhibitory G-proteins termed Gs and Gi respectively, was aided by the isolation of certain exotoxins isolated from cultures of both *Vibrio cholerae* and *Bordetella pertussis*. 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 was an ADP-ribosylation, similar to that already detailed for the action of diptheria 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.

In a similar manner, pertussis toxin, which is also termed Islet activating protein (IAP), isolated from *Bordetella pertussis*, was shown to produce alterations in receptor mediated control of cyclic AMP production (Katada and Ui, 1979, 1981). Initial experiments with this toxin in rat C6 glioma cells demonstrated an enhancement of GTP activation of adenylyl cyclase, concomitant with the transfer of ADP-ribose from 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 polypeptide as the putatively proposed, but 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 effect of pertussis toxin catalysed ADP-ribosylation of Giα is to prevent productive coupling between receptor and 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 observation led to the prevalent hypothesis that any event which is attenuated by prior treatment with pertussis toxin, was indicative of a role for Gi in mediating the response, however 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 protein, as opposed to a guanine nucleotide binding site on the catalytic moiety of 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 system to dissect the adenylyl cyclase system since an increase in intracellular cyclic AMP 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 cyclic AMP (Bourne et al., 1975). Initially designated AC-, later cyc-, the clone was later shown to be deficient in Gs, and thus provided 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 had been inactivated (Ross and Gilman, 1977).

Purified Gs was shown initially to be composed of α subunits of either 52 or 45kDa and a 35kDa β-subunit (Northup et al., 1980). 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)

Parallel work on the mechanism of visual transduction in retinal photoreceptor rod cells led to the purification of a heterotrimeric G-protein termed transducin, which serves to couple the light receptor rhodopsin to cyclic GMP phosphodiesterases (Baehr et al., 1982). The ease of preparation of rod outer segment membranes and the comparative abundance of transducin, which may represent over 5% of the total membrane protein in rod outer segment membranes (Fung, 1985) allowed the purification of quantities large enough to.allow biochemical studies (Fung, 1983), generation of specific antisera, and amino-acid sequencing of the three G-protein subunits (Hurley et al., 1984a; Sugimoto et al., 1985; Fong et al., 1986).

Initial attempts at purification of Gi employed methods which were essentially identical to those employed for purification of Gs, since Gi comigrated with Gs during the initial chromatographic steps (Bokoch et al., 1983). Pertussis toxin catalysed ADP-ribosylation was used as a specific means of identifying purified Gi. Using tissues such as rabbit liver (Bokoch et al., 1983), and human erythrocytes (Codina et al., 1983) it was possible to resolve an apparently single polypeptide, although of apparently dissimilar molecular weight (41kDa and 39kDa respectively). The purified rabbit liver Gi was demonstrated to be functionally able to inhibit adenylyl cyclase, when reconstituted into S49 cyc- cell membranes, which suggested that the 41kDa polypeptide was indeed the G-protein involved in the inhibitory regulation of adenylyl cyclase (Katada et al., 1984a,b,c)

1.4 G-protein heterogeneity

Initial demonstrations that more than a single pertussis toxin sensitive G-protein existed came from attempts to purify 'Gi' from brain. Three groups of workers noted that purified preparations of pertussis toxin substrates from brain contained either two (Sternweis and Robishaw, 1984; Milligan and Klee, 1985) or

three (Neer et al., 1984) polypeptides in the 39-41kDa range along with β,γ subunits.

Subsequently, several additional heterotrimeric G-proteins have been identified and purified. Measurement of high-affinity specific guanine nucleotide binding in brain suggested that brain might be a rich source of GTP-binding proteins (Sternweis and Robishaw,1984). This was confirmed by the purification of a novel G-protein, Go, which comprised approximately 1 to 2% of total brain membrane protein (Sternweis and Robishaw, 1984; Neer et al., 1984; Milligan and Klee, 1985). In addition to these studies, immunochemical analysis of neutrophil plasma membranes, using antisera derived from injecting rabbits with purified transducin, suggested the existence of novel forms of pertussis toxin sensitive G-proteins (Gierschik et al., 1986a). It was thus becoming apparent that the G-protein family was not restricted to Gs, Gi and transducin, and that additional heterogeneity was evident. The fact that Go co-purified with Gi from brain, suggested that the increasing variety of G-proteins might share structural similarities (Milligan and Klee, 1985).

1.5 Mechanism of action of G-proteins

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 Sellinger were able to propose a model to account for G-protein function which is still applicable (Cassel and Selinger, 1978). The 'classical' G-proteins are thought to have a mechanism of action which is intrinsically similar; the binding of ligand to its receptor causes the α-subunit of the specific G protein to lose its bound GDP and bind GTP (Brandt and Ross, 1986). This results in the dissociation of Gα from its β,γ subunits in a Mg^{2+}-dependent process (Iyengar and Birnbaumer, 1982). The binding of GTP reduces the affinity of the receptor for agonist, resulting in dissociation of the ternary complex (see later). This permits one receptor to re-cycle and activate many G-

proteins (Pedersen and Ross, 1982) The Gα-GTP is now in an activated state and may interact with a specific effector protein. After this interaction, GTP is hydrolysed by the α-subunit's intrinsic GTPase activity to GDP (Rodbell, 1980). Gα-GDP is now in an inactive form and can re-associate with its β,γ subunits. This system is cyclical and dependent on the presence and binding of ligand, GTP and Mg^{2+} (Bourne, 1986) (Figure 1.1).

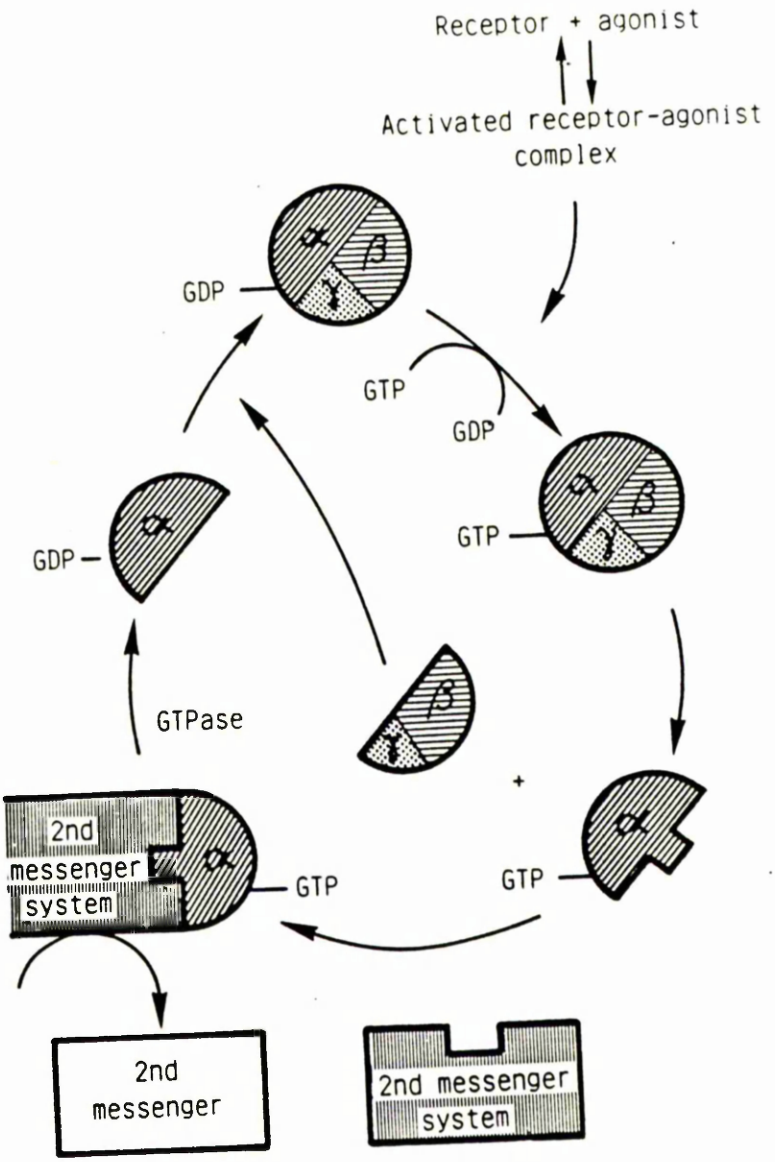
The dissociation which follows binding of GTP, means that the activation process is essentially irreversible (Fung, 1985), however it has not yet proved possible to demonstrate the dissociation of the α-subunit from the β,γ subunits either in a membrane system, or in response to the G-protein's physiological ligand, GTP. In the absence of an agonist-receptor complex, the rate of dissociation of GDP limits the GTPase cycle, since the catalytic rate (kcat) is approximately ten-fold higher than the GDP-dissociation rate (Ferguson et al., 1986). The purified α-subunit of Gs, has a kcat of approximately 10 min^{-1} (at 30°C), thus an activated α-subunit has a lifespan of many seconds, a facet which may allow interaction and activation of a number of effector moieties, and hence amplification of the signal relayed by the binding of hormone to receptor.

G-proteins can be activated by the use of non-hydrolysable analogues of GTP, such as Gpp(NH)p and GTPγS in a reaction which is no longer dependent on agonist occupancy of receptor, but still requires magnesium (Codina et al., 1983). In addition, aluminium fluoride (AlF_4^-) is able to activate G-proteins by mimicking the terminal phosphate of GTP when GDP is in the G-protein guanine nucleotide binding site, thus removing the requirement for GDP-dissociation for G-protein activation (Bigay et al., 1985).

Fig. 1.1 The role of GTP binding and hydrolysis in the activation and deactivation of a G-protein.

The function of the G-proteins is to couple agonist activated receptors to intracellular effector systems. As this process is of limited duration, then the G-protein is required to undergo a cyclical pattern of activation followed by a deactivation. Figure 1.1 is a diagramatic representation of the mechanism of G-protein action, as described in section 1.5, and is reproduced from Milligan, (1988).

Figure 1.1


Receptor + agonist
Activated receptor-agonist complex
GDP
GTP
GDP
GTP
GDP
GTPase
GTP
GTP
2nd messenger system
2nd messenger
2nd messenger system

1.6 The identification of a receptor which functions through G-protein activation

G-proteins have been either identified or implicated in mediating a wide variety of transmembrane signals. This has led to an accepted rationale for determining whether a receptor of interest functions through G-protein activation. In addition to showing an absolute requirement for GTP (Rodbell et al., 1971; Litosch, 1987), together with the attenuation of a receptor-mediated response by pretreatment with either pertussis or cholera toxin, the remaining methods currently available for detecting G-protein involvement in mediating a receptors' response are ; a) ligand binding assays and, b) GTPase assays.

1.6(i) Ligand binding assays

The effect of guanine nucleotides on ligand binding to receptor was firstly noted by Rodbell et al. (1971b) , 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 et al. (1976) were later able to demonstrate that in rat C6G1A glioma cell membranes, the binding of β-adrenergic agonists, but not antagonists was 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 are steep with 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 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, wheras 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 et al. (1980) proposed a model involving a ternary complex. In the unstimulated state, the inactive G-protein α-subunit (G-inact) may interact with 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 receptor.

Although the ternary complex model 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 et al., 1981) and led to the general conclusion that if a receptors' affinity for agonist is altered by the presence of guanine nucleotides, then that receptor functions through activation of a G-protein.

1.6(ii) Receptor stimulated GTPase activity

The steady state of GTP hydrolysis by the G-protein α-subunit is in the range of 0.2 to 0.5 min-1 (Gilman, 1987). When G-protein α-subunits are depleted of GDP, which is tightly bound under physiological conditions, and GTPase activity assessed, the rate of GTP hydrolysis is markedly increased. In kinetic experiments, the rate of association of GTPγS closely mirrors the rate of dissociation of GDP. These observation led to the hypothesis that the rate limiting step for G-protein activation is the dissociation of bound GDP (Ferguson et al., 1986). The role of the receptor is to accelerate the rate of guanine nucleotide exchange.

Apart from the visual transduction systems, receptor regulation of G-protein GTPase activity has mainly been characterised for receptors mediating the inhibition of adenylyl cyclase (Aktories et al., 1984). For many receptors known to function through G-protein activation, it has not been possible to assay GTPase activity. This is particularly true for receptors which function to stimulate the

rate of hydrolysis of inositol containing phospholipids in a pertussis toxin insensitive manner, and also for receptors which stimulate adenylyl cyclase activity through activation of Gs (Jakobs et al., 1984). Indeed, although GTPase activity was firstly identified as Gs mediated, it is only possible to measure receptor stimulated high-affinity GTPase activity by Gs in a very limited number of model systems studied, namely the turkey erythrocyte (Cassel and Sellinger, 1978) and the platelet (Houslay et al., 1986a). In contrast, stimulation of GTPase activity by receptors which interact with pertussis toxin sensitive G-proteins has been reported for a wide variety of membrane systems. The reason for this discrepancy may lie in the relative proportion of G-proteins which are present and able to interact with the receptor which is under scrutiny. In most signal transduction model systems, the expression of Gs is lower than that of Gi. (Ransnas and Insel, 1988). In addition the intrinsic GTPase activity of purified Gs is lower than that of purified Gi (Gilman, 1987). It is also conceivable that receptor number is a limiting factor since data exists to suggest that the greater the number of receptors present, the greater the GTPase stimulation obtained (see later). As such, receptor mediated stimulation of GTPase activity may indeed occur for all receptors which function through G-protein activation, but the proportion of substrate which is hydrolysed is too low for detection using current techniques of GTPase measurement.

Application of the above considerations has led to the identification of a wide variety of signalling pathways which are G-protein mediated. These include; the receptor mediated stimulation and inhibition of adenylyl cyclase, (Krupinski et al., 1989; Levitzki, 1986), the stimulation of cyclic GMP phosphodiesterases (Stryer, 1988) the stimulation of phosphoinositidase C (Litosch, 1987), the stimulation of phospholipase A2 (Burch et al., 1986), the inhibition of voltage operated Ca^{2+} channels (Yatani et al., 1987b; Rosenthal et al., 1988), and the stimulation of certain voltage operated K^{+} channels (Brown and Birnbaumer, 1988). In addition, less well characterised events such as secretion and exocytosis (Fernandez et al., 1984), olfactory transduction (Lancet and Pace., 1987), glucose transport (Schurmann et al., 1989) and Na^{+} channel activity (Cantiello et al., 1989) have been demonstrated to involve G-protein activation.

1.7 The mechanism of G-protein interaction with effector

The G-protein dissociation model was originally developed under the assumption that the G-protein α-subunit is the site of functional interaction with both receptor and intracellular effector. This implies that the intrinsic function of each G-protein is encoded by the α-subunit, with the β,γ subunits having a more passive role, being required to recognise the GDP-bound form of the α-subunit and to anchor the α-subunit to the plasma membrane (Sternweis, 1986). However, there is evidence to suggest that the β,γ complex is a requirement for interaction of Gα with receptor, since guanine nucleotide exchange is diminished in the absence of β,γ subunits (Cerione et al., 1985) and a receptor G-protein complex will not form in the absence of β,γ subunits (Fung, 1983). In the last few years it has become apparent that the β,γ subunits may fulfill a more complex role in the control of transmembrane signalling, (see below).

Although it has been appreciated that the function of Gi in transmembrane signalling is the transduction of agonist binding to inhibition of adenylyl cyclase (Klee et al., 1985). The mechanism by which this occurs remains an open question, with two main mechanisms of action being proposed. In the first of these proposals, the β,γ complex is thought to be primarily responsible for mediating the inhibition of adenylyl cyclase activity. Herein, Gi activation and dissociation would leave an increased concentration of free β,γ.in the membrane millieu. By mass-action, this increase in free β,γ would potentiate reassociation of any free Gsα with β,γ, effectively attenuating stimulation of adenylyl cyclase (Katada et al., 1984a,b,c). This hypothesis is supported by work on S49 wild type membranes, where the addition of purified β,γ complex results in a dose dependent inhibition of Gsα stimulated adenylyl cyclase activity. In addition, for this theory to be correct, one would expect to find Gi present in larger amounts than Gs. As such, activation of Gi would release a proportionately large amount of free β,γ complex which could de-activate Gsα. This has been shown to be the case (Gilman et al., 1987). Interestingly, the addition of purified unliganded Giα can actually activate adenylyl

cyclase activity in both platelet and S49 cell membranes, presumably by binding to β,γ subunits and shifting the equilibrium to release free Gsα (Katada et al., 1984a,b,c).

Secondly, it is possible that the Giα subunit inhibits cAMP production directly by acting at a specific regulatory site on adenylyl cyclase (Hildebrandt et al., 1983). Evidence for the latter has been obtained from work on S49 cyc^- cells where Gsα is not expressed but inhibition of adenylyl cyclase can occur through an inhibitory agonist, somatostatin (Ross and Gilman, 1977). This inhibition cannot be explained by a β,γ deactivation of Gsα. Interestingly, kinetic experiments have shown Gi to inhibit Gs stimulated adenylyl cyclase activity in a non-competitive manner, suggesting that if Giα is indeed able to directly inhibit adenylyl cyclase, it acts at a different site on adenylyl cyclase to that of Gsα (Hildebrandt et al., 1983). It is possible to reconcile both theories by suggesting that in wild type cells which express functional Gs, inhibition of adenylyl cyclase by Gi is a consequence of both direct α-subunit inhibition and β,γ subunit inhibition, thus implying a specific role for both Giα and the β,γ subunits in signal transduction.

The role of the β,γ subunits in the control of signal transduction has been the subject of several recent reports. Katada et al. (1987) have reported an interaction of the β,γ subunits with the calcium binding protein, calmodulin, which has previously been noted to stimulate specific forms of adenylyl cyclase. It is not known whether the interaction between the β,γ subunits and calmodulin is physiologically relevant, or if it may simply be explained by a hydrophobic interaction between the proteins. However, in the presence of Ca^{2+}, purified β,γ subunits were able to inhibit Ca^{2+}/calmodulin stimulation of adenylyl cyclase, but not Gs or forskolin stimulation, suggesting a broader role for β,γ subunits in the control of adenylyl cyclase activity.

In rod outer segment membranes, photon activation of phospholipase A2 activity has been shown to occur by a transducin-dependent mechanism, requiring dissociation of the heterotrimer. The addition of purified β,γ subunits to

transducin-depleted rod outer segments produced a stimulation of phospholipase A2 activity which was attenuated by addition of purified transducin α-subunits, suggesting a dual role for transducin in the stimulation of both cyclic GMP phosphodiesterases and phospholipase A2 (Jelsema and Axelrod, 1987).

A role for β,γ subunits as direct activators of certain K^+ channels has been proposed by Neer and co-workers, (Logothetis et al., 1987). Chick embryonic atrial cells contain muscarinic acetylcholine receptors which are involved in the opening of an inwardly rectifying potassium channel. The addition of poorly hydrolysable GTP analogues has been demonstrated to open the K^+ channel in the absence of receptor agonist. Pretreatment with IAP abolished agonist-induced channel opening which suggested the involvement of an IAP substrate such as Gi (Pfaffinger et al., 1985). Perfusion of embryonic chick atrial cells with purified Giα, Goα and β,γ subunits, unexpectedly showed that addition of β,γ subunits alone was enough to produce opening of the K^+ inflow channel, and that purified α-subunits from either erythrocytes (40kDa polypeptide), or brain (39kDa polypeptide), were also able to promote opening of the K^+ channel, but were less efficacious than purifed β,γ subunits from brain (Logothetis et al., 1987). The situation has been complicated by the work of Birnbaumer and colleagues, who have purified a G-protein from human red blood cells which is capable of opening a subset of potassium channels present in guinea pig atrial cells (Yatani et al., 1987a; Yatani et al., 1988a,b). Although originally termed Gk, the G-protein responsible has been identified as Gi3 (Codina et al., 1988), and when activated with GTPγS, opens K^+ channels in an essentially irreversible manner. Most experiments conducted in patch clamp systems have involved the addition of purified G-proteins to reconstitute receptor-mediated effects on the channel under study. However, many 'pure' G-protein samples may contain a heterogeneous population of α-subunits which cannot be resolved under standard SDS-polyacrylamide gel electrophoresis and may therefore be significantly impure. To circumvent this problem, Birnbaumer and colleagues have expressed recombinant Gα fusion polypeptides with an additional nine N-terminal amino-acids in *Escherichia coli*. The recombinants may comprise up to 10% of total cellular protein, enabling easy purification. Surprisingly, when either recombinant Giα

1,2 or 3 were activated with GTPγS and applied to atrial membrane patches, all three subunits were equally capable of opening K^+ channels (Yatani et al., 1988c). Controversy has continued subsequent to two reports which identify arachidonic acid metabolites produced by β,γ stimulation of phospholipase A2 (Kim et al., 1989), as being able to open K^+ channels in atrial cells (Kurachi et al., 1989), thus the identification of the G-protein which can interact with K^+ channels in atrial cells, together with the elucidation of the respective subunit which mediates the effect, remains to be resolved.

1.8 G-protein cloning and sequence homology

With improved G-protein purification protocols, sequence data quickly emerged, allowing the cloning of complementary DNA's encoding putative G-protein α-subunits The heterogeneity of the G-proteins thus identified structurally was rapidly becoming apparent, and to date there are at least 30 amino-acid sequences corresponding to at least 9 different classes of α-subunits (Table 1.1).

Amongst the best characterised G-proteins are; Gs, transducin and Gi. Two forms of transducin (Td) occur, Td1 and Td2, which are encoded by separate mRNA's, are found in rod and cone cells respectively (Lerea et al, 1986) and serve to couple rod and opsins to cyclic GMP phosphodiesterases. The two forms of transducin show a very limited distribution and it has been suggested that one transducin variant is involved in mediating colour vision, whilst the other mediates mono-chromatic vision. (Stryer, 1988). Gs and Gi are thought to be ubiquitously expressed and function to couple various receptors to the stimulation and inhibition of adenylyl cyclase respectively. At least four forms of Gsα, exist which are obtained from alternate splicing of a transcript produced from a single gene (Bray et al., 1986). These have been grouped into two subtypes which are termed long and short Gsα. The cDNA's that encode two of the forms of Gsα have been isolated from a bovine adrenal cDNA library and are identical except for a

Table 1.1 The form and function of the α-subunits of the 'classical' G-proteins.

The 'classical' G-proteins can be defined according to their sensitivity to either pertussis toxin or cholera toxin (section 1.2), their function (section 1.6ii) and their distribution (section 1.8). Table 1.1 provides a summary of the form and function of the 'classical' G-proteins, and is taken from Milligan (1989).

Table 1.1 The structure and function of the α subunits of the 'classical' G-proteins.

G-Protein	Sensitivity to ADP-ribosylation by bacterial toxins	Function	Distribution
Gs	Yes, cholera toxin	stimulation of adenylyl cyclase, activation of dihydropyridine-sensitive Ca2+ channels.	universal
Golf	Yes, cholera toxin	stimulation of adenylyl cyclase	olfactory sensory neurones
Gi1	Yes, pertussis toxin	undefined	limited; high levels in brain
Gi2	Yes, pertussis toxin	inhibition of adenylyl cyclase stimulation of phospholipase C?	universal
Gi3	Yes, pertussis toxin	regulation of K+ channels	undefined; universal ?
Go	Yes, pertussis toxin	regulation of Ca2+ channels	limited
Gz	No	undefined, stimulation of phospholipase C ?	Undefined but restricted
TD1	Yes, cholera and pertussis toxins	activation of cGMP phosphodiesterase	rod outer segments
TD2	Yes, cholera and pertussis toxins	activation of cGMP phosphodiesterase	cone outer segments

sequence of 46 nucleotides in which the shorter forms 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 can vary (Mumby et al., 1986). Functional differences between the two forms of Gs have been difficult to assess since current protein purification protocols fail to fully resolve long and short Gsα, however a recent report suggests that the shorter 42kDa form of Gsα may have greater functional activity than the larger 52kDa form (Walseth et al., 1989). To circumvent the poor resolution of the various forms of Gsα by current purification techniques, cDNA's which encode both long and short forms of Gsα have been expressed in *Escherichia coli*. When partially purified, both forms of Gsα can reconstitute, albeit with low intrinsic activity, receptor, AlF_4^- and guanine nucleotide stimulation of adenylyl cyclase activity in S49 cyc$^-$ cells with equal efficacy, suggesting that there is no functional difference between the long and short forms of Gsα (Graziano et al., 1987). The failure to produce more active Gsα in *Escherichia coli* may be due to the lack of a co- or post-translational modification of the protein which is a requirement for interaction with the effector, adenylyl cyclase (Graziano et al., 1989). A hypothesis supported by the ability of rabbit reticulocyte lysates to synthesise Gsα which is fully active but which migrates anomalously upon SDS-Page (Olate et al., 1988). Although Gs was originally identified as the G-protein required for receptor mediated stimulation of adenylyl cyclase, the protein has a more recently defined role as an activator of dihydropyridine-sensitive Ca^{2+} channels (Yatani et al., 1987b).

Initial attempts to isolate cDNA's corresponding to Gi were performed by a number of groups, who employed a variety of different tissues such as bovine brain (Nukada et al., 1986), bovine pituitary (Michel et al., 1986), human monocyte (Didsbury et al., 1987), mouse macrophage (Sullivan et al, 1986) and rat C6 glioma cells (Itoh et al., 1986). The cDNA's isolated were termed Gi cDNA's either on the basis of identity in amino-acid sequence with a known 41kDa pertussis toxin substrate from brain, or on the basis of a potential site for pertussis toxin modification (Kim et al., 1988). Comparison of two different cDNA clones obtained from a human brain cDNA library (λgt11) suggested that two types of Gi existed

(Bray et al., 1987). The first type, isolated from bovine brain libraries, was found to correspond exactly to the 41kDa pertussis toxin substrate from brain (Michel et al., 1986; Nukada et al., 1986) and has been termed Gi-1 (Bray et al., 1987; Jones and Reed, 1987). The most commonly found sequence is that reported by Itoh et al. (1986), which has been designated Gi-2 by Jones and Reed (1987). Sequences which are essentially identical to this Gi-2 have been found in a mouse monocyte library (Sullivan et al., 1986), a bovine pitiutary library (Michel et al., 1986) and a human monocyte library (Didsbury et al., 1987). More recently, a sequence corresponding to a third form of Gi has been isolated and termed Gi-3 (Jones and Reed, 1987; Suki et al., 1987; Itoh et al., 1988; Kim et al., 1988). Although the name Gi is derived from an initial association with adenylyl cyclase inhibition (Katada et al., 1986; Katada et al., 1987), the protein products of the genes which encode Gi1, Gi2 and Gi3 seem to play a role in the opening of K^+ channels (Yatani et al., 1987a) and possibly in the activation of phosphoinositidase C (PLC) (Ohta et al., 1985) and phospholipase A2 (Bokoch and Gilman, 1984). Less well characterised G-proteins include Go, (o for other) which is abundant in neural tissue such as brain (Sternweis and Robishaw, 1984). At present, only one sequence corresponding to Go has been identified in mammals (Itoh et al., 1986; Jones and Reed, 1987), however two separate mRNA's which hybridize to a Go specific probe have been identified (Jones and Reed, 1987), suggesting that heterogeneity in the forms of Go which may be expressed is possible. This is borne out by purification data which demonstrate the occurence of more than one form of G-protein immunologically corresponding to Go (Goldsmith et al., 1988; Kobayashi et al., 1989). The function of Go remains unresolved, however evidence is accumulating to suggest that the G-protein may be involved in the regulation of receptor-mediated inhibtion of voltage operated Ca^{2+} channels (Hescheler et al., 1987; Harris-Warrick et al., 1988; Ewald et al., 1988). More recently, a sequence for a G-protein termed Gx (also Gz, Fong et al., 1988) has been isolated from a human retinal cDNA library. This sequence lacks an apparent ADP-ribosylation site for pertussis toxin and when translated, would be similar in size to the pertussis toxin sensitive G-proteins (Matsuoka et al., 1988). Although a particular function has yet to be ascribed to Gz (Gx), it has been suggested that this

G-protein may mediate signal transduction in signalling systems which are not blocked by pertussis toxin, such as the control of the pertussis toxin insensitive forms of linkage to phosphoinositidase C. However this remains to be experimentally assessed (Fong et al., 1988). A subtype of Gs, termed G_{olf} has been cloned from olfactory epithelia (Jones and Reed, 1989). It has been suggested that G_{olf} may function in a manner analogous to that of Gs, but in contrast to Gs, which appears to be expressed ubiquitously, G_{olf} shows a different pattern of distribution, being located solely in olfactory epithelia, (Jones and Reed, 1989).

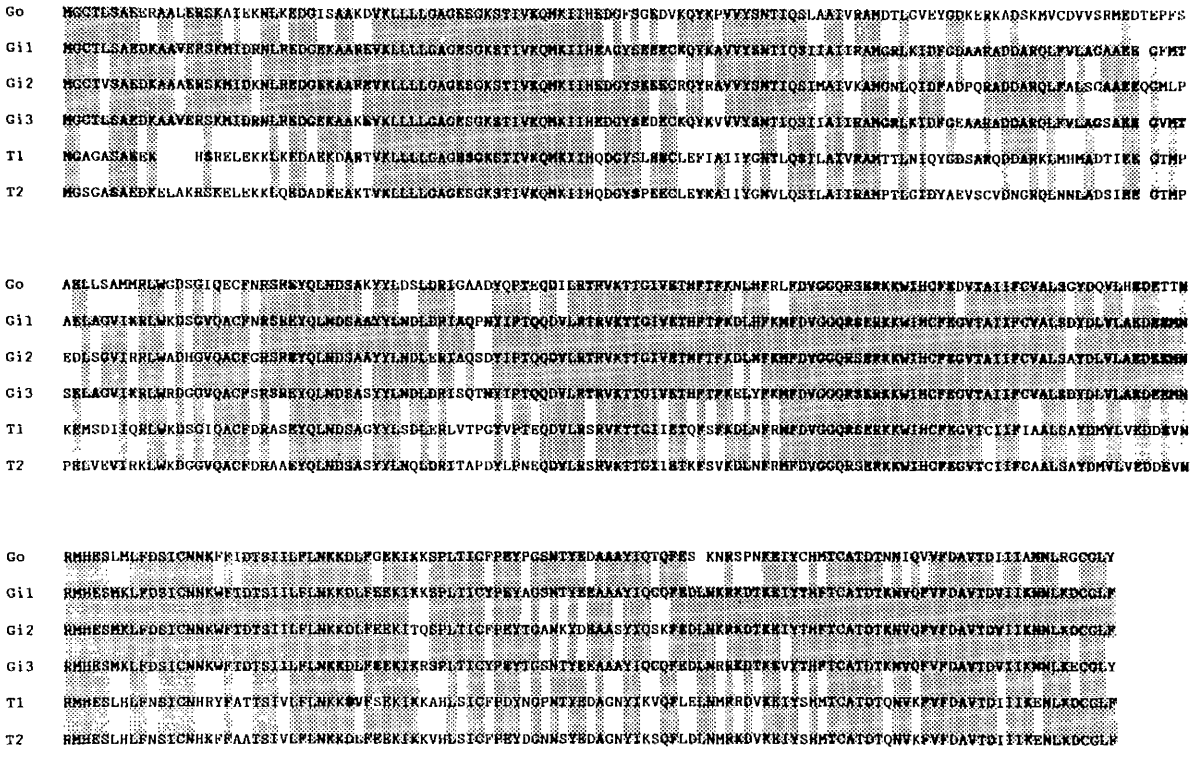
The above G-protein sequences do not represent the entire family of G-proteins which may be expressed. Simon and colleagues have recently made use of polymerase chain reaction technology to detect additional gene products in mouse brain. The group failed to detect two of the known Gi sequences and as such the G-protein sequences detected do not represent a comprehensive screen of α-subunit sequences in mouse, however four previously undetected sequences were observed, suggesting that an even greater number of unique G-protein α-subunits may be expressed and involved in transmembrane signalling (Strathmann et al., 1989).

When the predicted protein sequences of Go, Gi1, 2, 3, Td1 and Td2 (rat) are aligned (Figure 1.2), the G-protein α-subunits show impressive amino-acid sequence homology (Jones and Reed, 1987). Gsα is the most divergent, containing several insertions that the others lack, and is approximately 40 % identical to any of the other six α-subunits. The three Gi's and Go share greater homology, with Go 73% identical to Gi1, 68% with Gi2 and 70% with Gi3. Gi2 shares 88% identity to Gi1 and 85% identity with Gi3. Gi1 and 3 are most homologous, with 94% identity. Gz (Gx) shows strongest homology to Gi1, Gi2 and Gi3 (66-67%) whilst being 60% homologous to Go and only 41% homologous to Gs. Between species, amino-acid homology is even more startling, with at least 98% identity of Gi2, Gi3, Gx and Go from rat, bovine and human sources.

Fig. 1.2 Primary sequences of the α-subunits of the currently identified pertussis toxin sensitive G-proteins expressed in rat.

Areas of identity are shaded. In cases in which identity at particular residues does not extend across all the polypeptides, then the closest homology to the 'Gi-like' subfamily is indicated. Where each of Gi1, Gi2 and Gi3 is represented by a different amino-acid at one position then no further homology to Go, Td1 or Td2 is noted. The sequences for Go, Gi1, Gi2 and Gi3 are taken from Jones and Reed (1987), that for Td1 from Tanabe et al. (1985), and that for Td2 from Lochrie et al. (1985). Figure 1.2 is reproduced from Milligan (1988).

Figure 1.2



It thus appears that according to amino-acid homology the G-proteins may be grouped into several categories, the Gi-like G-proteins, which are Gi1, 2, 3 and Go, the Gs-like G-proteins, Gs and Golf. The transducins Td_1 and Td_2, and Gz (Gx), which is as divergent from the Gi group as are the transducins and is therefore best categorized as a separate class of G-protein (Fong et al., 1988).

In contrast to the diversity in the number of α-subunits, a similar degree of diversity in β-subunits has not been observed. Purification of G-proteins from a variety of sources had previously demonstrated that at least two forms of the β-subunit existed (Sternweis et al., 1981; Sternweis and Robishaw, 1984) which were immunologically distinct (Evans et al., 1987), and had molecular masses of 35 and 36kDa. In visual transduction systems, expression of β-subunits is limited to the 36kDa form, however both forms may be expressed in other tissues. To date the sequences of three cDNA clones corresponding to β1, β2 and β3 have been determined (Sugimoto et al., 1985; Fong et al., 1987; Gao et al., 1987; Levine et al., 1990). All three β-subunits are highly homologous at the primary sequence level however β1,(35kDa) and β2 (36kDa) are 10% divergent in amino acid sequence. The functional significance of these differences is unknown.

At present, one γ-subunit sequence is available (Ovchinnikov et al., 1985; Hurley et al., 1984a). The cloned transducin γ subunit cDNA encodes a peptide with a calculated molecular weight of 8400. Data exists to suggest that there are other γ-subunits to be characterised. Antisera raised against purified transducin α,β,γ, are capable of recognising the β-subunit in purified preparations of Gi or Gs, but do not recognise the γ subunit of Gi or Gs, implying that the β-subunits of transducin, Gs and Gi may be homologous, but the γ subunits of transducin are not (Gierschik et al., 1986b). In addition, both immunological studies on the β,γ complex purified from human placenta (Evans et al., 1987) and peptide mapping of purified γ-subunits (Hildebrandt et al., 1985), suggest that there is more than one non transducin γ-subunit, and therefore at least three γ-subunits exist. Under normal physiological conditions the β,γ subunits which are tightly, though not covalently bound, do not dissociate, and may be thought of as a single entity.

The G-protein α-subunits have molecular masses of between 39 to 46 kDa, as calculated from primary sequence data. However, a variety of other proteins exist which are capable of specifically binding and hydrolysing guanine nucleotides. Initiation and elongation factors required for protein synthesis, such as Ef-Tu, utilise GTP hydrolysis as part of their activity. This has proved extremely beneficial to current understanding of G-protein structure, since Ef-Tu was the first GTP-binding protein to be crystallised in its GDP bound form (Jurnak, 1985; La Cour et al., 1985). Proteins involved in maintaining cellular structure such as the tubulins, are capable of the binding and hydrolysis of GTP, however these proteins do not appear to function in an analogous manner to the G-proteins which have been characterised to date (Seckler et al., 1990).

1.9 Small molecular weight G-proteins

A range of smaller molecular weight G-proteins exists, with molecular masses of between 21-27kDa (Chardin, 1988). The 21kDa protein products of the *ras* oncogene may also be designated as belonging to the G-protein family since these proteins have the ability to bind and hydrolyse GTP. Overexpression of this oncogene results in stimulation of inositol phospholipid hydrolysis and uncontrolled cell growth, which led to the suggestion that *ras* may be involved in signal transduction (Wakelam et al., 1986). However it has recently been demonstrated that the uncontrolled cell growth elicited by expression of mutated forms of *ras*, cannot be explained by constitutive activation of inositol lipid breakdown, so the exact site of *ras* action remains elusive (Seuwen et al., 1988). Although a specific role for small molecular weight G-proteins has yet to be defined, the high degree of evolutionary conservation of amino-acid sequence suggests that they have a definite role in cellular function. Several members of this family have been purified including ARF, a protein cofactor required for cholera toxin catalysed ADP-ribosylation of Gs, (Kahn and Gilman, 1984a), and Gp, not to be confused with the G-protein mediating stimulation of phosphoinositidase C, has

been purified from human placenta (Evans et al., 1986). Gp was reported to co-purify with β,γ subunits indistinguishable to those present in heterotrimeric G-protein complexes. This remains the only example of a small molecular weight G-protein being able to interact with β,γ subunits. Brain tissue has proved to be a rich source of low molecular weight G-proteins, with a report claiming as many as six distinct polypeptides (Kikuchi et al., 1988). In contrast to the 'classical' G-proteins, little is known about the mechanism of action of these smaller G-proteins. However, as well as the obvious difference in size they are not susceptible to either cholera or pertussis toxin mediated modifications. In contrast, several low molecular weight G-proteins including the *rho* gene product, serve as substrates for ADP-ribosylation catalysed by certain botulinum toxins (Yamamoto et al., 1988; Bokoch et al., 1988). Although small molecular weight G-proteins are capable of binding and hydrolysing GTP, a putative nucleotide exchange reaction is lacking, which is perhaps related to the striking difference shown by the kinetics of guanine nucleotide binding to *ras*. When compared to its larger counterparts, the rate of GTP hydrolysis by *ras in vitro* is at least ten fold lower (McCormick, 1989). However the rate of GTP hydrolysis by *ras in vivo* has been demonstrated to occur very much faster than *in vitro*. This is due to the presence of a GTPase-activating protein, GAP, which has been found in the cytoplasm of all vertebrate cells (Trahey and McCormick, 1987). GAP has been purified and cloned and demonstrated to have a molecular mass of 116kDa. From *in vitro* experiments, the protein appears to function enzymatically by converting the GTP bound form of *ras* to the GDP bound form (Gibbs et al., 1988).

1.10 G-proteins in yeast and invertebrate transmembrane signalling systems.

Much of the biochemical characterisation of G-proteins has been centred on vertebrate signal transduction systems, however several recent studies have focussed on the mating pheromone response pathway of the yeast *saccharomyces cerevisiae*, which contains components sharing strong structural similarities to

the mammalian G-protein signalling pathway. Haploid yeast cells of the MATa and MATα designation express cell-type specific pheromone receptors encoded by the STE2 and STE3 genes (Burkholder and Hartwell, 1985; Nakayama et al., 1985, Hagen et al., 1986; Dietzel and Kurjan, 1987). These receptors, although not homologous to each other, have seven hydrophobic, potential membrane spanning, domains as well as a serine/threonine rich C -terminal domain and as such are homologous to mammalian G-protein linked receptors. Mutations in the STE2 and STE3 genes leads to a loss of responsiveness to pheromones and a sterile phenotype. Yeast has also been shown to encode a gene termed either SCG1 (Dietzel and Kurjan, 1987) or GPA1 (Nakafuku et al., 1987) which encodes a 54kDa polypeptide homologous to the α-subunits of Gs, Go, and Td, and is thought to be involved in the mating pheromone response pathway. Disruption of the SCG1 gene is lethal to haploid cells due to constitutive activation of the pheromone respone pathway along with cell cycle arrest. Interestingly, expression of rat Gsα in yeast can suppress growth arrestment, suggesting that SCG1 functions in a manner analogous to mammalian Gs. The products of several other STE genes are required for pheromone response. Two such genes, STE4 and STE18, have been cloned and sequenced and shown to be homologous to the β1 and β2 subunits of bovine and human Td (STE4) and to the γ-subunit of bovine Td (STE18). Mutations in either gene supress the lethality conferred by mutation in the SCG1 gene. This data has led to a model whereby binding of pheromone to receptor (STE2 and STE3 gene products) promotes dissociation of the α-subunit (SCG1 gene product) from β,γ (STE4 and STE18 gene products), with the free β,γ subunits interacting with effector. Although speculative, this model suggests a definite role for the β,γ subunits in transmembrane signalling.

In addition to the data obtained from studies on yeast, it is apparent that invertebrates employ G-proteins in cellular signalling. Non-hydrolysable guanine nucelotides have produced phototransduction effects in limulus (Fain, 1986), squid (Tsuda et al., 1986) and house flies (Blumenfield et al., 1985). In addition, a 39-40kDa pertussis toxin substrate has been observed in nervous tissue of a variety of invertebrates (Hopkins et al., 1988). This protein cross-reacts with

polyclonal antibodies generated to vertebrate Goα (Homburger et al., 1987), and may be involved in mediating a dopamine -induced decrease in neuronal calcium currents in snails (Harris-Warrick et al., 1988). Perhaps one of the most widely studied invertebrates is the fruit fly, *Drosophila melanogaster*, the nervous system of which has been shown to express ion channels (Salkoff et al., 1987), visual pigments (Zuker et al., 1985) as well as adenylyl cyclase (Chen et al 1986). More importantly, a range of G-proteins have been identified in *Drosophila*,the nomenclature of which is based on the respective homology to mammalian G-protein sequences. Thus, G-proteins which have been termed; Gi1α, Gi2α, Gsα, and Goα, have been described (Provost et al., 1988; Quan et al., 1989; deSousa et al., 1989; Thambi et al., 1989). In addition a putative β-subunit has been isolated (Yarfitz et al., 1988). As yet the function of these mammalian G-protein homologues in *Drosophila* remains to be determined.

When deprived of nutrients, *Dictyostelium discoideum* amoebae cease growth and initiate a developmental program to form a multicellular organism (Gerisch, 1987). The cells move in a chemotactic manner in response to extracellular cyclic AMP, which activates two signal transduction pathways by binding to transmembrane receptors. The primary structure of the cyclic AMP receptor(s) displays seven putative transmembrane domains, homologous to mammalian G-protein linked receptors (Klein et al., 1988). Biochemical studies indicate that the cAMP activated signal transduction pathways involve G-proteins, and indeed the cDNA for two such G-proteins has been isolated and designated Ga1 and Ga2. Both proteins are homologous to rat Gi1α.

As yet, the respective function of invertebrate G-protein homologues remains to be ascertained, however the high degree of amino-acid sequence conservation over the long evolutionary distance between yeast, insects and mammals suggests that function may also be conserved. The ability to produce mutants, together with the well characterised genetics in invertebrate signal transduction systems, suggest that such systems are likely to provide an increasingly powerful tool in the analysis of mammalian G-protein function.

1.11 Structural and functional G-protein characteristics

In addition to sharing extensive amino-acid homology (Figure 1.2), the G-proteins which have been most widely studied are thought to share similar functional characteristics, these include;

(i) the ability to bind and hydrolyse GTP
(ii) interaction with β,γ subunits
(iii) interaction with receptor
(iv) functional interaction with effector

(i) GTP binding

If the amino-acid sequence of the α-subunits of each of Gi 1,2 and 3, Go, Gs (long form) from rat, human Gz and bovine rod and cone transducin are aligned, then some 82 invariant amino acids can be identified (Figure 1.2). A majority of the most highly conserved sequences are near regions predicted to be involved in the binding of guanine nucleotides. The basis for these predictions comes from studies of genetic mutations in *ras* which affect guanine nucleotide interactions, and from biophysical data obtained from the crystal structures of the GDP-bound form of Ef-Tu (Jurnak, 1985; LaCour et al., 1985), It has been possible to identify four concensus sequence elements which have been termed A, C, E, and G, using the Halliday classification (Halliday, 1983), which are sequentially arranged along the primary protein structure from the amino to carboxy terminal. The A concensus sequence is Gly-X-X-X-X-Gly-Lys. In EF-Tu this region is near the α-phosphate of GDP. Mutations in an analogous region of *ras*, for example replacement of glycine at position 12 with a variety of amino-acids, reduces GTP binding and GTPase activity (Der et al., 1986) as well as increasing transforming ability (Seeburg et al., 1984). The C-region concensus sequence is Asp-X-X-Gly. A mutation of Asp-X-Ala-Gly to Asp-X-Thr-Gly in *ras* results in auto-phosphorylation of threonine if GTP is used as substrate. The E region is hydrophobic and often contains an alanine residue which is 26 amino-acids

removed from the aspartic acid residue in the C-region. In *ras*, mutation of the analogous alanine to threonine results in a 30-fold reduction in GTP-affinity (Feig et al., 1986). The G-region has the concensus sequence Asn-Lys-X-Asp and confers nucleotide binding specificity. A mutation of the aspartic acid in the G-region of EF-Tu to asparagine alters the nucleotide specificity to xanthine diphosphate over guanosine diphosphate (Hwang and Miller, 1987). Mutations in this region in *ras* reduce, alter or abolish nucleotide binding (Feig et al., 1986; Sigal et al., 1986; Walter et al., 1986).

Several recent reports have taken advantage of the above described predictions of regions of the G-protein α-subunit required for GTP binding and hydrolysis to produce a variety of G-protein mutants which have been expressed in either S49 cyc- cells or in *Escherichia coli*. Expression of Gsα in S49 cyc- cells in which leucine replaces glutamine 227 (designated Q227L, and corresponding to glutamine 61 in *ras*) results in the constitutive activation of adenylyl cyclase and reduction of GTPase activity of Gs. In another mutant in which valine replaces glycine 49 (designated G49V and corresponding to glycine 12 in ras), there is also a reduction in GTPase activity, however the G-protein cannot stimulate adenylyl cyclase due to an inability to adopt an active confirmation (Masters et al., 1989). When the same mutated Gsα-subunits are expressed in *Escherichia coli*, purified and reconstituted into S49 cyc- cells, similar results are obtained (Graziano and Gilman, 1989). This confirms the inferences drawn from similarities in the primary structure of both EF-Tu and *ras* with the G-protein α-subunit, in that the amino-acid residues surrounding glycine 49 and glutamine 227 of Gsα are involved in guanine nucleotide binding and hydrolysis.

Amino-acid sequences outside of the Halliday regions are thought to be necessary for α-subunit specific interactions such as interaction with β,γ subunits, interaction with receptor and interaction with effector.

(ii) Interaction with β,γ subunits

There is some evidence to suggest that the N-terminus is important for interaction with the β,γ subunits. The α-subunit of many G-proteins contains a site sensitive to trypsin, which is close to the N-terminus (Hurley.et al., 1984b; Winslow et al., 1987) In the presence of GTPγS, trypsin cleaves a 2kDa peptide from the amino terminus of Gi1α and Goα, the remaining 39 and 37kDa proteins being resistant to further proteolysis. Gi1 and Go are virtually identical in this region, and the tryptic site has been demonstrated to be after residue 21 in Gò, a site present in both α-subunits. After tryptic cleavage at the N-terminal region, the α-subunits of Gi1 and Go are no longer able to interact with the β,γ subunits, since both subunits are no longer substrates for pertussis toxin (Neer et al., 1988). Limited proteolysis of transducin α, involving the removal of a 1-2kDa fragment from the N-terminus, results in the diminuition of a number of reactions which are thought to require α,β,γ interaction, such as ADP-ribosylation by pertussis toxin, rhodopsin binding, Gpp(NH)p binding (Fung and Nash, 1983), and immunoprecipitation of Td α,β,γ by a monoclonal antibody which identifies Td α (Navon and Fung, 1987).

(iii) Interaction with receptor.

The C-terminus of Gα has been implicated in the interaction with receptor. The main evidence for this statement lies in the role of pertussis toxin to ADP-ribosylate Gi. This covalent modification, at a residue close to the extreme C-terminus of Gi, attenuates productive coupling between receptor and G-protein, with no impairment of the ability of Gi to dissociate in response to activating guanine nucleotides (Katada et al., 1986). In addition, a mutant ofthe S49 murine lymphoma exists which has been termed *unc* (uncoupled) (Haga et al., 1977). In this cell line, it is possible to obtain stimulation of adenylyl cyclase using agents which are able to directly activate Gs, such as ALF_4^- and non-hydrolysable analogues of GTP. However, it is not possible to demonstrate receptor mediated stimulation of adenylyl cyclase by either β-adrenergic receptor agonists, or PGE1.

In addition, the sensitivity of β-adrenergic agonist binding to guanine nucleotides is no longer apparent (Haga et al., 1977). Two-dimensional electrophoretic analysis has demonstrated that in the *unc* mutant, Gsα migrates as a more acidic species than Gsα from wild type S49 cells, by approximately one charge, suggesting that the impairment in signal transduction lies in the α-subunit of Gs (Schleifer et al., 1980). The subsequent sequencing of complementary DNAs encoding Gsα in both *unc* and wild type S49 cells showed that in the *unc* mutant, a point mutation in Gsα results in the replacement of an arginine residue by a proline residue, at a position six amino-acids from the C-terminus. The functional consequence of this mutation is the inability of the G-protein to interact with receptor. (Sullivan et al., 1987; Rall and Harris., 1987).

(iv) Interaction with effector

The mechanism by which a G-protein may interact with an effector is probably the least well understood aspect of G-protein function. Sequences thought to be involved in the interaction with effector lie between the Halliday regions A and C where there is most variability between the G-protein α-subunits. The analogous region in Ef-Tu binds aminoacyl-tRNA in a GTP-dependent reaction, and mutations in *ras* in this region disrupt its biological activity without interfering with either nucleotide or membrane interaction (Stein et al., 1986). A mutant of Gs termed H21a is able to interact with receptor and bind guanine nucleotides, but is unable to interact with adenylyl cyclase, suggesting that the mutation is in the domain of interaction of Gsα with effector, however it has since been demonstrated that the mutation, which involves the replacement of a glycine with an alanine at position 226, prevents Gsα adopting an active conformation (Miller et al., 1988). Thus the domain of G-protein interaction with effector remains unresolved.

A conserved restriction endonuclease site, present in almost all mammalian α-subunit cDNA's, separates the putative domains involved in receptor and effector interaction (Masters et al., 1986). This allows the production of chimeric cDNA's in which a hybrid G-protein α-subunit derived from two different α-subunits is encoded by a single reading frame. In this manner it has been possible to produce a

Gs/Gi2 hybrid, which is derived from the amino-terminal 212 amino-acids of Gi2 and the C-terminal 160 amino-acids of Gs. The C-terminal region of Gs is thought to be the domain of interaction with receptor, wheras the 212 N-terminal amino-acids encoded by the Gi2 section of the chimera is thought to contain the domain of interaction with effector. It was thus theorised that the chimeric G-protein would be capable of interaction with receptors which stimulate adenylyl cyclase, and couple to the inhibition of adenylyl cyclase. When the chimeric G-protein was expressed in S49 cyc- cells, a polypeptide of approximately 46kDa which is immunologically identifiable as both Gs and Gi, is prevalent (Masters et al., 1988). Surprisingly, the Giα/Gsα chimera was shown to mediate β-adrenergic receptor stimulation of adenylyl cyclase activity to a similar extent as wild type Gs, and be incapable of mediating inhibition of adenylyl cyclase activity by either stimulatory or inhibitory receptors.

