The stoichiometry of interaction between receptors, G-proteins and effector species.

A thesis presented for

the degree of

DOCTOR OF PHILOSOPHY

by

Gun-Do Kim

Institute of Biomedical & Life Sciences,

Division of Biochemistry and Molecular Biology,

University of Glasgow.

March 1996.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements.

I would like to thank my supervisor, Prof. Graeme Milligan, for introducing me to the field of G-proteins, and for his support, advice, encouragement and patience in all aspects of the production of this thesis. Thanks also to Drs. Christine M. Brown, who gave me good advice and comfort like my sister and my auditor, Dr. R. H. C. Strang for his concern and kindness. Thanks to my colleagues in lab A20 my thesis has been possible: Andrew Burt, Craig Carr, Sandra Gardner, Morag A. Grassie, Fiona R. Kennedy, Tae-Weon Lee, David J. MacEwan, Fraser J. McCallum, Fiona M. Mitchell, Ian Mullaney, Bukhtiar Shah, Alan Wise and very especially to Elaine J. Adie and Petr Svoboda for help and encouraging conversations. Thanks also to everyone in Prof. Miles D. Houslay's lab A3 and Dr. Gwyn Gould's C36.

At the moment of writing and so close to leaving, I feel the presence of many friends who made these past three years in Glasgow far from home such an enjoyable experience and without whose support this thesis would probably have not been possible: outside the members of A20, a lonely single friends Y. S. Han, and J. S. Lee, Aadne, Ase, Dimos, Alan's mother, uncle Steven Henderson and Leo W. Millican,--- unfortunately, too many names to be included here. To all of the hours we shared talking about so many things. The beautiful scene around the gothic main building of glasgow university, peculiar accent but intimate glaswegian and peaceful contryside of Scotland will be described in a page of reminiscence.

Finally, I would like to give very special thank to my parents for their unfailing love and support during all these years in which I have been far away and this thesis is dedicated to mom and dad.

Abbreviations.

AC	Adenylyl cyclase.
ADP	Adenosine 5'-diphosphate.
ARF	ADP ribosylation factor.
ATP	Adenosine 5'-triphosphate.
BAAM	Bromoacetyl alprenolol methane.
B_{max}	Maximal binding capacity.
BSA	Bovine serum albumin.
cAMP	Adenosine 3', 5'-cyclic monophosphate.
cDNA	Complimentary deoxyribonucleic acid.
CHO	Chinese hamster ovary.
CMV	Cytomegalovirus.
cpm	Counts per minute.
C-terminus	Carboxy terminus.
CTX	Cholera toxin.
DADLE	D-Ala^2, D-Leu^5-enkephalin.
DAG	*sn*-1, 2-diacylglycerol.
DHA	Dihydroalprenolol.
DMEM	Dulbecco's modification of Eagle's medium.
DMSO	Dimethylsulphoxide.
DPM	Disintegrations per minute.
EC_{50}	Median effective dose.
E. coli	*Escherichia coli*.
EDTA	Ethylenediaminetetraacetic acid.
EF-Tu	Bacterial elongation factor-Tu.
FCS	Foetal calf serum.
GDP	Guanosine 5'-diphosphate.
GNRP	Guanine nucleotide releasing protein.

Gpp(NH)p	Guanylyl 5'-[βγ imido] diphosphate.
G-protein	Guanine nucleotide binding protein.
GSAC	Active complex formed between $G_S\alpha$ and adenylyl cyclase.
GTP	Guanosine 5'-triphosphate.
GTPγS	Guanosine 5'-[3-o-thio] triphosphate.
h	Hour.
HAT	Hypoxanthine-aminopterin-thymidine.
HBG	Hank's buffered saline solution with 1% (w/v) BSA and 10 mM glucose.
HEK293 cells	Human embryonic kidney 293 cells.
HEPES	4-(2-Hydroxyethyl)-1-piperazine-N' 2-ethane-sulphonic acid.
IC_{50}	Median inhibitory dose.
IgG	Immunoglobulin G.
I_{KACH}	Muscarinic gated, inwardly rectifying K^+ channel.
Ins(1,4,5)P3 or IP3	D-*myo*-inositol (1, 4, 5) trisphosphate.
K_{cat}	Catalytic rate constant.
kDa	Kilodaltons.
K_d	Equilibrium dissociation constant; representing the concentration of a ligand that half-maximally occupies the receptor at equilibrium.
MAP	Mitogen activated protein.
mRNA	Messenger ribonucleic acid.
NAD	Nicotinamide adenine dinucleotide.
NECA	5'-N-Ethylcarboxamidoadenosine.
NP-40	Nonidet P-40 detergent.
N-terminus	Amino (NH_2) terminus.
$p21^{ras}$	Product from the *ras* family of genes coding for 21 kDa

	GTP-binding proteins.
PBS	Phosphate-buffered saline.
PGE1	Prostaglandin E1.
PKC	Ca^{2+} and phospholipid dependent protein kinase.
PLA_2	Phospholipase A_2.
PLC	Phospholipase C.
PMSF	Phenylmethylsulphonyl fluoride.
PTX	Pertussis toxin.
R	Receptor.
RT-PCR	Reverse transcriptase-polymerase chain reaction.
S.D.M	Standard deviation of the mean.
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis.
S.E.M	Standard error of the mean.
SV40	Simian virus 40.
TCA	Trichloracetic acid.
TEMED	N, N, N', N'-tetramethylethylenediamine.
TRH	Thyrotropin-releasing hormone.
Tris	Tris (hydroxymethyl) aminomethane.

List of contents.

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This thesis includes data from the following;

1. Kim, G. D., Adie, E. J. and Milligan, G. (1994) Quantitative stoichiometry of the proteins of the stimulatory arm of the adenylyl cyclase cascade in neuroblastoma x glioma hybrid, NG108-15 cells. Eur. J. Biochem. 219, 135-143.

2. Kim, G. D., Carr, C., Anderson, L. A., Zabavnik, J., Eidne, K. A. and Milligan, G. (1994) The long isoform of the rat thyrotropin-releasing hormone receptor down-regulates G_q proteins. J. Biol. Chem. 269, 19933-19940.

3. Kim, G. D. and Milligan, G. (1994) Concurrent specific immunological detection of both primate and rodent forms of the guanine nucleotide binding protein $G_{11}\alpha$ following their coexpression. Biochim. Biophys. Acta 1222, 369-374.

4. Kim, G. D. and Milligan, G. (1994) Receptor availability defines the extent of agonist-mediated G-protein down-regulation in neuroblastoma x glioma hybrid cells transfected to express the β_2-adrenoceptor. FEBS Lett. 355, 166-170.

5. Kim, G. D., Carr, I. C. and Milligan, G. (1995) Detection and analysis of agonist-induced formation of the complex of the stimulatory guanine nucleotide binding protein with adenylyl cyclase in intact wild type and β_2-adrenoceptor expressing NG108-15 cells. Biochem. J. 308, 275-281.

6. MacEwan, D. J., Kim, G. D. and Milligan, G. (1996) Agonist regulation of adenylyl cyclase in neuroblastoma x glioma hybrid NG108-15 cells transfected to co-express adenylyl cyclase type II and the β_2-adrenoceptor (Evidence that adenylyl cyclase is the limiting component for receptor mediated stimulation of adenylyl cyclase.). Biochem. J. (in press).

7. Svoboda, P., Kim, G. D., Eidne, K. A. and Milligan, G. (1996) Thyrotropin releasing hormone induced subcellular redistribution and down-regulation of the guanine nucleotide binding protein $G_{11}\alpha$ (Analysis of differences in agonist regulation of co-expressed G_{11} alpha species variants). Mol. Pharmacol. (in press).

Summary.

G-proteins have been demonstrated to be necessary to allow transduction of information from hormone-activated cell surface receptors to a variety of effector systems. This study has focused on their expression and regulation in response to agonists at receptors which produce stimulation of adenylyl cyclase or stimulation of phospholipase C activity.

To understand receptor regulation of cyclic AMP generation, it is important to know the relative or absolute levels of expression of each component (receptor, G-protein (G_S) and adenylyl cyclase) in individual cells, their cellular disposition and how alteration in levels of each component might alter the effectiveness of cellular signaling.

In chapter 3, neuroblastoma x glioma hybrid, NG108-15, cells are shown to express $G_S\alpha$ in a substantial molar excess over its effector adenylyl cyclase. Sustained exposure of a cell to an agonist for a G-protein coupled receptor can lead to the down-regulation of levels of the receptor and the G-protein and this process can play a substantial part in the regulation of cellular sensitivity to the presence of agonist. Regulation of agonist access to the receptor population by pretreatment of NG108-15 cells transfected to express the human β_2-adrenoceptor with varying concentrations of an irreversible β-adrenoceptor antagonist (BAAM) demonstrated that the extent of agonist-mediated $G_S\alpha$ down-regulation was dependent upon the availability of receptor to agonist and that the levels of receptor expression defines the intrinsic activity and potency of agonists.

As noted above, however, the availability of sufficient $G_S\alpha$ to interact with the total cellular population of adenylyl cyclase suggests that the adenylyl cyclase is likely to be the limiting component for information transfer. As a means to examine quantitative aspects of the expression of adenylyl cyclase, human β_2-adrenoceptor expressing NG108-15 cells were further transfected to overexpress adenylyl cyclase type 2. The results demonstrated that receptor-mediated maximal output of the

stimulatory arm of the adenylyl cyclase cascade can be increased by increasing total levels of adenylyl cyclase but this did not result in any significant alteration in the concentration-effect curves for stimulation of adenylyl cyclase activity produced by either the transfected human β_2-adrenoceptor or any of the endogenously expressed (IP prostanoid, A_2 adenosine or secretin) $G_s\alpha$-linked receptors.

In chapter 4, human embryonic kidney (HEK-293) cells which express the long splice variant form of the rat thyrotropin-releasing hormone (TRH) receptor (clone E2) were examined to address whether both $G_q\alpha$ and/or $G_{11}\alpha$ and $G_s\alpha$ are involved in TRH-stimulation of phospholipase C β1 and/or adenylyl cyclase or whether different splice variants of the receptor selectively interact with different G-proteins to regulate different signal transduction cascades. Activation of this receptor with TRH caused only a large stimulation of production of inositol phosphates in a manner which was mediated by the pertussis-toxin-insensitive phospholipase C-linked G_q and G_{11} proteins despite the fact that TRH has been reported to stimulate adenylyl cyclase activity via activation of $G_s\alpha$.

In addition, with development of a 6 M urea containing SDS-PAGE (10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide) system which allowed resolution of $G_q\alpha$ and $G_{11}\alpha$ I assessed whether a single receptor type could utilise species variants of the same G-protein equivalently? Treatment of clone E2M11 (which expresses the long isoform of the rat TRH receptor and both the endogenous human and exogenous introduced murine $G_{11}\alpha$) with TRH resulted in a qualitatively similar pattern of cellular redistribution and down-regulation of $G_{11}\alpha$ isoforms. Quantitative analysis indicated, however, that the murine isoform of $G_{11}\alpha$ was less effectively regulated by the long isoform of the rat TRH receptor than the endogenous human form of $G_{11}\alpha$.

Chapter 1.

Introduction.

1.1. G-protein coupled receptors.

1.1.1. The identification of a G-protein coupled receptor.

Cell surface receptors may be functionally classified into several categories. Growth factor receptors have an intrinsic tyrosine kinase activity, such as receptors for insulin and epidermal growth factor (EGF) (Hunter *et al.*, 1985). Receptors like the nicotinic acetylcholine receptor are multi-subunit ion channels (Changeux *et al.*, 1987), whereas the transferrin receptor functions as a carrier protein (Goldstein *et al.*, 1985). However, the largest class of receptors are those which interact with and activate G-proteins to mediate a variety of transmembrane signals.

The effect of guanine nucleotides on ligand binding to receptor initially demonstrated the possibility of a G-protein link to a rat liver glucagon receptor: the affinity of glucagon binding was decreased in the presence of guanine nucleotides (Rodbell *et al.*, 1971). Analysis of competition curves at β-adrenoceptors suggested the existence of two receptor affinity states for an agonist as opposed to one affinity state for an antagonist. It was proposed that in the presence of guanine nucleotides receptors adopted a low affinity state, whereas a high affinity state for agonist persisted in the absence of guanine nucleotide (De Lean *et al.*, 1980). In an attempt to account for the data emerging on guanine nucleotide effects on ligand binding the ternary complex model was proposed. In the unstimulated state the inactive G-protein α subunit (Gα), coupled to βγ, interacts with the receptor (R), leading to agonist (H) promoted formation of a high affinity ternary complex (HRGα). In the presence of guanine nucleotides which activate Gα, the ternary complex is destabilized and both agonist and G-protein can dissociate from the receptor. Although the ternary complex was originally based on the observations of agonist-induced stimulation of adenylyl cyclase, receptors linked to the inhibitory arm of this transduction pathway were also found to fit this interpretation (Koski *et al.*, 1981a, b). These observations led to the general conclusion that guanine nucleotide-induced agonist affinity alterations implicate a G-protein coupled receptor.

Another property characteristic of a G-protein linked receptor is agonist stimulated GTPase activity. Although GTPase activity was firstly identified as $G_s\alpha$ mediated, it was only possible to measure receptor stimulated high affinity GTPase activity by $G_s\alpha$ in a very limited number of model systems, namely the turkey erythrocyte (Cassel *et al.*, 1976) and the human platelet (Houslay *et al.*, 1986). In contrast, stimulation of GTPase activity by receptors which interact with pertussis toxin-sensitive G-proteins has been reported for a variety of membrane systems, in particular those mediating inhibition of adenylyl cyclase (McClue *et al.*, 1992).

Agonist-stimulated GTPase activity has also recently been demonstrated for pertussis toxin-insensitive G-proteins. Thromboxane A2 stimulated a small, yet significant, increase in GTPase activity in human platelet membranes mediated via the G_q-like G-proteins (Shenker *et al.*, 1991). The low level of stimulated GTPase activity may explain the lack of success of previous attempts to measure this activity upon stimulation of receptors that couple to pertussis toxin-insensitive G_q-like G-proteins. It is becoming evident that the intrinsic GTP hydrolytic properties of the different $G\alpha$ subtypes may differ. Indeed, the intrinsic GTPase activity of purified $G_s\alpha$ is lower than that of purified $G_i\alpha$ (Gilman *et al.*, 1987) and purification data suggests that the $G_q\alpha/G_{11}\alpha$ proteins do bind GTPγS with slow rates. Thus, with these caveats, receptor-stimulated high affinity GTPase activity is a useful indication of a G-protein coupled receptor.

1.1.2. The structural features of G-protein coupled receptors.

Although G-protein linked receptors have numerous structural adaptations to their extracellular domains to allow them to respond to diverse signals, characterisation by amino acid sequence has shown that all these receptors are likely to share the same basic structure in which seven hydrophobic segments of 20-25 amino acids in length form transmembrane helices. Protease digestion studies and detailed immunological mapping have provided supporting evidence for the existence of seven transmembrane

spanning domains linking an extracellular N-terminus and an intracellular C-terminus (Dohlman *et al.*, 1987).

Figure 1.1 shows a scheme in which the receptor has been flattened to simplify description of its primary features. I and II; The tertiary structure and relative positioning of the transmembrane segments depends not only on connecting extra- and intracellular loops, but also on interactions among transmembrane segments. Co-expression of an N-terminal half (N-terminus plus TM's I-V plus part of the 3rd intracellular loop) and a C-terminal half lead to an active receptor (Kobilka *et al.*, 1988); proteolytic digestion of purified receptor protein leads to a limit digest formed of all seven TM's (Rubenstein *et al.*, 1987); mutations that interfere with TM-I: TM-VII interaction prevent receptor folding (Suryanaryana *et al.*, 1992).

A) The NH_2-terminus.

The higher apparent molecular mass of receptors than that predicted from their amino acid sequences suggests that many are glycosylated. This is supported by the observations that nearly all G-protein coupled receptors have one or more asparagine residues (usually in the N-terminal region), which are present in glycosylation consensus sequences. Direct evidence for glycosylation has been shown for a number of receptors, including the purified β_2-adrenoceptor, in which endoglycosidase treatment decreases its molecular mass from some 65 kDa to 49 kDa (Rands *et al.*, 1990). For most receptors it is believed that glycosylation has little role in agonist binding but that it may be of importance in determining the correct distribution of the receptor in the cell. For example, treatment of the β_2-adrenoceptor with endoglycosidases or mutational alteration of the two asparagine residues predicted to undergo glycosylation has little effect on agonist or antagonist binding or coupling of the receptor to adenylyl cyclase (O'Dowd *et al.*, 1989).

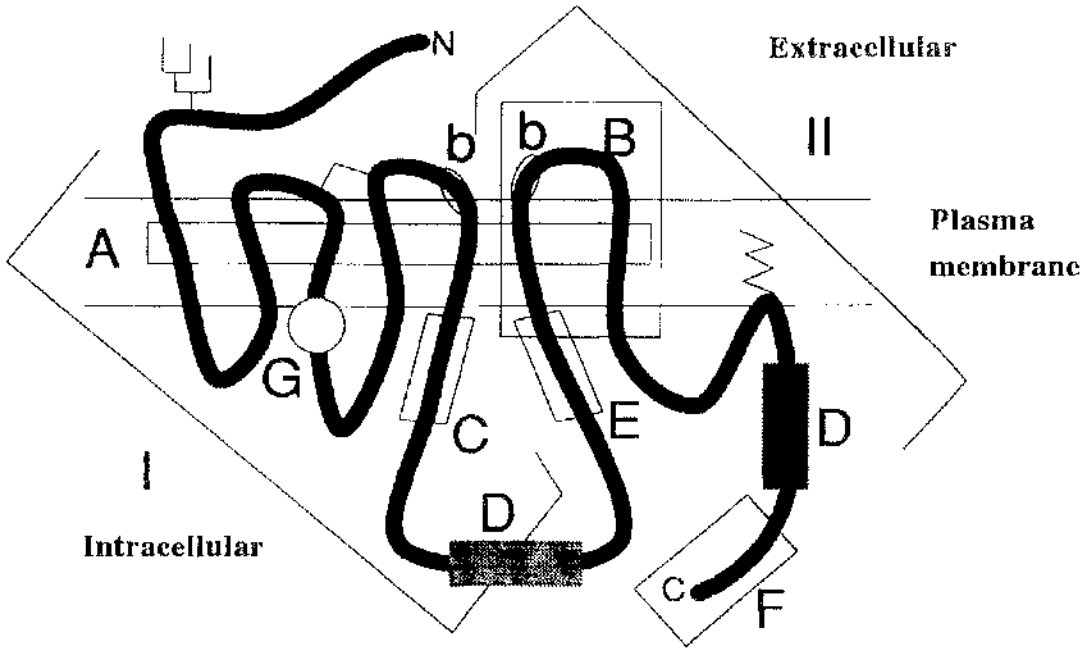
B) Cysteine disulphide bridges.

The presence of a cysteine residue towards the carboxy end of the first extracellular loop and a second one towards the middle of the second extracellular loop is a constant finding in many G-protein coupled receptors. Direct chemical evidence

Figure 1.1.

Common structural features of G-protein coupled receptors.

A schematic diagram of the seven transmembrane helices of a G-protein coupled receptor is adapted from Birnbaumer *et al.*, 1995. The structural elements and their functions are discussed in section 1.1.2.



has been obtained for a disulphide linkage between these two cysteine residues in rhodopsin and it has been shown that this is essential for achieving the correct tertiary structure (Karnik *et al.*, 1988). Two further cysteine residues located next to each other in the third extracellular loop of the β_2-adrenoceptor are also linked by a disulphide bond (Ostrowski *et al.*, 1992).

C) Ligand binding domains.

The seven hydrophobic domains represented in box A participate in the formation of the ligand binding pocket. Small hormone and neurotransmitter molecules, such as catecholamines, acetylcholine, histamine and serotonin are assumed to interact with the transmembrane domains to trigger a response (Strader *et al.*, 1989). Photoaffinity labeling studies have been used to identify the ligand binding site of the turkey β-adrenoceptor, the hamster β_2-adrenoceptor (Dohlman *et al.*, 1988), and the human platelet α_2-adrenoceptor (Matsui *et al.*, 1989).

In the β_2-adrenoceptor, the carboxyl group on the conserved aspartate residue in TM-III is postulated to act as a counter-ion for the catecholamine nitrogen in the presence of an agonist or antagonist containing a protonated amine group, like isoprenaline and propranolol. Two conserved serines (204 and 207) in TM-V of the receptor were found to form a hydrogen bond with the para- and meta- hydroxyl of isoprenaline respectively (Strader *et al.*, 1989). This gives a model of isoprenaline binding to the β_2-adrenoceptor through a carboxy-amine salt bridge at aspartate 113 and hydrogen bonds at serines 204 and 207.

Additional studies of the ligand binding domain have suggested that antagonists and agonists bind to distinct sites, and although these sites are not identical there is likely to be some overlap. Site B (Figure 1.1) on transmembrane region VI and VII define specificities for antagonists in biogenic amine receptors. A chimera that comprises the β_2-adrenoceptor for TM's I-V and the α_2-AR for TM's VI-VII (plus C-terminal tail) activates adenylyl cyclase (β_2-effect) but is blocked by yohimbine (α_2-adrenoceptor antagonist) (Kobilka *et al.*, 1988). In area b, antagonist binding may be more important than B domain (Figure 1.1). For example, a non-peptide NK1

tachykinin receptor antagonist, interacts with epitopes that are on top of both TM-VI and TM-VII, even though neither epitope is required for NK1 (substance P) binding (Gether *et al.*, 1993).

Although the general features of ligand binding are beginning to be mapped, the residues involved in determining ligand specificity and receptor subtype ligand selectivity remain to be fully defined.

D) G-protein coupling domains.

The first and second cytoplasmic loops are reasonably well conserved between different receptors, but the third cytoplasmic loop and the carboxy terminal tail are quite divergent, possibly reflecting a molecular basis for variable ligand and G-protein specificity (Cotecchia *et al.*, 1990; Kobilka *et al.*, 1988).

The N-terminal segment of the 3rd intracellular loop (domain C) may be involved in defining specificity of G-protein interactions (Figure 1.1). Replacement of 17 amino acids of the N-terminal end of intracellular loop 3 of the PLC activating M3 muscarinic acetylcholine receptor with the cognate 16 amino acid stretch of the adenylyl cyclase inhibiting M2 muscarinic acetylcholine receptor resulted in loss of PLC stimulation and acquisition of adenylyl cyclase inhibitory activity to a level that was 25% of control (Wess *et al.*, 1990).

Domain E within the carboxy terminus of the third intracellular loop may also be a potential protein: protein interaction site for receptor: Gα interactions (Figure 1.1). Point mutations in the C-terminal end of the 3rd intracellular loop may lead to constitutive (agonist independent) activation of receptors as shown by artificial mutations of the α_{1B}-(Cotecchia *et al.*, 1990; Kjelsberg *et al.*, 1992), α_{2A}- (Ren *et al.*, 1993) and β_2-adrenoceptors (Samama *et al.*, 1993). Two natural mutations of the TSH receptor in this region result in a similar effect (Parma *et al.*, 1993). Peptides derived from this region of receptors can activate purified G-proteins *in vitro* (Dalman *et al.*, 1991; Cheung *et al.*, 1991). Although the third intracellular loop appears to be essential for G-protein coupling, the first and second loops may also play a minor role

(O'Dowd *et al.*, 1988; Cotecchia *et al.*, 1992), possibly through allosteric interactions with the third intracellular loop and other components of the receptor.

The C-terminal tail (designated F in figure 1.1) may also contribute to interaction with G-proteins for some receptors such as the EP3-prostaglandin receptor (Sugimoto *et al.*, 1993).

A "DRY" motif (consensus D/e-R-Y/f/h/c of which the R is invariant) designated as G (Figure 1.1) is found in some 200 G-protein coupled receptors. Exceptions exist however, including receptors for the glucagon-related peptides (glucagon, secretin, vasoactive intestinal peptide (VIP) type 1 and 2, pituitary adenylyl cyclase activating peptide (PACAP)). The DRY motif is thought to be important for coupling to and activation of G-proteins but this cannot be an invariant feature because of the exceptions noted above. Mutation of this "R" in the vasopressin receptor type 2 (VP2) results in loss of coupling to $G_S\alpha$ without changes in agonist binding or loss of sequestration in response to hormone binding (Rosenthal *et al.*, 1993).

E) The carboxy (COOH-) terminus.

The β_2-adrenoceptor and rhodopsin have one and two cysteine residues, respectively, in their C-terminal sequence which have been shown to bind palmitate via a thioester linkage (O'Dowd *et al.*, 1989). It is believed that the covalently bound palmitate becomes intercalated into the membrane bilayer, thereby creating a 'fourth cytoplasmic loop'. Removal of the cysteine residue in the β_2-adrenoceptor uncouples the receptor from its associated G-protein, $G_S\alpha$ (O'Dowd *et al.*, 1989; Moffett *et al.*, 1993), but equivalent mutations caused no functional effect in rhodopsin (Karnik *et al.*, 1988) or the α_{2A}-adrenoceptor (Kennedy *et al.*, 1993). The presence of one or two cysteines in a similar position in most other G-protein coupled receptors suggests that this may be a common structural feature.

F) Regulation of receptor function.

Responses to G-protein coupled receptors undergo rapid desensitisation following prolonged exposure to agonists and there is a great deal of evidence to support a role of protein phosphorylation in this process (Hausdorff *et al.*, 1990).

Serine-threonine rich regions in either or both the 3rd intracellular loop and the C-terminal tail as shown in figure 1.1 (designated as D) are substrates for G-protein coupled receptor kinases (GRKs) and second messenger activated protein kinases such as protein kinase A (PKA) and C (PKC). These sites are thought to be exposed upon activation of receptor by agonist. Except for rhodopsin kinase (also named GRK1) which is anchored to the membrane through C-terminal polyisoprenylation and phosphorylates light-activated rhodopsin without requiring other proteins, the other GRKs appear to phosphorylate G-protein coupled receptors only in the context of an HR-$\beta\gamma$ complex (Inglese *et al.*, 1993; Lohse *et al.*, 1992; Haga *et al.*, 1992; Benovic *et al.*, 1989).

Exposure of the β_2-adrenoceptor to agonist is followed by a rapid uncoupling of the receptor from $G_s\alpha$ and desensitization mediated by protein kinase A (PKA) (Benovic *et al.*, 1985) and β-adrenoceptor kinase (βARK) (also named GRK2) (Benovic *et al.*, 1987). Phosphorylation of the receptor by PKA interferes with the interaction of the receptor and $G_s\alpha$. Phosphorylation of the receptor by βARK is not sufficient to interfere with the activation of $G_s\alpha$ by the receptor. β-arrestin recognizes βARK phosphorylated receptor and blocks activation of $G_s\alpha$ (Lohse *et al.*, 1990; 1993). With sustained exposure to agonist the receptor may then be removed from the plasma membrane by sequestration and/or down-regulation. Sequestration is the rapid (within five minutes), reversible removal of functional receptors from the plasma membrane to distinct membrane vesicles. On removal of agonist the receptor quickly returns to the plasma membrane and is fully functional (Hertel *et al.*, 1983). Down-regulation, on the contrary, occurs more slowly than sequestration and leads to the irreversible loss of receptors from the plasma membrane. The rate of receptor loss is greatest during the first four hours of down-regulation (Bouvier *et al.*, 1989). Within 24 hours, the process usually approaches a new steady state. However, it is not entirely clear whether receptor sequestration and down-regulation are distinct processes mediated via different pathways, or if the two processes are sequential.

Apparently several different mechanisms contribute to the process of down-regulation. Down-regulation of the β_2-adrenoceptor can be mediated via reduction of the receptor mRNA (Hadcock *et al.*, 1988; Bouvier *et al.*, 1989). This reduction is due to a destabilization of the mRNA since it can also be observed after inhibition of mRNA synthesis by actinomycin D (Hadcock *et al.*, 1989). Agonist-induced reduction of receptor mRNA has been described for a number of G-protein coupled receptors including the TSH receptor (Akamizu *et al.*, 1990), α_{1B}-adrenoceptor (Izzo *et al.*, 1990), M1, M2, M3-muscarinic acetylcholine receptors (Wang *et al.*, 1990) and the thyrotropin releasing hormone (TRH) receptor (Fujimoto *et al.*, 1992). It is not yet clear whether this agonist-induced mRNA destabilization is agonist specific, or whether it is protein kinase mediated.

The marked increase in intracellular cAMP observed following agonist binding to the β_2-adrenoceptor leads to the activation of many cellular substrates, including members of CRE-binding protein (CREB) family of transcription factors (Karpinski *et al.*, 1992). Activation of CREB, through a cascade of events, results in its binding to the cAMP response element (CRE) in the 5' promotor region of the β_2-adrenoceptor gene (Collins *et al.*, 1990), which in turn increases the rate of transcription, leading to a transient increase in receptor expression. This could appear to be counter to the concept of agonist-mediated down-regulation of the receptor but the increase in receptor transcriptional rate is transient.

1.2. Guanine nucleotide binding proteins (G-proteins).

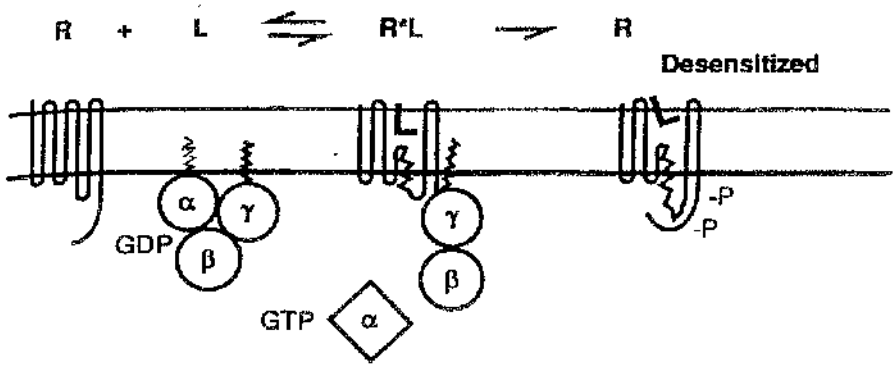
1.2.1. Signal transduction mediated by G-protein subunits.

A large number of neurotransmitters, peptide hormones, neuromodulators, growth factors and ordorants induce changes in cellular metabolism by interaction with cell membrane associated receptors that are coupled to intracellular effector enzymes by guanine nucleotide binding regulatory proteins (G-proteins). G-proteins, which are heterotrimeric proteins composed of α, β and γ subunits encoded by distinct genes,

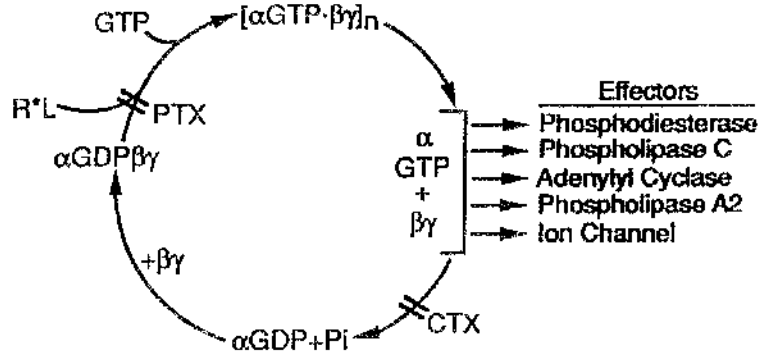
Figure 1.2.

Signal transduction mediated by G-protein subunits.

A: Model of G-protein dependent transmembrane signaling.



B: The G-protein cycle.



An activated receptor (R*) produced by a specific ligand (L) binding to its appropriate receptor (R) can catalyze the exchange of GTP for GDP bound to the α subunit of a specific G-protein, and the subsequent separation of α-GTP from the βγ complex. Activated α subunits (α-GTP) and βγ complex can interact with different effectors. Wavy lines emerging both from α and γ subunits denote myristoylation or palmitoylation and prenylation respectively. The receptor is desensitized by specific phosphorylation (-P). Pertussis toxin (PTX) blocks the catalysis of GTP exchange by the receptor while cholera toxin (CTX) blocks the GTPase activity of some α subunits fixing them in an activated form. The figures are adapted from Simon *et al.*, 1991.

are localized to the inner surface of the plasma membrane where they serve as receptor-mediated signal transducers.

A model for G-protein signal transduction is described in Figure 1.2. When GDP is bound the α subunit associates with the βγ subunit to form an inactive heterotrimer that binds to the receptor. Monomeric GDP-liganded α subunits can interact with receptors but their association is greatly enhanced by βγ. Activation follows interaction with ligand bound receptor and the GDP-liganded α subunit responds with a conformational change that decreases GDP affinity, so that GDP comes off the active site. The guanine nucleotide release proteins (GNRPs) catalyse release of bound GDP, promoting its replacement with GTP in a Mg^{2+} dependent manner (Brandt *et al.*, 1986). The GNRP for the signal transducing G-proteins can be considered as a contribution from both the ligand-activated receptor and the βγ subunit. The binding of GTP reduces the affinity of the receptor for the agonist, resulting in a Mg^{2+} dependent dissociation of the Gα-GTP both from the receptor and the βγ. Agonists act to reduce the concentration of Mg^{2+} required for activation (Iyengar *et al.*, 1982). The free Gα-GTP (active conformation) and βγ subunits can specifically interact with effector proteins such as adenylyl cyclase, phospholipases, ion channels etc. The activated state lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α subunit (Rodbell *et al.*, 1980). The intrinsic rate of GTP hydrolysis varies greatly from one type of α subunit to another. K_{cat} values of 2-5 min^{-1} have been reported for $G_s\alpha$ and $G_i\alpha$ (Brandt *et al.*, 1986; Linder *et al.*, 1990; Carty *et al.*, 1990) while considerably lower values have been detected for $G_q\alpha$ (K_{cat} 0.8 min^{-1}) (Berstein *et al.*, 1992) and $G_z\alpha$ (K_{cat} 0.05 min^{-1}) (Casey *et al.*, 1990). Once GTP is hydrolysed to GDP, the α and βγ subunits reassociate, become inactive, and return to the receptor. Although the βγ subunit does not bind GTP its active lifetime depends on the rate of GTP hydrolysis by an α subunit.

Fluoride (present as AlF_4^-) is a GTP-independent G-protein activator because it binds the nucleotide site adjacent to the β-phosphate of GDP. This mimics the γ-phosphate of bound GTP and triggers G-protein activation (Bigay *et al.*, 1985). In

addition, poorly-hydrolyzed analogues of GTP such as guanosine-5'-o-(3-thiotriphosphate) (GTPγS) and guanylyl-imidodiphosphate (Gpp(NH)p) also produce G-protein activation due to their resistance to GTPase activity although the reaction still requires Mg^{2+} (Codina *et al.*, 1983).

Cholera toxin (CTX) and pertussis toxin (PTX) interfere with the GTPase cycle as indicated in Figure 1. 2B. Cholera toxin (from *Vibrio cholerae*) transfers the ADP-ribose moiety from NAD^+ to a conserved Arg residue within $G_s\alpha$ and $G_t\alpha$. This modification requires the presence of an additional protein, ADP-ribosylating factor (ARF) (Donaldson *et al.*, 1994) and supresses GTPase activity, thereby locking the G-protein in the active GTP bound conformation (Kahn *et al.*, 1984). Pertussis toxin (from *Bordetella pertussis*) ADP-ribosylates G-protein α subunits at a Cys located four residues from the C-terminal of $G_{i1-3}\alpha$, $G_o\alpha$ and $G_t\alpha$. This modification appears dependent upon an $\alpha\beta\gamma$ heterotrimeric state and results in G-protein uncoupling from receptor (Navon *et al.*, 1987).

1.2.2. Structure of G-proteins.

A) α subunits.

Models of G-protein α subunits have been proposed based on the crystal structure of GTP- and GDP-liganded transducin and $G_{i1}\alpha$ and allowed identification of potentially important residues in guanine nucleotide binding and the GTP hydrolysis reaction (Noel *et al.*, 1993; Lambright *et al.*, 1994; Coleman *et al.*, 1994).

The structure of the GTP binding domain comprises of a hydrophobic core of alternating β-sheets and hydrophilic α-helices. This structure has been implicated in regulating both guanine nucleotide release (GDP) and the binding and hydrolysis of GTP. A key residue (Arg-178 in $G_t\alpha$) is needed for GTP hydrolysis and so help to set the GTPase activity (Conklin *et al.*, 1993b). The helical domain is postulated to restrict movement out of and into the guanine nucleotide binding pocket of the core domain. To promote the replacement of GDP by GTP the activated receptor potentially moves the helical domain, via a conformational change, allowing the exchange of guanine

nucleotides. The helical domain may also contribute to the effector binding site (Coleman *et al.*, 1994), along with other regions of the GTPase domain but its function is still not clear.

The N-terminal region of G-protein α subunits is important for membrane anchorage, lipid modification and interaction with βγ subunits. Tryptic digestion releases soluble α subunits missing an N-terminal fragment of 1-2 kDa, suggesting an important role for this region in membrane anchorage (Eide *et al.*, 1987). Proteolytic cleavage of the 20-29 N-terminal residues of $G_t\alpha$ and $G_o\alpha$ or deletion of 2-29 from $G_s\alpha$ inhibits α subunit interaction with βγ subunits and receptor dependent activation (Navon *et al.*, 1987; Neer *et al.*, 1988; Journot *et al.*, 1991). Mutational studies of the N-terminal 20 residues of $G_o\alpha$ have suggested that residues 7-10 have an important role in βγ interaction independent of myristoylation (Denker *et al.*, 1992a).

The effector binding region has been mapped only for the pairs $G_s\alpha$/adenylyl cyclase and $G_t\alpha$/cGMP phosphodiesterase (Conklin *et al.*, 1993b). The effector binding region of $G_s\alpha$ includes and partially overlaps the putative βγ binding surface. Therefore, it is unlikely that the α subunit can simultaneously bind effector and βγ. Further, when an effector, such as type 2 adenylyl cyclase, is activated both by α and by βγ subunits, it is likely that α and βγ bind to distinct sites on the enzyme.

The C-terminus is an important site of interaction with receptors and in defining the specificity of G-protein-receptor interactions (Conklin *et al.*, 1993a; Neer *et al.*, 1994a). An activated receptor triggers an intracellular response by dramatically decreasing the affinity of the α subunit for GDP, perhaps by moving or twisting the C-terminal α helix to loosen the grip of the α subunit on GDP. This effect is mimicked by deletion of 14 amino acids from the C-terminus of $G_o\alpha$ (Denker *et al.*, 1992b). The $G_s\alpha$ point mutation R389P (responsible for the *unc* S49 phenotype) located six residues from C-terminus results in a G-protein uncoupled from receptor (Sullivan *et al.*, 1987). In addition, mutational replacement of α subunit C-terminal residues alters specificity for receptor interaction (Cerione *et al.*, 1986). For example, the $G_{16}\alpha$ subunit interacts with the complement C5a receptor, while the $G_{11}\alpha$

subunit does not. Chimeras of $G_{11}\alpha/G_{16}\alpha$ with a large portion of the $G_{16}\alpha$ C-terminus did not function as $G_{11}\alpha$ unless an additional region of $G_{16}\alpha$ (residues 220-240) was present (Lee *et al.*, 1995).

B) β and γ subunits.

The β subunit is predicted to contain two types of structures: an N-terminal region thought to form an amphipathic α helix (Lupas *et al.*, 1992) followed by seven repeating units of approximately 40 amino acids (WD-40 repeat) (Simon *et al.*, 1991). The WD-40 repeat, $\{X_{6\text{-}94}\text{----[GH---}X_{23\text{-}41}\text{--WD]}\}^{N4\text{-}8}$ (X = variable length loop; WD = constant length core), consists of a conserved core of 23-41 residues usually bounded by Gly-His and Trp-Asp (Neer *et al.*, 1994b).

The β and γ subunits bind very tightly to each other and can only be separated by denaturants. The putative α helix at the N-terminus of β makes up part, but not all, of the βγ interaction site (Lupas *et al.*, 1992; Garritsen *et al.*, 1994). The N-terminal portion of β must lie close to γ because it contains a Cys that can be cross-linked to a Cys in γ (Bubis *et al.*, 1990). However, selectivity (the ability of β1 to dimerize with γ1 but not γ2) is determined by multiple sites in the WD repeat region, especially residues 215-255 in repeat 5 (Pronin *et al.*, 1992; Katz *et al.*, 1995).

The γ subunit is predicted to be largely α helical (Lupas *et al.*, 1992). The γ subunit is extended along the repeating units of β subunit and held in place by the N-terminal α helix of the β subunit. The prenyl group at the C-terminal of γ is likely to be on the outer surface of βγ because it is essential for membrane attachment (Casey *et al.*, 1994). Selectivity of the γ subunits for different β subunits is determined by a stretch of 14 amino acids in the middle of the γ subunit (Spring *et al.*, 1994). This 14 amino acid region contains the Cys that can be cross-linked to β (Bubis *et al.*, 1990). Because the specificity region of γ is in the middle of the γ molecule, βγ could be oriented C-terminus to N-terminus or N-terminus to N-terminus.

The α subunit seems to be able to interact with γ as well as β (Rahmatullah *et al.*, 1994), so perhaps the surface of βγ that binds α subunit to βγ blocks the ability of βγ to stimulate effectors, either because Gα–GDP induces a conformational change

in βγ, or because it covers the site of βγ-effector interaction. The relationship of the Gα–GDP binding site on βγ to the effector-binding site remains to be defined.

1.2.3. Membrane association of G-proteins.

A) Myristoylation and palmitoylation of α subunits.

G-protein α subunits are localized predominantly to membrane fractions despite their hydrophilic nature. It has been suggested that βγ complexes may serve as a hydrophobic anchor holding hydrophilic α subunits (Sternweis *et al.*, 1986). Interestingly, some α subunits ($G_o\alpha$, $G_{i1\text{-}3}\alpha$, $G_t\alpha$, $G_{gust}\alpha$ and $G_z\alpha$) have recently been shown to undergo co-translational modification by addition of the 14-carbon fatty acid myristate on glycine 2 after cleavage of the N-terminal methionine. Transducin undergoes a similar N-terminal modification although by heterogenous fatty acids. The consensus sequence for fatty acylation is unclear but includes a prerequisite for glycine 2 and a preference for a hydroxyamino acid such as serine at position 6 (MGXXXS) (Jones *et al.*, 1990). Mutagenenesis of glycine 2 to alanine in both $G_{i1}\alpha$ and $G_o\alpha$ results in non-myristoylated α-subunits whose localization is shifted from the plasma membrane to the cytosol, suggesting myristoylation to be necessary for membrane attachment and to facilitate binding of βγ (Mumby *et al.*, 1990; Casey *et al.*, 1994).

However, myristoylation is an irreversible modification and thus does not serve a regulatory role. The G_q, G_{12} and G_s families of G-protein α subunits are membrane bound although they lack the consensus sequence (MGXXXS) for myristoylation (Wu *et al.*, 1992a). For these (and other) α subunits, palmitoylation of cysteine residues within the N-terminal region has been recorded (Parenti *et al.*, 1993; Degtyarev *et al.*, 1993a, b; Milligan *et al.*, 1995a). In contrast to myristoylation, palmitoylation is reversible. Activation of the β_2-adrenoceptor leads to rapid depalmitoylation of $G_s\alpha$, and depalmitoylated $G_s\alpha$ does not activate adenylyl cyclase. Depalmitoylation might thus be a mechanism to turn off $G_s\alpha$ and so to desensitize the cell to β_2-adrenergic stimulation (Mouilliac *et al.*, 1992).

B) Prenylation and carboxymethylation of γ subunits.

G-protein β and γ subunits remains tightly associated under non-denaturing conditions and γ subunits have been shown to be isoprenylated in the Cys-A-A-X, (A= aliphatic residue, X=unspecified) of the C-terminus which increases protein hydrophobicity. Firstly, a long chain isoprenyl group is attached to the Cys after which a protease cleaves the AAX residues and the Cys is then carboxymethylated (Fung *et al.*, 1990). Different γ subunits are modified with different isoprenyl groups as the rod photoreceptor γ subunit (γ1) is modified with the C15 farnesyl moiety whereas brain γ subunit (γ2) has a C20 geranylgeranyl modification (Fukada *et al.*, 1990; Mumby *et al.*, 1990). Blocking isoprenoid synthesis or mutagenesis of the crucial Cys in the γ subunit prevents C-terminal processing and results in production of a βγ dimer which is both localized to the soluble fraction and functionally inactivated (Katz *et al.*, 1992). Prenylation of γ subunits is not necessary for βγ formation, but is necessary for membrane attachment of the βγ dimer and, perhaps, for association with the α subunit.

1.2.4. Diversity of G-proteins and interaction with effectors.

A) Gα subunits.

Molecular cloning and sequencing techniques have been most productive in identifying and classifying new α subunits. Mammalian α subunits can be grouped into four classes based on amino acid identity (Figure 1.3).

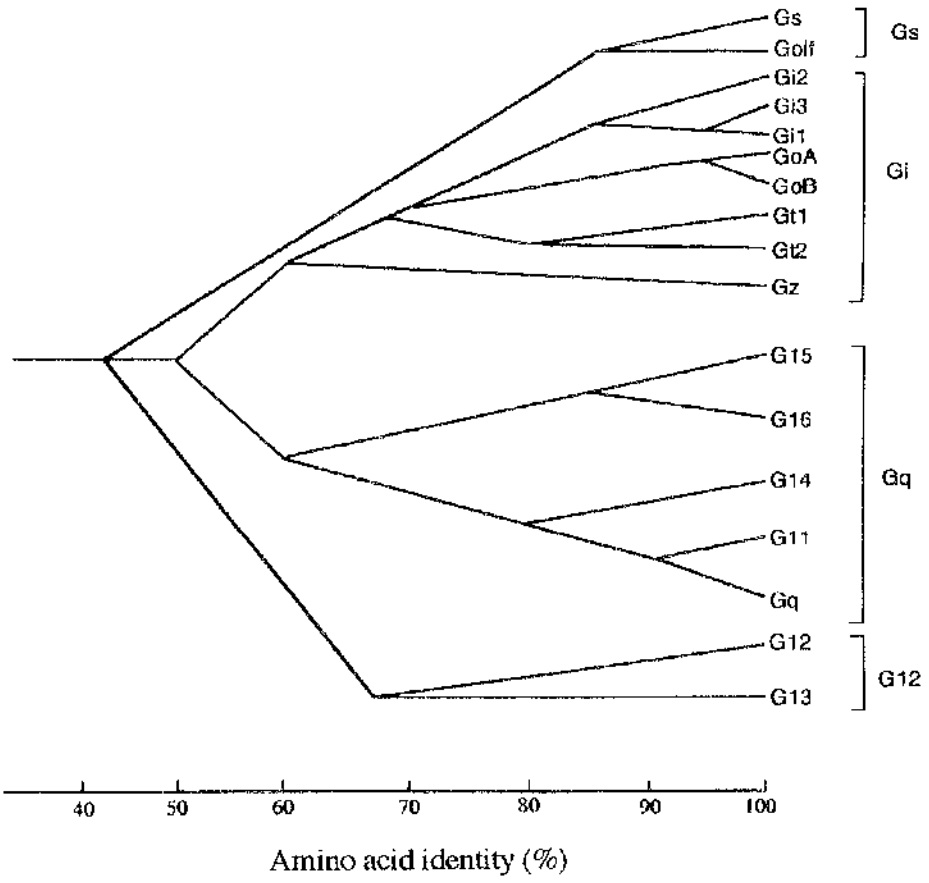
a) Gs family.

The G_S family includes both the $G_S\alpha$ and $G_{olf}\alpha$ isotypes, which are adenosine diphosphate (ADP)-ribosylated by cholera toxin (CTX). So far, four molecularly distinct forms of $G_S\alpha$ resulting from differential splicing of a single gene have been identified (Bray *et al.*, 1986). The $G_S\alpha$ gene contains 13 exons and while all become incoporated into the long forms ($G_S\alpha$-L), exon 3, encoding 15 amino acids, is excluded from the short form ($G_S\alpha$-S). These splice variants probably account for the diverse molecular weight forms of $G_S\alpha$ but spliced forms appear similarly active. Although $G_S\alpha$ was identified as the G-protein required for receptor-

Figure 1.3.

Relationship between mammalian Gα subunits.

The α subunits are grouped according to amino acid sequence identity with branch junctions corresponding to approximate comparative values for the α subunits indicated. The splice variants of $G_s\alpha$ and Ggustα are not included. This diagram is adapted from Simon *et al.*, 1991.



mediated stimulation of adenylyl cyclase, the protein has also been shown to couple to activation of dihydropyridine-sensitive Ca^{2+} channels in cardiac (Mattera *et al.*, 1989) and skeletal muscle (Yatani *et al.*, 1987a), inhibition of the Ca^{2+} pump in liver membranes (Jouneaux *et al.*, 1993) and inhibition of Na^{+} channels in cardiac myocytes (Schubert *et al.*, 1989).

$G_{olf}\alpha$ shows 88% amino acid homology to $G_s\alpha$. Expression of $G_{olf}\alpha$ is restricted to specific neural tissues and it is enriched in neurons in the olfactory neuroepithelium where it is responsible for olfactory regulation of adenylyl cyclase (type 3) (Jones *et al.*, 1987). $G_{olf}\alpha$ is also co-localized with D1 dopamine receptors in striatonigral neurons and with adenylyl cyclase type 5 in corpus striatum (Wong *et al.*, 1992).

b) Gi family.

Members of G_i family of α subunits contain sites susceptible to modification by pertussis toxin (PTX) (except for $G_z\alpha$) and are therefore expected to mediate activation of PTX-sensitive processes. The G_i family is composed of at least three closely related G_i-like α subunits ($G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$), $G_o\alpha$, $G_t\alpha$ and $G_z\alpha$.

G_i-like G-proteins are widely distributed and are highly expressed in the central nervous system and neuronal cell lines (Kim *et al.*, 1988). Sensitivity to PTX as well as reconstitution and heterologous expression have suggested several roles including adenylyl cyclase inhibition (Wong *et al.*, 1992), PLC-dependent phosphoinositide hydrolysis (Ohta *et al.*, 1985), K^{+} channel activation (Yatani *et al.*, 1988; Codina *et al.*, 1987), and inhibition of Ca^{2+} channel opening (Linder *et al.*, 1990). In addition, $G_{i3}\alpha$ stimulated activity of Na^{+} channels via an activation of phospholipase A_2 in retinal epithelial cells (Ausiello *et al.*, 1992) and of large conductance renal apical Cl^{-} channels has been noted (Schwiebert *et al.*, 1990). $G_{i2}\alpha$ has been reported to be phosphorylated by PKC (Yatomi *et al.*, 1992) and to interact with mitogen-activated protein (MAP) kinase with mediation via $\beta\gamma$ (Crespo *et al.*, 1994).

$G_o\alpha$ is the major G-protein in brain, where it constitutes about 1% of membrane protein (Neer *et al.*, 1984). Closely related to the G_i-like group, nevertheless, its kinetics of guanine nucleotide binding is about an order of magnitude faster than that determined for the G_i-like proteins. Two variants forms ($G_{oA}\alpha$ and $G_{oB}\alpha$) both with 354 residues, result from differential RNA splicing of a single $G_o\alpha$ gene with the C-terminal 113 amino acids encoded by alternative use of duplicated exons 7 and 8 (Kaziro *et al.*, 1991). The function of $G_o\alpha$ remains unsolved, the majority of the evidence suggests that this G-protein is involved in the regulation of receptor-mediated inhibition of voltage operated Ca^{2+} channels (Heschler *et al.*, 1987; Kleuss *et al.*, 1991). Recent studies have demonstrated selective interactions of $G_o\alpha$ splice variants with different receptors, β- and γ-subunits (Kleuss *et al.*, 1992; 1993). A role for $G_o\alpha$ coupling to the α_{1B}-adrenoceptor to cause stimulation of PLC-dependent phosphoinositide hydrolysis (Blitzer *et al.*, 1993) and in K^+ channel activation have also been reported (Kobayashi *et al.*, 1990).

Two forms of transducin, transducin 1 ($G_{t1}\alpha$) and 2 ($G_{t2}\alpha$), were found in retinal rod and cone cells respectively (Lerea *et al.*, 1986). These mediate activation of rod- and cone-specific cyclic GMP phosphodiesterases following rhodopsin and opsin photoactivation (Stryer *et al.*, 1986a, b; 1991). A novel transducin-like G-protein, gustducin ($G_{gust}\alpha$) has been identified from tongue where it is enriched in circumvallate, foliate and fungiform papillae (McLaughlin *et al.*, 1992) and it is considered that a cyclic GMP phosphodiesterase may be involved in its means of signal transduction, due to sequence homology in those regions of G-protein α subunits known to interact with receptor and effectors (Hoon *et al.*, 1995). $G_t\alpha$ and $G_{gust}\alpha$ each have the amino acid sites for ADP-ribosylation by both PTX and CTX.

The $G_z\alpha$ subunit is found predominantly in brain and platelets (Fong *et al.*, 1988), however, it mediates inhibition of adenylyl cyclase (types 1 and 5) in a PTX-insensitive manner (Wong *et al.*, 1992; Taussig and Gilman 1995)). $G_z\alpha$ exhibits biochemical properties distinct from other G-protein α subunits, including unusual Mg^{2+} ion dependence and very slow guanine nucleotide exchange and GTPase

activity (Casey *et al.*, 1990). In a series of experiments, it has been shown that $G_z\alpha$ becomes phosphorylated when platelets are activated by phorbol ester or thrombin (Carlson *et al.*, 1989; Loundsbury *et al.*, 1991), suggesting that $G_z\alpha$ is a substrate for PKC. Subsequent reconstitution studies and site-directed mutagenesis of $G_z\alpha$ demonstrated that PKC stoichiometrically phosphorylates $G_z\alpha$, predominantly on a serine residue near the N-terminus, and that this effect is selective for $G_z\alpha$, since PKC did not lead to phosphorylation of $G_i\alpha$ or $G_q\alpha$ (Loundsbury *et al.*, 1993).

c) Gq family.

The G_q family contains the widely distributed $G_q\alpha$ and $G_{11}\alpha$ (Strathmann *et al.*, 1990) as well as $G_{14}\alpha$, $G_{15}\alpha$ and $G_{16}\alpha$ which are more restricted in distribution. They lack the site for PTX modification and thus mediate agonist stimulated hydrolysis of membrane phosphoinositides by PLC-β1, PLC-β2, PLC-β3 (Wu *et al.*, 1992b; Lee *et al.*, 1992; Sternweis *et al.*, 1992) in a PTX-insensitive manner. $G_{14}\alpha$ is found primarily in stromal and epithelial cells (Wilkie *et al.*, 1991; Nakamura *et al.*, 1991), while $G_{15}\alpha$ and $G_{16}\alpha$ are found in cells of haematopoietic lineage. $G_{15}\alpha$ is found in murine B lymphocytes (Wilkie *et al.*, 1991) and $G_{16}\alpha$ in human T lymphocytes (Amatruda *et al.*, 1991). These are likely to represent species homologues of the same G-protein.

d) G12 family.

The G_{12} family includes $G_{12}\alpha$ and $G_{13}\alpha$. They are not substrates for PTX and like both G_s and G_q family members are not myristoylated. Both $G_{12}\alpha$ and $G_{13}\alpha$ are widely distributed (Strathmann *et al.*, 1991). $G_{12}\alpha$ has exhibited an increase in arachidonic acid mobilization via phospholipase A_2 (PLA_2) activity (Xu *et al.*, 1993) and coupling to thromboxane A2 (TXA2) and thrombin receptors in platelet membranes provides evidence that both proteins indeed play a functional role in receptor-mediated transmembrane signal transduction (Offermanns *et al.*, 1994a, b). The involvement of $G_{13}\alpha$ in activating Na^+-H^+ exchange has been suggested (Voyno-Yasenetskaya *et al.*, 1994). Other effector functions regulated by members of the G_{12} family however, remains to be elucidated.

B) β and γ subunits.

In mammals, four distinct β subunit isotypes have been found; β1 (Fong *et al.*, 1987); β2 (Fong *et al.*, 1987); β3 (Levine *et al.*, 1990); β4 (von Weizsacker *et al.*, 1992). They share more than 80% amino acid sequence identity and molecular mass in the range of 35-36 kDa. All of the β subunits share eight amino acid segments. Each segment shares a repetitive 40 amino acid sequence motif (WD-40 repeat). β1, β2 and β3 are ubiquitously expressed while β4 is abundant in brain and lung tissues. To date, seven γ subunits have been identified from a variety of tissues with molecular masses in the range of 6-10 kDa. γ1 is highly localized to rod cells of the retina (Gautam *et al.*, 1990), while other γ subunits are expressed at different levels in all tissues (Cali *et al.*, 1992).

β1 interacts with retinal $G_{t1}\alpha$ (Lerea *et al.*, 1986) and γ1 or γ3 (Peng *et al.*, 1992) to mediate stimulation of rod cell cyclic GMP phosphodiesterase by photoactivated rhodopsin (Stryer *et al.*, 1991). In GH_3 cells β1 interacts specifically with $G_{oB}\alpha$ and γ3 to form a G-protein mediating somatostatin receptor-dependent inhibition of Ca^{2+} channel activity (Kleuss *et al.*, 1991; 1992; 1993). β1 forms dimers with each of γ1, γ2, γ3 or γ5. β1γ1, β1γ2 and β1γ5 dimers stimulate phosphoinositide hydrolysis by PLC-β2 (Katz *et al.*, 1992). β2 forms dimers with either γ2 or γ3 subunits and the β2γ5 dimer can activate PLC-β2 (Wu *et al.*, 1993). Specific co-expression of β3 has demonstrated interaction with retinal $G_{t2}\alpha$ (Lerea *et al.*, 1989) and γ2 or γ3 to mediate stimulation of cone cyclic GMP phosphodiesterase by phosphoactivated opsin (Peng *et al.*, 1992). In GH_3 cells, β3 interacts with $G_{oA}\alpha$ and γ4 to induce muscarinic M4 receptor dependent inhibition of Ca^{2+} channel activity. It has also been reported that βγ activates a cardiac K^+ channel (I_{KACh}) which is activated by the M2 muscarinic acetylcholine receptor (Wickman *et al.*, 1995). In addition, βγ dimers regulate certain types of adenylyl cyclase; while βγ inhibits AC-1 activity, they stimulate AC-2 and AC-4 and have no effect on the others (AC-3, 5, and 6). Inhibition by βγ is independent of $G_s\alpha$, whereas stimulation by βγ has an underlying prerequisite for the stimulation of adenylyl cyclase by $G_s\alpha$ (Tang *et*

al., 1991; 1992). The βγ subunit may also act through $p21^{ras}$ or interact with raf-1 directly to activate mitogen-activated protein (MAP) kinase regulated pathways (Blumer *et al.*, 1994; Crew *et al.*, 1993; Crespo *et al.*, 1994; Pumiglia *et al.*, 1995). On the other hand, the βγ dimer forms a complex with G-protein coupled receptor kinases (GRKs) by binding to a βγ binding domain, which shares sequence homolgy with the pleckstrin homology (PH) domain. This results in translocation to a variety of G-protein coupled receptors (GPCRs) and their phosphorylation and inactivation (Inglese *et al.*, 1995). Phosphoinositide 3 kinase (PI3K) is another enzyme involved in signaling processes that can be modulated by βγ subunits. PI3K phosphorylates different substrates on the 3 position of the inositol ring and is regulated by several protein tyrosine kinases. Stimulation of PI3K activity by βγ subunits has been reported in platelets and neutrophils (Stephens *et al.*, 1994).

1.3. Specificity of receptor-G-protein-effector interactions.

A variety of different approaches have been used to define the specificity of coupling between receptors and effectors provided by G-proteins. The methods to address this issue include reconstitution of purified proteins (Yatani *et al.*, 1987a; Cerione *et al.*, 1991), transfection of cDNA (Conklin *et al.*, 1992; Cotecchia *et al.*, 1990), PTX sensitivity (Moriarty *et al.*, 1990; Nakamura *et al*, 1991), the use of C-terminal α subunit antibodies (McFadzean *et al.*, 1989; Milligan *et al.*, 1990), solubilization of native receptor-G-protein complexes followed by immunoblotting (Senogles *et al.*, 1987; Murray-Whelan *et al.*, 1992), agonist-dependent labeling of G-proteins with [^{32}P]GTP azidoanilide or [^{32}P]ADP-ribose plus bacterial toxins (CTX, PTX) (Milligan *et al.*, 1991a; Offermans *et al.*, 1990; Wange *et al.*, 1991) and selective antisense oligonucleotide technology (Kleuss *et al.*, 1991).

Reconstitution and transfection experiments may measure interactions between signaling components that are occurring unnaturally compared to native membranes but can be controled. The use of reconstitution and transfection methods can be exploited to explore the structural basis of receptor-G-protein-effector coupling by

using site-directed mutagenesis of the components. The use of selective antisense oligonucleotides was designed to perturb functional coupling of the specific components and this is suitable for determining the function of any protein provided that assay of its activity is possible in a single cell.

One cell is distinguished from another by the particular complement of receptors, G-proteins and effectors that it expresses, although the complement may change substantially with developmental or metabolic state. The precise stoichiometry among the signaling components can determine the predominant response pathway. For example, when the porcine M2 muscarinic receptor was transfected into CHO cells, the stimulation of phosphoinositol turnover was very dependent on receptor number, while inhibition of adenylyl cyclase was similar regardless of receptor number (Ashkenazi *et al.*, 1987). The results suggested that in these cells, adenylyl cyclase inhibition is limited by enzyme or G-protein, while PLC activation is limited by receptor number.

Conclusions about coupling specificity have been shown to depend on the level of receptor expression and the choice of cell used for transformation, including the type of G-proteins and effectors the cell contains.

1.4. Aims of research.

The experiments of this study were focused on two questions; one, the stoichiometry of interaction among the receptor, G-protein and effector species in individual cells and how does this relate to the absolute levels of expression of these polypeptides? Further, can the effectiveness of cellular signaling be altered by regulating the levels of expression of these components in a cell?

The other, how is the specificity and selectivity of interaction between receptors and effectors via G-proteins defined, especially in terms of the interaction of the long splice variant of the thyrotropin releasing hormone (TRH) receptor with species variants of the $G_{11}\alpha$ protein?

Chapter 2.

Materials and Methods.

2.1. Materials.

All reagents used in this study were of analytical or similar grade and were purchased from the following suppliers;

2.1.1. General reagents and Chemicals.

Appligene, Birtley, Durham.

Aquaphenol.

Boehringer Mannheim Ltd., Lewes, East Sussex, England.

Aprotinin, Bovine serum albumin (BSA), Creatine phosphate, Creatine-phosphokinase, Dithiothreitol, DNase free RNase, DOTAP (transfection reagent), Gpp(NH)p, GTPγS, Thymidine, Triethanolamine hydrochloride, Tris.

Calbiochem-Novabiochem Ltd., Beeston, Nottingham, England.

Pansorbin cells.

Cinna Biotecx, Texas, U.S.A.

RNAzol ^{TM} B.

Costal Scientific Corporation, Cambridge, MA, U.S.A.

Nitrocellulose (0.45 μM pore size).

Difco, Detroit, Michigan, U.S.A.

Bacto agar, Bacto tryptone, Yeast extract.

Formachem (Research International) plc., Strathaven, Scotland, U.K.

D-Glucose.

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

Acetic acid (glacial), Acrylamide, Folin-Ciocalteu's phenol reagent, N, N'-methylene bisacrylamide, Hydrogen peroxide.

Genetic Research Instrumentation Ltd., Essex, England.

Fuji RX X-ray film.

Gibco Life Technologies, Paisley, Scotland, U.K.

Agarose, 1 kb DNA ladder, 123 bp DNA marker, Lipofectin reagent, pSV2-neo, Ultrapure urea.

Invitrogen, San Diego, CA, U.S.A.

MC1061/P3, pcDNA1, pcDNA3.

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

Dimethylsulphoxide (DMSO), Magnesium sulphate, Sodium potassium tartrate.

May and Baker, Dagenham, England.

Ammonium persulphate.

National Diagnostics, Aylesbury, Buckinghamshire, England.

"Ecoscint" scintilation fluid.

Pharmacia Biotech Inc., U.S.A.

First strand cDNA synthesis kit.

Porton Products, Porton Down, Wiltshire, England.

Pertussis toxin.

Promega Ltd., Southampton, England.

Bacterial strain JM109, Calf intestinal alkaline phosphatase (CIAP), DNA Clean-Up system, DNA maxipreps, DNA minipreps, PCR preps, T4 Ligase, all restriction endonucleases.

Research Biochemicals International, Natick, MA, U.S.A.

Bromoacetyl alprenolol methane (BAAM).

Schering Health Care Ltd., Sussex, England.

Iloprost.

Scottish Antibody Production Unit, Lanarkshire, Scotland, U.K.

Horseradish peroxidase conjugated donkey anti-rabbit IgG.

Sigma Chemical Company, Poole, Dorset, England.

Alumina (neutral), Antipain, Ampicillin, Aprotinin, Arginine hydrochloride, ATP (disodium salt), Benzamidine, Bromophenol blue, Cholera toxin, Coomassie blue R-250, cAMP (sodium salt), Dowex AG50 W-X4 (200-400 mesh), [D-Ala^2, D-Leu^5]-Enkephalin (acetate salt) (DADLE), Forskolin, Geneticin, Gelatin (Type A), Imidazole, Isoprenaline, Low melting point agarose, Nicotinamide adenine

dinucleotide (NAD), N-ethylmaleimide, Nonidet P-40 (NP-40), 7-deoxycholic acid, Ortho-dianisidine dihydrochloride, Phenyl-methyl-sulfonyl fluoride (PMSF), Protein A Sepharose, Propranolol, Sodium azide, Soybean trypsin inhibitor, N, N, N', N',-Tetramethylethylene-diamine (TEMED), Thimerosal, Thymidine, Triton X-100, Trypsin, Prestained SDS Molecular Weight Markers consisting of α_2-Macroglobulin (180 kDa), β-Galactosidase (116 kDa), Fructose-6-phosphate kinase (84 kDa), Pyruvate kinase (58 kDa), Fumarase (48.5 kDa) Lactic dehydrogenase (36.5 kDa), Triosephosphate isomerase (26.6 kDa).

Whatman International Ltd., Maidstone, England.

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

All other reagents and chemicals used were analytical grade and obtained from **BDH (Dorset, Poole, England) and Fisons Scientific Equipment (Loughborough, Leicestershire, England).**

2.1.2. Radiochemicals.

Amersham plc., Amersham, Buckinghamshire, England.

[8-^{3}H]Adenosine 3', 5'-cyclic phosphate, ammonium salt, Adenosine 5'-[α-^{32}P] triphosphate, triethylammonium salt, [propyl-2, 3-^{3}H]Dihydroalprenolol, [12-^{3}H]Forskolin, [2-^{3}H]D-myo-Inositol, [5, 6(N)-^{3}H]Prostaglandin E1.

Du Pont NEN Ltd., Stevenage, Hertfordshire, England.

[Adenylate-^{32}P]Nicotinamide adenine dinucleotide, tri-ethanol-ammonium salt, [^{125}I]Goat anti-rabbit IgG, [L-proline 3, 4-^{3}H(N), histidyl-^{3}H(N)]Thyrotropin releasing hormone (TRH).

ICN Biomedicals, Inc., Irvine, CA, U.S.A.

Tran [^{35}S]-Label^{TM}.

2.1.3. Tissue Culture Plasticware.

Bibby Science Products Ltd., Stone, Staffordshire, England.

Tissue culture flasks (25, 75 cm^2).

Costar, Cambridge, MA, U.S.A.

Bio-freeze vials.

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

Centrifuge tubes (50 ml).

Nunc, Roskilde, Denmark.

Bio-freeze vials, Centrifuge tubes (50 ml),

Tissue culture flasks (25, 75 cm^2).

2.1.4. Cell culture media.

Gibco Life Technologies, Paisley, Scotland, U.K.

Dulbecco's modification of Eagle's medium (10 x) (DMEM), Inositol free DMEM, Newborn calf serum, Glutamine (200 mM), Ham's F-10 medium, HAT (50 x): Hypoxanthine (0.1 mM), Aminopterin (1 mM), Thymidine (16 mM), Penicillin (100 I.U./ml) and Streptomycin (100 mg/ml) solution (100 x), Sodium bicarbonate (7.5 %).

ICN Biomedicals, Inc., CA, U.S.A.

DMEM: (-) methionine and cysteine.

Imperial Labs., West Portway, Andover, Hants, England.

Foetal calf serum (FCS), Newborn calf serum.

Sigma Chemical Company, Poole, Dorset, England.

Controlled process serum replacement-3 (CPSR-3).

2.1.5. Standard buffers.

Hank's bufferred saline (Hank's) (pH 7.4).

1.26 mM Calcium chloride (hexahydrate), 0.5 mM Magnesium chloride (hexahydrate), 0.4 mM Magnesium sulphate (heptahydrate), 5.37 mM Potassium chloride, 137 mM Sodium chloride, 4.2 mM Sodium hydrogen carbonate, 0.35 mM Sodium di-hydrogen phosphate.

Hank's buffered saline with glucose and BSA (HBG).

Hank's buffer (pH7.4) containing 10 mM D-Glucose and 1% (w/v) BSA (fraction V).

Phosphate buffered saline (PBS) (pH 7.5).

2.7 mM Potassium chloride, 137 mM Sodium chloride, 1.5 mM Potassium dihydrogen orthophosphate, 8 mM Disodium hydrogen orthophosphate (heptahydrate).

Sterile trypsin solution for tissue culture.

Trypsin was prepared as a solution of 0.1 % (w/v) trypsin, 0.025 % (w/v) EDTA and 10 mM Glucose in PBS (pH 7.5). The solution was filtered through a sterile 0.22 mM membrane (flow pore D), prior to aliquoting into sterile tubes and storing at -20 °C.

2.2. Experimental Methods.

2.2.1. Cell Culture.

A) Cell growth.

The cells were grown in continuous monolayer culture in 75 cm^2 sterile tissue culture flasks in sodium pyruvate free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 100 I.U./ml penicillin, 100 µg/ml streptomycin, 5 or 10% (v/v) serum (as indicated in Table 2.2.1) which had been heat inactivated at 56 °C for 90 min. Buffering of the medium was achieved by the addition of 0.375% (v/v) sodium bicarbonate and growing the cells in an atmosphere of air: CO_2 in the ratio of 95%: 5%. Cells were incubated at 37 °C in a VSL incubator (Scotlab, Coatbridge, Scotland) with change of medium regularly until confluent.

NG108-15 mouse neuroblastoma x rat glioma hybrid cells, a kind gift from Dr. W. Klee (N.I.H., Bethesda, M.D., U.S.A.) were grown to confluency according to the method of Hamprecht *et al.*, 1985. NG108-15 cells, βN clones, and AC clones were grown in DMEM which containing 5% (v/v) (for NG108-15) or 10% (v/v) (for βN and AC clones) controlled process serum replacement-3 (CPSR-3). The medium was further supplemented with HAT; Hypoxanthine (0.1 mM), Aminopterin (1 mM)

and Thymidine (16 mM) as a selection marker. Mouse NCB20 neuroblastoma (Leigh *et al.*, 1985), human SH SY5Y and human IMR-32 neuroblastoma cells were grown in tissue culture as described previously (Ross *et al.*, 1985).

Table 2.2.1. Characteristics of the cell lines used.

Cell types	Selection markers	Remarks
NG108-15	5% CPSR-3, HAT.	Mouse/Rat hybrid cell.
βN Clones (βN17, βN22)	10% CPSR-3, HAT, 800 μg/ml geneticin.	NG108-15 expressing β_2-adrenoceptor.
AC clones (AC 2.4, AC2.7)	10% CPSR-3, HAT, 800 μg/ml geneticin, 200 μg/ml hygromycin B.	NG108-15 expressing β_2-adrenoceptor and adenylyl cyclase type 2.
HEK-293	5% Foetal calf serum.	Human embryonic kidney cell.
Clone E2	5% Foetal calf serum, 800 μg/ml geneticin.	HEK-293 expressing the long isoform of the rat TRH receptor.
Clone E2M11	5% Foetal calf serum, 800 μg/ml geneticin, 200 μg/ml hygromycin B.	HEK-293 expressing the long isoform of the rat TRH receptor and mouse $G_{11}\alpha$.
GH_3	5% Foetal calf serum.	Rat pituitary cell.
COS-1	10% Foetal calf serum.	Monkey cell for transient transfection.

Human embryonic kidney HEK-293 and rat pituitary GH_3 cells were obtained from the American Tissue Type Collection. HEK-293 cells, E2 clone (HEK-293 cells: co-transfected with the long splice variant of the rat TRH receptor and geneticin sulphate resistance genes) and E2M11 clone (E2 clone: co-transfected with the mouse $G_{11}\alpha$ and hygromycin B resistance genes) were grown in DMEM containing 5% foetal calf serum. GH_3 cells were grown in Ham's F-10 medium (Ham *et al.*, 1963) supplemented with 5% foetal calf serum (FCS) as described by Tashjian *et al.*, 1979. Monkey COS-1 cells were grown to confluency in DMEM medium supplemented with 10% (v/v) foetal calf serum.

To minimise cell reversion, several transfected clones were routinely passaged in DMEM containing geneticin sulphate (800 µg/ml) or hygromycin B (200 µg/ml).

B) Passaging of confluent cell cultures.

Confluent cells (approximately 1 $x10^7$ cells per 75 cm^2 flask) were trypsinized using 0.1% (w/v) trypsin, 0.67 mM EDTA and 10 mM glucose in PBS (pH 7.5). When the cells were detached from the surface of the flask, trypsinisation was terminated by the addition of two volumes of growth medium. This cell suspension was centrifuged at 800 x *gav* in an MSE centaur centrifuge for two minutes to pellet the cells. The cell pellet was resuspended in growth medium and plated out as required. Each flask of cells was split 1: 7 usually into new flasks.

C) Preservation of cell lines.

Confluent cells were removed from the surface of the flask by trypsinisation and the cells resuspended in freezing medium, which consisted of 8% (v/v) dimethyl sulphoxide (DMSO) in CPSR-3 (for NG108-15 cells, βN clones and AC clones) or 8% (v/v) DMSO in FCS (for COS-1 cells, HEK293 cells, E2 and E2M11 clones). This suspension was aliquoted in 1 ml volumes into Bio-freeze vials. The cells were frozen overnight at -80 °C, packed in cotton wool to prevent formation of ice crystals in the cells and then transferred to liquid N_2 for storage.

Cells to be brought up from liquid N_2 storage were thawed immediately at 37 °C, resuspended in 10 ml of appropriate growth medium, and centrifuged at 800 x *gav*

in an MSE centaur centrifuge for two minutes to pellet the cells. The cell pellet was resuspended in growth medium and plated out in a final volume of 10 ml in a 75 cm^2 flask.

D) Cell harvesting.

When adherent cells were confluent or the particular treatment time had elapsed, growth medium was removed from the flask and 10 ml of ice-cold PBS added. Cells were harvested by gentle washing of the surface of the flask or by scraping the monolayer into the PBS, collected in a 50 ml conical tube and centifuged at 800 x *gav* 4 °C in a Beckman TJ6 centrifuge for 10 min. The resulting cell pellet was resuspended in 40 ml of ice-cold PBS and re-centrifuged. The final pellet was stored at -80 °C until required. Pellets which had been stored for up to one year were found to retain full activity.

2.2.2. Preparation of membrane fractions.

A) Preparation of crude plasma membranes.

Membranes were prepared according to the method of Koski *et al.*, 1981b. Frozen cell pastes were thawed and suspended in 4 ml of ice-cold TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) and transferred to a pre-chilled glass homogeniser tube. The cells were then homogenised in a Potter-Elvejhem homogenisor with 25 up/down strokes of a teflon pestle, on ice. The homogenates were centrifuged at 500 x *gav* for 10 min at 4 °C in a Beckman L5-50B ultracentrifuge with a type Ti 50 rotor to remove unbroken cells and nuclei. Crude plasma membranes were obtained by centrifugation of the supernatant at 48, 000 x *gav* for 10 min at 4 °C, resuspension of the resulting pellet in 5 ml of the ice-cold TE buffer and a repeat centrifugation at 48, 000 x *gav* for 10 min at 4 °C. The pellet was resuspended in TE buffer to give an approximate protein concentration of 1-3 mg/ml, aliquoted and stored at -80 °C until required.

In some experiments membranes were prepared in the presence of an anti-proteolytic cocktail consisting of benzamidine (0.5 mg/ml), leupeptin (11.5 μM),

soybean trypsin inhibitor (2 µg/ml), PMSF (0.5 mM), pepstatin A (1.5 µM) and antipain (1.5 µM).

B) Preparation of plasma membrane and cytoplasmic fractions.

Cell pastes were homogenised in 1 ml ice-cold TE buffer to prevent excessive dilution of the resulting cytoplasmic protein. Homogenates were centrifuged at 500 x *gav* for 10 min as described above. The resulting supernatant was transferred to 11 mm x 34 mm centrifuge tubes and placed in a TL100 rotor and centrifuged at 200, 000 x g_{av} for 30 min at 4 °C in a Beckman TL100 tabletop-ultracentrifuge. The supernatant (designated "cytoplasmic fraction") was retained and aliquoted. The pellet (designated " plasma membrane fraction") was resuspended in TE buffer to give an approximate protein concentraton of 1-3 mg/ml and aliquoted. Both fractions were stored at -80 °C until required.

2.2.3. Subcelluar fractionation by sucrose density gradient.

Subcellular fractionation was performed as described by Svoboda *et al.*, 1994. Cells were harvested by centrifugation at 900 x *gav* 4 °C for 10 min in a Beckman TJ-6 benchtop centifuge, washed twice in 140 mM NaCl, 20 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4 and then frozen at -80 °C for at least 2 h. Frozen cell pastes were thawed, resuspended in 2.5 ml of ice-cold TME buffer (20 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and then homogenized using a Potter-Elvejhem homogenizer (Teflon pestle-Glass homogenizer) on ice. 2 ml homogenate was layered on the top of a discontinuous sucrose density gradient consisting of (from top to bottom) 19% (by mass; 5 ml), 23% (5 ml), 27% (5 ml), 31% (5 ml), 35% (5 ml) and 43% (10 ml) sucrose, 20 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 8.0. The gradients were centrifuged for 30 min at 27, 000 rpm in a Beckman L5-50B ultracentrifuge with a type SW 28 rotor (acceleration and deceleration speed set to 4). The density gradient was fractionated manually from the meniscus (fraction 1-7, 5 ml each). The first 5 ml (fraction 1) represented an interphase between the overlayed homogenate and 19% (w/v) sucrose. The gradient pellet (fraction 8) was suspended

by rehomogenation in 1 ml TME buffer. The gradient fraction were frozen at -80 °C until use.

2.2.4. Solubilization of membrane protein.

This assay was performed using a modification of the reconstitution assay described by Milligan *et al.*, 1985. 200-400 µl of the solubilization buffer consisting of 1% (w/v) sodium cholate, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA was added directly to the particulate cell fraction (crude membrane) described in chapter 2.2.2. and resusupended by passing the sample a few times through a 200 µl pipette tip. Samples were incubated for 1 h at 4 °C and centifuged at 200, 000 x *gav* 4 °C for 30 min in a Beckman TL100 tabletop ultracentrifuge to generate a solubilized fraction (supernatant fraction) and a particulate fraction containing any insoluble material such as proteins which had formed aggregated masses. The solubilized supernatant fraction and insoluble pellet were retained and resuspended in a known volume of Laemmli sample buffer (5 M urea, 0.17 M SDS, 0.4 M DTT, 50 mM Tris-HCl (pH 8.0), 0.01% bromophenol blue) so that exactly equivalent amounts of solubilized and insolubilized fractions could be analysed by western blotting.

2.2.5. Determination of protein concentration.

Protein concentrations were determined by the method described by Lowry *et al.*, 1951.

Stock solutions :

A) 2% (w/v) Sodium carbonate in 0.1 M 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 various amounts of 1 mg/ml bovine serum albumin (fraction V), and a standard curve constructed up to 30 µg BSA, in duplicate. Unknown amount of proteins were

assayed in 5 and 10 µl volumes in duplicate. 1 ml of solution D was added to each sample, mixed and left at room temperature for 10 min. 100 µl of Folin's Ciocalteau reagent diluted 1: 1 with dH_2O was added to each sample, mixed and allowed to stand for a further 20 min. The absorbance of each sample was assessed spectrophotometrically at 750 nm in an LKB Biochrom Ultraspec II spectrophotometer.

2.2.6. Production of antipeptide antisera.

All antisera were generated against synthetic peptides, essentially as described by Goldsmith *et al.*, 1987. This section is included for information purposes, as I did not perform this tecnique.

Synthetic peptides were obtained from Dr. C. G. Unson, the Rockerfeller University, New York, NY, U.S.A. with the exception of the peptide NLKLEDGISAAKDVK (IM), which was synthesised by Dr. A. I. Magee, N.I.M.R., Mill Hill, London; the peptide KNNLKECGLY (I3B) was obtained from Biomac Ltd., Glasgow, and the peptide YLTDVDRIATVGY (E976) which was a gift from Dr. John Exton, Vanderbilt University, Nashville, TN, U.S.A. An anti-peptide antiserum raised against the sequence IGARKPQYDIWGNT which is highly conserved in all currently and molecularly identified hormone regulated adenylyl cyclase isoforms was obtained from Prof. Ravi Iyengar, Mount Sinai School of Medicine, New York, NY, U.S.A.

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

The 1.5 ml solution was mixed with an equal volume of complete Freund's adjuvant and briefly sonicated with a Branson 'soniprobe' (Type 7532B). 1 ml 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 15 ml taken from the ear artery and collected in a glass universal. Blood was left to clot overnight at 4 °C and the plasma removed and centrifuged at 1000 x *gav* in a Beckman TJ6 centrifuge for 10 min to pellet any remnants of the clot. The supernatants thus produced were aliquoted into 250 µl volumes and stored at -80 °C until use.

A range of antisera used in this study were produced in a similar manner. These are summarised in table 2.2.2.

Table 2.2.2. Anti G-protein antipeptide antisera.

Antiserum	Peptide Sequence	G protein Sequence	Antiserum identifies
IM	NLKEDGISAAKDVK	$G_o\alpha$ 22-35	$G_o\alpha$
SG	KENLKDCGLF	Tdα 341-350	Tdα, $G_{i1}\alpha$, $G_{i2}\alpha$
I3B	KNNLKECGLY	$G_{i3}\alpha$ 345-354	$G_{i3}\alpha$
CS	RMHLRQYELL	$G_s\alpha$ 385-394	$G_s\alpha$
CQ	QLNLKEYNLV	$G_q\alpha$ 349-359	$G_q\alpha$, $G_{11}\alpha$
IQB	EKVTTFEHQYVNAIKT	$G_q\alpha$ 119-134	$G_q\alpha$
E976	YLTDVDRIATVGY	$G_{11}\alpha$ 160-172	$G_{11}\alpha$
βN	MSELDQLRQE	Gβ 1-10	β1, β2
AC	IGARKPQYDIWGNT		Adenylyl cyclase.

2.2.7. Labelling of cells with Tran [^{35}S]-label^{TM}.

When cells were approximately 60% confluent on 6 well tissue culture plates, the growth medium was replaced with 1.0-1.5 ml of 1: 3 (v/v) DMEM: (-) methionine and (-) cysteine DMEM (ICN Biomedicals, Inc.) supplemented with 50 µCi/ml Tran

[^{35}S]-label^{TM} and serum and then incubated for 20-48 h. For treatment of agonist, the cells were washed twice with 1 ml of growth medium and then a fresh 1.5 ml of DMEM containing agonist was added to each well for an appropriate time. To lyse the cells, 200 µl/well of 1% SDS was added to the cells, the cells were scraped and transferred to 2 ml screw-cap eppendorf tubes. These were boiled in a heating block for 20 min to denature the proteins and tubes left at room temperature for 2 min to allow to cool. After a pulse spin on a micro-centrifuge, the lysates were kept at -80 °C until further use.

2.2.8. Immunoprecipitation of G-protein α subunits.

Immunoprecipitations were perfomed using a modification of the method described by Rothenburg *et al.*, 1988. Membranes were extracted by the addition of 100 µl Pansorbin and 800 ml Kahn's solubilization buffer (1% Triton X-100, 10 mM EDTA, 100 mM NaH_2PO_4, 10 mM NaF, 100 µM Na_3VO_4, 50 mM HEPES (pH 7.2)) containing 2 mM phenylmethane sulphonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin. After incubation for 1 h at 4 °C, non-solublilized material was removed by centrifugation at 13, 000 rpm, 4 °C for 10 min. Immunoprecipitation was achieved by the addition of 10 µl of the relevant G-protein antiserum and incubation for 12 h at 4 °C. Subsequently, 100 µl of the protein A conjugated to agarose (50% slurry in PBS, Sigma) was added and the incubation continued for a further 2-5 h with occasional mixing. Immuno-complexes were collected as a protein A-agarose pellet by centrifugation at 13, 000 rpm for 2 min. The pellet was washed 2 times with 1 ml of Kahn's immunoprecipitation washing buffer (1% Triton X-100, 0.5% SDS, 100 mM NaCl, 100 mM NaF, 50 mM NaH_2PO_4, 50 mM HEPES (pH 7.2)) and resuspended in Laemmli sample buffer (5 M urea, 0.17 M SDS, 0.4 M DTT, 50 mM Tris-HCl (pH 8.0), 0.01% bromophenol blue). After boiling for 5 min, samples were resolved by SDS-PAGE. The gels were stained with Coomassie blue R-250, destained, dried and then autoradiographed as described in 2.2.11.

2.2.9. Preparation of samples for electrophoresis by TCA/deoxy-cholate precipitation.

The required amount of crude plasma membranes or cytoplasmic fractions, as indicated for each experiment, were taken and prepared for SDS-PAGE by sodium deoxycholate/trichloroacetic acid precipitation; 6.25 µl of 2% (w/v) sodium deoxycholate was added to each sample, followed by 750 µl of dH_2O, then 250 µl of 24% (w/v) trichloroacetic acid. The samples were vortexed briefly and incubated on ice for 15 min before being centrifuged on an MSE microcentrifuge at 13, 000 rpm for 5 min. The supernatants were removed and the pellets dissolved in 20 µl of 1 M Tris base followed by 20 µl of Laemmli buffer (mentioned in 2.2.8). The sample was then ready for loading onto the SDS-polyacrylamide gel.