A similar chimeric construct in which the 356-amino-terminal amino acid residues are contributed by Gsα and the 36 residues at the carboxy terminus are derived from Gi2α has been produced. Expression of this chimera in mammalian cos cells resulted in a constitutive elevation of intracellular cyclic AMP, presumably as a result of increased stimulation of adenylyl cyclase. Kinetic studies suggest that the increased ability of the chimeric G-protein to interact with adenylyl cyclase may be due to an enhanced rate of GDP-dissociation. This implies that in addition to interacting with receptor, the C-terminal region of the G-protein α-subunit may also play a role in the regulation of guanine nucleotide exchange (Woon et al., 1989).

1.12 Covalent modifications of G-proteins.

G-protein heterotrimers are known to be associated with the cytoplasmic surface of the plasma membrane, and with the exception of transducin, can only be extracted from membranes using detergents. This is in keeping with their known

functions, to modulate the level of activity of intracellular effectors at the plasma membrane. However the exact basis for membrane association has not been rigorously defined. Purified α-subunits do not readily associate with phospholipid vesicles unless β,γ subunits are present, suggesting that the β,γ subunits functionally serve to anchor the G-protein to the plasma membrane (Sternweis, 1986). Recent reports have indicated that G-protein α-subunits need not be continuously associated with the plasma membrane, since long term incubation of membrane preparations with non-hydrolysable GTP analogues promotes the release of the α-subunits of Gs, Gi2 and Go into a supernatant fraction (Milligan and Unson, 1989; Milligan et al., 1988; McArdle et al., 1988). Further, in S49 cells, agonist occupation of β-adrenergic receptors promotes a redistribution of Gsα from the plasma membrane into the cytosol (Ransnas et al., 1989). These observations suggest that dissociation of the G-protein α-subunit from the β,γ subunit attenuates the ability of the α-subunit to remain at the plasma membrane, and that the β,γ subunits must therefore play a role in G-protein membrane attachment. However this leaves the question of the method of attachment of the β,γ complex to the plasma membrane. The possibility of either of these subunits being transmembrane spanning proteins is unlikely, since, in common with the α-subunits, a suitable hydrophobic stretch of amino-acids is not immediately apparent (Chabre, 1988).

The α-subunits of both Gi and Go have recently been shown to be myristoylated (Buss et al., 1987; Schultz et al., 1987). This is a covalent modification which is thought to occur co-translationally and results in the attachment of the 14-carbon fatty acid, myristate to the N-terminal glycine residue of the α-subunit (Sefton and Buss, 1987). This covalent modification has been described for other proteins, many of which are membrane attached, such as the *src* oncogene (Garber et al., 1985) and is thought to be a requirement for protein function. Indeed, mutations which remove the N-terminal glycine from *src* result in a protein which is no longer myristoylated, can no longer associate with the plasma membrane, and is incapable of promoting cellular transformation (Kamps et al., 1985). Studies with purified N-methyltransferases on synthetic peptides have revealed the concensus sequence required for myristoylation to be;

Met, Gly, X, X, X, Ser. The methionine is not present in the mature polypeptide, having been removed presumably by an aminopeptidase. All G-protein subunits which have been cloned to date, share the above concensus sequence, with the exception of Gsα, β35, β36 or the γ subunits. Gsα has the prerequisite N-terminal glycine, but has an asparagine residue at position 6, this difference could reduce the Km of Gsα for N-methyltransferase by several thousand fold (Towler et al., 1988). If the amino-terminal glycine of Gi1 is modified by site directed mutation to an alanine residue, Gi1 can no longer be myristoylated, and can be detected predominately in the cytoplasmic region of cells in which it is expressed. (Jones et al., 1990). This may be regarded as strong evidence for myristoylation as the mechanism of G-protein attachment to the plasma membrane, however, since it has not been possible to detect a similar covalent modification of either Gsα, β35, β36 or γ subunits which are undoubtedly localised at the plasma membrane, it may be suggested that myristoylation is not the only mechanism of membrane attachment employed by G-proteins (Buss et al., 1987).

Ras proteins are acylated with various lipids, primarily palmitate, which was originally thought to be present on a cysteine residue, (cys186), four amino-acids from the C-terminus (Grand et al, 1987). Mutational analysis has revealed that cys186 is essential for membrane localisation (Willumsen et al., 1984). Examination of a number of proteins which are thought to be palmitoylated at this position has led to the identification of a concensus sequence; Cys-Al-Al-X, where Al represents an aliphatic amino-acid and X represents any amino-acid. Goα, Gi1,2,3α, Td1α, Td2α and Tdγ all possess this so-called CAAX box and as such may be subject to other lipid modifications. Recent reports have noted that instead of being palmitoylated, the cysteine present in the CAAX box of p21ras is both farnesylated and methylated, with palmitate present on a cysteine residue which is several residues further upstream. The remaining three amino-acids are cleaved by a carboxypeptidase (Hancock et al., 1989). It has recently been reported that the γ-subunit of transducin is methylated on the carboxyl group of a C-terminal cysteine residue (Fung et al., 1990). This modification is likely to be associated with both proteolytic removal of the three C-terminal amino-acids in the CAAX

sequence and lipidation of the methylated cysteine (Fung et al., 1990). Lipidation at the cysteine would serve to anchor the β,γ complex and assist in the anchorage of the G-protein α-subunit to the plasma membrane. It is therefore possible that G-proteins may be substrates for a range of lipid modifications which may be required for anchorage to the plasma membrane.

1.13 Phosphorylation

The activity state of a large number of cellular enzymes is controlled by phosphorylation/dephosphorylation events. Regulation of receptor function through phosphorylation has been well documented (Sibley et al., 1987). As central mediators of transmembrane signalling, it might well be hypothesised that G-protein function may also be regulated by kinase and phosphatase activity. The phosphorylation of Giα and transducin-α has been demonstrated *in vitro* by protein kinase C (Katada et al., 1985; Zick et al., 1986), the preferred substrate being the GDP-liganded α-subunit. Phosphoamino analysis revealed incorporation to be on serine residues. The exact residue(s) involved were not determined, but Ser at position 12 is a likely candidate (Zick et al., 1986), being conserved in all G-protein α-subunits except Gsα. As previously described, the N-terminal region of the G-protein α-subunit has been postulated to be the site of interaction with the β,γ subunits, as such it is of interest to note that the phosphorylation of Giα by protein kinase C was inhibited by addition of the β,γ complex (Katada et al., 1985). In addition to these *in vitro* studies, it has recently been demonstrated that the α-subunit of Gi can be phosphorylated in intact hepatocytes, (Pyne et al., 1989; Bushfield et al., 1990), platelets (Crouch and Lapetina, 1988) and U937 cells (Issakani et al., 1989). This may be presumed to be mediated through activation of protein kinase C, since the effect was obtained when cells were treated with agents which activate protein kinase C either directly, or through the stimulation of inositol phospholipid metabolism. In both hepatocytes and U937 cells, Gi2 seems to be the G-protein modified, whereas in platelets both Gi2 and Gz(x) may be

phosphorylated (Carlson et al., 1989). The functional effect of G-protein phosphorylation has yet to be rigo rously defined, however it is likely that G-protein activation is attenuated.

1.14 The interaction of receptors with G-proteins

The first step in the conveyance of information from the outside to the inside of a 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 an intrinsic tyrosine kinase activity (for example, receptors for insulin, PDGF and EGF)(Hunter and Cooper, 1985), b) multisubunit receptors which are ion channels (the nicotinic acetylcholine, γ-amino butryic acid and glycine receptors)(Changeux et al., 1987), c) receptors which function as carrier proteins (transferrin receptor)(Goldstein et al., 1985), and (d) receptors which activate G-proteins (Strader et al., 1989; Dohlman et al., 1987). Based on this functional classification, the largest class of receptors are those which interact with and activate G-proteins, and as such, these receptors have been the subject of much research in recent years (Weiss et al., 1988).

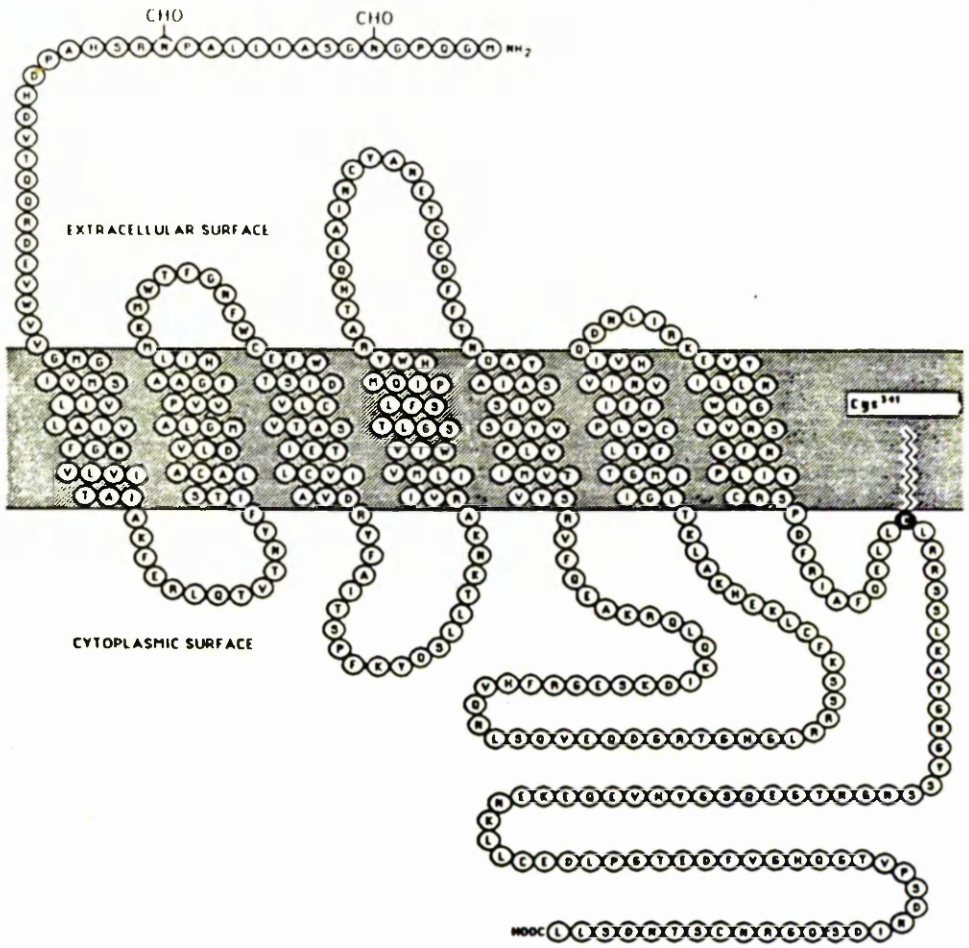
Recent reports have suggested that this delineation of receptor-G-protein linked signalling pathways may not be absolute, since the EGF receptor, which is a tyrosine kinase, has been shown to stimulate adenylyl cyclase activity in perfused rat hearts (Nair et al., 1989), and by the demonstration that EGF is able to stimulate phospholipase A2 activity in rat inner medullary collecting tubule cells, in a manner stimulated by GTPγS and inhibited by pertussis toxin pretreatment (Teitelbaum, 1990). However, it is still generally accepted that receptors which function through an intrinsic tyrosine kinase activity do not directly interact with G-proteins.

Although the G-protein linked signal transduction systems are diverse in nature, the cloning and sequencing of several G-protein linked receptors has demonstrated strong structural homology, and a number of key features have emerged. The predicted membrane topography of the human β_2 adrenergic receptor is shown (Figure 1.3.). Hydropathy analysis of G-protein linked receptors has revealed the existence of seven hydrophobic stretches of between 20 to 25 amino acids. Each of these stretches is proposed to form an alpha-helical membrane spanning domain. The intervening hydrophilic sections are exposed extra and intracellularly (alternately). This results in the localisation of the N-terminus extracellularly, with the C-terminus being intracellular (Strader et al., 1989). All G-protein linked receptors which have been sequenced, are known have between one to three concensus sites for N-linked glycosylation at the amino-terminal region of the protein (Dohlman et al., 1987). The functional significance of these glycosylation sites is currently unknown, although they may be required for localisation of the receptor to the plasma membrane. One of the most interesting questions regarding G-protein linked receptors, lies in the identification of the domains responsible for both ligand binding and G-protein interaction. Current data suggests that ligand binds to receptor within the plane of the lipid bilayer (Findlay and Pappin, 1986; Dixon et al., 1986). The interaction between receptors and G-proteins has to be both transient and specific (Chabre, 1987), for this reason, it was thought that G-proteins and receptors must interact at the inner surface of the plasma membrane. Both proteolysis and deletion mutagenesis experiments have shown that the third intracellular loop is required for receptor interaction with G-protein (Kuhn and Hargrave, 1981; Dixon et al., 1987; Strader et al., 1987). This region is one of the most divergent (Dohlman et al., 1987). Interestingly though, substitution of the entire third intracellular loop and the surrounding helices 5 and 6 of the $\alpha 2$ adrenergic receptor (adenylyl cyclase inhibitor) with the analogous region of the beta adrenergic receptor only resulted in a small degree of adenylyl cyclase stimulation by the chimeric receptor, suggesting that some level of G-protein specificity is controlled by the rest of the receptor (Koblika et al., 1988). Further work on this aspect has produced contradictory results (Dixon et al., 1987; O'Dowd et al., 1988), which leaves the

Fig. 1.3 The proposed organisation of the human β_2-adrenergic receptor in the plasma membrane.

Hydropathy analysis of the amino-acid sequence of G-protein linked receptors has revealed the existence of seven hydrophobic stretches of between 20 to 25 amino-acids. Each of these stretches is proposed to form an α-helical membrane spanning domain (section 1.14). The proposed organisation of the human β_2 adrenergic receptor in the plasma membrane is shown. Potential sites for N-linked glycosylation are marked (CH0), along with the cysteine residue which may be palmitoylated in this receptor (Cys 341). Figure 1.3 is reproduced from O'Dowd et al., (1989).

Figure 1.3



situation presently unresolved.

The activation of G-proteins by a receptor is catalytic in nature, in that one receptor may activate numerous G-proteins (Levitzki, 1988). However unlike conventional enzymes, receptors which activate G-proteins do not seem to catalyse the formation or cleavage of covalent bonds, but instead serve to stabilise a conformational change which allows guanine nucleotide exchange on the G-protein α-subunit (Wessling-Resnick et al., 1987). The mechanisms of interaction which assist guanine nucleotide exchange are not well defined, however reconstitution studies with purified proteins suggests that the β,γ subunits are required for receptor mediated guanine nucleotide exchange , and that one β,γ complex may recycle between α-subunits, allowing many α-subunits to be activated by a single agonist occupied receptor (Fung, 1985). Evidence exists that the β,γ subunits have a site of interaction with receptor, since both β,γ and transducin α-subunits are capable of preventing the phosphorylation of the light receptor rhodopsin by rhodopsin kinase (Fung, 1985). A single receptor may function catalytically to activate many G-proteins, as has been reported for the β-adrenergic receptor (Pederson and Ross, 1982). A single photobleached rhodopsin may activate up to 500 transducins in vertebrate rods (Stryer, 1986).

1.15 Interaction of G-proteins with effectors.

The least studied area of G-protein function is the mechanism of interaction with effector. It is generally thought that G-proteins interact with intracellular effectors in a transient manner, and that one G-protein may be able to interact with more than one effector before the intrinsic GTPase activity of the α-subunit deactivates the G-protein. This model allows for signal amplification as well as explaining why G-protein activation does not result in the constitutive activation or inhibition of an effector under G-protein control. In contrast, the activation of atrial K+ channels by Gi3 (see earlier) appears be essentially irreversible, since

repeated washings do not attenuate the ability of Gi3 which has been activated with GTPγS ,to open the channel (Yatani et al., 1987a).

Gs is known to co-purify with adenylyl cyclase at a stoichiometric ratio of one mole per mole (Arad et al., 1984), and it is posible to immunoprecipitate adenylyl cyclase with antisera which identify the C-terminal region of Gsα, when Gs is in the activated state (Simonds et al., 1989b). This suggests that Gsα may form a strong association with adenylyl cyclase and that the C-terminal region of Gs is not the site of interaction with effector. The recent isolation of cDNA's which encode adenylyl cyclase should aid research in this neglected area (Krupinski et al., 1989) and thus assist in our understanding of G-protein function.

1.16 Regulation of G-protein levels

Few studies have addressed the regulation and control of G-protein levels in signal transduction model systems, however it has been appreciated that the expression of G-proteins may alter during aging (Green and Johnson, 1989), and organ development (Luetje et al., 1987). It is not known whether these changes represent alterations in mRNA transcription, translation or stability, or in protein degradation. A recent study addressed to the steady state levels and degradation rate of Go in GH_4 cells and rat cardiocytes suggests that protein transiational controls may be important in determining the concentration of G-protein α-subunit in a given tissue (Silbert et al., 1990).

In several tissues, corticosteroids have been shown to play a role in regulating G-protein levels. Dexamethasone has been shown to increase the expression of Gsα in GH_3 cells as measured by an increase in both mRNA and protein (Chang and Bourne, 1987). This is in keeping with the ability of glucocorticoid hormones to enhance hormonal stimulation of cyclic AMP accumulation in a variety of cells and tissues (Davies and Lefkowitz, 1984). The glucocorticoid hormone corticosterone has also been shown to increase the levels of

Gs (both 45 and 52kDa forms) at both mRNA and protein levels, and to decrease protein and mRNA levels of both Gi1 and Gi2 in rat cerebral cortex. The levels of both Go and β-subunits did not change (Saito et al., 1989).

Treatment of a variety of cells with "differentiation' agents has been demonstrated to dramatically alter the steady state G-protein levels. The 3T3-L1 fibroblast cell line may be induced to differentiate into a more adipocyte-like cell type, using the glucocorticoid dexamethasone (Watkins et al., 1987). Accompaning this differentiation is an apparent increase in Gsα, β-subunits and Giα, however, these α-subunit increases were assessed by cholera and pertussis toxin catalysed ADP-ribosylation, which may be unreliable indicators of G-protein levels (Mullaney et al., 1988). Treatment of NG108-15 cells with the cyclic AMP analogue dibutryl cyclic AMP, which is capable of traversing the plasma membrane, results in a four-fold increase in Go levels and a concomitant 2 to 3 fold decrease in Gi levels (Mullaney et al., 1988). In a similar manner, differentiation of HL-60 cells with dimethyl sulphoxide produces an increase in the levels of Gi2 (Uhing et al., 1987). Functional correlates between changes in G-protein levels and changes in the ability to interact with intracellular effectors have not been convincing enough to determine the functional role for each G-protein.

1.17 Research Aims

It is therefore apparent that although G-proteins play a pivotal role in the process of transmembrane signalling, the mechanism by which they functionally interact with other protein members of the signal transduction cascade is largely unresolved. The aim of this thesis is to examine both the mechanism and the specificity of interaction between G-proteins receptors and effectors in a widely used signal transduction model system, and thus ascertain if G-proteins are selective in their interactions or if they are more promiscuous and able to interact with a variety of different effectors in the same tissue. The model system under study, along with the strategy employed to investigate G-protein specificity will be described in detail in chapter three.

Chapter 2

Materials and Methods.

2.1.Materials

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

a.General reagents

Sigma Chemical Co.	Cholera toxin
Poole, Dorset.	Bovine Serum Albumin
	NAD
	Naloxone hydrochloride
	TEMED
	Trypsin
	Norit-A charcoal
	Ouabain
	Theophylline
	Arginine hydrochloride
	N-ethylmaleimide
	DADLE
	DALAMID
	0-dianisidine hydrochloride
	ATP disodium salt
	cAMP sodium salt
	Keyhole limpet Haemocyanin
	Freund's complete adjuvant
	Freund's incomplete adjuvant
	Coomassie blue R-250.
	Bromophenol Blue.
	Thimerosal.
	Protein-A Sepharose.
	Tween 20
	Dowex AG50W-X4 (200-400 mesh)

	Alumina (neutral)
	Imidazole
Boehringer (U.K.) Ltd	GTP
Lewes, East Sussex	GTPγS
	Gpp(NH)P.
	GDP
	GDPβS
	App(NH)p
	Dithiothreitol
	Creatine phosphate.
	Creatine phosphokinase
	Triethanolamine hydrochloride
	Tris
	Thymidine
Calbiochem	Forskolin
Cambridge.	
National Diagnostics	'Ecoscint" scintillation fluid
Aylesbury,	
Buckinghamshire	
Porton Products	Pertussis toxin
Porton Down	
Salisbury	
Wiltshire.	
Koch-Light Lab. Ltd	DMSO
Haverhill	sodium potassium tartrate
Suffolk.	

F.S.A. Lab. Supplies	N,N'-methylenebisacrylamide
M & B Dagenham.	Ammonium persulphate
Camlab Cambridge, England	Polygram Sil-G 20cm x 20cm precoated plastic sheets Polygram CEL 300 PEI/UV 5cm x 20cm precoated plastic sheets
Whatman International Ltd. Maidstone	GF/C Glassfibre filters 3mm Chromatography paper. No. 1 Filter paper.
B.R.L. Paisley, Scotland.	Prestained molecular weight markers.

b.Tissue culture plasticware

Costar 205 Broadway, Cambridge M.A., U.S.A..	Biofreez vials
Bibby Science Products Ltd Stone, Staffordshire.	75cm^2 tissue culture flasks.
Elkay Products Shrewsbury, M.A., U.S.A.	50ml centrifuge tubes

2.2.Cell culture media

Gibco Life Technologies	Dulbecco's modification of Eagle's
Paisley, U.K.	medium (10x)
	Foetal Calf Serum
	Glutamine (200mM)
	Sodium Bicarbonate
	Hypoxanthine (0.1mM), Aminopterin
	(1μM), Thymidine (16μM) (50x).
	Penicillin (100 I.U./ml),
	Streptomycin (100mg/ml) (100x)
Imperial Labs	Foetal Calf Serum
West Portway, Andover, Hants.	

2.3.Radiochemicals

Amersham plc,	γ[^{32}P]GTP
Amersham,	[5', 8-^{3}H] Adenosine 3', 5'-cyclic
Buckinghamshire	phosphate
	α[^{32}P]ATP
	[15, 16 (n)-^{3}H] Diprenorphine
New England Nuclear,	(2-D-Alanine-5-D-Leucine), [Tyrosyl-
Boston, Mass., U.S.A	3,5-^{3}H(N)]-Enkephalin.
	Adenylate-[^{32}P] NAD.

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

2.4.Radiochemical Purity

a DADLE.

Purity of the radiolabelled enkephalin DADLE, was determined by thin later chromatography. 5µl of stock radiolabelled peptide was spotted onto plastic-backed plates of silica-gel (polygram sil-G), which were then placed in a chromatography tank lined with filter paper which had been saturated with solvent (chloroform; methanol; ammonia, 12:9:4 by volume) for at least one hour before development of the chromatograms. Plates were removed after the solvent had moved 10cm from the origin, and the gel sectioned into vertical strips of 0.5cm tall x 2cm wide. Each strip was placed in a scintillation vial with 10mls of ecoscint scintillation fluid and counted in the tritium channel of a rackbeta scintillation counter. The above resolving conditions give an RF value of 0.55 for DADLE (James, I., 1980, PhD. thesis, Glasgow University). Routinely approximately 90% of the radioactivity in the sample was the appropriate radiolabelled peptide.

b $\gamma[^{32}P]GTP$

1 µl of stock $\gamma[^{32}P]GTP$ was spotted onto a 10cm x 5cm sheet of PEI cellulose which had been pre-run in water to take most of the yellow soluble material to the top, and left to dry at room temperature for 5 minutes. The sheet was placed in a chromatography tank containing 0.75M potassium phosphate buffer (pH 3.5) until the solvent front had travelled three-quarters of the way up the sheet. The cellulose was then sectioned vertically into 0.5cm tall x 2cm wide strips which were put in scintillation vials with 10mls of ecoscint scintillation fluid and counted in the ^{32}P channel of a rackbeta scintillation counter. Under these conditions $\gamma[^{32}P]GTP$ migrates with a RF value of approximately 0.15 to 0.20, with the major contaminant being ^{32}Pi. Typically,greater than 80% of the total radioactivity present in the sample was $\gamma[^{32}P]GTP$.

c.α[^{32}P]ATP

α[^{32}P]ATP purity was assessed in a manner identical to that for γ[^{32}P]GTP described above, however α[^{32}P]ATP migrated with an RF value of approximately 0.35,and typically greater than 70% of the total radioactivity present was α[^{32}P]ATP.

2.5.Standard Buffers

Phosphate Buffered Saline. (PBS)	2g potassium chloride 80g sodium chloride 2g potassium dihydrogen orthophosphate 21.6g of disodium hydrogen orthophosphate heptahydrate to a final volume of 1 litre, pH7.4.
Tris Buffered Saline (TBS)	500mM sodium chloride, 20mM Tris-HCL pH 7.5.
Tris Buffered Saline with Tween 20 (TTBS)	500mM sodium chloride, 20mM Tris-HCL pH 7.5, 0.05% (v/v) Tween 20.

2.6.Cell culture

a.Cell growth.

NG108-15 neuroblastoma x glioma hybrid cells were a kind gift from Dr. W. Klee (N.I.H., Bethesda, M.D. USA) and were grown in 75cm^2 tissue culture flasks in 0.0375% (w/v) sodium bicarbonate buffered Dulbecco's modification of Eagle's Medium (DMEM), containing 10% (v/v) foetal calf serum (FCS) which had been heat inactivated at 56^{o}C for 30 minutes. The medium was supplemented with glutamine (2mM), hypoxanthine (0.1mM), aminopterin (1μM) and thymidine (16μM). Both penicillin (100 units/ml) and streptomycin (100μg/ml) were routinely included. The growth media will henceforth be termed DMEM/10% (v/v) FCS. Cells were grown in a humidified atmosphere of 5% CO_2/95% air.

b.Cell subculture.

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

c.Cell maintenance.

Confluent cells were removed from the surface of the flask by trypsinisation as described above and the cells resuspended in freezing medium, which consisted of 8% (v/v) DMSO in FCS. The suspension was aliquoted into 0.5 ml volumes into biofreez vials, frozen overnight at -80^{o}C packed in cotton wool, and then transferred to liquid nitrogen for storage. Cells to be brought up from liquid nitrogen storage were thawed immediately at 37^{o}C, resuspended in 10 mls of DMEM/ 10% (v/v) FCS, and centrifuged at 800 x g in a MSE centaur for two minutes to pellet the cells. The cell pellet was resuspended in DMEM/ 10% (v/v) FCS and plated out in a final volume of 10mls of DMEM/ 10% (v/v) FCS in a 75 cm^2 flask.

d.Toxin treatment of cells.

Confluent cells to be treated with toxin were given a change of media into DMEM/10% (v/v) FCS, supplemented with either pertussis toxin to a final concentration of 25ng/ml or cholera toxin to a final concentration of 100ng/ml. A parallel set of control flasks were treated with an equal volume of toxin vehicle. After 16 hours treatment, cells were harvested as described below.

e.Cell Harvesting.

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

f.Production of plasma membranes.

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

The homogenate was centrifuged at 500 x g for 10 minutes in a Beckman L5-50B centrifuge with a Ti 50 rotor, to remove unbroken cells and nuclei. Plasma membranes were collected by centrifugation of the supernatant fluid at 48,000 x g for 10 minutes, washed in 10 volumes of the same buffer and after a second centrifugation, were resuspended in the same buffer to a final protein concentration of between 2 to 4 mg/ml, aliquoted, and stored at -80^{o}C until required.

2.7.Protein Determination.

The method used is based on that described by Lowry and co-workers, (Lowry et al, 1951).

Stock Solutions;

A)	2% (w/v) sodium carbonate in 0.1M sodium hydroxide.
B)	1% (w/v) copper sulphate.
C)	2% (w/v) sodium potassium tartrate.

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

Protein standards were prepared using a 1mg/ml bovine serum albumin fraction 5, and a standard curve constructed for a maximum of 30µg of protein per sample. Unknowns were assayed in 2, 4 and 8µl volumes in duplicate. 1ml of

solution D was added to each sample, mixed and left to stand for 10 minutes. 100µl of 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 a LKB Ultrospec 2.

2.8.Antibody Production.

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

3mg of the appropriate peptide and 10mg of keyhole limpet haemocyanin were dissolved slowly in 1ml of 0.1M phosphate buffer pH 7.0. 0.5ml of 21mM glutaraldehyde (also in 0.1M phosphate buffer pH 7.0) was then added dropwise with stirring and the combined 1.5ml incubated overnight at room temperature.

The 1.5ml solution was mixed with an equal volume of complete Freund's adjuvant and briefly sonicated with a Branson 'soniprobe' (Type 7532B).1ml aliquots of the resulting emulsion were injected in multiple subcutaneous sites in New Zealand white rabbits. Four weeks later each animal received a booster immunization with material identically prepared, except one half as much keyhole limpet haemocyanin and peptide were injected in incomplete Freund's adjuvant.

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

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

Table 2.1

Antiserum	Peptide Used	G-protein sequence	Antiserum Identifies
AS7	KENLKDCGLF	Tdα 341-350	Td, Gi1, Gi2
OC1	ANNLRYCGLY	Goα 345-354	Go
I3B	KNNLKECGLY	Gi3α 345-354	Gi3
CS1	RMHLRQYELL	Gsα 385-394	Gs
LE2	LERIAQSDYI	Gi2α 160-169	Gi2
I1C	LDRIAQPNYI	Gi1α 159-168	Gi1
BN1	MSELDQLRQE	Gβ. 1-10	β1, β2

2.9.Antibody Purification

Crude antisera were chromatographed on a 1.5cm x 2 cm column of protein-A-sepharose 4B. A 5ml volume of antiserum in glycine buffer (1.5M Glycine, 3M NaCl, pH 8.9) was added to the column and allowed to equilibrate. The column was washed with glycine buffer until the eluate had an A_{280} of 0.0 and was then eluted with 100mM citric acid, pH 4.0 into 2M Tris/HCL, pH 7.5. The eluted IgG fractions were dialysed overnight against 1000 volumes of 10mM Tris-HCl, 0.1mM EDTA pH 7.5, and lyophilised. Just prior to use, samples were reconstituted to the required dilution with the same buffer.

In some cases, antisera AS7 was affinity purified on a column of bovine transducin as described by Gierschik and colleagues (Gierschik et al.,1986b). 1.5 mg of purified bovine transducin was coupled to cyanogen bromide activated sepharose 4B to a final concentration of 1mg of protein per mg of settled gel. Whole serum diluted 1 : 3 with 50mM Glycine, 500mM NaCl, pH 7.5 was incubated

overnight at 4°C with the transducin linked sepharose and after washing with the same buffer, specifically bound immunoglobulins were eluted with 100mM glycine, 500mM NaCl, pH 2.5 The eluate was immediately restored to pH 7.5, aliquoted and stored at -80°C until use.

2.10.Gel Electrophoresis

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

a.Resolving Gel Preparation.

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

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

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

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

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

To a final volume of 24ml with H_2O.

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

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

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

To a final volume of 36ml with H_2O

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

b.Stacking gel preparation.

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

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

To a final volume of 15 ml with H_2O.

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

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

c.Sample preparation.

(i).Protein precipitation

Samples were prepared for gel electrophoresis by sodium deoxycholate/ trichloroacetic acid precipitation;- 6.25µl of 2% (w/v) sodium deoxycholate was added to each sample, followed by 750µl of H_2O, and then 250µl of 24% (w/v) trichloroacetic acid. Samples were centrifuged in a Hettich Mikro Rapid/K at12000 x g for 20 minutes, after which time the supernatants were removed and the pellets dissolved in 20µl of 1M Tris base followed by 20µl of Laemmli buffer which consisted of 5M Urea, 0.17M SDS, 0.4M DTT, 50mM Tris-HCl pH 8.0, 0.01% Bromophenol Blue.

(ii).Protein Alkylation

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

Samples were centrifuged in a Hettich Mikro Rapid/K at12000 x g, the supernatants discarded and the pellets resuspended in 20µl of 10mM Tris-HCL,1mM EDTA pH 7.5. 10µl of 5% (w/v) SDS,50mM DTT was added to each sample, mixed, and placed in a boiling water bath for 15 minutes. Samples were then cooled to hand temperature and 10µl of 100mM NEM added. After 15 minutes at room temperature 20µl of Laemmli buffer was added to each sample, leaving the samples ready for gel electrophoresis.

d.Gel protein staining.

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

e.Autoradiography.

Gels to be autoradiographed were firstly stained for protein with coomassie blue as described above, and dried down onto Whatman 3mm chromatography paper at 60°C for 1 hour under vacuum. The dried gel was placed next KODAK X-OMAT X-ray film in a cassette with intensification screens for up to 1 week at -80°C. Films were developed by hand using Kodak LX 24 developer and FX 40 fixer.

f. Densitometry.

Analysis of incorporation of radioactivity into polypeptides of interest was performed by scanning the autoradiograms with a Bio-rad scanner driven by an Olivetti M24 personal computer. Absorption was measured in arbitrary units as detailed by Gawler and co-workers (Gawler et al., 1987).

2.11.Western blotting.

Proteins were separated under appropriate resolving conditions on SDS/polyacrylamide gels overnight at 50V. The proteins were transferred to a nitrocellulose sheet (Schleicher and Schuell) for 2 hours at 1.5mA in a 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 3% (w/v) gelatin in TBS, after which time, gelatin was washed off with distilled H_2O, and the nitrocellulose sheet incubated overnight at 30°C with the appropriate dilution of antisera in 1% (w/v) gelatin in TBS. Next day, the antisera was removed and the sheet subjected to a series of washes in distilled H_2O; TTBS, and TBS for 10 minutes each, after which, the blot was incubated with a second antibody (peroxidase conjugated goat anti-rabbit IgG) for 2 hours at room temperature. The second antibody was then removed, and the sheet subjected to the series of washes in distilled H_2O, TTBS and TBS as before. The blot was then developed in 40mls of 10mM Tris pH 7.5 with 0.025% (w/v) 0-dianisidine as substrate.

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

2.12.ADP-Ribosylation.

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

250mM potassium phosphate buffer pH 7.0
3µM ^{32}P NAD (4 x 10^6 c.p.m.)
20mM Thymidine
1mM ATP pH 7.5
100µM GTP pH 7.5 (`When the presence of GTP was required)
20mM Arginine/HCL

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

The ribosylation assay was initiated by addition of membranes and transferral of tubes to a 37°C water bath. Assays proceeded for either 1 hour, or for 2 hours (in the absence of GTP) and were terminated by removal to ice followed by sodium deoxycholate/ trichloroacetic acid precipitation as detailed (2.10c). Samples were then resolved under appropriate SDS/polyacrylamide gel electrophoresis.

2.13.GTPase assays.

The assay is used to monitor the release of ^{32}Pi from $\gamma[^{32}P]GTP$ and is essentialy performed as by Koski and Klee, (1981), which is a modification of the method described by Cassel. and Selinger, (1976). except the concentration of 5' adenyl imidodiphosphate (App(NH)P), was lowered to 0.1mM.

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

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

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

In some experiments, membrane protein was preincubated with synthetic peptides which were homologous to, or corresponded to the C-terminal decapeptides of Gi2α and Goα for 30 minutes at $37^{o}C$, in the presence of the GTPase assay reagents, with the exception of $\gamma[^{32}P]GTP$ and receptor ligands. After this time, both $\gamma[^{32}P]GTP$ and suitable ligands were added, and the assay proceeded as normal. In other experiments, antibodies which had been purified on a column of either bovine transducin or protein-A sepharose and reconstituted to the required

concentration were preincubated with membranes from NG108-15 cells at 37°C for 1 hour in the presence of the GTPase assay components excluding γ[^{32}P]GTP and receptor ligands. After this time, both γ[^{32}P]GTP and suitable ligands were added, and the assay proceeded as normal.

2.14.Binding assays.

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 pH7.5, containing 50-150μg of membrane protein and suitable radiolabelled ligand in a final volume of 250μl. Non-specific binding was assessed in parallel tubes containing an excess of non-radiolabelled ligand. Blank values were determined by replacement of membrane protein with buffer.

The assay was initiated by transferral of tubes to a 30°C water bath for 25 minutes, after which time the tubes were removed to ice and a 200μl volume rapidly filtered through Whatman GF/C glassfibre filters which had been presoaked in assay buffer, followed with three 5ml washes of the filter with ice-cold buffer. Filters were soaked overnight in ecoscint scintillation fluid, prior to counting in a rackbeta scintillation counter. In some assays, membranes were pre-incubated with an IgG fraction purified from either normal rabbit serum or an anti-G-protein antiserum (as detailed in section 2.9) for 60 minutes at 37°C prior to addition of radiolabelled and non-radiolabelled ligand. After the incubation period the assay was continued as normal. In binding assays designed to determine the rate at which equilibrium binding was achieved, the assay volume was increased to 1000μl, with protein and ligand concentrations altered accordingly. After transferr al to a 30°C water bath, aliquots (100μl) were removed and filtered at varying time intervals, filters were then treated as described.

In some instances, data were manipulated according to Scatchard. (1949)

2.15.Adenylate Cyclase assays.

a.Binding protein method

Assays were performed as described by Houslay et al., (1986a,b).

The assay system contained 1.5mM ATP, 5mM magnesium sulphate, 10mM theophylline, 1mM EDTA, 20mM creatine phosphate, 600U/ml creatine phosphokinase, 25mM triethanolamine hydrochloride, 1mM dithiothreitol and 0.8 mg/ml bovine serum albumin final pH 7.4. Membrane protein (10 to 30μg) and suitable drugs were added to a final volume of 100μl.

The reaction was initiated by transferral of tubes to a 30°C water bath for 15 minutes and terminated by removing tubes to a boiling water bath for approximately 5 minutes. Precipitated protein was pelleted by centrifugation in a MSE microcentaur at 12000 x g for 10 minutes and the supernatant liquid removed for cAMP determination.

(i).cAMP determination

This was based on the saturation binding assay as described by Brown et al., (1972), as modified by Tovey et al., (1974). All subsequent procedures were performed at 4°C.

A series of unlabelled cAMP standards ranging from 0 to 320 pmoles/ml were prepared in assay buffer consisting of 50mM Tris-HCl, 4mM EDTA (pH 7.4 at 4°C). 100μl of ^{3}H-cAMP (5,8-^{3}H-adenosine 3': 5'-cyclic monophosphate) diluted in assay buffer to give approximately 500,000 c.p.m./ml, was placed in an eppendorf with 50μl of either unkown sample or cAMP standard,100μl of a cAMP binding protein (prepared as described by Rubin et al., (1974), and a gift from Professor M.D. Houslay) diluted 1:70 in assay buffer, and assay buffer to a final volume of 300μl. Tubes were mixed and incubated at 4°C for 2 to 3 hours. 10 minutes prior to the end of incubation, a charcoal suspension of 2% (w/v) Norit-A Charcoal, 1%(w/v) BSA in ice-cold assay buffer was prepared and allowed to mix at 4°C.

250µl of the charcoal suspension was added to each tube, the tubes were mixed and centrifuged at 12000 x g in a MSE microcentaur to pellet the charcoal. 400µl of the supernatant was counted in ecoscint scintillation fluid in a Rackbeta scintillation counter with an RIA curve fitting program. The program constructed a standard curve and calculated the cAMP content of each sample. The sensitivity of the assay was routinely found to be lower than of the Salomon assay, with cAMP levels of and between 5 to 15 pmoles/ml/sample detectable.

b.Two step chromatography (Salomon) Method

This assay monitors the production of [^{32}P] cAMP from the substrate α[^{32}P]ATP and was performed in a manner similar to that described by Salomon (1979), except that the amount of α[^{32}P]ATP was reduced to 1µCi per sample

(i).Sample preparation.

Briefly, reaction mixtures of 50µl containing; 5mM creatine phosphate, 100mM NaCl, 100U/ml creatinephosphokinase, 25mM Tris acetate pH7.0, 5mM Mg acetate, 0.5mM ATP pH 7.0, 0.05mM cAMP, 1mM DTT, 0.1mg/ml BSA, 10mM GTP pH 7.0, α[^{32}P]ATP (1 x 10^6 c.p.m.), between 5 to 20µg of membrane protein., together with the ligand(s) of interest. Reactions tubes were kept on ice at all times and the reaction started by removal to a 30^{o}C water bath. After 15 mins, the reaction was terminated by removal to ice and the addition of 100µl of stopper solution which was 2% (w/v) SDS, 45mM ATP, 1.3mM 3'5'cAMP. 50ml of [8-^{3}H]3'5'cAMP (approx. 13,000 c.p.m) was added to each tube prior to boiling for 15 mins. 750µl of water was then added to each sample, and the ^{32}P cAMP content of each tube determined immediately.

(ii). 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^+ 50 x 4 (200-400) was washed in twice its packed volume with 1M Hydrochloric acid and then with the same volume of water four times. The Dowex was mixed with water to a slurry (1:1 v/v ratio) and then 3mls removed and added to glass wool stoppered columns. The water was allowed to drain out and the columns washed with 2mls of 1M hydrochloric acid and stored at room temperature. Prior to use, the columns were washed with 1ml of 1M hydrochloric acid followed by 20mls of water.

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

(iii). Separation of cAMP on dowex columns

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

Freeze-dried cAMP was reconstituted in water to give approximately 10,000 c.p.m. in 50µl. [^{32}P]ATP was diluted from stock of 1m Ci/ml to give approximately 2000 c.p.m. in 50µl. 50µl of each of the cAMP and ATP solutions were added to 900 µl of water and the mixture applied to a dowex column. The ATP and cAMP were eluted from the column by successive washes of the column with 0.5mls of water. Fractions were collected in a vial with 5mls of ecoscint and radioactivity determined by scintillation counting in a dual label programme.

The elution volumes required to elute the cAMP from the Dowex columns were then determined from a graph as shown (Figure 2.1). Typical recovery from the Dowex columns was always greater than 90%.

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

(iv).Determination of cAMP produced by membrane fractions.

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

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

2.16.Data Analysis

Where appropriate, data were analysed for statistical significance using either Student's two-tailed t-test (paired) or by a one way analysis of variance.

Fig. 2.1 The elution profiles of ATP and cAMP standards from dowex 50W (H^+ form) and neutral alumina columns.

The elution profiles of [α^{32}P]ATP and [^{3}H]cAMP from dowex (50W H^+ form) columns (2.1a) and the elution of [^{3}H]cAMP from neutral alumina columns (2.1b) was determined. Firstly, by the addition of a [^{3}H]cAMP standard (15,000 c.p.m., 500µl)(closed circles) and a [α^{32}P]ATP standard (18,000 c.p.m., 500µl)(open circles) to a dowex column (as detailed in section 2.15(iii)), followed by elution from the column by repeated addition of 1ml volumes of H_2O (Figure 2.1a). Secondly, a [^{3}H]cAMP standard (14,000 c.p.m., 1000µl)(2.1b) was added to a dowex column and eluted by the addition of 3.5mls of H_2O, into an alumina column. The standard was eluted from the alumina column by repeated addition of 1ml volumes of 0.1M imidazole/HCl, pH7.3 (Figure 2.1b). The progress of both standards was monitored by counting 1ml fractions of eluate in a scintillation counter as detailed in section 2.15(iv).

Figure 2.1(a)

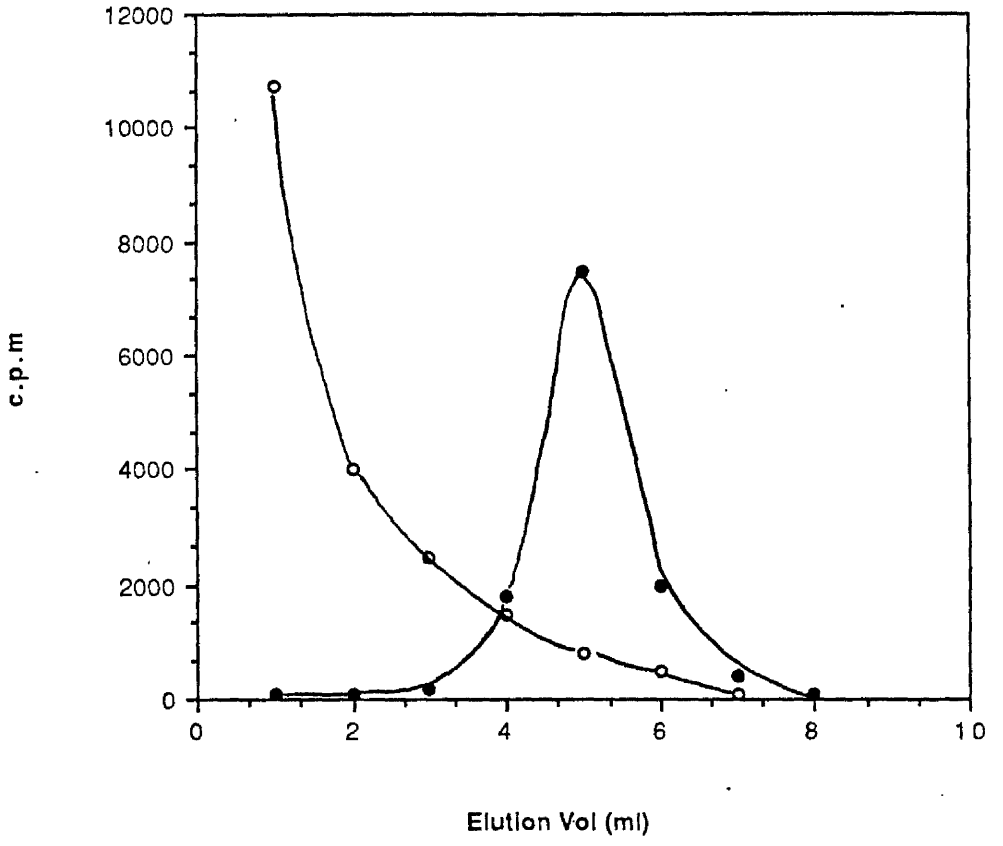
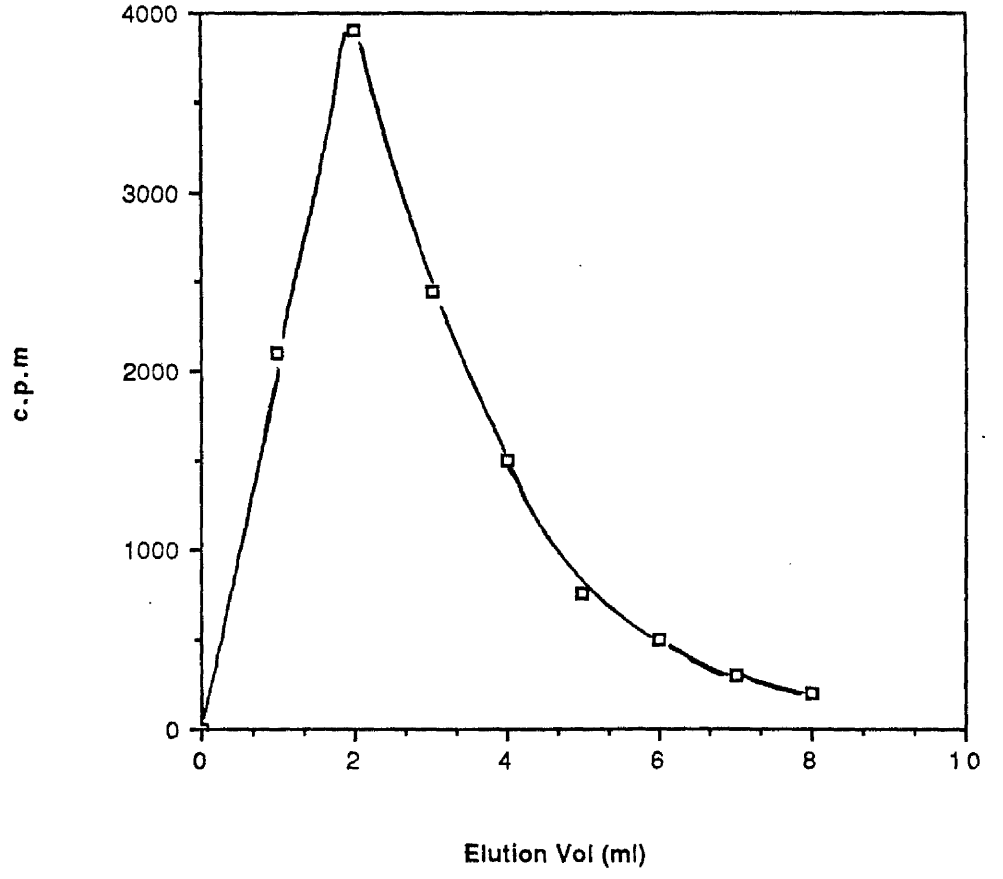


Figure 2.1(b)



Chapter 3

The NG108-15 cell line as a model system to study G-protein signal transduction mechanisms.

Introduction.

The neuroblastoma x glioma hybrid cell line, NG108-15, (also called 108CC15) was generated by the fusion of the 6-thioguanine-resistant clonal mouse neuroblastoma cell line N18TG2 with the bromodeoxyuridine-resistant rat glioma cell line C6-BU1, followed by selection with hypoxanthine, aminopterin, thymidine (HAT) medium and cloning (Hamprecht et al., 1977, 1985). These cells display a considerable range of neuronal features and possess many of the functions of differentiated neurones, including the expression of excitable membranes, the formation of functional synapses and the presence of neurotransmitter enzymes. In addition, NG108-15 cells are able to synthesise neurotransmitters such as acetylcholine, leucine and methionine enkephalins, and are known to secrete both adenosine and catecholamines (Mochly-Rosen et al., 1988).

The NG108-15 cell line has been widely used as a model system to examine transmembrane signalling processes in the nervous system. These cells are particularly suitable for such studies as they express a considerable range of receptors which can be demonstrated to couple to a variety of effector systems including the stimulation and inhibition of adenylate cyclase (Klee et al., 1985), activation of phosphoinositidase C (Higashida et al, 1986), regulation of voltage-sensitive Ca^{2+} channels (VOCC) (Tsunoo et al., 1986) and activation of a Ca^{2+} dependent K^{+} channel (Fukuda et al., 1988). Each of these effector systems is coupled to relevant receptors by members of the family of guanine nucleotide binding proteins (G-proteins), as detailed in chapter 1.

The best characterised receptors expressed by NG108-15 cells are coupled to the inhibition of adenylyl cyclase and include those for δ-opioids (Sharma et al., 1975; Klee and Nirenberg, 1974; Chang et al., 1978; Chang and Cuatrecasas, 1979; Law et al., 1985a,b) and α-2 adrenergic receptors (Sabol and Nirenberg, 1979) which have been designated as the α-2B subtype (Bylund, 1988; Bylund et al., 1988). In addition, bradykinin (Higashida et al., 1986; Osugi et al., 1987),

adenosine A_2 receptors (Kenimer and Nirenberg, 1981), and muscarinic receptors (Nathanson et al., 1978) of the M4 designation (Fukada et al., 1988; Peralta et al., 1988) are expressed by this cell line. NG108-15 cells do not express a wide variety of receptors linked to stimulation af adenylyl cyclase, however prostanoid receptors of the IP classification are expressed (Carroll and Shaw, 1989) as well as receptors for glucagon (Traber et al., 1975a), and secretin (Traber et al., 1975a,b).