2.2.10. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

Protein separation was achieved using the discontinuous SDS- polyacrylamide gel electrophoresis (SDS-PAGE) system described by Laemmli *et al.*, 1970 and modified by Milligan *et al.*, 1985.

A) Lower resolving gel : 10% (w/v) SDS-PAGE.

Stock solutions (stored at 4 °C)

Solution A: 1.5 M Tris, 0.4% (w/v) SDS, pH 8.8 with HCl.

Solution B: 0.5 M Tris, 0.4% (w/v) SDS, pH 6.8 with HCl.

Solution C: 30% (w/v) Acrylamide, 0.8% (w/v) N, N'-methylene bisacrylamide.

Solution D: 50% glycerol

Solution E: 10% (w/v) Ammonium persulphate (freshly prepared).

Solution F: N, N, N', N'-Tetramethylenediamine (TEMED).

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

10% (w/v) acrylamide/0.25 % (w/v) N, N'-methylene-bis-acrylamide gels were prepared from the stock solutions as follows:

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

To a final volume of 24 ml with dH_2O.

The solution was immediately mixed and poured into a Bio-Rad Protean ™ I electrophoresis system (Bio-Rad Laboratories Ltd., Watford, Herts), which consisted of 180 mm x 160 mm glass plates with 1.5 mm spacers. The gel was layered with 0.1% (w/v) SDS to exclude air and left to set at room temperature for approximately 2h.

B) Lower resolving gel : 4-8 M Urea gradient SDS-PAGE.

In order to maximise separation of each G-protein α subunit, urea gradient SDS-PAGE was carried out as a modification of the method described by Scherer *et al.*, 1987 and Codina *et al.*, 1991 using 160 mm x 200 mm gels and a Bio-Rad Protean ™ II system. Resolving gels containing a linear 4-8 M urea gradient were fashioned by preparing stock solutions of acrylamide (30% (w/v) acrylamide, 0.15% (w/v) N, N'- methylene bisacrylamide) and solution A (as above) in both 4 M and 8 M urea. This gave final concentrations of 12.5% (w/v) acrylamide, 0.0625% (w/v) N, N'-methylene-bisacrylamide, 0.375 M Tris HCl (pH 8.8), 0.1% (w/v) SDS. The two mixtures were polymerized by the addition of 3 µl of TEMED and 15 µl of fresh 10% (w/v) ammonium persulphate, then the 4 M and 8 M urea mixtures were quickly added to the two 20 ml chambers of a gradient maker separately. The gradient was pumped into the gel plates and the gel allowed to polymerize overnight. Upper stacking gels were as described in 2.2.10.D and electrophoresis was carried out as described in 2.2.10.E.

C) Lower resolving gel : 6 M urea SDS-PAGE.

In order to separate species variants of $G_q\alpha$ and $G_{11}\alpha$, membranes were resolved by SDS-PAGE in 160 mm x 200 mm slab gels using 10 % (w/v) acrylamide, 0.0625% (w/v) N, N'-methylene-bisacrylamide gels containing a final concentration of 6 M urea. The gel was layered with 0.1% (w/v) SDS to exclude air and left to set at room temperature for 5-6 h. After polymerisation, upper stacking gel was layered as described in 2.2.10.D and electrophoresis was carried out as described in 2.2.10.E.

D) Upper stacking gel.

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

To a final volume of 15 ml with dH_2O.

The solution was mixed, layered on top of the resolving gel and allowed to polymerize around a 15 well teflon comb. After polymerization, samples were loaded onto each well using a Hamilton microsyringe (Hamilton Co., Reno, Nevada, U.S.A.).

E) Electrophoresis.

Molecular mass determinations were based on prestained SDS molecular weight markers (Sigma Chemical Company). The electrophoresis of 10% (w/v) acrylamide gels was performed at 60 volts until the bromophenol blue dye front was 0.5 cm from the bottom, while, 12.5% acrylamide 0.0625% bis-acrylamide gels containing a linear gradient of 4-8 M urea or 6 M urea were exposed to 100 volts until the prestained lactic dehydrogenase molecular weight marker was 5 cm from the bottom.

F) Staining of SDS-PAGE gels.

After electrophoresis, the gel was soaked in staining solution which consisted of 0.1% (w/v) Coomassie blue R-250 in 50% (v/v) H_2O, 40% (v/v) methanol, 10% (v/v) glacial acetic acid and gently shaken on a rotary shaker for 1 h. The stain solution

was discarded and then frequently changed with destaining solution (identical to staining solution except lacking Coomassie blue R-250) until the proteins were apparent on the gel as discrete bands.

2.2.11. Autoradiography.

After staining gels as described above, the gels containing [^{32}P]-labelled proteins or Tran [^{35}S]-labelled proteins were dried onto Whatman No. 3 chromatography paper under a vacuum line attached to a Bio-Rad 583 gel drier at 80 °C for 1 h. The dried gel was placed next to Fuji X-ray film in a cassette with intensification screens for various periods at -80 °C, or Kodak X-O-mat X-ray film in a cassette for up to three days at room temperature. Films were developed using either a Kodak X-o-mat developing machine with Kodak LX 24 developer and FX 40 fixer for Fuji X-ray film, or a Fuji BAS 1000 phosphorimager linked to an Apple Macintosh Quadra 650 microcomputer for Kodak X-O-mat X-ray film.

Analysis of incorporation of radioactivity into the polypeptides of interest was performed by scanning the autoradiographs with a Bio-Rad scanner driven by an Olivetti M 24 personal computer or with a Bio-Rad Model GS-670 Imaging Densitometer which linked to a Canberra Packard Instant Imager model 2024 and an Apple Macintosh Quadra 800 microcomputer. Absorption was measured in arbitrary units.

2.2.12. Western Blotting.

Proteins were separated under appropriate resolving conditions on SDS-polyacrylamide gels overnight at 60 V or 100 V and then transferred to nitrocellulose membranes for 2 h at 1.5 mA in an LKB transblot apparatus with blotting buffer which consisted of 0.192 M glycine, 25 mM Tris, 20% methanol using a modification of the method of Towbin *et al.*, 1979. The nitrocellulose membrane was then "blocked" in 5% (w/v) porcine gelatin in PBS (pH 7.5), containing 0.004% (w/v) thimerosal as an anti-bacterial agent at 37 °C for a minimum 2 h. The gelatin/PBS was removed and the membrane was washed three times with 100 ml dH_2O. The

nitrocellulose was then incubated with the appropriate dilution of antipeptide antiserum, diluted in 1% (w/v) porcine gelatin in PBS (pH 7.5), containing 0.2% (v/v) non-ionic detergent Nonidet P-40 (NP-40) for at least 2 h at 37 °C. The primary antiserum was then removed and the nitrocellulose membrane given two washes with PBS (pH 7.5)/0.2% (v/v) NP-40 for 10 min, after which the blot was incubated with a second antibody (horseradish peroxidase conjugated donkey anti-rabbit IgG) at a dilution of 1: 200 in 1% (w/v) gelatin/PBS (pH 7.5)/0.2% (v/v) NP-40 and the nitrocellulose membranes were incubated for 2 h at 37 °C. The secondary antiserum was removed and the blots washed three times with PBS (pH 7.5) containing 0.2% (v/v) NP-40 for 10 min and then three times with PBS for 10 min. The blot was then developed in 40 ml of PBS (pH 7.5) with 0.02% (w/v) *O* -dianisidine dihydrochloride as substrate for detection of the antibody complex. Development of the immunoblot was initiated by the addition of 20 µl of H_2O_2 (30% (v/v)), and then stopped by immediately immersing the blot in 1% (w/v) sodium azide. The developed immunoblot was rinsed in dH_2O and allowed to dry.

2.2.13. Quantification of the primary immunoreactive signal with an [^{125}I]-labelled secondary antibody.

After incubation of the immunoblot with a non-radioactive primary antiserum, an [^{125}I]-labelled goat anti-rabbit IgG (0.05-0.1 µCi/ml) was prepared in a small tube as described in 2.2.12 for the primary antipeptide antiserum and the immunoblot then replaced in this secondary antibody. The immunoblot was then incubated at 37 °C for a minimum of 2 h, the antibody was discarded and the nitrocellulose washed as described in 2.2.12. During the washing procedure the immunoblot was monitored to assess the background levels of incoporation of [^{125}I] and the wash conditions altered accordingly to achieved a low background signal. The immunoblot was dried and autoradiography was then performed as described in 2.2.11.

2.2.14. Quantitation of immunoblots.

Following western blotting, the developed immunoblots were scanned with either a Shimadzu CS-9000 dual wavelength flying-spot laser densitometer on reflectance mode at 450 nm and analysed on a Shimadzu FDU-3 central processing unit or with a Bio-Rad GS-670 imaging densitometer to enable quantitation of the immunoblots. Background was subtracted by scanning equivalent sized areas of nitrocellulose which did not contain immunoreactive protein. Absorption was measured in arbitrary units and the results were expressed as a percentage of control values.

Quantification of the levels of the $G_s\alpha$ or $G_q\alpha$ in membranes were achieved in the same manner on immunoblots which contained varying concentrations of *E. coli* expressed $G_s\alpha$ (Freissmuth *et al.*, 1989) (this material was a kind gift of Dr. M. Freissmuth, Dept. of Pharmacol., Univ. of Vienna, Austria) or $G_q\alpha$ (a kind gift of Dr. S. Arkinstall, Galaxo Institute for Molecular Biology, Geneva, Switzerland). On such immunoblots, added quantities of *E. coli* generated recombinant $G_s\alpha$ or $G_q\alpha$ ranged over 0-30 ng. Densitometric analysis of the developed immunoblots allowed construction of a standard curves for recombinant $G_s\alpha$ or $G_q\alpha$, which were used to assess the level of these G-proteins in membranes of the cells.

2.2.15. *In vitro* mono ADP-ribosylation.

In vitro mono ADP-ribosylation of G-proteins in membranes was performed using a modification of the method described by Hudson *et al.*, 1980. Prior to use, both cholera toxin (1 mg/ml in H_2O) and pertussis toxin (200 µg/ml in 50 mM phosphate buffer (pH 7.2), 500 mM NaCl, 50% (v/v) glycerol) were activated by preincubating with an equal volume of freshly prepared 100 mM DTT at room temperature for 90 min. The appropriate toxin was added at a final concentration of either 10 µg/ml for pertussis toxin or 50 µg/ml for cholera toxin.

Membranes to be ADP-ribosylated were diluted in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) to a protein concentration of between 1 to 3 mg/ml. 20 µl of aliquots were assayed in a final volume of 50 µl containing the following:

250 mM Sodium phosphate buffer (pH 7.5),

[^{32}P]NAD^+ (2 µCi/assay tube)

20 mM Thymidine

1 mM ATP (pH 7.5),

100 mM GTP (pH 7.5),

20 mM Arginine/HCl.

Assay :

Protein (membranes)	20 µl
Activated toxin (or DTT as negative control)	5 µl
Agonist (or dH_2O as negative)	5 µl
Mix (as above)	20 µl
	total volume 50 µl

After the assay was initiated by addition of the membranes, the tubes were incubated at 37 °C for 2 h. The reaction was terminated by the transferral of the tubes to an ice slurry followed by sodium deoxycholate/TCA precipitation as described in 2.2.9. The samples were then subjected to appropriate SDS-PAGE conditions, as described in 2.2.10 and the [^{32}P]ADP-ribosylated proteins visualized by autoradiography of the dried gel as mentioned in 2.2.11.

2.2.16. Radioligand Binding Assay.

Radioligand binding assays were performed by a rapid filtration method as described by Pert *et al.*, 1973. The conditions of different assays are indicated in the relevant figure legends.

Membrane binding assays were performed routinely with [^{3}H]-labelled ligand and 5-300 µg of membrane protein at either 20 °C (for [^{3}H]forskolin) or 30 °C (for [^{3}H]DHA) for 60 min in TSM binding buffer consisted of 20 mM Tris-HCl (pH 7.4), 50 mM sucrose, 20 mM $MgCl_2$. In [^{3}H]ligand binding experiments, the absence and presence of unlabelled ligand was used to define maximal and non-specific binding

respectively. Blank values were determined by replacement of membrane protein with TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4).

The specific high affinity binding of [^{3}H]forskolin to the GSAC complex in whole cells was performed as described by Alousi *et al.*, 1991. NG108-15 cells and the β_2-adrenoceptor expressing clones derived from these cells were harvested and resuspended in DMEM buffered to pH 7.4 at 4 °C with 20 mM HEPES (DMEM-H). Cells were then counted using a haemocytometer and maintained on ice for 20 min prior to use in whole cell binding assays. Resuspended cells were added to tubes containing [^{3}H]forskolin (approximately 10 nM) in either the absence or presence of 10 μM cold forskolin to define non-specific binding. The tubes also contained varying concentrations of either the β-adrenoceptor agonist isoprenaline or the IP prostanoid receptor agonist iloprost. In cases in which cells were pretreated with BAAM, following harvest of the cells and removal of the growth medium as above they were subjected to three wash and centrifugation cycles with 20 ml volumes of DMEM-H prior to final resuspension. They were then incubated at 4 °C for 60 min.

All binding experiments were terminated by rapid filtration through Whatman GF/C filters, which had been pre-soaked in ice cold TSM binding buffer, followed by three times 5 ml washes with ice-cold TSM binding buffer using a Brandell cell harvester. The filters were soaked overnight in 10 ml of ecoscint scintillation fluid prior to counting in a Rackbeta scintillation counter. Binding data was analysed by either a non-linear least squares analysis or the Kaleidograph curve fitting programme driven by an Apple McIntosh computer. The K_d and B_{max} of a receptor population, was estimated either by the equation of DeBlasi *et al.*, 1989 ($K_d = IC_{50} - L$ and $B_{max} = B_O (K_d+L) / L$; B_O, specific binding; L, the concentration of radiolabelled ligand used; IC_{50}, the concentration of unlabelled ligand that displaced half the specific binding of radioligand) or by the method of Scatchard *et al.*, 1949.

2.2.17. Assay of adenylyl cyclase catalytic activity.

Adenylyl cyclase assays were performed as described by Milligan *et al.*, 1987a. Separation of radiolabeled cyclic AMP and ATP was achieved by the method of Salomon *et al.*, 1979; Johnson *et al.*, 1991, except that the amount of [α-^{32}P]ATP was reduced to 1 µCi per sample. The catalytic activity of adenylyl cyclase is determined by methods which rely on the measurement on cAMP formed from unlabeled substrates, with cAMP-binding proteins and radio-immunoassay procedures, or by methods that rely on the use of radioactively labeled substrate followed by isolation and determination of the radioactively labelled product. These two different approaches have different purposes, different sensitivities, and different ease of use. The procedures described here focus on measuring the production of [^{32}P]cAMP from the substrate [α-^{32}P]ATP. The cAMP thus produced is separated from the unreacted [α-^{32}P]ATP by a two-step column method.

A) Preparation of Sample.

Reaction mixtures of 50 µl containing 2.0 µM GTP (pH 7.5), 2.0 mM cAMP, 0.4 mM ATP (pH 7.5), 200 mM Tris HCl (pH 7.5), 4 mM creatine phosphate, 100 U/ml creatine phosphokinase, 10 mM $MgCl_2$, [α-^{32}P]ATP ($1x10^6$ cpm), 100 mM NaCl were added to tubes containing between 5-20 µg membrane protein and appropriate ligands to a final volume of 100 µl. Reaction tubes were kept on ice slurry at all times and the reaction started by incubation at 30 °C in a water bath. After 15 min incubation, the reaction was terminated by placing the tubes back into the ice-slurry and addition of 100 µl of stopper solution (2% (w/v) SDS, 45 mM ATP, 1.3 mM 3' 5'-cAMP). 50 µl of [8-^{3}H]3' 5' cAMP (approximately 10, 000 cpm) was added to each tube prior to boiling for 10 min. 750 µl of water was then added to each sample, and the [^{32}P]cAMP content of each tube determined.

B) 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 *et al.*, 1979 and involves the separation of cyclic AMP from other nucleotides by dowex and then alumina chromatography. The dowex H^+ 50 x 4 (200-400) was mixed with water to a slurry (1: 1 (v/v)) and then 2 ml

removed and added to glass wool stoppered columns. The dH_2O was allowed to drain out and the columns washed with 2 ml of 1 M hydrochloric acid, then stored at room temperature. Prior to use, the columns were washed with 4 ml of 1 M NaOH then 4 ml of 1 M hydrochloric acid and approximately 20 ml of dH_2O. After each use, dowex H^+ 50 columns were regenerated by washing with 4 ml of 1 M HCl, then stored until re-use.

The alumina columns were prepared by the addition of dry neutral alumina : 0.1 M imidazole (pH 7.3) slurry (1: 1 v/v) to glass wool stoppered columns. The columns were washed with 10 ml of 1 M imidazole (pH 7.3) followed by 15 ml of 0.1 M imidazole (pH 7.3) and stored at room temperature until used. After each use, the columns were washed with 10ml of 0.1 M imidazole (pH 7.3).

C) Nucleotide elution profile for both dowex and alumina columns.

Before intial use, the nucleotide elution profiles for each column must be determined. This was performed by applying a mixture of [^{3}H]cAMP and [^{32}P]ATP to the column and determining the elution volume.

To quantitate sample recovery, stock [^{3}H]cAMP was diluted in dH_2O to give approximately 10, 000 cpm in 50 µl and [^{32}P]ATP was diluted from a stock of 1 µCi/ml to give approximately 2, 000 cpm in 50 µl. 50 µl of each of the cAMP and ATP solutions were added to 950 µl of water and then the mixture applied to a dowex column. The ATP and cAMP were eluted from the column by successive washes of the column with 0.5 ml of dH_2O. Fractions were collected in a vial with 5 ml of Ecoscint and radioactivity determined by liquid scintillation counting using a dual label programme. The elution volumes required to elute the cAMP from the dowex columns were then determined graphically. The elution volume required to elute the cAMP from the alumina columns was determined as for the dowex columns except that only [^{3}H]cAMP was used, and the eluting buffer was 0.1 M imidazole (pH 7.3). Recoveries were similar to that obtained for the dowex columns.

During the experiments, typical recovery from the dowex columns was always greater than 70%. When recovery fell below 60% the columns were discarded and fresh columns prepared. The life of columns is approximately 6 months.

D) Determination of cAMP production from membrane proteins.

Samples (total volume 950 ml) were loaded to prepared dowex columns and the ATP eluted with 1.5 ml of dH_2O. 6 ml of dH_2O was then added to the dowex columns and this eluate allowed to run directly onto the alumina columns. The cAMP fraction was eluted into vials containing 14 ml of Ecoscint, with 6 ml of 0.1 M imidazole (pH 7.3).

The cAMP fractions obtained were counted on a dual label liquid 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 that represented 100% recovery of the added [^{3}H]cAMP. Data was then calculated as pmoles of cAMP formed per minute per milligram of membrane protein, and the assay was sensitive to approximately 5 pmoles/min/mg.

2.2.18. Measurement of stimulated inositol phosphate generation.

Occupation of a cell surface receptor by a hormone stimulates the generation of a second messenger. Many hormones, neurotransmitters and growth factors stimulate the breakdown of phosphatidylinositol 4, 5-bisphosphate (PIP2) by activating a specific phospholipase C, this generates the two second messenger molecules inositol 1, 4, 5-trisphosphate (IP3) and sn-1, 2-diacylglycerol (DAG). IP3 can be measured at the mass level by a specific binding assay but it is easier to make use of the "Li^+ assay". The normal pathway of IP3 degradation is by specific phosphatases to generate inositol which can be re-incorporated into PIP2. Inositol monophosphatase is an enzyme inhibited by Li^+, therefore in the presence of this ion the inositol phosphates will accumulate. If the cells are prelabelled with [^{3}H]inositol the inositol phosphates formed will be [^{3}H]-labelled and thus can be measured. In order to do this

the inositol phosphates are separated from inositol by ion-exchange chromatography on dowex-1-formate columns.

A) Preparation of dowex formate.

Dowex 1 x 8 resin has a quaternary group attached (compound formed from an amine by addition of a proton to produce a +ve ion) but in the presence of Cl^- there is an overall neutral charge. Dowex Cl^- cannot be used because there is too strong an electrovalent bond between dowex and Cl^- such that there would be no displacement by the phosphate group of the inositol phosphates of Cl^-. To convert dowex chloride to dowex formate, 100 g of dowex chloride (Dowex 1 x 8-200) were washed twice with 1 L dH_2O and the beads gently allowed to settle. These were transfered to a scintered glass funnel and washed with 2 L 2 M NaOH. This was followed by 5-6 L dH_2O to remove NaCl and excess NaOH and then with 0.5 L 1 M formic acid. The formate form of dowex was finally washed with about 20 L dH_2O until the pH was constant at 5-5.5 as measured with narrow range pH paper (pH 4-6) and stored in a covered beaker at room temperature in an slurry of approximately 1: 1 dowex : dH_2O.

B) Preparation of cells.

Cells were seeded in 24 well plates for 2-3 days in DMEM containing 5-10% serum. When cells were approximately 70% confluent, medium was replaced with inositol-free DMEM containing 1 % dialyzed foetal calf serum and [2-^{3}H]myo-inositol (1 µCi/ml) and the cells incubated for a further 48 h.

C) Agonist regulation of inositol phosphate production.

Prior to agonist addition, the labelling medium was removed and the cells washed twice with 0.5 ml Hank's buffered saline (pH 7.4) containing 1% (w/v) BSA and 10 mM glucose (HBG) as described in 2.1.5. The cells were incubated for 10 min with HBG containing 10 mM Lithium chloride (HBG/LiCl) and stimulation carried out with agonist in HBG/LiCl for 20 min. All incubations were performed at 37 °C. Reactions were terminated by the addition of 0.5 ml ice cold methanol. The cells in each well were then scraped and transferred to vials. Chloroform was added in a ratio of 1: 2 ($CHCl_3$: MeOH) and the samples extracted overnight. The phases were split

by the addition of chloroform and dH_2O to a final ratio of 1: 1: 0.9 ($CHCl_3$: MeOH: dH_2O) and the upper phase was taken for analysis of total inositol phosphates. Total inositol phosphates were analysed by batch chromatography on dowex-1-formate as described by Plevin *et al.*, 1990.

2.2.19. Data analysis.

Where appropriate, data was analysed for statistical significance using either the paired or unpaired Student's t-distribution test with "n" numbers representing the degree of freedom, as indicated. In the paired test this "n" value was calculated from the number of experiments (n') performed-1, whereas in the unpaired test it was calculated from n1, +n2'-1. Dose response and displacement curves were analyzed using the Kaleidograph Curve Fit programme, courtesy of Syntex Research Centre, Edinburgh, Scotland, U.K.

2.2.20. *Escherichia coli* strains and plasmids.

All strains of *E. coli* were grown in L broth (10 g/L Bacto-tryptone, 5 g/L yeast extract, 0.17 M NaCl; pH 7.5 with NaOH), containing appropriate antibiotics at 37 °C with continuous shaking (120-150 rpm).

The plasmid pCMV containing the cDNA for mouse $G_{11}\alpha$, cloned into the Cla I/Xho I sites, was a kind gift from Dr. Melvin I. Simon, Califonia Institute of Technology, Pasadena, CA, U.S.A. The full length cDNA for type 2 adenylyl cyclase was a gift from Dr. Paul Herzmark and Prof. Henry Bourne, Department of Pharmacology, University of Califonia, San Francisco, CA, U.S.A and PBS II containing the full length cDNA for type 6 adenylyl cyclase cloned into the BamH I (N-terminal)/EcoR I (C-terminal) sites was a kind gift from Dr. Joseph P. Pieroni, and Prof. Ravi Iyengar, Mount Sinai, School of Medicine, New York, NY, U.S.A.

E. coli strains and plasmids used in the experiments are listed in Table 2.2.3.

Table 2.2.3. *E. coli* strains and plasmids.

Strains and Plasmids	Genotype or Phenotype	Sources
JM109	endA1, recA1, gyrA96, thi, hsdR17, (r_k^-,m_k^+), relA1, supE44, D(lac-proAB), [F', traD36, proAB, $lacI^qzDM15$].	Promega Corporation.
MC1061/P3	$deoR^+$, rpsL, thi, mcrB, galK, D lacX74, (r^{-k},m^{+k}), strA, araD139, D(araABC-leu)7679. {p3:amber Amp^R, amber Trt^R, Km^R}.	Invitrogen.
pSV2-neo	$G418^R$, Amp^R, 5.6 kb, SV40 (COS), pMB1 *(E. coli).*	Gibco/BRL.
pBABE Hygro	Amp^R, Hygromycin ^{R}, 5.2 kb, SV 40.	
LK444 (pJM16)	$G418^R$, Amp^R, β-actin promoter, 10.0 kb.	Gunning *et al.*, 1987.
pcDNA1	CMV promoter, SV 40, M 13 origin, Sup F, Col E1 origin, T7 and Sp6 RNA promoter, 4.1 kb.	Invitrogen.
pcDNA3	CMV promoter, SV40, $G418^R$, Amp^R, Col E1 origin, f1 origin, T7 and Sp6 RNA promoter, BGHpA, 5.4 kb.	Invitrogen.
pCMV4	CMV promoter, SV 40 ori, Amp^R, f1 origin, hGH fragment, 4.9 kb.	Andersson *et al.*, 1989.

2.2.21. Transformation of competent *E. coli* with plasmid DNA.

E. coli strains (JM109 or MC1061/P3) were grown in 500 ml L-broth until OD_{600} was approx 0.25 (equivalent to approx $1x10^8$ cells/ml). The cell suspension was allowed to cool on ice for approximately 30 min and spun at 10, 000 rpm for 10 min at 4 °C on a Beckman J2-21 centrifuge using a JA14 rotor. The supernatant was discarded and the pellets resuspended in 20 ml each of ice-cold sterile buffer I (100 mM $RbCl_2$, 50 mM $MnCl_2.4H_2O$, 30 mM potassium acetate, 10 mM $CaCl_2.2H_2O$, 15 % (w/v) glycerol, pH 5.8). The suspension was left on ice for 15 min and recentrifuged as before. The supernatants were discarded again and the pellets resuspended in a total volume of 3.5 ml of ice-cold buffer II (10 mM $RbCl_2$, 10 mM MOPS, 75 mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, pH 6.8) and pooled into one tube. The suspension was left on ice for 15 min before 200 µl aliquots were frozen in dry ice and stored at -80 °C.

200 µl of competent cells were thawed and added to an appropriate amount of plasmid DNA, 10-100 ng of supercoiled plasmid DNA or 5 µl of a ligation reaction. After gently mixing, the mixture was incubated on ice for 60 min before being heat shocked at 42 °C for 90 sec and returned to ice for 2 min. 800 µl of L-broth was added and the tubes incubated, with shaking at 150 rpm, 37 °C for 45-60 min. Different densities of the mixtures were spread onto L-broth agar plates containing appropriate antibiotics to select the colonies which had taken up the plasmid. The plates were incubated overnight at 37 °C and antibiotic resistant colonies picked and grown overnight in 5 ml L-broth containing the required antibiotics as above. 3 ml of this was used to prepare plasmid DNA and the remainder used to make glycerol stocks (Sterile 85% (v/v) cultured *E. coli* and 15% (v/v) glycerol were mixed). The glycerol stocks were snap frozen in dry ice and then stored at -80 °C until required.

2.2.22. Transfection of eukaryotic genes into mammalian cells.

A) Preparation of plasmid DNA.

Plasmid DNA was prepared using either the alkaline lysis procedure of Sambrook *et al.*, 1989, or Promega Mini-preps, Promega Maxi-preps Kits according to the manufacturer's instructions.

Most of subcloning experiments including digestion of plasmid DNA with restriction endonucleases, calf intestinal alkaline phosphatase (CIAP) treatment of DNA fragments, separation of digested plasmid DNA by electrophoresis and ligation of the desired DNA fragments of plasmid vector and insert DNA were also performed according to Sambrook *et al.*, 1989 but purification of the desired DNA fragments from low melting agarose gel electophoresis followed the protocol of a Promega PCR purification kit.

The amount of DNA in a given sample was quantitated by measuring the absorbance at 260 nm (A_{260}) in a UV-1201 UV-VIS spectrophotometer with a 1: 200 dilution of the sample in sterile Milli Q dH_2O. An A_{260} of 1 was assumed to be equal to 50 µg/ml of double stranded DNA or 20 µg/ml of single stranded oligo-nucleotide. The purity of the DNA was assessed by measuring the A_{280} in parallel and calculating the A_{260}/A_{280} ratio. A ratio of approximately 1.8 was considered to be sufficiently pure for use otherwise a phenol:chloroform extraction and then EtOH precipitation was carried out as described in Sambrook *et al.*, 1989.

B) Lipid-mediated DNA-transfection.

Transfections using mouse neuroblastoma x rat glioma hybrid NG108-15 cells, human embryonic kidney (HEK-293) cells and monkey COS-1 cells were performed using either Lipofectin reagent (Gibco/BRL) or DOTAP (Boehringer Mannheim Biochemica) according to the manufacturer's instructions. Firstly, the minimum concentration of selection markers (geneticin or hygromycin B) to kill the wild type cells within 7 days was determined prior to transfection.

Cells were grown to approximately 40% confluency on 100 mm tissue culture dishes in an appropriate culture medium containing serum. 10 µg (for stable) or 100 µg (for transient) of DNA was diluted with dH_2O up to 250 µl in a sterile polypropylene tube. 70 µl of DOTAP or Lipofectin was separately diluted up to 250

µl with dH_2O in a 13 ml sterile polypropylene tube. Both DNA and transfection reagent complexes were incubated for 15 min during which time the cells were washed twice with serum containing DMEM (DOTAP) or serum free DMEM (Lipofectin). 10 ml serum containing DMEM (DOTAP) or serum free DMEM (Lipofectin) was added to the complexes and then added to the cells. Cells were then incubated for 10-24 h to allow the DNA/transfection reagent complexes to adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm before the medium was replaced with serum containing DMEM. Cells were then incubated for approximately 72 h. For transient transfection the cells were then harvested whereas, the cells for stable transfection were plated out in the appropriate selection medium either containing 800 µg/ml geneticin sulphate or 200 µg/ml hygromycin B alone, or both 800 µg/ml geneticin sulphate and 200 µg/ml hygromycin B. Stable colonies were selected on the basis of resistance to antibiotics and expanded for detailed analysis.

2.2.23. Reverse transcriptase-polymerase chain reaction (RT-PCR).

A) Isolation of RNA.

Total RNA was isolated according to the acid phenol/guanidium thiocyanate method of Chomczynski *et al.*, 1987. Cells grown in monolayer were lysed by the addition of 1 ml of RNA zol ^{TM} B per flask (75 cm^2). RNA was solubilized by passing the lysate a few times through the pipette. 200 µl of chloroform was added to the homogenate. The homogenate was vortexed briefly and put on ice for 5 min. The suspension was centrifuged on an MSE microcentrifuge at 13, 000 rpm for 15 min (4 °C). After addition of chlorform and centrifugation, the homogenate forms two phases: the lower chloroform and the colourless upper aqueous phase whereas DNA and proteins are in the interphase and organic phase. The aqueous phase was transferred to a fresh tube, an equal volume of isopropanol added and the samples stored for 15 min at 4 °C. After centrifuging the samples for 15 min at 13, 000 rpm (4 °C), the supernatant was discarded and the RNA pellet was washed once with 1ml of 75% EtOH by vortexing and subsequent centrifugation at 13, 000 rpm for 5 min (4 °C).

The pellet was dried briefly under vacuum and then dissolved in 1 mM EDTA (pH 7.0) by vortexing or by passing a few times through a pipette tip. All solutions and materials were treated with 0.1% Diethylpyrocarbonate (DEPC). Purity and quantitation were assessed by OD_{260}/OD_{280} ratios.

B) Reverse transcription.

0.2-2 µg samples of DNAsed RNA were heat-denatured by incubation at 65 °C for 10 min followed by chilling on ice and reverse transcribed in either 15 µl or 33 µl of reaction mixture consisting of 50 mM Tris/HCl (pH 8.3), 40 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, RNAguard (17 units), 2 µg Oligo d(T)12-18 and 13 units of reverse transcriptase with a first strand cDNA synthesis kit (Pharmacia Biotech Inc.) as detailed by the manufacturer. Incubation was carried out at 37 °C for 1 h and stopped by incubation at 95 °C for 5 min to denature the RNA-cDNA duplex and inactivate the reverse transcriptase. Reaction mixtures were not extracted and were stored at -20 °C prior to amplification.

C) Polymerase chain reaction (PCR) amplication.

PCR was carried out using the following 24 mers (synthesized on an Applied Biosystems Synthesizer) according to a modification of the method described by Steel *et al.*, 1993.

TRH-963 sense 5'-CAACCTCATGTCTCAGAAGTTTCG;

TRH-1333 anti-sense 5'-AATGAAGACCTTCGATCAGTTGG;

Type 2 Adenylyl cyclase-2122 sense

5'-CACGGATCTCCCTCACAATCGTCA;

Type 2 Adenylyl cyclase-2356 anti-sense

5'-GCCCATGGTTGATACCCACTCGCA;

Type 6 Adenylyl cyclase-2059 sense

5'-CGGAAAGTAGACCCTCGTTTCGGA;

Type 6 Adenylyl cyclase-2652 anti-sense

5'-GCCAAGCCATGGACGCTAAGCA;

PCR for the TRH receptor isoforms was performed in the presence of 20 pmols of sense and antisense primer, dNTPs (0.2 mM each of dATP, dCTP, dGTP, dTTP), 6 mM $MgCl_2$ and *Taq* polymerase (2.5 units) in a final volume of 100 µl. Cycling conditions were 95 °C/ 2 min, 48 °C/1 min, 72 °C/1 min (1 cycle); 95 °C/30 sec, 48 °C/1 min, 72°C/1 min (33 cycles); 95 °C /30 sec, 48 °C/30 sec, 72 °C/1 min (1 cycle), followed by a final extension at 72 °C for 2 min.

In addition, amplifications for the type 2 and type 6 adenylyl cyclases were performed over 35 cycles in the presence of 20 pmols of sense and antisense primer, dNTPs (0.2 mM each of dATP, dCTP, dGTP, dTTP), 6 mM $MgCl_2$ and *Taq* polymerase (2.5 units) in a final volume of 100 µl. Cycling conditions were as follows; 95 °C/ 5 min, 56 °C/1 min, 72 °C/1 min (1 cycle); 95 °C/30 sec, 56 °C/1 min, 72°C/1 min (30-35 cycles); 95 °C /30 sec, 56 °C/1 min, 72 °C/5 min (1 cycle).

Reaction mixtures were covered with 50 µl mineral oil and amplication was performed in a Hybaid Omnigene Thermocycler. Reaction products were then resolved by 1-1.5 % (w/v) agarose gel electrophoresis. No signal was observed in samples that were amplified without prior reverse transcription. To confirm the specificity of the primers to amplify only mRNA corresponding to the anticipated signals, PCR was done under identical conditions using cDNA species corresponding to receptor, G-proteins, or adenylyl cyclase as positive controls.

Chapter 3.

The stoichiometry and the regulation of the levels of protein components of the stimulatory adenylyl cyclase cascade in NG108-15 cell lines.

3.1. Introduction.

Many hormones, neurotransmitters and growth factors exert their effects on target cells and tissues by modulating the intracellular levels of various second messengers, via members of a family of G-proteins. It has been known that receptor mediated generation of cyclic AMP requires the participation of at least three classes of proteins, which are receptors, G-proteins and adenylyl cyclases. As I mentioned in chapter 1 molecular analysis has indicated that all receptors of this class are represented by a single polypeptide which topographically is predicted to have seven transplasma membrane helices to provide an extracellular N-terminus, a cytoplasmic C-terminus and a series of intracellular and extracellular loops (Savarese *et al.*, 1992). In contrast, G_S is a heterotrimeric protein in which the α subunit can exist as four distinct splice variants derived from a single gene (Bray *et al.*, 1986; Robishaw *et al.*, 1986). This complex is located at the intracellular face of the plasma membrane. The mammalian adenylyl cyclase family of proteins is expanding as new molecular forms continue to be identified as novel cDNA species but a common feature of those identified to data is the presence of two highly hydrophobic areas which are each predicted to contain six transplasma membrane helices linked by a large cytoplasmic loop (Tang *et al.*, 1992).

While receptor regulation of cyclic AMP generation has been widely examined, very little information is available on the relative or absolute levels of expression of G_S and adenylyl cyclase in individual cell types, on what proportions of these proteins become activated following agonist occupation of a receptor and whether different G_S-linked receptors have access to the total cellular population of G-protein and effector enzyme or if each receptor may be restricted to utilizing different pools of either G_S and/or adenylyl cyclase. In this study, the neuroblastoma x glioma hybrid cell line, NG108-15 (Hamprecht *et al.*, 1985) has been studied as a model system to analyse signal transduction by the adenylyl cyclase cascade as these cells express a considerable range of receptors which act in both a stimulatory and inhibitory manner on the generation of cyclic AMP (Milligan *et al.*, 1990).

The neuroblastoma x glioma hybrid NG108-15 cell, (also called 108CC15) was generated by the fusion of the 6-thioguanine-resistant clonal mouse neuroblastoma cell line N18TG2 and the bromodeoxyuridine-resistant rat glioma cell line C6-BU1, followed by selection with hypoxanthine, aminopterin, thymidine (HAT) medium and cloning (Hamprecht *et al.*, 1985). The transmembrane signaling systems which have been examined in detail in NG108-15 cells are stimulation and inhibition of adenylyl cyclase (Klee *et al.*, 1985), activation of phosphoinositidase C and regulation of voltage-sensitive Ca^{2+} channels (Tsunoo *et al.*, 1986) and activation of a Ca^{2+} dependent K^+ channel (Fukuda *et al.*, 1988). Each of these effector system is coupled to relevant receptors by G-proteins (Milligan *et al.*, 1990). Activation of the α_{2B}-adrenoceptor (Bylund *et al.*, 1988), M4 muscarinic (Fukuda *et al.*, 1988) and δ-opioid receptors expressed by NG108-15 cells inhibits adenylyl cyclase activity and voltage-dependent Ca^{2+} flux through coupling to G_{i2} and G_o respectively (McClue *et al.*, 1990; McKenzie *et al.*, 1990; McFadzean *et al.*, 1989; Hescheler *et al.*, 1987). In contrast, NG108-15 cells express receptors for adenosine A_2 (Kenimer *et al.*, 1981) and IP prostanoid receptors (Carroll *et al.*, 1989) which couple to the activation of adenylyl cyclase via activation of $G_s\alpha$ as well as receptors for glucagon and secretin (Traber *et al.*, 1975).

Sustained exposure of cells or tissues to agonists which activate a G-protein-linked receptor frequently results in a time- and concentration-dependent decrease in plasma membrane, and subsequently cellular, levels of that receptor. This process is called down-regulation, and can contribute to desensitization, which limits cellular responsiveness to the maintained presence of extracellular signaling molecules (Benovic *et al.*, 1985; 1987; Sibley *et al.*, 1985). In NG108-15 cells, agonist-mediated selective down-regulation of $G_s\alpha$, activated upon receptor occupancy, has been produced by treatment of the cells with either prostanoid receptor agonists (McKenzie *et al.*, 1990b) or with chronic exposure to ethanol (Mochly-Rosen *et al.*, 1988) and results in a heterologous desensitization of receptor mediated generation of cyclic AMP.

In chapter 3.2, therefore, I have examined the quantitative stoichiometry of the cascade which leads from activation of a prostanoid receptor which has pharmacology consistent with it being of the IP prostanoid subtype (Carroll *et al.*, 1989) via $G_S\alpha$ to adenylyl cyclase and the generation of cyclic AMP. I have used the stimulation of specific high-affinity binding of [^{3}H]forskolin produced by either NaF or Gpp(NH)p to total membranes and intact cells of NG108-15 to attempt to define the maximal number of GSAC complexes that can be formed in these cells.

The diterpene forskolin binds with high affinity to the complex of $G_S\alpha$ and adenylyl cyclase (GSAC) but with substantially lower affinity to isolated adenylyl cyclase (Laurenza *et al.*, 1989; 1991). This property has been utilized as a means to purify the complex of these two polypeptides, following treatment with either fluoroaluminate or a poorly hydrolysed analogue of GTP to persistently activate $G_S\alpha$, by passage of cellular detergent extracts over a forskolin affinity resin (Pfeuffer *et al.*, 1985; Marbach *et al.*, 1990) although adenylyl cyclase in the absence of G_S has also been purified by use of a forskolin affinity column to which membrane extracts have been applied without incubation with persistent G-protein activators (Smigel *et al.*, 1986).

In addition, NG108-15 cells transfected to express the human β_2-adrenoceptor (Adie *et al.*, 1994 a, b) have been used in chapter 3.3 to define the regulation of agonist access to the receptor population by pretreatment of the cells with varying concentrations of an irreversible β-adrenoceptor antagonist bromoacetyl alprenolol methane (BAAM) which covalently attaches to receptor protein through bromoalkylation of sulfhydryl group and thus irreversible occupancy of the receptor binding site (Mahan *et al.*, 1985). Prior to the availability of cDNA species encoding G-protein-linked receptors (Bouvier *et al.*, 1988b; Johnson *et al.*, 1979; Whaley *et al.*, 1994), measurements of differences in agonist efficacy and intrinsic activity could be gained only either from comparing responses to an agonist in different tissues or by use of irreversible antagonists (e.g. BAAM) to block varying proportions of the receptor population. The terms efficacy (Stephenson *et al.*, 1956) and intrinsic activity

relate to the maximal ability of a drug-receptor complex in evoking a response in a cell or tissue. Partial agonists display lower intrinsic activity compared to full agonists as assessed by their inability to produce the maximal response of a system even at maximal receptor occupancy. However, receptor theory anticipates that the observed intrinsic activity of ligands will also depend on the level of receptor expression and receptor reserve, and potentially on the level at which the transmitted signal is measured (Kenakin *et al.*, 1989; Hoyer *et al.*, 1993).

Data on the levels of an adenylyl cyclase stimulatory receptor, the G-protein, G_s and adenylyl cyclase have previously been presented by Alousi *et al.*, 1991. It was demonstrated that, in S49 lymphoma cells, the absolute numbers of β_2-adrenoceptor: $G_s\alpha$: adenylyl cyclase are 1,500: 100,000: 3,500. In the cells studied herein case the measured levels of the adenylyl cyclase catalytic subunit are substantially lower than those of the corresponding G-protein, suggesting that the adenylyl cyclase is likely to be the limiting component for information transfer. Furthermore, expression of higher levels of the receptor do not result in greater adenylyl cyclase activity but only in a progressive shift in the agonist concentration-response curve to lower concentrations (Adie *et al.*, 1994b), indicative of the presence of spare receptors. Increased expression of $G_s\alpha$ in this genetic background, as anticipated from the basal stoichiometries noted above, does not result in any significant alteration in the maximal effectiveness of the adenylyl cyclase cascade, although receptors can access and activate the introduced $G_s\alpha$ as effectively as the endogenous G-protein pool (Mullaney *et al.*, 1994). These findings are in accord with studies in which transgenic mice were produced over-expressing $G_s\alpha$ in a cardiac specific manner (Gaudin *et al.*, 1995). In chapter 3.3, the levels of expression of adenylyl cyclase have been altered by transfection of adenylyl cyclase type 2 in this genetic background to evaluate whether this would result in a greater maximal output following agonist activation of the adenylyl cyclase cascade and to examine how potencies of agonists might be regulated.

3.2. Analysis of agonist-induced formation of the complex of $G_s\alpha$ with adenylyl cyclase in NG108-15 cell lines.

3.2.1. Results.

Levels of the IP prostanoid receptor in a total membrane fraction (P1) of NG108-15 cells were measured using [^{3}H]prostaglandin E1 (PGE1) as ligand in the presence of a high concentration of Mg^{2+} (20 mM) (Figure 3.2.1). 0.22 ± 0.03 mg (mean ± S.E.M., n = 6) of the total membrane protein (P1) was generated from 1 x 10^6 NG108-15 cells. Non-specific binding was assessed by parallel assays containing 10 μM PGE1 because it was previously established that this concentration is sufficient to fully displace specific binding of [^{3}H]PGE1 under these assay condition (Adie *et al.*, 1992). As saturation analyses generated from such self-displacement studies are prone to error at concentrations of competitor considerably greater than that of the [^{3}H]ligand used, I applied the formalisms of DeBlasi *et al.*, 1989 [$K_d = IC_{50}$-L; $B_{max} = B_O (K_d+L) / L$; IC_{50}, median inhibitory dose; L, the concentration of radiolabelled ligand used; B_O, the specific binding (total binding - non specific binding)] to assess K_d and B_{max} at this site. Non-linear least-squares analysis indicated that this site bound [^{3}H]PGE1 with a K_d of 34 ± 2 nM (mean ± S.E.M., n = 10) and was present at some 850 ± 70 fmol/mg membrane protein (mean ± S.E.M., n = 10). This is equivalent to the presence of some 113,000 ± 9,000 receptors/cell. Transformation of the specific binding by the method of Rosenthal *et al.*, 1967 indicated the presence of a single high-affinity site with similar binding characteristics (Figure 3.2.1B).

Analysis of levels of $G_s\alpha$ was achieved by immunoblotting the P1 membrane fraction of NG108-15 cells with an antiserum (CS) which was raised against a synthetic peptide predicted to represent the C-terminal decapeptide of all of the splice variants of this G-protein (Milligan *et al.*, 1989). Quantitation of the levels of $G_s\alpha$, which in NG108-15 cells is represented predominantly by a 45 kDa polypeptide (McKenzie *et al.*, 1990b; Adie *et al.*, 1992), was accomplished by concurrent immunoblotting of known amounts of recombinant $G_s\alpha$ purified following expression

in *E. coli* (Figure 3.2.2A). Such analysis demonstrated the $G_S\alpha$ 45 kDa protein to be expressed at some 9.6 ± 0.7 pmol/mg membrane protein (mean ± S.E.M., n = 5). This is equivalent to some 1,275,000 ± 93,000 copies/cell. Quantification of the levels of $G_S\alpha$ based on the radioactivity incorporated into this polypeptide by cholera toxin-catalysed [^{32}P]ADP ribosylation produced considerably lower values (maximum = 550 ± 30 fmol/mg membrane protein). Measured levels of cholera toxin-catalysed [^{32}P]ADP ribosylation of this polypeptide varied with the presence or absence of guanine nucleotides in the assay. Maximal incorporation was achieved in the presence of the poorly hydrolysed analogue of GTP, GTP[S] (Table 3.2.1).

[^{3}H]forskolin binds with high affinity to the complex of adenylyl cyclase and $G_S\alpha$. As flouride ions [due to the formation of the fluoroaluminate ion (AlF_4^-) which is able to interact with GDP-ligated, inactive G-proteins and hence mimic the presence of the γ phosphate of GTP] causes persistent activation of G-proteins, I performed [^{3}H]forskolin binding experiments designed to detect the maximal number of $G_S\alpha$/adenylyl cyclase complexes in the presence of NaF. Preliminary experiments demonstrated that 10 mM NaF was sufficient to produce the greatest level of [^{3}H]forskolin binding (Figure 3.2.3) and that, in the absence of NaF, only some 10% of the maximal specific binding of the radioligand could be detected (Figure 3.2.3). A linear increase in specific high affinity NaF-dependent [^{3}H]forskolin binding was observed with membrane amounts up to 500 µg and no difference in non-specific [^{3}H]forskolin binding (measured by the presence of 10 µM forskolin) was noted in the presence or absence of NaF (data not shown). Dose response curves for NaF activation of adenylyl cyclase and for stimulation of high affinity [^{3}H]forskolin binding were similar (Figure 3.2.3). Addition of the poorly hydrolysed analogue of GTP, Gpp(NH)p stimulated specific high affinity [^{3}H]forskolin binding (Figure 3.2.4) and dose response curves for both this parameter and for Gpp(NH)p-mediated stimulation of adenylyl cyclase were essentially identical with median effective (EC_{50}) values close to 100 nM (Figure 3.2.4). Non-radioactive forskolin displaced the NaF-dependent binding of [^{3}H]forskolin (approximately 20 nM) were detected under these

conditions with IC_{50} of some 30 nM (Figure 3.2.5A). Further displacement of [^{3}H]forskolin by non-radiolabelled forskolin was not obtained at concentrations beyond 10 μM and, as such, this concentration was used routinely to define non-specific binding. In such self-competition studies (Figure 3.2.5), using the formalism of DeBlasi *et al.*, 1989, the estimated K_d for the high affinity binding of [^{3}H]forskolin to membranes of NG108-15 cells was 14 nM. Analysis of the specific binding of [^{3}H]forskolin in self-competition experiments was also performed by the method of Rosenthal *et al.*, 1967 following correction of the specific binding for dilution of the specific activity of the radiolabel. Curve fitting of such data clearly fitted a biphasic-curve consistent with both a high affinity (K_d = 18 nM) lower capacity site (B_{max} = 144 fmol/mg membrane protein) and a low-affinity (K_d = 550 nM) but higher capacity site (approximately 750 fmol/mg membrane protein) although the characteristics of the low-affinity site were difficult to measure accurately due to the low affinity of the ligand for this site. The biphasic nature of [^{3}H]forskolin binding was not unexpected as computer analysis of the self-displacement curves had indicated a pseudo-Hill coefficient of 0.80 ± 0.07, suggestive of the likely existence of two sites. The nature of the low-affinity [^{3}H]forskolin binding site is unknown, but the interaction of forskolin with a variety of cellular proteins including glucose transporters has been reported and it is not believed to represent binding to adenylyl cyclase (Laurenza *et al.*, 1989).

In a range of experiments, the high-affinity binding component of [^{3}H]forskolin binding to membranes of NG108-15 cells in the presence of 10 mM NaF demonstrated the presence of some 130 ± 7 fmol/mg membrane protein (mean ± S.E.M., n = 12) of this complex, equivalent to some 17,300 ± 900 copies/cell. Similar levels of specific high-affinity [^{3}H]forskolin binding was detected when experiments were performed and defined by the presence of the poorly hydrolysed analogue of GTP, Gpp(NH)p (100 μM; data not shown).

Such results indicated that the $G_s\alpha$/adenylyl cyclase complex is quantitatively the limiting component for stimulatory regulation of cyclic AMP production in

NG108-15 cells. Such results, however, present a paradox as it has been noted that IP prostanoid receptor agonist treatment of NG108-15 cells results in down-regulation of not only receptor but also of $G_s\alpha$ (McKenzie *et al.*, 1990b; Adie *et al.*, 1992; Kelly *et al.*, 1990) and that down-regulation results in a heterologous form of desensitization of receptors which cause activation of adenylyl cyclase (Kelly *et al.*, 1990).

When NG108-15 cells were treated with the IP prostanoid receptor agonist, iloprost (1 μM, 16h) and membranes subsequently prepared from these cells, membrane levels of the IP prostanoid receptor were substantially reduced in comparison to membranes prepared in parallel from untreated cells (untreated 900 ± 100 fmol/mg membrane protein; iloprost treated 290 ± 50 fmol/mg membrane protein, each mean ± S.E.M., n = 4). Immunologically detectable membrane-associated levels of $G_s\alpha$ were also reduced significantly ($p = 0.011$) by some 35% from 9.6 ± 0.7 pmol/mg membrane protein (mean ± S.E.M., n = 5). The remaining $G_s\alpha$ appears as if it should be sufficient to generate unchanged amounts of the $G_s\alpha$/adenylyl cyclase complex, particularly as the remaining G-protein appears to be fully active as a regulator of adenylyl cyclase (Adie *et al.*, 1992). However, maximal NaF-dependent high-affinity [^{3}H]forskolin binding in membranes derived from iloprost-treated cells (110 ± 6 fmol/mg membrane protein) was significantly lower (25%; $P = 0.02$) than in membranes from the untreated cells (130 ± 7 fmol/mg membrane protein; each mean ± S.E.M., n = 4).

Furthermore, the specific binding of [^{3}H]forskolin (approximately 10 nM), defined by the presence and absence of 10 μM non-radioactive forskolin, to a total membrane preparation of both clones βN22 and βN17 were assessed in the presence of Gpp(NH)p (100 μM) as previously performed with membranes of parental NG108-15 cells. The derivation of clones βN22 (which expresses 3990 ± 700 fmol of human β_2-adrenoceptor/mg of membrane protein) and βN17 (which expresses 292 ± 62 fmol of the human β_2-adrenoceptor/mg of membrane protein) by transfection of NG108-15 cells has been described in detail previously (Adie *et al.*, 1994a, b). Displacement of Gpp(NH)p-stimulated specific [^{3}H]forskolin binding was achieved

by forskolin in βN22 membranes (Figure 3.2.6A) and in βN17 membranes (data not shown) with high affinity. Use of the formalism of DeBlasi *et al.*, 1989 ($K_d = IC_{50}$-[(^{3}H) L] when the radioligand and displacing drug are one and the same) provided estimates of the affinity of binding of [^{3}H]forskolin (K_d) in these membranes in the presence of Gpp(NH)p of between 20 and 30 nM in a range of experiments.

The specific high affinity binding of [^{3}H]forskolin and its regulation by G_s-linked receptor agonists were assessed in intact wild type NG108-15 cells and those of clones βN22 and βN17 using the method of Alousi *et al.*, 1991. Preliminary time courses for agonist stimulation of [^{3}H]forskolin binding demonstrated that maximal levels of iloprost (10 μM)-stimulated [^{3}H]forskolin binding in NG108-15 cells were achieved by 60 min (Figure 3.2.7A), as was maximal stimulation of specific [^{3}H]forskolin binding by either iloprost (10 μM) or the β-adrenoceptor agonist isoprenaline (10 μM) in cells of clone βN22 (Figure 3.2.7B). This length of incubation was subsequently used for all the other studies. Time courses of [^{3}H]forskolin binding achieved in the absence of agonist also suggested that maximal levels were obtained within 60 min (Figures 3.2.7A and 7B), but these were not generally amenable to computer analysis because of the low level of specific binding obtained. Little specific binding of 10 nM [^{3}H]forskolin (1,600 ± 800 sites per cell; mean ± S.E.M., n = 3) was observed at 60 min in wild type NG108-15 cells in the absence of a receptor ligand but treatment of NG108-15 cells with the IP prostanoid receptor agonist iloprost (10 μM) resulted in a large increase in specific [^{3}H]forskolin binding to 15,000 ± 180 sites per cell (mean ± S.E.M., n = 3; p = 0.04). Addition of the β-adrenoceptor agonist isoprenaline (10 μM) failed, however, to increase specific [^{3}H]forskolin binding (1,900 ± 1,100 sites per cell; mean ± S.E.M., n = 3; p = 0.86). When similar experiments were performed on cells of clone βN17, the specific binding of [^{3}H]forskolin in the absence of a receptor ligand was similar to that observed in wild type NG108-15 cells (2,700 ± 530 sites per cell, mean ± S.E.M., n = 3; p = 0.34) but iloprost (10 μM) produced a larger stimulation of specific [^{3}H]forskolin binding than observed in wild type NG108-15 (31,000 ± 3,900 sites

per cell; mean ± S.E.M., n = 3; p = 0.047) and now addition of isoprenaline (10 μM) resulted in a substantial increase in specific [^{3}H]forskolin binding (26,000 ± 2,500 sites per cell; mean ± S.E.M., n = 3; p = 0.001). A similar pattern was observed in βN22 cells except that the specific binding of [^{3}H]forskolin in the absence of receptor agonist (11,000 ± 1,200 sites per cell; mean ± S.E.M., n = 3) was substantially elevated in comparison to both wild type NG108-15 cells (p = 0.009) and βN17 cells (p = 0.02). However, the maximal level of specific binding of [^{3}H]forskolin produced by isoprenaline (10 μM) (p = 0.21) or by iloprost (p = 0.68) was not significantly greater than in βN17 cells (Table 3.2.3). The ability of forskolin to displace isoprenaline-stimulated [^{3}H]forskolin binding in intact βN22 cells showed that the K_d for specific binding of [^{3}H]forskolin was the same in whole cells as in cell membranes (Figure 3.2.8). Applying the formalism of DeBlasi *et al.*, 1989, as noted above, to such data a K_d for [^{3}H]forskolin of 29 ± 6 nM was determined.

To assess whether the elevated levels of basal specific binding observed in clone βN22 reflect spontaneous empty-receptor activation of GSAC formation by the expressed β_2-adrenoceptor, I performed [^{3}H]forkolin-binding experiments with both NG108-15 cells and clone βN22 cells in the absence of agonist or in the presence of either isoprenaline (10 μM) or propranolol (10 μM) (Table 3.2.4). Propranolol has previously been shown to be an inverse agonist, able to cause a reduction in basal adenylyl cyclase activity in membranes of βN22 cells (Adie *et al.*, 1994b). Furthermore, although isoprenaline had no ability to stimulate specific [^{3}H]forskolin binding in NG108-15 cells, isoprenaline stimulation of specific [^{3}H]forskolin binding in βN22 cells was competed for by propranolol (Table 3.2.4).

Iloprost treatment of wild type NG108-15 cells resulted in a dose-dependent increase in specific binding of [^{3}H]forskolin. A maximum was observed with 100 nM iloprost and this was not increased further by incubation with concentrations of iloprost up to 10 μM. Half-maximal stimulation of specific [^{3}H]forskolin binding was achieved with some 5 nM iloprost (Figure 3.2.9A). Identical dose-effect curves for iloprost stimulation of high affinity [^{3}H]forskolin binding were recorded for cells of

both clones βN22 (EC_{50} = 5.1 ± 1.5 nM) and βN17 (EC_{50} = 2.5 ± 0.3 nM) and the parental NG108-15 cells (Figure 3.2.9B). The β-adrenoceptor agonist isoprenaline also stimulated specific high affinity [^{3}H]forskolin binding in both clones βN22 and βN17 in a dose-dependent manner (Figure 3.2.10) which reached a similar maximal level. Unlike the situation with iloprost, however, the dose-effect curves for isoprenaline were substantially different in the two clonal cell lines. In clone βN22 cells the measured EC_{50} for isoprenaline (1.5 ± 0.4 nM; mean ± S.E.M., n = 3) was some 15 fold lower than in clone βN17 (20.0 ± 1.5 nM; mean ± S.E.M., n = 3).

It has previously been demonstrated that the basal cellular levels of $G_s\alpha$ are not different in clones βN22 and βN17 from these in parental NG108-15 cells (Adie *et al.*, 1994a). To examine if a possible reason for the elevated levels of basal high affinity [^{3}H]forskolin binding in βN22 cells compared to βN17 and parental NG108-15 cells might relate to an elevated level of expression of the adenylyl cyclase catalytic moiety specifically associated with this clone, I immunoblotted membranes of each of these cell types with a antipeptide antibody raised against a section of primary amino acid sequence which is highly conserved in all currently identified hormonally-regulated adenylyl cyclase isoforms. These immunoblots demonstrated a single polypeptide of some 120 kDa, the levels of which were not substantially different in the three cell lines (Figure 3.2.11).

To assess whether IP prostanoid receptor-mediated stimulation of high affinity [^{3}H]forskolin binding in NG108-15 cells would be compromised by the activation of a receptor that mediates inhibition of adenylyl cyclase in these cells, I measured stimulation of high affinity [^{3}H]forskolin binding by iloprost (10 μM) in the presence and absence of a receptor saturating concentration (10 μM) of the peptide DADLE, which in NG108-15 cells mediates inhibition of adenylyl cyclase via the δ opioid receptor and activation of $G_{i2}\alpha$ (McKenzie *et al.*, 1990a). However, even with submaximal stimulation of [^{3}H]forskolin binding by iloprost, the opioid peptide was unable to reduce iloprost-stimulated [^{3}H]forskolin binding and DADLE had no effect in isolation on basal [^{3}H]forskolin binding (Table 3.2.5). Similar results were

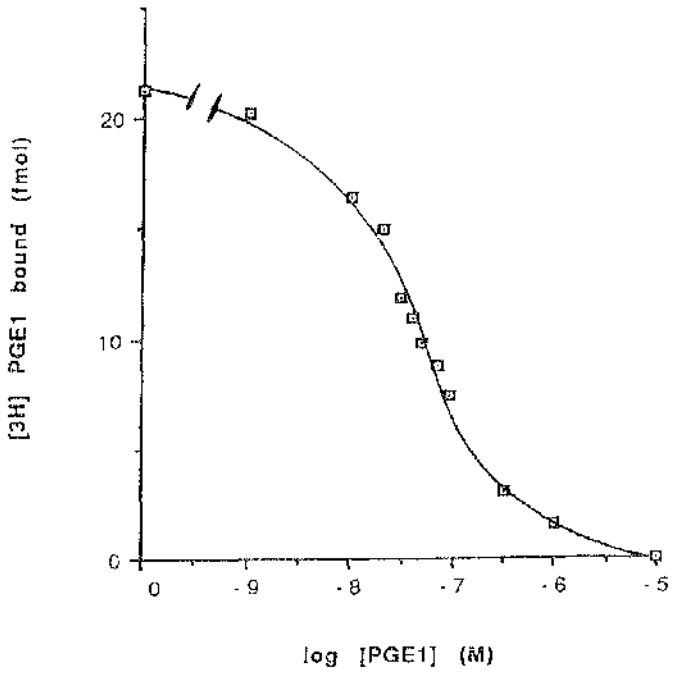
obtained for both iloprost- and isoprenaline-stimulated high affinity [^{3}H]forskolin binding in βN17 and βN22 cells (data not shown).

Figure 3.2.1.

Analysis of levels of the IP prostanoid receptor in membranes of NG108-15 cells.

The specific binding of [^{3}H]PGE1 (defined by the presence and absence of 10 nM PGE1) to total (P1) membranes (100 μg) of NG108-15 cells was performed at 30 °C for 1 h and assessed in self-competition studies as described in chapter 2.2.16. Data was analysed either by the method of DeBlasi *et al.*, 1989 (panel A) or by the method of Rosenthal *et al.*, 1967 (panel B). In the example displayed, [^{3}H]PGE1 was 10.1 nM. Calculated data varied between B_{max} = 849 fmol/mg membrane protein, K_d = 30 nM (panel A) and B_{max} = 985 fmol/mg membrane protein, K_d = 39 nM (panel B).

A


[3H] PGE1 bound (fmol)
20
10
0
0
-9
-8
-7
-6
-5
log [PGE1] (M)

B

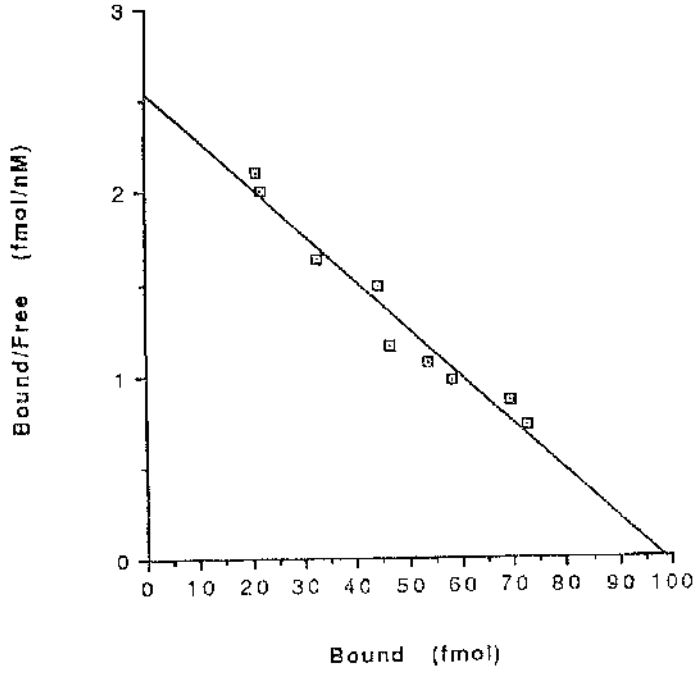
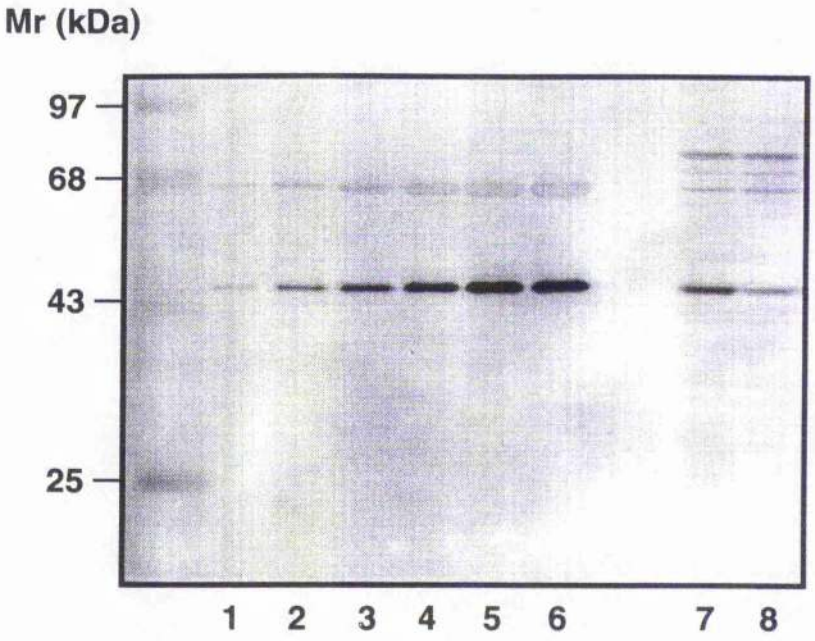

Bound/Free (fmol/nM)
3
2
1
0
0 10 20 30 40 50 60 70 80 90 100
Bound (fmol)

Figure 3.2.2.

Quantitation of $G_s\alpha$ levels in membranes of NG108-15 cells.

Differing amount of *E. coli* generated recombinant $G_s\alpha$ long form (lane 1, 2.5 ng; lane 2, 5 ng; lane 3, 10 ng; lane 4, 15 ng; lane 5, 20 ng; lane 6, 25 ng) were resolved in SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) along with membranes (25 μg) from untreated (lane 7) and iloprost-treated (1 μM, 16h) (lane 8) NG108-15 cells. The gel was then transferred to nitrocellulose and immunoblotted using antiserum CS (which identifies all splice variants of $G_s\alpha$) as primary reagent. The developed immunoblot (panel A) was scanned to construct a standard curve of amounts versus immunological signal (panel B). A similar standard curve was used in each experiment in which quantitation of $G_s\alpha$ was performed. The standard curve was routinely sigmoidal but only values encompassed by the linear section of the curve were used for quantitation. The negatively stained polypeptide observed at 67 kDa in the standard curve samples in panel A is bovine serum albumin, which was added to the purified recombinant G-protein at a final concentration of 1 μg/ng $G_s\alpha$ to limit losses of the G-protein during dilution and acid precipitation. In the example displayed, $G_s\alpha$ in the untreated membranes was 11.2 ng/25 μg membrane protein and in membranes from iloprost treated cells was 7.2 ng/25 μg membrane protein.

A


Mr (kDa)
97
68
43
25
1 2 3 4 5 6 7 8

B

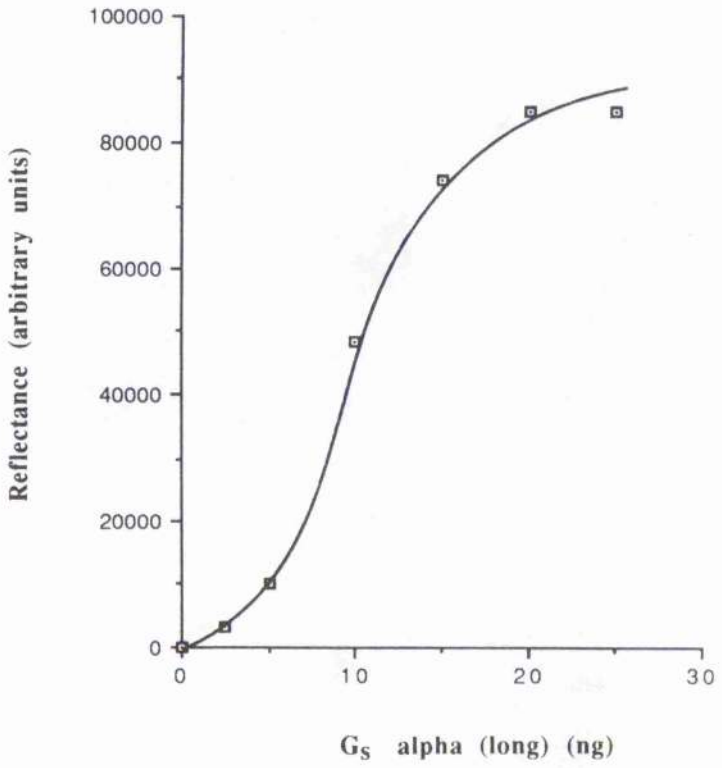

100000
80000
60000
40000
20000
0
Reflectance (arbitrary units)
0
10
20
30
G_S alpha (long) (ng)

Figure 3.2.3.

Sodium fluoride stimulates high affinity binding of [^{3}H]forskolin and activates adenylyl cyclase activity with similar dose response curves.

Adenylyl cyclase activity (filled squares) and the high affinity binding of [^{3}H]forskolin (open squares) were measured on total (P1) membranes of NG108-15 cells in the presence of NaF (10^{-5} - $10^{-1.7}$ M) as described in chapter 2.2.17 and 2.2.16 respectively. Results are plotted as % of the maximal stimulation over basal levels. In the examples displayed for adenylyl cyclase, the basal activity was 15.9 ± 5.8 pmol/min/mg membrane protein, while the maximum activity was 166.9 ± 9.6 pmol/min/mg membrane protein. In this experiment, [^{3}H]forskolin was 12.5 nM. Basal specific [^{3}H]forskolin binding measured was 148 d.p.m. and maximal binding was 1147 d.p.m.

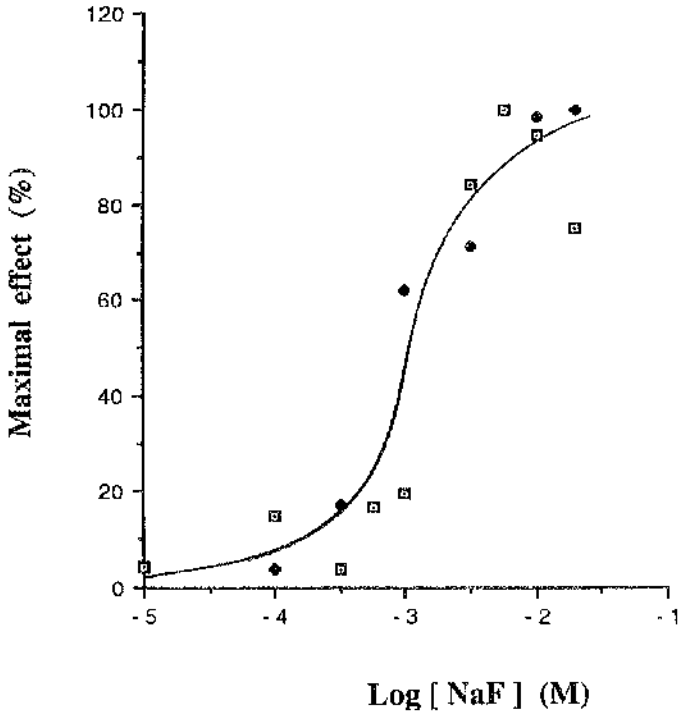


Figure 3.2.4.

Gpp(NH)p stimulates high affinity binding of [^{3}H]forskolin and activates adenylyl cyclase with similar dose response curves.

The high affinity binding of [^{3}H]forskolin (filled squares) and adenylyl cyclase activity (open squares) was measured on total (P1) membranes of NG108-15 cells in the presence of Gpp(NH)p (10^{-10} - 10^{-4} M). Results are plotted as % of the maximal stimulation over basal levels. In the examples displayed, for adenylyl cyclase, the basal activity was 19.1 ± 2.0 pmol/min/mg membrane protein, while the maximum activity was 156.3 ± 0.8 pmol/min/mg membrane protein. In this experiment, the membranes of NG108-15 cells were incubated with 12.5 nM [^{3}H]forskolin. Basal specific [^{3}H]forskolin binding was 169 d.p.m. and maximal binding was 1003 d.p.m.

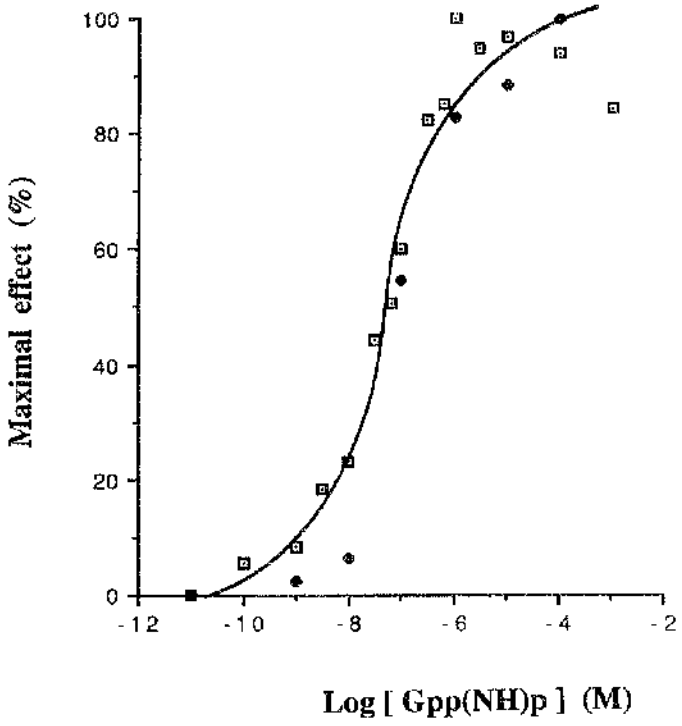
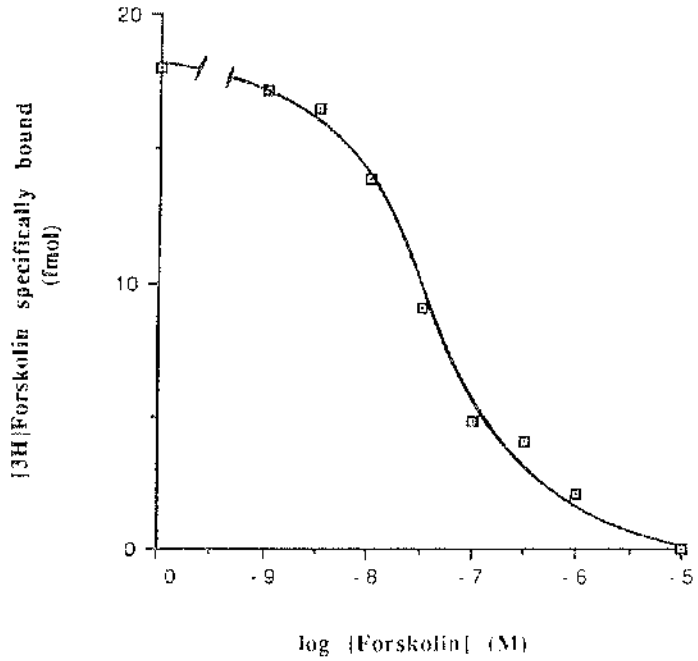


Figure 3.2.5.

[^{3}H]forskolin binding studies and self competition with forskolin to total (P1) membranes of NG108-15 cells.

The binding of [^{3}H]forskolin to total (P1) membranes (250 μg) of NG108-15 cells was performed in the presence of 10 mM NaF and varying concentrations of unlabeled forskolin. Non-specific binding was defined by the presence of 0.1 mM forskolin. Analysis of such data was performed by the methods of either DcBlasi *et al.*, 1989 (panel A) or Rosenthal *et al.*, 1967 (panel B) to define B_{max} and K_d for [^{3}H]forskolin binding. In the experiment displayed, [^{3}H]forskolin was 18 nM and the observed specific binding was 18.0 fmol. Manipulation of this data according to DeBlasi *et al.*, 1989 provides an estimate of B_{max} = 129 fmol/mg membrane protein and K_d = 14 nM. Analysis of the high affinity [^{3}H]forskolin binding site as in Rosenthal *et al.* 1967 estimated that B_{max} = 145 fmol/mg membrane protein and K_d = 18 nM.

A


20
10
0
0
-9
-8
-7
-6
-5
[3H]Forskolin specifically bound (fmol)
log [Forskolin] (M)

B

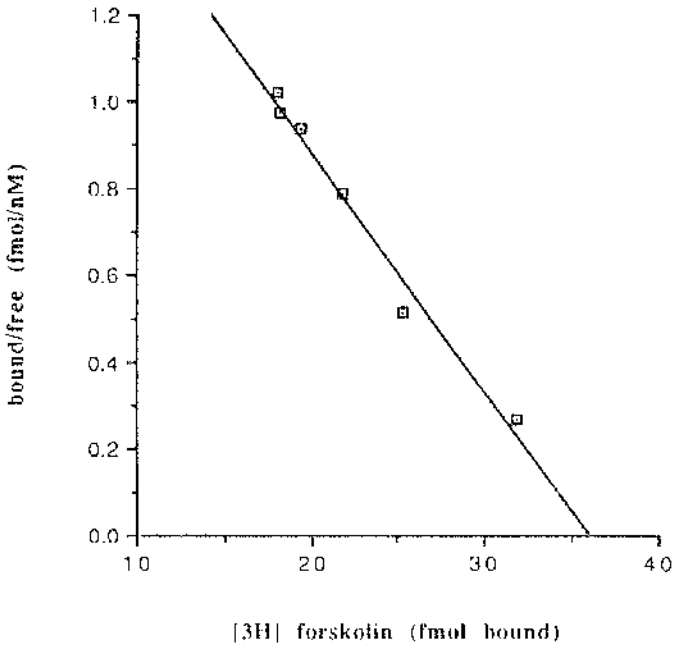

1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
20
30
40
bound/free (fmol/nM)
[3H] forskolin (fmol bound)

Figure 3.2.6.

Analysis of the affinity of the GSAC complex for [^{3}H]forskolin in membranes of clone βN22 cells.

Displacement of guanine nucleotide-stimulated [^{3}H]forskolin binding to membranes of βN22 cells by unlabeled forskolin was performed. Aliquots of a total (P1) membrane fraction (250 µg) of clone βN22 cells were incubated with 100 µM Gpp(NH)p and [^{3}H]forskolin (15.8 nM) along with varying concentrations of forskolin at 20 °C for 60 min as described in chapter 2.2.16 and then bound [^{3}H]forskolin was measured. In the example displayed, the estimated IC_{50} for forskolin was 42.8 nM. Application of the formalism of DeBlasi *et al.* 1989 (K_d = IC_{50} -[(^{3}H)L]) resulted in an estimation of K_d for [^{3}H]forskolin of 27.0 nM.

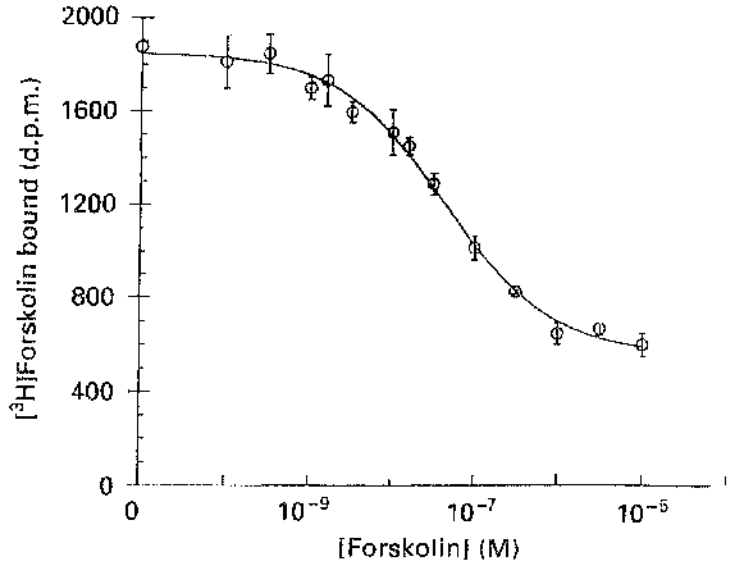


Figure 3.2.7.

Time course of agonist stimulated specific [^{3}H]forskolin binding in intact NG108-15 derived cell lines.

NG108-15 cells (panel A): The ability of iloprost (10 μM) (filled circles) to promote specific [^{3}H]forskolin (13.2 nM) binding in intact NG108-15 cells (4.0 x 10^5/assay) with time was assessed as described in chapter 2.2.16 as was the rate of specific binding of [^{3}H]forskolin in the absence of agonist (open squares). Results are mean ± S.E.M. for three determinations. Maximal levels of binding were achieved by 60 min.

Clone βN22 cells (panel B): The ability of iloprost (10 μM) (open diamonds) or isoprenaline (10 μM) (filled squares) to promote specific [^{3}H]forskolin (11.0 nM) binding in intact clone βN22 cells (4.5 x 10^5/assay) with time was assessed as described in chapter 2.2.16 as was the rate of specific binding of [^{3}H]forskolin in the absence of agonist (filled circles). Results are mean ± S.E.M. for three determinations. Maximal levels of binding were achieved by 60 min.

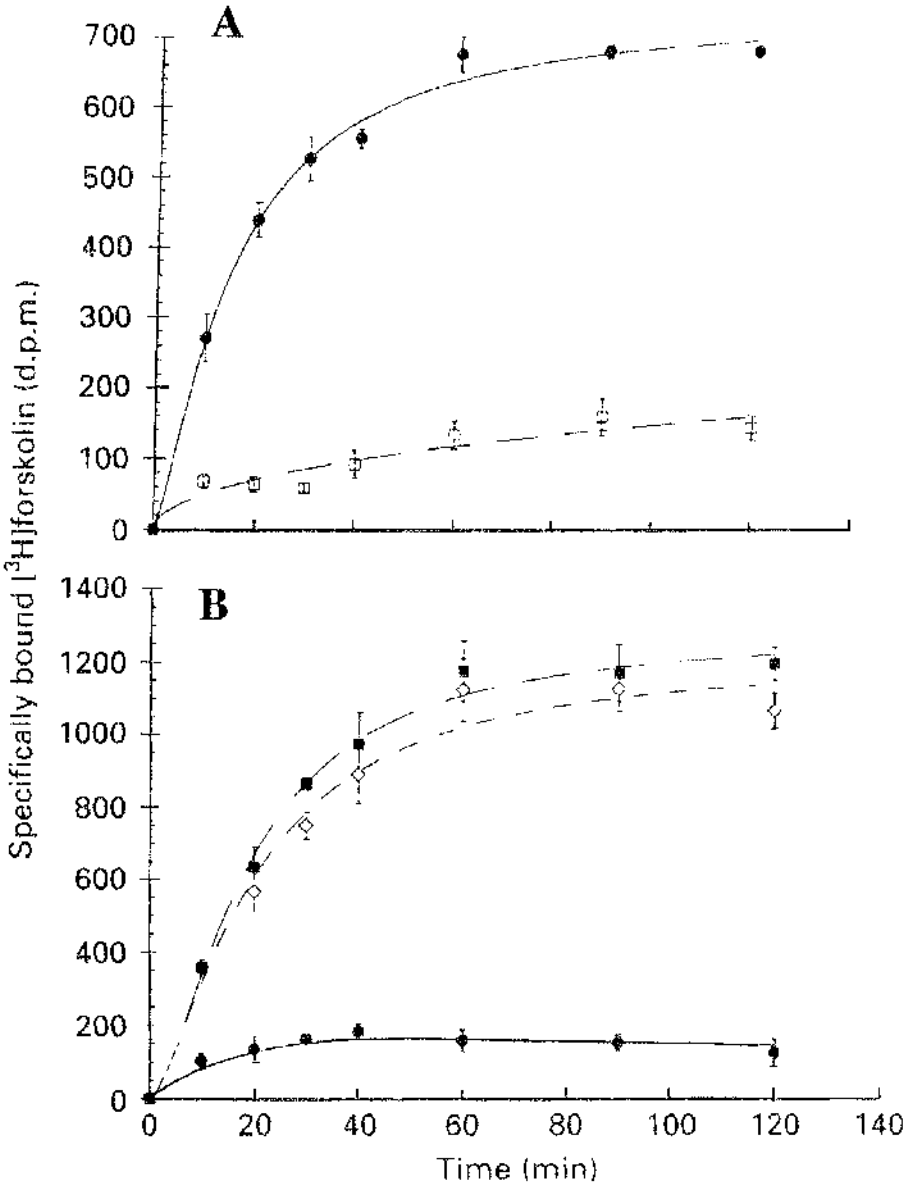

A
B
700
600
500
400
300
200
100
0
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
Time (min)
Specifically bound [3H]forskolin (d.p.m.)

Figure 3.2.8.

Displacement of isoprenaline stimulated [^{3}H]forskolin binding in intact clone βN22 cells by forskolin.

Clone βN22 cells (3.1 x 10^5/assay) were incubated with isoprenaline (10 μM), [^{3}H]forskolin (11.1 nM) and various concentrations of forskolin at 4 °C for 60 min as described in chapter 2.2.16 and bound [^{3}H]forskolin subsequently measured. The estimated IC_{50} was 33.4 nM. Application of the formalism of DeBlasi *et al.*, 1989 as in Figure 3.2.6 resulted in an estimation of K_d for [^{3}H]forskolin of 22.3 nM.

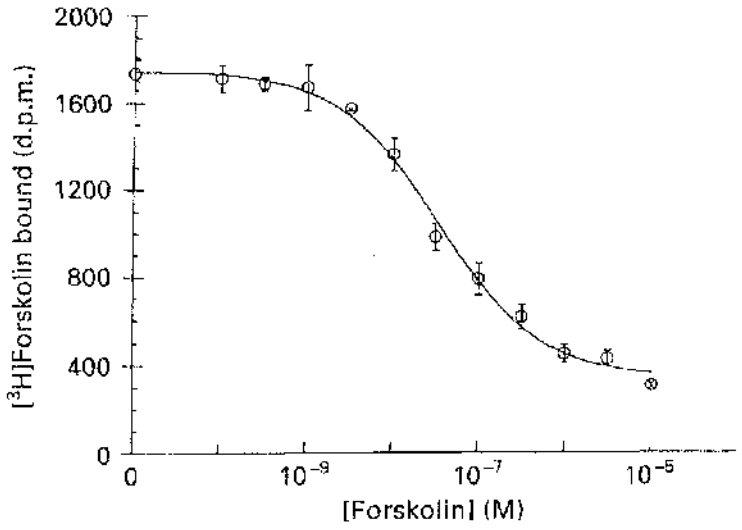


Figure 3.2.9.