Prior to undertaking a study of the nature of receptor- G-protein coupling in NG108-15 cells, it was felt necessary to firstly characterise which members of the G-protein family are expressed by NG108-15 cells. This was accomplished by using both pertussis and cholera toxins, whose effects have been described in chapter1, as well as employing immunological methods, utilising the antisera which are described in table 2.1 in chapter 2. As detailed in chapter 1, the G-protein α-subunits and the three β-subunits which have been sequenced to date are highly homologous at the primary sequence level, and it was felt that the most worthwhile approach to adopt in attempting to produce specific antisera, was to employ synthetic peptides rather than purified protein, since proteins which are highly homologous at the primary sequence level are likely to have similar epitopes, which in turn would lead to a high degree of G-protein antisera cross reactivity (Milligan, 1989). The peptides utilised corresponded to either regions of variation between the G-protein α-subunits, or regions which have been proposed to represent sites of functional interaction between the G-protein and other polypeptides of the signal transduction pathway (Masters et al., 1987). In the case of the β-subunits, a peptide corresponding to the extreme amino-terminal region of the protein was chosen since it was likely to produce antisera which demonstrate cross-reactivity with all β-subunits. The N-terminal peptide had little homology to any known sequence in the G-protein α-subunits which have been cloned and sequenced to date, and as such was more likely to produce specific β-subunit antisera.

Fig. 3.1 Pertussis toxin-catalysed ADP-ribosylation of membranes of neuroblastoma x glioma hybrid NG108-15 cells.

NG108-15 membranes (20 µg) from either untreated cells (lanes a,c) or cells which had been pretreated *in vivo* with pertussis toxin (lane b) as described in section 2.6d were ADP-ribosylated with thiol-preactivated pertussis toxin and [^{32}P]NAD^{+} as described in section 2.12. The sample was resolved on either SDS-PAGE (10% acrylamide, 0.25% bisacylamide, 18cm x 16cm resolving gel) (Figure 3.1 a, b), or SDS-PAGE (12.5 % acrylamide, 0.0625% bisacrylamide, 20 x 20 cm resolving gel) (Figure 3.1 c) as detailed in section 2.10a. The dried gel was autoradiographed for 24 hours with intensifying screens exactly as described in section 2.10f.

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

Figure 3.1

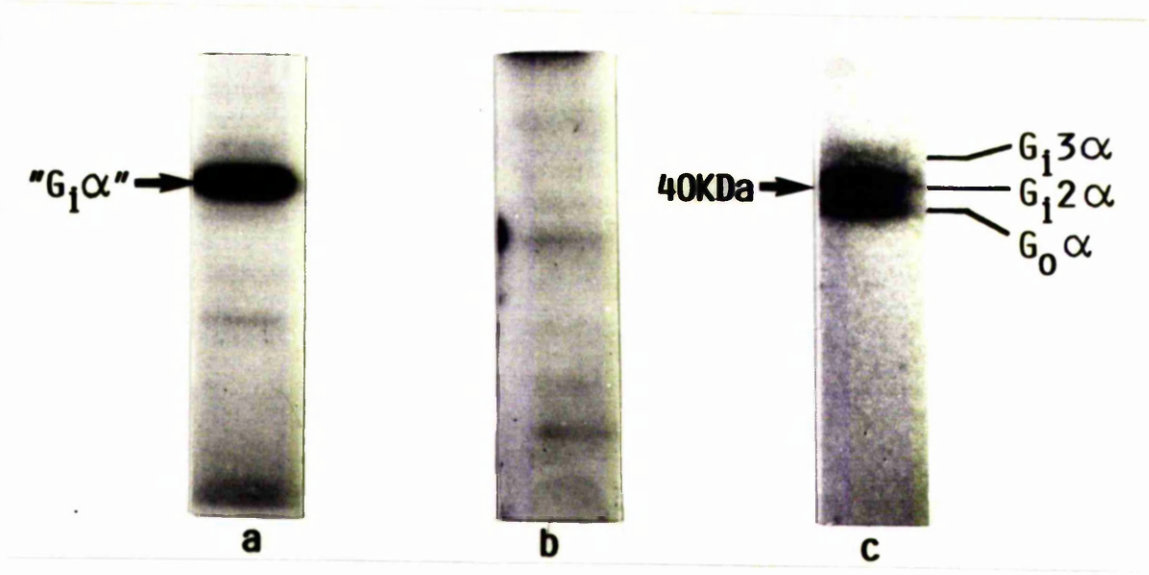


Fig. 3.2 **Cholera toxin-catalysed ADP-ribosylation of membranes of neuroblastoma x glioma hybrid NG108-15 cells.**

NG108-15 membranes (20 µg) from either untreated cells (lanes a,c) or cells which had been pretreated *in vivo* with cholera toxin (lanes b,d) as described in section 2.6d, were ADP-ribosylated with thiol-preactivated cholera toxin and [^{32}P]NAD^{+}. The sample was resolved on either SDS-PAGE (10% acrylamide, 0.25% bisacylamide, 18cm x 16cm resolving gel) (Figure 3.2 a,b), or SDS-PAGE (12.5 % acrylamide, 0.0625% bisacrylamide, 20 x 20 cm resolving gel) (Figure 3.2 c,d). The dried gel was autoradiographed for 24 hours with intensifying screens. All procedures were performed as detailed in the legend to Figure 3.1.

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

Figure 3.2

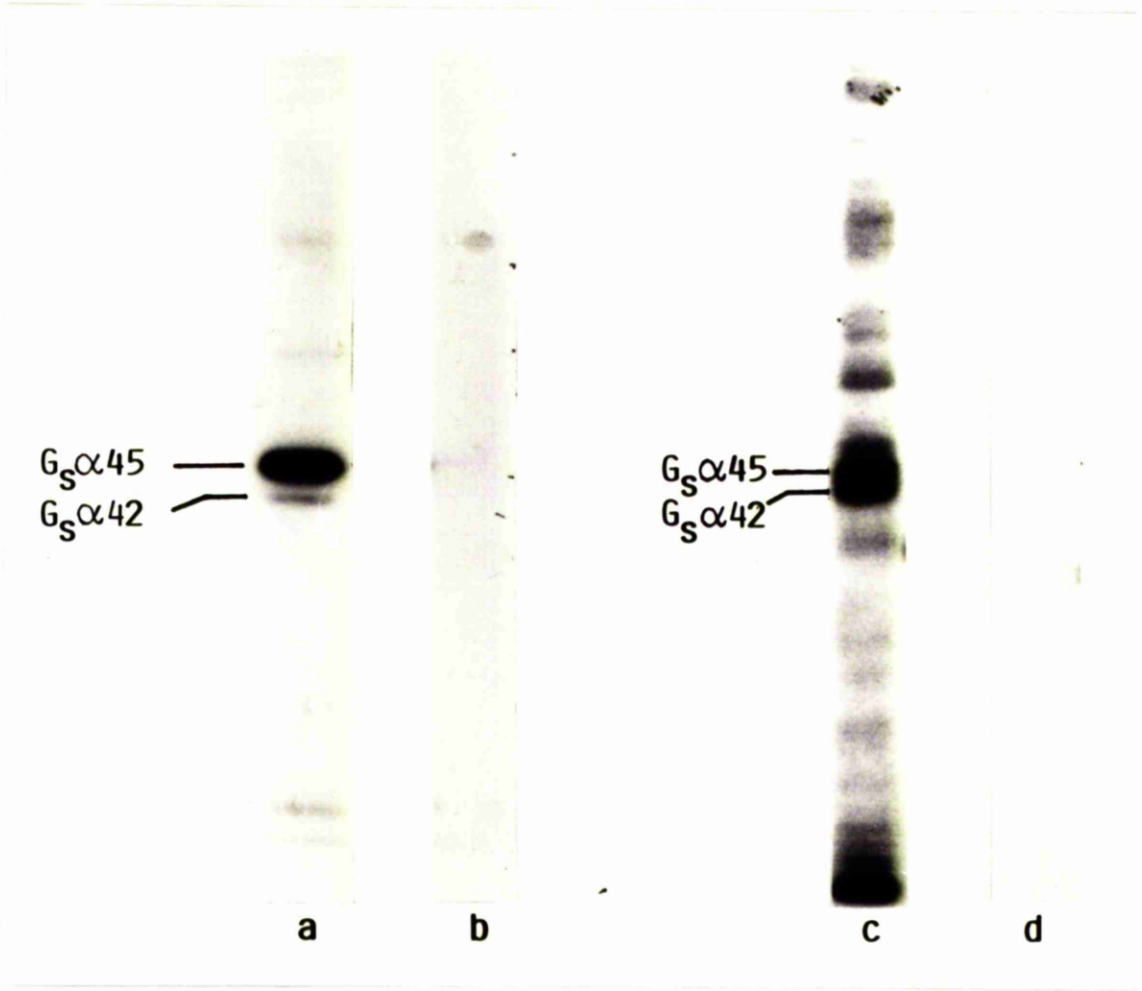


Fig. 3.3 Expression of multiple pertussis toxin-sensitive G-proteins in neuroblastoma x glioma hybrid cells. Immunological demonstration of their molecular identity.

Membranes of NG108-15 cells and of rat glioma C6 BU1 cells, rat cerebral cortex and rat white adipocytes were resolved on SDS-PAGE as detailed in the legend to Figure 3.1 and transferred to nitrocellulose. The choice of tissue to compare to NG108-15 membranes was based on previous identification of particular G-proteins in those tissues (Goldsmith et al., 1987; Mitchell et al., 1989). Immunoblotting was performed as detailed in section 2.11) with 1: 200 dilutions of (A) antiserum OC1 (1; 100 µg NG108-15 membranes, 2; 10 µg rat cerebral cortex), (B) antiserum CS1 (1; 100 µg NG108-15 membranes, 2; 50 µg rat white adipocyte membranes), (C) antiserum I3B (1; 100 µg NG108-15 membranes, 2; 50 µg rat white adipocytes, 3; 100 µg rat glioma C6 BU1 cell membranes) (D) antiserum AS7 (1; 100 µg NG108-15 membranes, 2; 50 µg rat cerebral cortex, 3; 50 µg rat glioma C6 BU1 membranes).

Detection of the primary antiserum was achieved using an IgG fraction from a donkey anti-rabbit antiserum which was coupled to horseradish peroxidase as detailed in section 2.11. The substrate for the enzyme was o-dianisidine.

Figure 3.3

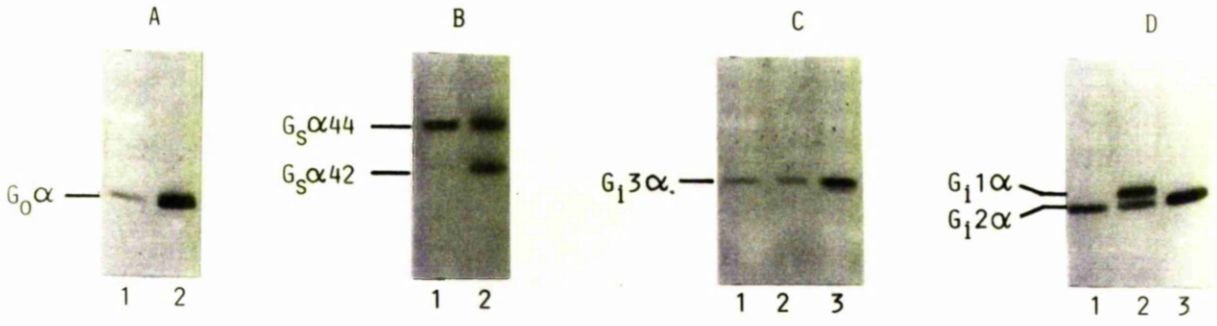

A
B
C
D
$G_o\alpha$
$G_s\alpha 44$
$G_s\alpha 42$
$G_i3\alpha$
$G_i1\alpha$
$G_i2\alpha$
1 2
1 2
1 2 3
1 2 3

Fig. 3.4 Immunological detection of Gi2 but not Gi1 in NG108-15 cell membranes.

Membranes of NG108-15 cells (100μg, panels A,B,C , lane 1) and rat cortex (75μg, panels A,B,C, lane 2) were resolved on SDS-PAGE as described in the legend to Figure 3.1 and transferred to nitrocellulose. Immunoblotting was performed with a 1: 200 dilution of either antiserum LE2 (panel A), SG1 (panel B) or antiserum I1C (panel C) as detailed in section 2.11. The primary antiserum was detected as described in the legend to Figure 3.3.

Figure 3.4

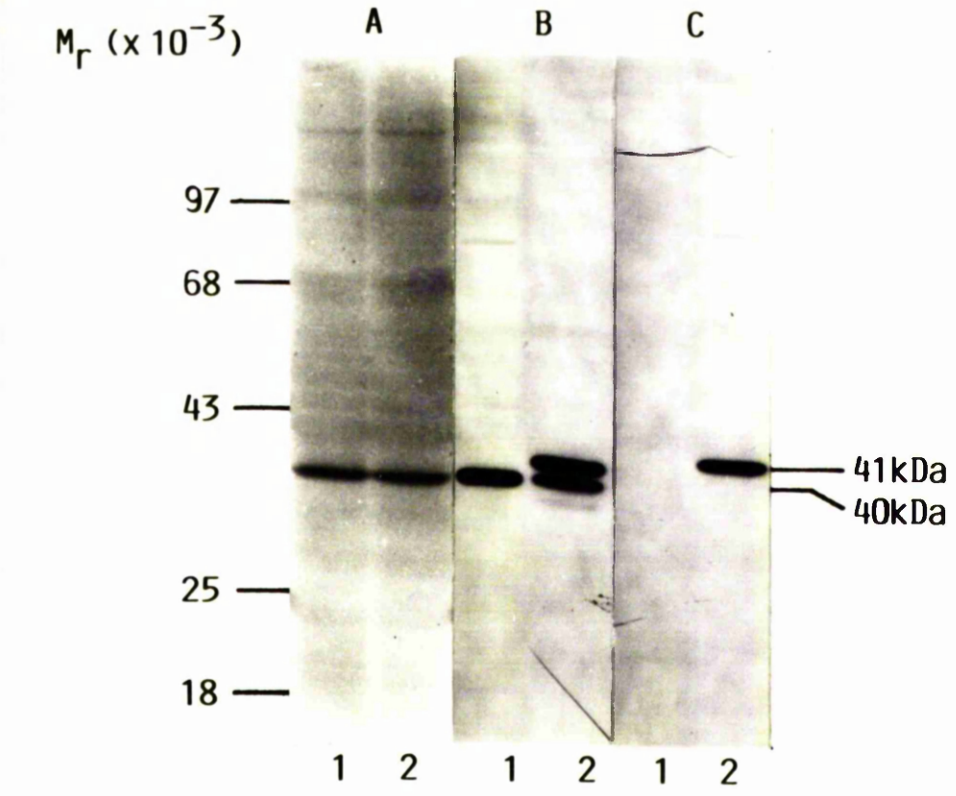


Fig. 3.5 Immunological detection of the G-protein β-subunit in NG108-15 cell membranes.

Membranes of NG108-15 cells (100μg, lane a) and rat glioma C6BU1 cells (75μg, lane b) were resolved on SDS-PAGE as detailed in the legend to Figure 3.1 and transferred to nitrocellulose. Immunoblotting was performed as described in section 2.11, with a 1: 200 dilution of antiserum BN1. The primary antiserum was detected as described in the legend to Figure 3.3.

Figure 3.5

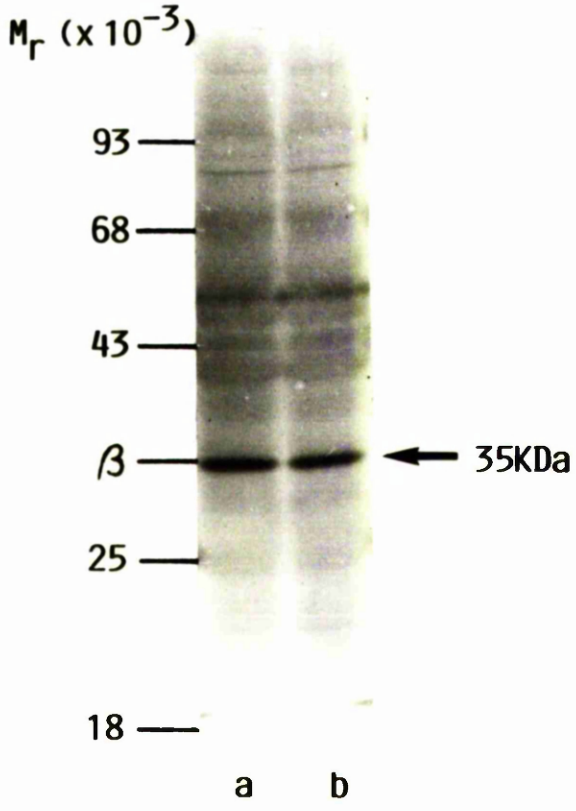


Fig. 3.6 Immunoblotting of NG108-15 membranes with affinity purified antibodies from antiserum AS7.

Membranes of NG108-15 cells (50µg, lane a) or rat cortex (60µg, lane b) were separated on a SDS 10% polyacrylamide gel at 50V overnight as described in section 2.10. The resolved proteins were transferred to nitrocellulose and blocked and immunoblotted with a 1:100 dilution of antibodies affinity purified from antiserum AS7 as detailed in section 2.9. The primary antiserum was detected as described in the legend to Figure 3.3.

Figure 3.6

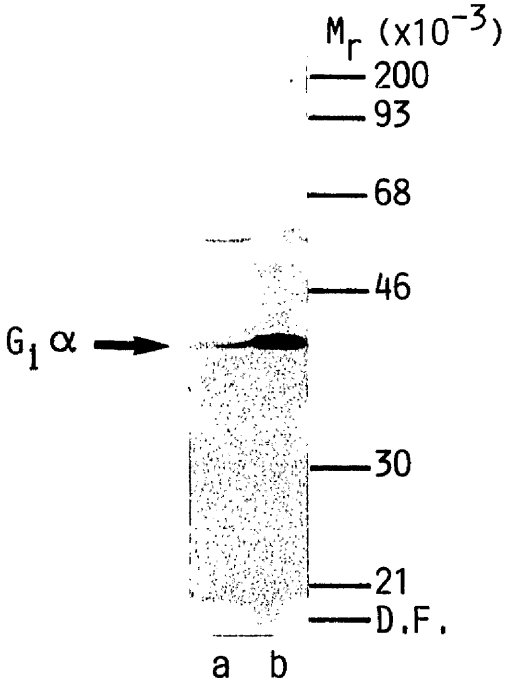


Fig. 3.7. IgG purified from antiserum AS7 displays the same specificity for G-proteins as the crude antiserum.

Membranes from NG108-15 cells and of rat glioma C6BU1, rat cerebral cortex and rat white adipocytes were resolved on 20cm x 20cm SDS-PAGE gels as described in the legend to Figure 3.1. Immunoblotting was performed as described in the legend to Figure 3.3 ,except an IgG fraction purified by passage over protein-A sepharose was used as the primary reagent (section 2.9). Immunoblotting was performed with 1: 200 dilution of an IgG fraction from AS7 (1; 100 µg NG108-15 membranes, 2; 50 µg rat cerebral cortex, 3; 50 µg rat glioma C6 BU1 membranes).

The primary antiserum was detected as described in the legend to Figure 3.3.

Figure 3.7

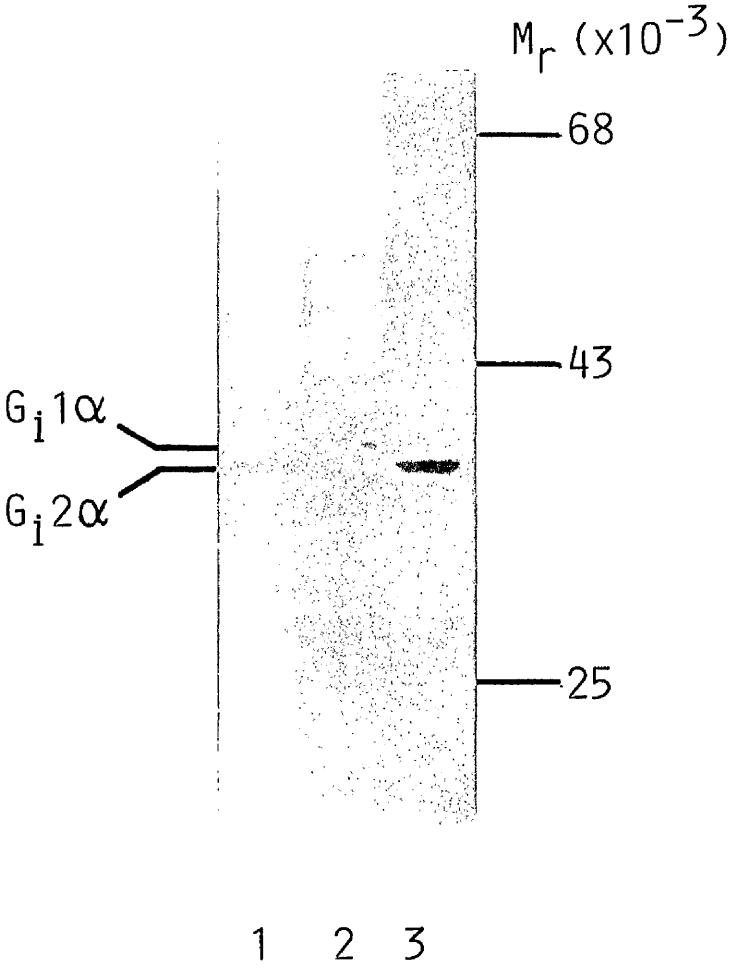


Fig. 3.8 Identification of the pertussis toxin substrates expressed in NG108-15 cell membranes as Gi2, Gi3 and Go.

NG108-15 membranes (20 µg) from untreated cells were ADP-ribosylated with thiol-preactivated pertussis toxin and [^{32}P]NAD^{+} as detailed in section 2.12. The sample was resolved by SDS-PAGE (12.5 % acrylamide, 0.0625% bisacrylamide, 20 x 20 cm resolving gel) as detailed in the legend to Figure 3.1. The gel obtained was then western blotted and probed with a combination of a 1:200 dilution of antiserum, I3B, a 1: 1000 dilution of antiserum OC1 and a 1: 3000 dilution of antiserum AS7. The primary antiserum was detected as described in the legend to Figure 3.3 (Figure 3.8, a). After development, the dried nitrocellulose was autoradiographed for 24 hours with intensifying screens as detailed in section 2.10f (Figure 3.8, b). None of the polypeptides shown were labelled in the absence of activated pertussis toxin.

Figure 3.8

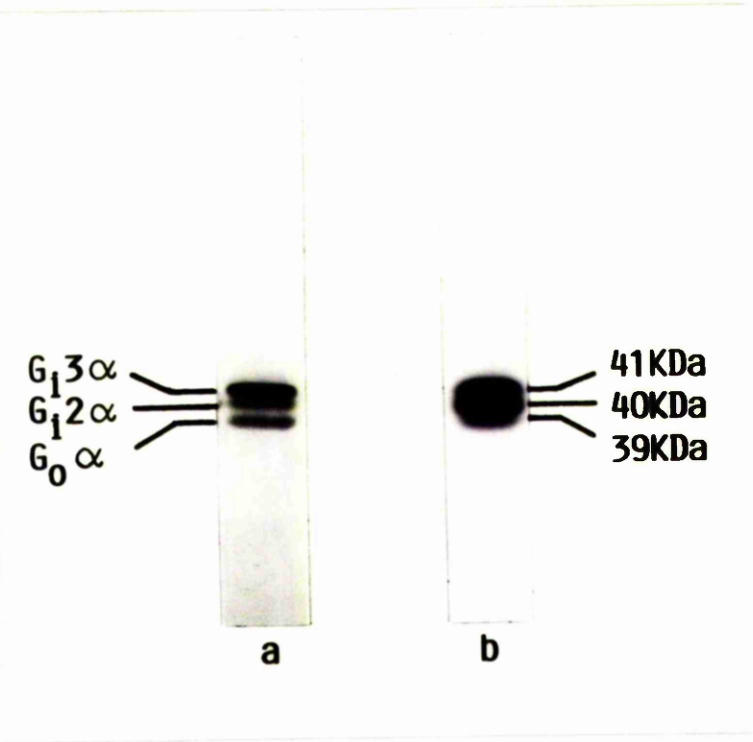
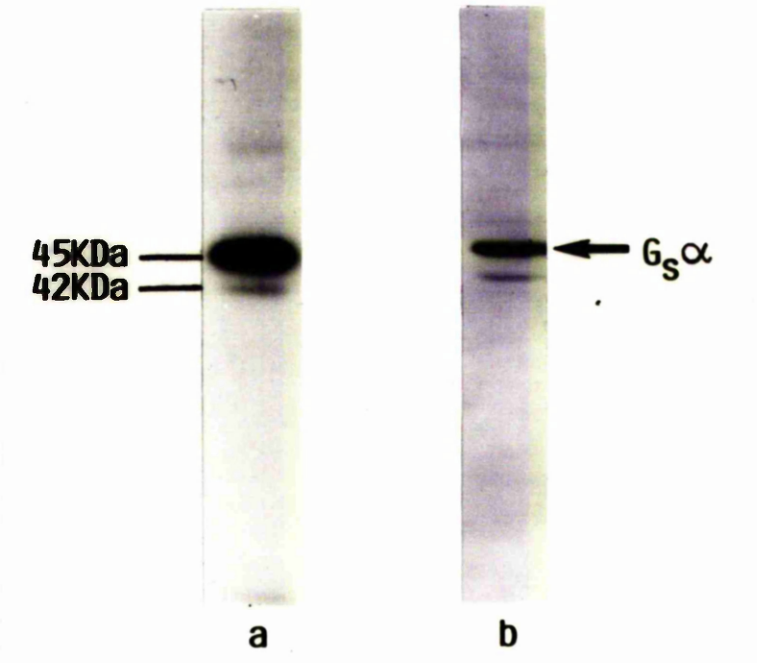


Fig. 3.9 Identification of the cholera toxin substrates expressed in NG108-15 cell membranes as two distinct forms of Gs

NG108-15 membranes (20 µg) from untreated cells were ADP-ribosylated with thiol-preactivated cholera toxin and [^{32}P]NAD^{+}. The sample was resolved by SDS-PAGE (12.5 % acrylamide, 0.0625% bisacrylamide, 20 x 20 cm resolving gel) as detailed in the legend to Figure 3.1. The gel obtained was then western blotted and probed with a 1:200 dilution of antiserum CS1. The primary antiserum was detected as described in the legend to Figure 3.3 (Figure 3.9, b). After development, the dried nitrocellulose was autoradiographed for 24 hours with intensifying screens (section 2.10f) (Figure 3.9, a). None of the polypeptides shown were labelled in the absence of activated cholera toxin.

Figure 3.9



Results

The treatment of membranes of NG108-15 cells with activated pertussis toxin and [^{32}P]NAD$^+$ led to the incorporation of radioactivity into an apparently single, broad band of 40kDa, presumably representing "Gi" (Figure 3.1 a). Several other polypeptides incorporated radioactivity, however in no case was the incorporation as great as that demonstrated in the 40kDa region. When membranes produced from cells which had been pretreated *in vivo* with pertussis toxin (as described in section 2.6d) were challenged with fresh pertussis toxin and [^{32}P]NAD$^+$, no incorporation of radioactivity into the 40kDa band was obtained.(Figure 3.1 b). This demonstrated that the entire complement of pertussis toxin sensitive G-proteins had been ADP-ribosylated by *in vivo* treatment with pertussis toxin. If the SDS-PAGE resolving conditions were altered to obtain better resolution of proteins with a molecular mass of between 35 to 45kDa, then the single broad band of radioactivity seen at approximately 40 kDa in Figure 3.1a, could be resolved into three separate polypeptides with molecular masses of 41, 40 and 39kDa (Figure 3.1 c), the labelling of which was dependent on the presence of pertussis toxin (*not shown*). This demonstrated that NG108-15 cells express at least three different pertussis toxin sensitive G-proteins.

When membranes produced from NG108-15 cells were treated with thiol-activated cholera toxin in the presence of [^{32}P]NAD$^+$, incorporation of radioactivity into two polypeptides with molecular masses of 45 and 42kDa was routinely obtained. These presumably represent two of the four possible variants of Gsα (Figure 3.2a). Several other polypeptides also demonstrated incorporation of radioactivity, however it was difficult to routinely demonstrate radiolabelling of the same polypeptides from different NG108-15 membrane preparations in a toxin-dependent manner. In membranes produced from NG108-15 cells which had been pretreated *in vivo* with cholera toxin, re-exposure to cholera toxin along with [^{32}P]NAD$^+$ did not result in the incorporation of radioactivity into the two polypeptides (Figure 3.2b), demonstrating that pre-treatment of NG108-15 cells *in vivo* with cholera toxin ADP-ribosylated the entire pool of the stimulatory G-

protein, Gs. When the SDS-PAGE resolving conditions were altered to increase the resolution of proteins with molecular masses of approximately 35 to 45kDa (Figure 3.2c), a result essentially identical to that shown in Figure 3.2a was obtained, where only two bands of approximately 45 and 42 kDa incorporated radioactivity in a toxin dependent manner.

In an attempt to identify the pertussis toxin sensitive G-proteins which were expressed by NG108-15 cells, a series of immunobiots were performed using the antisera described in chapter 2, (Table 2.1). Antiserum OC1 specifically identifies the α-subunit of Go, and identified a single polypeptide of 39kDA in NG108-15 membranes which co-migrated with an equivalent polypeptide in rat brain (Figure 3.3a), a tissue known to express high levels of this polypeptide (Milligan and Klee, 1985). Antiserum AS7 interacts equally with the α-subunits of Gi1 and Gi2 as these G-proteins have identical C-terminal decapeptides (Goldsmith et al., 1987). This antiserum identified two polypeptides of 41 and 40kDa in both rat brain and adipocytes, but identified only a single polypeptide in NG108-15 membranes (Figure 3.3d). This polypeptide co-migrated with the polypeptide of greater mobility in adipocytes and brain, which has previously been shown to be Gi2 (Mitchell et al., 1989). A Gi2 specific antiserum (LE2) also identified the 40kDa polypeptide in NG108-15 cell membranes (Figure 3.4, panel A, lane1) and in adipocytes (results not shown) but did not identify the 41kDa polypeptide of brain (Figure 3.4, panel A, lane2). It has been demonstrated previously that the 41kDa polypeptide of brain and adipocytes corresponds immunologically to Gi1.(Mitchell et al., 1989). The Gi1 specific antiserum I1C was able to specifically recognise this polypeptide in membranes derived from brain, but not from NG108-15 cells (Figure 3.4, panel B, lanes 1, 2). Antiserum I3B identifies Gi3α; this antiserum identified a single polypeptide of 41kDa in NG108-15 membranes which co-migrated with a polypeptide in rat glioma C6BU1 cells and in rat adipocytes (Figure 3.2c), which has been demonstrated to be Gi3α (Mitchell et al., 1989). This immunological data confirmed that the antisera described are indeed selective and can be used as specific probes to monitor the expression of the various G-proteins. In addition, it may also be noted that NG108-15 cell express immunologically detectable levels of Gi2, Gi3, Go and Gs, but do not express Gi1.

A similar range of western blots to those described in Figure 3.3 were carried out using IgG fractions derived from primary antisera by chromatography on a column of protein-A sepharose as described in section 2.9. This purification was carried out for two reasons. Firstly, due to a desire to employ these antisera in functional assays (see chapters 5 and 6) and thus obtain a purer starting material, and secondly to remove the antibodies from the serum they were in, since serum is a potent activator of certain G-proteins (see chapter 5). The specificity of each purified IgG fraction, as compared to the primary antiserum had to be determined prior to use. Figure 3.7 demonstrates that IgG fractions purified from antiserum AS7, showed the same specificity for G-proteins as the crude antiserum. IgG fractions purified from antisera CS1, I3B, OC1 and BN1 also demonstrated the same G-protein specificity (results not shown). In some instances, functional assays were performed using a fraction affinity purified from antiserum AS7 (see later). When immunoblots were performed using a fraction from antiserum AS7 which had been affinity purified on a column of bovine transducin, a polypeptide corresponding to Gi2 was identified, demonstrating that the affinity purified fraction retained the same specificity as the primary antiserum (Figure 3.6).

In order to rigorously identify the three pertussis toxin sensitive G-proteins identified in Figure 3.1 as Gi2, Gi3 and Go, NG108-15 cell membranes were ADP-ribosylated with pertussis toxin and resolved by SDS-PAGE. The resultant gel was then immunoblotted with a combination of antisera AS7, I3B and OC1. Figure 3.8 demonstrated that the most slowly migrating pertussis toxin sensitive G-protein in NG108-15 cell membranes was solely recognised by antiserum I3B, the antiserum which specifically identifies Gi3. The G-protein which migrated the greatest distance under these conditions is recognised by the anti-Go antiserum OC1, and the G-protein which migrated between these two polypeptides was exclusively recognised by the Gi2 specific antiserum AS7.

When similar ADP-ribosylation/immunoblotting experiments were carried out on NG108-15 cell membranes which had been ADP-ribosylated with cholera toxin and then immunoblotted with antiserum CS1, which exclusively recognises

Gsα, we see that two forms of Gsα are expressed in this cell line, and that the cholera toxin substrates co-migrate with the polypeptides which are specifically identified by antiserum CS1.

Discussion.

As cDNA cloning experiments have demonstrated the potential expression (outwith photoreceptor containing tissues) of four distinct G-proteins which are substrates for pertussis toxin, namely Gi1, Gi2, Gi3 and Go (Jones and Reed, 1987; Itoh et al., 1988) along with the cholera toxin substrate, Gs and the toxin insensitive Gz (x), it is apparent that a given cell type may express a considerable repertoire of highly homologous G-proteins.

Few studies have attempted a rigorous definition of the members of the G-protein family which may be expressed by a given cell line or tissue. This is primarily due to difficulty in selectively identifying each member of the G-protein family. However, the generation of a series of antipeptide antisera which are able to discriminate between each of the currently identified G-proteins, along with the development of electrophoresis conditions able to resolve the individual α-subunits, has allowed several groups to examine a variety of cell lines which are commonly used as a model of signal transduction (Mitchell et al., 1989; Scherer et al., 1987; McKenzie and Milligan, 1990).

The major pertussis toxin sensitive G-proteins expressed by neutrophils and other haemopoietically derived cells have thus been identified as Gi2 and Gi3 (Murphy et al., 1987). Antipeptide antisera have failed to detect either Gi1 or Go in such cells, a conclusion supported by the detection of mRNA's corresponding to Gi2 and Gi3, but not Gi1 or Go (Murphy et al., 1987). In a similar manner, the G-proteins which are expressed by human platelets have been identified by antipeptide antisera as being Gs, Gi2 , Gi3 and Gx(z). Gi1 and Go cannot be detected (Simonds et al., 1989a).
It can therefore be appreciated that the distribution of the individual G-protein α-subunits is not universal, with the exception of Gs, Gi2 and Gi3 (Milligan, 1989).

The NG108-15 cell line has been widely used as a model system to study signal transduction since it expresses a variety of receptors which are able to modulate the level of activity of various intracellular effectors through G-protein

activation (Hamprecht et al., 1985). The most widely studied signal transduction system in NG108-15 cells is adenylyl cyclase, which may be both stimulated or inhibited by a variety of transmembrane receptors (Klee et al., 1985). Receptor mediated inhibition of adenylyl cyclase is always attenuated by prior treatment with pertussis toxin, defining a role for a pertussis toxin sensitive G-protein(s) in mediating the response (Clapham and Neer, 1987).

In order to rigorously assess which of the pertussis toxin sensitive G-proteins is coupled to the inhibition of adenylyl cyclase in NG108-15 cells, the complement of G-proteins which are expressed by this cell line were firstly defined. This was accomplished by the generation of anti-peptide antisera against synthetic peptides which correspond to the C-terminal decapeptides of the pertussis toxin sensitive G-proteins, and the stimulatory G-protein, Gs. Using these antisera it has been possible to demonstrate the expression of three pertussis toxin sensitive G-proteins, Gi2, Gi3 and Go in the NG108-15 cell line, as well as two forms of Gs. High-titre antisera which have been demonstrated to recognise the α-subunit of Gi1 (Figure 3.4, panel c, lane 2), fail to detect Gi1 in NG108-15 cell membranes (Figure 3.4, panel c, lane 1).

The characterisation of human cDNA's corresponding to four different forms of Gsα has been reported, suggesting that in a given tissue, two larger and two smaller forms of Gsα may be present (Bray et al., 1986). The exact complement of Gsα expressed by NG108-15 cells has not yet been fully defined, although one dimensional gel electrophoresis suggests that two forms are expressed, with molecular masses of 45 and 42kDa. It may be noted that based upon both immunoblotting and ADP-ribosylation, the 45kDa form is more prevalent than the 42kDa form.

In addition to the immunological techniques described, a recent report has examined Northern blots of total RNA isolated from NG108-15 cells, using synthetic oligonucleotide probes derived from the coding sequence for amino-acids 125-135 of the α-subunits of each of Gi1, Gi2 and Gi3. These probes confirmed the presence of

mRNA coding for Gi2 and Gi3 in NG108-15 cells, however mRNA corresponding to Gi1 could not be detected (McKenzie and Milligan, 1990). These observations are therefore in agreement with the immunological data presented in this chapter.

The data presented herein demonstrate that NG108-15 cells express detectable levels of Gi2, Gi3 and Go, as well as two forms of the stimulatory G-protein Gs. Gi1 is either not expressed by these cells, or the levels of both peptide and relevant mRNA are below current methods of detection. In addition it may be noted that it is possible to generate antisera which can specifically recognise members of the G-protein family. It is of interest to note that IgG fractions produced as detailed in section 2.9 demonstrate the same specificity as the primary anti-G-protein antisera and as such may be employed as specific tools to study G-protein function (chapters 5 and 6).

The aim of this thesis is to examine the interaction of G-proteins with effectors, with a view to determining whether G-proteins are specific signal transducers or whether they are more promiscous and can interact with a wide variety of intracellular effectors. In the NG108-15 cell line, a variety of receptors are coupled to the inhibition of adenylyl cyclase in a pertussis toxin sensitive manner, this means that the G-protein which mediates the inhibition of adenylyl cyclase in NG108-15 cells must be one of Gi2, Gi3 or Go, or a combination therof. The data presented in the following chapters will attempt to define which of these G-proteins is the true 'Gi" of the adenylyl cyclase system and thus ascertain whether individual G-proteins can be defined as having a specific role in transmembrane signalling.

Chapter 4

Cholera toxin catalysed ADP-ribosylation of Gi.

Introduction

The original identification of Gs and Gi, the G-proteins which stimulate and inhibit adenylyl cyclase respectively, was aided by the isolation of both cholera toxin from cultures of *Vibrio cholerae* and pertussis toxin, from cultures of *Bordetella pertussis.*

Pertussis toxin catalyses the mono-ADP-ribosylation of Giα at a cysteine residue four amino acids removed from the C-terminus. This covalent modification attenuates receptor mediated inhibition of adenylyl cyclase (Katada and Ui, 1982; Hsia et al., 1984). With the exception of the protein product of the *ras* protooncogene, all G-protein α subunits with a cysteine in this position have been demonstrated to be pertussis toxin substrates. Gsα does not have a cysteine residue in this position and is not a substrate for pertussis toxin. In contrast, cholera toxin catalyses the mono-ADP-ribosylation of Gsα at an arginine residue, which is either at position 187 or 188 (short forms of Gs), or position 201 or 202 (long forms of Gs). This results in the permanent activation of Gsα (Cassel and Pfeuffer, 1978; Northup et al., 1980). It is generally held that cholera toxin does not affect Giα, however the arginine residue modified by cholera toxin in Gsα, is conserved in an apparently equivalent position in all G-protein α subunits which have been isolated to date by cDNA cloning, and indeed under the appropriate conditions, Tdα may be ADP-ribosylated by both pertussis and cholera toxins. It would therefore appear that cholera toxin's lack of ability to ADP-ribosylate Giα is not a consequence of Giα lacking a suitable acceptor site, but is due to either constraints imposed by the three dimensional structure of Giα, or by the use of assay conditions which are not conducive to the mono-ADP-ribosylation of Giα by cholera toxin.

The data presented in this chapter will attempt to analyse whether cholera toxin may catalyse the ADP-ribosylation of Gi in membranes derived from NG108-15 cells under appropriate conditions and thus determine possible constraints which prevent cholera toxin from ADP-ribosylating a homologous region of Gi to that of Gs which is subject to cholera toxin catalysed ADP-ribosylation.

Fig. 4.1 ADP-ribosylation of NG108-15 membranes by cholera or pertussis toxin in the presence of guanine nucleotides.

Membranes (30μg) of NG108-15 cells were treated with [^{32}P]NAD^{+} and activated cholera toxin (lane a), activated pertussis toxin (lane b) or vehicle (lane c) in the presence of 100μM GTP for 2 hours at 37^{o}C, as detailed in section 2.12. The incubation was terminated by removal to ice, followed by protein precipitation with sodium deoxycholate/TCA (section 2.10c). The membranes were then resolved on a 10% polyacrylamide / SDS gel as detailed in section 2.10. An autoradiogram (section 2.10e) of a dried gel which is similar to at least ten others obtained.is displayed.

Figure 4.1

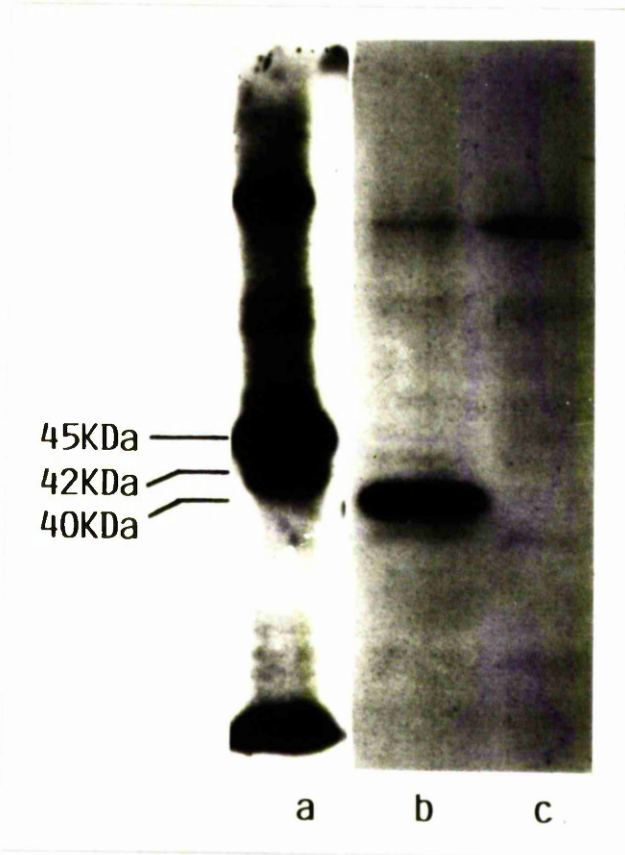


Fig. 4.2 ADP-ribosylation of membranes of untreated NG108-15 cells by cholera or pertussis toxin in the absence of guanine nucleotides.

Membranes (30μg) of NG108-15 cells were treated with [^{32}P]NAD^{+} and activated cholera toxin (a), activated pertussis toxin (b) or without toxin (c) in the absence of exogenously added guanine nucleotide for 2 hours at 37^{o}C as detailed in section 2.12. The incubation was terminated by removal to ice, followed by protein precipitation with sodium deoxycholate/TCA (see section 2.10c) Samples were then resolved on a10% polyacrylamide/SDS gel. An autoradiogram of the dried gel, which is typical of three others obtained, is displayed. The only bands labelled in a toxin dependent manner were of apparent molecular mass 45, 42 and 40kDa.

Figure 4.2

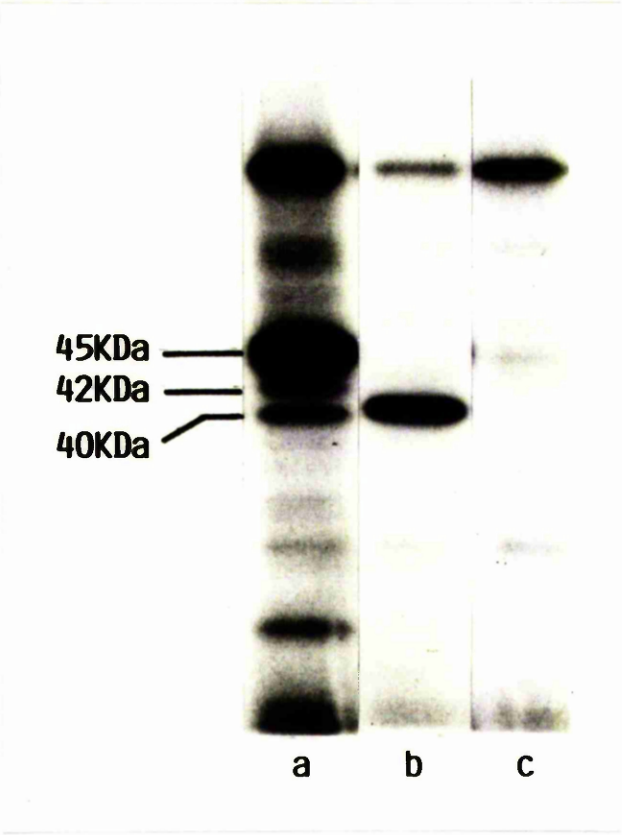


Fig. 4.3 ADP-ribosylation of NG108-15 cell membranes by cholera and pertussis toxin in the absence of exogenously added guanine nucleotides.

Membranes were prepared from NG108-15 cells which had been pretreated with either pertussis toxin, cholera toxin, or without toxin, as described in section 2.6d.

These membranes (40μg) were then treated with [^{32}P]NAD^{+} and activated cholera or pertussis toxin, and resolved on a 10% polyacrylamide/SDS gel and autoradiographed as described in the legend to Figure 4.1.

Lanes (a) and (d) contain membranes prepared from untreated cells, lane (b), membranes prepared from cells pretreated *in vivo* with cholera toxin (section 2.6d) and lanes (c) and (e), membranes from cells pretreated *in vivo* with pertussis toxin (section 2.6d). Lanes (a), (b) and (c) show the labelling of membranes with cholera toxin, lanes (d) and (e) show labelling of membranes with pertussis toxin. The autoradiograph shown is representative of three others which gave essentially identical results.

Figure 4.3

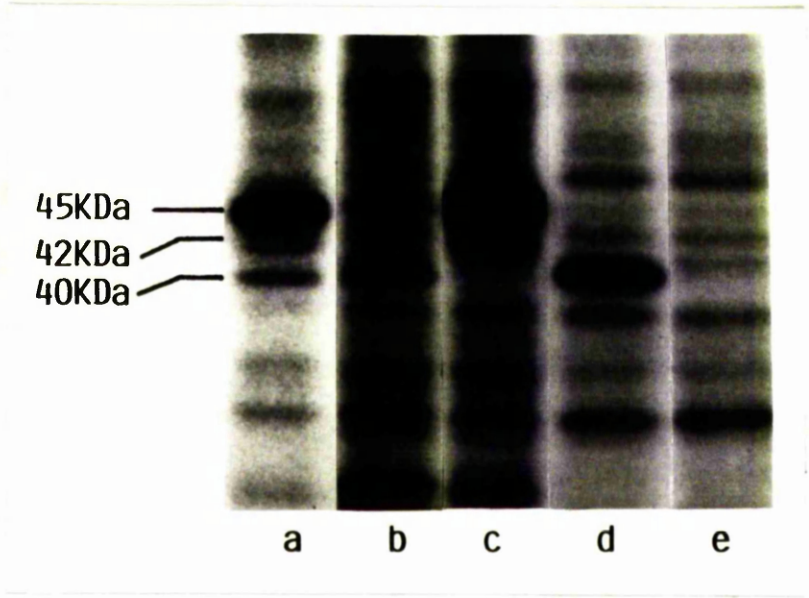


Fig. 4.4 Pharmacology of the opioid-peptide stimulation of cholera toxin catalysed ADP-ribosylation of a 'Gi-like" protein in membranes of untreated NG108-15 cells.

4.4 (a) Membranes of untreated NG108-15 cells (30μg) were treated with either pertussis toxin (lane a) or cholera toxin (lanes b to f) in the absence of guanine nucleotides for 2 hours at 37°C as described in section 2.12.

Lanes (a) and (f) contained no ligand, lane (b) contained naloxone (1mM), lane (c) naloxone (1mM) plus DADLE (0.1μM), lane (d) contained DADLE (0.1μM), and lane (e) DADLE (10μM). The samples were resolved on a 10% polyacrylamide/SDS gel and autoradiographed as described in the legend to Figure 4.1; D.F., Dye Front. 4.4 (b) Incorporation of radioactivity was assessed densitometrically as described in section 2.10f. Taking the incorporation of radioactivity catalysed by pertussis toxin under these conditions (lane a) as 100%, and incorporation of radioactivity by cholera toxin into this band in the absence of ligand (lane f) as a blank, then lane (b) = 4%, lane (c) = 7%, lane (d) = 41% and lane (e) = 66%. Similar data was obtained in three separate experiments.

Figure 4.4(a)

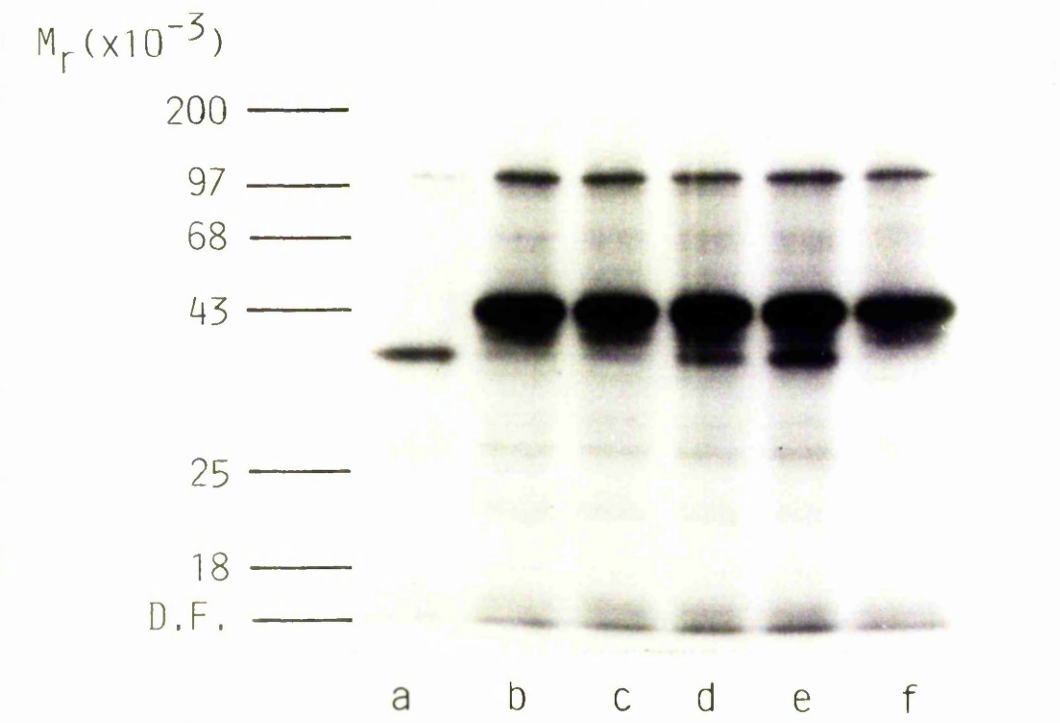


Figure 4.4(b)

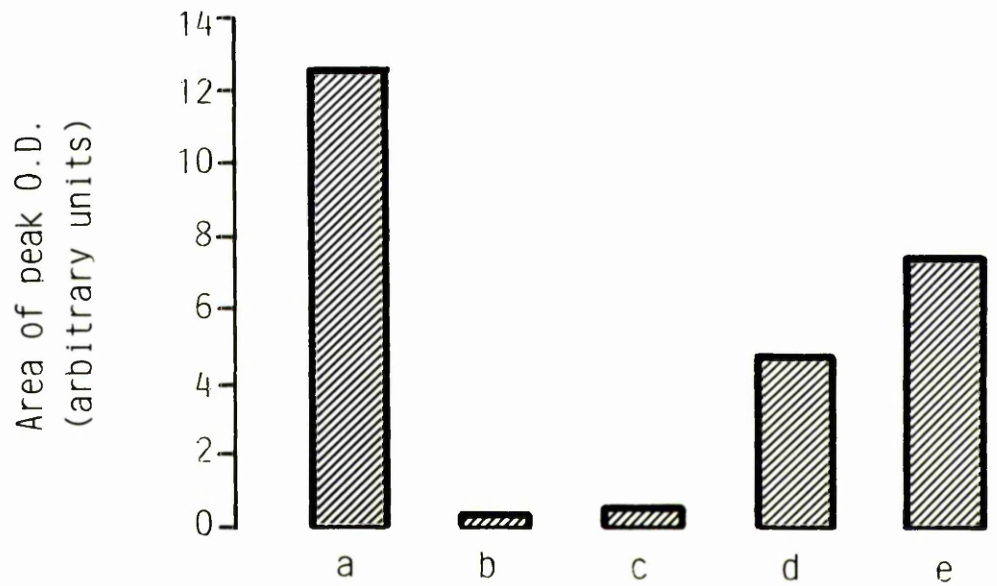


Fig. 4.5 Pharmacology of the opioid-peptide stimulation of cholera toxin catalysed ADP-ribosylation of a 'Gi-like' protein in membranes of cholera toxin pretreated NG108-15 cells.