Iloprost stimulation of specific [^{3}H]forskolin binding to intact wild type NG108-15, βN22 and βN17 cells: Dose-effect data.

Panel A represents iloprost stimulation of specific [^{3}H]forskolin binding data to intact NG108-15 cells. The specific binding of [^{3}H]forskolin (10.6 nM) and its regulation by various concentrations of iloprost was measured in NG108-15 cells (2.1 x 10^5 cells/assay). In the example shown the EC_{50} for iloprost was 3.6 nM.

Panel B represents iloprost stimulation of specific [^{3}H]forskolin binding data to intact βN22 and βN17 cells. The specific binding of [^{3}H]forskolin (10.0 nM) and its regulation by various concentrations of iloprost were measured in both βN22 (2.3 x 10^5 cells/assay) (filled symbols) and βN17 (2.4 x 10^5 cells/assay) cells (open symbols). In the example shown the estimated EC_{50} for iloprost in βN22 cells was 5.1 nM and in βN17 cells 2.5 nM.

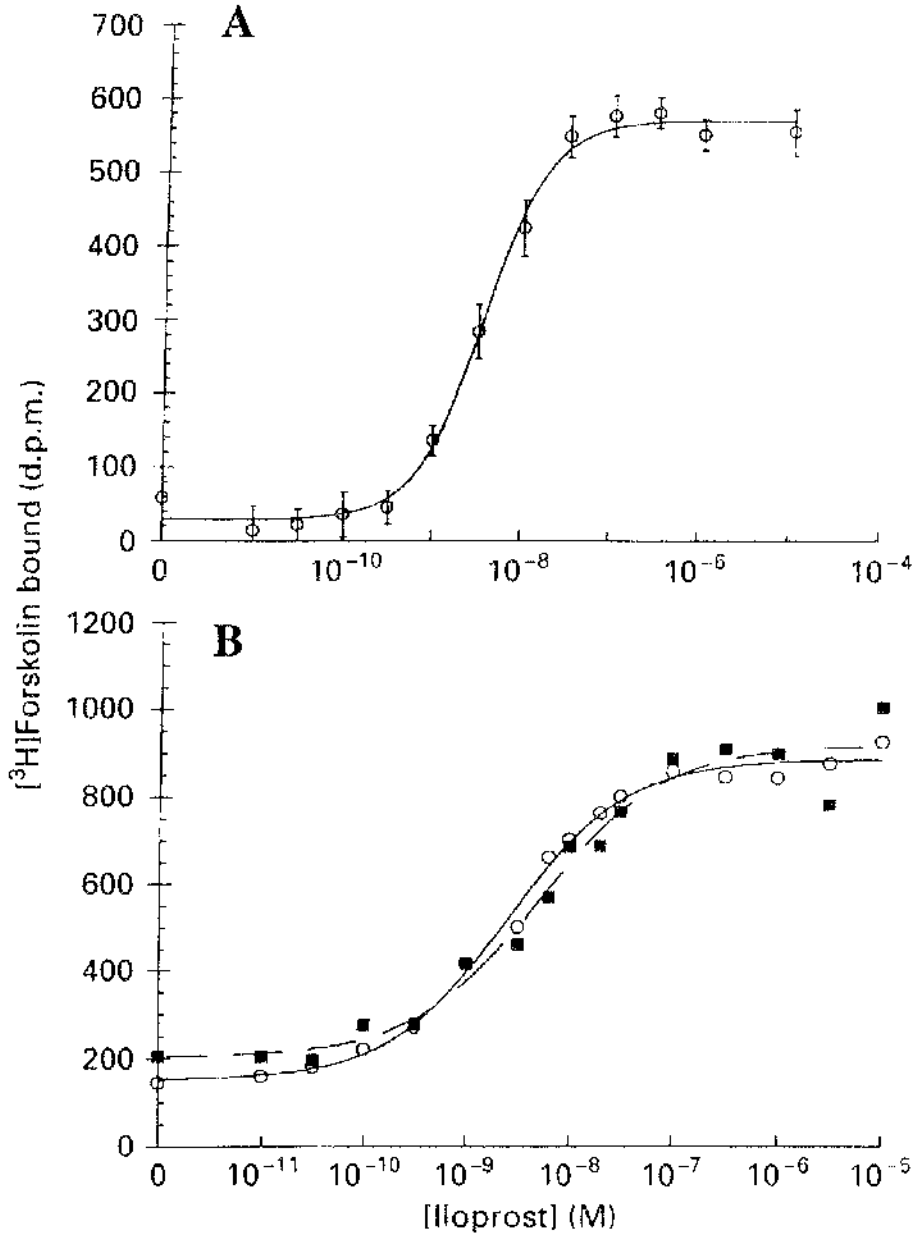

A
B
700
600
500
400
300
200
100
0
1200
1000
800
600
400
200
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[^{3}H]Forskolin bound (d.p.m.)
[Iloprost] (M)

Figure 3.2.10.

Differences in dose-effect curves for isoprenaline stimulation of specific [^{3}H]forskolin binding to βN22 and βN17 cells.

The specific binding of [^{3}H]forskolin (9.7 nM) and its regulation by various concentrations of isoprenaline was measured in both βN22 (2.3 x 10^5 cells/assay) (filled symbols) and βN17 (2.0 x 10^5 cells/assay) cells (open symbols). In the example shown the EC_{50} for isoprenaline in βN22 cells was 0.8 nM and in βN17 cells 18.4 nM.

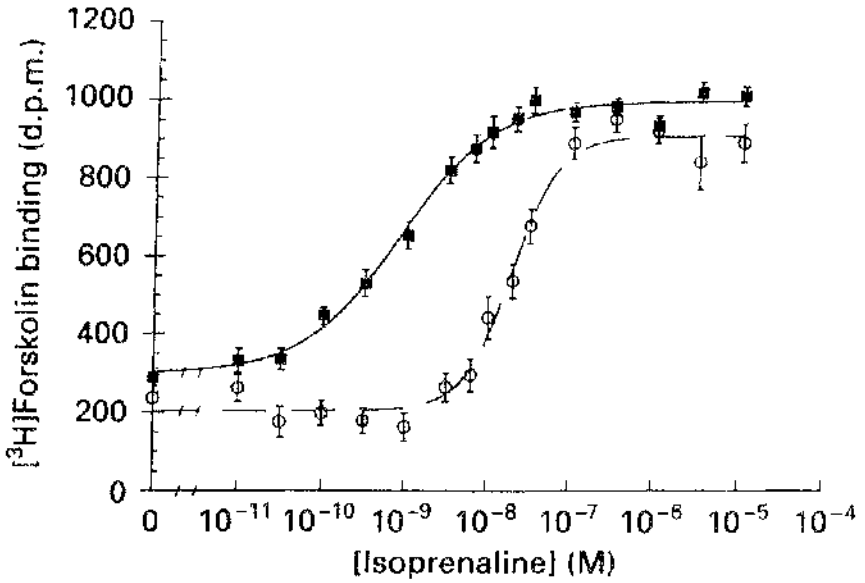


Figure 3.2.11.

Immunological detection of adenylyl cyclase in membranes of wild type NG108-15 and clones βN22 and βN17 cells.

Membranes (100 μg) of NG108-15 (lanes 1 and 4) and clone βN17 (lanes 2 and 5) or βN22 (lanes 3 and 6) cells derived from untreated (lanes 1-3) or isoprenaline (10 μM, 16h) treated (lanes 4-6) cells were resolved by SDS-PAGE (7.5% (w/v) acrylamide 0.25% (w/v) bisacrylamide), transferred to nitrocellulose and immunoblotted with the anti-adenylyl cyclase common peptide serum mentioned in chapter 2.2.6 (1:500 dilution). The nitrocellulose filter was subsequently treated as described in chapter 2.2.13 and detection was achieved by treatment with [^{125}I] protein A (0.04 mCi/ml) followed by exposure to a phosphor storage plate.

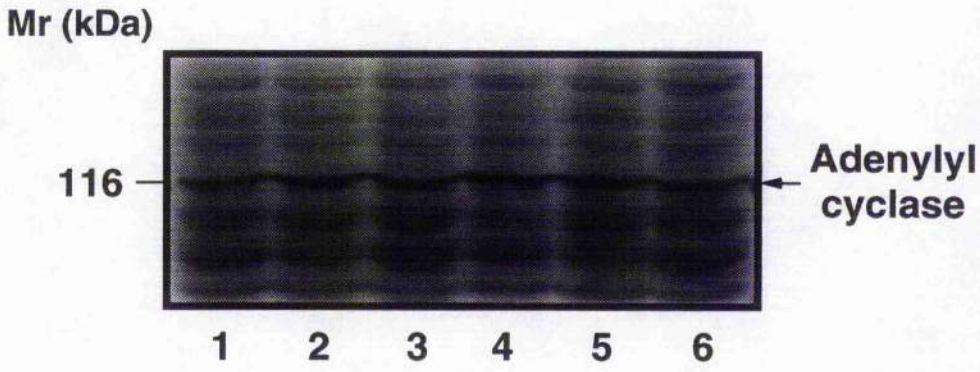

Mr (kDa)
116
Adenylyl
cyclase
1
2
3
4
5
6

Table 3.2.1.

Quantitation of $G_s\alpha$ in membranes of NG108-15 cells using cholera toxin-catalysed [^{32}P]ADP ribosylation.

Cholera toxin-catalysed [^{32}P]ADP ribosylation was performed on membranes of NG108-15 cells as described in chapter 2.2.15. Data represent mean ± S.E.M. (n = 4).

Condition	Apparent $G_s\alpha$ levels
	fmol/mg membrane protein
Control	254 ± 11
GDP[S] (100 μM)	300 ± 12
GTP[S] (100 μM)	547 ± 29

Table 3.2.2.

Quantitation of components of the stimulatory arm of the adenylyl cyclase cascade in NG108-15 cells.

The levels of each polypeptide component expressed as copies/cell (mean ± S.E.M.; n = 10 for IP prostanoid receptor, n = 5 for $G_S\alpha$, n = 12 for $G_S\alpha$/adenylyl cyclase complexes).

Components	Copies/cell
IP prostanoid receptor	113,000 ± 9,000
$G_S\alpha$	1275,000 ± 93,000
$G_S\alpha$/adenylyl cyclase complex	17,300 ± 900

Table 3.2.3.

Agonist stimulated maximal specific [^{3}H]forskolin binding in intact βN17 and βN22 cells.

Specific binding of [^{3}H]forskolin (10.0 nM) to intact βN17 and βN22 cells was assessed as described in the chapter 2.2.16 in the absence (basal) or presence of either iloprost (10 μM) or isoprenaline (10 μM). Data are presented as mean ± S.E.M., n = 3 for the calculated number of high affinity [^{3}H]forskolin binding site per cell detected by this concentration of [^{3}H]labelled ligand.

	[^{3}H]forskolin bound (sites/cell)	
	βN17	βN22
Basal	2,700 ± 530	11,000 ± 1,200
Isoprenaline (10 μM)	26,000 ± 2,500	35,000 ± 4,900
Iloprost (10 μM)	31,000 ± 3,900	34,000 ± 4,100

Table 3.2.4.

Effect of propranolol on basal specific [^{3}H]forskolin binding in intact NG108-15 and clone βN22 cells.

The specific binding of [^{3}H]forskolin (13.0 nM) was determined in either intact parental NG108-15 cells or clone βN22 cells (2.2 x 10^5/assay). Data are presented as mean ± S.E. of triplicate determinations from a single experiment. Similar data were observed in a further experiment. N.D., Not determined.

	[^{3}H]forskolin binding (d.p.m.)	
	NG108-15	βN22
Basal	110 ± 19	260 ± 11
Isoprenaline (10 μM)	97 ± 3	840 ± 160
Propranolol (10 μM)	100 ± 3	160 ± 26
Isoprenaline (10 μM) + Propranolol (10 μM)	N. D.	430 ± 16

Table 3.2.5.

Effect of the opioid agonist DADLE on agonist-stimulated specific [^{3}H]forskolin binding to NG108-15 cells.

The specific binding of [^{3}H]forskolin (12.0 nM) was assessed in intact NG108-15 cells (2.0 x 10^5 cells/assay) as described in the chapter 2.2.16 either in the absence of agonist or in the presence of the IP prostanoid receptor agonist iloprost (10 μM), the opioid receptor agonist DADLE (10 μM) or both. Data are presented as mean ± S.D. of quadruplicate determinations from a single experiment. Similar results were obtained in two further experiments.

	[^{3}H]forskolin binding to NG108-15 cells (d.p.m.)
Basal	79 ± 4
Iloprost (10 μM)	320 ± 8
DADLE (10 μM)	85 ± 11
Iloprost (10 μM) + DADLE (10 μM)	310 ± 14

3.2.2. Discussion.

The absolute or even relative levels of the individual polypeptides which comprise cellular signaling cascades across the plasma membrane of individual cells have hardly been investigated despite enormous interest in the mechanisms of agonist-mediated second-messenger generation. Considerable information is available for the receptors because of the availability of radiolabelled drugs of high selectivity, but the same is not true for the G-proteins and/or effectors. However, to appreciate the potential role of regulation of levels of these polypeptides in the control of cellular sensitivity, it is necessary to know their amounts. In this study, I have measured levels of each of IP prostanoid receptor, $G_s\alpha$ and the complex of $G_s\alpha$ and adenylyl cyclase in membranes of neuroblastoma x glioma NG108-15 cells (Table 3.2.2). Furthermore, I have examined the ability of receptors to activate adenylyl cyclase in NG108-15 cells and in clones generated from these cells that have been transfected to express different levels of the human β_2-adrenoceptor.

Levels of the IP prostanoid receptor were assessed by analysis of self-competition experiments using [^{3}H]PGE1. The traditional approach used to measure receptor levels is to perform saturation experiments using increasing concentrations of radioligand but in situations in which the ligand has relatively low affinity or the specific binding represents a low proportion of the measured total binding, such an approach is not feasible. In this case, I have analysed the specific binding of [^{3}H]PGE1 both taking into account the dilution of the specific activity of the radioligand by the presence of unlabelled PGE1 and using the formalisms described by DeBlasi *et al.*, 1989 to calculate both receptor number and the affinity of the receptor for PGE1. Both of these approaches have limitations. In the case of recalculation for the reduction in specific activity, this is primarily due to the potential for high errors in calculations in which the bulk of the radioligand has been displaced and which, therefore, require a large dilution-factor correction to be applied to a relatively small number of specific counts. In the case of the analysis of DeBlasi *et al.*, 1989, if the measured IC_{50} for the unlabelled ligand is high in comparison to the

ligand concentration employed, errors can be large as $K_d = IC_{50}-L$. Despite these concerns, the two approaches provided very similar results, indicating that the receptor is expressed at approximately 100,000 copies/cell and with a K_d of some 30 nM for [^{3}H]PGE1. The receptor number data are very much higher than those originally reported for this receptor on NG108-15 cells by Keen *et al.*, 1991 but are in good agreement with more recent data from both Adie *et al.*, 1992 and Donnelly *et al.*, 1992.

Potentially, two distinct approaches are available to measure levels of the stimulatory G-protein, $G_s\alpha$. The first of these is cholera toxin-catalysed [^{32}P]ADP ribosylation of this polypeptide and the second is by immunological means. Quantitation of cholera toxin-catalysed [^{32}P]ADP ribosylation of $G_s\alpha$ is subject to a number of constraints including the nature of the guanine nucleotide bound to the polypeptide and hence the interaction of the α subunit with the $\beta\gamma$ complex and the availability of polypeptides of the ADP ribosylation factor (ARF) family (Donaldson *et al.*, 1992). I made no attempt to add purified $\beta\gamma$ subunits to the membranes to assess the effect of these on the extent of cholera toxin-catalysed ADP ribosylation. Using an equivalent quantitative immunoblotting approach to that used herein to measure levels of $G_s\alpha$ levels of the steady-state level of the β subunit in membranes of NG108-15 cells is some 80 pmol/mg membrane protein (Adie and Milligan unpublished results) and, as lower levels of incorporation of [^{32}P]ADP-ribose into $G_s\alpha$ 45 kDa protein were obtained in the presence of GDP[S] (which should promote the interaction of G-protein α subunits with $\beta\gamma$) than with GTP[S] (which will dissociate the complex), then I would anticipate that addition of $\beta\gamma$ would even reduce incorporation of radiolabel and hence the apparent levels of $G_s\alpha$ measured by this approach even further. As such, cholera toxin-catalysed [^{32}P]ADP ribosylation can only provide a minimum estimate of the cellular levels of $G_s\alpha$. Immunological quantification offers a more quantitatively rigorous approach. In these studies, I have used varying amounts of the long form of $G_s\alpha$ expressed in and subsequently purified from *E. coli* to construct standard curves against which amount of $G_s\alpha$ present in membranes of

NG108-15 cells could be measured. Prokaryotically expressed G-proteins lack post-translational modifications which are essential for the effective functioning of these proteins in mammalian cells, but there is no known modification of $G_s\alpha$ in the region of the polypeptide which serves as the epitope for recognition by the antiserum used in these studies. As such, it must be anticipated that immunoreactivity of the recombinant G-protein and that expressed in NG108-15 membranes will be equivalent. As with other uses of quantitative immunoblotting, the measured signal versus amount of the G-protein produced a sigmoidal curve (Figure 3.2.2B) a linear region of approximately 2.5-20 ng recombinant protein was routinely recorded which allowed quantitation of $G_s\alpha$ and demonstrated the presence of some 1,250,000 copies of this polypeptide/cell, a value some 12 fold in excess of the IP prostanoid receptor. As it has previously been calculated that agonist activation of this receptor is able to result in the concurrent down-regulation of some 8 mol $G_s\alpha$/mol receptor in a cyclic AMP independent manner $G_s\alpha$ and it has been argued that this ratio may represent stoichiometry of interaction of the IP prostanoid receptor with $G_s\alpha$ in the presence of agonist (Adie *et al.*, 1992), then full receptor activation might thus be anticipated to be able to activate some 66% of the total G-protein pool. The level of $G_s\alpha$ assessed using cholera toxin-catalysed [^{32}P]ADP ribosylation was only 6% of that measured immunologically and thus further confirms the problems associated with using this approach for quantitative purposes. The number of copies of $G_s\alpha$/cell calculated here is some 5 fold higher than that reported for $G_s\alpha$ in S49 lymphoma cells (Levis *et al.*, 1992) but it must be considered that NG108-15 cells are larger that S49 cells. Similarly, higher numbers, but a similar 5 fold higher ratio of $G_s\alpha$/adenylyl cyclase complexes/cell was detected in these studies in comparison to S49 cells (Alousi *et al.*, 1991).

The activation of multiple G-proteins by a receptor allows for amplification of the signal but how effective this amplification is must also depend upon the level of expression of the effector moiety. The effective adenylyl cyclase catalytic moiety is a functional complex of $G_s\alpha$ and the adenylyl cyclase polypeptide. This complex is the

high affinity target for forskolin binding (Laurenza *et al.*, 1989; Levis *et al.*, 1992). Thus, to measure levels of this complex in NG108-15 cell membranes, I have assessed specific high-affinity binding of [^{3}H]forskolin. As both the fluoroaluminate ion (AlF_4^-; provided as NaF) and the GTP analogue, Gpp(NH)p, are able to activate adenylyl cyclase in a pseudo-constitutive fashion, I initially assessed whether the dose response curves for activation of adenylyl cyclase and for promotion of high affinity binding of [^{3}H]forskolin in NG108-15 cells membranes by both of these agents were identical. As they were (Figures 3.2.3 and 4), this provided strong support for the notion that maximal high affinity [^{3}H]forskolin binding under such conditions provides a quantitative measure of the $G_s\alpha$/adenylyl cyclase complex. A low affinity (approximately 550 nM) but higher capacity site was also evident upon computer analysis of the forskolin self-competition binding experiments. The nature of this site is undefined (Laurenza *et al.*, 1989) but it is possible that the low affinity [^{3}H]forskolin site does represent interaction with a population of adenylyl cyclase and that the actual level of adenylyl cyclase in the cells is some 5 fold greater than I have estimated. However, if so, this adenylyl cyclase does not appear to be relevant to either guanine nucleotide or NaF-stimulated adenylyl cyclase activity, as the dose response curves of these two agents to activate adenylyl cyclase and to promote the high affinity binding of [^{3}H]forskolin were identical (Figures 3.2.3 and 4). Others have also argued that this site, which is observed in a wide range of cells and tissues (Laurenza *et al.*, 1989) is not relevant to adenylyl cyclase regulation by agonists (Alousi *et al.*, 1991).

Recently, isolation of cDNA clones encoding distinct molecular forms of adenylyl cyclase have indicated the expression of multiple forms of this enzyme. These individual forms are regulated to varying degree (and in both a stimulatory and inhibitory fashion) by a number of factors which include $\beta\gamma$ subunits and Ca/calmodulin (Tang *et al.*, 1992; Pieroni *et al.*, 1993). Within these studies, I have not been able to address the nature of the adenylyl cyclase isoforms expressed by NG108-15 cells. However, as the membranes of the cells were generated by

homogenization in a buffer to which no calcium was specifically added and which contains EDTA, then levels of this ion must be low. As such, I may have, to some extent, depleted the membranes of calmodulin. This may have reduced the level of adenylyl cyclase I have measured if a calmodulin-sensitive form of the enzyme represents a significant fraction of the cellular adenylyl cyclase pool in these cells.

The measured ratio of $G_s\alpha$ to the complex of this polypeptide and adenylyl cyclase might appear to provide evidence for the effector providing the limiting component for the effectiveness of stimulatory regulation of adenylyl cyclase in NG108-15 cells. However, it has previously been noted that reduction in cellular levels of $G_s\alpha$ following treatment with either the prostanoid agonist iloprost (Kelly *et al.*, 1990) or with ethanol (Monchly-Rosen *et al.*, 1988) can be associated with the development of sustained heterologous desensitization of the stimulatory arm of adenylyl cyclase in NG108-15 cells. Such studies have been used to imply that $G_s\alpha$ might represent the limiting component for this cascade, at least in these cells. Morover, similar studies in rat white adipocytes, where treatment with either prostaglandin E1 or phenyl-isopropyladenosine leads to a down-regulation of the G_i-like G-proteins (Green *et al.*, 1990) and heterologous desensitization to all antilipolytic hormones (Green *et al.*, 1992) also indicate the potential for the regulation of G-protein level to act as a locus to control cell signaling sensitivity.

One of the aims of these experiments was therefore to assess whether the reduction of $G_s\alpha$ following prostanoid treatment of NG108-15 cells would be sufficient to reduce the ability to form the complex of $G_s\alpha$ and adenylyl cyclase. In these studies, I found that sustained treatment of NG108-15 cells with iloprost resulted in a 35% reduction in cellular amounts of $G_s\alpha$ but that despite leaving some 45 fold excess of $G_s\alpha$ in comparison to the measured levels of high affinity [^{3}H]forskolin binding sites, this treatment resulted in a decline by 25% of these sites. These observations suggest that it is possible that only a limited fraction of the $G_s\alpha$ in the cell has access to adenylyl cyclase or that the stoichiometry of G-protein/effector must be high to generate a maximal signal. There are a number of potential reasons for

both of these possibilities. It has previously been shown in brain that $G_s\alpha$ can interact both strongly and selectively with tubulin (Wang *et al.*, 1990a). Recent studies on S49 lymphoma cells clearly indicates that agents (e.g. colchicine and vinblastine) which are able to act to disrupt microtubules increase receptor-mediated cyclic AMP formation (Leiber *et al.*, 1993). Furthermore, these agents can increase the amount of $G_s\alpha$/adenylyl cyclase complex in cells as assessed by the high affinity binding of [^{3}H]forskolin (Leiber *et al.*, 1993). Such observations provide clear evidence for a role of the cytoskeletal network in delineating and limiting the physical interactions of $G_s\alpha$ with adenylyl cyclase. Both in the visual system, where the G-protein transducin has been reported to be present in some 10 fold molar excess of the effector, cyclic GMP phosphodiesterase (Stryer *et al.*, 1986b) and in S49 lymphoma cells where values of 30-80 fold molar excess of $G_s\alpha$ over the adenylyl cyclase/$G_s\alpha$ complex has been calculated (Alousi *et al.*, 1991) the G-protein appears to be more prevalent that the effector system it regulates. This may be a reflection of the life time of the GTP-bound and hence activated G-protein. Studies with the G-protein $G_q\alpha$ and its effector phospholipase C β1 indicate that the effector can act as a GTPase activating protein for the G-protein (Berstein *et al.*, 1992b), thus limiting the period for which a single G-protein can regulate the effector. The same appears to be true for the interactions of transducin with the cyclic GMP phosphodiesterase of the visual system (Arshavsky *et al.*, 1992). Although it is yet to be demonstrated directly, these reports suggest that all effector systems, including adenylyl cyclase, may act as GTPase-activating protein for their relevant G-proteins. If this is true, it may provide a rationale for why a high stoichiometry of G-protein/effector is required to provide maximally effective regulation of a signaling cascade.

On the other hand, data on the levels of an adenylyl cyclase stimulatory receptor, the G-protein, G_s and adenylyl cyclase have been presented concurrently only for two cell lines. Alousi has demonstrated that, in S49 lymphoma cells, the absolute numbers of β_2-adrenoceptor: $G_s\alpha$: adenylyl cyclase are 1,500: 100,000: 3,500 (Alousi *et al.*, 1991), while in neuroblastoma x glioma hybrid, NG108-15 cells,

I have shown levels of IP prostanoid receptor: $G_s\alpha$: adenylyl cyclase are 113,000: 1,275,000: 17,000. In both these cases the measured levels of the adenylyl cyclase catalytic subunit are very much lower than those of the corresponding G-protein, suggesting that the adenylyl cyclase is likely to be the limiting component for information transfer. If this is so, receptor activation of the cascade might be expected to be able to result in virtually complete activation of the theoretically available adenylyl cyclase. Therefore, I have examined the ability of receptors to activate adenylyl cyclase in NG108-15 cells and in clones generated from these cells which have been transfected to express differing levels of the human β_2-adrenoceptor. To do so I have adopted the strategy of Alousi *et al.*, 1991 in which the ability of agonist to promote the formation and maintenance of a high affinity binding site for the diterpene [^{3}H]forskolin provides a measure of the formation of the physical complex between $G_s\alpha$ and adenylyl cyclase (GSAC). However, one potential concern with these studies is that agonist regulation of [^{3}H]forskolin binding had to be performed at 4 °C. I have attempted to measure agonist-stimulation of [^{3}H]forskolin binding in both membranes and whole cells at higher temperatures, but with a lack of success. Such a limitation was also noted by Alousi *et al.*, 1991. This is likely to be a reflection that, as noted by a variety of workers G-protein GTPase activity is negligible at 4 °C and therefore, following stimulation, the GSAC complex will be maintained because the G-protein will remain in the active GTP-liganded form, whereas at higher temperatures the G-protein would be deactivated and the high affinity complex for [^{3}H]forskolin will thus not be stably maintained.

In this study I have demonstrated in membranes of NG108-15 cells that both NaF and Gpp(NH)p stimulation of high affinity [^{3}H]forskolin binding (K_d approximately 20 nM) have similar dose-effect curves as stimulation of adenylyl cyclase activity by these two agents (Figures 3.2.3 and 4). I also demonstrate that the measured K_d for the binding of [^{3}H]forskolin to the GSAC complex is similar whether it is assessed by examining forskolin competiton curves for the [^{3}H]forskolin

binding site following activation of the complex in membranes with the GTP analogue Gpp(NH)p or in whole cells by addition of receptor agonist (Figures 3.2.6 and 8).

Incubation of parental NG108-15 cells with [^{3}H]forskolin in the absence of receptor agonists resulted in very little specific binding of this ligand (Figure 3.2.7A). However, addition of the IP prostanoid receptor agonist iloprost produced a substantial elevation of specific [^{3}H]forskolin binding. Time courses of these effects indicated that maximum levels of binding were obtained by 60 min, both in these cells and in the clonal cell lines transfected to express the β_2-adrenoceptor (Figure 3.2.7B). Dose-effect curves for iloprost demonstrated an EC_{50} for this effect of some 5 nM (Figure 3.2.9). The lack of significant levels of high affinity [^{3}H]forskolin binding in the absence of agonist may be taken to indicate that there is little preformed, precoupled GSAC complex in NG108-15 cells. The question of whether receptors and G-proteins and G-proteins and effectors exist in some form of precoupled arrangement is one which has been argued over the years from a number of points of view (Levitski *et al.*, 1990; 1993). In the case of $G_S\alpha$ and adenylyl cyclase, the ability to co-purify (Pfeuffer *et al.*, 1985; Marbach *et al.*, 1990) or co-immunoprecipitate (Morris *et al.*, 1990) a complex containing both adenylyl cyclase and $G_S\alpha$ in an apparently 1:1 stoichiometric ratio would appear to provide evidence for the precoupling of these polypeptides. These experiments, however, have usually been performed on samples following preactivation of the G-protein with either a poorly hydrolysed guanine nucleotide (e.g. Gpp(NH)p or GTPγ(S)) or with a mixture of Al^{3+}, Mg^{2+} and F^- ions. These agents are all predicted to promote an essentially irreversible formation of a GSAC complex as they persistently activate adenylyl cyclase by producing a quasi-constitutive activation of $G_S\alpha$. Furthermore it is following such pretreatments that forskolin affinity columns have been noted to be most useful for the retention and hence purification of adenylyl cyclase (Pfeuffer *et al.*, 1985; Marbach *et al.*, 1990). Other studies which have detected the presence of preformed GSAC complex have been performed at low ionic strength, an inappropriate approach when compared to the intact cell studies reported herein. It has

also been argued from kinetic considerations that a GSAC complex must exist in the absence of hormonal stimulation (Levitski *et al.*, 1990; 1993). However, if the high affinity state for [^{3}H]forskolin binding indeed represents this complex, then it cannot exist to any significant degree either in unstimulated whole NG108-15 cells or membranes from these cells as I observed little or no high affinity binding in the absence of hormonal or direct stimulation in the parental cells. An alternative possibility is that the high affinity state for [^{3}H]forskolin is only produced by a complex of GTP-GSAC and not by GDP-GSAC. It is difficult, however, to devise experiments in whole cells which would allow these possible models to be differentiated.

Whereas few specific high affinity [^{3}H]forskolin binding sites could be measured either in the absence of hormonal stimulation in intact wild type NG108-15 cells or cells of clone βN17 which express relatively low levels of the β_2-adrenoceptor, this was not the case in cells of clone βN22 in which levels of this receptor are considerably higher (Adie *et al.*, 1993; 1994a). The basal level of high affinity [^{3}H]forskolin binding was substantially (some 3 fold) higher than in the other two cell lines (Tables 3.2.3 and 4). However, it has previously been demonstrated that the basal cellular levels of $G_s\alpha$ are the same in these cells (Adie *et al.*, 1994a). Furthermore, to exclude that βN22 cells endogenously express higher levels of the adenylyl cyclase catalytic moiety, membranes from βN22, βN17 and parental NG108-15 cells were immunoblotted with an antiserum raised against a sequence predicted to be highly conserved amongst the known mammalian adenylyl cyclase isoforms (Premont *et al.*, 1993). Although there are at least ten known molecular forms of mammalian hormonally regulated adenylyl cyclase, all are targets for regulation by forskolin (Premont *et al.*, 1993; Tang *et al.*, 1992). This antiserum identified an apparently single polypeptide of some 120 kDa which was present at the same level in the three cell lines (Figure 3.2.11). This indicates that there is either a greater preponderance of preformed GSAC complex in the absence of hormonal stimulation in these cells or that more of this complex, if it exists preformed, is in the GTP-occupied

state. These findings are entirely in accord with previous observations that membranes of βN22 cells have a substantially elevated basal adenylyl cyclase activity compared to membranes from either wild type NG108-15 or clone βN17 cells, an effect which was interpreted as reflecting the spontaneous intrinsic activation of a fraction of the cellular $G_S\alpha$ population in clone βN22 due to the high levels expression of the β_2-adrenoceptor (Adie *et al.*, 1994b). This is a general concept which has found support in a number of other recent studies which have examined 'negative agonism' at the β_2-adrenoceptor following expression of this receptor in either mammalian cell lines or in insect Sf9 cells (Samama *et al.*, 1994a; Chidiac *et al.*, 1994). Furthermore, after expression of the human β_2-adrenoceptor in *E. coli* and reconstitution of membranes of these cells with G_S, the β-adrenoceptor antagonist propranolol has been shown to be able to reduce the rate of binding of [^{35}S] GTPγS below basal levels (Schutz *et al.*, 1993). Studies of transgenic mice expressing the β_2-adrenoceptor in high levels in a cardiac tissue specific manner have also provided data consistent with such a model as in these animals a spontaneous increase in β_2-adrenoceptor function was observed (Milano *et al.*, 1994). This observation is consistent with propranolol either displaying 'negative intrinsic activity' (Milano *et al.*, 1994) or simply suppressing the spontaneous activity resulting from the high levels of expression of the receptor. An alternative explanation for these observations might be that the cells generate sufficient endogenous catecholamine during the incubations to activate the receptor population. If this were so, it would be expected to result in elevated levels of basal specific [^{3}H]forskolin binding in clone βN22 cells by competing with the endogenously produced ligand. Although such a hypothesis is consistent with the observations made, there are a number of reason why such reasoning is incorrect in this case. (1) If propranolol were competing with endogenously generated catecholamine for the ligand binding site, then it would be expected to totally reverse the elevated 'basal' level if present in sufficiently high concentration. This was not observed; high concentrations of propranolol were used in these assays specifically to address this point. Basal specific [^{3}H]forskolin binding in clone βN22 cells was reduced by propranolol but not

to the levels observed in the parental NG108-15 cells. (2) Exactly the same partial reversal of elevated activity by propranolol has been observed in membranes derived from these cells (Adie *et al.*, 1994b). (3) Previous attempts to measure levels of endogenously generated catecholamines in the tissue culture medium of βN22 cells at the moment of cell harvest to assess whether carryover from this could account for the increased adenylyl cyclase activity via agonist mediated stimulation of the receptor have indicated that both adrenaline and noradrenaline were below detectable levels, whereas standards at concentrations as low as 125 pM were easily detected (Adie *et al.*, 1994b). (4) I have extended these studies to determine whether the cells produce detectable levels of catecholamines during the period of the [^{3}H]forskolin binding assay. I have failed to detect measurable levels of catecholamine in the assay medium after such incubations.

In both βN22 and βN17 cells, but not in parental NG108-15 cells, the addition of the β-adrenoceptor agonist isoprenaline resulted in a substantial increase in high affinity [^{3}H]forskolin binding. Maximally effective concentrations of isoprenaline caused not significantly greater maximal stimulation of high affinity [^{3}H]forskolin binding in βN22 cells than that produced in βN17 cells (Table 3.2.3) but the dose-effect curve for isoprenaline indicated an EC_{50} of only 1.5 nM in βN22 cells in contrast to an EC_{50} of some 20 nM in βN17 cells (Figure 3.2.10). It has been noted previously that the EC_{50} for isoprenaline-stimulation of adenylyl cyclase in membranes of βN22 cells is also substantially to the left of that observed in βN17 cells (Adie *et al.*, 1994b). The absolute EC_{50} values noted in the [^{3}H]forskolin binding assay were somewhat lower than those recorded for stimulation of adenylyl cyclase but this may be a reflection of the differing assay conditions used (whole cells vs. membranes, different temperatures). The differences in isoprenaline dose-response curves for stimulation of high-affinity [^{3}H]forskolin between these cells indicates that a lower fractional occupancy of the receptor population is required to cause stimulation of GSAC complex formation in clone βN22 than in clone βN17. It does provide

evidence for a large β_2-adrenoceptor reserve for activation of the adenylyl cyclase cascade in clone βN22 cells.

The addition of iloprost (acting at the endogenously expressed IP prostanoid receptor) also led to a dose-dependent increase in formation of the GSAC complex in both clone βN17 and clone βN22 cells (Figure 3.2.9B). Iloprost produced a similar maximal activation of [^{3}H]forskolin binding in these two cells lines but in contrast with the situation for isoprenaline, similar dose-effect curves for iloprost were observed. It has been noted previously that clones βN17 and βN22 express similar levels of the IP prostanoid receptor (Adie *et al.*, 1994a). A similar dose-effect curve for iloprost-stimulation of [^{3}H]forskolin binding was also observed in wild type NG108-15 cells (Figure 3.2.9A).

Analysis of the mechanism(s) of receptor-mediated inhibition of adenylyl cyclase have led to a variety of proposals (Hildebrandt *et al.*, 1993), but recent studies have indicated that at least both a direct inhibition by $G_i\alpha$ and an inhibition via receptor-generated free G-protein βγ subunits may occur in specific situations. One model for direct inhibition would envisage competition between $G_s\alpha$ and $G_i\alpha$ for a common site on the adenylyl cyclase enzyme. To assess this I added the opioid agonist DADLE, which causes inhibition of adenylyl cyclase in NG108-15 cells via binding to the δ opioid receptor and subsequent activation of $G_{i2}\alpha$ (McKenzie *et al.*, 1990a) and determined whether this altered the increase in specific [^{3}H]forskolin binding induced by varying concentrations of iloprost (Table 3.2.5). The failure of DADLE to alter either iloprost stimulation of [^{3}H]forskolin binding or to itself promote [^{3}H]forskolin binding can be interpreted to indicate that $G_s\alpha$ and $G_i\alpha$ do not share a common or overlapping site on the adenylyl cyclase polypeptide.

The results presented in this chapter demonstrate the absolute levels of each polypeptide component of G-protein linked transmembrane cellular signaling cascades in membranes of wild type NG108-15 cells. In addition, it also demonstrates that agonist-stimulation of high affinity binding of [^{3}H]forskolin in intact wild type and β_2-adrenoceptor expressing NG108-15 cells can provide a good measure of formation

of the GSAC complex and can be used to quantitatively evaluate the stoichiometry of receptor activation of adenylyl cyclase. The data provided herein argue that previously observed elevated levels of basal adenylyl cyclase activity in cells expressing high levels of the β_2-adrenoceptor reflect empty receptor stimulation of the cascade, as these cells basally contain more preformed, activated copies of the GSAC complex. Further information on the cellular location and levels of expression of receptors, G-proteins and effectors in a wide range of systems will be required to increase our understanding of why cells appear to express considerably greater levels of G-proteins than of the relevant effector enzymes and whether there is physical or functional pooling or compartmentalization of these polypeptides as has been suggested in a number of recent studies (Ott *et al.*, 1989; Monchly-Rosen *et al.*, 1990).

3.3. Regulation of the levels of protein components of the stimulatory adenylyl cyclase cascade in NG108-15 cells transfected to express the β_2-adrenoceptor.

3.3.1. Results.

Membranes derived from cells of clones βN22 and βN17 which were generated following transfection of neuroblastoma x glioma hybrid, NG108-15, cells with a plasmid containing a cDNA encoding the human β_2-adrenoceptor (Adie *et al.*, 1994a, b), were examined for expression of this receptor by measuring the specific binding of the β-adrenoceptor antagonist [^{3}H]dihydroalprenolol (DHA) (2 nM). βN22 and βN17 cells were treated with varying concentrations of the irreversible β-adrenoceptor antagonist bromoacetyl alprenolol methane (BAAM) for 4 h prior to cell harvest. Membranes prepared from these cells were then assessed for the number of remaining specific [^{3}H]DHA binding sites (Figure 3.3.1A). Half-maximal reduction in the number of available β_2-adrenoceptors was achieved by treatment with some 25 nM BAAM and treatment with 10 μM BAAM reduced the number of β_2-adrenoceptors binding sites detected by [^{3}H]DHA by some 90%. Saturation binding studies using [^{3}H]DHA demonstrated that the effect of BAAM (100 nM) treatment was to reduce the available β_2-adrenoceptor population (B_{max}) in membranes prepared from the treated-cells without altering the measured affinity of the remaining receptors for [^{3}H]DHA (K_d in control membranes was 0.84 ± 0.1 nM and in membranes of BAAM-pretreated cells was 0.90 ± 0.23 nM) (Figure 3.3.1B). Such data indicated that BAAM had been effectively removed during the production and washing of the membrane preparations and thus was not present to compete and potentially interfere in subsequent functional assays.

Sustained (8 h) exposure of clone βN22 cells to maximally effective concentrations of either the IP prostanoid receptor agonist, iloprost (10 μM) or the β-adrenoceptor agonist isoprenaline (10 μM) resulted in some 50% down-regulation of cellular levels of the α subunit of the stimulatory G-protein of the adenylyl cyclase

cascade, $G_s\alpha$ (Figure 3.3.2). Such treatments did not alter cellular levels of other G-proteins expressed by these cells (Adie *et al.*, 1994a). Following pretreatment of the cells with BAAM (10 μM, 4h) no alteration in the ability of this concentration of iloprost to down-regulate $G_s\alpha$ was noted. By contrast, a substantial reduction in the effect of 10 μM isoprenaline was observed (Figure 3.3.2). This pattern of reduced effectiveness of isoprenaline to cause down-regulation of $G_s\alpha$ was observed over a range of isoprenaline concentrations (Figure 3.3.3) following treatment with 10 μM BAAM. At all concentrations of isoprenaline tested the degree of down-regulation of $G_s\alpha$ was reduced and there was a substantial shift (some 50 fold) to higher concentrations in the requirement for isoprenaline to cause half-maximal reduction in $G_s\alpha$ levels (Figure 3.3.3). However, no significant alteration in the dose-effect curve for iloprost-mediated $G_s\alpha$ down-regulation was observed following such treatment with BAAM (data not shown).

Preincubation of cells of clone βN22 with varying concentrations of BAAM (1 nM - 10 μM) followed by subsequent exposure to a maximally effective dose of isoprenaline (10 μM) demonstrated that the ability of the agonist to cause $G_s\alpha$ down-regulation was compromised as increasing numbers of the β_2-adrenoceptor were eliminated (Figure 3.3.4). Half-maximal reduction in the ability of 10 μM isoprenaline to cause elimination of $G_s\alpha$ was obtained following treatment with 100 nM BAAM (Figure 3.3.4). Even following treatment with 10 μM BAAM the maximal ability of isoprenaline to stimulate adenylyl cyclase activity in membranes of βN22 cells was only slightly lower than in membranes of untreated cells although the dose-effect curve for isoprenaline was shifted to significantly higher concentrations (Figure 3.3.5).

In addition, adenylyl cyclase assays were performed on membranes of both βN22 and βN17 cells using a range of β-adrenoceptor ligands. Basal adenylyl cyclase activity varied some two fold in membrane preparations from individual cell passages (an observation not unusual for cultured cell lines) but in the absence of receptor ligands was routinely higher in membranes of βN22 cells than in those from βN17 cells as reported previously (Adie *et al.*, 1994b). Concentration-response curves for

adenylyl cyclase stimulation in βN22 and βN17 membranes by each of isoprenaline, salbutamol and ephedrine demonstrated that each drug had greater potency (as well as higher measured intrinsic activity in the cases of ephedrine and salbutamol) in membranes of βN22 than in βN17 and that the maximal effectiveness of stimulation of adenylyl cyclase which could be achieved by each of these agents was produced with concentrations at or below 10 μM (Table 3.3.1). It was observed that isoprenaline (10 μM) stimulated adenylyl cyclase activity to similar maximal levels in membranes from cells of clones βN22 and βN17 (βN17 isoprenaline-stimulated adenylyl cyclase activity was 82.0 ± 8.2% (mean ± S.D., n = 6) of that seen in βN22 cells); see also (Adie *et al.*, 1994a). Using the values obtained for isoprenaline (10 μM) as a reference (100%), the other β-adrenoceptor ligands were observed to display a range of intrinsic activities in these membranes (Table 3.3.1). A similar profile to that obtained in the adenylyl cyclase assay was also generated by use of agonist-driven high affinity [^{3}H]forskolin binding in whole cells (MacEwan *et al.*, 1995). Concentration-response curves for epinephrine indicated similar potency in βN22 cells in both the adenylyl cyclase and [^{3}H]forskolin binding assays (EC_{50} = 30.3 ± 3.1 nM and 7.5 ± 0.9 nM receptively, mean ± S.D. of triplicate determinations) I have previously noted that the [^{3}H]forskolin binding assay also resulted in a somewhat lower EC_{50} value for isoprenaline in cells of clone βN22 and βN17 compared to the adenylyl cyclase assay (chapter 3.2 and Adie *et al.*, 1994b).

If the alteration in measured intrinsic activity of the β-adrenoceptor partial agonists between clones βN22 and βN17 was truly a reflection of differences in receptor levels between these two clones rather than a trivial difference based on specific features of these particular clonal isolates, I predicted that functional ablation of varying proportions of the β_2-adrenoceptor population in cells of clone βN22 would decrease the potency and also the measured intrinsic activity of β-adrenoceptor partial agonists. Adenylyl cyclase assays performed on membranes derived from control and BAAM (10 μM, 4h)-treated βN22 cells demonstrated that while there was a significant decrease in potency for isoprenaline in the BAAM-treated cells, the

intrinsic activity of isoprenaline was reduced to only some 80% of that observed in membranes from the untreated cells (Table 3.3.1). BAAM (1 μM) treatment of βN22 cells resulted in a 14 fold shift in the epinephrine concentration-effect curve, with minimal diminution in the maximal epinephrine-stimulated adenylyl cyclase activity, demonstrating a receptor reserve. However, for each of salbutamol and ephedrine both a reduction in potency and in measured intrinsic activity was observed following BAAM (1 μM) treatment (Table 3.3.1 and Figure 3.3.6). To examine this point in more detail the intrinsic activity of isoprenaline, salbutamol and ephedrine was measured in either adenylyl cyclase assays (Figure 3.3.6A) or using agonist-driven high affinity [^{3}H]forskolin binding (Figure 3.3.6B) in βN22 cells and membranes following treatment with a range of concentrations of BAAM for 4h. At the highest concentration of BAAM examined (10 μM) the measured intrinsic activity of isoprenaline was reduced, as noted above, by only some 15-20% in either assay, but the measured intrinsic activity of the partial agonists ephedrine (10% of maximal isoprenaline response) and salbutamol (approximately 25% of maximal isoprenaline response) were reduced substantially compared with those observed in untreated cells (Figure 3.3.6). The intrinsic activity of ephedrine, which was demonstrated to be lower than that of salbutamol in untreated βN22 cells (Table 3.3.1) was clearly compromised following treatment with lower concentrations of BAAM than was that of salbutamol (Figure 3.3.6). Furthermore, the data indicated that isoprenaline would require elimination of a greater amount of the receptor population than could be achieved in these experiments to significantly compromise its intrinsic activity (Figure 3.3.6).

To examine to what extent the β_2-adrenoceptor population would have to be reduced to restrict the intrinsic activity of isoprenaline to 50% of that observed in untreated cells, cells of clone βN17 were treated with varying concentrations of BAAM. Subsequently the ability of a concentration of isoprenaline sufficient to fully occupy the available receptors to stimulate adenylyl cyclase or to promote the specific high affinity binding of [^{3}H]forskolin was then assessed (Figure 3.3.7). A reduction

in the intrinsic activity of isoprenaline to 50% of that in untreated βN17 cells was achieved following treatment with approximately 2 μM BAAM. As this concentration of BAAM was demonstrated to eliminate binding of [^{3}H]DHA to 90% of the expressed β_2-adrenoceptors on these cells (Figure 3.3.1), this indicates that maximal occupancy of a β_2-adrenoceptor population of approximately 50 fmol/mg membrane protein (corresponding to around 3000 receptors/βN17 cell) with this agonist would be sufficient to generate a half-maximal adenylyl cyclase response in these cells. To extend such analysis I assessed the levels of receptor required to be occupied by each of isoprenaline, salbutamol and ephedrine to obtain different degrees of intrinsic activity for these ligands (Figure 3.3.8). Ephedrine, as mentioned above, failed to act as a full agonist even in membranes and cells of clone βN22 but 50% of the maximal possible receptor-mediated activation of the adenylyl cyclase was achieved by occupancy by maximally effective concentrations of ephedrine of some 1800 fmol/mg membrane protein of the β_2-adrenoceptor while salbutamol required occupancy of some 500 fmol/mg membrane protein of the receptor (Figure 3.3.8). Such data demonstrate why isoprenaline and salbutamol act as full agonists in cells and membranes of clone βN22 while isoprenaline but not salbutamol is a full agonist in clone βN17. It further explains why ephedrine is not a full agonist in either system.

I have already demonstrated, in NG108-15 cells transfected to express varying levels of the human β_2-adrenoceptor, that maximal adenylyl cyclase activity can be achieved by occupation by isoprenaline of only some 200 fmol/mg membrane protein of this receptor. Expression of higher levels of the receptor do not result in greater adenylyl cyclase activity but only in a progressive shift in the agonist concentration-response curve to lower concentrations (Adie *et al.*, 1994b), indicative of the presence of spare receptors. Increased expression of $G_s\alpha$ in this genetic background, as anticipated from the basal stoichiometries noted above, does not result in any significant alteration in the maximal effectiveness of the adenylyl cyclase cascade (I. Mullaney and G. Milligan, unpublished observations), even though receptors can access and activate the introduced $G_s\alpha$ as effectively as the endogenous G-protein

pool (Mullaney *et al.*, 1994). To further extend such analyses, I have isolated stable clonal cell lines following transfection of adenylyl cyclase type 2 (Feinstein *et al.*, 1991; Lustig *et al.*, 1993) into this genetic background and examined how this alters the effectiveness of signal transduction from both endogenously expressed and stably transfected $G_s\alpha$-coupled receptors in clones in which the total adenylyl cyclase content of the cell is increased by up to ten fold.

To do so, clone βN22 was further transfected in a 10:1 ratio with a combination of a cDNA encoding adenylyl cyclase type 2 in plasmid pcDNA1 and plasmid pBABEhygro which allows expression of resistance to the antibiotic hygromycin B. Colonies were selected following exposure to hygromycin B (200 μg/ml) and expanded. The presence of mRNA encoding adenylyl cyclase type 2 in both parental βN22 cells and a number of the isolated colonies was assessed by reverse transcriptase-polymerase chain reaction performed on RNA isolated from these cell lines with a primer pair (chapter 2.2.23) designed to amplify a 980 bp fragment specifically from the type 2 isoform of adenylyl cyclase. At this level of sensitivity no adenylyl cyclase type 2 mRNA could be detected in clone βN22 cells but a clear signal, of varying magnitude, was detected in a number of the clones isolated following transfection with the adenylyl cyclase type 2 cDNA (Figure 3.3.9). As a positive control for the effectiveness of the primers in these experiments, the adenylyl cyclase type 2 cDNA in plasmid pcDNA1 was used. Such reactions also generated an 980 bp fragment (Figure 3.3.9). Equivalent polymerase chain reaction experiments performed with primer pairs designed to detect the presence and expression of either type 1 or type 6 adenylyl cyclase have demonstrated the expression of mRNA encoding the type 6 but not the type 1 adenylyl cyclase in these cells (E. Kellett and G. Milligan unpublished observations).

I have previously made use of the stimulation of specific high affinity binding of [^{3}H]forskolin to cell membrane preparations by the poorly hydrolysed analogue of GTP, Gpp(NH)p, to detect the presence and formation of a complex of $G_s\alpha$ and adenylyl cyclase in NG108-15 and βN22 cells (chapter 3.2). Equivalent studies on

membranes of clone βN22 and of clones designated AC2.4, AC2.5, AC2.7 and AC2.9 demonstrated that the guanine nucleotide-stimulated binding of [^{3}H]forskolin to membranes of each of these cells was substantially higher than in clone βN22, with the highest levels of binding observed in clone AC2.7 (Table 3.3.2). Confirmation that this elevated specific binding of [^{3}H]forskolin represented an increase in the total number of $G_S\alpha$-adenylyl cyclase complexes which could be formed in clone AC2.7 by exposure to Gpp(NH)p was produced by performing such binding assays on membranes of clones βN22 and AC2.7 in the presence of Gpp(NH)p (100 μM) with a fixed concentration of [^{3}H]forskolin and increasing concentrations of non-radioactive forskolin. Specific [^{3}H]forskolin binding to 100 μg of membranes from clone AC2.7 was displaced by self-competition with unlabelled forskolin in a concentration-dependent manner (Figure 3.3.10A). Greater amounts of membranes (300 μg) from clone βN22 were needed to generate enough specific [^{3}H]forskolin binding for analysis. Subsequent correction of the specific binding data for dilution of the specific activity of [^{3}H]forskolin and display of the derived data as a linear transformation (Figure 3.3.10B) allowed demonstration of at least 6 fold higher levels of $G_S\alpha$-adenylyl cyclase complexes in clone AC2.7 compared to clone βN22. Slightly different best estimates of the binding affinity of [^{3}H]forskolin to the membranes of these two clones was observed (Figure 3.3.10B) but I cannot be certain if this indicates somewhat different binding affinities of [^{3}H]forskolin to the complex of $G_S\alpha$ with different adenylyl cyclase isoforms or simply reflects the quantitative difficulties inherently associated with estimations of such parameters when they can only be obtained via such self competition studies (DeBlasi *et al.*, 1989).

Both clones AC2.4 and AC2.7 were demonstrated to have retained similarly high levels of the β_2-adrenoceptor as clone βN22, as measured by the specific binding of the β-adrenoceptor antagonist [^{3}H]DHA (Table 3.3.3). Furthermore, immunodetectable level of the adenylyl cyclase stimulatory G-protein, $G_S\alpha$ or the phosphoinositidase C-linked G-proteins, $G_q\alpha/G_{11}\alpha$ were not different in membranes of clones AC2.4 and AC2.7 compared to clone βN22 (MacEwan *et al.*, 1996).

Basal adenylyl cyclase activity in membranes of clones AC2.7 and AC2.4 was substantially elevated in comparison to clone βN22 (Figure 3.3.11, (also Figures 3.3.12A and 13A)) and addition of forskolin (100 μM), Gpp(NH)p (100 μM) or NaF (10 mM) produced markedly higher adenylyl cyclase activities in each of these two clones compared to clone βN22 (Figure 3.3.11). Isoprenaline (acting at the β_2-adrenoceptor) or iloprost (acting at the endogenously expressed IP prostanoid receptor) (both at 10 μM) resulted in an activation of adenylyl cyclase activity in membranes of both clones AC2.4 and AC2.7 far above that obtained in membranes from clone βN22 (Figure 3.3.12A). However, agonist occupancy of the endogenous bradykinin B2 receptor (a receptor which stimulates the activity of phosphoinositidase C) resulted in no stimulation of the adenylyl cyclase activity in membranes derived from any of the clones studied (Figure 3.3.12). A similar pattern was observed by measuring the ability of isoprenaline or iloprost to stimulate the specific high affinity binding of [^{3}H]forskolin in whole cells of these clones (Figure 3.3.12B). Concentration-response curves to isoprenaline indicated, however, little difference in EC_{50} values for adenylyl cyclase stimulation in clones βN22, AC2.4 and AC2.7 (EC_{50}s of 12.8, 12.1 and 13.8 nM respectively) (Figure 3.3.13). The β-adrenoceptor partial agonist ephedrine caused greater adenylyl cyclase activity in clones AC2.4 and AC2.7 than in membranes of clone βN22 (data not shown). Despite this, when compared to the effect of isoprenaline the measured efficacy of ephedrine was unaltered between the clones (Figure 3.3.14) and concentration-effect curves for adenylyl cyclase stimulation by ephedrine were very similar in membranes of clone βN22 and clone AC2.7 (Figure 3.3.14). A variety of β-adrenoceptor ligands of differing efficacy again showed little alteration in their intrinsic activities when stimulating adenylyl cyclase activity in membranes from clone βN22 and AC2.7 (Figure 3.3.15).

Both secretin and A_2 adenosine receptors are expressed endogenously by NG108-15 cells. Agonists at each of these receptors (secretin and 5'-N-ethylcarboxamidoadenosine (NECA) respectively) also produced substantially greater

total adenylyl cyclase activity in clone AC2.7 compared to clone βN22 (Table 3.3.4). However, when compared to the effect of isoprenaline the activities of these two agonists was again very similar and concentration-effect curves displayed no significant alterations in the EC_{50}s for these agents in stimulation of adenylyl cyclase activity (Figure 3.3.16).

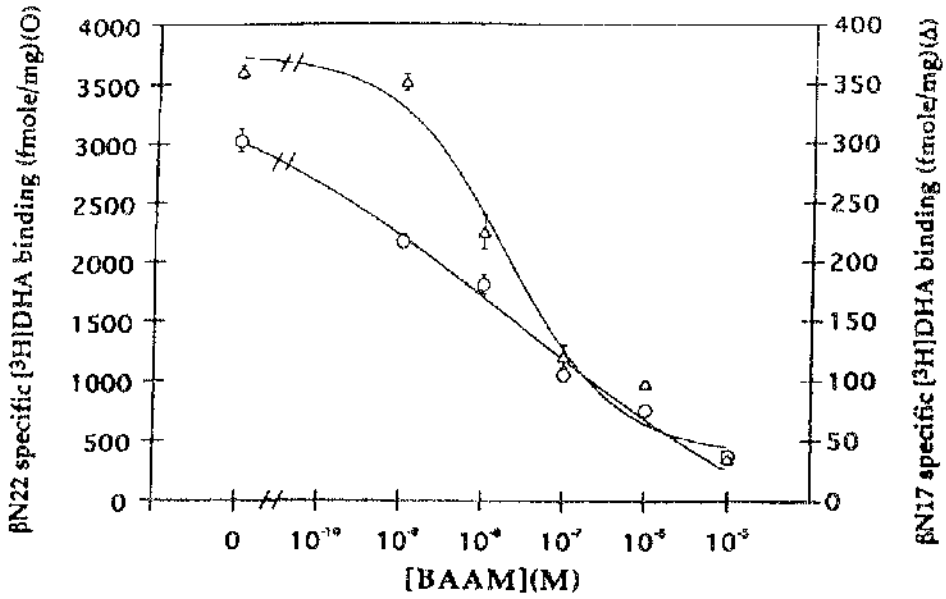
Figure 3.3.1.

The effect of BAAM treatment on specific binding of [^{3}H]DHA to membranes of βN22 and βN17 cells.

Panel A shows that BAAM pretreatment reduces the detected number of specific [^{3}H]DHA binding sites. Cells of clone βN22 and βN17 were treated in culture with varying concentrations of BAAM (up to 10 μM) for 4 h. Following cell harvest and membrane preparation the specific binding of a single concentration of [^{3}H]DHA to available β_2-adrenoceptors was measured as described in chapter 2.2.16. The results are the mean ± S.D. of triplicate determinations, from 2 experiments.

Panel B shows that BAAM pretreatment reduces maximal binding of [^{3}H]DHA without altering the affinity of the remaining receptors for [^{3}H]DHA. Cells of clone βN22 were untreated (circles) or treated with BAAM (100 nM) for 4 h (squares). Membranes were subsequently prepared and saturation binding studies performed with [^{3}H]DHA to determine both the maximal capacity and affinity of this ligand for the available receptors. Data is presented as a Scatchard analysis. In the example displayed B_{max} was 3796 fmol/mg membrane protein in the control membranes and 1142 fmol/mg membrane protein in the BAAM-pretreated cells while the K_d for [^{3}H]DHA was 0.74 nM in the controls and 0.67 nM following treatment with BAAM.

A


βN22 specific [3H]DHA binding (fmole/mg)(O)
βN17 specific [3H]DHA binding (fmole/mg)(Δ)
4000
3500
3000
2500
2000
1500
1000
500
0
400
350
300
250
200
150
100
50
0
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[BAAM](M)

B

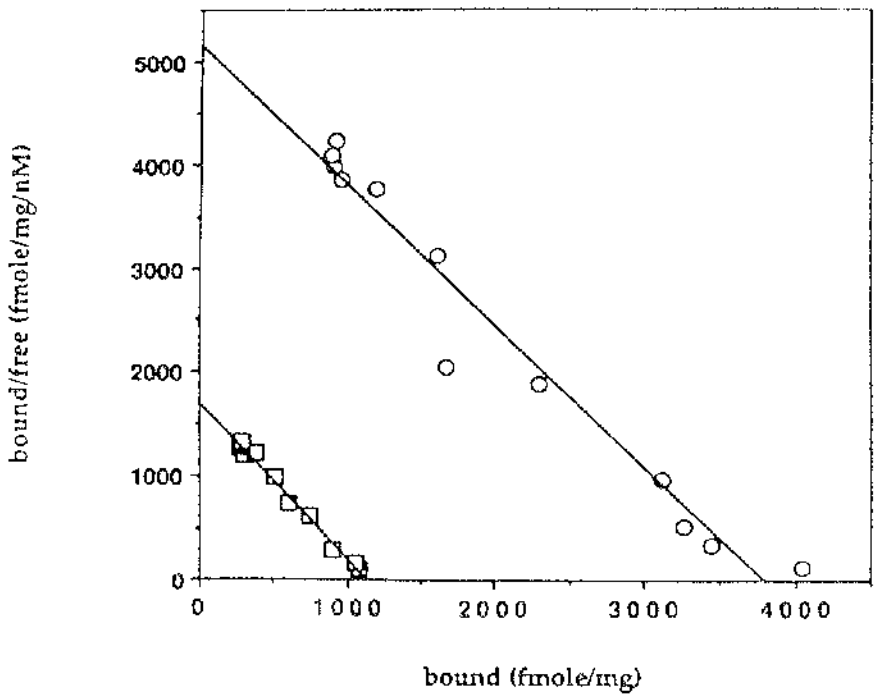

bound/free (fmole/mg/nM)
5000
4000
3000
2000
1000
0
0
1000
2000
3000
4000
bound (fmole/mg)

Figure 3.3.2.

Treatment of clone βN22 cells with BAAM restricts β_2-adrenoceptor but not IP prostanoid receptor agonist-mediated down-regulation of $G_s\alpha$.

Clone βN22 cells were untreated (control) or treated with BAAM (10 μM, 4h), washed extensively and then either left in the absence of ligand (CON) or treated with iloprost (10 μM) (ILO) or isoprenaline (10 μM) (ISO) for 8 h. The cells were harvested, membranes generated and immunoblotted for the presence of $G_s\alpha$. Data are presented as mean ± S.D.M. from 3 separate experiments.

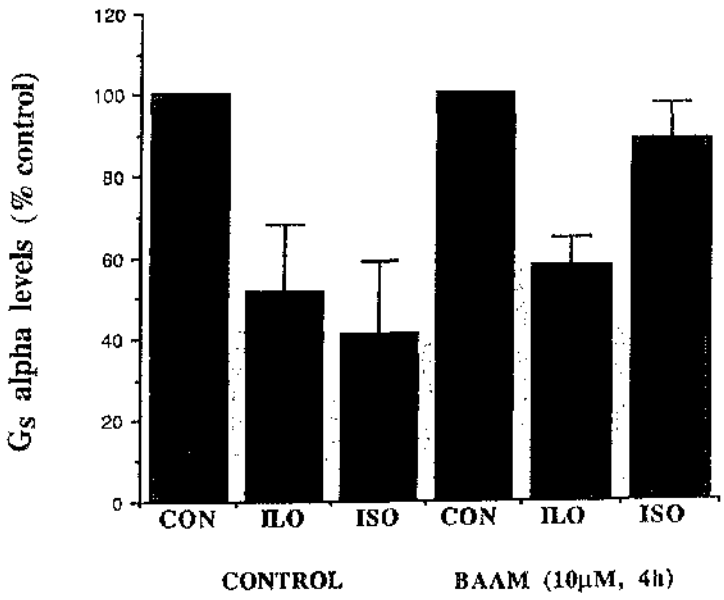


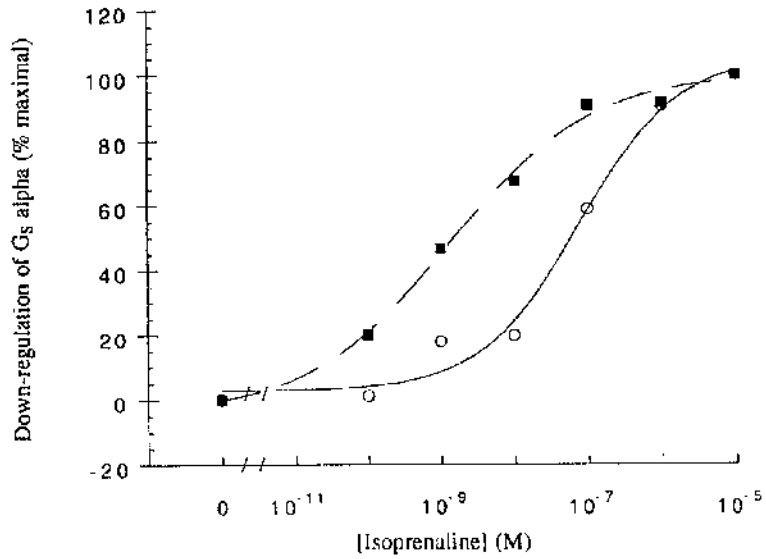
Figure 3.3.3.

BAAM treatment of clone βN22 cells reduces the potency of isoprenaline-mediated $G_s\alpha$ down-regulation.

Clone βN22 cells were untreated or treated with BAAM (10 μM, 4h) and subsequently challenged with varying concentrations of isoprenaline for 8 h. Membranes were prepared and the relative levels of $G_s\alpha$ determined immunologically using antiserum CS, at dilution of 1: 250, as described in chapter 2.2.12.

Immunoblots A and B represents cells of clone βN22 which were treated and untreated with BAAM respectively. Subsequent treatment with isoprenaline: lane 1, control; lane 2, 100 pM; lane 3, 1 nM; lane 4, 10 nM; lane 5, 100 nM; lane 6, 1 μM; lane 7, 10 μM.

The data of immunoblots A and B was analysed and is displayed as the % of maximal effect of isoprenaline (produced by 10 μM) on membrane levels of $G_s\alpha$. In the example displayed the estimated EC_{50} for isoprenaline-induced down-regulation of $G_s\alpha$ was 1.3 nM in the cells which had not been exposed to BAAM (filled symbols) and 78 nM in the cells which had been pretreated with BAAM (open symbols). Similar results were obtained in two other independent experiments.



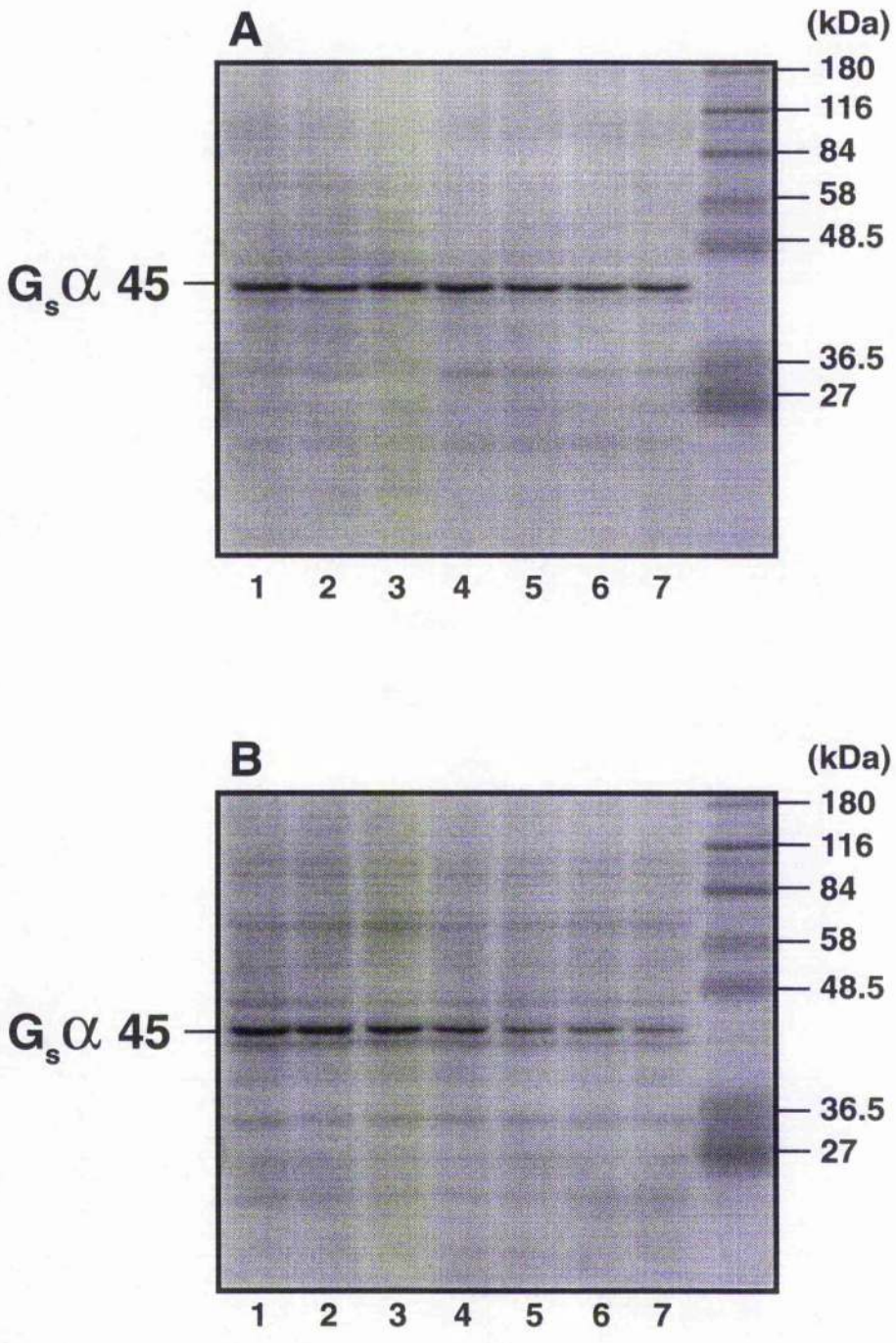

A
(kDa)
180
116
84
58
48.5
36.5
27
$G_s\alpha$ 45
1 2 3 4 5 6 7
B
(kDa)
180
116
84
58
48.5
36.5
27
$G_s\alpha$ 45
1 2 3 4 5 6 7

Figure 3.3.4.

The effect of BAAM on isoprenaline-induced down-regulation of $G_S\alpha$; Dosc-effect analysis.

Clone βN22 cells were untreated or treated for 4 h with varying concentrations of BAAM and subsequently with isoprenaline (10 μM, 8h). Relative levels of $G_S\alpha$ in membranes from these cells were then assessed immunologically. Data is presented as the % of the membrane $G_S\alpha$ which was down-regulated by isoprenaline at each concentration of BAAM.

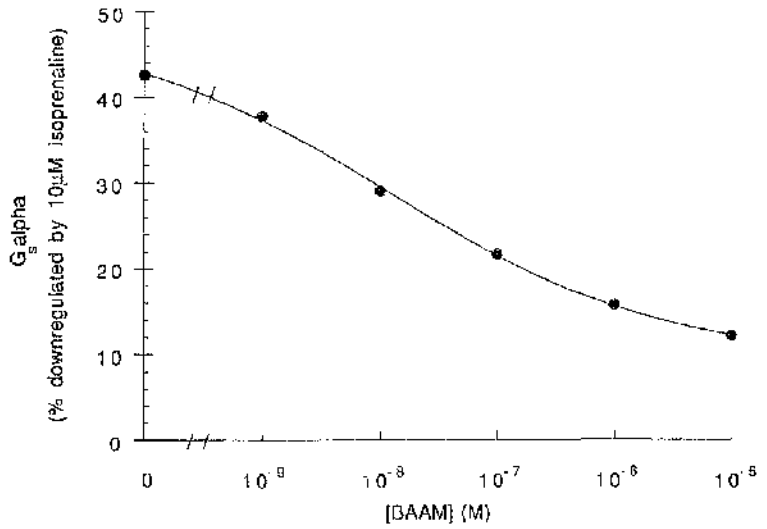


Figure 3.3.5.

The effect of BAAM on isoprenaline stimulation of adenylyl cyclase activity.

Membranes were prepared from control (open symbols) and BAAM (10 μM, 4h)-treated (filled symbols) βN22 cells and the ability of varying concentrations of isoprenaline to stimulate adenylyl cyclase activity measured as described in chapter 2.2.17. Results are presented as % of the maximal effect of isoprenaline in membranes of untreated cells. This was a representative experiment (mean ± S.D.) of which two others were performed which gave similar results.

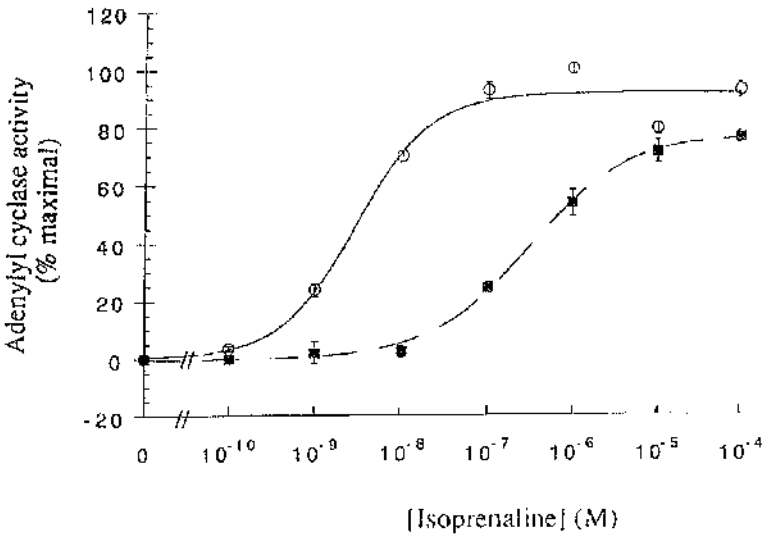


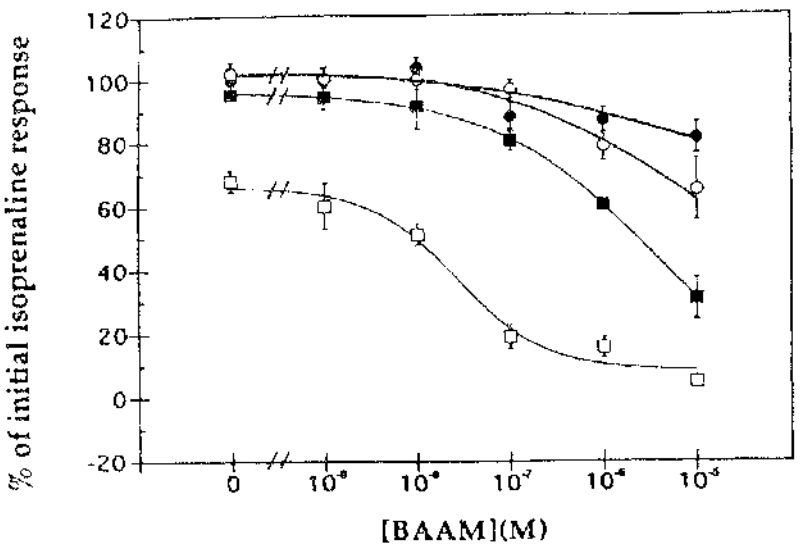
Figure 3.3.6.

Receptor availability regulates the measured intrinsic activity of β-adrenoceptor active ligands in βN22 cells.

Panel A represents adenylyl cyclase assays. Membranes were prepared from clone βN22 cells which had been pretreated with varying concentrations of BAAM for 4 h. Adenylyl cyclase activity and its regulation by maximally effective concentrations (10 μM) of isoprenaline (filled circles), epinephrine (open circles), salbutamol (filled squares) and ephedrine (open squares) was subsequently assessed. Results are presented as % of the effect obtained in membranes of untreated cells by 10 μM isoprenaline. In the experiments displayed, basal adenylyl cyclase activity in βN22 and βN17 preparations was 61.4 ± 4.2 and 40.5 ± 5.2 pmol/min/mg membrane protein respectively. 10 μM isoprenaline-stimulated adenylyl cyclase activity in βN22 and βN17 preparations was 186.4 ± 6.4 and 136.3 ± 23.9 pmol/min/mg membrane protein respectively (mean ± S.D.M., n=3).

Panel B represents [^{3}H]forskolin binding assays. The ability of maximally effective concentrations (10 μM) of isoprenaline (filled circles), salbutamol (open circles) and ephedrine (filled squares) to stimulate high affinity [^{3}H]forskolin binding in clone βN22 cells which had been pretreated with varying concentrations of BAAM for 4 h was assessed as described in chapter 2.2.16. Results are presented as % of the effect produced in untreated cells by 10 μM isoprenaline. Control specific [^{3}H]forskolin binding represented 643 ± 103 dpm/10^5 cells (mean ± S.D.M., n = 3).

A


% of initial isoprenaline response
120
100
80
60
40
20
0
-20
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[BAAM](M)

B

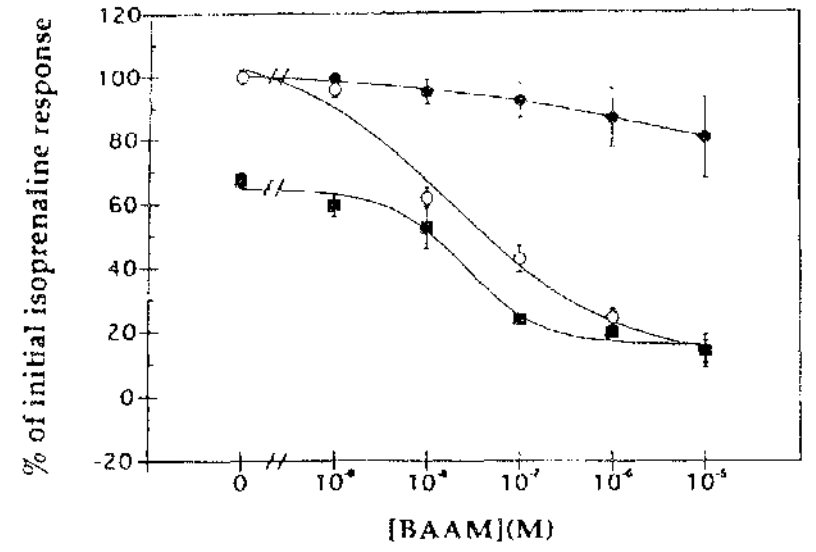

% of initial isoprenaline response
120
100
80
60
40
20
0
-20
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[BAAM](M)

Figure 3.3.7.

Regulation by receptor availability of the measured intrinsic activity of isoprenaline in βN17 cells.

Clone βN17 cells were pretreated with varying concentrations of BAAM for 4h. Either these were used directly in [^{3}H]forskolin binding assays (squares) or membranes were prepared from these cells and adenylyl cyclase activity (circles) measured as described in chapter 2.2.16 and 2.2.17 respectively. The effect of a maximally effective concentration of isoprenaline (10 μM) was assessed in both these assays. Results are presented as % of the effect obtained in membranes of untreated cells by 10 μM isoprenaline. The results are the mean ± S.E. of triplicate determinations.

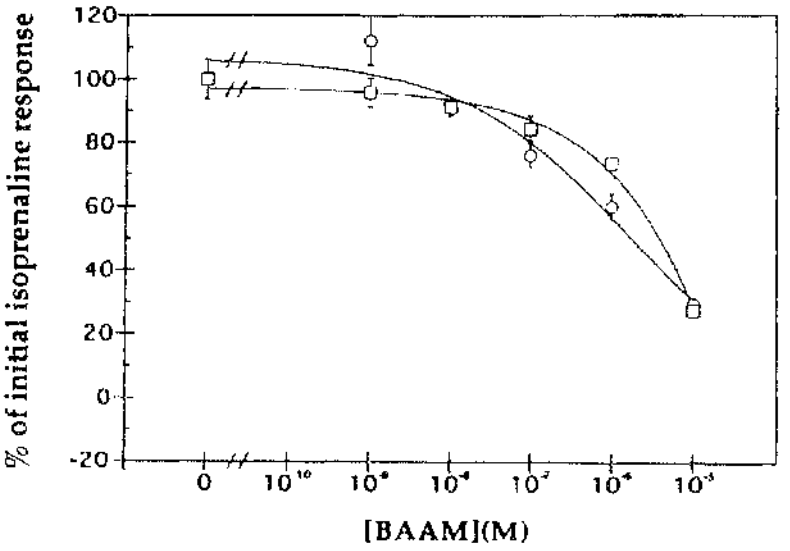


Figure 3.3.8.

Analysis of receptor levels in defining the observed intrinsic activity of β–adrenergic ligands.

Adenylyl cyclase activity (filled symbols) and agonist regulation of [^{3}H]forskolin binding (open symbols) was measured in the presence of 10 μM isoprenaline (circles), salbutamol (squares) or ephedrine (triangles) in membranes (adenylyl cyclase activity) or whole cells ([^{3}H]forskolin binding) of clone βN22 and βN17 which were either untreated or exposed to varying concentrations of BAAM. Data is presented for each ligand as the % of the maximal response which could be achieved in this genetic background (10 μM isoprenaline, untreated βN22 cells and membranes) vs. the number of β_2-adrenoceptors available to be occupied.

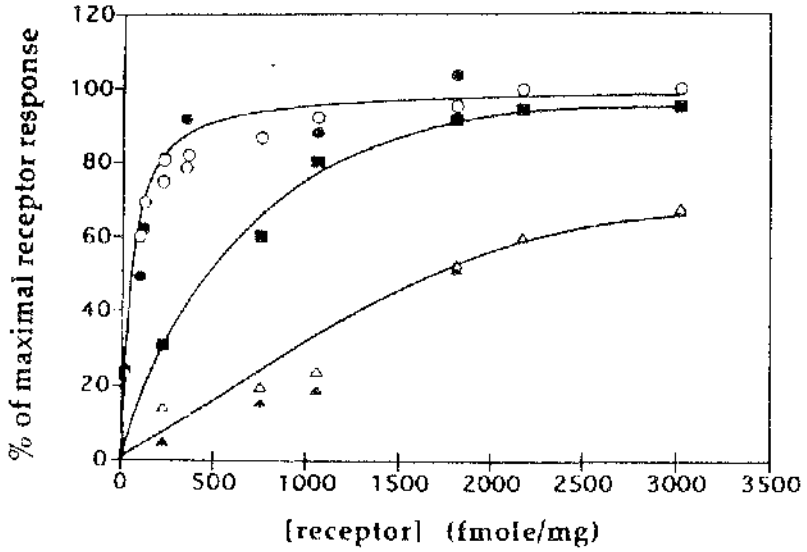


Figure 3.3.9.

Generation and isolation of clones derived from βN22 cells which express adenylyl cyclase type 2.

A cDNA encoding adenylyl cyclase type 2 (4.0 kb) (a kind gift from Dr. H.R. Bourne, UCSF, CA) was subcloned into the EcoR1 site of the eukaryotic expression vector pcDNA1 (4.1 kb). Cells of clone βN22 were co-transfected with a 10: 1 ratio of the cDNA encoding adenylyl cyclase type 2 in plasmid pcDNA1 and plasmid pBABEhygro, which is able to direct expression of the hygromycin B resistance marker, by using DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer's instructions. After transfection the cells were maintained in medium containing hygromycin B (200 μg/ml). Individual colonies were selected, expanded and subsequently examined for the maintained expression of the β_2-adrenoceptor and novel expression of adenylyl cyclase type 2.

Expression of adenylyl cyclase type 2 in clone βN22 cells was detected by RT-PCR. RNA was isolated from clone βN22 cells (lane 2) and from clones AC2.5 (lane 3), AC2.7 (lane 4), AC2.9 (lane 5) and AC2.4 (lane 6). These RNA preparations were reverse transcribed and polymerase chain reaction then performed using the primers for adenylyl cyclase type 2 noted in chapter 2.2.23 (C). These were designed to generate a 980 bp fragment from adenylyl cyclase type 2. As a positive control a cDNA encoding adenylyl cyclase type 2 in plasmid pcDNA1 was also subjected to polymerase chain reaction under identical conditions (lanes 7 and 8). Size markers (1 kb DNA ladder) in bp are displayed in lane 1.

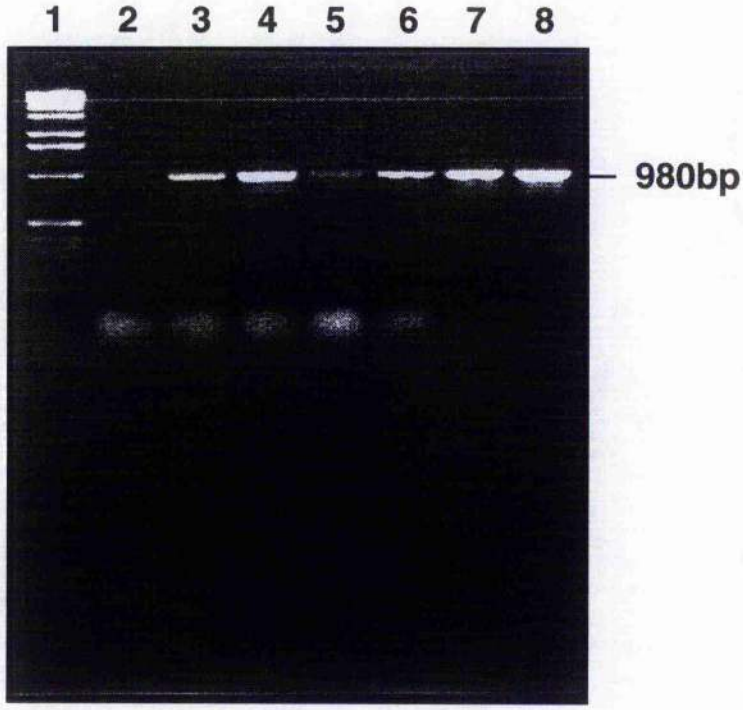

1 2 3 4 5 6 7 8
980bp

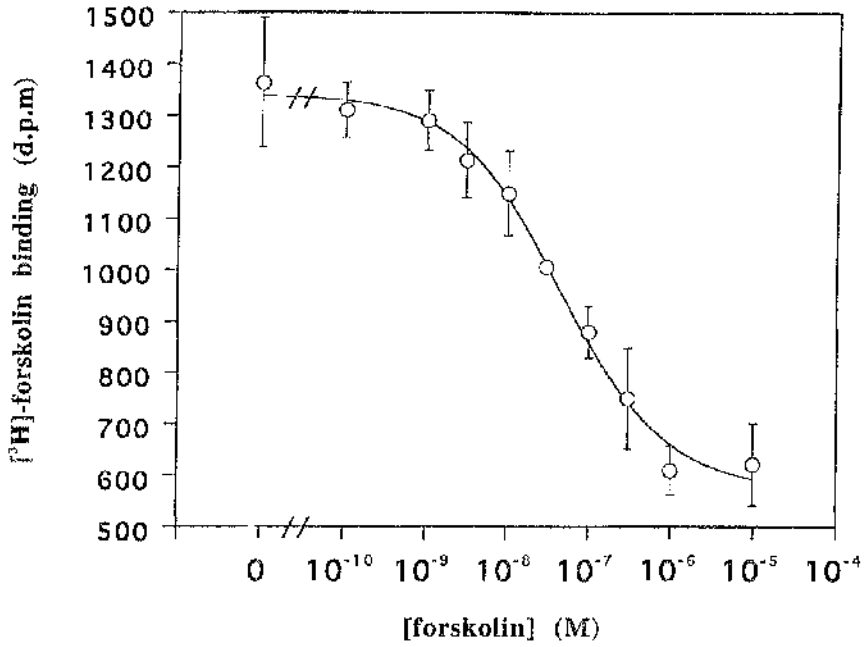
Figure 3.3.10.