4.5 (a) Membranes of cholera toxin pretreated NG108-15 cells (30μg) were treated with either pertussis toxin (lane a) or cholera toxin (lanes b-f) in the absence of guanine nucleotides for 2 hours at 37°C as described in section 2.12.

Lanes (a) and (f) contained no ligand, lane (b) contained naloxone (1mM), lane (c) naloxone (1mM) plus DADLE (0.1μM), lane (d) contained DADLE (0.1μM), and lane (e) DADLE (10μM). The samples were collected and autoradiographed as detailed in the legend to Figure 4.1; D.F., Dye Front. 4.5 (b) Incorporation of radioactivity was assessed densitometrically as described in section 2.10f. Taking the incorporation of radioactivity catalysed by pertussis toxin under these conditions (lane a) as 100%, and incorporation of radioactivity by cholera toxin into this band in the absence of ligand (lane f) as a blank, then lane (b) = 3%, lane (c) = 8%, lane (d) = 51% and lane (e) = 69%. Similar data was obtained in three separate experiments.

Figure 4.5(a)

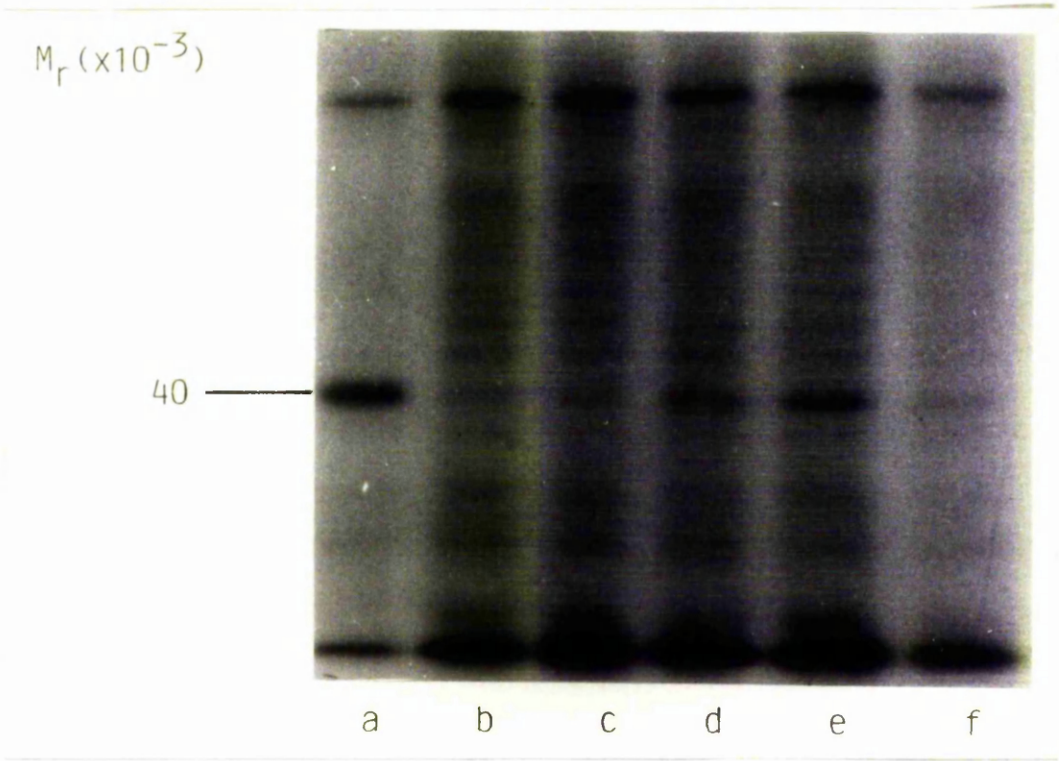


Figure 4.5(b)

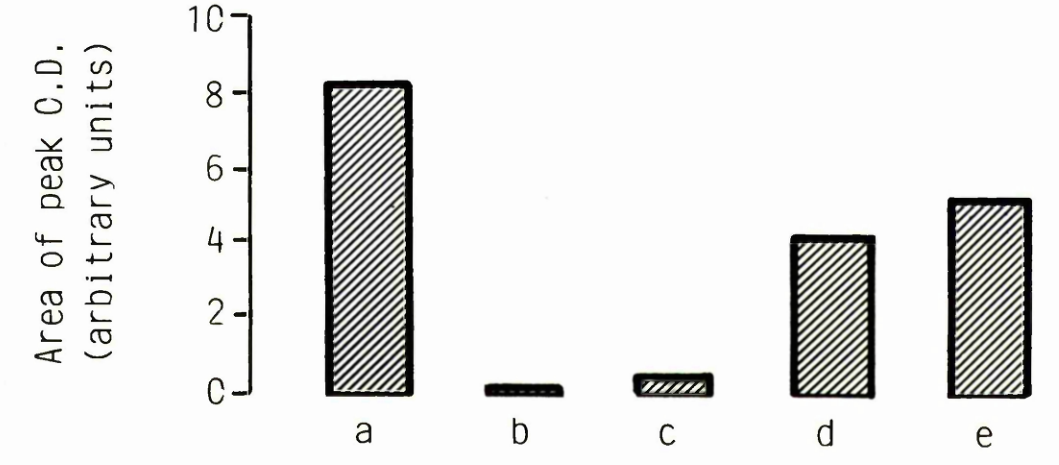


Fig 4.6 Dose/response curve of DADLE stimulation of the cholera toxin catalysed ADP-ribosylation of a Gi-like protein in membranes of untreated NG108-15 cells.

4.6(a) Cholera toxin catalysed ADP-ribosylation of membranes of untreated NG108-15 cells (25μg) was carried out in the absence of guanine nucleotides as described in section 2.12. DADLE was included at a final concentration of; 0.01nM (lane b), 0.1nM (lane c), 1nM (Lane d), 10nM (Lane e), 0.1μM (Lane f), 1μM (Lane g) and 10μM (Lane h). Densitometric analysis of the 40kDa band was performed as in section 2.10f and incorporation of radioactivity into this polypeptide in the absence of ligand (Lane a) was subtracted as a blank. In three separate experiments, a representative example of which is shown, the estimated EC_{50} for DADLE was 16±10nM. (mean ± S.D.)

4.6 (b).

Figure 4.6(a)

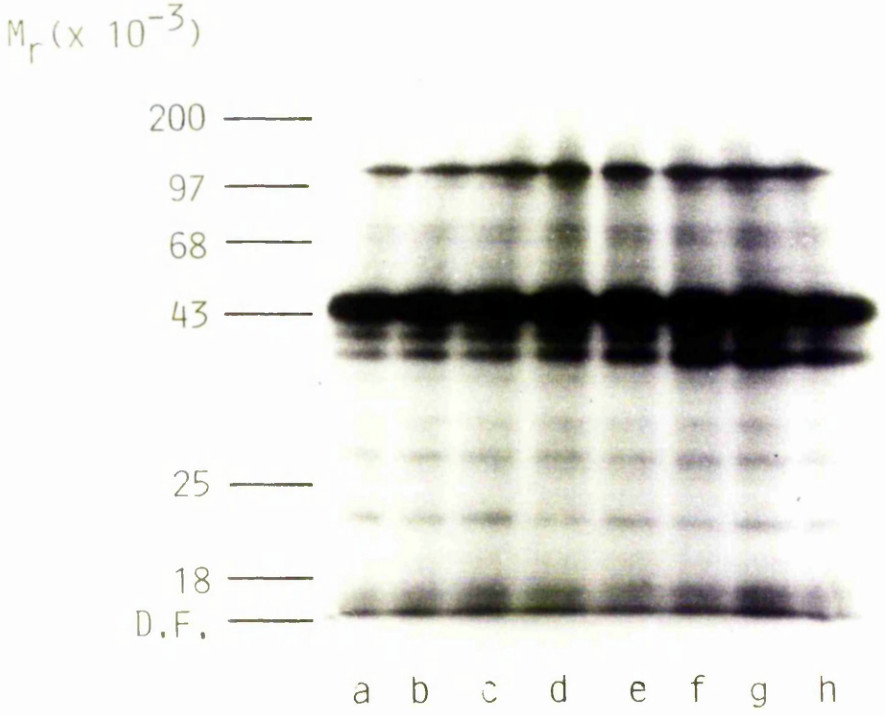


Figure 4.6(b)

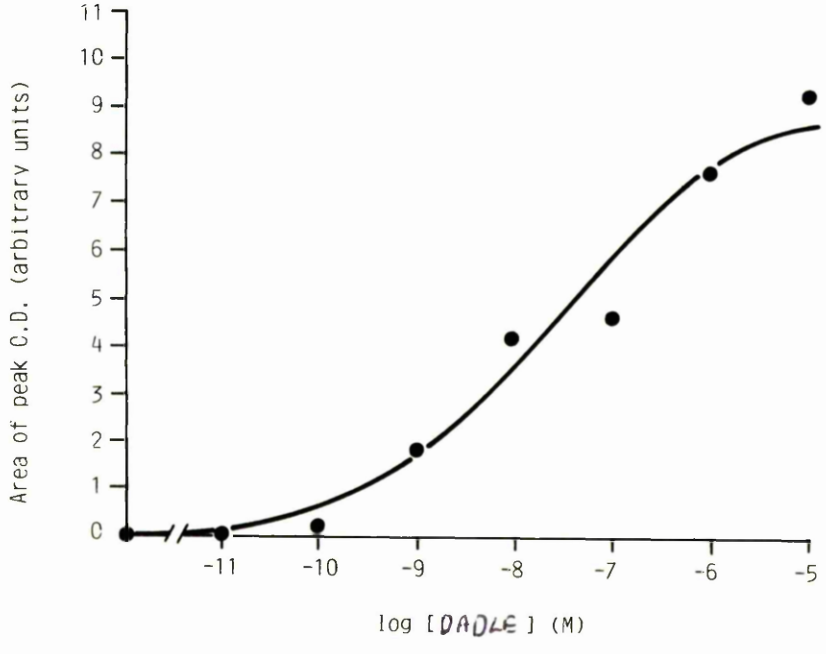


Fig 4.7 Dose/response curve of DADLE stimulation of the cholera toxin catalysed ADP-ribosylation of a Gi-like protein in membranes of cholera toxin pretreated NG108-15 cells.

4.7 (a) Cholera toxin catalysed ADP-ribosylation of membranes of cholera toxin pretreated NG108-15 cells (30μg) (section 2.6d) was carried out in the absence of guanine nucleotides as described in section 2.12. DADLE was included at a final concentration of; 0.01nM (lane b), 0.1nM (lane c), 1nM (Lane d), 10nM (Lane e), 0.1μM (Lane f), 1μM (Lane g) and 10μM (Lane h). Densitometric analysis of the 40kDa band was performed as described in section 2.10f and incorporation of radioactivity into this polypeptide in the absence of ligand (Lane i) was subtracted as a blank (Lane a contained no toxin). In three separate experiments, a representative example of which is shown, the estimated EC_{50} for DADLE was 18 ± 9nM (mean ± S.D.) 4.7 (b).

Figure 4.7(a)

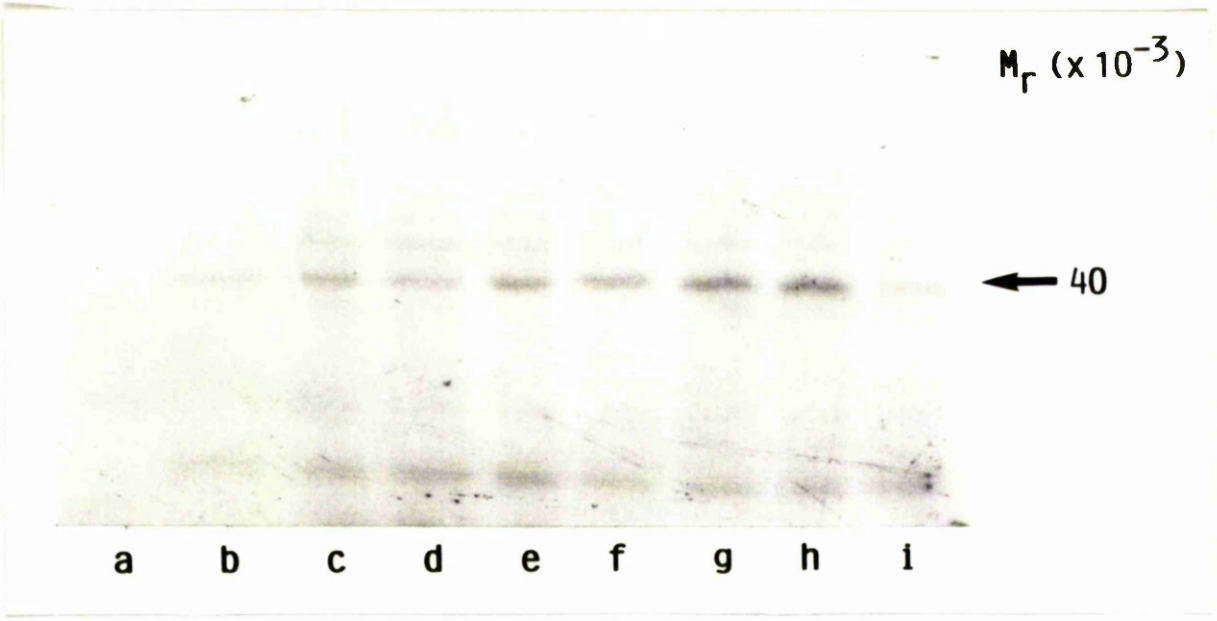
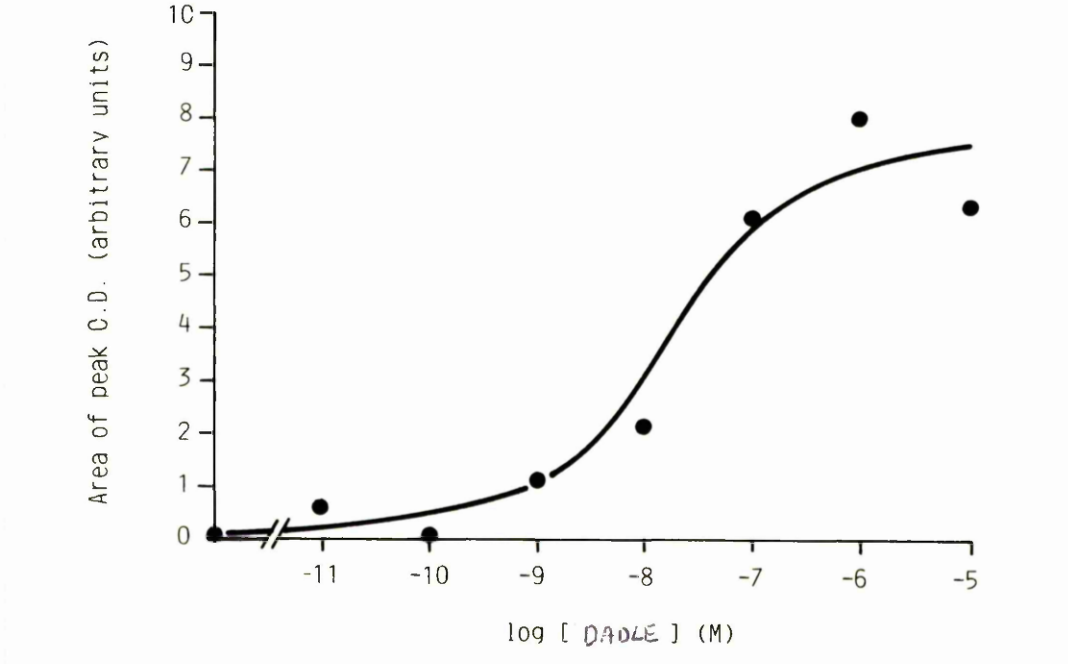


Figure 4.7(b)



Results

When NG108-15 cell membranes were ADP-ribosylated in the presence of added guanine nucleotide (GTP), cholera toxin catalysed the incorporation of radioactivity from [^{32}P] NAD^+ predominantly into a 45kDa polypeptide. (Figure 4.1, lane a), This band co-migrated with the α–subunit of Gs, as determined by immunological means (chapter 3, Figure 3.7). In addition, a 42kDa polypeptide was also seen to be labelled, and could be identified immunologically as being a smaller form of Gsα. However, due to the low abundance of this 42kDa protein in comparison to the 45kDa form of Gsα, it was difficult to routinely observe the 42kDa form of Gsα in NG108-15 membrane preparations. In addition to cholera toxins' effect, under these conditions, pertussis toxin was able to catalyse the incorporation of radioactivity from [^{32}P] NAD^+ into a 40kDa polypeptide, (Figure 4.1, lane b), It can be noted that cholera toxin was unable to catalyse the incorporation of radioactivity into this polypeptide, and similarly, pertussis toxin was unable to catalyse the incorporation of radioactivity into either the 45 or 42kDA polypeptides which were modified by cholera toxin. It is therefore apparent that in the presence of GTP, both cholera and pertussis toxins are apparently specific in their ability to ADP-ribosylate Gs and Gi respectively

When a similar experiment was performed in membranes from NG108-15 cells in the absence of any exogenously added guanine nucleotides, a different picture emerged. Cholera toxin catalysed the incorporation of radioactivity from [^{32}P] NAD^+ into two polypeptides with apparent molecular weights of 45 and 42kDa and in addition a 40kDa poypeptide was modified in a cholera-toxin dependent manner (Figure 4.2, lane a). Radioactivity was incorporated into certain other polypeptides, but in no other case was this dependent on the presence of toxin in the assay. The 40kDa band modified by cholera toxin co-migrated with the only apparent polypeptide to be specifically ADP-ribosylated by pertussis toxin in these membranes (Figure. 4.2, lane b).

After pretreatment of NG108-15 cells *in vivo* with pertussis toxin, cholera toxin in the absence of GTP was able to catalyse ADP-ribosylation of both the 45 and 42kDa polypeptides, but could no longer modify the 40kDa species in membranes prepared from these cells (Figure. 4.3, lane c). Pertussis toxin equally, was unable to catalyse incorporation of radioactivity into the 40kDa band *in vitro* following treatment of the cells *in vivo* with pertussis toxin, (Figure 4.3 lane e). In contrast, however, after treatment of the cells *in vivo* with cholera toxin, fresh cholera toxin in the absence of GTP was able to incorporate radioactivity from [^{32}P]NAD^{+} into the 40kDa polypeptide but not the 45 and 42kDa proteins in membranes prepared from these cells (Figure 4.3 , lane b). Pertussis toxin also catalysed ADP-ribosylation of a 40kDa protein under these conditions (Figure 4.3, lane d). This data suggests that the polypeptide modified by both cholera and pertussis toxin in the absence of GTP was identical.

Addition of the synthetic opioid peptide DADLE, enhanced the cholera-toxin-catalysed ADP-ribosylation of the 40kDa polypeptide, but did not substantially affect the incorporation of radioactivity into either the 45 or 42 kDa bands when the reaction was performed on membranes of untreated control NG108-15 cells in the absence of exogenously added GTP (Figure.4.4 , lane e). Over the time course employed in these experiments 0.1μM DADLE promoted less incorporation of radioactivity into the 40 kDa band than did 10μM DADLE (Figure. 4.4, lanes,d,e). This effect was not mimicked by the opioid antagonist naloxone, and the antagonist blocked the DADLE-mediated effect (Figure. 4.4, compare lanes c and d). Essentially the same results were obtained in membranes of NG108-15 cells produced from cells which had been pretreated *in vivo* with cholera toxin, except that the 45 and 42kDa polypeptides no longer supported cholera toxin catalysed incorporation of radioactivity (Figure. 4.5). Dose-response curves of the enhancement of cholera-toxin-catalysed ADP-ribosylation of the 40 kDa polypeptide showed that no effect of DADLE could be observed at concentrations below 1nM and that a maximal increase in the cholera-toxin-catalysed ADP-ribosylation of the 40kDa polypeptide was achieved by 1μM. The EC 50 for DADLE was estimated to be some 16 ±10 nM (mean ± S.D.) (Figure 4.6 b), a value in close agreement with the EC 50 estimated for

DADLE-mediated stimulation of high-affinity GTPase activity in these membranes as detailed in chapter 5. Similar results in terms of both the pharmacological profile of the response and the dose-response curves to DADLE (Figure 4.6 a,b) were obtained when membranes of cholera-toxin-pretreated NG 108-15 cells were used as the acceptor system for the cholera toxin catalysed ADP-ribosylation *in vitro* in the absence of GTP (Figure 4.7 a,b). The cholera-toxin-pretreated system had, however, the distinct advantage that essentially no radioactivity was incorporated into the 45 and 42 kDa bands. Thus the 40 kDa band was the sole polypeptide to be ADP-ribosylated in a cholera-toxin-sensitive manner in membranes from the cholera-toxin-pretreated cells.

In contrast to the effect of DADLE on cholera toxin labelling of the 40kDa polypeptide, DADLE had no effect on pertussis toxin catalysed ADP-ribosylation of the 40kDa polypeptide (results not shown).

Discussion

In classical terms, it has been assumed that pertussis toxin catalysed ADP-ribosylation provides a specific means of identification of Gi, and cholera toxin ribosylation allows a similar identification of Gs. Particularly with pertussis toxin, this is now recognized to be an oversimplification. Three genes coding for 'Gi-like' proteins have been identified (Jones and Reed, 1987; Suki et al., 1987), as well as a gene for Go (Itoh et al., 1986). All of the polypeptides are potential substrates for pertussis toxin. The amino acid substrates of cholera and pertussis toxin have been identified as being Arg-174 and Cys-347 respectively in Transducin α.(Van Dop et al., 1984; West et al., 1985). The corresponding amino-acid residue modified by cholera toxin in Gsα is arginine 187/188 or 201/202 (Medynski et al., 1985); the exact number of this residue depends on which of the four splice variants of this protein is under study (Bray et al., 1986).

The target site for cholera toxin modification is conserved in all G-protein α-subunits which have been cloned to date, wheras the pertussis toxin substrate is found in Gi1, Gi2, Gi3, Go, Td1 and Td2. and not in Gs or Gx (Kaziro, 1990). The precise toxin recognition sequences are unknown, which means that the ability or inability of a particular toxin to ADP-ribosylate a G-protein may not simply depend on the presence of the substrate amino-acid in a homologous position. In experiments designed to address whether Gs may be modified to become a pertussis toxin substrate, the extreme C-terminal region of Gsα has been altered from the wild type (Gln-Tyr-Glu-Leu-Leu) to a more Gi-like C-terminus containing a cysteine residue four amino-acids removed from the C-terminus, (Asp-Cys-Gly-Leu-Leu). Despite these changes, the mutant protein was a poor substrate for pertussis toxin (Freissmuth and Gilman, 1989). Gz is thought not to be a substrate for pertussis toxin since it does not have a cysteine residue in the appropriate location. However this remains to be rigorously assessed as it does have a cysteine residue three amino-acids removed from the cysteine position in the identified pertussis toxin sensitive G-proteins.

The possibility that cholera toxin was not absolutely specific in its action on Gsα, but was also able to ADP-ribosylate Giα, was firstly raised by Graves and co-workers (Graves et al., 1983). In rat adipocyte membranes, cholera toxin was demonstrated to catalyse the incorporation of radioactivity from [^{32}P]NAD^{+} into two proteins with molecular masses of 54 and 45kDa, presumably representing two forms of Gsα. However, when the assay was carried out in the absence of GTP, cholera toxin was also found to catalyse the incorporation of radioactivity into a 41kDa polypeptide. The 41kDa protein labelled by cholera toxin was identified as being Giα (Owens et al., 1985), since ADP-ribosylation of the 41kDa protein by both cholera and pertussis toxin had the same functional consequence of attenuating receptor mediated inhibition of adenylyl cyclase.

It has previously been demonstrated in a macrophage cell line (Aksamit et al., 1985), neutrophils (Verghese et al., 1986) and the rat glioma cell line C6BU1 (Milligan, 1987) that cholera toxin can catalyse ADP-ribosylation of a 40kDa protein in assays to which no guanine nucleotide has been added. It has also been noted that cholera toxin can produce functional effects on transducin (Fung, 1985). Transducin may be ADP-ribosylated by both cholera and pertussis toxins, an effect which is dependent upon the guanine nucleotide bound to the G-protein α-subunit. Indeed it is possible to ADP-ribosylate transducin with both toxins, but only in the sequence pertussis toxin, followed by cholera toxin. If cholera toxin is used first, transducin is no longer a substrate for pertussis toxin (Stryer, 1988).

The data presented in this chapter demonstrate that cholera toxin can indeed catalyse ADP-ribosylation of a G-protein which is also a substrate for pertussis toxin, namely the inhibitory G-protein of the adenylyl cyclase system, Gi. The evidence presented in support of this is threefold. Firstly; cholera toxin in the absence but not in the presence of guanine nucleotides, catalysed ADP-ribosylation of a 40kDa protein which co-migrated with a 40kDa pertussis-toxin substrate in membranes of the neuroblastoma x glioma hybrid cell line NG108-15. Secondly, In membranes prepared from NG108-15 cells which had been pretreated *in vivo* with pertussis toxin before cell harvest, to inactivate the pertussis toxin sensistive G-

proteins present, cholera toxin was unable to catalyse ADP-ribosylation of the 40kDa protein. However, this toxin was still able to modify the 45 and 42kDa polypeptides which represent forms of Gs (chapter 3). Thirdly, activation of the population of δ-opioid receptors on the surface of these cells with synthetic enkephalin agonists enhanced the cholera-toxin modified ADP-ribosylation of the 40kDa band. This effect was blocked by the opioid antagonist naloxone. In addition, it is noteworthy that the EC_{50} for DADLE promotion of cholera-toxin catalysed ADP-ribosylation of Giα was very similar to that for DADLE stimulation of high-affinity GTPase activity in this system (chapter 5, Figure 5.13a).

Although pertussis toxin pretreatment of NG108-15 cells *in vivo* prevented both pertussis and cholera-toxin-catalysed ADP-ribosylation of Giα *in vitro*, this effect is not because both toxins have the same target amino-acid. As stated above, pertussis toxin catalyses the transfer of ADP-ribose onto a cysteine residue, and cholera toxin onto an arginine residue. The explanation may lie in the preferred states of the substrates for each toxin. The ability of either cholera or pertussis toxin to catalyse the ADP-ribosylation of their respective substrates has been demonstrated to be modified by the state of association of the G-protein α-subunit with the β,γ subunits (Tsai et al., 1984). The ability of pertussis toxin to covalently modify the purified α-subunit of Gi is negligible, however, upon addition of purified β,γ subunits, a ten fold increase in ADP-ribosylation is apparent (Neer et al., 1988). In the presence of either GTPγS or Gpp(NH)p, non-hydrolysable analogues of GTP which have been demonstrated to bind to Giα and promote formation of the active, dissociated form of Gi (Katada et al., 1986), the ability of pertussis toxin to ADP-ribosylate Giα is reduced, suggesting that the dissociated α-subunit of Gi is a poor substrate for pertussis toxin. In contrast, the inclusion of GDPβS, an analogue of GDP thought to promote association and hence inactivation of Gi (Gilman, 1984), increased the ADP-ribosylation of Giα by pertussis toxin. From similar experiments on transducin, which is a substrate for both cholera and pertussis toxins, the preferred substrate of cholera toxin is thought to be the dissociated form of the α-subunit, since cholera toxin catalysed ADP-ribosylation of transducin is increased by the presence of either GTPγS or Gpp(NH)p and inhibited by the addition of GDP (Abood et al., 1982). In similar experiments, the ability of cholera toxin to ADP-

ribosylate Gs has been shown to be increased by the presence of Gpp(NH)p, suggesting that in addition to transducin, the preferred substrate for cholera toxin is the monomeric α-subunit of Gs (Owens et al., 1985). However, the existence of a preferred conformational state of Gi to be ADP-ribosylated by pertussis toxin has recently been questioned by two reports, which have demonstrated that pertussis toxin itself, has a nucleotide requirement (Lim et al., 1985), and that both adenine and guanine nucleotides can bind to the toxin and induce a conformational change which results in increased ADP-ribosyltransferase activity. This data suggests that experiments conducted to assess the ability of guanine nucleotides to alter pertussis toxin catalysed ADP-ribosylation of Gi, have to be analysed with care (Mattera et al., 1986; Ribeiro-Neto et al., 1987).

If it is assumed that pertussis toxin does indeed prefer to interact with the holomeric heterotrimeric forms of its substrates and that cholera toxin interacts preferentially with the isolated α-subunit of its substrates then after pertussis toxin pretreatment, the holomeric form of Gi will be stabilised and might then represent a poor substrate for cholera toxin catalysed ADP-ribosylation. A second possibility is based on the observation that, for pertussis-toxin-sensitive G-proteins to serve as substrates for cholera toxin, no guanine nucleotide should be bound to the protein. Pertussis-toxin catalysed ADP-ribosylation of Gi attenuates coupling between receptor and G-protein and therefore prevents the receptor driven stimulation of GTP hydrolysis and guanine nucleotide exchange. The ADP-ribosylation of Gi by pertussis toxin would therefore be expected to diminish guanine nucleotide exchange and thus maintain the presence of GDP in the nucleotide binding site of Giα (Gilman, 1987). The presence of GDP in the guanine nucleotide binding site of Giα may be sufficient to prevent cholera-toxin catalysed ADP-ribosylation of the protein. Further evidence in favour of the requirement for a nucleotide free form of Giα as a substrate for cholera toxin was provided by the observation that agonist activation of a receptor which stimulates the activity of an inhibitory G-protein(s), promoted the cholera toxin mediated ADP-ribosylation of this G-protein. The rate limiting step in the cycle of G-protein activation and deactivation appears to be the rate of release of GDP (Ferguson et al., 1986). The function of an agonist occupied

receptor is to promote a conformational change in the G-protein α-subunit which reduces its affinity for GDP. This will increase the rate of release of GDP, and therefore enhance the rate of binding of the activating guanine nucleotide, GTP (Gilman, 1987). In the absence of exogenously added GTP, the GDP will not be replaced, producing a protein stripped of bound nucleotide.

Evidence from other signal transduction model systems suggests that cholera toxin may still be able to ADP-ribosylate 'Gi', even in the presence of guanine nucleotides or their analogues. In a macrophage like cell line, RAW 264, pretreatment with pertussis toxin has been shown to inhibit receptor mediated chemotaxis by the chemotatic peptide formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe), suggesting a role for a pertussis toxin sensitive G-protein in mediating the response. In membranes produced from RAW 264 cells, pertussis toxin has been demonstrated to catalyse the incorporation of radioactivity from [^{32}P]NAD$^+$ into a 41kDa polypeptide, presumably the α-subunit of Gi, wheras cholera toxin catalyses the incorporation of radioactivity from [^{32}P]NAD$^+$ into a 45kDa polypeptide, presumably the α-subunit of Gs (Backlund et al., 1985). When cholera toxin catalysed ADP-ribosylation is performed in the absence of guanine nucleotide, a different picture emerged. As well as the incorporation of radioactivity into Gs, radioactivity is also incorporated into Gi. Interestingly, when the non-hydrolysable guanosine triphosphate analogue Gpp(NH)p is present, labelling of Gi by cholera toxin still occurs. Pretreatment of RAW 264 cells *in vivo* with cholera toxin prevented incorporation of radioactivity into Gs, but did not prevent the incorporation of radioactivity into Gi. However, pretreatment of the cells with pertussis toxin, whilst not altering ADP-ribosylation of Gs, prevented the *in vitro* ADP-ribosylation of Gi by cholera toxin.

In HL60 cells, F-Met-Leu-Phe has been shown to bind to specific receptor(s) and stimulate the activity of phosphoinositidase C and hence protein kinase C, in a pertussis toxin sensitive manner (Dougherty et al., 1984), suggesting that the receptor(s) for F-Met-Leu-Phe stimulate the activity of intracellular effectors by interacting with a pertussis toxin sensitive G-protein(s). Gierschik and

Jacobs (1987) have demonstrated that in membranes produced from HL 60 cells, cholera toxin is able to ADP-ribosylate a 40kDa protein which migrates in an identical manner to the 40kDa polypeptide(s) which becomes ADP-ribosylated after pertussis toxin treatment. Interestingly, the ability of cholera toxin to ADP-ribosylate what was originally thought to be a single polypeptide but has since been resolved into a doublet of Gi2 and Gi3 (Gierschik et al., 1989a) is absolutely dependent upon the presence of the G-protein activating ligand, F-Met-Leu-Phe. Prior treatment of HL60 cells with pertussis toxin *in vivo* prevented the *in vitro* ADP-ribosylation of Gi2α and Gi3α by both toxins, suggesting that the substrates for both cholera and pertussis toxin were identical.

The data discussed above was obtained from membrane preparations, in contrast, a recent report from Katada and co-workers has examined the ability of cholera toxin to ADP-ribosylate Gi in both whole HL-60 cells and membranes (Iiri et al., 1989). In membranes produced from HL60 cells a 40kDa protein was ADP-ribosylated by cholera toxin, but only in the presence of the chemotactic peptide agonist, F-Met-Leu-Phe. Prior treatment of HL60 cells *in vivo* with pertussis toxin prevented subsequent incorporation of ADP-ribose into the 40kDa protein by either cholera or pertussis toxins, suggesting that the target protein for each toxin was the α-subunit of Gi. Cholera toxin mediated ADP-ribosylation of Gi was found to be stimulated by guanine nucleotides such as GTPγS, Gpp(NH)p and GTP and also by the inclusion of Mg^{2+}, in a biphasic manner, such that low concentrations of both guanine nucleotide and Mg^{2+} were stimulatory and higher concentrations inhibitory. Interestingly, in membranes produced from cells pretreated with both cholera toxin and F-Met-Leu-Phe, it was no longer possible to ADP-ribosylate Gi with cholera toxin although the protein still supported ADP-ribosylation by pertussis toxin. This led the authors to conclude that cholera toxin could catalyse the ADP-ribosylation of the α-subunit of Gi *in vivo*. However, it should be noted that when the cells were pretreated with both cholera toxin and F-Met-Leu-Phe, the chemotactic peptide was only present in the assay for five minutes. Based upon data obtained in membrane systems where the timecourse of ADP-ribosylation of both Gsα and Giα has been examined, it does not seem feasible to ADP-ribosylate a significant proportion of the G-protein pool in a five minute time span (Milligan, 1987). This data is more

con with an alternative G-protein modification, such as a phosphorylation. It has recently been appreciated that treatment of rat hepatocytes with agents which stimulate the activity of protein kinase C, results in the phosphorylation of the α-subunit of Gi2 (Bushfield et al., 1990). In addition, treatment of human monocyte U937 cells with lipopolysaccharide for five minutes elicits the phosphorylation of Gi2α and attenuates the ability of Gi2α to support pertussis toxin catalysed ADP-ribosylation (Issakani et al., 1989). The effect of treating HL-60 cells with both cholera toxin and F-Met-Leu-Phe may not be due to the *in vivo* ADP-ribosylation of Giα by cholera toxin, but may instead be due to the phosphorylation of Giα, induced by agonist stimulation of protein kinase C.

In membranes produced from HL-60 cells, cholera toxins' ability to ADP-ribosylate the classically defined pertussis toxin sensitive G-proteins is not limited to Giα, since in the presence of F-Met-Leu-Phe the toxin was able to ADP-ribosylate purified α-subunits of Gi1, Gi2 and Go, in the order; endogenous Giα > Gi2α = Gi1α > Goα, when each α-subunit was reconstituted into membranes produced from pertussis toxin pretreated HL60 cells (Iiri et al., 1989). The differing degree of ADP-ribosylation may reflect differing abilities of each G-protein α-subunit to couple to the F-Met-Leu-Phe receptor and hence suggest that some measure of specificity is maintained in this reconstitution system.

A range of pertussis toxin sensitive G-proteins are known to be expressed in the NG108-15 cell line, these are Gi2, Gi3 and Go (Chapter 3). The data presented in this chapter do not define which of these G-protein α-subunit(s) is represented by the 40kDa polypeptide(s) which is ADP-ribosylated by pertussis toxin and also by cholera toxin in a manner which is enhanced by opioid peptides. This was primarily due to the difficulty of incorporating sufficient radioactivity into the 40kDa cholera toxin substrate to allow resolution and identification using polyacrylamide gel electrophoresis. It may be noted that the stoichiometry of cholera toxin catalysed ADP-ribosylation of Giα, was considerably lower than that of pertussis toxin labelling of Giα in NG108-15 cell membranes. The reason for this discrepancy is unclear, however it may simply be a reflection of the difficulty in converting the entire pool of Giα into a suitable substrate for cholera toxin catalysed

ADP-ribosylation.

Although the precise nucleotide requirements and the mechanism by which cholera toxin is able to ADP-ribosylate 'Gi-like' G-proteins in membrane systems remains to be resolved, agonist stimulation of the cholera-toxin catalysed ADP-ribosylation of Giα may be a general phenomenon for receptors coupled to 'Gi-like' G-proteins. In this regard, it has recently been noted that foetal calf serum activation of a poorly characterised growth factor receptor on C6BU1 cells stimulated cholera-toxin-catalysed ADP-ribosylation of Gi2 (Milligan, 1988). If some degree of specificity is maintained, as is suggested from the work of Iiri et al., (1989) then experiments similar to those described in this chapter may be of general use in defining the nature of receptor to G-protein coupling.

Chapter 5

The interaction of receptors linked to adenylyl cyclase inhibition with G-proteins in the NG108-15 cell line.

Introduction

The control of cell growth is mediated by the concerted action of a variety of growth factors and hormones which regulate the level of activity of intracellular effectors such as adenylyl cyclase, phosphoinositidase-C, or various ion channels. It may therefore be appreciated that the control and regulation of the second messenger systems which are modulated by hormones and growth factors must be subject to a high level of control (Pouyssegur, 1989).

With G-proteins acting as central mediators in the signal transduction process, one of the most important questions relating to G-protein function lies within the specificity of interaction between G-proteins, transmembrane receptors and the effector systems with which they interact. Although it has been assumed that all receptors which regulate the activity of one type of effector do so through the activation of only one species of G-protein, it is currently unclear whether one receptor will interact exclusively with one G-protein species, or if more than one G-protein may interact with the same receptor.

In membrane systems, there appears to be tight regulation of G-protein coupling, whereby receptors which are known to interact with different effectors do so through the activation of different G-proteins, as assessed by measurements of GTP hydrolysis (Houslay et al., 1986a,b; McKenzie et al., 1988a,b). However, studies which employ the reconstitution of purified G-proteins, receptors and effectors into phospholipid vesicles, suggest that one type of receptor may interact with a large number of homologous, but non-identical G-proteins (Florio and Sternweis 1985; Cerione et al., 1986; Kurose et al., 1986; Asano et al., 1984). Coupling of a given receptor-G-protein pair in artificial phospholipid vesicles cannot be taken as evidence for a physiologically relevant interaction (Okajima et al., 1985), but may simply reflect a high degree of structural similarity in the domains involved in receptor to G-protein coupling and as such, describe possibilities of interaction rather than giving a definition of interaction. In addition, it should be noted that the early reconstitution studies described were performed

using purified "Gi" which was in all probability a non-homogeneous population of 'Gi-like' G-proteins.

In chapter 3, an immunological approach has been employed to define the various species of G-protein expressed by NG108-15 cells, these are Gs, Gi2, Gi3 and Go. Gz (Gx) may also be expressed, but current techniques have not as yet allowed its rigorous identification in this cell line. The most widely studied effector system which functions in NG108-15 cells is adenylyl cyclase, which is subject to both stimulatory and inhibitory inputs from a variety of G-protein linked receptors (Klee et al., 1985). Receptor mediated inhibition of adenylyl cyclase in NG108-15 cells is attenuated by pretreatment with pertussis toxin (Kurose et al, 1983), thus defining a role for a pertussis toxin sensitive G-protein in mediating the response. The G-protein which mediates inhibition of adenylyl cyclase in NG108-15 cell must be one of Gi2, Gi3, Go or a combination thereof. The data presented in this chapter will address the specificity of receptor to G-protein interactions in membranes produced from the NG108-15 cell line and thus ascertain which of the pertussis toxin sensitive G-proteins is linked to the inhibition of adenylyl cyclase.

Fig. 5.1 Stimulation of high-affinity GTPase activity in membranes of NG108-15 cells in response to GTP.

5.1(a) High-affinity GTPase activity was measured in membranes of NG108-15 cells (8μg) in response to increasing concentrations of GTP, as described in section 2.13, except that GTP was excluded from the mix. 5.1(b) The data obtained were replotted in the manner described by Hofstee (1952). Points represent means ± S.D. for quadruplicate determinations obtained from four independent experiments (n=16).

Figure 5.1(a)

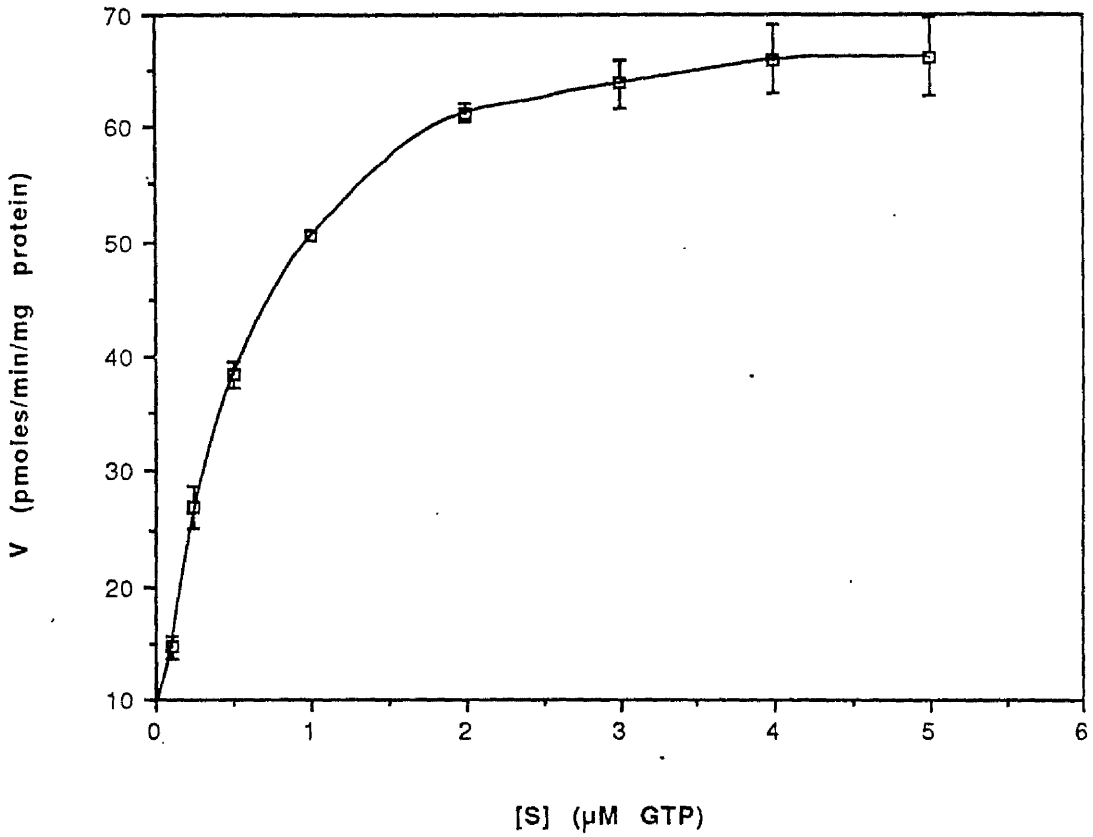


Figure 5.1(b)

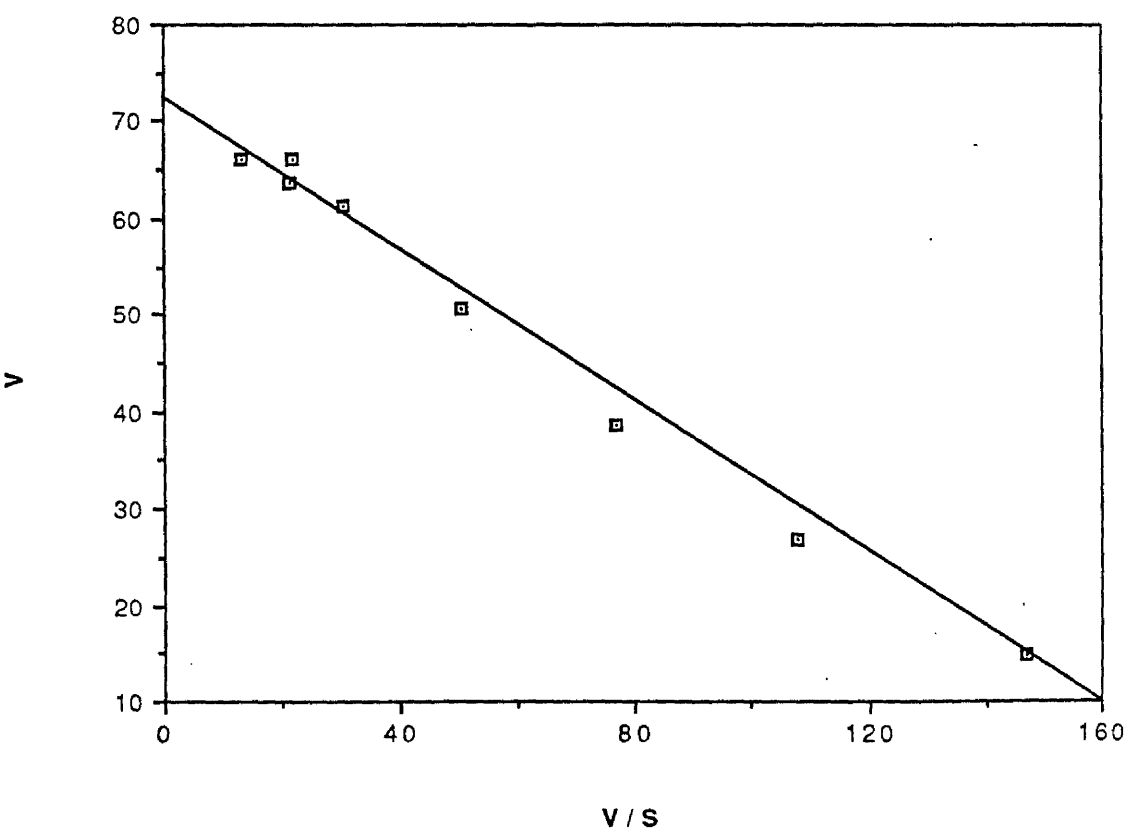


Table. 5.1 Stimulation of high-affinity GTPase activity in NG108-15 membranes by various agents.

High-affinity GTPase activity was measured in membranes (8μg) of NG108-15 cells in response to a range of different agonists, as described in the legend to Figure 5.1, except GTP was included in the assay at a final concentration of 0.5μM. The data shown are means.± S.D. taken from quadruplicate determinations in a single experiment which is representative of five others performed. These gave essentially identical results.

Table 5.1

Addition	Conc. used	GTPase activity (pmol/min/mg protein)	Stimulation (pmol/min/mg protein)
None		14.2 ± 0.1	
DADLE	(10μM)	23.1 ± 0.15	8.9
DALAMID	(10μM)	22.9 ± 0.76	8.7
Bradykinin	(10μM)	18.6 ± 0.1	4.4
Adrenaline	(10μM)	15.8 ± 0.12	1.6
FCS	(10%, v/v)	20.7 ± 1.0	6.5
PGE1	(10μM)	14.4 ± 0.4	0.2

Fig. 5.2 Stimulation of High affinty GTPase activity in NG108-15 membranes; the effect of pertussis toxin pretreatment.

High affinity GTPase activity was measured in NG108-15 cell membranes (7μg) which had been pretreated with vehicle (empty bars) or pertussis toxin (hatched bars) as detailed in section 2.6d. Ligands were either; vehicle (A); DADLE (10μM) (B), bradykinin (10μM) (C), adrenaline (10μM) (D) or FCS (10% v/v) (E). The assay was conducted as described in the legend to Table 5.1. The data shown are means ± S.D. pooled from quadruplicate determinations obtained in three separate experiments (n=12).

Figure 5.2

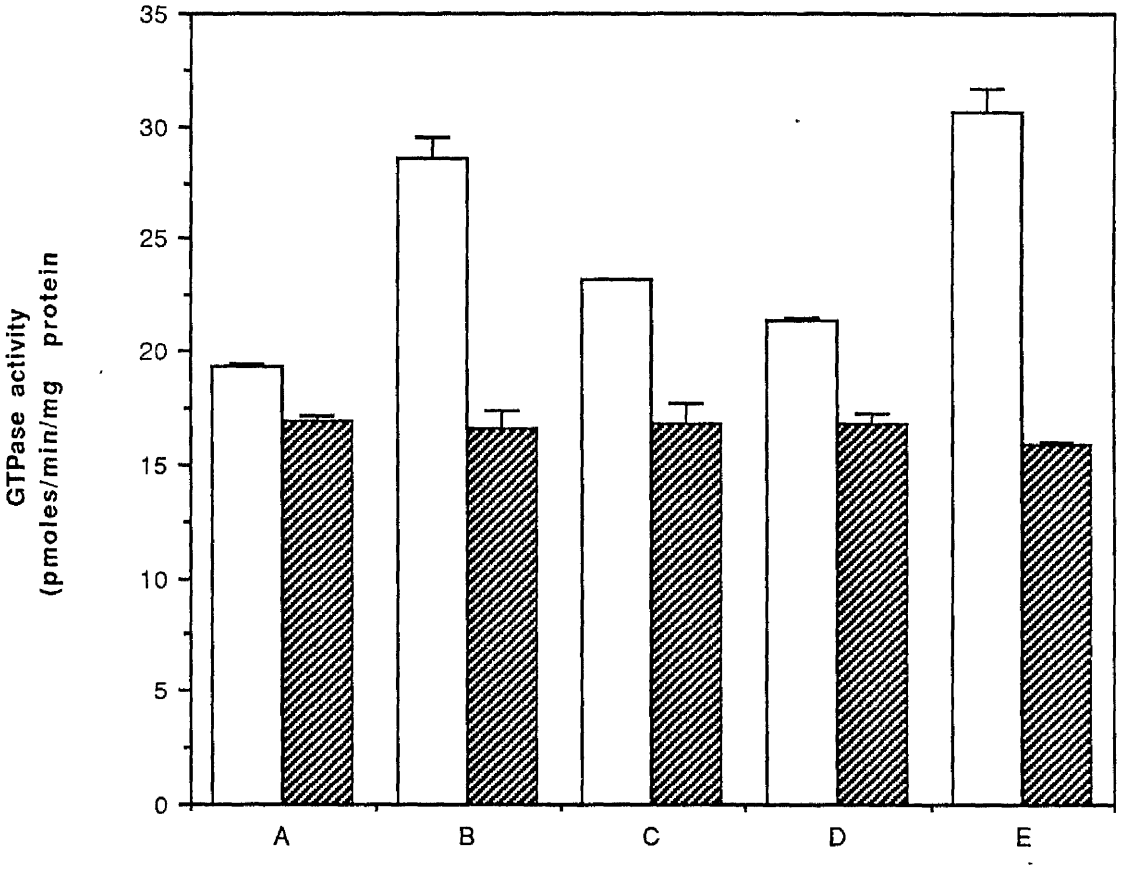

GTPase activity
(pmoles/min/mg protein
35
30
25
20
15
10
5
0
A
B
C
D
E

Fig. 5.3 **Stimulation of high-affinty GTPase activity in NG108-15 membranes: additivity studies with DADLE, bradykinin and adrenaline.**

High-affinity GTPase activity was assessed in NG108-15 cell membranes (7μg) exactly as described in the legend to Figure 5.2. Ligands were; DADLE (1), bradykinin (2), adrenaline (3), DADLE + bradykinin (4), DADLE + adrenaline (5), bradykinin + adrenaline (6) and DADLE + bradykinin + adrenaline (7). All ligands were used at a final concentration of 10μM. Data represent means ± S.D. from quadruplicate determinations obtained from a single experiment which was repeated three times with essentially identical results. In the experiment shown, basal high-affinity GTPase activity (in the absence of added agonist) was 15.8 ± 0.2 pmoles/min/mg protein.

Figure 5.3

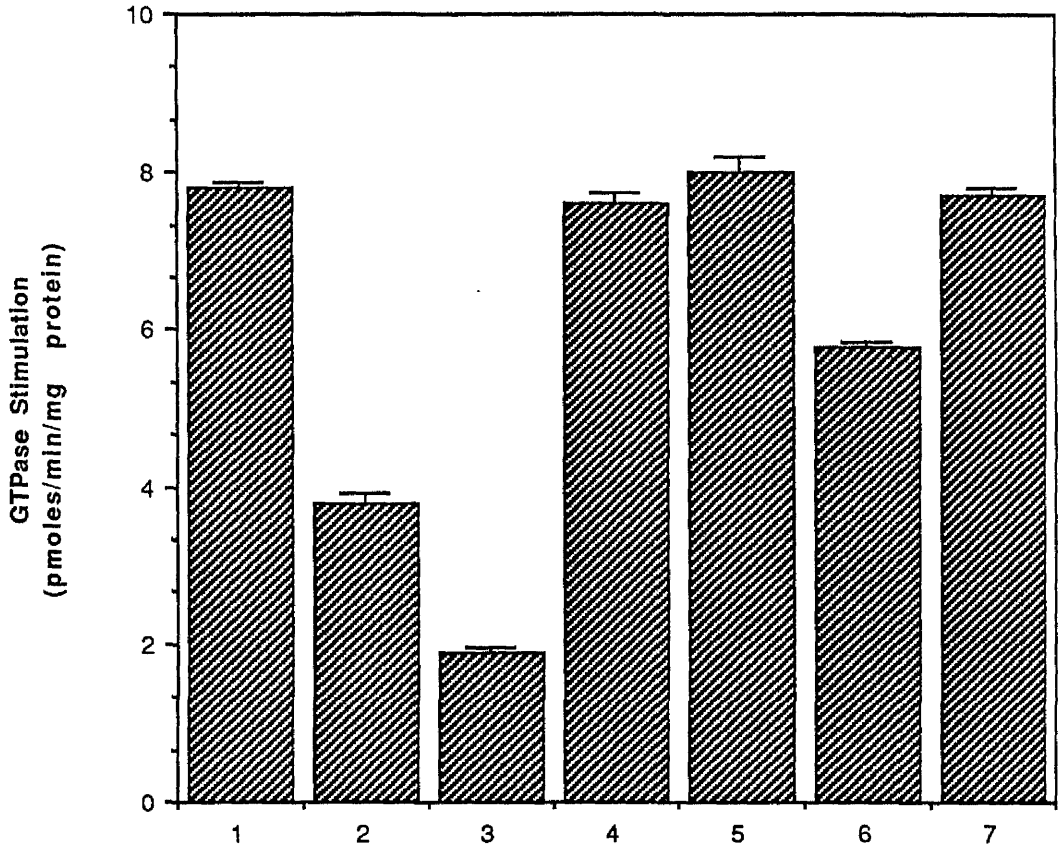


Fig. 5.4 **Stimulation of high-affinity GTPase activity by DADLE in NG108-15 cell membranes is specifically blocked by naloxone.**

High-affinity GTPase activity was assessed in NG108-15 cell membranes (7μg) exactly as described in the legend to Figure 5.2, in the presence of vehicle (A), or concentrations of DADLE (B), bradykinin (C) or adrenaline (D) which elicited the maximal stimulation of high-affinity GTPase activity which could be produced by that ligand (10μM). Naloxone was either absent (empty bars) or included (hatched bars) in parallel tubes at a final concentration of 1mM. Data represent means ± S.D. from quadruplicate determinations obtained from a single experiment which was repeated twice with essentially identical results.

Figure 5.4

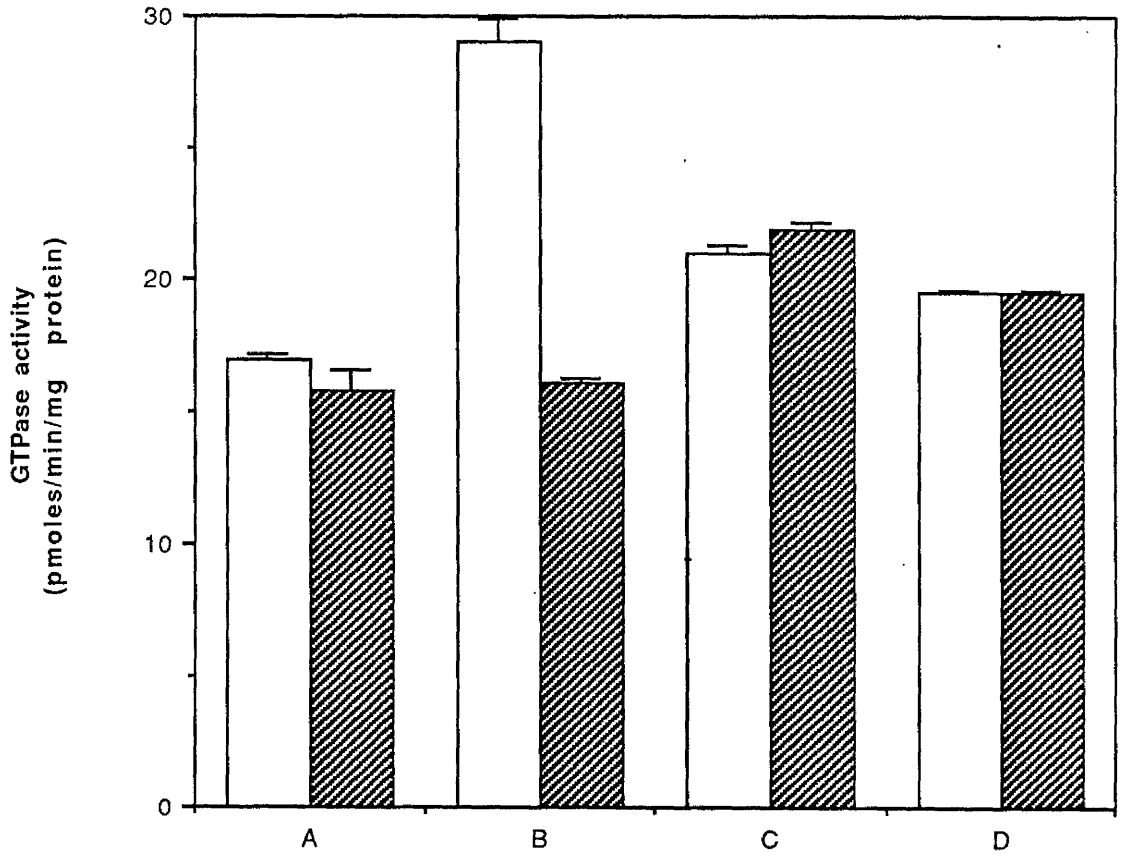


Fig. 5.5 **Stimulation of high-affinity GTPase activity by adrenaline in NG108-15 cell membranes is specifically blocked by yohimbine.**

High-affinity GTPase activity was assessed in NG108-15 cell membranes (7μg) exactly as described in the legend to Figure 5.2, in the presence of vehicle (A), or a concentration of either DADLE (B), bradykinin (C) or adrenaline (D) which elicited the maximal stimulation of high-affinity GTPase activity which could be produced by that ligand (10μM). Yohimbine was either absent (empty bars) or included (hatched bars) in parallel tubes at a final concentration of 1μM. Data represent means ± S.D. from quadruplicate determinations obtained from a single experiment which was repeated twice with essentially identical results.

Figure 5.5

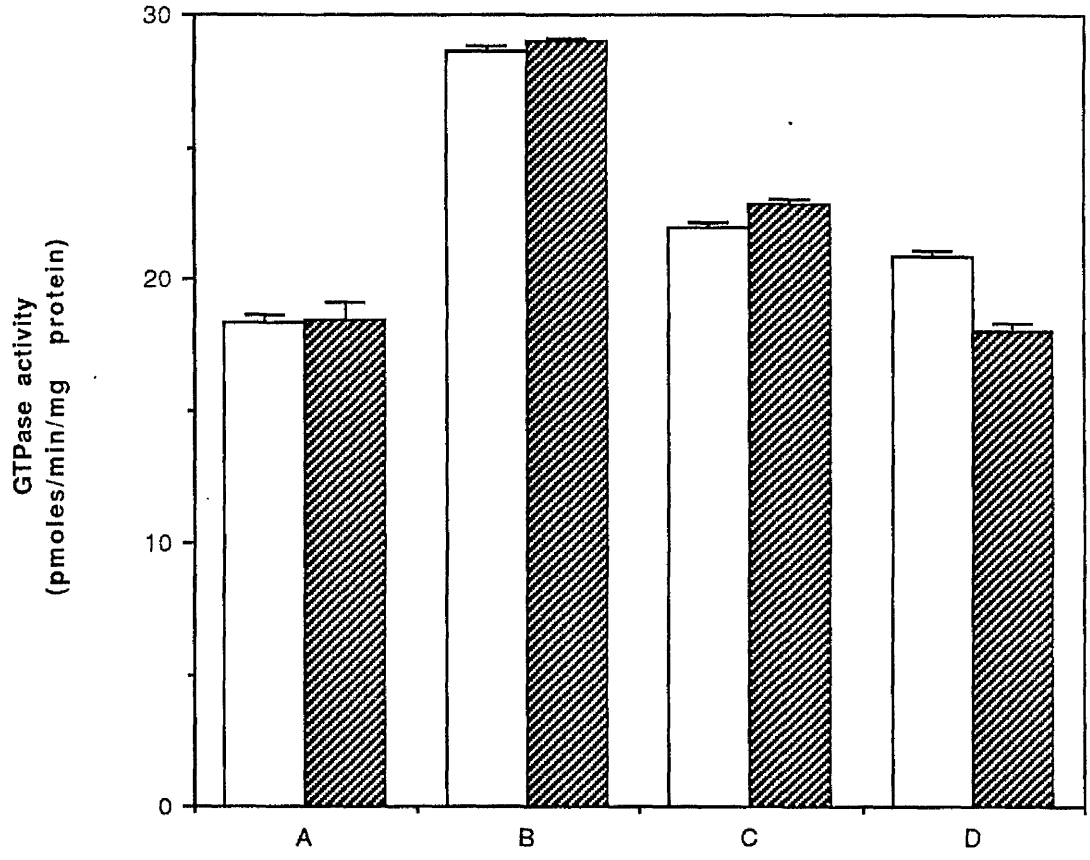


Table 5.2 The stimulation of adenylyl cyclase activity in membranes of NG108-15 cells in response to a variety of agents.

Adenylyl cyclase activity was measured in membranes of NG108-15 cells (20μg) by the method of Salomon (1979), as described in section 2.15b. The agonists present were used either singly or in concert at the following final concentrations; forskolin (10μM), PGE1 (10μM), NaF (10mM), $MnCl_2$ (10mM). The data shown represent means ± S.D taken from quadruplicate determinations pooled from three independent experiments (n=12).

Table 5.2

Addition	Adenylyl cyclase activity (pmol/min/mg protein)	Stimulation (pmol/min/mg protein)
None	40.0 ± 2.1	
Forskolin	613.9 ± 7.2	573.9
PGE1	219.4 ± 5.8	179.4
NaF	106.2 ± 4.9	66.2
MnCl2	65.8 ± 2.0	25.6
Forskolin + PGE1	915.8 ± 21.0	875.8
Forskolin + $MnCL_2$	721.6 ± 17.8	681.7

Fig. 5.6 **The stimulation of adenylyl cyclase in NG108-15 cell membranes in response to increasing concentrations of forskolin.**

Adenylyl cyclase activity was measured in membranes of NG108-15 cells (20μg) as described in the legend to Table 5.2, in response to concentrations of forskolin ranging from 0.1nM to 100μM. Results shown are means ± S.D taken from quadruplicate determinations in a single experiment which was performed twice with essentially identical results being obtained.

Figure 5.6

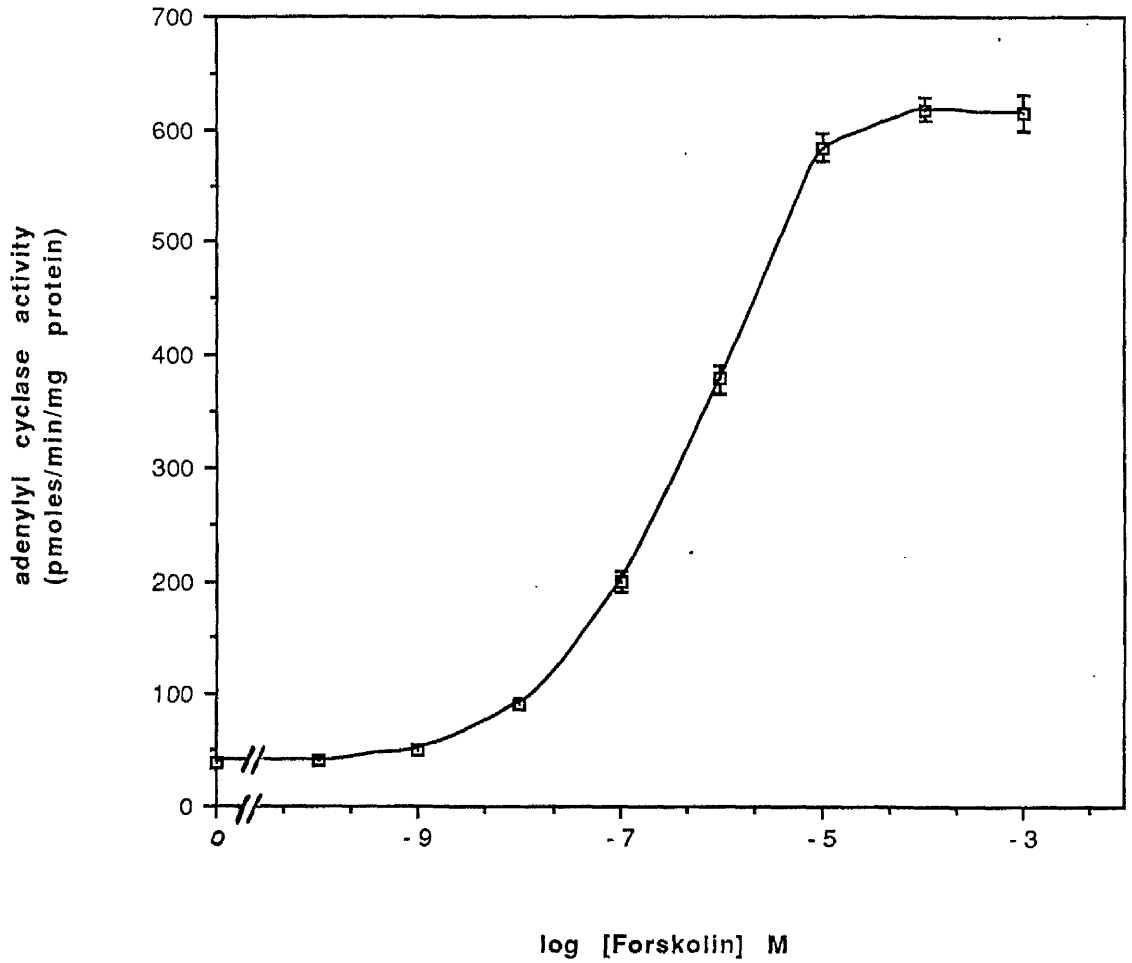

700
600
500
400
300
200
100
0
0
-9
-7
-5
-3
adenylyl cyclase activity
(pmoles/min/mg protein)
log [Forskolin] M

Fig. 5.7 The stimulation of adenylyl cyclase in NG108-15 cell membranes in response to increasing concentrations of Prostaglandin E1.

Adenylyl cyclase activity was measured in membranes of NG108-15 cells (20μg) as described in the legend to Table 5.2, in response to concentrations of PGE1 which ranged from 0.1nM to 10μM. Results shown are means ± S.D taken from quadruplicate determinations in a single experiment which was performed three times with essentially identical results being obtained.

Figure 5.7

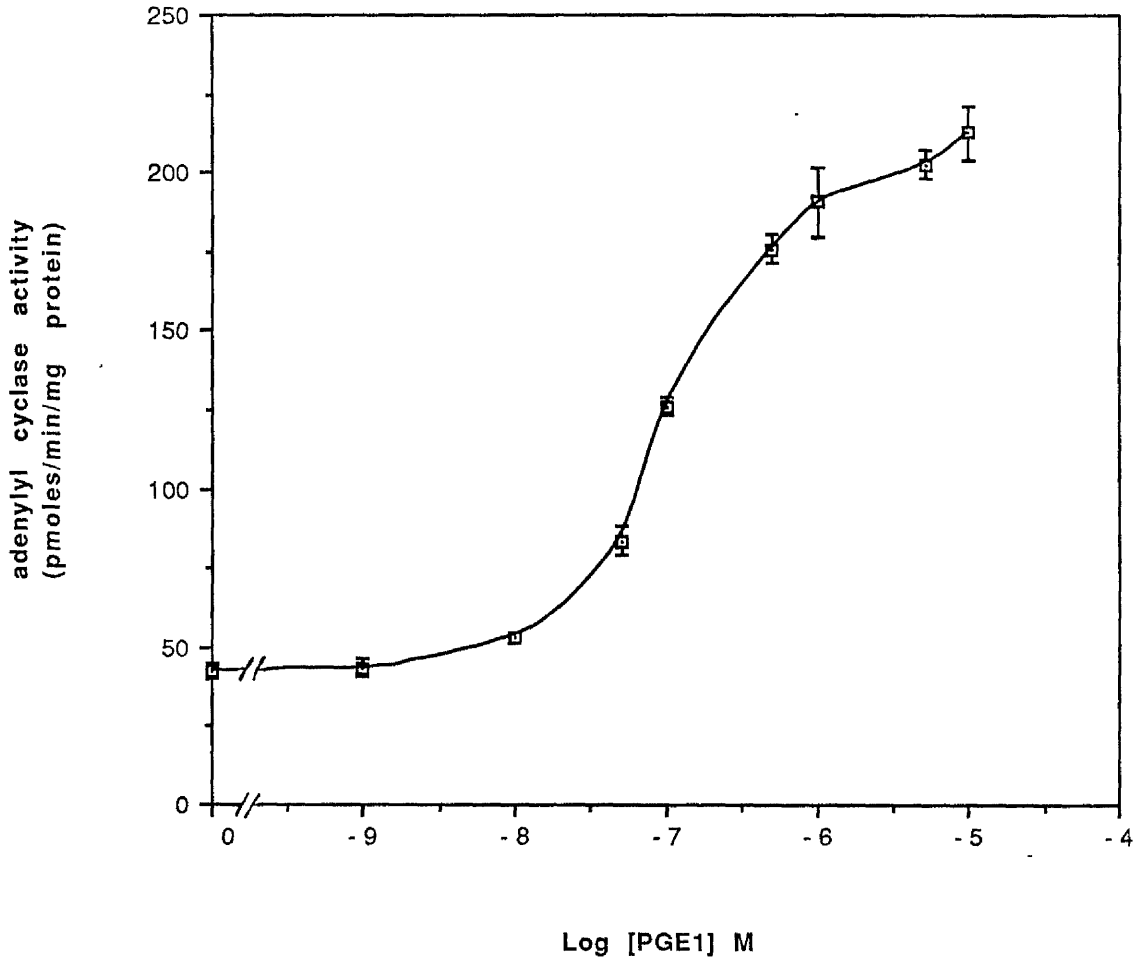

250
200
150
100
50
0
adenylyl cyclase activity
(pmoles/min/mg protein)
0
-9
-8
-7
-6
-5
-4
Log [PGE1] M

Table 5.3 The inhibition of stimulated adenylyl cyclase activity in NG108-15 cell membranes: the effect of DADLE, bradykinin and adrenaline.

Adenylyl cyclase activity was measured in membranes of NG108-15 cells (20μg) as described in the legend to Table 5.2. Adenylyl cyclase activity was stimulated by the inclusion of either forskolin (10μM) or PGE1 (10μM), and the effect of each of; DADLE , bradykinin, adrenaline (all at a final concentration of10μM) and FCS (10% v/v final concentration) was assessed. In the experiment shown, the unstimulated adenylyl cyclase activity was 43.1 ± 2.0 pmoles/min/mg protein. Data shown are means ± S.D. of quadruplicate determinations taken from a single experiment which was performed three times with identical results.

Table 5.3

Addition	Forskolin stimulation (pmol/min/ mg protein)	% Inhibition	PGE1 stimulation (pmol/min mg protein)	% Inhibition
None	675.8 ± 15.5		243.4 ± 1.6	
DADLE	459 ± 9.2	32	179.1 ± 5.4	26.4
Bradykinin	472 ± 14.2	30	174.0 ± 7.9	28.5
Adrenaline	456 ± 6.0	33	166.5 ± 6.6	31.3
FCS	680 ± 17.2	-0.1		
DADLE + Bradykinin	463 ± 9.6	31.8	167.3 ± 8.5	31.3
DADLE + Adrenaline	461.1 ± 10.1	32.1	161.5 ± 12.1	33.8
Adrenaline + Bradykinin	462.4 ± 9.1	32	170.0 ± 5.8	30.1
DADLE + Bradykinin+ Adrenaline	470.9 ± 5.0	30.0	175.8 ± 4.5	28.0

Fig. 5.8 The effect of pertussis toxin pretreatment on adenylyl cyclase activity in NG108-15 cell membranes.

Adenylyl cyclase activity was assessed in NG108-15 cell membranes which had been pretreated with vehicle (empty bars) or pertussis toxin (hatched bars) as detailed in section 2.6d, in response to; vehicle (A), forskolin (B), forskolin and DADLE (C), forskolin and bradykinin (D) or forskolin and adrenaline (E). All ligands were present at a concentration of 10μM. The assay was performed as described in the legend to Table 5.2. Data shown are means ± S.D.(n=4) taken from a single representative experiment which gave essentially identical results to three others performed.

Figure 5.8

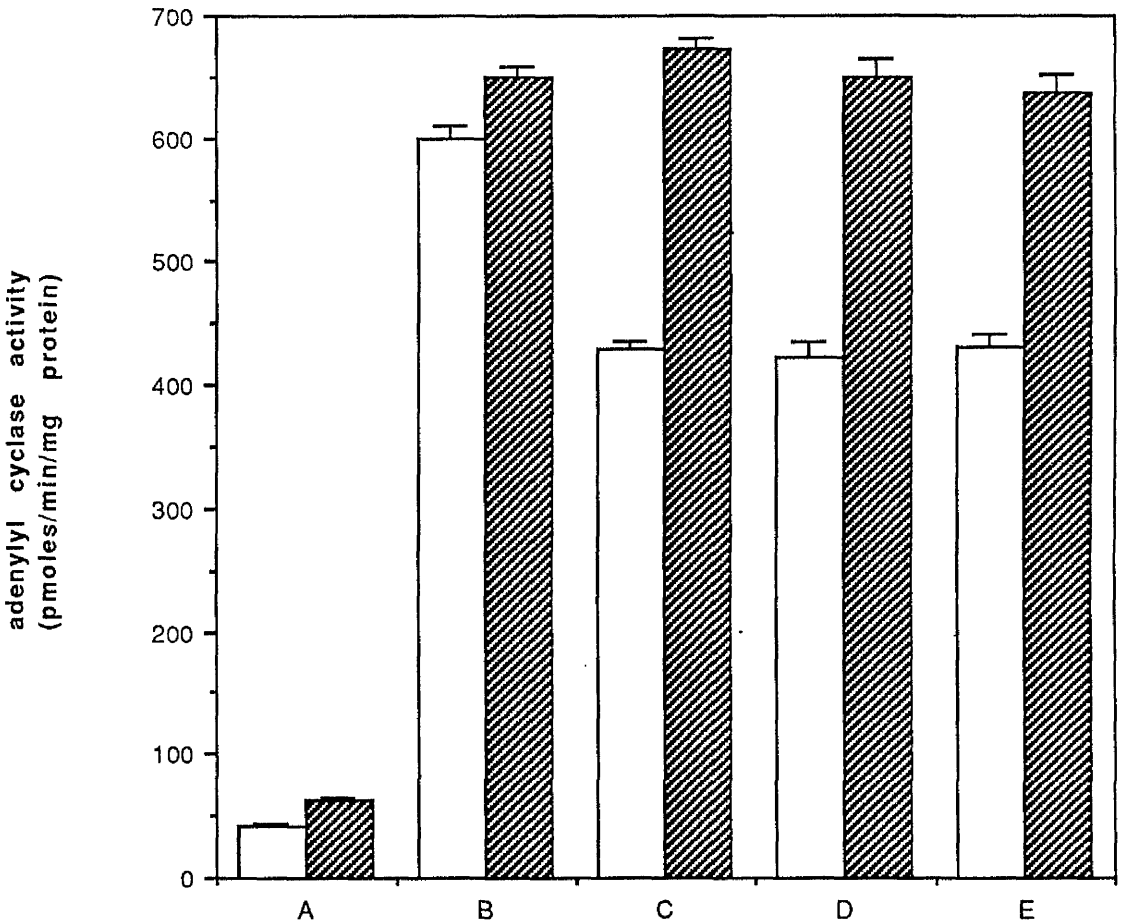

adenylyl cyclase activity
(pmoles/min/mg protein)
700
600
500
400
300
200
100
0
A
B
C
D
E

Fig. 5.9 The inhibition of adenylyl cyclase activity in NG108-15 cell membranes in response to increasing concentrations of DADLE.

Adenylyl cyclase activity was assessed in membranes produced from NG108-15 cells (20μg), in response to concentrations of DADLE which ranged between 0.1nM and 10μM, as described in the legend to Table 5.2. Adenylyl cyclase activity was amplified by the inclusion of forskolin (10μM). The data shown are means ± S.D. taken from quadruplicate determinations obtained in a single experiment which was repeated three times. In the experiment shown, the basal adenylyl cyclase activity was 43.2 ± 3.5 pmoles/min/mg protein.

Figure 5.9

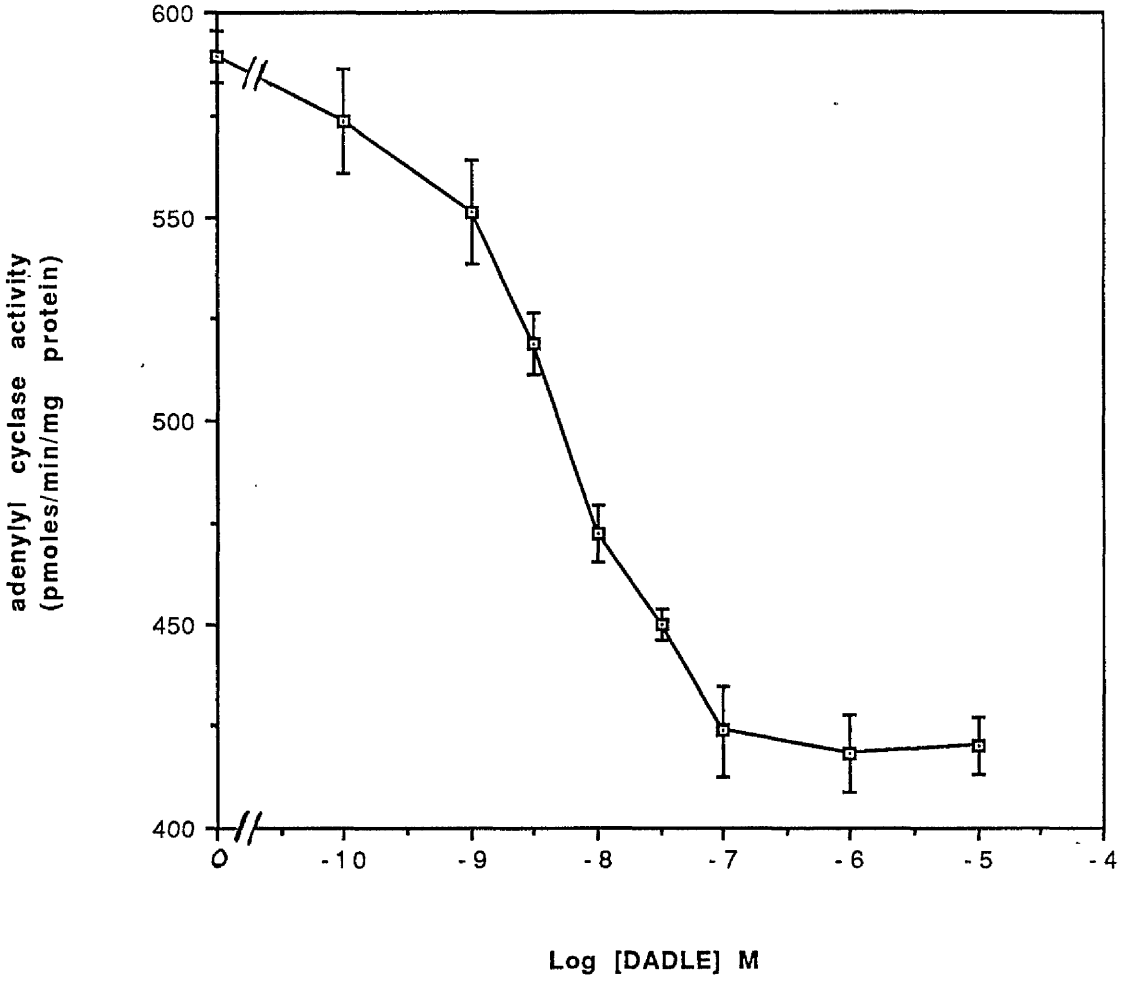

600
550
500
450
400
adenylyl cyclase activity
(pmoles/min/mg protein)
0
-10
-9
-8
-7
-6
-5
-4
Log [DADLE] M

Fig. 5.10 The inhibition of adenylyl cyclase activity in NG108-15 cell membranes in response to increasing concentrations of bradykinin.

Adenylyl cyclase activity was assessed in membranes produced from NG108-15 cells (20μg), in response to concentrations of bradykinin which ranged between 0.1nM and 10μM, as described in the legend to Table 5.2. Adenylyl cyclase activity was amplified by the inclusion of forskolin (10μM). The data shown are means ± S.D. taken from quadruplicate determinations obtained in a single experiment which was repeated three times. The basal adenylyl cyclase activity in the experiment shown was 40.6 ± 2.8 pmoles/min/mg protein.

Figure 5.10

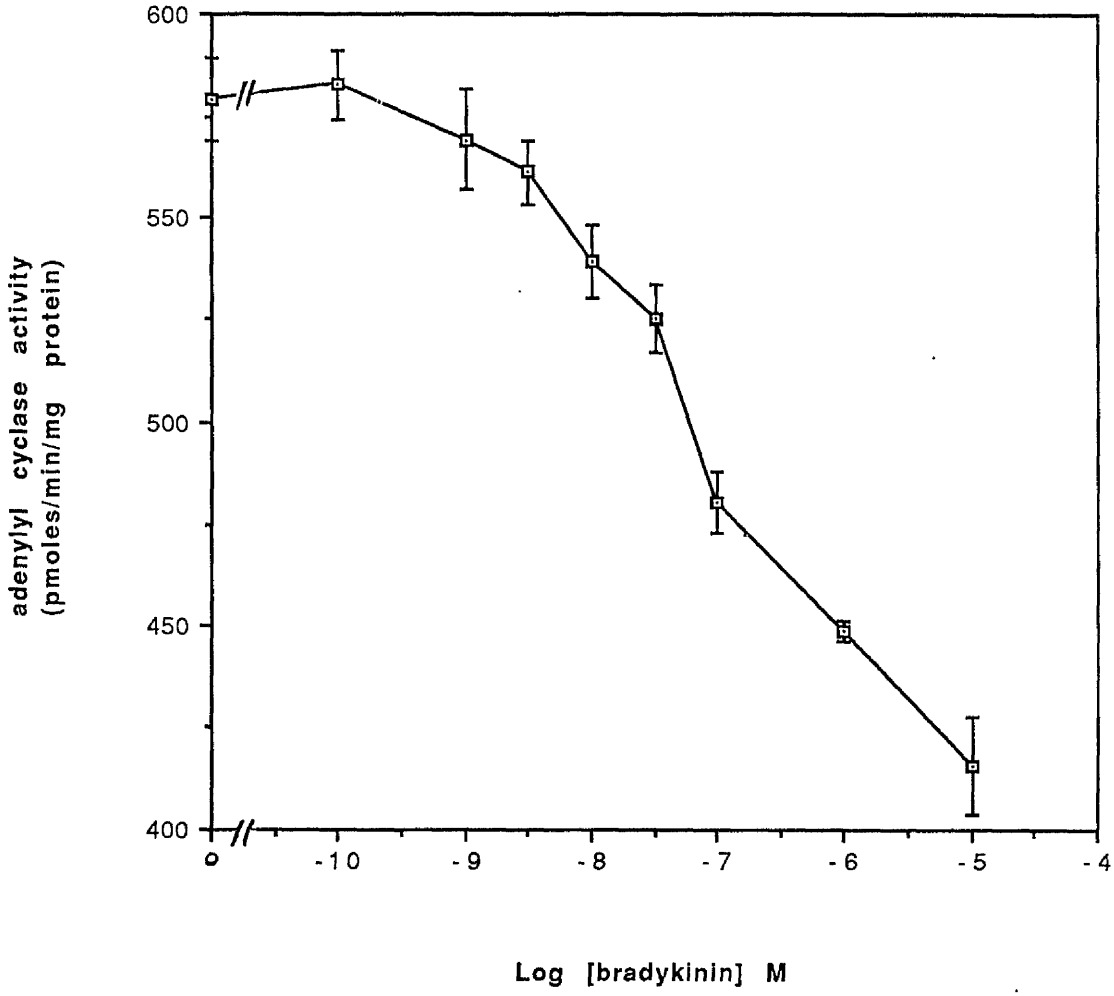

600
550
500
450
400
adenylyl cyclase activity
(pmoles/min/mg protein)
0
-10
-9
-8
-7
-6
-5
-4
Log [bradykinin] M

Fig. 5.11 **The inhibition of adenylyl cyclase activity in NG108-15 cell membranes in response to increasing concentrations of adrenaline.**

Adenylyl cyclase activity was assessed in membranes produced from NG108-15 cells (20μg), in response to concentrations of adrenaline which ranged between 0.1nM and 10μM, as described in the legend to Table 5.2. Adenylyl cyclase activity was amplified by the inclusion of forskolin (10μM). The data shown are means ± S.D. taken from quadruplicate determinations obtained in a single experiment which was repeated three times. The basal adenylyl cyclase activity in the experiment shown was 45.5 ± 1.4 pmoles/min/mg protein.

Figure 5.11

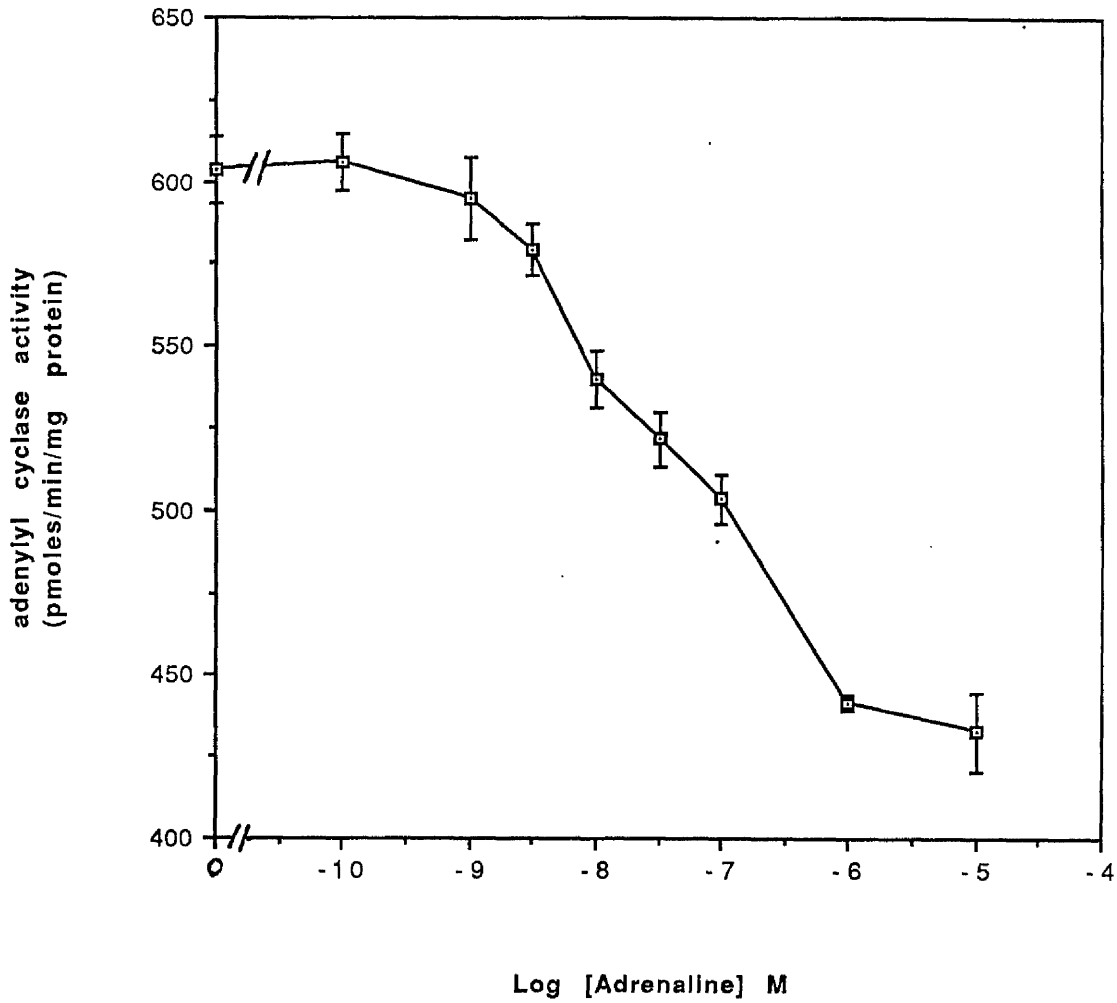

650
600
550
500
450
400
0
-10
-9
-8
-7
-6
-5
-4
adenylyl cyclase activity
(pmoles/min/mg protein)
Log [Adrenaline] M

Fig. 5.12 Inhibition of adenylyl cyclase activity in NG108-15 cell membranes: additivity studies with DADLE, bradykinin and adrenaline.

Adenylyl cyclase activity was measured in membranes of NG108-15 cells (20μg) exactly as described in the legend to Table 5.2, in response to either forskolin (10μM) (A), or forskolin (10μM) and either; DADLE (B); bradykinin (C); adrenaline (D); DADLE + adrenaline (E); DADLE + bradykinin (F); adrenaline + bradykinin (G) or DADLE, bradykinin and adrenaline (H). DADLE, bradykinin and adrenaline were used at a concentration which produced 50% of the maximum observable inhibition of forskolin (10μM) stimulated adenylyl cyclase activity which could be obtained by that ligand (5nM, 50nM and 50nM respectively) (Figures 5.9, 5.10 and 5.11). Data represent means ± S.D. of quadruplicate determinations taken from three pooled independent experiments (n=12). The basal adenylyl cyclase activity for the data shown was 35.7 ± 1.7 pmoles/min/mg protein.

Figure 5.12

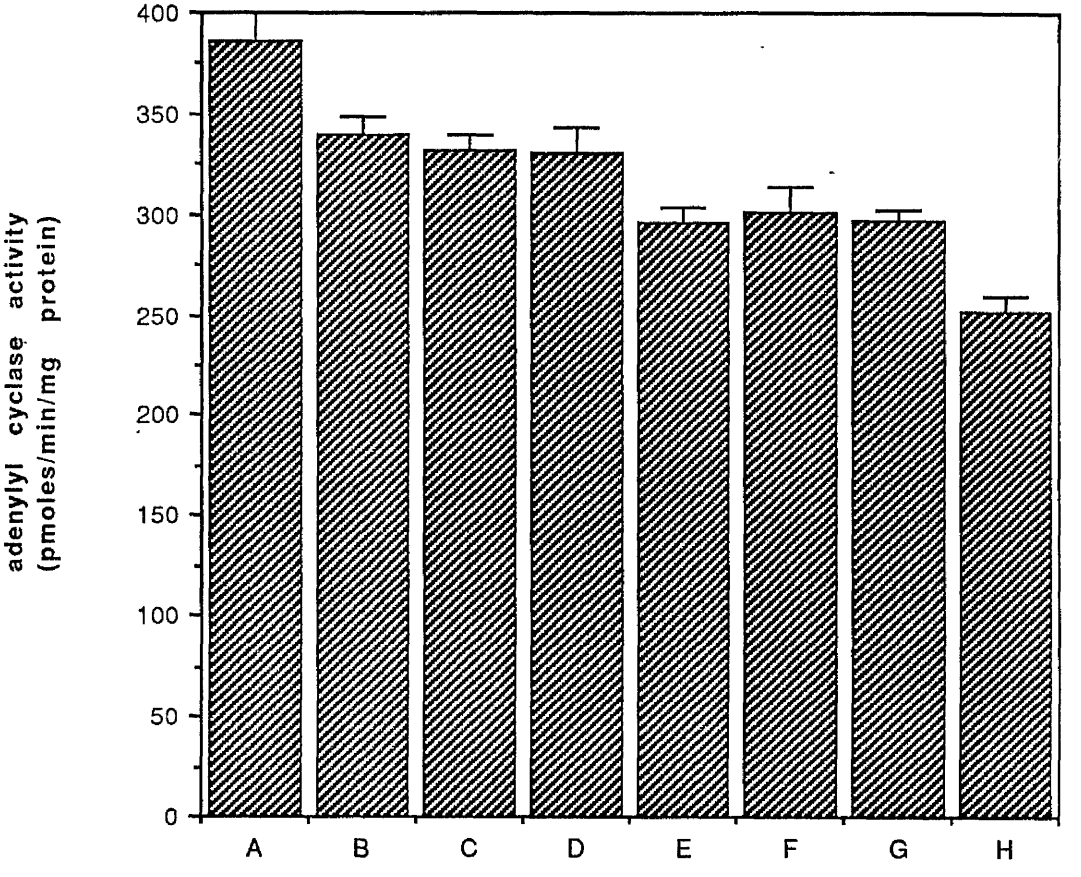

adenylyl cyclase activity
(pmoles/min/mg protein)
400
350
300
250
200
150
100
50
0
A
B
C
D
E
F
G
H

Fig. 5.13 Stimulation by DADLE and foetal calf serum of high affinity GTPase activity in NG108-15 membranes: the effect of pertussis toxin.

High affinity GTPase activity was measured in membranes produced from NG108-15 cells (8μg) which had been treated either with pertussis toxin (open circles) or vehicle (closed circles) (section 2.6d) as described in the legend to Table 5.2, in response to different concentrations of either; 5.13(a) DADLE (0.1nM to 1μM). or 5.13(b) foetal calf serum (0.01% to 20%). Data represent means ± S.D. of quadruplicate determinations from a single experiment which gave identical results to two others performed.

Figure 5.13(a)

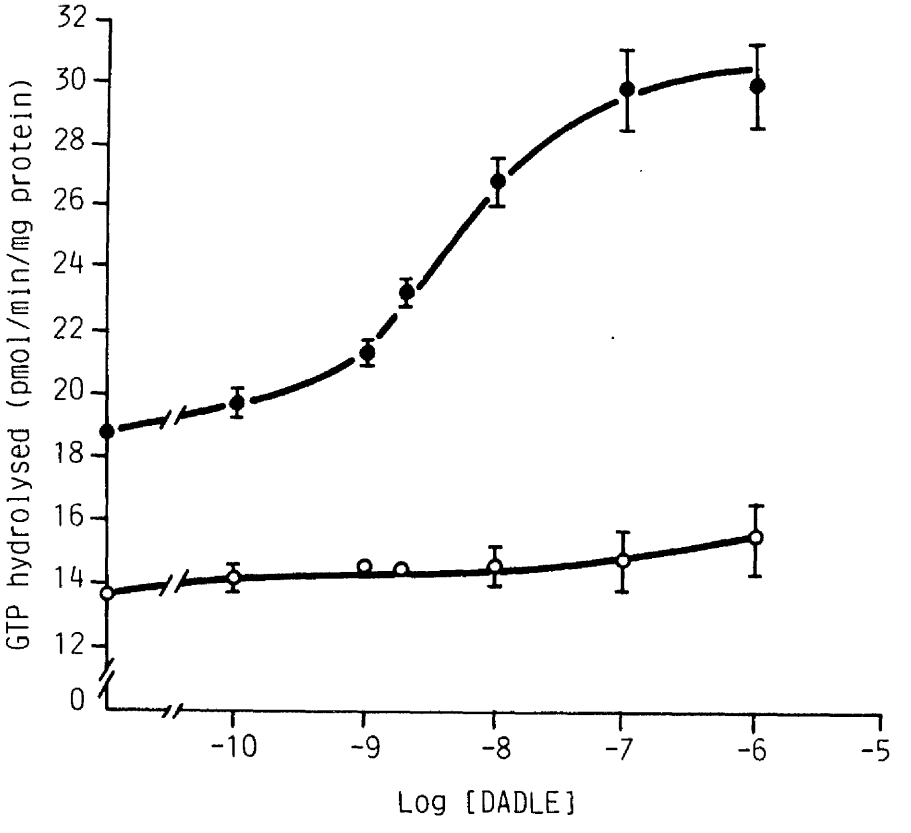


Figure 5.13(b)

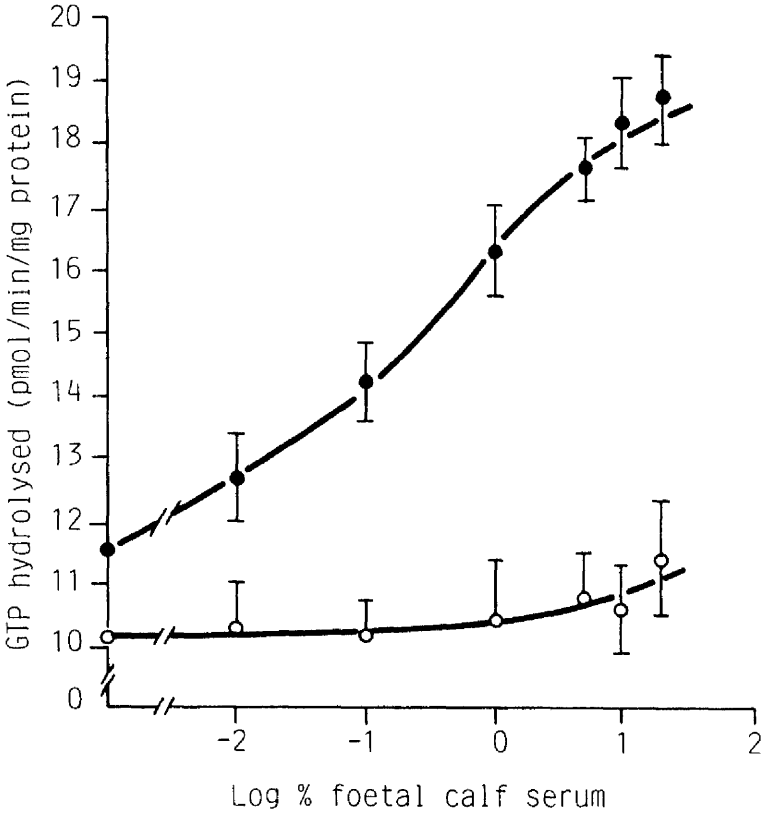


Fig. 5.14 High affinity GTPase activity in NG108-15 membranes: additivity studies with DADLE and foetal calf serum.

5.14(a) DADLE (0.1nM to 1μM) stimulation of high affinity GTPase activity was measured in NG108-15 cell membranes (8μg) exactly as described in the legend to Table 5.2, in the presence (open circles) or absence (closed circles) of a concentration of foetal calf serum which gave maximal stimulation of GTPase activity by this ligand (20%). 5.14(b) Foetal calf serum (0.01% to 20%) stimulation of high affinity GTPase activity was measured in NG108-15 cell membranes (8μg) exactly as described in the legend to Table 5.2, in the presence (open circles) and absence (closed circles) of a concentration of DADLE which gave maximal stimulation of GTPase activity by this ligand (10μM). Points are means ± S.D. of quadruplicate assays. The experiment was performed three times and yielded identical results.