Adenylyl cyclase type 2 positive clone AC2.7 expresses higher levels of guanine nucleotide-stimulated [^{3}H]forskolin binding than parental βN22 cells.

Panel A: Membranes (100 μg) from clone AC2.7 were incubated with 10 nM [^{3}H]forskolin and Gpp(NH)p (100 μM) as described in the chapter 2.2.16 in the presence of varying concentrations of non-radioactive forskolin as indicated. The data represent the mean ± S.D. of triplicate determinations. Similar self-competition displacement assays for βN22 clone were performed as here, except using 300 μg of membranes per assay and 10 nM [^{3}H]forskolin (data not shown but see chapter 3.2).

Panel B: Membranes from clones βN22 (300 μg) and clone AC2.7 (100 μg) were incubated with [^{3}H]forskolin and Gpp(NH)p (100 μM) as described in chapter 2.2.16 in the presence of varying concentrations of forskolin. The specific binding data from such experiments were corrected for dilution of the specific activity of the [^{3}H]forskolin and analysed by the method of Rosenthal *et al.*, 1967. The number of high affinity binding sites for [^{3}H]forskolin were markedly higher in membranes of clone AC2.7 compared to clone βN22 (B_{max} estimated at 609 and 125 fmol/mg respectively in the example displayed). Estimates of the K_d for binding of forskolin to membranes of clones βN22 and AC2.7 were 36 and 63 nM respectively. The data represent the mean of determinations performed in triplicate.

A


[³H]-forskolin binding (d.p.m)
1500
1400
1300
1200
1100
1000
900
800
700
600
500
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
[forskolin] (M)

B

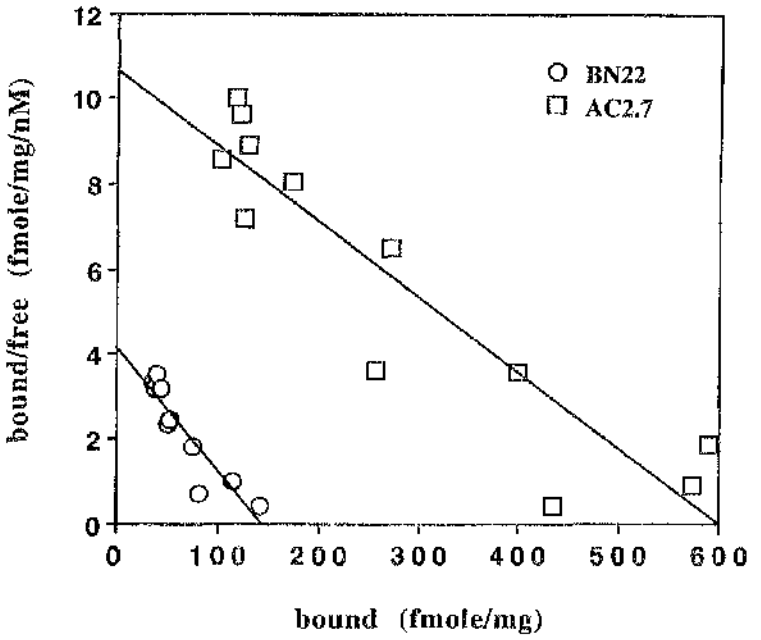

bound/free (fmole/mg/nM)
12
10
8
6
4
2
0
0
100
200
300
400
500
600
bound (fmole/mg)
BN22
AC2.7

Figure 3.3.11.

Increased basal and $G_s\alpha$-stimulated adenylyl cyclase activity in membranes of adenylyl cyclase type 2 expressing cells.

Adenylyl cyclase activity was measured in membranes (10 µg) of clones βN22, AC2.4 and AC2.7 in the absence (basal) or presence of forskolin (100 µM), isoprenaline (10 µM), Gpp(NH)p (100 µM) or AlF_4^- (provided as NaF (10 mM)). In the experiment shown, the basal adenylyl cyclase activities in clones βN22, AC2.4 and AC2.7 were 60 ± 2, 605 ± 24 and 896 ± 21 pmol/min/mg respectively. The data represent the mean ± S.D. of triplicate determinations.

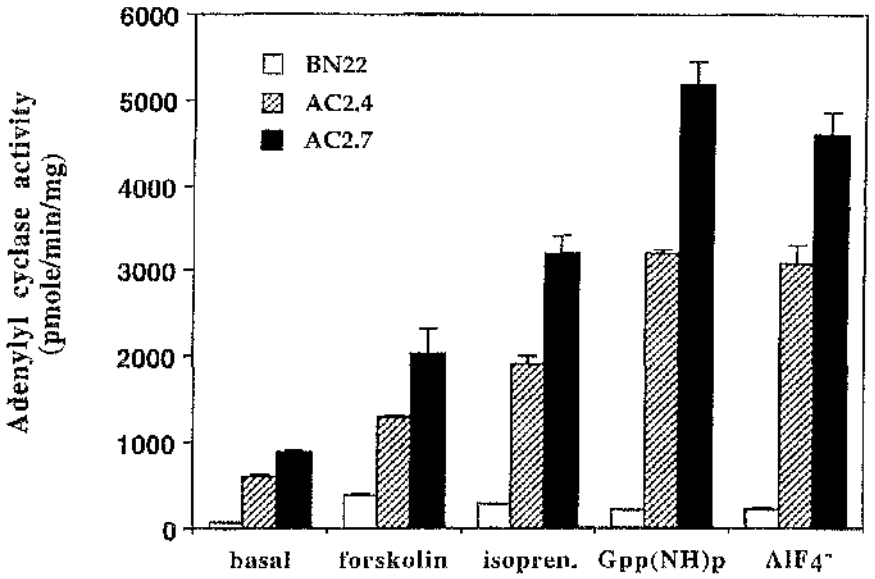


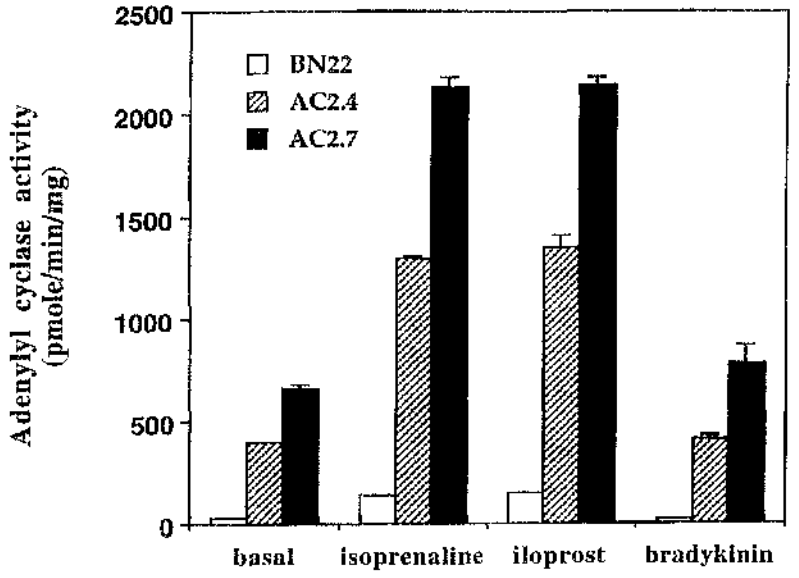
Figure 3.3.12.

Increased adenylyl cyclase activity and numbers of $G_S\alpha$-adenylyl cyclase complexes formed in reponse to isoprenaline or iloprost in adenylyl cyclase type 2 expressing cells.

Adenylyl cyclase activity (panel A) was measured in membranes (10 μg) of clones βN22, AC2.4 and AC2.7 in the absence or presence of isoprenaline (10 μM), iloprost (10 μM) or bradykinin (10 μM). The data represent the mean ± S.D. of triplicate determinations.

Whole cell specific [^{3}H]forskolin (14 nM) binding (panel B) was measured in clones βN22, AC2.4 and AC2.7 in the absence (basal) or the presence of isoprenaline (10 μM), iloprost (10 μM) or bradykinin (10 μM). The presence of 10 μM forskolin determined non-specific binding which was subtracted from the remaining conditions. The data represent the mean ± S.D. of quadruplicate determinations.

A


Adenylyl cyclase activity (pmole/min/mg)
2500
2000
1500
1000
500
0
BN22
AC2.4
AC2.7
basal
isoprenaline
iloprost
bradykinin

B

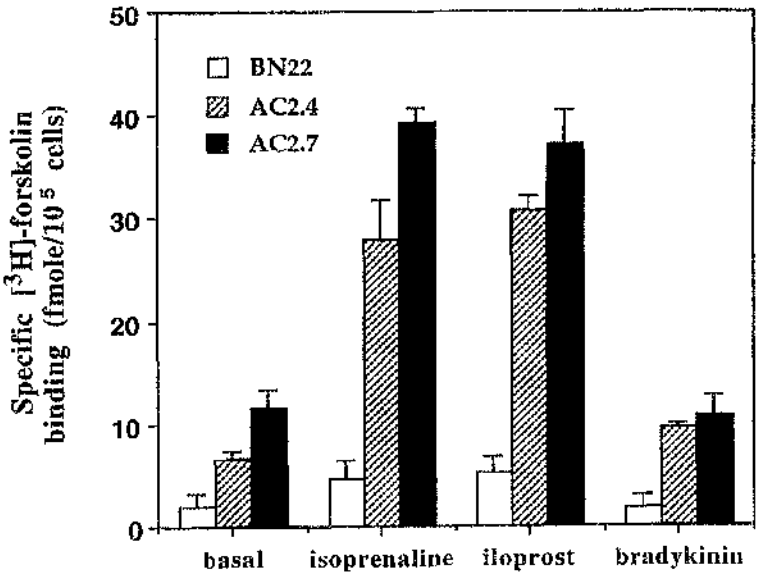

Specific [3H]-forskolin binding (fmole/10 5 cells)
50
40
30
20
10
0
BN22
AC2.4
AC2.7
basal
isoprenaline
iloprost
bradykinin

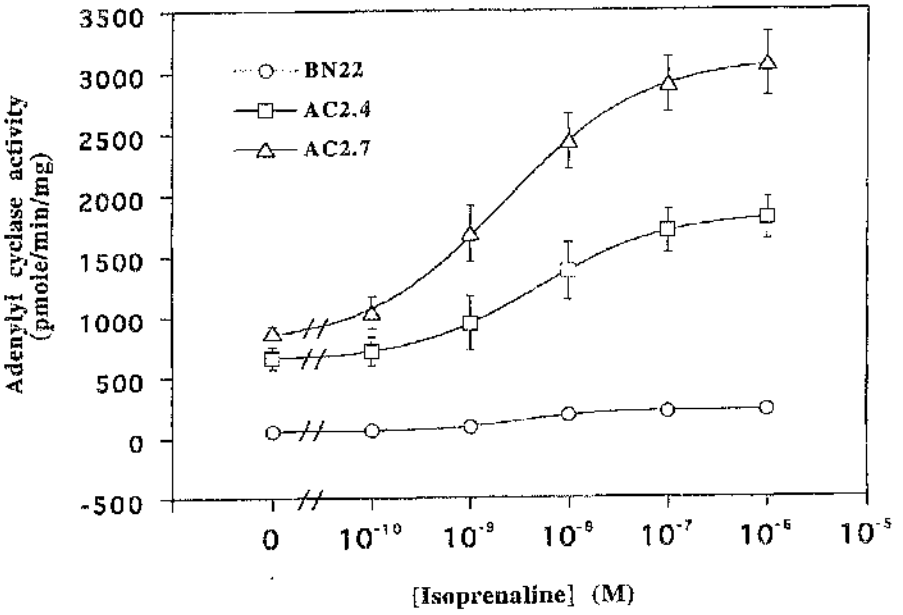
Figure 3.3.13.

Isoprenaline-stimulated adenylyl cyclase activity: concentration-effect analysis in membranes from clones βN22, AC2.4 and AC2.7.

Adenylyl cyclase activity (panel A) was measured in membranes (10 μg) of clones βN22, AC2.4 and AC2.7 in the absence or presence of varying concentrations of isoprenaline. The data represent the mean ± S.D. of triplicate determinations from a typical experiment.

In panel B, the data from panel A was normalised relative to adenylyl cyclase activity produced by a maximally effective concentration of isoprenaline (10 μM in each case).

A


Adenylyl cyclase activity (pmole/min/mg)
3500
3000
2500
2000
1500
1000
500
0
-500
BN22
AC2.4
AC2.7
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[Isoprenaline] (M)

B

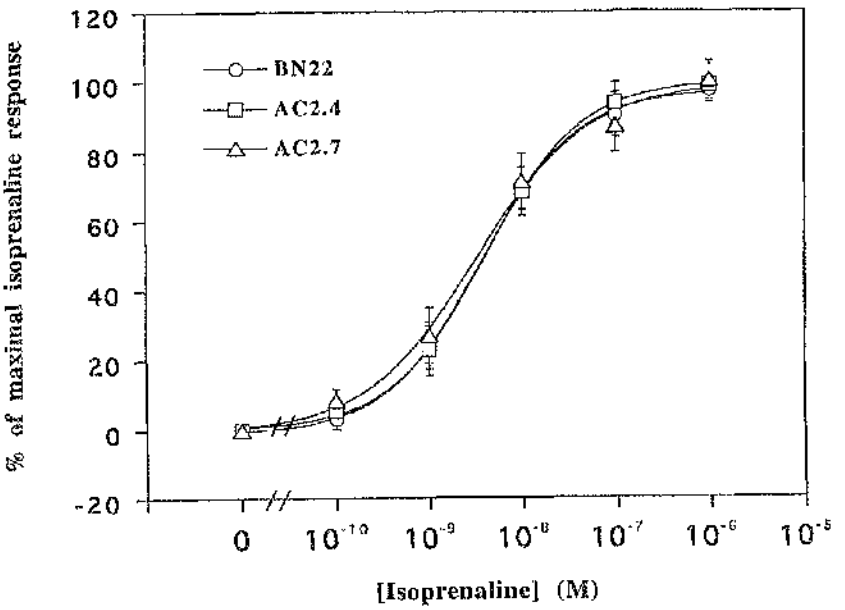

% of maximal isoprenaline response
120
100
80
60
40
20
0
-20
BN22
AC2.4
AC2.7
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[Isoprenaline] (M)

Figure 3.3.14.

The efficacy and potency of ephedrine to stimulate adenylyl cyclase is not altered by overexpression of adenylyl cyclase type 2.

Ephedrine concentration-response curves for adenylyl cyclase activity in membranes (10 μg) of clones βN22 and AC2.7 were compared. Data is normalised to the response concurrently observe to a maximal concentration (10 μM) of isoprenaline. In the experiment shown, basal adenylyl cyclase activity was 54 ± 8 and 693 ± 20 pmole/min/mg and 10 μM isoprenaline-stimulated activity was 169 ± 7 and 3006 ± 45 pmole/min/mg in clones βN22 and AC2.7 respectively. The data represent the mean ± S.D. of triplicate determinations.

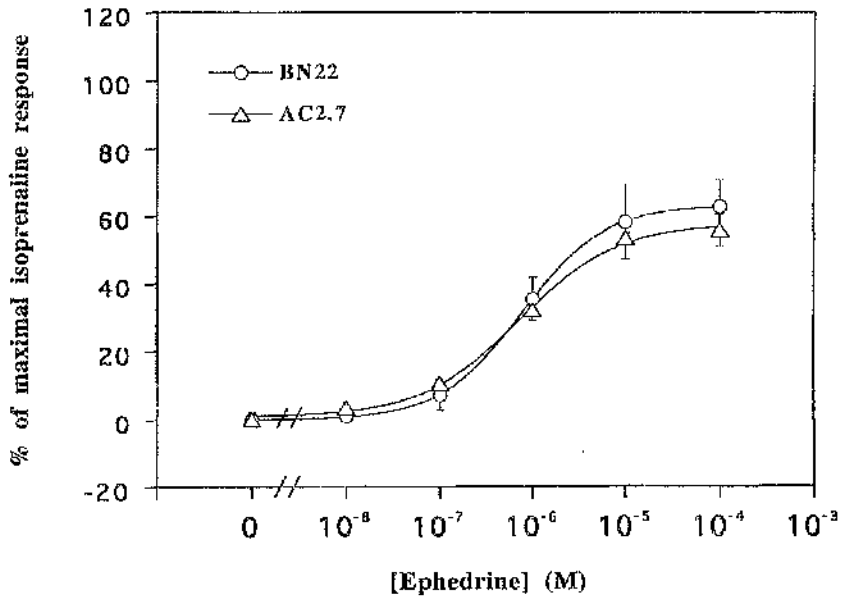


Figure 3.3.15.

The efficacies of β-adrenoceptor agonists are not altered by overexpression of adenylyl cyclase type 2.

The effect on adenylyl cyclase activity of a maximally effective concentration (10 μM) of agonists acting at the β_2-adrenoceptor displaying a range of efficacies was compared in membranes (10 μg) of clones βN22 and AC2.7. Data is normalised to that seen with isoprenaline. In the experiment shown, basal adenylyl cyclase activity was 33 ± 3 and 491 ± 14 pmole/min/mg and 10 μM isoprenaline-stimulated activity was 160 ± 6 and 1748 ± 43 pmole/min/mg in clones βN22 and AC2.7 respectively. The data represent the mean ± S.D. of triplicate determinations.

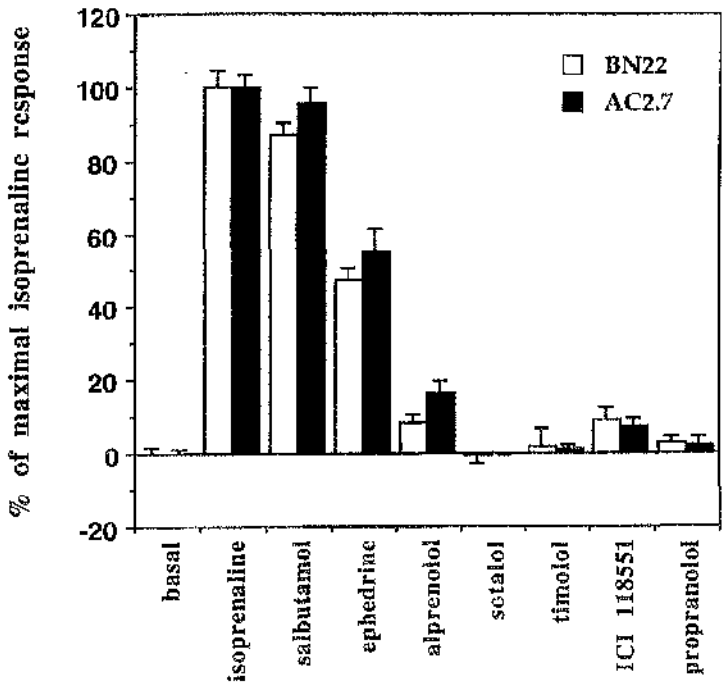


Figure 3.3.16.

The efficacy and potency of secretin and the adenosine receptor agonist NECA are not altered by overexpression of adenylyl cyclase type 2.

Secretin and 5'-N-Ethylcarboxamidoadenosine (NECA) concentration-response curves for adenylyl cyclase activity in membranes (10 μg) of clones βN22 and AC2.7 were compared. Data is normalised to the response concurrently observe to a maximally effective concentration (10 μM) of isoprenaline. The data represent the mean ± S.D. of triplicate determinations. The data is representative of a single experiment. Three further experiments produced similar results.

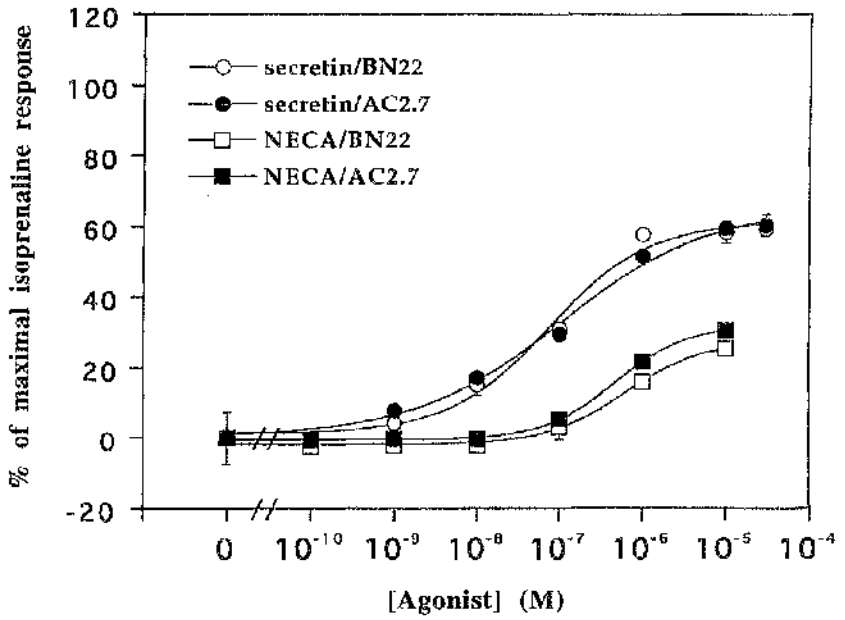


Table 3.3.1.

The potency and intrinsic activity of β-adrenergic agonists in βN17 and βN22 (with and without BAAM treatment) cell membranes.

Concentration-response curves were performed in the adenylyl cyclase activity assay to assess the potency (EC_{50}) and intrinsic activity of full and partial agonists under conditions of varying receptor number. BAAM (1 μM, 10 μM for isoprenaline experiments) pretreatment of βN22 cells and data analysis were as described in chapter 2.2.19. Data represent the mean ± S.D.M. of between 2 and 10 separate experiments, each averaging triplicate determinations. N.D., Not determined.

	EC_{50} (nM) and [intrinsic activity (%)]		
Agonist	**βN22**	**βN17**	**βN22/BAAM**
Isoprenaline	3.6 ± 0.6 [100]	100 ± 12 [82.0 ± 8.2]	310 ± 44 [76.5 ± 1.8]
Epinephrine	30.3 ± 3.1 [90.1 ± 1.6]	136 ± 75 [83.4 ± 8.1]	405 ± 61 [95.0 ± 2.5]
Salbutamol	109 ± 18 [101.9 ± 2.4]	449 ± 114 [71.8 ± 1.9]	882 ± 29 [77.3 ± 0.4]
Dobutamine	111 ± 27 [83.2 ± 3.7]	486 ± 121 [42.0 ± 1.9]	ND [ND]
Ephedrine	1330 ± 300 [66.2 ± 2.9]	7700 ± 667 [20.0 ± 0.6]	10283 ± 1667 [17.5 ± 1.2]

Table 3.3.2.

Adenylyl cyclase type 2 positive clones express elevated levels of guanine nucleotide-stimulated [^{3}H]forskolin binding.

Membranes (100 μg) prepared from each of clone βN22 and the adenylyl cyclase type 2 mRNA positive clones used in Figure 3.3.9 were examined for 100 μM Gpp(NH)p-dependent specific high affinity [^{3}H]forskolin (10 nM) binding as described in chapter 2.2.16. The data represent the mean ± S.D. of triplicate determinations, from a single experiment. Three further experiments produced similar results.

Clones	Gpp(NH)p-stimulated adenylyl cyclase activity (pmole/min/mg)
βN22	48.4 ± 0.6
AC2.4	294.2 ± 5.4
AC2.5	292.1 ± 4.2
AC2.7	432.6 ± 11.9
AC2.9	160.5 ± 8.9

Table 3.3.3.

Clones βN22, AC2.4 and AC2.7 express similar levels of the β_2-adrenoceptor.

β_2-adrenoceptor expression was measured in membranes (10 μg) of clones βN22, AC2.4 and AC2.7 as judged by the specific binding of a single concentration of [^{3}H]DHA (see chapter 2.2.16). The data represent the mean ± S.D. of triplicate determinations. The data is representative of two experiments performed on separate membrane preparations.

Clones	Specific [^{3}H]DHA binding (fmole/mg protein)
βN22	2790 ± 150
AC2.4	2740 ± 150
AC2.7	3040 ± 160

Table 3.3.4.

Stimulation of adenylyl cyclase activity in clones βN22 and AC2.7 by secretin and NECA.

Adenylyl cyclase activity was measured in membranes (10 μg) of clones βN22 and AC2.7 in the absence (basal) or presence of a maximally effective concentration of secretin, 5'-N-Ethylcarboxamidoadenosine (NECA) or isoprenaline. The data represent the mean ± S.D. of triplicate determnations, from a single experiment. Three other experiments produced similar results.

	Adenylyl cyclase activity (pmole/min/mg)	
Condition	**βN22**	**AC2.7**
Basal	85 ± 20	1320 ± 60
Secretin (30 μM)	245 ± 10	3530 ± 190
NECA (10μM)	155 ± 10	2430 ± 65
Isoprenaline (10 μM)	350 ± 15	4970 ± 380

3.3.2. Discussion.

Although agonist-induced down-regulation of receptors has been studied for many years and is appreciated to provide a means to regulate cellular sensitivity to agonist ligands (Lohse *et al.*, 1993) the concept that agonist treatment can also regulate the cellular content of G-proteins has taken longer to be accepted and has been more recalcitrant to analysis. In a variety of circumstances sustained exposure of a cell to an agonist ligand does result in a substantial reduction in cellular levels of the G-protein(s) which is activated by the receptor (Milligan *et al.*, 1993c). However, such effects are not observed for all systems and for all agonists. For example, in the neuroblastoma x glioma hybrid NG108-15 cell, treatment with agonists at the IP prostanoid receptor but not with agonists at either the A_2 adenosine or secretin receptors results in a substantial down-regulation of $G_s\alpha$ even though all of these receptors couple to $G_s\alpha$ and thus cause activation of adenylyl cyclase (Kelly *et al.*, 1990; McKenzie *et al.*, 1990b). As addition of analogues of cAMP or agents able to elevate intracellular cAMP in a non-receptor-dependent fashion is unable to mimic the effect of prostanoid agonists (McKenzie *et al.*, 1990b) it has been argued that the differences between the receptor ligands is unlikely to represent the ability of each receptor to activate adenylyl cyclase and may reflect the levels of cellular expression of each receptor (Milligan *et al.*, 1991b). This, however, has been difficult to establish clearly in these cells as only agonist [^{3}H]ligands are available for use in binding studies for the IP prostanoid and secretin receptors and there have been concerns as to whether ligands for the A_2 adenosine receptor may label other sites (Keen *et al.*, 1989) such as the adenosine transporter.

Following transfection of NG108-15 cells with a cDNA encoding the human β_2-adrenoceptor (Adie *et al.*, 1994 a, b) a clone expressing high levels of the receptor (some 3000 fmol/mg membrane protein) responded to challenge with isoprenaline by down-regulating levels of $G_s\alpha$ whereas little effect was observed in a clone expressing much lower levels (some 300 fmol/mg membrane protein) of the receptor (Adie *et al.*, 1994a). While such results provide evidence for the concept that agonist-

mediated G-protein down-regulation is likely to reflect levels of expression of a receptor they suffer from the fact that they had to be performed on different individual clonal isolates. To counteract this concern in this study I have used a clone βN22 which expresses relatively high levels of the β_2-adrenoceptor and then limited access of isoprenaline to the receptor by pretreating the cells with varying concentrations of the irreversible β-adrenoceptor antagonist BAAM. BAAM undergoes covalent attachment to receptor protein through bromoalkylation of sulfhydryl groups and thus irreversible occupancy of the recptor binding site. BAAM (10 μM, 4h) treatment of clone βN22 cells results in a substantial reduction in the maximal down-regulation of $G_s\alpha$ which can be achieved by isoprenaline without altering the amount of down-regulation of $G_s\alpha$ produced by occupancy of the IP prostanoid receptor by iloprost (Figure 3.3.2). Half-maximal down-regulation of $G_s\alpha$ which can be produced by isoprenaline required substantially higher levels of the agonist following treatment with BAAM (10 μM) compared to the control cells (Figure 3.3.3). By contrast, dose-effect curves for iloprost-mediated down-regulation of $G_s\alpha$ were unaffected by treatment with BAAM. Treatment of clone βN22 cells with a concentration of BAAM sufficient to reduce the amount of [^{3}H]DHA to some 300 fmol/mg membrane protein substantially reduced, but did not eliminate entirely, isoprenaline-induced down-regulation of $G_s\alpha$ (Figure 3.3.4). Such treatment, however, resulted in only a small reduction in the maximal ability of isoprenaline to stimulate adenylyl cyclase activity (Figure 3.3.5). A 50% reduction in the maximal ability of isoprenaline to regulate $G_s\alpha$ levels was produced by treatment of the cells with 100 nM BAAM, a concentration able to block agonist access to some 70% of the receptor population (Figure 3.3.1).

These data demonstrate that the degree of G-protein down-regulation observed is related to the number of receptors available for the agonist ligand to occupy and not to the ability of this receptor occupancy to result in the activation of adenylyl cyclase. This should be anticipated as previous studies have shown that such effects are restricted to the G-protein activated by the receptor, that the effect is a reflection of enhanced degradation of the G-protein without significant transcriptional or

translational control (Milligan *et al.*, 1993c; Mitchell *et al.*, 1993), that it is not mimiced by treatment with analogues of cAMP (McKenzie *et al.*, 1990b) and because mutationally activated G-protein α subunits are known to have reduced half-lives compared with the wild type proteins (Milligan *et al.*, 1993c; Levis et al., 1992). Thus, if greater levels of available receptors are able to activate more copies of the G-protein then greater down-regulation of this polypeptide should be anticipated. However, the observations that reduction of β_2-adrenoceptor availability levels in clone βN22 cells to some 300 fmol/mg membrane protein still resulted in a detectable down-regulation of $G_S\alpha$ whereas this was not observed in clone βN17 which expresses this level of receptor endogenously (Adie *et al.*, 1994a) demonstrates that it is unwise to extrapolate results from data obtained in different clonal isolates.

$G_S\alpha$ is expressed at some 1.25×10^6 copies per cell and the IP prostanoid receptor at some 10^5 copies per cell in NG108-15 cells and in the transfected clonal cell lines derived from it (chapter 3.2). In clone βN22 the β_2-adrenoceptor is expressed at some 3-4 fold higher levels than the IP prostanoid receptor (Adie *et al.*, 1994a, b). It has previously been calculated that the IP prostanoid receptor can activate some 65% of the cellular $G_S\alpha$ in NG108-15 cells (Adie *et al.*, 1992) and therefore if the β_2-adrenoceptor is able to activate the G-protein with similar stoichiometry then less than maximal occupancy of this receptor would be expected to result in maximal down-regulation of $G_S\alpha$ as I observe herein. The inability to observe detectable G-protein down-regulation in a range of systems may thus be a reflection of a combination of the levels of receptor expression and that of the G-protein. Clearly in cells which express high levels of a G-protein, agonist may be able to cause down-regulation of only a small fraction of the polypeptide and this would be virtually undetectable when measured immunologically. Equally it is unlikely to be observed with agonists at receptors which are expressed at only low levels. This may then provide the explanation for the inability of either secretin or an A_2 adenosine receptor agonist to alter $G_S\alpha$ levels detectably in NG108-15 cells (Kelly *et al.*, 1990; McKenzie *et al.*, 1991).

In this study, furthermore, I have examined that the importance of receptor levels in defining the intrinsic activity and potency of agonist drugs without alteration in amounts of the other components of the signal transduction cascade in both clones βN22 and βN17 by treatment with BAAM to vary receptor availability to ligands. The concept that different degrees of intrinsic activity of agonist drugs should be observed in cells and tissues expressing different levels of a G-protein-linked receptor is a classical element of receptor theory (Stephenson *et al.*, 1956; Kenakin *et al.*, 1989; Hoyer *et al.*, 1993). Experimental examination of this has traditionally focused on the observations that many drugs act as full agonists in some tissues but not in others or by the use of irreversible antagonists to limit access of agonists to receptors. The first of these approaches is limited by the fact that levels of both the G-protein(s) and the effector systems which are coupled to a particular receptor are likely to vary widely between the cell type and are generally unknown, and the second by the possibility that irreversible antagonism of a fraction of the receptors might be anticipated to limit access of the remaining receptors to a proportion of the G-protein population. In cells in which G-protein levels are comparable to those of the receptor this might alter receptor output.

The standard approach to alter available receptor levels in a tissue is to treat it with varying concentrations of an irreversible antagonist at that receptor. When βN22 cells were treated with the irreversible β-adrenoceptor antagonist BAAM (1 μM, 4h), a treatment sufficient to prevent binding of [^{3}H]DHA to some 75% of the initial receptor population (Figure 3.3.1), the intrinsic activities and EC_{50} values of ephedrine and salbutamol were changed to levels close to those observed in untreated βN17 cells (Table 3.3.1). Maximally effective concentrations of isoprenaline (up to 10 μM) resulted in a similar adenylyl cyclase activity in membranes of clone βN22 and βN17 (Adie *et al.*, 1994b). However, when a series of traditional β-adrenoceptor partial agonists were examined, the measured intrinsic activity of these compared to isoprenaline was observed to be routinely higher in membranes of clone βN22 than βN17 (Table 3.3.1). For example, salbutamol which in membranes of clone βN17

was a partial agonist displaying an intrinsic activity of around 70% compared to isoprenaline, acted as a full agonist in membranes of clone βN22; while ephedrine, with an intrinsic activity of some 20% in membranes of clone βN17 was also considerably more efficacious in clone βN22 membranes (intrinsic activity of 66%).

As noted above, there are some relationship between the measured EC_{50} for a drug response and the nature and level of receptor expression (Whaley *et al.*, 1994; Samama *et al.*, 1994). Whaley *et al.*, 1994 observed increased potency (decreased EC_{50}) of the natural ligand epinephrine with increasing receptor number. I also have a similar observation for full agonists and extend this relationship to partial agonists. Differences in EC_{50} values in whole tissues or in systems in which the receptor population for a ligand is poorly characterised are often interpreted to reflect receptor subtypes (Ashkenazi *et al.*, 1987; Milligan *et al.*, 1993b), although this would not be the likely conclusion if, for example, activation occurred via distinct G-proteins, or if the agonist structure activity profile was to remain unaltered across a variety of chemical structures. Mathematical predictions have recently been developed to analyse alterations in β-adrenoceptor full agonist EC_{50} values with receptor number (Whaley *et al.*, 1994) and, in systems in which the effector species is quantitatively the limiting component of a signal transduction cascade, it is often observed that elevation in receptor number will result in a leftward shift in the dose-effect curve (i.e. a reduction in EC_{50} value), consistent with the notion of a receptor population reserve. It have previously recorded exactly this phenomenon when measuring isoprenaline-stimulation of adenylyl cyclase activity in membranes of βN22 and βN17 cells (Adie *et al.*, 1994b) and a number of other reports have recorded similar data with cells expressing various levels of this receptor (Whaley *et al.*, 1994; Bouvier et al., 1988b) although one report in S49 lymphoma cells did not observe this effect (Johnson *et al.*, 1979). This is rather surprising as S49 lymphoma cells are the one other cell system apart from NG108-15 cells in which the absolute number of copies of receptor, $G_s\alpha$ and adenylyl cyclase have been calculated (Alousi *et al.*, 1991), and like NG108-15 cells (chapter 3.2), a large molar excess of $G_s\alpha$ over adenylyl cyclase has been

observed. It may be that in this system the differences in levels of receptor expression in the individual clones were insufficient to allow detection of the effect.

The relationship of intrinsic activity of partial agonists to receptor number was examined in detail using membranes and cells treated with varying levels of BAAM and noticeably and interestingly the measured regulation of agonist intrinsic activity was reduced by BAAM with greater potency the lower the measured intrinsic efficacy of the ligand in the untreated cells (Figure 3.3.6). As such, the measured intrinsic activity of ephedrine in membranes and cells of clone βN22 was reduced to half-maximal levels by treatment with approximately 25 nM BAAM, a concentration sufficient to eliminate 50% of the receptor population while this was not achieved for salbutamol until some 75% of the receptors were irreversibly blocked (i.e. by treatment with 1.5 μM BAAM) (Figure 3.3.6A). It was unable to use sufficiently high concentrations of BAAM to substantially reduce the measured intrinsic activity of isoprenaline in βN22 cells and membranes (Figure 3.3.6). To extend the generation of primary data I have made use of a subsequent assay, in which agonist activation of the β_2-adrenoceptor on intact clone βN22 and βN17 cells causes an increase in high affinity [^{3}H]forskolin binding because the complex formed between $G_s\alpha$ and adenylyl cyclase is the high affinity binding site for this ligand (Alousi *et al.*, 1991; Barber *et al.*, 1989). This assay allowed whole cell analysis of measured intrinsic activities and thus a comparison with data generated on cell membranes. As with the adenylyl cyclase assay, similar levels of [^{3}H]forskolin binding in βN22 and βN17 were observed (Figure 3.3.6B). However, as a means to address the question of how many receptors would need to be occupied by a full agonist to half-maximally activate the cellular adenylyl cyclase population, clone βN17 was treated with a range of concentrations of BAAM and then assessed the intrinsic activity of isoprenaline (Figure 3.3.7). I observed that 50% of the intrinsic activity for isoprenaline compared to that in untreated cells was produced by pretreatment of βN17 cells with 2 μM BAAM, a concentration consistent with access of isoprenaline to only some 50

fmol/mg membrane protein of the β_2-adrenoceptor in both the whole cells and membranes derived from them.

Presentation of data derived from such experiments in a form which allowed levels of receptor required to be occupied by each of isoprenaline, salbutamol and ephedrine to obtain different degrees of intrinsic activity for these ligands (Figure 3.3.8) allows direct comparison of the absolute intrinsic activities of these ligands. Some 35 fold higher levels of β_2-adrenoceptors had to be occupied with ephedrine than with isoprenaline to obtain 50% of the maximal activation of the adenylyl cyclase cascade whether this was measured as adenylyl cyclase activity in membranes or as stimulation of [^{3}H]forskolin binding in whole cells (Figure 3.3.8) while salbutamol required occupancy of only a third as many receptors as ephedrine. Such data demonstrate why isoprenaline and salbutamol act as full agonists in cells and membranes of clone βN22 while isoprenaline but not salbutamol is a full agonist in clone βN17. It further explains why ephedrine is not a full agonist in either system. Such results are also help to explain why certain drugs may act as full agonists in one tissue but as partial agonists in other tissues.

So far, I have mentioned that the alteration of levels of both a $G_s\alpha$-linked receptor and $G_s\alpha$ in NG108-15 cells transfected to express the human β_2-adrenoceptor. I have noted that in clones expressing above 200 fmol/mg membrane protein of this receptor full agonists were able to activate adenylyl cyclase to the same maximal level (Figure 3.3.8). At lower levels than this submaximal stimulation was achieved while at higher levels of the receptor the concentration-effect curve for stimulation of adenylyl cyclase was simply moved to lower concentrations of agonist (Adie *et al.*, 1994b). These observations are consistent with the notion of spare receptors at higher levels of expression. As such, the significant benefit of increasing receptor level beyond 200-300 fmol/mg membrane protein would be to induce a response at lower concentrations of agonist. It further observed that expression of higher levels of the β_2-adrenoceptor in these cells resulted in partial agonists displaying greater efficacy than produced in cells expressing lower levels of the

receptor. In addition, stable tranfection of NG108-15 cells with an epitope-tagged variant of $G_s\alpha$ and isolation of a clone in which levels of this G-protein was now increased to 2.5 x 10^6 copies per cell (more than doubling the $G_s\alpha$ levels in the cells) (Mullaney *et al.*, 1994) had little effect on the ability of iloprost to stimulate adenylyl cyclase activity either in terms of potency or maximal effect (I. Mullaney and G. Milligan, unpublished observations). It was able to demonstrate that the IP prostanoid receptor had both equal access to, and was able to interact with, both the endogenously expressed $G_s\alpha$ and the transfected epitope-tagged $G_s\alpha$ (Mullaney *et al.*, 1994). These results are in accord with recent studies in which trangenic mice were produced over-expressing $G_s\alpha$ in a cardiac specific manner (Gaudin *et al.*, 1995). Only minor differences were observed in regulation of the effectiveness of the adenylyl cyclase cascade (Gaudin *et al.*, 1995).

Therefore, in this study, I have also altered the levels of expression of adenylyl cyclase in this genetic background to evaluate whether this would result in a greater maximal output following agonist activation of the adenylyl cyclase cascade and to examine how potencies of agonists might be regulated. As mentioned in chapter 3.2, at least 10 individual adenylyl cyclase isoforms have now been identified and isolated as distinct cDNA species (Pieroni *et al.*, 1993; Taussig *et al.*, 1995). One of the most studied isoforms to date is adenylyl cyclase type 2 (Feinstein *et al.*, 1991; Lustig *et al.*, 1993; Jacobowitz *et al.*, 1994; Tsu *et al.*, 1995; Chen *et al.*, 1995) and thus for these studies I selected this isoform for transfection into clone βN22. As clone βN22 was selected based on its resistance to genetecin sulphate, this further transfection and selection of new clones derived from these cells was based on co-transfection of the adenylyl cyclase type 2 cDNA along with a plasmid which allows expression of resistance to the antibiotic hygromycin B. βN22 cells do not express detectable adenylyl cyclase type 2 mRNA (Figure 3.3.9). Following transfection of clone βN22 cells with a cDNA for adenylyl cyclase type 2 it was able to identify a series of clones exhibiting stable expression of adenylyl cyclase type 2 mRNA (Figure 3.3.9).