Figure 5.14(a)

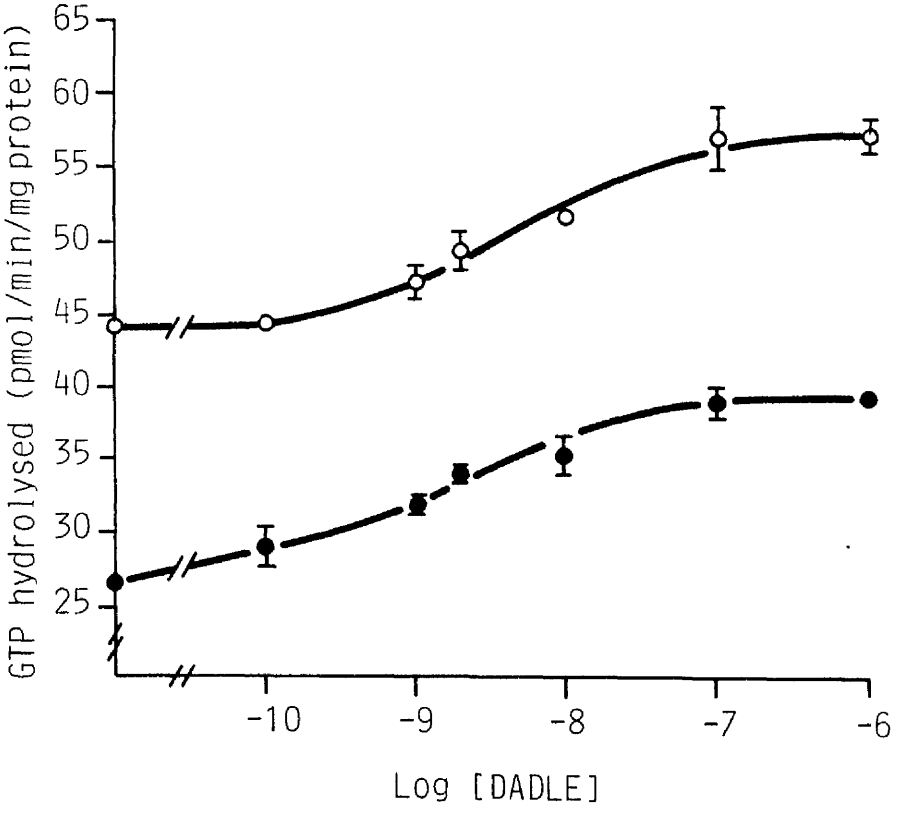


Figure 5.14(b)

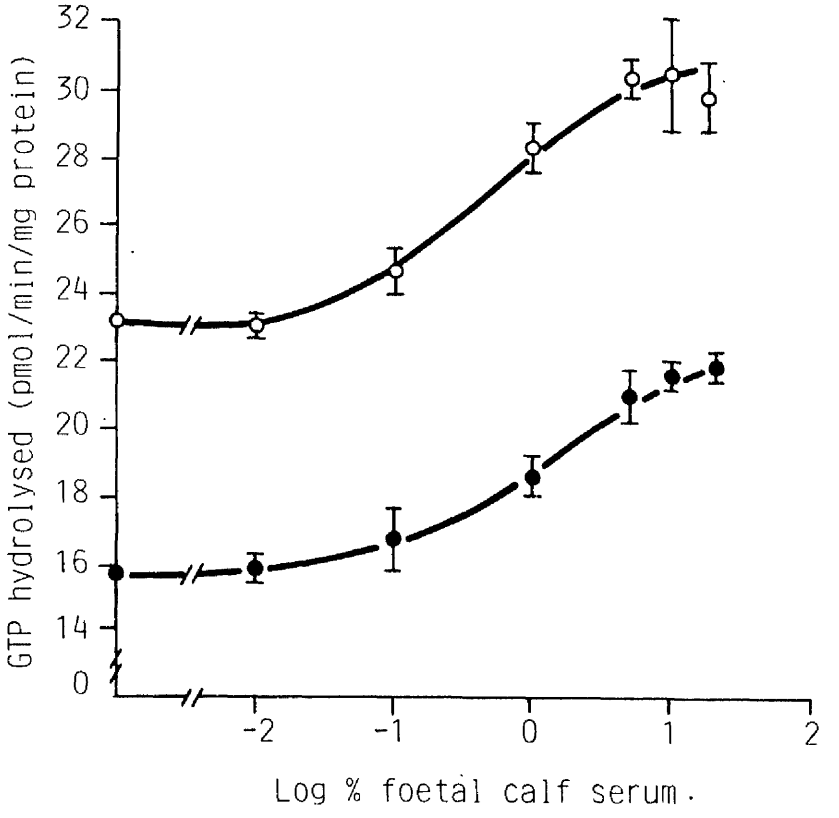


Fig. 5.15 Preincubation of NG108-15 cell membranes with synthetic peptides homologous to the C-terminus of Gi2α and Goα, reduces opioid receptor mediated high affinity GTPase activity.

NG108-15 cell membranes (7μg) were incubated with water, or varying concentrations (1nM to 0.1mM) of synthetic peptides corresponding to the last ten C-terminal amino-acids of either Tdα (5.15a) or Goα (5.15b) as described in section 2.13, for 30 minutes at 37°C in the presence of the GTPase assay reagents, but in the absence of [γ^{32}P]GTP. After this time both DADLE and [γ^{32}P]GTP were added, and the incubation continued for a further 20 minutes. Samples were then processed as described in section 2.13. Data which are means ± S.D.are taken from a single experiment which is representative of four experiments performed. 'GTPase stimulation' represents agonist stimulation over and above the basal high-affinity GTPase activities. For the data shown, the basal GTPase activity was 15.8 ± 1.0 pmoles/min/mg protein.

Figure 5.15(a)

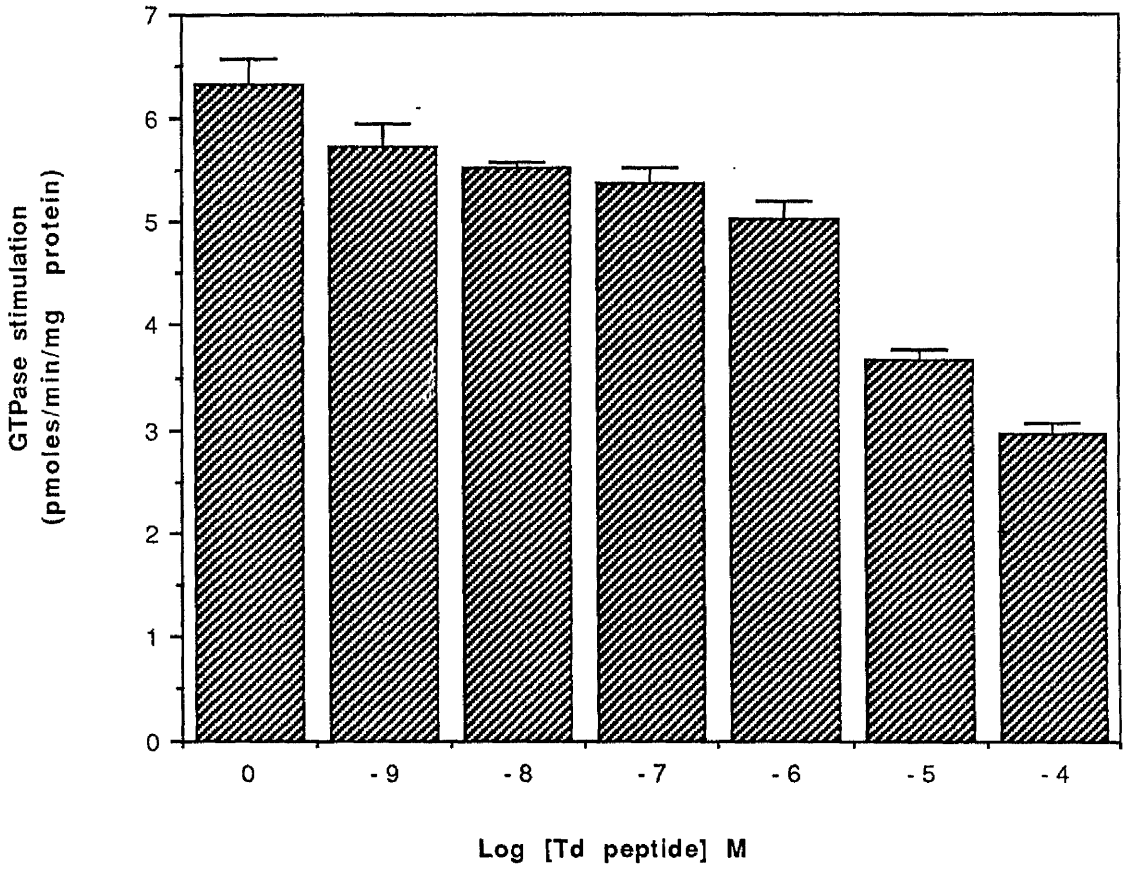


Figure 5.15(b)

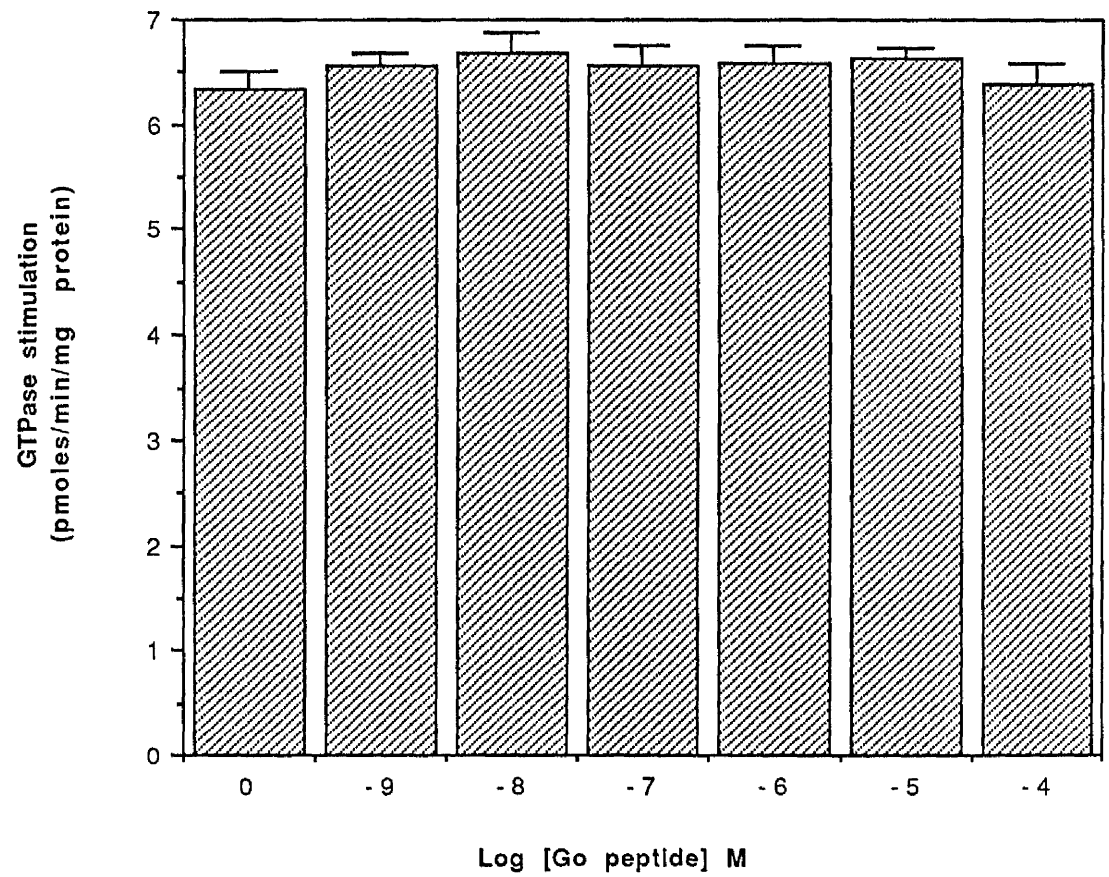


Table 5.4 Attenuation of opioid receptor stimulated high affinity GTPase activity in membranes of NG108-15 cells by affinity purified antibodies from antiserum AS7.

NG108-15 membranes (7μg) were incubated with water, normal rabbit serum or a 1:100 dilution, in normal rabbit serum, of antibodies which had been affinity purified from antiserum AS7 as detailed in section 2.9, for 1 hour at 37°C in the presence of the GTPase assay reagents, but in the absence of [γ^{32}P]GTP as described in section 2.13. After this time the appropriate receptor ligand and [γ^{32}P]GTP were added, and the incubation was continued for a further 20 minutes. Samples were then processed as described in section 2.13. Data which are means ± S.D.are taken from a single experiment which is representative of five experiments performed which gave identical results.

Table 5.4

Addition to Preincubation	Basal high affinity GTPase activity (pmol/min/mg protein)	Receptor-stimulated high-affinity GTPase activity (pmol/min/mg protein) DADLE (1μM)	Foetal calf serum (10%v/v)
Water	4.01 ± 0.29	3.01 ± 0.15	4.22 ± 0.13
Normal Rabbit Serum	6.50 ± 0.10	2.64 ± 0.09	6.27 ± 0.11
AS7 antibodies	5.41 ± 0.69	0.19 ± 0.11	4.21 ± 0.16

Fig. 5.16 Affinity purified antibodies: inhibition of δ-opioid-receptor-stimulated GTPase activity.

Antibodies which were affinity-purified from antiserum AS7 as detailed in section 2.9, were diluted with normal rabbit serum. NG108-15 membranes (5μg) were incubated with the GTPase assay mixture except for [γ^{32}P]GTP and various concentrations of the antibodies for 1hour at 37°C as detailed in section 2.13. After this time, the samples were placed on ice and DADLE (10μM) and [γ^{32}P]GTP were added. The samples were then incubated for a further 20 minutes at 37°C before being processed as in section 2.13. Points represent means ± S.D. of quadruplicate assays from a single experiment which was repeated four times.

Figure 5.16

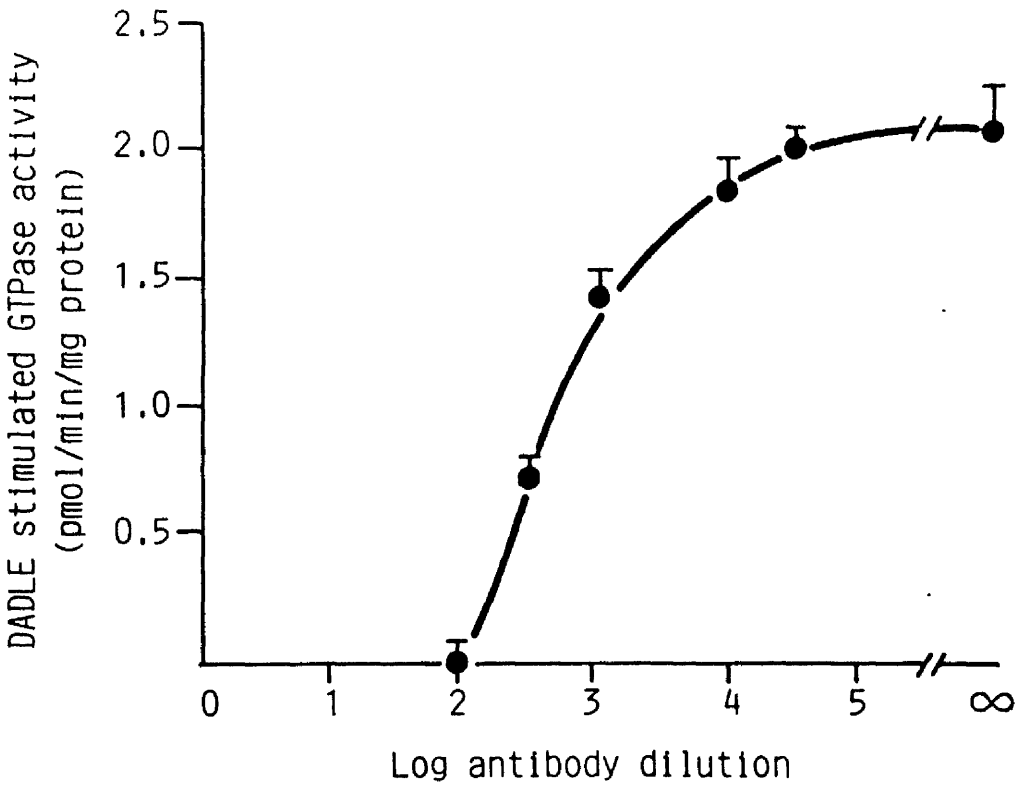

DADLE stimulated GTPase activity
(pmol/min/mg protein)
2.5
2.0
1.5
1.0
0.5
0
1
2
3
4
5
∞
Log antibody dilution

Fig. 5.17 Time course of the loss of basal and δ-opioid receptor stimulated high affinity GTPase activity during preincubation at 37°C.

NG108-15 cell membranes (6μg) were preincubated at 37°C for various times in the presence of all the components of the GTPase assay except [γ^{32}P]GTP as described in section 2.13. After this preincubation, [γ^{32}P]GTP, and where appropriate DADLE (1μM), were added, and the incubation was allowed to continue for a further 20 minutes. Samples were then processed as in section 2.13. Basal (open circles) and opioid stimulated above basal (closed circles) high affinity GTPase activities are presented. Points represent means ± S.D. of quadruplicate determinations obtained from a single experiment.

Figure 5.17

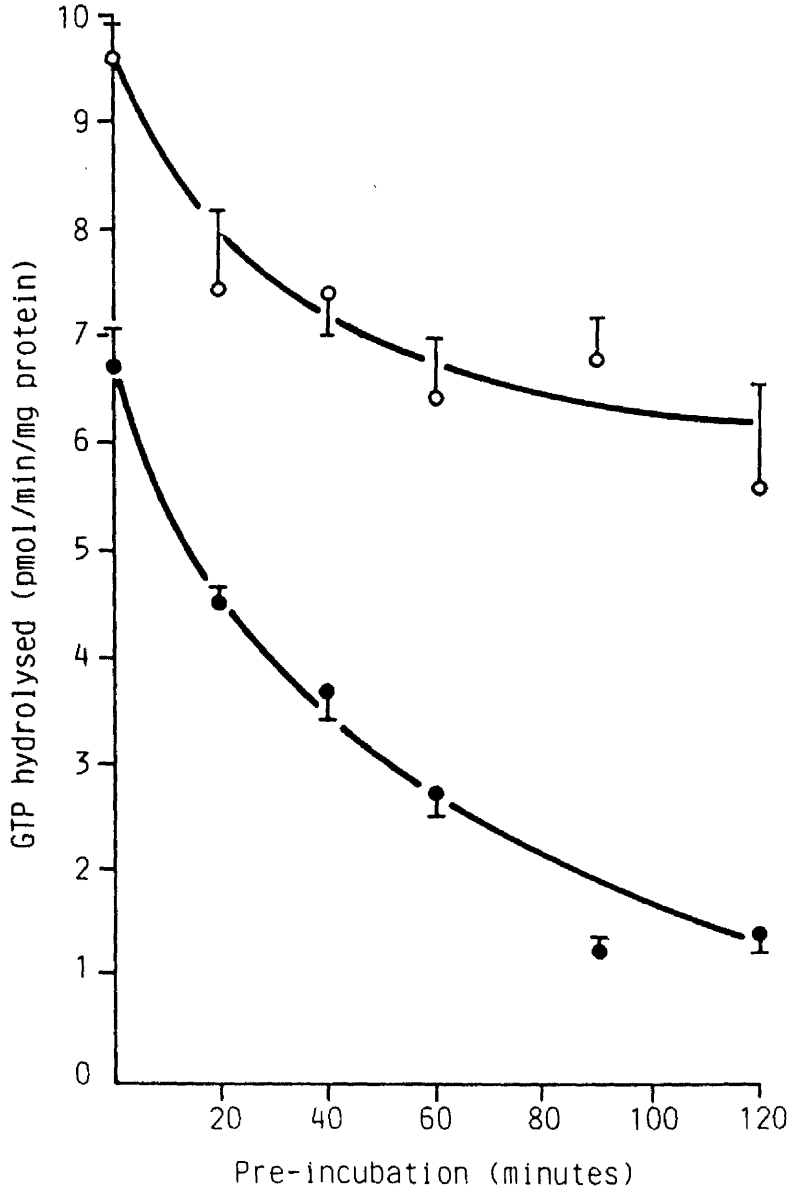

GTP hydrolysed (pmol/min/mg protein)
0
1
2
3
4
5
6
7
8
9
10
20
40
60
80
100
120
Pre-incubation (minutes)

Fig. 5.18. δ opioid receptor-stimulation of high affinity GTPase activity in membranes of neuroblastoma x glioma hybrid cells. The receptor interacts specifically with Gi2.

Membranes produced from NG108-15 cells (10μg) were incubated with IgG fractions produced from both normal rabbit serum and from the various antiserum described in chapter 2, Table 2.1 by chromatography on protein-A sepharose as described in section 2.9. These fractions were incubated with membranes of NG108-15 cells for 60 minutes at 37°C in the presence of all reagents used in the GTPase assay except [γ^{32}P] GTP and receptor agonist as detailed in section 2.13. Following the preincubation period both [γ^{32}P] GTP and DADLE (1μM) were added and samples incubated at 37°C for a further 20 minutes. Samples were then processed as detailed in section 2.13. Results are presented to display levels of both the basal high affinity GTPase activity (open bars) and high affinity GTPase activity in the presence of agonist (hatched bars). Results are means ± S.D. from 4 independent experiments. Quadruplicate determinations were performed for each point in each experiment (n=16).

Figure 5.18

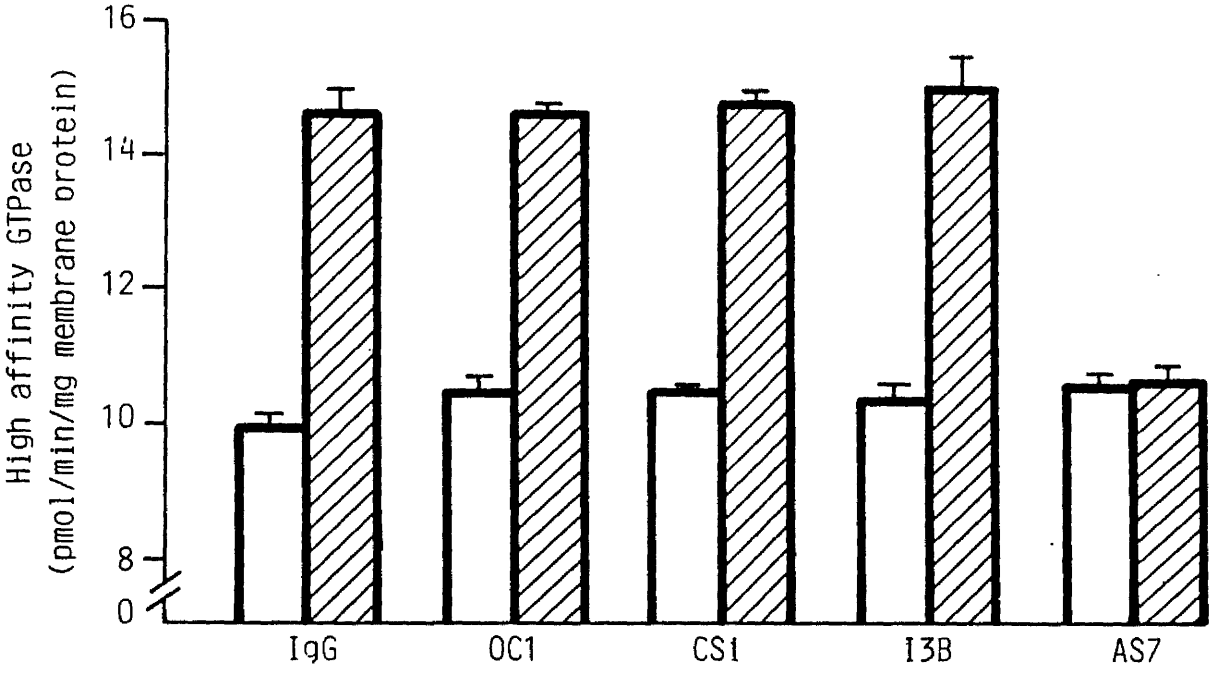

High affinity GTPase
(pmol/min/mg membrane protein)
16
14
12
10
8
0
IgG
OC1
CS1
I3B
AS7

Table 5.5 **Antibodies from antiserum AS7 attenuate opioid receptor mediated inhibition of adenylyl cyclase.**

Membranes of NG108-15 cells (20μg) were preincubated for 1 hour with IgG from either normal rabbit serum or antiserum AS7 (equivalent to a 1:100 dilution of the original antiserum) as detailed in section 2.15a. After this preincubation, the effect of DADLE (10μM) on forskolin (10μM) amplified adenylate cyclase was assessed by use of a binding protein for the determination of cyclic AMP as described in section 2.15. Results are means ± S.D. for assays performed in quadruplicate. The results are taken from a single experiment but two further experiments produced identical data.

Table 5.5

Incubation condition (IgG from)	Forskolin stimulated adenylyl cyclase (pmol/min/mg protein) - DADLE	+ DADLE	% inhibition by DADLE (10μM)
Normal rabbit serum	83.6 ± 5.6	54.8 ± 4.9	34.5
Antiserum AS7	79.1 ± 2.6	80.2 ± 1.1	-1.4

RESULTS

The rate of hydrolysis of GTP by high affinity GTPases in membranes produced from NG108-15 cells was increased in a dose-dependent manner by increasing concentrations of the substrate for such reactions, GTP (Figure 5.1a). When this data was replotted as an Eadie-Hofstee plot (Figure 5.1b), it is possible to assess the Km value for GTP by high affinity GTPases in NG108-15 membranes, a value of 0.4μM being routinely obtained (0.42 ± 0.04 μM, mean ± S.D., n=4). The hydrolysis of GTP by high affinity GTPases in NG108-15 cell membranes was linear over a 30 minute timecourse and with membrane protein concentrations of between 2 to 30μg. The total hydrolysis of GTP never exceeded 15% (results not shown). NG108-15 cells possess a wide range of receptors linked to second messenger systems, as detailed in chapter 3. When the ability of a variety of agonists to stimulate high affinty GTPase activity was assessed in NG108-15 membranes, the opioid peptides DADLE and DALAMID gave the greatest stimulation of the rate of hydrolysis of GTP (8.9 ± 0.1 pmol/min/mg protein, mean ± S.D., n=4) and 8.7 ± 0.3 pmol/min/mg protein, respectively) (Table 5.1). Foetal calf serum (FCS), also gave a significant stimulation of high affinity GTPase activity (6.5 ± 0.3 pmol/min/mg protein). Bradykinin and adrenaline, though capable of G-protein stimulation (Kurose et al., 1982), routinely gave a measurable, though poor response (4.4 ± 0.1 pmol/min/mg protein and 1.6 ± 0.1 pmol/min/mg protein, respectively). Prostaglandin E1 (PGE1) has been shown to stimulate adenylyl cyclase activity, presumably through activation of Gs, (Traber et al., 1975.), however high affinity GTPase activity in the presence of PGE1 (14.4 ± 0.4 pmoles/min/mg protein) was not significantly higher than the high affinity GTPase activity in the absence of PGE1 (14.2 ± 0.1 pmoles/min/mg protein) (Students' t-test, $P > 0.3$) (Table 5.1). The basal high affinity GTPase activity varied between different membrane preparations over an approximately two-fold range from 9.7 ± 0.2 pmoles/min/mg protein to 19.3 ± 0.2 pmoles/min/mg protein.

In addition to expressing a single subtype of opiate receptor of the δ-classification (Chang and Cuatrecasas, 1979), NG108-15 cells are known to express α-adrenergic receptors of the α-2B designation (Bylund, 1988). NG108-15 cells also express bradykinin receptors (Higashida et al., 1986), however the identity of the receptor which the factor(s) contained in FCS interacts with is poorly defined. To ascertain whether DADLE, adrenaline, bradykinin and FCS were stimulating the activity of a pertussis toxin sensitive G-protein(s), NG108-15 cells were pretreated with pertussis toxin as described in section 2.6d. After pertussis toxin pretreatment of NG108-15 cells *in vivo*, DADLE, adrenaline, bradykinin and FCS were found to be unable to stimulate high-affinity GTPase activity in NG108-15 membranes (Figure. 5.2), thus indicating that each of the ligands function through activation of a pertussis toxin sensitive G-protein(s). It is of interest to note that the basal GTPase activity is significantly reduced by pertussis toxin treatment from a value of 19.3 ± 0.2 pmol/min/mg protein in membranes from untreated cells to 16.9 ± 0.1 pmol/min/mg protein in membranes from pertussis toxin treated cells (means ± S.D., n=4, Students t-test, $P < 0.001$). In an attempt to analyse whether the agonists DADLE, adrenaline and bradykinin were stimulating the rate of hydrolysis of GTP through activation of the same pertussis toxin sensitive G-protein, or through activation of different species of pertussis toxin sensitive G-protein, additivity experiments were performed (Figure 5.3). When either DADLE, adrenaline or bradykinin were assayed for their ability to stimulate high-affinity GTPase activity on their own, DADLE always gave the greatest stimulation of GTPase activity, with bradykinin producing the second highest and adrenaline giving the lowest observable increase in high-affinity GTP hydrolysis of the three agonists tested (Figure 5.3, columns 1, 2 and 3). When either DADLE and adrenaline or DADLE and bradykinin were assayed together, no further increase in hydrolysis was noted as compared to the effect of DADLE on its own (Figure 5.3, columns 4 and 5). However, when adrenaline and bradykinin were assayed together, the stimulation obtained was fully additive (Figure 5.3, column 6). When all three agonists were included in the assay, the stimulation of GTPase activity observed (7.7 ± 0.1 pmoles/min/mg protein) was not greater than the stimulation produced by DADLE alone (7.8 ± 0.1 pmoles/min/mg protein) (mean ±

S.D., n=4, Student's t-test P > 0.4) (Figure 5.3, columns 1 and 7). Therefore of the ligands tested, DADLE was capable of eliciting the maximal stimulation of GTPase activity on its own.

The pharmacology of action of DADLE was examined, by including the opioid antagonist naloxone in GTPase assays (Figure 5.4). Naloxone was capable of preventing DADLE mediated stimulation of GTPase activity in NG108-15 cell membranes, without affecting the stimulation obtained by either adrenaline or bradykinin (Figure 5.4). In a similar manner, inclusion of the α-2 adrenergic antagonist yohimbine in GTPase assays resulted in the complete attenuation of adrenaline mediated GTPase stimulation with no effect on either DADLE or bradykinin's ability to stimulate GTPase activity (Figure 5.5). Thus DADLE and adrenaline stimulate GTPase activity by interacting with a different class of receptor in NG108-15 cell membranes. The pharmacological profile of bradykinin receptors expressed by the NG108-15 cell line to date, has not been examined in detail. Inclusion of the B_1 agonist desArg9-bradykinin, was without effect in stimulating GTPase activity, when used at concentrations up to 10μM. Inclusion of the B_1 antagonist desArg9-[Leu8] bradykinin in GTPase assays did not attenuate bradykinin mediated stimulation (results not shown), suggesting that the bradykinin receptor capable of stimulating GTPase activity was not of the B_1 designation (Regoli and Barabe, 1980). Interestingly, the B_2 selective bradykinin analogue [Thi5,8-D-Phe7]-bradykinin, which has been described as either having the properties of an agonist or antagonist, depending on the tissue under examination (Plevin and Owen, 1988), was found to be a partial agonist, when included in GTPase assays and compared to the effect of bradykinin. Thus the pharmacological identification of the bradykinin receptor(s) expressed by NG108-15 cell remains elusive.

It is possible to stimulate adenylyl cyclase activity in NG108-15 cell membranes in response to a variety of ligands (Table 5.2). Measurements of cAMP production by NG108-15 cell membranes using both the binding assay (section 2.14) and the two-step chromatography assay (section 2.15b) were linear when each assay was conducted from between 5 to 30 minutes, and over membrane protein concentrations of between 2 to 100 μg in the case of the binding assay, and between 5

to 30 μg for the two-step chromatography method (results not shown). Of the ligands tested at saturating concentrations, forskolin (10μM), which is thought to be able to activate the enzyme directly and also in concert with Gsα (Ho and Shi, 1984), produced the greatest stimulation, followed by PGE1. NaF was also capable of eliciting stimulation of adenylyl cyclase in these membranes, as was manganese chloride ($MnCl_2$), albeit poorly (Table 5.2). Interestingly, when forskolin and PGE1 were included in the same assay, the stimulation of adenylyl cyclase obtained was greater than when each agent was used singly, similarly, the inclusion of forskolin and $MnCl_2$ in the same assay, produced a greater stimulation of adenylyl cyclase activity than that seen for each agonist on its own (Table 5.2). Forskolin was shown to stimulate adenylyl cyclase activity in the concentration range 0.1nM to 100μM, half maximal stimulation occurred at approximately 1μM (Figure 5.6). In a similar manner, PGE1 increased adenylyl cyclase activity in NG108-15 membranes in a dose-dependent manner, when concentrations which ranged from 0.1nM to 10μM were applied. The half maximally effective concentration was approximately 50nM (Figure 5.7).

In an attempt to further examine the nature of the pertussis toxin sensitive G-proteins with which adrenaline, DADLE, bradykinin and FCS interact, the effect of each agonist on adenylyl cyclase activity was assessed in NG108-15 membranes. Table 5.3 displays the change in activity of adenylyl cyclase in NG108-15 membranes which have been stimulated with either forskolin or PGE1. The percentage of inhibition of adenylyl cyclase obtained by DADLE, adrenaline and bradykinin was in the order of 30%. For the data presented, an analysis of variance demonstrated that the inhibition of both forskolin and PGE1 stimulated adenylyl cyclase activty was not significantly different, irrespective of the ligands present in the assay (one-way analysis of variance, $P > 0.5$). In contrast to DADLE, adrenaline and bradykinin, FCS was unable to inhibit either forskolin or PGE1 stimulated adenylyl cyclase activity in these membranes. It may be noted that there is no additivity of inhibition when either DADLE, bradykinin or adrenaline are present in combination. Each agonist is fully capable of inhibiting adenylyl cyclase activity on

its own, when used at a saturating concentration (Table 5.3).

Since G-protein stimulation by each of DADLE, bradykinin and adrenaline was pertussis toxin sensitive, as assessed by GTPase activity (Figure 5.2), it was expected that the inhibition of adenylyl cyclase activity elicited by these agonist would also be pertussis toxin sensitive. This was shown to be the case (Figure 5.8). In membranes produced from untreated NG108-15 cells, DADLE, bradykinin and adrenaline were able to inhibit forskolin (10μM) stimulated adenylyl cyclase activity by approximately 30%, in close agreement with earlier observations (Table 5.3). However, pretreatment of NG108-15 cells *in vivo* with pertussis toxin abolished the ability of each of DADLE, adrenaline and bradykinin to inhibit adenylyl cyclase. Pertussis toxin pretreatment was routinely noted to produce a significant increase in the basal adenylyl cyclase activity from 41.0 ± 2.8 pmol/min/mg protein in membranes produced from untreated cells, to 62.0 ±2.5 pmol/min/mg protein in membranes from pertussis toxin treated cells (means ± S.D., n=4) (Student's t-test, $P < 0.005$). In addition, forskolin (10μM) stimulated adenylyl cyclase activity was significantly higher in membranes prepared from pertussis toxin pretreated cells, 650 ± 7.8 pmol/min/mg protein, compared to 600 ± 9.8 pmol/min/mg protein in membranes prepared from untreated NG108-15 cells (means ± S.D., n=4) (Student's t-test, $P < 0.05$). The inhibition of adenylyl cyclase produced by DADLE was dose dependent within the concentration range 0.1nM to 10μM, the concentration of DADLE which elicited 50% of the maximal inhibition which could be attained by this ligand was approximately 5nM (Figure 5.9). Similarly, the inhibition of adenylyl cyclase produced by bradykinin was dose dependent, within the concentration range 1nM to 100μM, the concentration of bradykinin which elicited 50% of the maximal inhibition which could be attained by this ligand was approximately 50nM (Figure 5.10). The inhibition of adenylyl cyclase produced by adrenaline showed a similar profile to that obtained with bradykinin, within the concentration range 1nM to 100μM, the concentration of adrenaline which elicited 50% of the maximal inhibition which could be attained by this ligand was approximately 50nM (Figure 5.11).

It can be noted in Table 5.3, that the ability of saturating concentrations of DADLE, adrenaline and bradykinin to inhibit either forskolin (10μM) or PGE1 (10μM) stimulated adenylyl cyclase activity was not additive, maximum inhibition was obtainable by each agonist on its own. To examine the lack of additivity of inhibition of adenylyl cyclase, the concentrations of each of DADLE, adrenaline and bradykinin which produced 50% of the maximal inhibition of adenylyl cyclase which could be produced by each ligand (Figures, 5.9, 5.10, 5.11) were employed in additivity studies (Figure 5.12). On its own, each agonist produced approximately 50% of the maximal inhibition of forskolin stimulated adenylyl cyclase which could be obtained by that ligand (DADLE, 16 ± 0.5 %; bradykinin, 13 ± 0.5 %; adrenaline, 14 ± 0.5 % inhibition, mean ±S.D., n=3) (Figure 5.12, columns, B, C, D). When any of the two agonists were used in combination, the inhibition of adenylyl cyclase activity obtained was significantly greater than the inhibition obtained by each agonist on its own (DADLE + bradykinin, 21 ± 0.9 %; DADLE + adrenaline, 23 ± 0.5 %; bradykinin + adrenaline, 25 ± 0.4 % inhibition) (Figure 5.12, columns E, F, G) (one way analysis of variance, $P < 0.05$). When all three agonists were present(Figure 5.12, column H), the inhibition of adenylyl cyclase activity obtained (32 ± 0.9 %, mean ± S.D., n=3) was similar in magnitude to the inhibition of adenylyl cyclase which could be obtained by each agonist on its own, when used at a saturating concentration (Table 5.3). Thus the ability of each of DADLE, bradykinin and adrenaline to inhibit adenylyl cyclase was additive, when each ligand was used at a concentration which did not elicit the maximal inhibition of adenylyl cyclase which could be produced by that ligand.

Returning to Table 5.2, it can be noted that the stimulation of high affinity GTPase activity produced by FCS was greater than all other ligands tested, with the exception of the opioid peptides, DADLE and DALAMID. In addition, both FCS and DADLE have been demonstrated to stimulate the GTPase activity of pertussis toxin sensitive G-proteins (Figure 5.2). It was thus decided to address the specificity of interaction between each of these ligands. Stimulation of high affinity GTPase activity by DADLE (Figure 5.13a) occurred over concentrations between 0.1nM and 1μM and was half maximal at 5nM (Fig 5.13a) Absolute values of the high affinity GTPase activity were increased by some 12.5 ± 0.8 pmol/min/mg protein by maximal

concentrations of the opioid peptides (mean ± S.D., n=4). Foetal calf serum stimulation of high-affinity GTPase activity in these same membranes occurred between 0.01 and 20% (v/v) foetal calf serum and was half maximal at 0.8% (v/v) (Figure 5.13b). Maximal increases in high-affinity GTPase activity in response to foetal calf serum were 6.3 ± 1.0 pmol/min/mg of membrane protein (mean ± S.D., n=4).

Membranes of pertussis toxin treated cells displayed decreased basal high-affinity GTPase activity, as described previously for the data shown in Figure 5.2, and the residual high-affinity GTPase activity was no longer stimulated by either the opioid peptide DADLE (Figure 5.13a) or foetal calf serum (Figure 5.13 b).

NG108-15 cells are known to express three different pertussis toxin sensitive G-proteins, Gi2, Gi3 and Go, as detailed in chapter 3. To examine whether both DADLE and foetal calf serum caused stimulation of the GTPase activity of the same pertussis toxin sensitive G-protein, additivity studies were performed. When dose response curves of GTPase stimulation by foetal calf serum were carried out in the presence or absence of a single concentration of DADLE (10μM) which had previously been shown to produce a maximal increase in GTPase activity, then the response to foetal calf serum was unaltered (Figure 5.14a). The response was merely shifted to higher high affinity GTPase activity owing to the stimulation produced by the opioid ligand. In the reciprocal experiment, high-affinity GTPase activity was measured in response to increasing concentrations of DADLE, in the absence or presence of 20% (v/v) foetal calf serum (Figure 5.14 b). This produced similar results to that obtained in Figure 5.14a, indicating that stimulation of high-affinity GTPase activity by foetal calf serum and opioid peptides was fully additive. As detailed in Table 5.3, DADLE produced inhibition of adenylyl cyclase with a similar concentration dependence to that required for stimulation of GTPase activity (Fig 5.9). In contrast however, foetal calf serum did not modulate adenylate cyclase activity at concentrations up to 20% (v/v), which produced a maximal stimulation of GTPase activity (Table 5.3). Thus, DADLE and foetal calf serum interact with receptors which stimulate two different classes of pertussis toxin sensitive G-protein; the receptor for DADLE interacts with the G-protein mediating inhibition of

adenylyl cyclase, whereas the receptor for foetal calf serum interacts with a different pool of G-proteins which do not inhibit adenylyl cyclase and by extension, represent a different class of G-protein.

When NG108-15 cell membranes were pre-incubated for 1 hour at 37°C with a synthetic decapeptide which was 100% homologous to the last ten C-terminal amino-acids of Tdα (KENLKDCGLF), and was one amino-acid different to the last ten C-terminal amino-acids of Gi2α (KNNLKDCGLF) (where the highlighted residue is not present in the Tdα peptide), the ability of DADLE to stimulate GTPase activity is reduced by approximately 60%, from 6.33 ± 0.2 pmoles/min/mg protein in the absence of peptide, to 2.96 ± 0.1 pmoles/min/mg protein in the presence of Tdα peptide (means ± S.D., n=4) (Figure 5.15a). In contrast, pre-incubation of the same membranes with a synthetic decapeptide 100% homologous to the extreme C-terminal region of Goα (ANNLRGCGLY) had no effect on the ability of DADLE stimulate GTPase activity (Figure 5.15b). Basal high affinity GTPase activity in the absence of added peptide (15.8 ± 1.0 pmoles/min/mg protein) was unaltered by the addition of either Tdα peptide (14.9 ± 0.3 pmoles/min/mg protein) or Goα peptide (15.9 ± 0.5 pmoles/min/mg protein), (means ± S.D., n=4, Student's t-test, $P > 0.5$ and $P > 0.6$ respectively), (Figure 5.15 a,b).

Immunoblotting membranes of NG108-15 cells with antiserum AS7 detected a polypeptide of some 40kDa, which has been defined as being the α-subunit of Gi2 (chapter 3, Figure 3.3). Affinity purified antibodies from antiserum AS7 also recognized the α-subunit of Gi2 (chapter 3, Figure 3.4). Pre-incubation of NG108-15 membranes for 1 hour at 37°C with a 1:100 dilution in normal rabbit serum of antibodies which had been affinity purified from antiserum AS7 completely abolished the ability of DADLE to stimulate high-affinity GTPase activity, but had little effect on the foetal calf serum stimulation of high-affinity GTPase activity (Table 5.4). Basal GTPase activity of the membranes was not inhibited by the affinity purified antibodies, and in a number of experiments the basal GTPase activity was higher after preincubation in the presence of normal rabbit serum than in its absence. Preincubation of the membranes with normal rabbit serum alone did not produce an attenuation of the response to DADLE (Table 5.4). Greater dilutions of

the affinity purified antibodies into normal rabbit serum decreased the antibody-mediated attenuation of opioid stimulation of GTPase activity (Figure .5.16), half-maximal effects of the antibodies affinity purified from AS7 were achieved at a 1:1000 dilution and were completely abolished at a 1:10 000 dilution (Fig 5.16). Preincubation of NG108-15 membranes at 37°C induced a time-dependent loss of both basal and opioid -stimulated high-affinity GTPase activity (Figure 5.17). After a pre-incubation of 2 hours, there was a 30% reduction in basal GTPase activity from 9.6 ± 0.2 pmoles/min/mg protein to 5.4 ± 0.7 pmoles/min/mg protein and a 75% loss of DADLE stimulated GTPase activity from 6.6 ± 0.4 pmoles/min/mg protein to 1.4 ± 0.2 pmoles/min/mg protein (means ± S.D., n=4), suggesting that a proportion of both the G-protein and receptor pool was being inactivated with time.

A more detailed attempt to identify the G-protein species that the δ-opiate receptor activates, involved the use of IgG fractions which were purifed from each of normal rabbit serum, and antisera AS7, I3B, OC1, and CS1 by passage over protein A-sepharose as described in chapter 2, section 9. The IgG fractions displayed the same specificity as the original antiserum as defined by immunoblotting procedures (chapter 3, Figure 3.5 and results not shown). Membranes of untreated NG108-15 cells were preincubated with these IgG fractions for 60 minutes at 37°C (concentrations corresponding to a 1: 100 dilution of the original antiserum), as detailed in section 2.13. Subsequently the ability of a receptor-saturating concentration of DADLE (1μM) to stimulate high-affinity GTPase activity was ascertained. Following treatment of the membranes with the IgG fraction from non-immune rabbits, DADLE stimulated high-affinity GTPase activity over the basal level (4.7 ± 0.3 pmol/min/mg protein, mean ± S.D., n=4) to the same degree as in the absence of the non-immune IgG fraction. IgG fractions from each of OC1 (anti-$G_o\alpha$) (4.1 ± 0.3 pmol/min/ mg protein. n=4), I3B (anti-$G_i3\alpha$) (4.6 ± 0.4 pmol/min/mg protein, n=4) and CS1 (anti-$G_s\alpha$) (4.3 ± 0.3 pmol/min/mg protein, n=4) did not affect the function of the opioid peptide. However, the IgG fraction purified from antiserum AS7 (anti-$G_i1\alpha$ and $G_i2\alpha$) completely attenuated the ability of the agonist to stimulate the high affinity GTPase activity (0.1 ± 0.1 pmol/min/mg protein, n=4) (Figure 5.18).

In contrast to receptor-stimulated GTPase activity, the IgG fraction of antiserum AS7 did not modulate the basal high-affinity GTPase activity (AS7, 10.5 ± 0.2 pmol/min/mg protein, n=4, normal rabbit serum, 9.9 ± 0.2 pmol/min/mg protein, n=4) or the non-G-protein related, low-affinity GTPase activity of these membranes. The same was true of the IgG fractions of all of the antisera employed in this study (Figure 5.18). As such, the effect of the IgG fraction of antiserum AS7 was solely to prevent receptor activation of high affinity GTPase activity.

If productive coupling between G-protein and receptor has been attenuated, then coupling of receptor to effector should also be attenuated. To address this question, NG108-15 membranes were incubated for 1 hour with either an IgG fraction isolated from normal rabbit serum, or an IgG fraction isolated from antiserum AS7. After this preincubation, the inhibition of forskolin stimulated adenylyl cyclase activity in NG108-15 cell membranes by DADLE, which was some 35% in both the presence and absence of IgG derived from normal rabbit serum, was also completely attenuated by preincubation of the membranes of NG108-15 cells with a 1:100 dilution of the IgG fraction from antiserum AS7 (Table 5.5). Indicating that the opioid receptor was no longer able to alter the activity of adenylyl cyclase.

Discussion

The identification of the stimulatory G-protein, Gs, as a protein involved in the regulation of adenylyl cyclase activity and the realization that certain hormones mediated a GTP dependent inhibition of adenylyl cyclase, led to the hypothesis that a similar protein might be involved in the inhibitory regulation of adenylyl cyclase (Rodbell, 1980). The purification of Gs, and later Gi, led to the suggestion that a wide variety of second messenger regulating effector systems might require a G-protein. Recently improved protein purification protocols, along with cDNA cloning techniques have revealed that a large family of G-proteins exists (Stryer and Bourne, 1987; Clapham and Neer, 1988; Gilman, 1987). These share extensive structural homology, indeed amino-acid homology varies from 94% identity between Gi1 and Gi3 to 68% between Gi2 and Go (Jones and Reed, 1987). On this basis it is pertinent to ask whether each of these G-proteins are able to serve distinct functions or whether they might be more appropriately viewed as isozymic forms. The situation has now been reached where the heterogeneity of G-protein α-subunits has in all probability exceeded the variety of effectors with which they may interact (Spiegel, 1987). However, this may simply be a reflection of the massive increase in G-protein research in recent years.

In view of the burgeoning numbers of G-proteins available to a cells' signal transduction apparatus, it is of prime concern to address the question, are individual G-protein species selective in their interactions with protein components of the signal transduction cascade, or are G-proteins more promiscuous, being able to interact with a wide variety of receptors and effectors. One of the earliest techniques employed to examine specificity of receptor to G-protein coupling was the reconstitution of purified G-protein and receptor into artificial phospholipid vesicles. Functional coupling was observed by the ability of agonist to stimulate either high-affinity GTPase activity (Pederson and Ross, 1982), or the binding of [^{35}S]GTPγS (Kurose et al., 1986). Such studies have suggested that Gsα is rather specific in that it interacts selectively with receptors which stimulate adenylyl cyclase activity but is barely able to interact with either the photon receptor,

rhodpsin, or the α2-adrenergic receptor (Cerione et al., 1985; Cerione et al., 1986). However it has also been demonstrated that rhodopsin can couple effectively with transducin, Gi and Go (Cerione et al., 1985; Cerione et al., 1986). Some specificity is preserved in that Gs is more efficient in coupling to the β-adrenergic receptor and transducin is relatively ineffective (Cerione et al., 1985). Surprisingly, Ross and co-workers have demonstrated that β-adrenergic receptors, which serve to stimulate adenylyl cyclase and can also stimulate certain classes of Ca^{2+} channel, interact not only with Gs (as expected), but also with Gi (Asano et al., 1984). More recently, the D_2 dopamine receptor has been purified from anterior pituitary, where it mediates inhibition of adenylyl cyclase (McDonald et al., 1984), and reconstituted into phospholipid vesicles along with purified Gi1, Gi2, Gi3 and Go (Senogles et al., 1990). The G-proteins were isolated from either; bovine brain (Gi1, Gi2, Go), anterior pituitary (Gi2) or human erythrocyte (Gi3), and their identity and apparent purity established by Western blotting with G-protein subtype specific antipeptide antibodies. The D_2 receptor was demonstrated to be able to functionally interact with each G-protein, as assessed by the stimulation of either [^{35}S]GTPγS binding or high affinity GTPase activity, with the exception of Go. However, the Go employed was functionally active, as it was able to be stimulated after reconstitution with purified rhodopsin. Gi2 was found to couple to the D_2 receptor with a ten fold higher affinity than any other Gi subtype. The source of Gi2 was not critical as brain and pituitary proteins were indistinguishable in their ability to couple to the D_2 receptor. This demonstrates that the D_2 dopamine receptor may be selective in its interaction with G-proteins, and couple preferentially with Gi2 (Senogles et al., 1990).

In addition to the reconstitution of purified G-proteins into phospholipid vesicles, it is possible to reconstitute functionally active G-proteins into purified plasma membranes, indeed the ability to reconstitute adenylyl cyclase stimulation to S49 cyc- membranes was employed in the initial purification of Gs (Northup et al., 1980). In HL 60 cells, the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe) stimulates the rate of hydrolysis of inositol containing phopholipids in a pertussis toxin sensitive manner, thus defining a role

for a pertussis toxin sensitive G-protein in mediating the response (Ohta et al., 1985). The addition of both purified Gi and Go to plasma membranes produced from cells which had been pretreated with pertussis toxin to inactivate the endogenous pertussis toxin sensitive G-proteins, was demonstrated to reconstitute receptor mediated inositol phospholipid hydrolysis. Gi and Go were found to be equally efficacious in reconstituting the response (Kikuchi et al., 1986). In rat brain membranes, the di-peptide kyotorphin (Tyrosine-Arginine) binds to specific receptors and stimulates both high-affinity GTPase activity and the activity of phosphoinositidase C, in a pertussis toxin sensitive manner (Ueda et al., 1989). It is possible to reconstitute kyotorphin mediated stimulation of both GTPase activity and phosphoinositidase C in pertussis toxin pretreated membranes by the addition of purified G-proteins. Interestingly, activity was recovered by reconstituting with Gi, but not with Go. The failure of Go to interact with the kyotorphin receptor was not as a result of the G-protein undergoing inactivation during the purification procedure, since the purified Go was demonstrated to possess GTPase activity This suggests that some specificity is maintained in the ability to reconstitute interaction with receptor (Ueda et al., 1989).

As mentioned in chapter 3, when NG108-15 cells are differentiated to a more neuronal-like cell type, they express voltage operated Ca^{2+} channels (Tsunoo et al., 1986) which are inhibited by agonist occupation of both δ-opioid and α- 2 adrenergic receptors, and stimulated by the addition of non-hydrolysable guanine nucleotides, In a pertussis toxin sensitive manner (McFadzean et al., 1989). The addition of purified G-proteins to whole NG108-15 cells using the whole cell clamp technique allows reconstitution of δ-opioid receptor mediated inhibition of the calcium current. The α-subunit of purified Go was found to be more efficacious than purified Giα in its ability to restore the response, suggesting that Go is involved in the coupling of specific receptors to neuronal voltage dependent calcium channels (Hescheler et al., 1987)

Reconstitution studies with purified proteins have therefore yielded contradictory results. In studies where the identity and purity of the G-proteins involved have been rigorously assessed, G-proteins appear to be selective in their

ability to interact with receptor. However, the majority of reconstitution studies imply that some members of the G-protein family are functionally interchangeable in their ability to interact with both receptor and effector. As detailed in chapter 1, the pertussis toxin sensitive G-proteins are highly homologous at the primary sequence level and as such, it may be expected that the domains of functional interaction between G-protein and other protein members of the signal transduction system are highly conserved between different members of the G-protein family (Masters et al., 1986). It is not unreasonable to suggest that when both G-proteins and receptors are removed from their normal enviroment and inserted into either artificial phospholipid vesicles or plasma membranes where they would not normally be expressed, that the specificity of interaction may be adversely affected. In addition, analysis of the above data is absolutely dependent on the purity of the G-protein and receptor fractions employed. The increasing variety of G-proteins which can now be identified in any given tissue has only recently been appreciated, as has the difficulty in resolving individual members of the G-protein family using current purification protocols (Yatani et al., 1988c). The majority of G-proteins used in reconstitution studies have been purified from brain, a tissue now known to express at least five different immunologically identifiable pertussis toxin substrates; Gi1, Gi2, Gi3 and at least two forms of Go (Jones and Reed, 1987; Goldsmith et al., 1988) It is therefore pertinent to suggest that some purified G-protein fractions employed for reconstitution work were in fact mixtures of a variety of highly homologous G-proteins, this would of course cast doubt on the results obtained in reconstitution systems.

In contrast to the lack of G-protein-receptor specificity observed when purified proteins are reconstituted into either phospholipid vesicles or toxin pre-treated plasma membranes, in native membrane systems, there appears to be tight regulation of G-protein coupling. Receptors which are known to interact with different effectors do so through the activation of different G-proteins, as assessed by the additivity of response to ligands which stimulate GTP hydrolysis (Houslay et al., 1986a, b; McKenzie et al., 1988a, b). The rationale for performing GTPase additivity experiments is that the activation of the entire population of receptors in

a membrane preparation with a saturating concentration of a full agonist will prospectively lead to the activation of the full complement of G-protein(s) present with which that receptor is able to interact. Thus additivity of receptor stimulated GTPase activity following the addition of two agonists which interact with independent receptors, would indicate the activation of separate pools of G-protein, and by extension, different G-proteins. This method has been utilised to examine a wide range of receptor-G-protein interactions in human platelets and has led to the conclusion that PGE1, adrenaline (in the presence of a β-adrenergic receptor antagonist) and vasopressin all stimulate GTPase activity through the activation of different species of G-protein (Houslay et al., 1986a, b).

The model system chosen to address the question of G-protein specificity was the NG108-15 cell line. Prior to undertaking an examination of the signalling pathways which may be assayed in this cell line, the G-protein complement was firstly defined. In chapter 3, it has been demonstrated, that within the limits of detectability, NG108-15 cells express two forms of the stimulatory G-protein Gs, along with three pertussis toxin sensitive G-proteins, Gi2, Gi3 and Go. Gi1 is either not expressed, or expressed in amounts not detectable with current western and northern blotting techniques. NG108-15 cells express a wide variety of receptors linked to the stimulation and inhibition of adenylyl cyclase (Klee et al., 1985), stimulation of phosphoinositidase C (Higashida et al., 1986) as well as inhibition of voltage operated Ca2+ channels (McFadzean et al., 1989). Therefore the G-protein which mediates the inhibition of adenylyl cyclase must be either Gi2, Gi3, Go, or a combination therof. To unambiguously identify the G-protein which mediates the inhibition of adenylyl cyclase, it is necessary to either block or delete the normal pathway. It is possible to block the normal pathway of adenylyl cyclase inhibition using pertussis toxin, however since the toxin will modify Gi2, Gi3 and Go in NG108-15 cells, it is not possible to identify which of these pertussis toxin sensitive G-proteins is required for receptor mediated inhibition of adenylyl cyclase.

The studies described in this chapter have attempted to block the regulation of adenylyl cyclase by the δ opioid receptor of neuroblastoma x glioma hybrid, NG108-15, cells with antisera which are directed against, and can specifically recognise the extreme C-terminus of the α subunit of the various G-proteins. This C-terminal region has been focussed upon since it appears to be the domain of functional interaction with receptor (as detailed in chapter 1). It was therefore hoped that it would prove possible to attenuate productive coupling between G-protein and receptor by placing an antibody on the extreme C-terminal region of the G-protein α-subunit.

All G-proteins are GTP hydrolysing enzymes (Gilman, 1987). As such, high-affinity GTPase activity in a membrane preparation is usually simple to assess, particularly when pertussis toxin sensitive G-proteins are known to be expressed. The ligands DADLE, adrenaline, bradykinin and FCS, are able to functionally interact with a δ-opioid , α2 adrenergic, bradykinin and a poorly characterised growth factor receptor respectively in this cell line. Each ligand is capable of stimulating high-affinity GTPase activity in NG108-15 cell membranes in a pertussis toxin sensitive manner, thus allowing an assessment of specificity to be made. It may be noted that in this cell line, pretreatment with pertussis toxin, as well as preventing receptor stimulation of GTPase activity by DADLE, adrenaline, bradykinin and FCS, also reduces the basal hydrolysis of GTP by high affinity GTPases (Figure 5.11 a, b). By contrast, non-specific hydrolysis of GTP was unaffected (results not shown). The effect of ADP-ribosylation of Gi by pertussis toxin is believed solely to prevent coupling between receptor and G-protein, indeed in the presence of the non-hydrolysable analogue of GTP, Gpp(NH)p it is still possible for Giα to dissociate from the β,γ subunits (Katada et al., 1986) and inhibit adenylyl cyclase (Gosse et al., 1989). After pertussis toxin pretreatment of NG108-15 cells, there is no reduction in the levels of either Gi (Law et al., 1985b, and results not shown), or in the levels of either the α-2 adrenergic, δ-opioid (Kurose et al., 1982) or bradykinin (Osugi et al., 1987) receptors expressed by NG108-15 cells. As such it is difficult to explain the reduction in basal GTPase activity seen after pertussis toxin pretreatment. In addition to the effect of pertussis toxin on the basal hydrolysis of GTP, pretreatment of NG108-15 cells with the toxin

was noted to produce an increase in both basal and forskolin stimulated adenylyl cyclase activity, when compared to control membranes. A possible explanation is that the unoccupied receptor is capable of G-protein activation, which would result in both a stimulation of GTPase activity and a concurrent inhibition of adenylyl cyclase. According to the original ternary complex model of interaction between agonist, receptor and G-protein, agonists but not antagonists promote the association of receptor to G-protein in membrane systems. The resulting complex can be de-stabilised by the addition of guanine nucleotides such as GTPγS and Gpp(NH)p (DeLean et al., 1980). The intrinsic activity of a ligand is therefore seen to represent its ability to stabilise the ternary complex of ligand, receptor and G-protein, and will range from null values for antagonists to positive values for partial and full agonists (DeLean et al., 1980). However binding studies on muscarinic (Burgisser et al., 1982), D2-dopaminergic (DeLean et al., 1982), and A1-Adenosine receptors (Green, 1984), reveal a more elaborate effect of guanine nucleotides, where agonist and antagonist binding to these receptors may be modulated in a reciprocal manner. To include these observations, the ternary complex model was altered to allow for the ability of an antagonist to be able to promote dissociation of the ternary complex (Wregget and DeLean, 1984). When purified G-proteins are reconstituted into phospholipid vesicles in the absence of receptor or effector, it is possible to measure high-affinity GTPase activity. Upon addition of purified receptor, an increase in the ability of the G-protein to hydrolysed GTP is observed, suggesting that an unoccupied receptor is capable of G-protein activation. The spontaneous association of empty receptor with G-protein may account for both the basal high-affinity GTPase activity and for the tonic inhibition of adenylyl cyclase activity which is observable in membrane systems. Receptor purification data support the argument for an interaction of unoccupied receptor with G-protein, since both D-2 dopamine and α-2 adrenergic receptors co-purify with a tightly bound G-protein (Senogles et al., 1987; Regan et al., 1986).

In further reconstitution experiments, it has been demonstrated that the inclusion of antagonist can inhibit receptor mediated G-protein activation in the absence of agonist (Cerione et al., 1984). Recently, it has been demonstrated in membranes produced from NG108-15 cells, that there are two types of antagonist for the δ-opioid receptor, antagonists with null activity, and antagonists which are capable of preventing interaction of receptor with G-protein. The latter was assessed by the ability of the δ-opioid antagonist ICI 174864 to inhibit basal high-affinity GTPase activity in NG108-15 cell membranes (Costa and Herz, 1989). It is thus possible to speculate that both the reduction in basal high-affinity GTPase activity and the increase in both basal and forskolin stimulated adenylyl cyclase activity produced by pretreatment with pertussis toxin is a consequence of Gi's inability to interact with unoccupied δ-opioid receptor after being modified by ADP-ribosylation.

As mentioned earlier, GTPase additivity experiments provide a means of determining whether different classes of receptor interact with different species of G-protein. When additivity experiments are carried out using saturating concentrations of each of DADLE, adrenaline and bradykinin, then some degree of additivity is obtained. The maximal stimulation of GTP hydrolysis is achieved by DADLE on its own. The presence of all three ligands does not give a further increase. However, adrenaline and bradykinin, which separately are poor stimulators of GTPase activity (Figure 5.3), when used in combination, give an additive response. This data may be interpreted in several ways; firstly, adrenaline and bradykinin although interacting with the same G-protein, are incapable of activating the entire pool of G-protein with which the receptors for each ligand interacts, secondly, that two receptors exist for DADLE, with each receptor interacting with a different pool of G-proteins or lastly, the δ-opioid receptor is capable of interacting with two separate pools of G-protein.

When additivity experiments are conducted with DADLE and FCS, a different picture emerges, the two ligands are perfectly additive, suggesting that the receptor for each of these ligands interacts with a different pool of G-proteins and by extension, different G-protein species. When the ability of each of DADLE,

adrenaline, bradykinin and FCS to inhibit forskolin or PGE1 stimulated adenylyl cyclase activity in NG108-15 cell membranes is determined, of the three ligands, only FCS is incapable of mediating inhibition, thus adding more weight to the hypothesis that DADLE and FCS do not function through activation of the same G-protein species. DADLE, adrenaline and bradykinin all inhibit adenylyl cyclase. activity in a pertussis toxin sensitive manner. It may be noted that each ligand is capable of acheiving the maximal inhibition of adenylyl cyclase when used on its own, when the three ligands are used in combination further inhibition is not observed.

An important aspect to be addressed is that of receptor heterogeneity. NG108-15 cells are thought to express only a single population of receptors for opiates, of the δ-classification. This rationale allowed ease of purification of the δ-opioid receptor from this cell line (Simonds et al., 1985). Fewer pharmacological studies have foccussed on the α2 adrenergic receptors expressed by NG108-15, however it is apparent that only a single population of α2 adrenergic receptors is expressed of the α2-B classification (Bylund, 1988). The nature of the bradykinin receptor(s) expressed by NG108-15 cells is poorly defined. If we examine the intracellular effector systems modulated by the binding of DADLE, adrenaline and bradykinin to their receptors on NG108-15 cells, we see that in addition to the ability to inhibit adenylyl cyclase, bradykinin, in contrast to both DADLE and adrenaline, is also able to stimulate an increase in the rate of hydrolysis of inositol containing phospholipids (Higashida et al., 1986; F.M. Mitchell, unpublished). Bradykinin's ability to stimulate phospholipase C is pertussis toxin insensitive (Osugi et al., 1987), which is in marked contrast to the ligands ability to inhibit adenylyl cyclase. This suggests that either NG108-15 cells express more than one type of bradykinin receptor, or NG108-15 cells express a single class of bradykinin receptor which is capable of interacting with two distinct G-proteins. One of the G-protein species is pertussis toxin sensitve and one is not. The B_1 receptor agonist desArg-bradykinin is incapable of stimulating either GTPase activity in NG108-15 cell membranes (results not shown), or of stimulating an increase in the rate of hydrolysis of inositol containing phospholipids in NG108-15 cells (R.J.Plevin, personnal communication).If NG108-15 cells express two different bradykinin

receptors, then there is a rationale to suggest that each type of receptor is capable of interacting with different effector systems and would do so through the activation of different species of G-protein. It would appear that if heterogeneity exists, then NG108-15 cells express more than one type of B_2 receptor.

Since DADLE, adrenaline and bradykinin are capable of full inhibition of adenylyl cyclase, it is of interest to note that the three ligands do not produce the same stimulation of GTPase activity. It may be suggested that full G-protein activation is not required for each receptor to maximally inhibit adenylyl cyclase, this would be apparent when dose-reponse curves of adenylyl cyclase inhibition were compared to the extent of receptor occupancy. In hepatocytes, the ability of vasopressin to stimulate the hydrolysis of inositol containing phospholipids occurs over a concentration range of 5nM to 1μM. The EC 50 value for the response is approximately 50nM. However when the physiological response, the activation of phosphorylase, is examined, the EC 50 value for phosphorylase activation by vasopressin is at least one log order lower than that required for phosphoinositidase C activation, suggesting that full receptor and hence full G-protein activation may not be required in order to obtain the maximal effect on the intracellular effector system under study.(Creba et al., 1983). When dose response curves for inhibition of adenylyl cyclase are analysed, the half maximally effective concentration of DADLE in inhibiting adenylyl cyclase (I.C. 50) (5nM) is very similar to the half maximally effective concentration of DADLE in stimulating GTPase activity (5nM). In addition, the concentration of [^{3}H]DADLE which is required to occupy 50% of the available δ-opioid receptors on NG108-15 cell membranes is approximately 2nM (see chapter 6, Figure 6.3). These data argue that receptor occupancy of half the available receptors for DADLE leads to activation of 50% of the G-proteins which may interact with the receptor and hence gives 50% of the maximal inhibition of adenylyl cyclase obtainable through maximal agonist occupancy of the δ-opioid receptor. Thus full agonist occupancy of the δ-opioid receptor is required to produce both the maximal stimulation of GTPase activity and the maximal inhibition of adenylyl cyclase. Unfortunately, the stimulation of GTPase activity produced by adrenaline and bradykinin in NG108-15 cell membranes is so low that useful dose-response data of

GTPase stimulation could not be obtained. Hence, although it may be suggested that maximal stimulation of GTPase activity by the receptors for both adrenaline and bradykinin is required for maximal inhibition of adenylyl cyclase, the inability of both bradykinin and adrenaline to produce a stimulation of GTPase activity which is comparable to that produced by DADLE means that this remains an open question. When concentrations of DADLE, adrenaline and bradykinin which produce 50% of the maximal inhibition of adenylyl cyclase which may be elicited by each agonist are used in combination, the inhibition of adenylyl cyclase activity obtained is greater than that obtained when each ligand is used singly. The presence of all three ligands at half-maximally effective inhibitory concentrations gives an even greater inhibition of adenylyl cyclase, comparable with the inhibition produced by a saturating concentration of any of the three ligands. This data may be explainable by each ligand activating a different species of G-protein, however this is not borne out by the lack of GTPase additivity seen with DADLE and adrenaline and with DADLE and bradykinin (Fig 5.3). A simpler explanation for additivity of adenylyl cyclase inhibition by half-maximally effective concentrations of DADLE, adrenaline and bradykinin is that the receptors for each ligand are activating the same G-protein species, so that by mass action, the greater the number of agonist occupied receptors, the greater the G-protein activation and hence the greater the inhibition of adenylyl cyclase activity obtained.

The use of synthetic peptides to identify domains of functional interaction between the G-protein α-subunit and other protein components of the signal transduction process has been employed by several groups (Takemoto et al., 1985; Hamm et al., 1988; Palm et al., 1990). Peptides which correspond to the C-terminal region of rhodopsin have been demonstrated to inhibit rhodopsin stimulated GTPase activity, whilst peptides corresponding to external loop regions were without effect. This suggested that a region of rhodopsin close to the C-terminus was required for coupling to transducin, a result which is not in total agreement with the current opinion that the site of interaction between receptor and G-protein is the third intracellular loop (see chapter 1) (Takemoto et al., 1985). A similar set of experiments have demonstrated that synthetic peptides corresponding to two regions of the α-subunit of transducin close to the C-terminus were capable of competing

with transducin for rhodopsin, as assessed by the the ability of transducin to stabilise the active metarhodopsin II conformation of rhodopsin (Hamm et al., 1988). Synthetic peptides homologous to the last sixteen C-terminal amino-acids of Gsα, Gi1α, Goα and Tdα have been employed in turkey erythrocyte membranes in an attempt to prevent receptor mediated stimulation of adenylyl cyclase activity. Preincubation of the turkey erythrocyte membranes for 60 minutes at 4°C with the peptide homologous to the C-terminus of Gsα, had little effect on AlF_4^- stimulated adenylyl cyclase activity, but abolished isoproterenol mediated stimulation of adenylyl cyclase. In contrast, preincubation with the peptides homologous to the C-terminus of Gi1α, Goα and Tdα were without effect (Palm et al., 1990).

The main thrust of these data is to demonstrate that it is possible to examine G-protein functional domains using synthetic peptides which correspond to suitable regions on the G-protein α-subunit.

The extreme C-terminal region of the G-protein α-subunit is the site of functional interaction with receptor (chapter 1). As such it was felt that anything which may disrupt or compete with the G-protein for the receptor at the site of interaction, might prevent the functional interaction of the G-protein with the receptor with which it interacts. The last ten amino acids of Tdα have the sequence KENLKDCGLF, which is one amino-acid different to the last ten amino acids of Gi2α, which has the sequence KNNLKDCGLF. Preincubation of NG108-15 cell membranes with a synthetic peptide which corresponds to the C-terminal decapeptide of transducin reduced DADLE stimulation of GTPase activity in a dose dependent manner. In contrast, preincubation with a synthetic peptide homologous to the last ten C-terminal amino-acids of Goα was without effect. An interesting hypothesisis for this observation is that the δ-opioid receptor functions through activation of Gi2, and that the addition of a synthetic peptide corresponding to the receptor interaction domain of Gi2α, results in competition between the peptide and the G-protein for the δ-opioid receptor, hence the resultant reduction in GTPase stimulation produced by DADLE after preincubation with the Gi2α peptide. This approach, whilst suggesting the possible interaction of the δ-opioid receptor with Gi2, and not with Go, proved to be limiting in that excessively high concentrations of peptide were required to

produce an attenuation of approximately 50% of receptor stimulated GTPase activity (Figure 5.13 a,b).

An antibody which can specifically identify and bind to the C-terminus of a G-protein α-subunit might be regarded as a potentially more specific tool in preventing coupling between receptor and G-protein. As detailed in chapter 3, antiserum AS7, which was raised to a synthetic decapeptide corresponding to the C-terminal region of Tdα, specifically identifies Gi2α in NG108-15 cell membranes. Antibodies affinity purified from antiserum AS7 retain specificity for Gi2α (chapter 3, Figure 3.4). Preincubation of NG108-15 cell membranes with a 1:100 dilution of antibodies affinity purified from AS7 completely abolished the ability of DADLE to stimulate high-affinity GTPase activity, whilst having little effect on the ability of FCS to stimulate high-affinity GTPase activity (Table 5.5). Normal rabbit serum did not mimic the effect of the affinity purified antibodies, whose effect could be titrated out. A 1:10,000 dilution of affinity purified AS7 in normal rabbit serum was found to be without effect. This data argues strongly that DADLE and FCS bind to receptors which function through activation of different G-protein species, but more importantly, that Gi2 is the G-protein reponsible for mediating the inhibition of adenylyl cyclase in NG108-15 cells.

As a more rigorous immunological definition of the G-protein with which the δ-opioid receptor interacts to inhibit adenylyl cyclase, a series of IgG fractions were produced from each of normal rabbit serum, antiserum AS7, OC1, CS1 and I3B (chapter 2, section 2.9). These antisera were raised against synthetic peptides corresponding to the C-terminal region of the G-protein α-subunit and can be used as specific tools to identify Gi2, Go, Gs and Gi3 respectively (chapter 3). Both opioid-inhibition of adenylate cyclase and opioid-stimulation of high affinity GTPase activity was completely abolished in membranes of these cells by pretreatment with an IgG fraction from antiserum AS7. IgG fractions from each of normal rabbit serum, the anti-G_i3, anti-G_o and anti-G_s C-terminal antisera had no effect. These data argue strongly that G_i2 must be the true "G_i" of the adenylate cyclase cascade, at least in NG108-15 cells. It is not possible to rule out the possibility that G_i1 might function

in a similar manner in other tissues in which it is expressed. It should be noted, however, that the expression of G_i2 (and G_i3) appears to be universal in mammalian tissues, whilst G_i1 is limited in distribution (Milligan, 1989) and as such does not mirror the distribution of adenylyl cyclase which is also essentially universal. *In situ* hybridisation experiments in rat brain slices have shown that distributions of mRNA for both Gs and Gi2 are very similar, whilst the distributions of mRNA for Go and Gi1 are different (Brann et al., 1987). Since adenylyl cyclase is under the dual control of both stimulatory and inhibitory hormones, it has been suggested that on the basis of the distribution pattern of Gs and Gi2, the inhibition of adenylyl cyclase is mediated by activation of Gi2 (Brann et al., 1987). However a similar correlation has observed in the distribution of both Go (as assessed immunochemically) and protein kinase C (assessed by the binding of phorbol ester), suggesting that Go might represent the G-protein linked to receptor mediated stimulation of phosphoinositidase C (Worley et al., 1986). This is probably incorrect, since Go is a pertussis toxin sensitive G-protein and the stimulation of phosphoinositidase C in brain is generally not attenuated by pertussis toxin treatment (Ueda et al., 1989). An additional caveat is that there may be little or no correlation between the relative abundance of a G-protein mRNA and the corresponding polypeptide (Silbert et al., 1990).

The G-protein antisera described above were employed under the assumption that the binding of antibody to the site of receptor interaction on the G-protein would prevent interaction of G-protein with receptor, however it is not possible to conclude that the C-terminus of Gi2 is indeed the domain responsible for receptor recognition. The effect of antibodies derived from antisera AS7 may be due to the cross linking of antibody to G-protein, which might be expected to produce sufficient steric hindrance to prevent interaction of G-protein with effector. This question could be resolved by the use of antigen binding fragments derived from the IgG fractions described, however this was not performed.

For each anti-G-protein antiserum described, it is not possible to assess the proportion of IgG which can specifically recognise the G-protein α-subunit, thus it is not possible to determine the stoichiometry of antibody to G-protein which results

in complete attenuation of coupling to receptor. However, other groups have performed a range of other experiments using similar approaches to those described in this chapter which may allow stoichiometric measurements to be made. As detailed in chapter1, the detection of light by the photon receptor rhodopsin promotes the activation of transducin which then stimulates cyclic GMP phosphodiesterases (cGMP PDE). The functional reconstitution of purified transducin, rhodopsin and cGMP PDE into phospholipid vesicles by a number of research groups has allowed the direct analysis of interactions between transducin and rhodopsin (Fung, 1985). Using this system, it has recently been shown that preincubation of purified transducin α-subunit with antibodies affinity purified from antiserum AS7 before incorporation into phospholipid vesicles, leads to a diminished ability of rhodopsin to stimulate GTPase activity, wheras preincubation with non-specific rabbit IgG had no effect (Cerione et al., 1988). Maximal inhibition of the rhodopsin stimulated GTPase activity occurred at a molar ratio of transducin α-subunit to AS7 of 1:1. Since the presence of transducin β,γ subunits during the preincubation did not alter the effect of AS7, it was concluded that the binding of AS7 to transducin did not alter the ability of the α-subunit of transducin to interact with β,γ. Further, the intrinsic basal GTPase activity of transducin α was not reduced by preincubation with AS7. An additional point obtained from the above experiments was that preincubation of antiserum AS7 with the α-subunit of transducin which had been pre-activated by the addition of the non-hydrolysable GTP analogue, GTPγS, enhanced the ability of transducin to interact with the cGMP PDE.

Whilst the interaction of G-proteins with receptors in reconstitution systems is more amenable to analysis, a range of similar experiments have been performed in plasma membrane systems using a monoclonal antibody, termed 4A, raised against the α-subunit of frog transducin (Hamm et al., 1987), which have been shown to recognise a region close to the C-terminus. When rod outer segment membranes are preincubated with monclonal antibody 4A, then light activation of transducin by rhodopsin is blocked (Hamm et al., 1987; Deretic and Hamm, 1987). As the antibody which produced inhibition was able to immunoprecipitate the holomeric transducin α,β,γ complex, it is unlikely that the effect was produced by

preventing the interaction of transducin α with the β,γ subunit. In addition, monoclonal antibody 4A has been shown to prevent muscarinic receptor activation of atrial potassium channels, a process which has ascribed to either the α-subunit of Gi3, or β,γ subunits, depending on ones point of view (Yatani et al., 1987a,b). In platelet membranes, which have been demonstrated to express Gs, Gi2, Gi3 and Gz(x), it is possible to attenuate α-2 adrenergic receptor mediated inhibition of adenylyl cyclase, by preincubation with antibodies affinity purified from antiserum AS7, which specifically recognises the extreme C-terminal region of Gi2 (see earlier). Antibodies affinity purified from antisera which specifically recognised Gi3 or Gz were without effect. (Simonds et al.,1989a). The ability of a Gi2 specific antibody to prevent productive coupling between inhibitory receptor and adenylyl cyclase has therefore been demonstrated in more than one signal transduction model system.

In addition to the ability to block receptor interaction with 'Gi"-like G-proteins, Simonds and colleagues have demonstrated that antibodies which were raised against the C-terminal decapeptide of Gsα, and specifically recognise Gsα, are able to block receptor-mediated activation of Gs and adenylyl cyclase in membranes produced from wild type S49 cells. The antibodies could also immunoprecipitate adenylyl cyclase activity from GTPγS or AlF_4^- activated bovine brain membranes, demonstrating that an activated conformation of Gsα was able to bind both antibody and effector (Simonds et al.,1989b).

The conclusions to be made from an analysis of the experiments described above, are that the extreme C-terminal region of the G-protein α-subunit is indeed the site of interaction with receptor, and that it is possible in membrane systems, to identify which G-proteins function through activation of defined receptors to interact with identifiable signalling pathways. As such, it is expected that similar protocols will allow the dissection of signal transduction pathways other than those linked to the stimulation and inhibition of adenylyl cyclase, and the opening of atrial potassium channels. Considerable research has been aimed at identifying the G-protein which is required for receptor-mediated stimulation of phosphoinositidase

C, there have been several major obstacles, most notably in the heterogeneity of G-protein(s) which mediate this response (pertussis toxin sensitive and insensitive signalling pathways), and in the difficulty in producing a cell free system to study inositol lipid breakdown. The development of a cell free system which allows measurement of the hydrolysis of inositol containing phospholipids, as has been described for membranes produced from HL 60 cells (Kikuchi et al., 1986), will facilitate the use of specific antibodies to uncouple G-proteins from receptors and will undoubtedly provide a powerful tool in the analysis of G-protein signal transduction.

Chapter 6

Uncoupling of the δ-opioid receptor in NG108-15 membranes from the G-protein with which it interacts, by antibodies which identify the C-terminus of the α-subunit of Gi2.

Introduction

One of the initial observations which allowed the identification of G-proteins as being involved in receptor mediated transmembrane signalling events, was the demonstration that guanine nucleotides such as GTP reduced the binding of glucagon to the plasma membrane of rat liver preparations (Rodbell et al.,1971b). The effects of GTP on the binding of ligand to receptor were later shown to be specific for agonists, since in rat C6G1A glioma cell membranes, the binding of β-adrenergic agonists, but not antagonists was reduced by the presence of the GTP analogue, Gpp(NH)p (Maguire et al., 1976). Further studies on β-adrenergic receptors suggested the existence of two affinity states for agonist, but only one affinity state for antagonist, since competition curves for antagonist *versus* radiolabelled antagonist are steep with pseudo Hill coefficients close to 1 and competition curves for agonist *versus* radiolabelled antagonist are shallower with pseudo Hill coefficients less than 1 (De Lean et al., 1980). The affinity state of a receptor for agonist has since been demonstrated to be dependent on the identity of the guanine nucleotide bound to the G-protein which interacts with the receptor under study. In the presence of non-hydrolysable analogues of GTP, such as Gpp(NH)p or GTPγS, a low affinity state of receptor for agonist is adopted, whereas in the absence of guanine nucleotides the receptor demonstrates a higher affinity for agonist (Wregget and DeLean., 1984).

As detailed in chapter 1, the C-terminus of the G-protein α-subunit has been identified as the functional domain of interaction with receptor. If NG108-15 cells are pretreated with pertussis toxin, then as well as an attenuation of receptor mediated inhibition of adenylyl cyclase, there is concomittant loss of guanine nucleotide sensitivity of agonist binding (Kurose et al., 1982). This may be regarded as an axiom applicable to all receptors which function through the activation of pertussis toxin sensitive G-proteins (Milligan, 1988). Thus, if the G-protein is prevented from interacting with its receptor, either by modifying the C-terminal region of the α-subunit, or by promoting the dissociation of the α-subunit from the β,γ subunits, a form of receptor with a lowered affinity for agonist is produced.

In chapter five, the effect of pre-incubating membranes from NG108-15 cells with antiserum AS7, an antiserum which specifically identifies the C-terminus of Gi2 in this cell line, was described. The preincubation had the same functional effect as pretreatment with pertussis toxin, in that receptor mediated inhibition of adenylyl cyclase via the δ-opioid receptor was abolished. This led to the speculation that the antibody uncoupling of the δ-opioid receptor from Gi2, may also be identified as it would result in an altered afffinity state of the receptor for agonist. To examine this hypothesis directly, a series of binding assays on membranes produced from NG108-15 cells have been performed with the aim of assessing the effect of the range of G-protein specific antisera described in chapter 2, on the affinity state of the δ-opioid receptor in this cell line.

Fig. 6.1 Timecourse of binding of [^{3}H]DADLE to membranes of NG108-15 cells.

Membranes of NG108-15 cells (100μg) were incubated with a fixed concentration of [^{3}H]DADLE (10 nM), as described in section 2.14. At times varying between 0 and 30 minutes, incubations were terminated by removal to ice, followed by rapid filtration through Whatman GF/C filters, and three 5ml washes with buffer, as detailed in section 2.14. A further series of incubations were performed in the presence of 10μM DADLE to define non-specific binding. Data shown are means ± S.D. from eight replicates taken from two separate experiments.

Figure 6.1

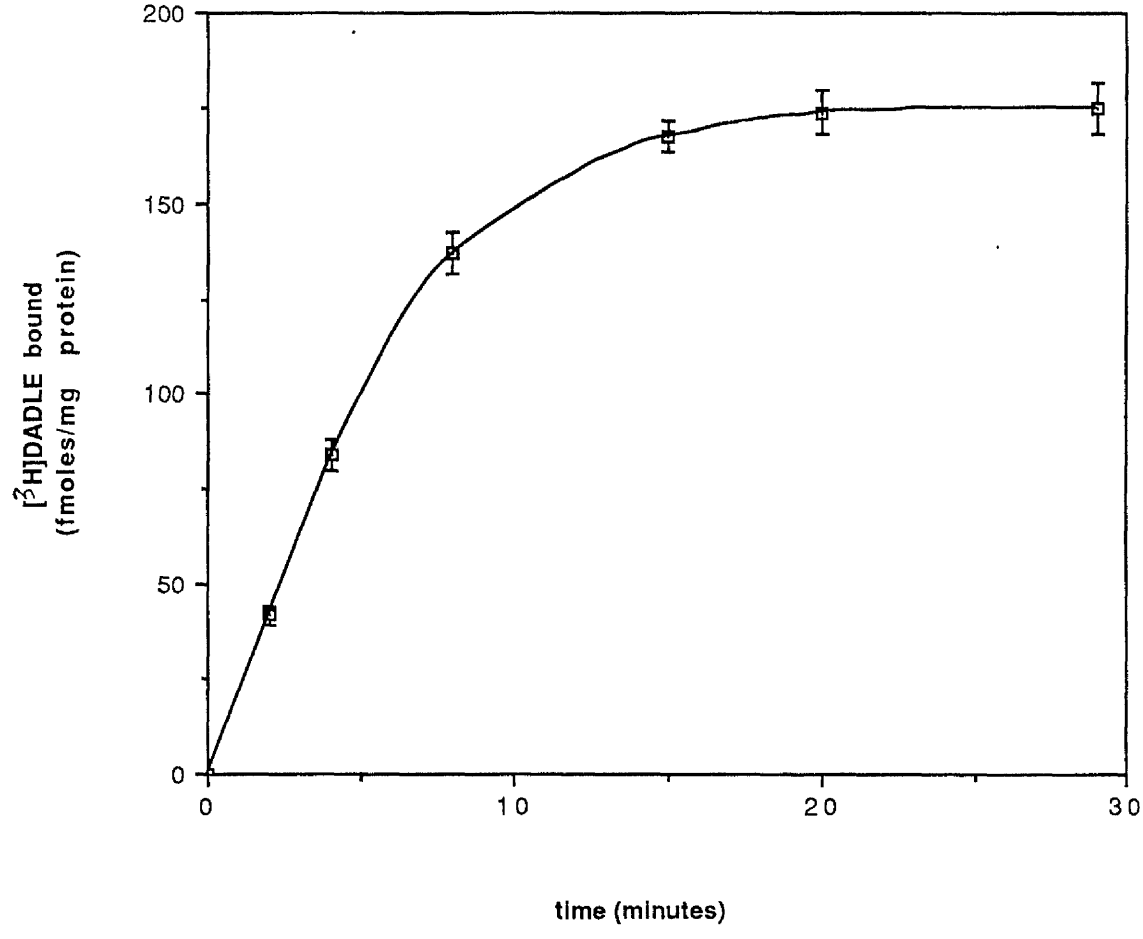

200
150
100
50
0
0
10
20
30
[3H]DADLE bound
(fmoles/mg protein)
time (minutes)

Fig. 6.2 The binding of [^{3}H] DADLE to NG108-15 cell membranes in response to increasing protein concentration.

NG108-15 cell membranes (0 - 300μg) were incubated with a fixed concentration of [^{3}H]DADLE (10 nM). After 30 minutes, incubations were terminated by removal to ice, followed by rapid filtration through Whatman GF/C filters and three 5ml washes with buffer, as detailed in section 2.14. A parallel series of incubations were performed in the presence of 10μM DADLE for each membrane concentration used, to define non-specific binding. Data shown are means ± S.D. from eight replicates taken from two separate experiments.

Figure 6.2

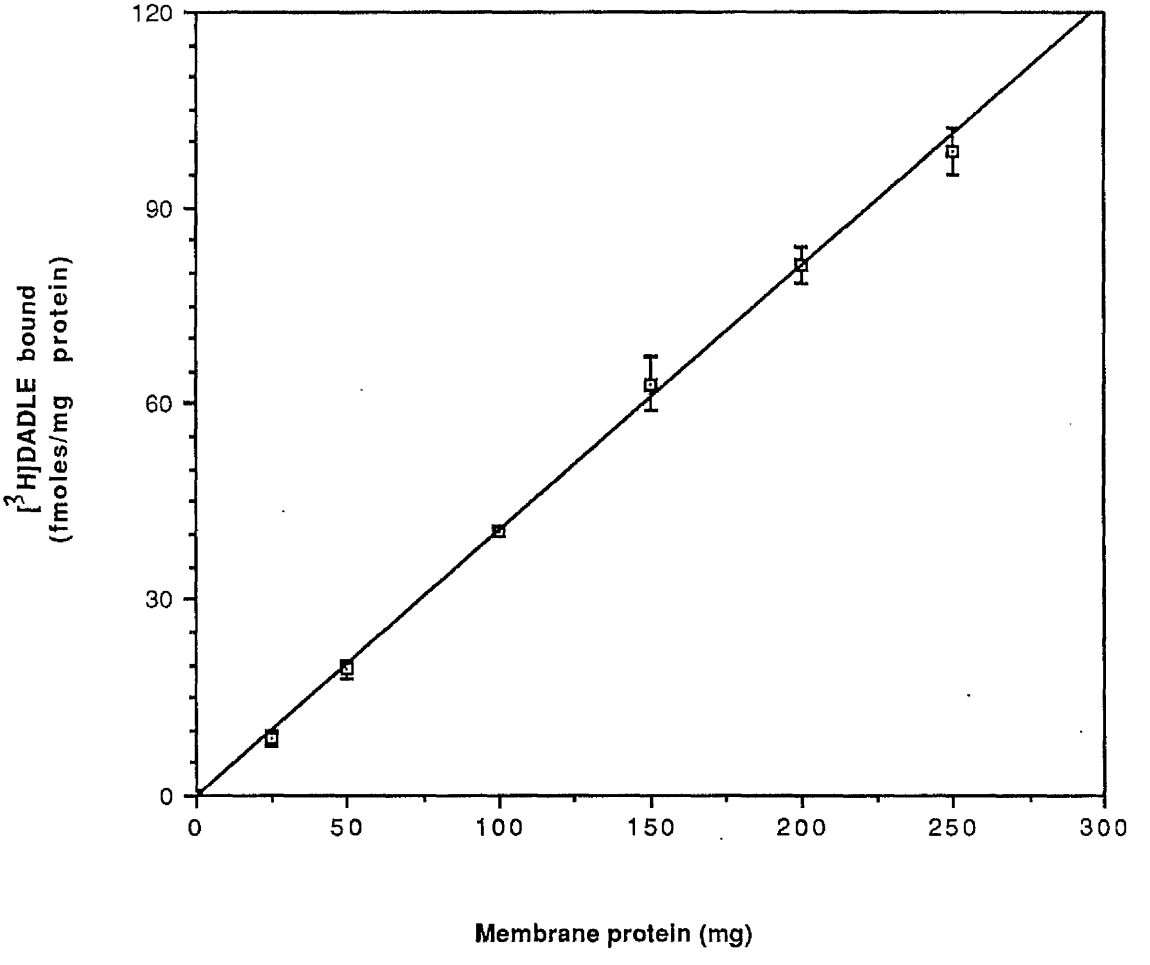

120
90
60
30
0
0
50
100
150
200
250
300
[3H]DADLE bound
(fmoles/mg protein)
Membrane protein (mg)

Fig. 6.3 Saturation binding analysis of [^{3}H] DADLE to membranes of NG108-15 cells.

Membranes of NG108-15 cells (100µg) were incubated with varying concentrations of [^{3}H]DADLE (0.5-20 nM), as described in section 2.14. A further series of incubations were performed in the presence of 10µM DADLE to define non-specific binding.(Figure 6.3 a). Specific binding data was treated as described by Scatchard (1949). and replotted (Figure 6.3 b). Scatchard analysis of the data presented in Figure 6.3 b indicated the presence of some 390 fmoles receptor/mg membrane protein, with an apparent dissociation constant (Kd) for the ligand of 2.2 ± 0.2nM . Data are means ± S.D. taken from quadruplicate determinations in a single experiment which was performed four times and gave essentially identical results.

Figure 6.3(a)

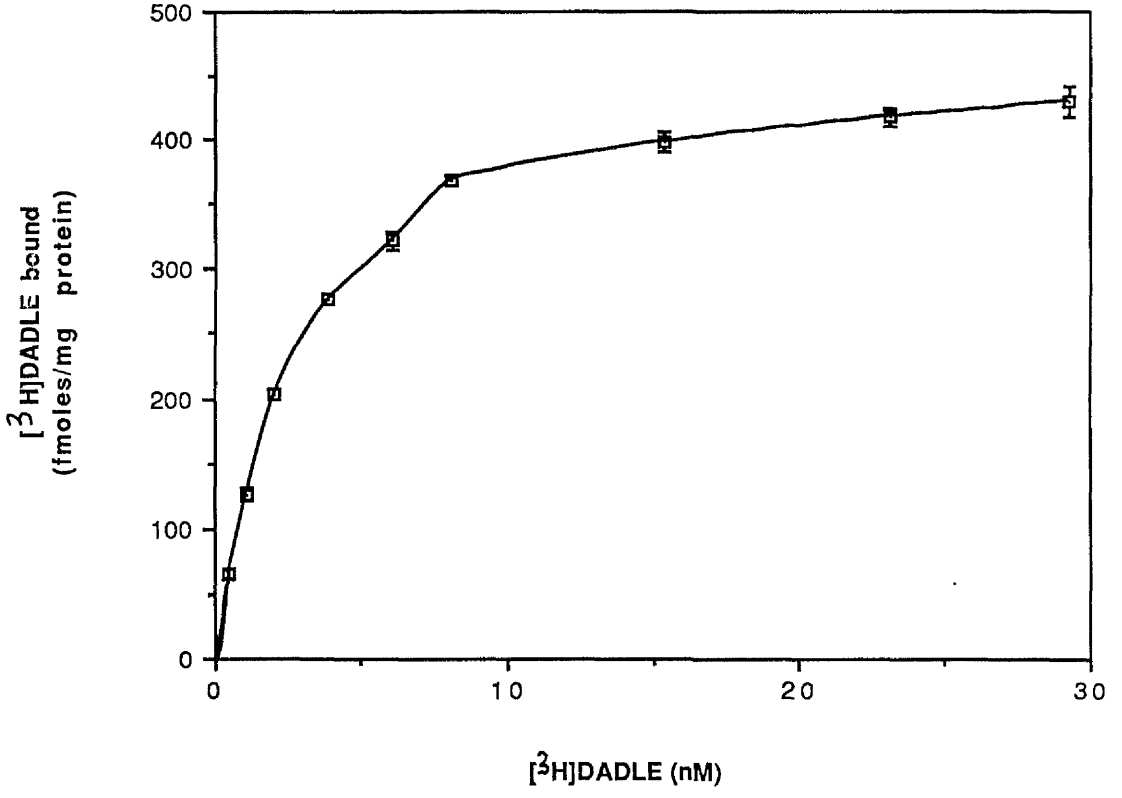


Figure 6.3(b)

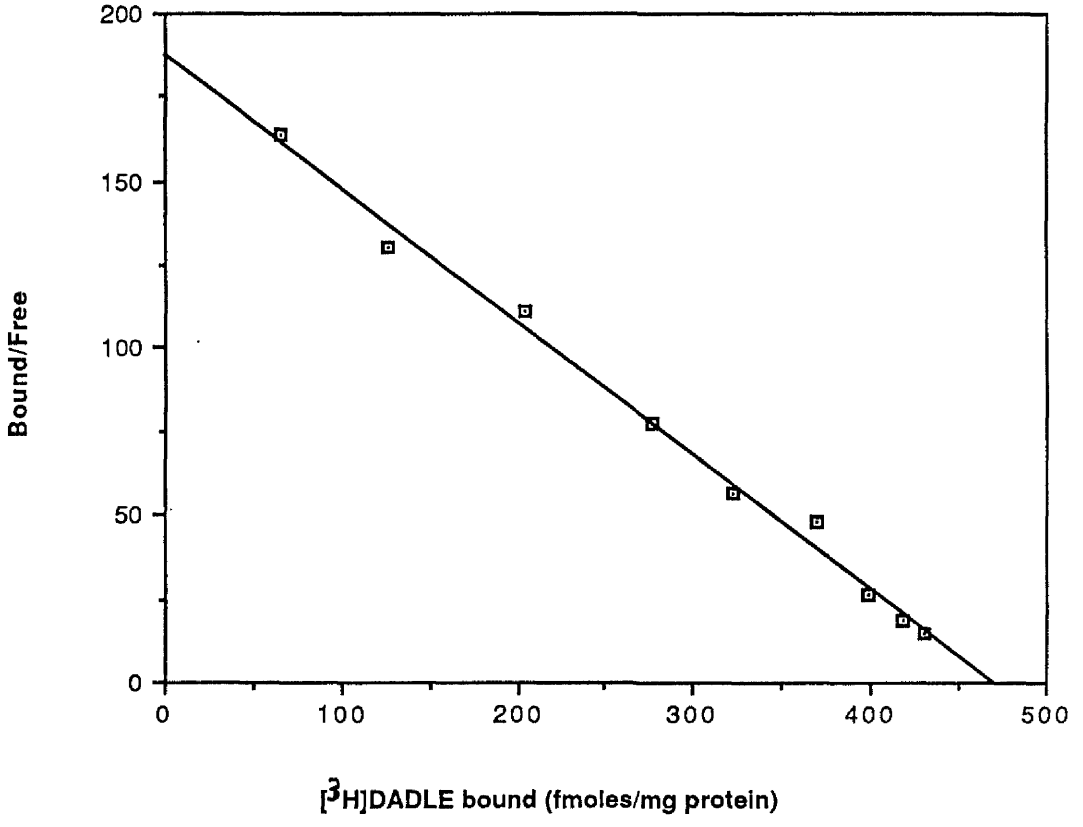


Fig. 6.4 The binding of [^{3}H]DADLE to membranes of NG108-15 cells: the effect of Gpp[NH]p.

Membranes of NG108-15 cells (100μg) were incubated with varying concentrations of [^{3}H]DADLE (0.5-20 nM), as described in section 2.14, in the absence (open circles) or presence (closed circles) of Gpp[NH]p (100 μM). A further series of incubations were performed in the presence of 10μM DADLE to define non-specific binding. Specific binding data was treated as described by Scatchard (1949). In the experiment shown, binding parameters were; Bmax 309 ± 7 fmol/mg protein, Kd 3.2 nM, in the absence of Gpp[NH]p and Bmax 283 ± 15 fmol/mg protein, Kd 6.4 nM in the presence of Gpp[NH]p. Data are means ± S.D. taken from a single experiment which gave essentially identical results to three others performed.

Figure 6.4

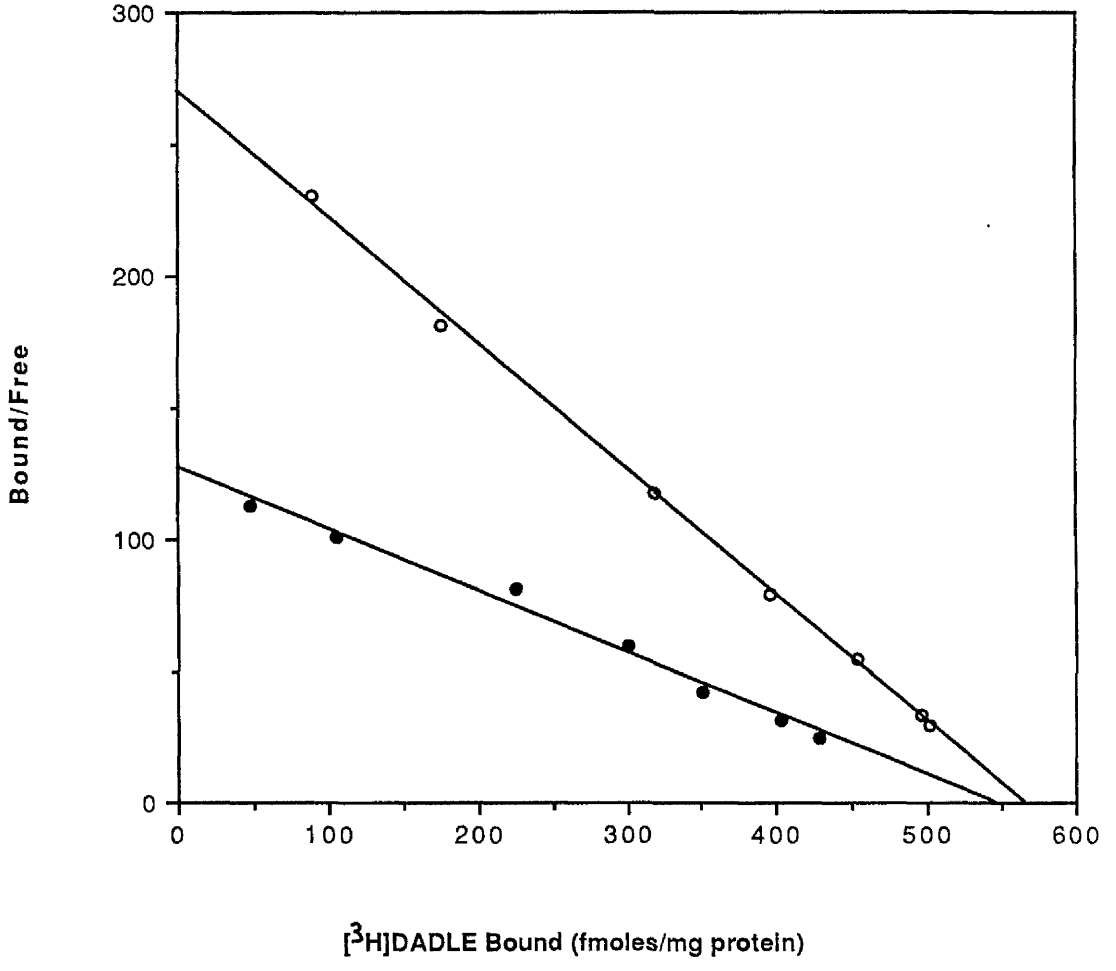

300
200
100
0
Bound/Free
0
100
200
300
400
500
600
[3H]DADLE Bound (fmoles/mg protein)

Fig. 6.5 **The binding of [^{3}H]DADLE to membranes of untreated and pertussis toxin pretreated NG108-15 cells.**

NG108-15 cell membranes (100μg) from either untreated cells (open circles) or from cells which had been pretreated *in vivo* with pertussis toxin (closed circles) as detailed in section 2.6d, were incubated with varying concentrations of [^{3}H]DADLE, in the absence of Gpp[NH]p, as described in the legend to Figure 6.4. Non-specific binding was defined as in Figure 6.1. Binding parameters in the experiment displayed were: membranes from untreated cells, Bmax. 331 ± 6 fmol/mg protein, Kd 1.8 nM, membranes from pertussis toxin-pretreated cells, Bmax. 293 ± 16 fmol/mg protein, Kd 4.2 nM. Data are means ± S.D. from pooled determinations from three separate experiments (n=12).

Figure 6.5

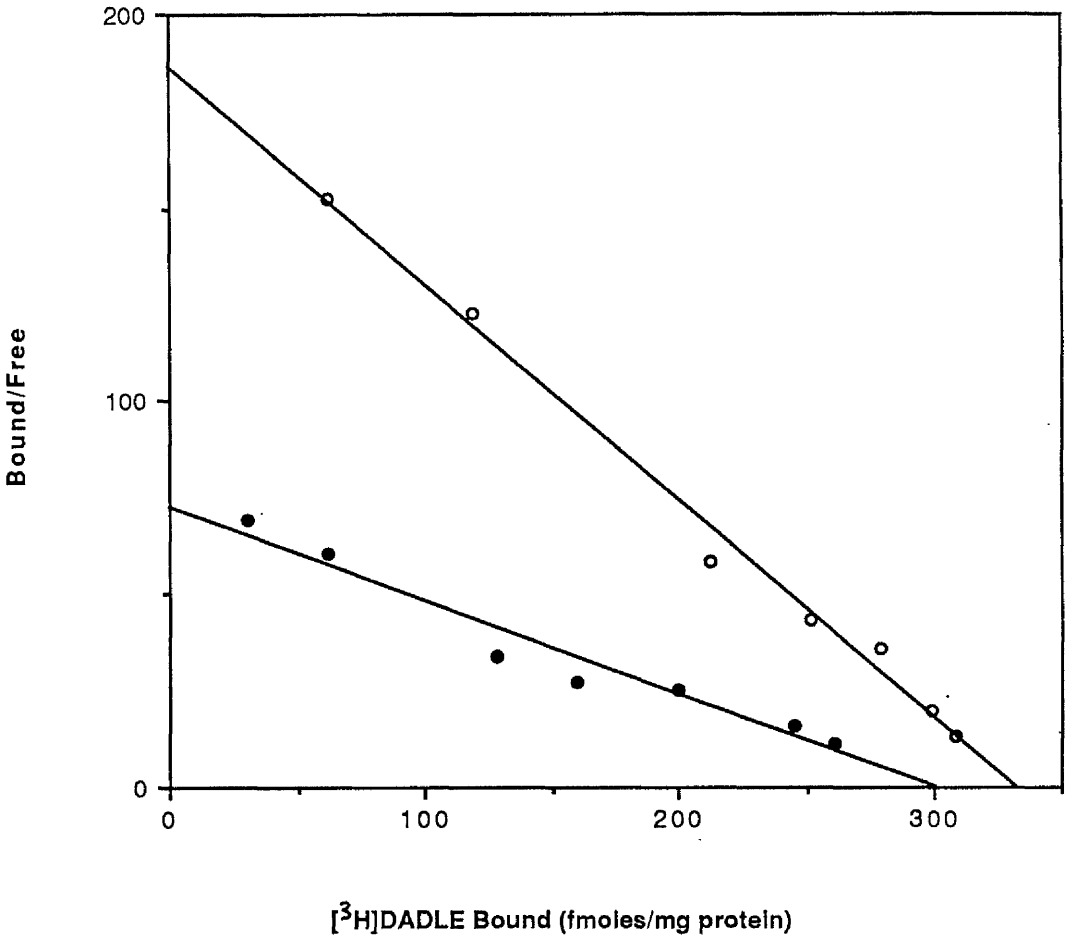

200
100
0
0
100
200
300
Bound/Free
[3H]DADLE Bound (fmoles/mg protein)

Fig. 6.6 Timecourse of reduction in available δ-opioid receptors produced by pre-incubation of NG108-15 cell membranes at 37°C

Membranes (100μg) of NG108-15 cells were incubated either on ice for 75 minutes (A), or at 37°C for 0 minutes (B), 15 minutes (C), 45 minutes (D), 60 minutes (E) or 75 minutes (F) in the presence of binding assay buffer but in the absence of radiolabelled or non-radiolabelled ligand as described in section 2.14. After pre-incubation, binding assays were performed as described in the legend to Figure 6.2. Data are means ± S.D. taken from a single experiment.

Figure 6.6

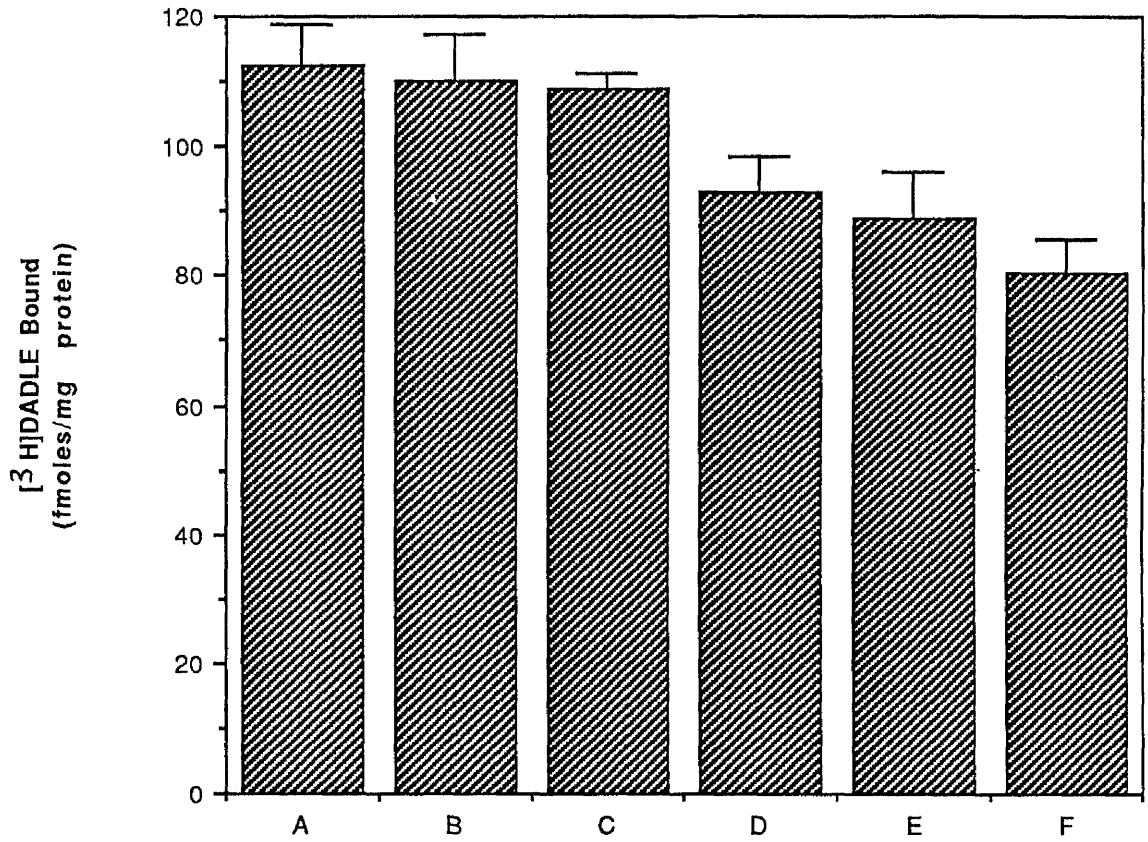
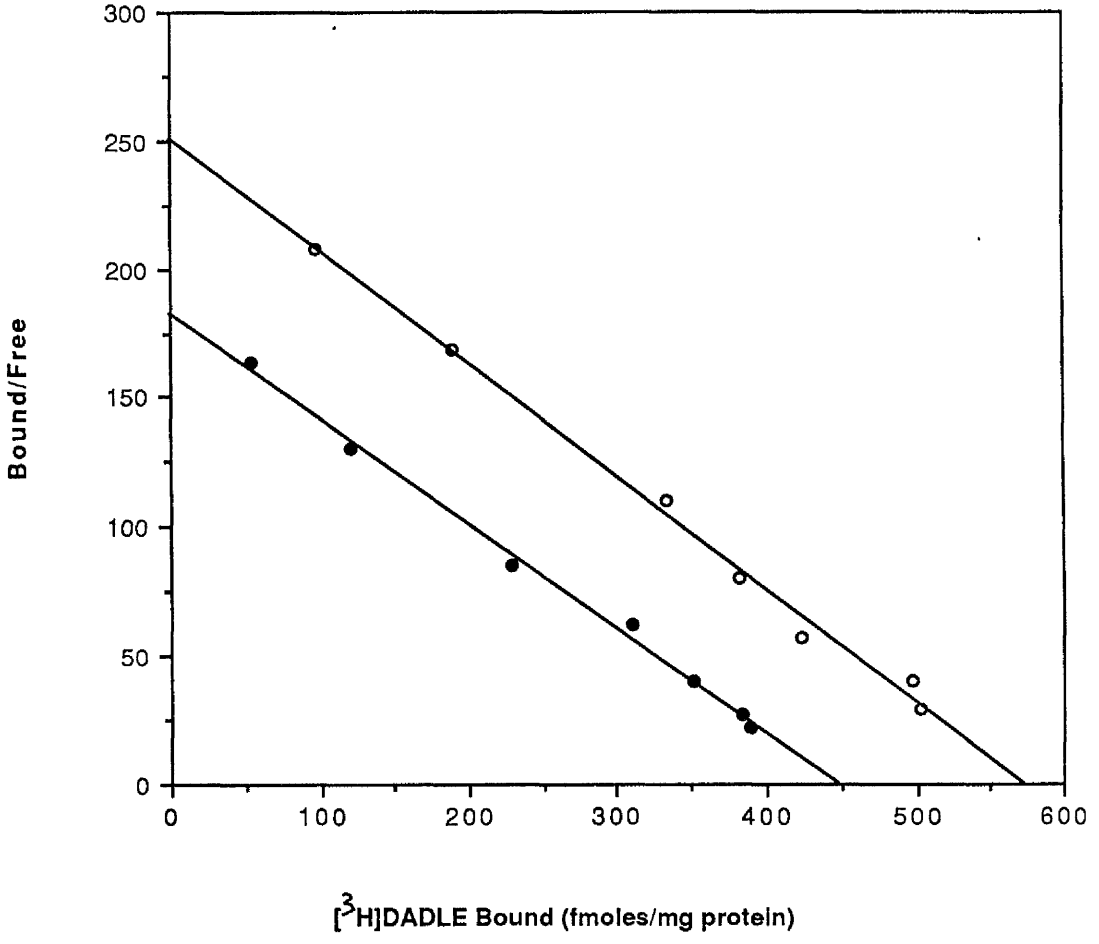


Fig. 6.7 **Preincubation of membranes of NG108-15 cells at 37°C produces a reduction in available δ opioid receptors, with no alteration in affinity for agonist.**

Membranes (100μg) of NG108-15 cells were incubated for 60 minutes either on ice (open circles) or at 37°C (closed circles) in the presence of binding assay buffer but in the absence of both [^{3}H] DADLE or DADLE as detailed in the legend to Figure 6.6. Binding assays were then performed as described in section 2.14. Binding parameters in this experiment were: Bmax 578 ± 11 fmol/mg protein, Kd 2.5 nM for the samples preincubated on ice and Bmax. 429 ± 9 fmol/mg protein, Kd 2.4 nM for the samples preincubated at 37°C. Data are means ± S.D. from quadruplicate determinations in a single experiment which was performed twice with identical results.

Figure 6.7


300
250
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100
50
0
Bound/Free
0
100
200
300
400
500
600
[3H]DADLE Bound (fmoles/mg protein)

Fig. 6.8 Antiserum AS7 specifically reduces the binding of [^{3}H]DADLE to membranes of NG108-15 cells.

Membranes of NG108-15 cells (100μg) were incubated for 60 minutes at 37^{o}C in the presence of (2) Gpp[NH]p (100μM) or the IgG fractions (equivalent to a 1:100 dilution of the original antiserum) isolated from either (1) normal rabbit serum (control) , (3) antiserum AS7 (anti- $G_i2\alpha$) , (4) antiserum OC1 (anti- $G_o\alpha$) or (5) antiserum I3B (anti- $G_i3\alpha$) as detailed in section 2.9. The binding of a single concentration of [^{3}H] DADLE (2 nM) close to the calculated Kd for this ligand was measured and the non-specific component of this binding defined by parallel incubations in the presence of 10 μM DADLE. Only the IgG fraction isolated from antiserum AS7 was able to mimic Gpp[NH]p in reducing the specific binding of the [^{3}H]DADLE. Data are presented as means ± S.D. for quadruplicate determinations in a single experiment which was repeated four times and gave identical results.

Figure 6.8

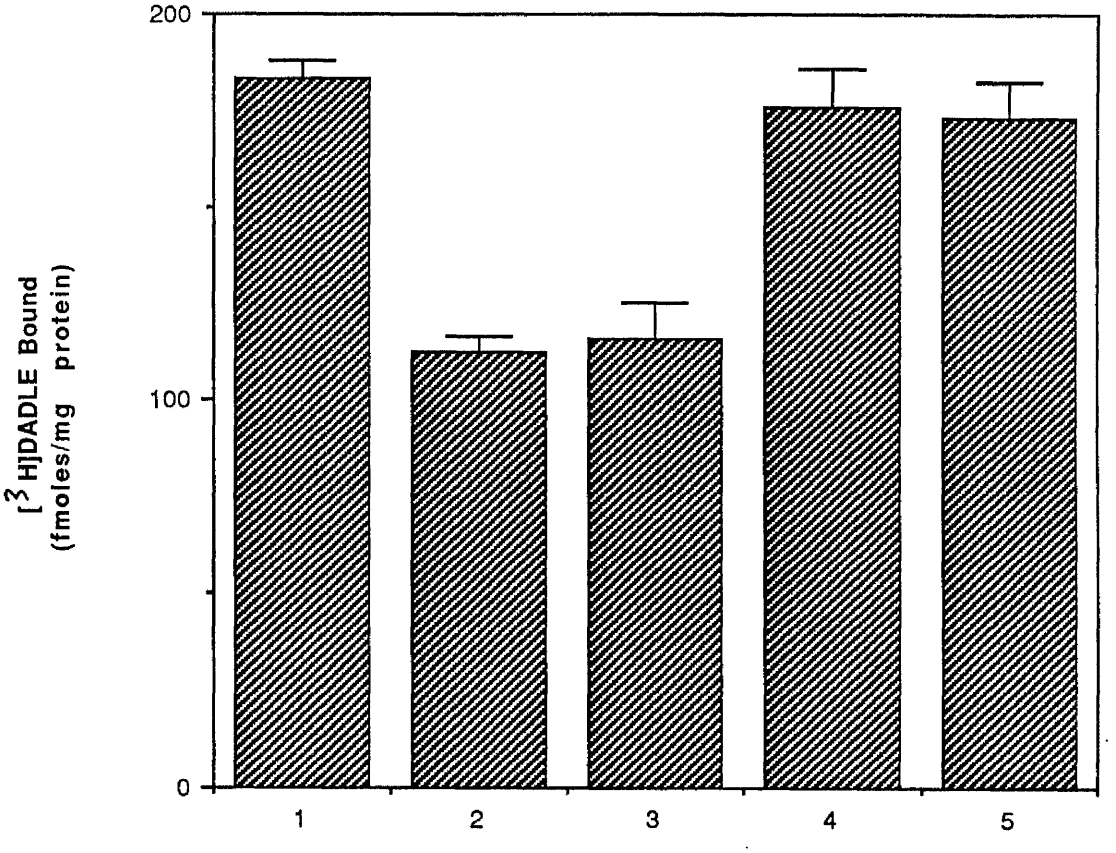


Fig. 6.9 The effects of Gpp[NH]p and antiserum AS7 on [^{3}H]DADLE binding to membranes of NG108-15 cells. Lack of additivity.

Membranes of NG108-15 cells (100μg) were pre-incubated for 60 minutes at 37^{o}C with either; (1) the IgG fraction of normal rabbit serum (concentration equivalent to a 1:100 dilution of the original serum), (2) IgG fraction from normal rabbit serum (as in 1) + Gpp[NH]p (100μM), (3) The IgG fraction of antiserum AS7 (equivalent to a 1:100 dilution of the crude antiserum) or (4) The IgG fraction of antiserum AS7 (as in (3)) + Gpp[NH]p (100μM). as detailed in the legend to Figure 6.8. Following the preincubation, specific binding of a single concentration of [^{3}H]DADLE (2 nM) was assessed as in Figure 6.1 Results are presented as mean ±S.D. for quadruplicate determinations pooled from three independent experiments (n=12).

Figure 6.9

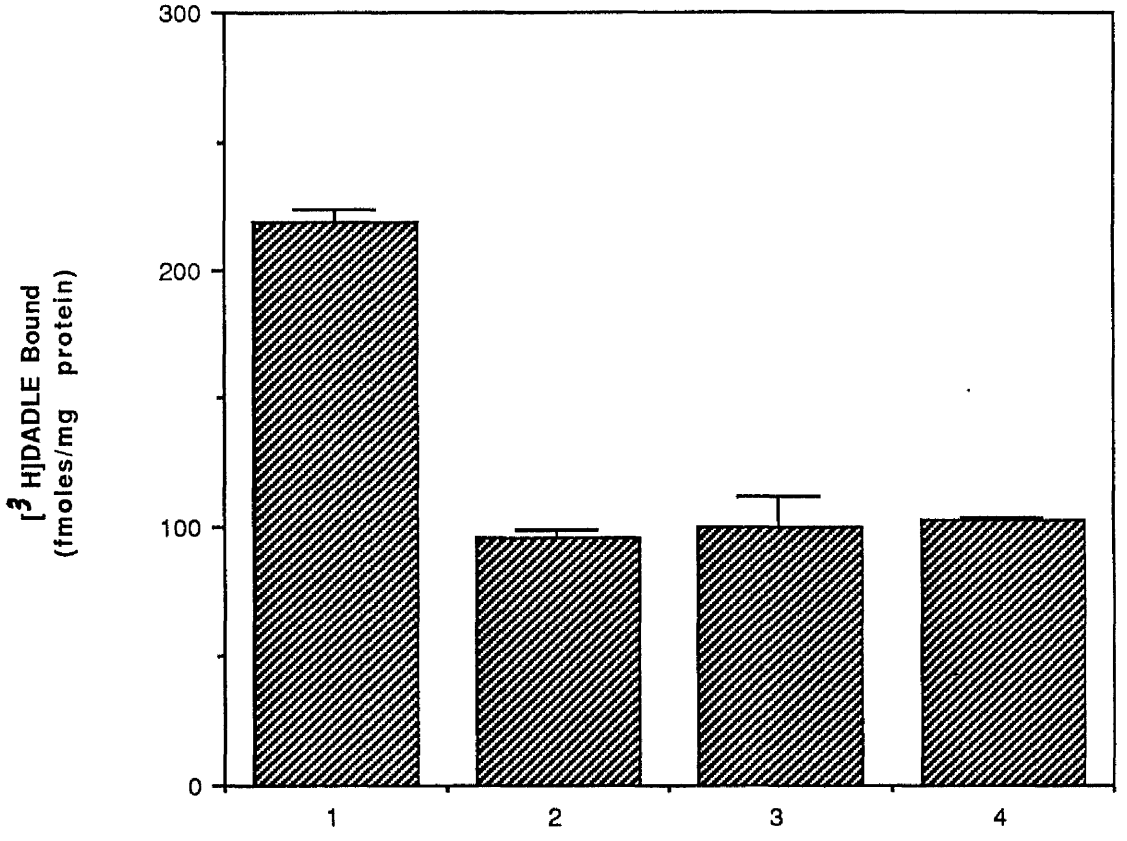
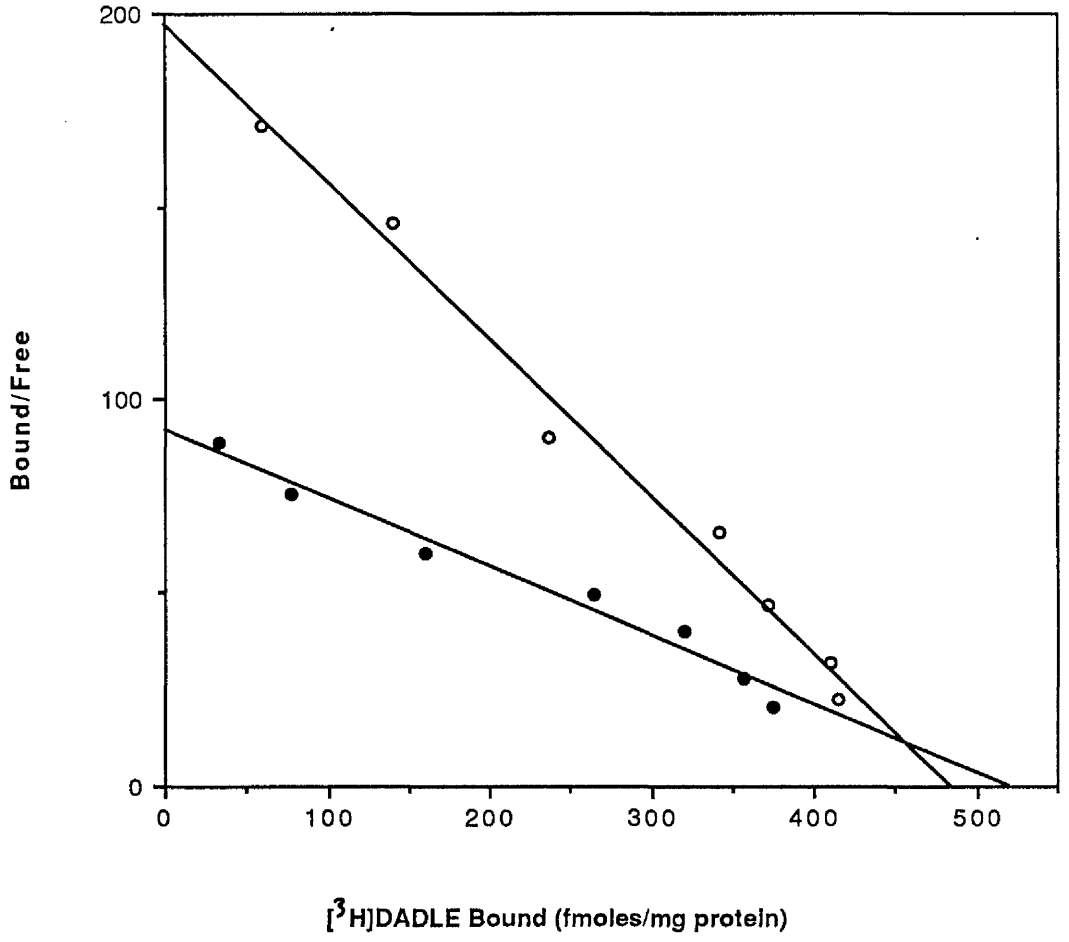


Fig. 6.10 Antiserum AS7 converts δ opioid receptors in membranes of NG108-15 cells from high affinity for agonist binding to low affinity.

Membranes of NG108-15 cells (100μg) were pre-incubated at 37°C for 60 minutes with an IgG fraction from either normal rabbit serum (open circles) or antiserum AS7 (closed circles) as in the legend to Figure 6.8. The specific binding of a range of concentrations of [^{3}H]DADLE were then assessed as detailed in section 2.14. Binding parameters in this experiment were Bmax. 481 ± 12 fmol/mg protein, Kd 2.4 nM for membranes incubated with normal rabbit serum and Bmax. 510 ± 23 fmol/mg protein, Kd. 5.6 nM for membranes incubated with antiserum AS7. Data are means ± S.D. from pooled triplicate determinations from three independent experiments.(n=9).

Figure 6.10


200
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0
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100
200
300
400
500
Bound/Free
[3H]DADLE Bound (fmoles/mg protein)

Fig. 6.11 [^{3}H] diprenorphine binding to membranes of NG108-15 cells is reduced both by Gpp[NH]p and antiserum AS7.

NG108-15 cell membranes (100μg) were treated as detailed in the legend to Figure 6.6 except that the opioid receptor ligand was [^{3}H] diprenorphine (2nM). The apparent Kd for this ligand in this system was estimated in saturation binding experiments to be some 2.4 nM (see Table 6.1). (A) membranes preincubated with the IgG fraction of normal rabbit serum. (B) preincubation with normal rabbit serum IgG + Gpp[NH]p (100μM). (C) preincubation with the IgG fraction of antiserum AS7. (D) preincubation with AS7 IgG + Gpp[NH]p (100uM). Data for the specific binding are presented as means ± S.D. from quadruplicate determinations in a single experiment.

Figure 6.11

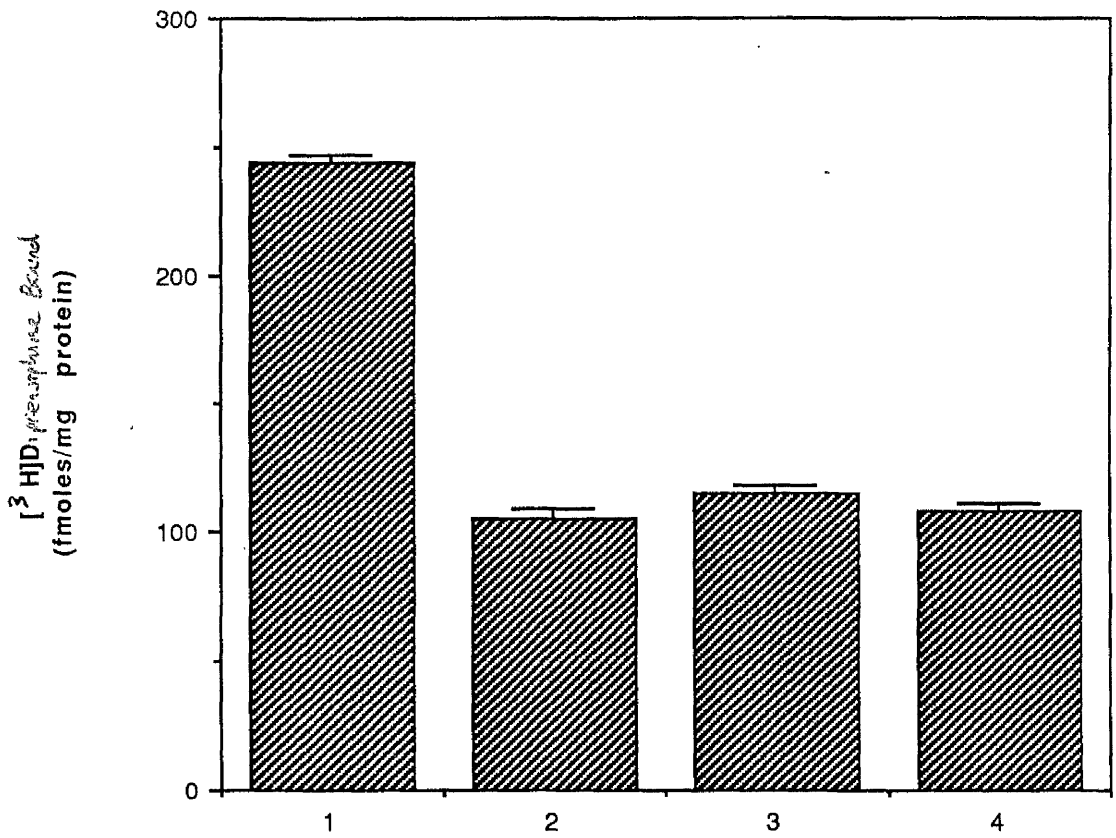

Table 6.1 The effect of Gpp[NH]p on the specific binding of [^{3}H] diprenorphine to membranes of NG108-15 cells.

Membranes of NG108-15 cells (100μg) were incubated with varying concentrations of [^{3}H] diprenorphine (0.5-20 nM), as described in section 2.14, in the absence or presence of Gpp[NH]p (100 μM). A further series of incubations were performed in the presence of 10μM DADLE to define non-specific binding. Binding parameters were calculated as described by Scatchard (1949). Data are means ± S.D. from quadruplicate determinations in a single experiment. Two further experiments produced essentially identical results.

Table 6.1 The effect of Gpp[NH]p on the specific binding of [^{3}H] diprenorphine to membranes of NG108-15 cells.

	[^{3}H]diprenorphine	
	BMax. (fmol/mg protein)	Kd (nM)
Untreated	471.0	2.6
+ Gpp[NH]p	462.0	4.5

Results.

The binding of [^{3}H] DADLE (10nM) to NG108-15 cell membranes reached an equilibrium after approximately 15 minutes incubation at 30^{o}C (Figure 6.1). The data shown in Figure 6.1 and for all subsequent figures is for specific binding of [^{3}H] DADLE. All subsequent binding assays were performed with an incubation period of 20 minutes, to ensure that a binding equilibrium had been reached. The equilibrium obtained is related to both the rate of association and dissociation of the ligand. The rates of association and dissociation have previously been assessed in membranes produced from NG108-15 cell membranes, the rate of association, k_{on} of, [^{3}H] DADLE has been shown to be linear with respect to time, with an observed constant of 0.022+/- 0.0007 min^{-1}, being obtained when DADLE was used at a concentration of 2.1nM, close to the apparent Kd. In the presence of 20mM magnesium, the dissociation curve of [^{3}H] DADLE is monophasic, indicative of a single binding component (Law et al., 1985a). Both the specific and non-specific binding of [^{3}H] DADLE to NG108-15 membranes was linear between membrane protein concentrations of 25 to 250μg (Figure 6.2). All subsequent assays were performed using 100μg of membrane protein.

In saturation binding experiments, in the presence of Mg^{2+} (20 mM), specific binding of [^{3}H] DADLE to membranes of neuroblastoma x glioma hybrid, NG108-15, cells could be adequately defined as being to a single population of high affinity sites (Figure 6.3). Scatchard analysis of the data indicated the presence of some 400 fmols receptor /mg membrane protein which displayed an apparent dissociation constant (Kd) for the ligand of 2.2 $\pm$ 0.2 nM (mean $\pm$ S.D., n = 9). In a series of membrane preparations from individual cultures, the apparent number of receptors varied from 290 to 580 fmoles/mg protein. When a similar assay was performed in the presence of Gpp[NH]p (100 μM), Scatchard analysis of the specific binding indicated a reduction in affinity of [^{3}H] DADLE binding but no alteration in the total number of available sites (Figure 6.4).

Specific binding of [^{3}H] DADLE to membranes of NG108-15 cells which had been pretreated with pertussis toxin as detailed in section 2.6d also produced a reduction in [^{3}H] ligand affinity in comparison to that in membranes from untreated, control cells but did not alter the total number of sites (Figure 6.5). These two pieces of data confirmed previous studies (Milligan et al., 1985; Burns et al., 1983) and indicated that the opioid receptor in these cells interacts with a pertussis toxin-sensitive G-protein(s) (Koski and Klee, 1981; Milligan et al., 1985; Kurose et al., 1982).

Immunoblotting membranes of NG108-15 cells, as well as membranes of a series of other cells and tissues in which the complement of G-proteins expressed has previously been defined with a series of antipeptide antisera which are able to identify the extreme C-terminal region of the individual pertussis toxin-sensitive G-proteins, demonstrated the expression of Gi2, Gi3 and Go in these cells (see chapter 3). By contrast it was not possible to detect expression of Gi1.

To define which of these G-proteins was linked to the δ opioid receptor, a similar technique to that used in chapter 5 was employed. Membranes of NG108-15 cells were incubated either with Gpp[NH]p (100 μM) or with IgG fractions isolated from each of normal rabbit serum, and antisera AS7 (anti-$G_i2\alpha$), OC1 (anti-$G_o\alpha$) and I3B (anti- $G_i3\alpha$) for 60 minutes at 37^{o}C, after which time the ability of a single concentration of [^{3}H] DADLE (2nM), which was close to the apparent Kd for the ligand, to bind specifically to the δ opioid receptor was assessed. As anticipated, Gpp[NH]p reduced the specific binding of this concentration of the opioid ligand, however, of the various IgG fractions, only that derived from antiserum AS7 was able to produce a similar inhibition (Figure 6.8). The IgG fractions from each of normal rabbit serum and antisera OC1 and I3B did not affect the binding of [^{3}H]DADLE (Figure 6.8).

The preIncubation phase itself had only a small inhibitory effect on [^{3}H] DADLE binding (< 20%), which occurred in a time and temperature -dependent manner (Figure 6.6). Pre-incubation of NG108-15 membranes at 4^{o}C had no apparent effect on the binding of [^{3}H[DADLE (Figure 6.6). Scatchard analysis of the

specific binding (Figure 6.7) demonstrated the reduction in [^{3}H] DADLE binding was due to a reduction in the total number of available receptors without effect on the affinity of the [^{3}H] ligand for the remaining receptors. Indeed after the pre-incubation at either 4^{o}C or 37^{o}C, it was still possible to observe a Gpp(NH)p (100μM) mediated reduction in affinity of [^{3}H] DADLE binding with no alteration in the total number of available sites (data not shown)

The effects of Gpp[NH]p and the IgG fraction of antiserum AS7 on reduction of [^{3}H]DADLE binding were not additive, indeed no further reduction in binding of [^{3}H] DADLE was achieved by the combination of these agents in comparison the effects of either in isolation (Figure 6.9).

In an attempt to assess whether the inhibitory effect of IgG from antiserum AS7 on the binding of [^{3}H] DADLE was due to an alteration in the affinity of the ligand for receptor as would be anticipated if the antibodies were producing a functional uncoupling of the receptor from the G-protein signalling system, saturation binding experiments were performed. Scatchard analysis of the specific binding of a range of concentrations (0.5 - 20nM) of [^{3}H]DADLE to membranes of NG108-15 cells following preincubation in the presence or absence of the IgG fraction of antiserum AS7 indeed indicated that the affinity of the receptor for ligand was reduced but that the total number of binding sites was not affected (Figure 6.10). Equivalent results were produced when each of the experiments detailed above were repeated using [^{3}H] diprenorphine as ligand (Table 6.1, Figure 6.11 and results not shown).

Discussion.

Although the ternary complex model 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 and Snyder, 1978; Koski and Klee, 1981) and led to the general conclusion that if the affinity of a receptor for agonist is altered by the presence of guanine nucleotides, then that receptor functions through activation of a G-protein.

Although there are several examples to the contrary, (Rojas and Birnbaumer, 1985; Ott et al., 1988), there appears to be a direct relationship between the ability of guanine nucleotides to activate a G-protein and the ability to produce a negative allosteric effect on binding of agonist to receptor. Analogues of GTP such as GTPγS and Gpp(NH)p which are known to promote dissociation (Hildebrandt et al., 1984; Codina et al., 1983) and hence activation of G-proteins, are able to elicit a reduction in the affinity of receptor for agonist. However binding studies on muscarinic (Burgisser et al., 1982), D2-dopaminergic (DeLean et al., 1982), and A1-Adenosine receptors (Green, 1984), reveal a more elaborate effect of guanine nucleotides, where agonist and antagonist binding to these receptors may be modulated in a reciprocal manner. To include these observations, the ternary complex model was altered to allow for the ability of an antagonist to be able to promote dissociation of the ternary complex (Wregget and DeLean, 1984). Thus the effects of guanine nucleotides on ligand binding may be complex, depending on the receptor type under study

Opioid receptors of neuroblastoma x glioma cells appear to be comprised only of the δ subtype (Chang and Cuatrecasas, 1979). This receptor is able to produce both a stimulation of high affinity GTPase activity and a GTP-dependent inhibition of adenylate cyclase activity (Koski and Klee, 1981). Both of these effects are attenuated by pretreatment of the cells with pertussis toxin (Milligan et al., 1985; Kurose et al., 1983) and as the effects of a range of opioid ligands in each assay

correlate very highly in terms of both potency and efficacy of the agonist (see chapter 5), it is believed that the two effects represent consecutive stages of the same signalling cascade. Pertussis toxin is able to catalyse an NAD^+-dependent mono-ADP-ribosylation of a series of G-proteins which as a common feature have a cysteine residue located 4 amino acids from the C-terminus of the α subunit (Gilman, 1987; Jones and Reed, 1987). Whilst pertussis toxin prevents opioid peptide-mediated inhibition of adenylate cyclase and hence must cause ADP-ribosylation of "G_i", the presence of multiple substrates for this toxin in NG108-15 cells means that it cannot be used as a diagnostic probe to identify which of these G-proteins functions to couple the opioid receptor to adenylyl cyclase. The expression of three distinct pertussis toxin sensitive G-proteins in these cells and their identification as products of the Gi2, Gi3 and Go genes has been described in chapter 3.

The data described in this chapter, in addition to chapter 5, demonstrates through studies on the alteration of affinity of the δ-opioid receptor for agonist, that the receptor interacts directly and specifically with G_i2.

The first indications for the presence of G-proteins as transducers of information between a receptor and an effector system has frequently been produced by noting the ability of guanine nucleotides to reduce the affinity of agonists (Milligan, 1988), but not antagonists, for the receptor. This is usually interpreted to imply that a receptor-G-protein complex displays higher affinity for an agonist than does the receptor in isolation (DeLean et al., 1980). Pretreatment with pertussis toxin, which prevents productive coupling between receptor and G-protein, equally reduces the affinity of ligand for a receptor which is coupled to a pertussis toxin sensitive G-protein, and attenuates the ability of guanine nucleotides to alter the binding of agonist to receptor (Hsia et al., 1984). As detailed in chapter one, the extreme C-terminal region of the α subunit of all G-proteins appears to be a key region for interaction of G-protein and receptor. As such a range of antipeptide antisera against the individual pertussis toxin-sensitive G-proteins have been produced by using synthetic peptides corresponding to the C-terminal decapeptides of these polypeptides as antigen. These antisera have been employed in an attempt to

interfere with receptor-G-protein interactions. It is postulated that these antisera would be able to interfere with the coupling of particular G-proteins with receptors which interact with them and hence produce a low affinity state of the receptor for agonists which could be detected in ligand binding assays.

These experiments were performed in the presence of a high concentration of Mg^{2+} (20mM) as under such conditions all the receptors in untreated 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 of Mg^{2+} 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 a high concentration of Mg^{2+} seems to be a requirement for dissociation of GDP, association of GTP and subunit dissociation (Brandt and Ross, 1986). It has recently been demonstrated that neomycin, an inositol phospholipid binding aminoglycoside antibiotic is able to exert a similar effect on the binding of agonist to receptor as that produced by Mg^{2+} (Herrmann et al., 1989). The HL 60 cell line expresses a receptor(s) for the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe), which is coupled via a G-protein, to the stimulation of the rate of hydrolysis of inositol-containing phospholipids (Gierschik and Jakobs, 1987). In membranes of differentiated HL60 cells, neomycin was found to induce high-affinity binding of [^{3}H] F-Met-Leu-Phe, an effect attenuated by prior treatment with pertussis toxin. In close agreement, high affinity binding of isoproterenol to β-adrenoceptors in guinea-pig lung membranes was induced by both magnesium and neomycin. 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 is as a result of the binding of magnesium (and neomycin) to membrane lipid components, to produce an alteration in the local lipid enviroment surrounding the receptor and G-protein which facilitates the coupling of G-proteins to agonist bound receptors (Herrmann et al., 1989).

In addition to the effect of magnesium on G-protein function, It has been well documented that sodium chloride is a requirement for agonist-mediated inhibition of adenylyl cyclase (Koski et al., 1982; Klee et al., 1985). Recent data indicate that chloride and other monovalent cations, but not sodium, modulate the affinity of guanine nucleotides for purified G-proteins (Higashijima et al, 1987). The effects of both Na^+ and Cl^- can be resolved into distinct components, since in membrane systems, Na^+ in the presence of Mg^{2+} lowers GTPase activity in a manner which is reversed by the addition of inhibitory agonists (Koski et al., 1982). However studies on Gi purified from brain are contradictory, suggesting that Na^+ is able to stimulate GTPase activity in the absence but not in the presence of Mg^{2+} (Milligan and Klee, 1985). It is therefore unclear as to whether Na^+ is required to facilitate G-protein function, receptor function or the interaction between receptor and G-protein. It has been proposed that the effect of Na^+ is to prevent the activation of G-proteins with unoccupied receptors (as discussed in chapter 5), since in membranes produced from HL60 cells, basal GTPase activity is inhibited by the presence of Na^+ in a manner similar to the reduction elicited by pertussis toxin, and agonist stimulation of GTPase activity is markedly enhanced in the presence of Na^+ (Gierschik et al., 1989b). These observations are in agreement with the effect of Na^+ on GTPase activity in membranes produced from NG108-15 cells (Koski et al., 1982). Na^+ has been shown to increase the dissociation of agonist from opiate binding sites (Blume, 1978; Costa et al., 1990), suggesting that the ion may be acting at the

ligand binding component, or via a membrane sodium-binding component which can interact with the opiate receptor. The sodium binding site may be located intracellularly because the concentration of sodium required for half-maximal activity is 20mM (Blume, 1978), the intracellular sodium concentration, and in whole cell experiments removal of extracellular sodium does not attenuate opiate mediated inhibition of adenylyl cyclase (Law et al., 1982). Target size analysis experiments have suggested that the Na^+ effect is mediated by a component distinct from G-proteins and receptor, with an apparent molecular weight of 168kDa. The identity of the putative Na^+ binding component is unknown (Ott et al., 1988). Na^+ can therefore be seen to have complex effects on both the binding of inhibitory agonist to receptor, and in the activation of G-proteins.

Within currently defined receptor theory, a decrease in the affinity of a receptor for a ligand may result from several circumstances; 1) a decrease in the rate of association, k_{on}, 2) an increase in the rate of dissociation, k_{off}, or 3) a decrease in the association rate and an increase in the dissociation rate as the receptors are being occupied. In most studies of alterations in the affinity state of receptor for agonist, an observed decrease in the k_{on} rate is rare and an increase in k_{off} is responsible for decreased affinity of receptor for agonist (DeLean and Rodbard, 1979). This is in agreement with the mechanism by which the δ-opioid receptor expressed by NG108-15 cells alters its affinity for agonist. In the presence of magnesium, the dissociation rate of [^{3}H]DADLE -receptor complexes is slow, however, upon addition of Gpp(NH)p, dissociation is more rapid, a characteristic of G-protein linked receptors, resulting in a decrease in affinity for agonist.(Law et al., 1985a) The alteration in affinity produced by either Gpp(NH)p, pertussis toxin pretreatment, or pretreatment with an IgG fraction derived from antiserum AS7, was only of the order of two-fold. This is somewhat less than has been previously reported for the δ-opioid receptor in this cell type (Law et al., 1985a), and for other G-protein linked receptors (Brandt and Ross, 1986). The reason for this discrepancy is unclear, however using the assay conditions described, a greater than two-fold alteration in affinity for either DADLE or diprenorphine was never observed.

In the uncoupling experiments it was only antibodies derived from antiserum AS7 which converted the opioid receptor to a low affinity state. Whilst antiserum AS7 is able to identify both $G_i1\alpha$ and $G_i2\alpha$ equally (Goldsmith et al., 1987) as the C-terminal sequences of these two G-proteins are identical; $G_i1\alpha$ is not expressed in NG108-15 cells (chapter 3). Based on this observation, then the effect of antiserum AS7 must be due to its interaction with $G_i2\alpha$. Further, as pretreatment with the IgG fraction of antiserum AS7 produces a maximal degree of uncoupling, i.e. the effect mimics completely those produced both by Gpp[NH]p and by pretreatment of the cells with pertussis toxin, then these results imply that the δ-opioid receptor must interact selectively with G_i2. Since the effects of both IgG derived from AS7, and Gpp(NH)p were not additive, then it may be speculated that each agent elicits an alteration in affinity of the δ-opioid receptor for agonist by the same mechanism, the uncoupling of Gi2 from the receptor.

In parallel experiments using [^{3}H]diprenorphine as the opioid receptor ligand, equivalent results were obtained. Diprenorphine is not a full agonist at the opioid receptor in NG108-15 cells, but neither does it display antagonist characteristics. Indeed it appears to display an intrinsic activity of some 60% as compared to full agonists such as the opioid peptides and alkaloids such as etorphine (Koski and Klee, 1981). It would have been instructive to have performed the same set of experiments using a [^{3}H] antagonist as the receptor ligand because it would have been anticipated that each of Gpp[NH]p, pertussis toxin treatment and the IgG fraction of antiserum AS7 would be unable to reduce the affinity of such a ligand. However, [^{3}H]naloxone is the only readily available antagonist for this receptor and it is of sufficiently low affinity as to make it difficult to achieve a ratio of specific to non-specific binding in NG108-15 membranes which would allow the generation of useful data.

It has been demonstrated in chapter 5 that both opioid peptide-mediated stimulation of high affinity GTPase activity and inhibition of adenylate cyclase activity in membranes produced from NG108-15 cells are attenuated by an IgG fraction isolated from antiserum AS7, an antiserum which specifically recognises Gi2 in this cell line. The data presented in this chapter demonstrates that it is

possible to selectively uncouple the δ-opioid receptor from the G-protein with which it interacts using an IgG fraction derived from antiserum AS7. In addition, it may be noted that other G-protein specific antisera were without effect. It is therefore possible to conclude that the δ-opioid receptor functions exclusively through the activation of Gi2 in the NG108-15 cell line and that the true "G_i" of the adenylate cyclase cascade is the product of the Gi2 gene.

The ability to modify each of receptor-agonist binding affinity (herein), receptor-stimulation of high affinity GTPase activity (McKenzie and Milligan, 1990) and receptor-regulation of effector function (McKenzie and Milligan, 1990; McFadzean et al., 1989) using antisera which identify the C-terminus of various G-proteins implies that such tools are likely to be of widespread use in assessements of the specificity/selectivity of receptor-G-protein interactions. For example, it is difficult to produce cell -free systems in which coupling of receptor to G-protein to phosphoinositidase C is adequately maintained, but the coupling of such receptors to the relevant G-protein is often well maintained. As such, the use of antibody-induced production of low affinity interactions between receptor and [^{3}H]ligand may well provide a suitable assay for identification of the G-protein of this system, (G_p). As stated earlier, many other G-protein linked receptors show a much greater alteration of affinity for agonist when uncoupled from G-protein and as such, may provide a more sensitive system for examining possible alterations in affinity state elicited by antisera which can specifically recognise members of the G-protein family.

Conclusions

The data presented in chapter 3 demonstrated that NG108-15 cells do not express detectable levels of Gi1α, but detectable levels of Gi2α, Gi3α and Goα, as well as two forms of Gsα and the β-subunits common to all G-proteins are expressed. However, it is possible to resolve multiple charge variants of Gsα from S49 lymphoma cells using two dimensional electrophoresis (Schleifer et al., 1980). In addition, two charge variants of Go have been identified in both brain (Goldsmith et al., 1988) and NG108-15 cells (Mullaney and Milligan, 1990), which are not resolved under standard one dimensional polyacrylamide gel electrophoresis conditions. This raises the question, do NG108-15 cells express more that one isoform of Gi2α and Gi3α. At present, isolation of cDNA's from a variety of different tissues have identified only one cDNA sequence corresponding to Gi2α and Gi3α (Strathmann et al., 1989). In addition, screening of genomic libraries with rat cDNA clones has identified only one gene encoding Gi2α and Gi3α (Kaziro, 1990). However it may be possible that alternate splicing or post-translational modifications may produce variants of both Gi2 and Gi3 which are not resolved under the electrophoresis conditions employed in chapter 3. Attempts to analyse both Gi2α and Gi3α expressed by NG108-15 cells, using two-dimensional electrophoresis have met with little success (results not shown). The reason for this difficulty is unclear, however it may be a reflection of lipid modification of the G-protein α-subunit preventing entry into the first dimensional gel (results not shown). Two-dimensional analysis of Gi2α and Gi3α purified from bovine brain has revealed the presence of a single polypeptide corresponding to each of Gi2α and Gi3α (Goldsmith et al., 1988). it is therefore likely that only one form of Gi2α and Gi3α are expressed by NG108-15 cells.

G-proteins have been classically designated as belonging to one of two groups; pertussis toxin sensitive G-proteins and cholera toxin sensitive G-proteins. Td1 and Td2 are substrates for both toxins. Cholera toxin, which was originally thought to catalyse the ADP-ribosylation of only Gs, has been demonstrated to catalyse the ADP-ribosylation of 'Gi' in NG108-15 cell

membranes, when the experiments were performed in the absence of guanine nucleotides. The cholera toxin catalysed incorporation of radioactivity from [^{32}P]NAD^{+} into Gi was attenuated by pretreatment of the cells with pertussis toxin and stimulated by the addition of the δ-opioid agonist, DADLE, suggesting that interaction of the δ-opioid receptor with a pertussis toxin sensitive G-protein enables the G-protein to become a substrate for cholera toxin catalysed ADP-ribosylation. This provides a means of identifying the G-protein(s) which interacts with the δ-opioid receptor. However, after cholera toxin catalysed ADP-ribosylation of 'Gi', it was not possible to obtain adequate resolution of 'Gi" under resolving polyacrylamide/SDS electrophoresis conditions (section 2.10) to be able to unequivocally identify this G-protein (results not shown). It would be possible to circumvent this problem by specifically immunoprecipitating the G-protein in question, however this was not performed.

The C-terminal region of the G-protein α-subunit has been identified as the site of functional interaction with receptor. The antisera described in Table 2.1 were generated against synthetic peptides which were either homologous to, or corresponded to the C-terminal decapeptide of α-subunits of Gi2, Gi3, Go and Gs. The specificity of both primary antisera and IgG fractions derived from each antiserum was assessed, and it was hoped that these antisera would be useful as selective tools to prevent interaction of the corresponding G-protein with receptor. Antiserum AS7 was generated against a synthetic decapeptide homologous to the C-terminus of Td1 (KENLKECGLF). This region has one amino-acid difference from the last ten C-terminal amino-acids in the α-subunit of both Gi1 and Gi2, which are identical (KNNLKDCGLF). As such it was thought that AS7 would be able to specifically identify the α-subunits of Td, Gi1 and Gi2. Td1 and Td2 share an extremely restricted distribution, being expressed only in photoreceptor containing cells (Milligan, 1990). Fortuitously, NG108-15 cells do not express Gi1 (McKenzie and Milligan, 1990), which allows AS7 to be used as a specific probe for Gi2 in this cell line. Preincubation of NG108-15 cell membranes with an IgG fraction isolated from antiserum AS7 prevented productive coupling between the δ-opioid receptor and G-protein, as assessed by the attenuation of the ability of DADLE to both stimulate GTPase activity and

inhibit adenylyl cyclase.

As an additional means of detecting the uncoupling of receptor from G-protein, the binding of [^{3}H]DADLE was assessed in NG108-15 cell membranes in a variety of conditions. In membranes which have been either pretreated with pertussis toxin, or in the presence of non-hydrolysable analogues of GTP such as Gpp(NH)p, the δ-opioid receptor was uncoupled from the G-protein with which it interacts and adopted a lowered affinity for agonist (Kurose et al., 1982). Unfortunately this does not allow identification of the G-protein involved since pertussis toxin can catalyse the ADP-ribosylation of Gi2α, Gi3α and Goα in this cell line. When NG108-15 cell membranes were preincubated with an IgG fraction isolated from antiserum AS7, the δ-opioid receptor adopted a state with lowered affinity for agonist. The reduction in affinity produced by this preincubation was similar in magnitude to the alteration in affinity produced by either pertussis toxin pretreatment or the presence of Gpp(NH)p, suggesting that the δ-opioid receptor was no longer capable of interacting with a G-protein to form a ternary complex with high affinity for agonist.

In the data described above, IgG fractions generated from either antiserum specific for Gi3, Go and Gs, or IgG isolated from pre-immune serum, were ineffective in attenuating coupling between G-protein and the δ-opioid receptor. It may be suggested that each IgG fraction was inactive, however this is unlikely since antiserum OC1 has previously been demonstrated to attenuate the ability of α2 adrenergic receptors to inhibit voltage operated calcium channels in differentiated NG108-15 cells (McFadzean et al., 1989). In addition, antibodies affinity purified from an anti-Gs antiserum which was raised in an identical manner to the Gs specific antiserum, CS1 (Table 2.1) have been demonstrated to attenuate β-adrenergic receptor mediated stimulation of adenylyl cyclase in human platelet membranes (Simonds et al., 1989b). Antiserum CS1 has been demonstrated to attenuate isoproterenol stimulation of adenylyl cyclase in cardiac membranes (Milligan, personal communication).

Although there is no evidence to suggest that pertussis toxin sensitive G-proteins other than Gi2, Gi3 and Go are expressed by NG108-15 cells, it is conceivable that the δ-opioid receptor is capable of interacting with a G-protein other than Gi2, without stimulating the GTPase activity of the G-protein to a measurable level (see section 1.6ii). In such a stimulation, it would not be possible to assess the specificity of interaction of the δ-opioid receptor with the G-protein family. In addition if this were correct, then it would have to be assumed that the δ-opioid receptor was only capable of forming a ternary complex with Gi2, since pretreatment of NG108-15 cell membranes with an IgG fraction from AS7 produced a reduction in affinity for agonist which was of the same magnitude as agents which are known to uncouple G-proteins from receptors, such as Gpp(NH)p and pertussis toxin catalysed ADP-ribosylation (Hsia et al., 1984). It should be noted that the effects of Gpp(NH)p and IgG from AS7 were not additive, each reagent produced the maximal effect in isolation.

The most likely explanation for the results described herein is that the δ-opioid receptor interacts exclusively with Gi2 and that Gi2 must therefore represent the G-protein which mediates inhibition of adenylyl cyclase in NG108-15 cell membranes.

It may be suggested that the ability to uncouple receptor from G-proteins using G-protein specific antisera could be widely applicable to a variety of other G-protein mediated signalling systems.

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