High affinity [^{3}H]forskolin binding assay in the presence of Gpp(NH)p on membranes of these clones also demonstrated excess total stable expression of adenylyl cyclase in these same clones compared to clone βN22 (Table 3.3.2). Our estimates of the quantitative increases in total cellular levels of adenylyl cyclase in the type 2 expressing clones were based on the maximal levels of high affinity [^{3}H]forskolin binding which could be achieved. Although forskolin is able to interact with all of the currently examined adenylyl cyclase isoforms (Taussig *et al.*, 1995) it is possible that the individual isoforms may display rather different affinities for this ligand. It is also possible, though yet to be explored in any significant detail, that individual adenylyl cyclase isoforms will display different activities in the absence of receptor-mediated activation of $G_s\alpha$. As such, I cannot discriminate whether the potentially curvilinear Scatchard analyses for guanine nucleotide stimulated [^{3}H]forskolin binding to membranes of clone AC2.7 (Figure 3.3.10B) is an indication of the mixed complement of adenylyl cyclase isoforms now expressed by these cells and displaying somewhat different affinities to forskolin, or is simply a reflection of the technical limitations of the need to perform [^{3}H]forskolin versus forskolin self competition curves followed by corrections for isotopic dilution to assess B_{max}. It is impractical given the affinity of [^{3}H]forskolin for the $G_s\alpha$-adenylyl cyclase complex to perform conventional saturation curves. As discussed in chapter 3.2, it is also known that forskolin can interact with other cellular polypeptides (Laurenza *et al.*, 1989), particularly those such as the GLUT family of facilitative glucose transporters which share certain topographic features with the adenylyl cyclase polypeptides. However, the guanine nucleotide-stimulated high affinity binding of [^{3}H]forskolin appears to represent binding only to the active form of adenylyl cyclase. Analogues of forskolin which show higher selectivity between adenylyl cyclase and the GLUT proteins have been described (Appel *et al.*, 1992) but these are not widely available and certainly not in a radiolabelled form.

Activation of the β_2-adrenoceptor resulted in a markedly elevated adenylyl cyclase activity in the adenylyl cyclase type 2-expressing clones compared to clone

βN22, as did activation of the endogenously expressed IP prostanoid receptor (Figure 3.3.12). These results demonstrate that the transfected adenylyl cyclase type 2 can be activated in these clones by both of these receptors and that in this genetic background adenylyl cyclase expression is the limiting function for maximal output from this cascade. I have not, at this stage, examined the cellular distribution of the introduced adenylyl cyclase type 2 compared to the endogenously expressed type 6 isoform but it clearly has access to the activated $G_s\alpha$ produced upon relevant receptor occupation. This was an important observation given known constraints on free mobilities of polypeptides involved in inhibitory regulation of adenylyl cyclase in NG108-15 cells (Graeser *et al.*, 1993) and the emerging view that organisational structure exists in G-protein coupled signalling systems (Neubig *et al.*, 1994).

Although greater receptor-mediated maximal output from the adenylyl cyclase cascade could be produced in the adenylyl cyclase type 2 expressing cells it have been observed that this did not result in any significant alterations in the concentration-effect curves for stimulation of adenylyl cyclase activity produced by either the transfected β_2-adrenoceptor or any of the endogenously expressed (IP prostanoid, A_2 adenosine or secretin) $G_s\alpha$-linked receptors. On the other hand, the levels of expression and activity of downstream regulators of G-protein-linked signaling cascades such as the G-protein-linked receptor kinases are also likely to be able to modify the effectiveness of G-protein-linked receptor signaling. In a recent report transgenic overexpression of β-adrenoceptor kinase (βARK-1 also called GRK 2) (Premont *et al.*, 1995) in the heart of mice has been shown to be able to diminish the signaling capacity of β-adrenoceptors (Koch *et al.*, 1995). The modification of the level of expression of these kinases in the cell lines utilised herein will be a interesting subsequent studies.

The implication of these studies is that targeted efforts to improve the maximal signaling capacity of the stimulatory arm of the adenylyl cyclase cascade, as for example has been suggested as a strategy to combat the failing heart (Milano *et al.*, 1994) is likely to be achieved most effectively by increasing levels of the adenylyl cyclase polypeptide(s) rather than other components of the cascade. Future studies will

determine whether different adenylyl cyclase isoforms will be more or less efficient in this regard. Informed choices of alterations in levels of receptors, G-protein or effectors will be dependent upon far greater knowledge of levels of expression of the components of signal transduction cascades in individual cells and tissues which might be targeted for modification.

Chapter 4.

The coupling of the long splice variant of the rat TRH receptor to G_q family proteins.

4.1. Introduction.

Thyrotropin-releasing hormone (TRH) is a tripeptide (pyroglutamic acid-histidine-proline amide, <Glu-His-Pro-NH_2) that regulates functions of the anterior pituitary gland, central and peripheral nervous systems and may serve paracrine roles in retina, testis and endocrine pancreas. TRH has also been recognized as a potent stimulator of prolactin synthesis and secretion in lactotrophs, although its roles as a major regulator of normal prolactin physiology remains controversial (Metcalf *et al.*, 1989). Much of what is known regarding the mechanisms of TRH action derives from the use of clonal rat pituitary cell lines, of which the mammotropic /somatotropic tumour-derived GH cell lines are the most widely used (Tashjian *et al.*, 1968; Gershengorn et al., 1986; Hinkle *et al.*, 1989).

It has been indicated that TRH appears to interact with a single class of binding sites in each of normal and tumour pituitary cells or in brain (Hinkle *et al.*, 1989; Johnson *et al.*, 1989). However, a classification of brain and anterior pituitary TRH receptors as a heterogeneous group has been performed according to their equilibrium dissociation constants (Funatsu *et al.*, 1985) and binding of the hormone to GH_3 cells with two classes of K_d values has been reported (Gautvik *et al.*, 1981). Some differences in isoelectric point and regulation by guanyl nucleotides have also been observed for TRH receptors from pituitary tumour cells and brain (Johnson *et al.*, 1989). The possibility that these differences result from substantial divergences in the primary structure of the receptor molecules remains to be established.

So far several types of TRH receptor cDNA clones have been isolated from mouse pituitary (Straub *et al.*, 1990; Perlman *et al.*, 1992), human pituitary (Duthie *et al.*, 1993) and rat anterior pituitary GH_3 cells (de la Pena *et al.*, 1992a, b; Sellar *et al.*, 1993) which encode two different isoforms of the receptor produced by alternative splicing. The cloning and expression of TRH receptor cDNAs has confirmed them to be members of the seven putative transmembrane spanning element GPCR family which acts through binding to and activation of G-proteins. Each of the isolated

cDNAs predicts a similar amino acid sequence with a small degree of variance at the C-terminal region for each species.

Although specific sequences have not been identified, domains within the carboxy terminus (or cytoplasmic tail) of several GPCRs have been shown to be involved in internalisation (Bouvier *et al.*, 1988a; Strader *et al.*, 1987). The TRH receptor, like other GPCRs, also undergoes rapid internalisation after binding TRH (Hinkle *et al.*, 1989). The carboxyl terminus of the mouse TRH-R is comprised of 72 amino acid residues. By generating site-directed mutants with truncations of the carboxy terminus of different lengths (C335 Stop, K338 Stop) and substitution mutants in which Cys-335 and Cys-337 were substituted with Ser or Gly residues, Nussenzveig *et al.*, 1993 have found two domains within the carboxyl terminus of the mouse pituitary TRH-R that are involved in internalisation. These two Cys residues, which are located near the seventh transmembrane domain, are conserved in many GPCRs and have been shown to be acylated by palmitate in rhodopsin (Ovchinnokov *et al.*, 1988) and the β_2-adrenoceptor (O'Dowd *et al.*, 1989). Recently, ligand-induced internalisation and recycling of TRH receptor has been confirmed and visualized by conventional and confocal fluorescence microscopy (Ashworth *et al.*, 1995).

Since Rodbell's hypothesis (Rodbell *et al.*, 1971) for the existence of multiple guanine nucleotide-binding proteins, G-proteins have been identified as crucial intermediaries linking a specific cell surface receptor to an intracellular "second messenger" system. Purification studies and molecular cloning technology have shown the function and the variety of G-proteins. A great deal of evidence supports the idea of the TRH receptor coupling to G-proteins. Addition of GTP converts the membrane receptor to a lower affinity state (Hinkle *et al.*, 1984); TRH stimulates PT-sensitive and PT-insensitive high affinity GTPase in membranes of GH_3 cells (Offermanns *et al.*, 1989) and the TRH receptor shares the characteristic topological structure with other GPCRs. (Straub *et al.*, 1990).

Hinkle *et al.*, 1984 had suggested that the TRH receptor might be associated with a guanine nucleotide-binding regulatory protein which coupled the receptor to

activation of phospholipase C and subsequently a variety of studies have indicated the involvement of a G-protein distinct from the inhibitory (G_i) or stimulatory (G_s) GTP-binding protein (Aub *et al.*, 1986; Straub *et al.*, 1986; Martin *et al.*, 1986). A combination of biochemical purification and reconstitution approaches (Pang *et al.*, 1990; Taylor *et al.*, 1991b; Berstein *et al.*, 1992) and cDNA cloning strategies based on conserved elements which are known to play a key role in the function of all G-proteins has led to the realisation that G-proteins of the G_q family (Strathmann *et al.*, 1989; 1990) (which consists of three closely related members $G_q\alpha$, $G_{11}\alpha$ and $G_{14}\alpha$ (Simon *et al.*, 1991) as well as a rather more distantly related $G_{16}\alpha$ (Amatruda *et al.*, 1991) can fulfill this function. Indeed, the TRH receptor stimulates PI-PLC β1 by coupling to members of this family as has been established directly using an antiserum against a peptide derived from the C-terminal region of $G_q\alpha$ and $G_{11}\alpha$ (Hsieh *et al.*, 1992; Aragay *et al.*, 1992). Furthermore, the involvement of both $G_q\alpha$ and $G_{11}\alpha$ in PI-PLC β1 activation (Taylor *et al.*, 1991b; Wu *et al.*, 1992a) has been demonstrated by either transiently transfecting cells with $G_q\alpha$ and $G_{11}\alpha$ cDNAs or injection of antisense oligonucleotides to $G_q\alpha$ and $G_{11}\alpha$ (Lipinsky *et al.*, 1992; Quick *et al.*, 1994). These results strongly support $G_q\alpha$ and $G_{11}\alpha$ as the subunits that couple to the TRH receptor and mediate the activation of the phospholipase C-β which catalyzes the cleavage of the membrane lipid phosphatidyl-inositol 4, 5-bisphosphate to generate inositol 1, 4, 5-trisphosphate and 1, 2-diacylglycerol.

On the other hand, an essential role of PT-sensitive $G_i\alpha$ family G-proteins in Ca^{2+} channel stimulation by TRH has been hypothesized, based on findings in GH_3 cells where TRH stimulation of a Ca^{2+} channel in a PT-sensitive manner was observed (Gollasch *et al.*, 1991). This approach suggested that TRH modulated intracellular free Ca^{2+} through two mechanisms: if the Ca^{2+} stores were filled, hormone induced a PT-insensitive inositol trisphosphate-mediated Ca^{2+} release; if they were empty, the hormone stimulated voltage-dependent Ca^{2+} channels in a PT-sensitive manner.

Identification of the involvement of PT-sensitive $G_i\alpha$ subtypes became possible by intranuclear injection of antisense oligo-nucleotides specifically hybridizing with mRNAs of $G_i\alpha$ and $G_o\alpha$ subunit sequences to suppress the expression of these proteins in pituitary GH_3 cells (Kleuss *et al.*, 1991; 1992). Recent results have shown that stimulation of dihydropyridine sensitive (L-type) Ca^{2+} channels by TRH is mainly mediated by the widely distributed $G_{i2}\alpha$ with a minor contribution of $G_{i3}\alpha$ (Gollasch *et al.*, 1993). As revealed by the use of corresponding antisense oligonucleotides and PKC inhibitors, this pathway requires concurrent protein kinase C (PKC) activation mediated by PT-insensitive G-proteins. That is, TRH induced hydrolysis of phosphatidylinositol 4, 5,-bisphosphate to InsP3 and diacylglycerol with subsequent PKC activation is required to allow $G_{i2}\alpha$ mediated Ca^{2+} channel stimulation. It is likely that PKC represents the active component of the priming inositol phospholipid response and that $G_{i2}\alpha$ may serve as a target for PKC action, allowing stimulatory $G_i\alpha$-Ca^{2+}channel interaction (Gollasch *et al.*, 1993).

TRH has been also shown to increase levels of intracellular cAMP, possibly through a direct action on adenylyl cyclase (Gautvik *et al.*, 1983). It has previously been postulated that cholera toxin may act directly on a G-protein that is associated with TRH receptors in GH_3 cells (Yajima *et al.*, 1988). Recently, it has been shown that the expression of antisense $G_s\alpha$ RNA reduced levels of $G_s\alpha$ mRNA and resulted in reduction of membrane content of $G_s\alpha$ in GH_3 cells. The reduction of $G_s\alpha$ levels was paralleled by an attenuation of the adenylyl cyclase response to TRH. Furthermore, experiments employing antisera blocking the receptor coupling ability of different G-proteins confirmed that TRH-activated adenylyl cyclase involves coupling to $G_s\alpha$ and not to $G_o\alpha$ or $G_i\alpha$ (Paulssen *et al.*, 1992).

As mentioned above, the TRH receptor appears to be able to stimulate the phospholipase C β1 isoform via $G_q\alpha/G_{11}\alpha$ and either adenylyl cyclase via $G_s\alpha$ or a Ca^{2+} channel via $G_i\alpha$ under some circumstances. Many of the down-stream effects of TRH are believed to be dependent on mobilization of intracellular Ca^{2+} and activation of protein kinase C (Drust *et al.*, 1984). The association of Shc with growth factor

receptor bound protein 2 (Grb2), which contains src homology (SH) domains, has been implicated in activation of the $p21^{ras}$ pathway by tyrosine kinases via association with son of sevenless (SOS), the ras nucleotide exchange factor (Oliver *et al.*, 1993; Egan *et al.*, 1993). TRH induces the association of the 52 kDa Shc protein with Grb2, as well as increases in activity of raf-1 kinase, presumably activated as a consequence of $p21^{ras}$ stimulation (Wood *et al.*, 1992). It has been suggested that TRH can activate mitogen activated protein (MAP) kinase by two distinct pathways. One is protein kinase C dependent and the other involves tyrosine phosphorylation of Shc proteins (Ohmichi *et al.*, 1994). Furthermore, recent data indicate that cytosolic phospholipase A_2 may be phosphorylated and subsequently activated by MAP kinase (Lin *et al.*, 1993) indicating a potential role for arachidonic acid release (Nemennoff *et al.*, 1993) which is known to be stimulated by TRH (Ohmichi *et al.*, 1990).

The mechanisms of MAP kinase activation by TRH still remain unclear. MAP kinase activity possesses the potentiality for directly regulating gene expression by phosphorylating transcription factors (c-jun and c-fos) since c-jun and c-fos are members of a family of potent transcriptional regulators (Angel *et al.*, 1991; Blenis *et al.*, 1993; Karin *et al.*, 1994), some of which are direct DNA-binding proteins (Abate *et al.*, 1991; Curran *et al.*, 1988). As such, the activation of c-jun and/or c-fos has the potential to initiate a cascade of gene induction events (Blenis *et al.*, 1993). There is some evidence that TRH can regulate gene transcription by Ca^{2+} mobilization and protein kinase-C activation which represent distinct components of the signaling events initiated by TRH, resulting in increased c-jun and c-fos mRNA levels (Weisman *et al.*, 1987). Furthermore, TRH-induces prolactin (PRL) mRNA accumulation, possibly by a mechanism that involves increased PRL gene transcription (Morin *et al.*, 1984) but also alterations in PRL mRNA turnover (Laveriere *et al.*, 1983). Regulation of transcription of the PRL gene involves the pituitary specific transcription factor pit-1, for which responsive elements have been found (Day *et al.*, 1989; Yan *et al.*, 1991) and the responsive elements conferring TRH regulation may thus interact with pit-1 or other transcription factors. Based on

several studies, it is suggested that TRH stimulation of c-jun and/or c-fos may be a key element in mediating the intracellular processes regulating thyrotropin β-subunit (TSHβ) and prolactin (PRL) gene expression (Carr *et al.*, 1993), followed by synthesis and release of pituitary TSH as well as PRL.

As mentioned above, the TRH receptor has been assumed to be able to act as a bifunctional one which is able to regulate multiple signalling cascades by activating distinct G-proteins in pituitary GH3 cells. However, as GH3 cells express at least two splice variants of the TRH receptor it is thus clearly possible that the multiple signalling effects of TRH noted in pituitary GH3 cells are actually produced by pharmacologically similar but genetically distinct receptors. Differential signal transduction by splice variants of receptors has recently been described for both the EP3 prostanoid receptor (Namba *et al.*, 1993) where, like the TRH receptor, the splice variants occur in the C-terminal tail and the pituitary adenylyl cyclase activating polypeptide (PACAP) receptor (Spengler *et al.*, 1993).

Therefore, the main purpose of the study in chapter 4.2 was to assess whether both $G_q\alpha$ and/or $G_{11}\alpha$ and $G_s\alpha$ are involved in TRH stimulation of phospholipase C β1 and/or adenylyl cyclase or whether different splice variants of the receptor selectively interact with different G-proteins to regulate different signal transduction cascades. However, as GH3 cells have been reported to express at least two distinct splice variants of the TRH receptor it is difficult to use these cells to assess potential differences in the function of each variant. In this study human embryonic kidney HEK-293 cells, which do not endogenously express TRH receptors, were transfected to stably express the long variant of the rat TRH receptor (clone E2) to examine these questions.

In chapter 4.3 the studies focused on developing a 6 M urea containing SDS-PAGE (10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide) system to resolve a number of polypeptides which are recognized by an antiserum directed against the C-terminal decapeptide which is common to $G_q\alpha$ and $G_{11}\alpha$ to examine the expression of G_q-like G-proteins in cells. Because of the high degree of sequence identity (for

example, between $G_q\alpha$ and $G_{11}\alpha$, 89%; $G_{11}\alpha$ species variants, 97%) and heterologous expression of G protein-linked receptors into cell lines derived from different species regularly results in the expression of functionally coupled receptors, there has been little consideration as to whether individual receptors might display a degree of selectivity of interaction if concurrently offered the choice between species variants of the same G-protein.

Based on the 6 M urea incorporated SDS-PAGE system, I assessed whether a single receptor type can utilise species variants of the same G-protein equivalently? In this study, clone E2 HEK-293 cells were further transfected with a cDNA encoding murine $G_{11}\alpha$ (clone E2M11) to stably co-express the endogenous human and exogenously introduced murine $G_{11}\alpha$.

4.2. The long splice variant of the rat TRH receptor interacts with and down-regulates members of the G_q family of G-proteins only.

4.2.1. Results.

Human embryonic kidney HEK-293 cells were co-transfected with the plasmids pcDNA1 containing the long splice variant of the rat TRH receptor under control of the cytomegalovirus promoter and pSV2-neo which contains the neomycin resistance gene. Cells expressing this second gene product were selected by their resistance to geneticin sulphate (800 μg/ml) (Figure 4.2.1). A number of these clones were assayed for a TRH (1 μM) stimulation of inositol phosphate production and specific binding of [^{3}H]TRH to determine the cells stably expressing the TRH receptor.

Clone E2 which expressed the highest levels of the receptor was selected for examination in these studies. To confirm that the E2 HEK-293 cells contained only mRNA encoding the long isoform of the TRH receptor, RNA was isolated from these cells and subsequently reverse transcriptase-PCR performed using primer pairs which were designed to amplify mRNA encoding either the short or the long isoform as described in chapter 2.2.23. cDNAs of both the long and short isoforms of the rat TRH receptor were used as positive controls for the effectiveness of the primers in these experiments. As anticipated, the long isoform cDNA produced a band of 370 bases and the short isoform cDNA a band of 318 bases confirming the validity of the selected primers to generate fragments which would demonstrate the presence or absence of the 52-bp segment in the C-terminal tail which differs between the 412 amino acid (long) isoform and the 387 amino acid (short) isoform (de la Pena *et al.*, 1992b) (Figure 4.2.2, lanes 4 and 5). Only a product representing mRNA corresponding to the long isoform of the receptor was detected in E2 HEK-293 cells (Figure 4.2.2, lane 3). This band was absent when using RNA isolated from parental HEK-293 cells (Figure 4.2.2, lane 1). Reverse transcriptase-PCR of RNA isolated from our clone of GH_3 cells indicated that the expression of TRH receptor isoforms in

these cells was entirely the long isoform (Figure 4.2.2, lane 2). This was surprising as the short isoform of the TRH receptor was cloned from a GH_3 cell library and co-expression of the two forms in GH_3 cells has previously been noted (de la Pena *et al.*, 1992b). This may be a reflection of clonal variation between GH_3 cell lines.

1 mg of P2 membrane protein was generated from $51.6 \pm 10.8 \times 10^6$ untreated clone E2 HEK-293 cells (mean ± S.E., n = 4). The yield of membrane was not altered ($p = 0.51$) by pretreatment of the cells with TRH (1mg from $61.6 \pm 9.2 \times 10^6$ cells (mean ± S.E., n = 4)). The level of TRH receptor expression was measured in membranes of E2 HEK-293 cells by dilution of the [^{3}H]TRH (10 nM) with cold TRH and subsequent correction for alteration in the specific activity of the ligand (Figure 4.2.3). The experiments were performed in the presence of Gpp(NH)p (100 μM) to convert the population of TRH receptors to a single affinity state as no radiolabeled antagonists are available for this receptor and attempts to measure the total receptor population using [^{3}H]TRH in the absence of Gpp(NH)p produced data which were consistent with both high and low affinity sites for the [^{3}H]-ligand and generation of curvilinear data when presented as a Scatchard plot (data not shown). Displacement of approximately 10 nM [^{3}H]TRH under these conditions by cold TRH was achieved with as estimated IC_{50} of 98 nM when the Hill coefficient was restrained to 1.0 (Figure 4.2.3A). No further displacement was produced by concentrations of TRH above 100 μM, and this concentration was thus used subsequently to define non-specific binding. Correction of the specific binding of [^{3}H]TRH for dilution of specific activity in such self-displacement experiments (Figure 4.2.3A) allowed transformation of these data which indicated the B_{max} to be some 17.6 ± 0.8 pmol/mg membrane protein (Figure 4.2.3C) and the K_d for [^{3}H]TRH at this receptor binding site, under these conditions, to be some 133.7 ± 2.7 nM (Figure 4.2.3B).

Previous studies in GH_3 pituitary cells have reported that TRH can cause activation of both phosphoinositidase C and adenylyl cyclase activities (Paulssen *et al.*, 1992). In clone E2 HEK-293 cells, application of TRH resulted in the generation of high levels of inositol phosphates when the experiments were performed in the

presence of LiCl (10 mM) to limit the activity of inositol monophosphatases. The EC_{50} for TRH stimulation of inositol phosphate generation was 20 ± 10 nM (mean ± range, n = 2) (Figure 4.2.4A). This was markedly higher that the EC_{50} for TRH stimulation of inositol phosphate generation in GH_3 cells (3.0 ± 1.2 nM mean ± range, n = 2) (Figure 4.2.4B). TRH at maximally effective concentrations (1 μM and above) produced some 20 fold stimulation of inositol phosphate generation in comparison to that noted in the absence of TRH (Figure 4.2.4B). This effect was completely eliminated by pretreatment of E2 HEK-293 cells with TRH (10 μM, 16h) (Figure 4.2.4B). The ability of TRH to subsequently stimulate the generation of inositol phosphates was not modified (Table 4.2.1), however, by pertussis toxin treatment (25 ng/ml for 16h) of the cells under conditions which caused essentially complete ADP-ribosylation of the 'Gi-like' proteins ($G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$ and $G_o\alpha$) expressed in these cells (Figure 4.2.5). By contrast with its effect on inositol phosphate production TRH was unable to either significantly stimulate basal or to inhibit forskolin-amplified adenylyl cyclase activity in membranes derived from cells of clone E2. This was also the case, however, in our clone of GH_3 cells (Table 4.2.2).

As receptor regulation of pertussis toxin-insensitive phosphoinositidase C activity is transduced by members of the G_q family of G-proteins (Berstein *et al.*, 1992a; Pang *et al.*, 1990; Taylor *et al.*, 1991b) and the stimulation of adenylyl cyclase activity by the splice variants of $G_s\alpha$ then the absolute levels of expression of these G-proteins were examined in membranes of E2 HEK-293 and GH_3 cells (Figures 4.2.6 and 4.2.7, Table 4.2.3). $G_s\alpha$ immunoreactivity in membranes of E2 HEK-293 as detected by antiserum CS (an antipeptide antiserum directed against the C-terminal decapeptide which is common to all forms of $G_s\alpha$) (Milligan *et al.*, 1989) was provided by both 45 kDa and 42 kDa polypeptides which were expressed at similar steady state levels (Figure 4.2.6A). Construction of immunological standard curves of recombinant *E. coli* expressing the long splice variant isoform of $G_s\alpha$ (Figure 4.2.6B) allowed measurement of cellular levels of the two isoforms of $G_s\alpha$ (Table 4.3). $G_s\alpha$

45 kDa was expressed at some 10.3 ± 1.3 pmol/mg membrane protein (mean ± S.E., n = 6) and $G_s\alpha$ 42 kDa at some 11.3 ± 1.1 pmol/mg membrane protein (mean ± S.E., n = 6). Similar standard curves were constructed with recombinant *E. coli* produced $G_q\alpha$ (Figure 4.2.7B). Immunoblotting such curves with antiserum CQ (an antipeptide antiserum directed against the C-terminal decapeptide which is common to $G_q\alpha$ and $G_{11}\alpha$) (Mitchell *et al.*, 1991) in parallel with membranes of E2 HEK-293 cells demonstrated the expression of some 12.2 ± 1.2 pmol/mg membrane protein (mean ± S.E., n = 6) of total G_q-like G-proteins in these cells (Table 4.2.3).

On the basis of several reports that sustained activation of both G_q and G_s-linked receptors can result in a down-regulation of cellular levels of these G-proteins due to enhanced turnover of the activated G-protein (McKenzie *et al.*, 1990b; Milligan *et al.*, 1993c; Mitchell *et al.*, 1993; Shah *et al.*, 1995) the effect of the maintained presence of TRH on cellular levels of the G-proteins expressed by the E2 HEK-293 cells was examined. Incubation of these cells with TRH (10 μM, 16h) resulted in marked decrease in membrane levels of the G_q-like G-proteins. Quantitative immunoblotting demonstrated that levels fell from 12.2 ± 1.2 pmol/mg membrane protein to 5.7 ± 0.8 (mean ± S.E., n = 6) pmol/mg membrane protein (Table 4.2.4). This effect was highly significant (p = 0.001). In contrast, sustained treatment of clone E2 cells with TRH had no effect (p = 0.37) on levels of G_s (total $G_s\alpha$) 21.6 ± 1.7 pmol/mg membrane protein in untreated cells and 24.6 ± 2.6 pmol/mg membrane protein in untreated cells (mean ± S.E., n = 6) (Table 4.2.4). Parallel experiments also demonstrated that TRH treatment did not alter membrane associated levels of the α subunits of the pertussis toxin-sensitive G-proteins, $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$ or $G_o\alpha$ all of which are expressed by these cells (Figure 4.2.8 and Table 4.2.4). Furthermore, assessment of the relative levels of the α subunits of the pertussis toxin-sensitive G-proteins and of the isoforms of $G_s\alpha$ in membranes derived from untreated and TRH (10 μM, 16h) treated E2 HEK-293 cells by performing either pertussis or cholera toxin-catalysed [^{32}P]ADP-ribosylation demonstrated no obvious alterations produced by TRH treatment in the ability of these polypeptides to act as substrates for the ADP-

ribosyltranferase activities of these toxins (Figure 4.2.9). To assess whether TRH-induced down-regulation of G_q-like G-proteins was restricted to E2 HEK-293 cells or would also be observed in GH_3 cells, these cells were also treated with TRH (10 μM, 16h). As with E2 HEK-293 cells a substantial down-regulation of a combination of $G_q/G_{11}\alpha$ (20.4 ± 4.3 % (mean ± S.E., n = 3)) without any alteration in membrane associated levels of $G_s\alpha$ was recorded (Figure 4.2.10).

Exposure of E2 HEK-293 cells to 10 μM TRH resulted in half-maximal reduction in levels of the G_q-like G-proteins within 4 h and a new steady-state plateau of membrane associated levels was achieved by 10-12 h (Figures 4.2.11A and B). Treatment of E2 HEK-293 cells with varying concentrations of TRH for 16h followed by immunological detection of a combination of the G_q-like G-proteins in SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) demonstrated that half-maximal TRH-induced down-regulation of these polypeptides was produced by some 30 nM ligand (data not shown but see Figures 4.2.12A and B).

Resolution of membranes from untreated and TRH pretreated E2 HEK-293 cells in SDS polyacrylamide gels which contained a linear 4-8 M gradient of urea demonstrated the presence of two CQ reactive polypeptides in these cells (Figure 4.2.12). Treatment of E2 HEK-293 cells with varying concentrations of TRH caused an equivalent down-regulation of both of these G_q-like G-proteins (Figure 4.2.12A). Dose-response curves for the effect of TRH on down-regulation of these two G_q-like proteins were extremely similar (EC_{50} = 15-25 nM) (Figure 4.2.12B). Membranes from many rodent derived cell lines when resolved under these conditions can, however, be shown to express at least three distinct CQ reactive polypeptides (Figure 4.2.14A). It has previously been demonstrated that the most rapidly migrating of these polypeptides in a range of cells including GH_3 pituitary, CHO fibroblasts and NG108-15 neuroblastoma x glioma hybrid cells is the α subunit of rodent $G_{11}\alpha$ (Milligan *et al.*, 1993d). E2 HEK-293 cells did not express an immunodetectable polypeptide which migrated in this position (Figure 4.2.14A). This, however, is a reflection of a considerable difference in mobility of rodent and primate $G_{11}\alpha$ in such

gels (described in chapter 4.3) in which rodent $G_{11}\alpha$ migrates more rapidly than the primate form of this G-protein. Immunoblotting membranes of E2 HEK-293 cells with antisera both selective for $G_{11}\alpha$ (E976) and common for $G_q\alpha/G_{11}\alpha$ (CQ) demonstrated the co-expression of these two G-proteins in E2 HEK-293 cells. Furthermore antiserum E976 was able to identify two distinct polypeptides in membranes of these cells (Figure 4.2.14B). The relationship between these two E976 immunoreactive polypeptides however is unknown. Separation of GH_3 cell membranes in these gel conditions produced excellent resolution of $G_q\alpha$ and $G_{11}\alpha$ (Figure 4.2.14A) as described previously for other rodent cell lines (Milligan *et al.*, 1993e). Analysis under such conditions demonstrated that TRH treatment of GH_3 cells caused equivalent down-regulation of both $G_q\alpha$ (27.9 ± 7.4 %) and $G_{11}\alpha$ (31.2 ± 6.6 %) (mean ± S.E., n = 3) (Figure 4.2.13).

Figure 4.2.1.

Generation and isolation of clone E2 HEK-293 cells expressing the long splice variant of the rat TRH receptor.

The long splice variant of the rat TRH receptor (2.2 kb) was subcloned into the EcoR I site of the multiple cloning region in the eukaryotic expression vector pcDNA1 (4.1 kb) which is driven by the cytomegalovirus promoter. Monolayer cultures of HEK-293 cells (50% confluent) were co-transfected overnight with linearised pcDNA1/ TRH-receptor (800 ng) and pSV2-neo (5.6 kb) (200 ng) using lipofectin reagent in serum free Dulbecco's modified Eagle's medium (DMEM) as suggested by the manufacturer. After 24 hours medium was replaced with DMEM containing 800 µg/ml of geneticin sulphate. Resultant geneticin resistant clones were picked, expanded and assayed for the expression of the TRH receptor. Cells were trypsinized, transferred to 24 well plates and labelled with myo-[^{3}H] inositol (2 µCi/ml) in inositol free DMEM containing 1% dialysed foetal calf serum for 48 h. Total inositol phosphate (IP) production was then measured in TRH treated cells (1 µM for 30 min). TRH-receptor containing clones were identified as those in which TRH produced a rise in total IP production. Expression of the TRH receptor in membranes from these clones was assessed by the specific binding of [^{3}H]TRH. Clone E2 HEK293 cells was selected and maintained as described in chapter 2.2.1 for further analysis in these studies.

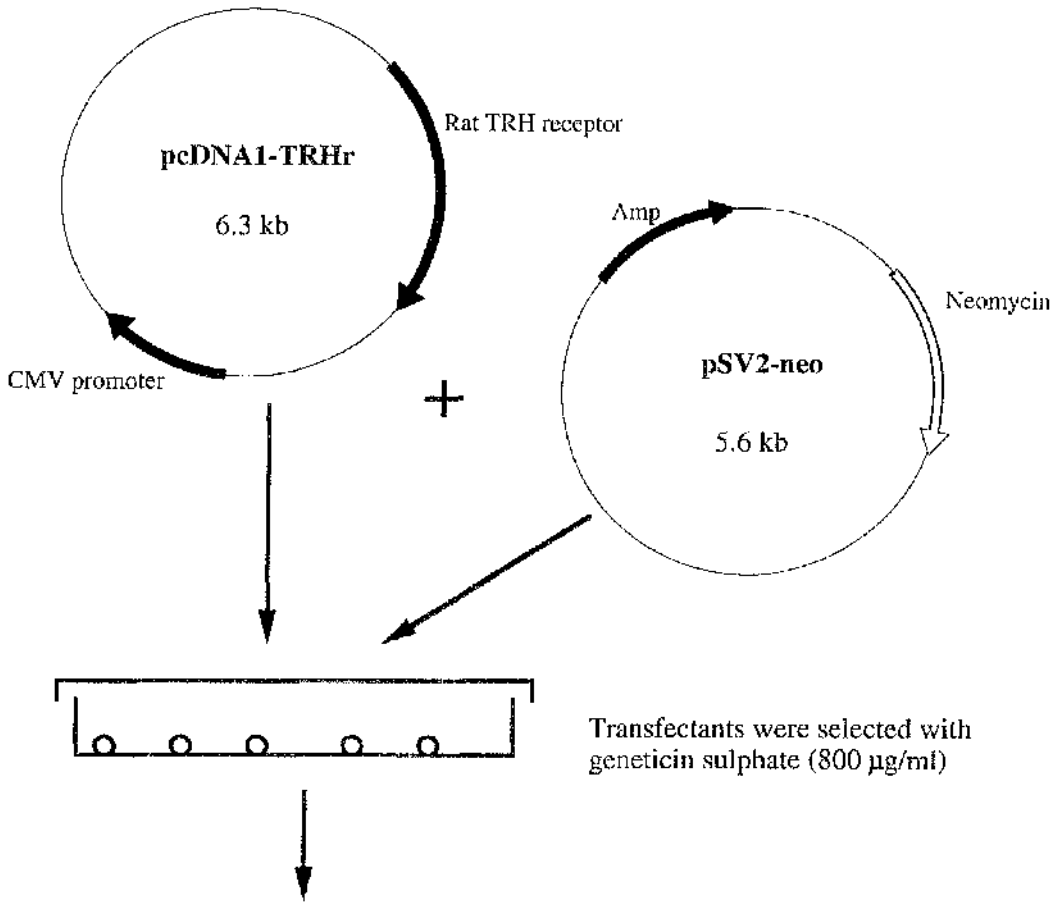

pcDNA1-TRHr
6.3 kb
Rat TRH receptor
CMV promoter
Amp
Neomycin
pSV2-neo
5.6 kb
+
Transfectants were selected with geneticin sulphate (800 µg/ml)
Clones were expanded and analysed for the expression of the long isoform of the rat TRH receptor.

Figure 4.2.2.

E2 HEK-293 cells express exclusively the long isoform of the rat TRH receptor.

Reverse transcriptase-PCR was performed using RNA isolated from either HEK-293 cells transfected with the plasmids pcDNA1/pSV2-neo only (lane 5), from GH_3 cells (lane 4), or from E2 HEK-293 cells (lane 3) as described in chapter 2.2.23. Amplification was also performed with plasmids containing either the short (lane 2) or long (lane 1) isoform of the rat TRH receptor. As anticipated amplification of the cDNA species representing the short and long isoforms resulted in the generation of fragments of 318 and 370 bases, respectively. Reverse transcriptase-PCR of RNA from both E2 HEK-293 cells and our clone of GH_3 cells resulted in the generation only of a product consistent with the expression of the long isoform of the TRH receptor by these cells.

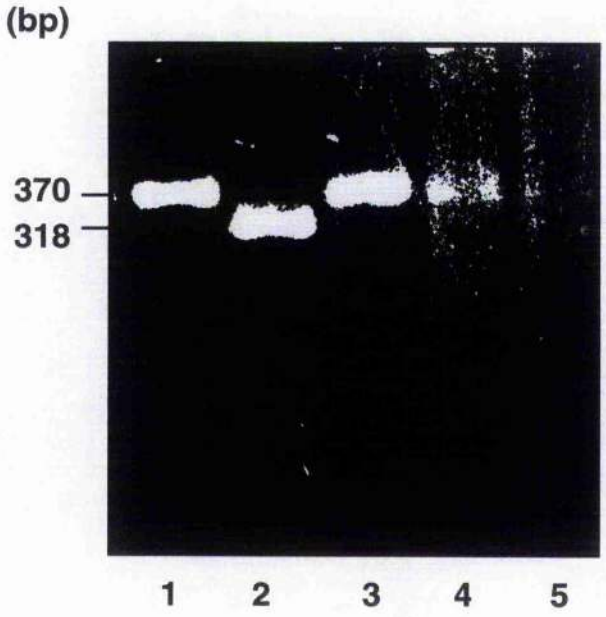
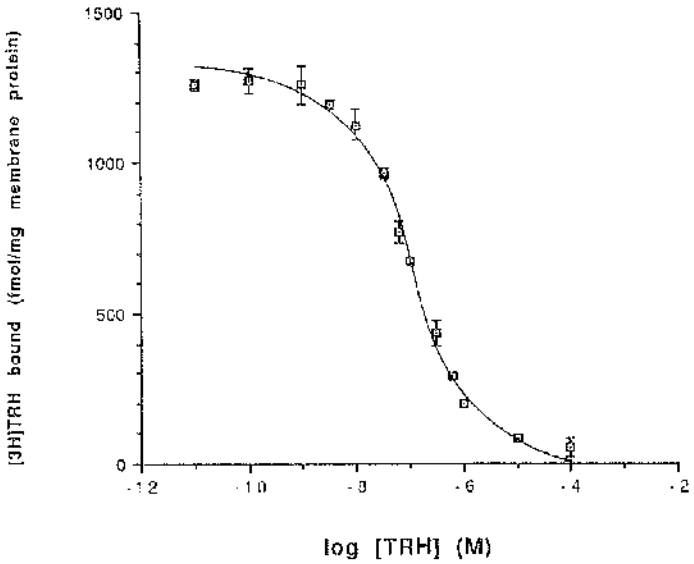

(bp)
370
318
1
2
3
4
5

Figure 4.2.3.

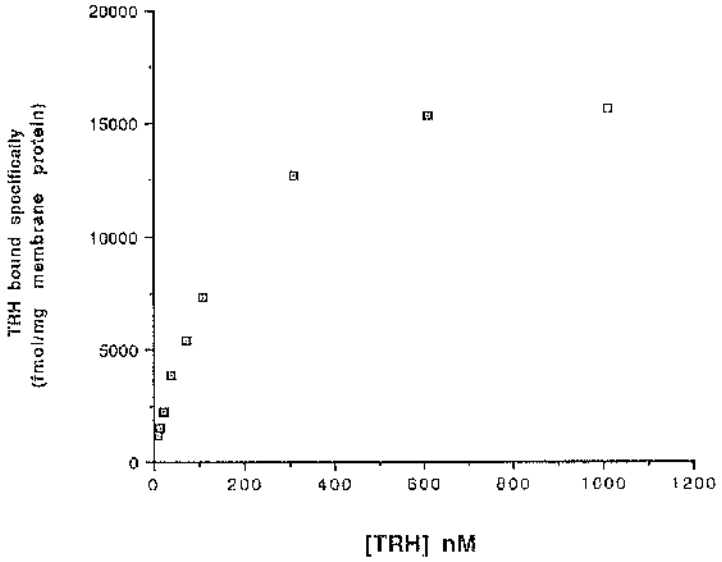
Binding characteristics of [^{3}H]TRH to membranes of E2 HEK-293 cells.

Panel A represents the ability of various concentrations of TRH to compete with [^{3}H]TRH (9.2 nM) for binding to the TRH receptors expressed in membranes (25 μg) of E2 HEK-293 cells in the presence of 100 μM Gpp(NH)p at 30 °C for 1 h as described in chapter 2.2.16. Maximal displacement, which was greater that 90% of the total binding, was achieved with concentrations of TRH at above 10 μM. No further displacement was produced by concentrations of TRH above 100 μM, and this concentration was thus used subsequently to define non-specific binding. Displacement of [^{3}H]TRH under these conditions by cold TRH was achieved with estimated IC_{50} of 98 nM when the Hill coefficient was restrained to 1.0. Linear transformation of the specific binding data was performed following correction for dilution of the specific activity of [^{3}H]TRH by addition of cold TRH. The estimated B_{max} was 17.6 ± 0.8 pmol/mg membrane protein (Panel C) and the K_d for [^{3}H]TRH at this receptor binding site, under these conditions, was 133.7 ± 2.7 nM (Panel B).

A


[3H]TRH bound (fmol/mg membrane protein)
1500
1000
500
0
-12
-10
-8
-6
-4
-2
log [TRH] (M)

B


TRH bound specifically
(fmol/mg membrane protein)
20000
15000
10000
5000
0
0
200
400
600
800
1000
1200
[TRH] nM

C

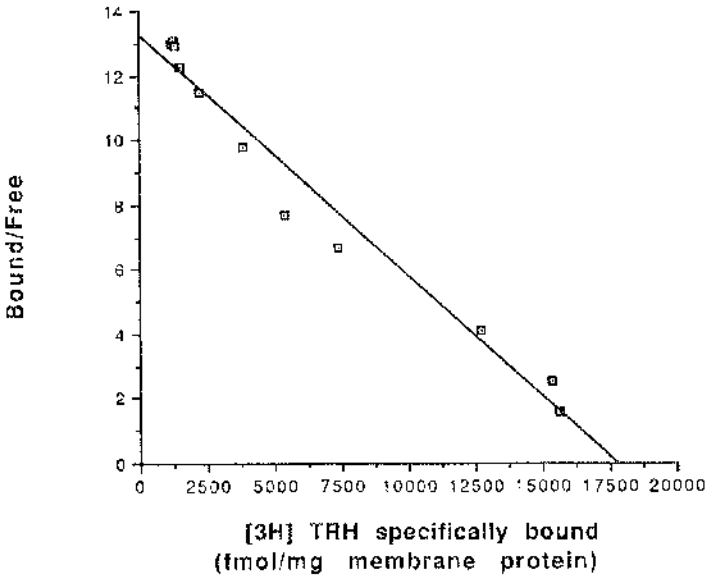

Bound/Free
14
12
10
8
6
4
2
0
0
2500
5000
7500
10000
12500
15000
17500
20000
[3H] TRH specifically bound
(fmol/mg membrane protein)

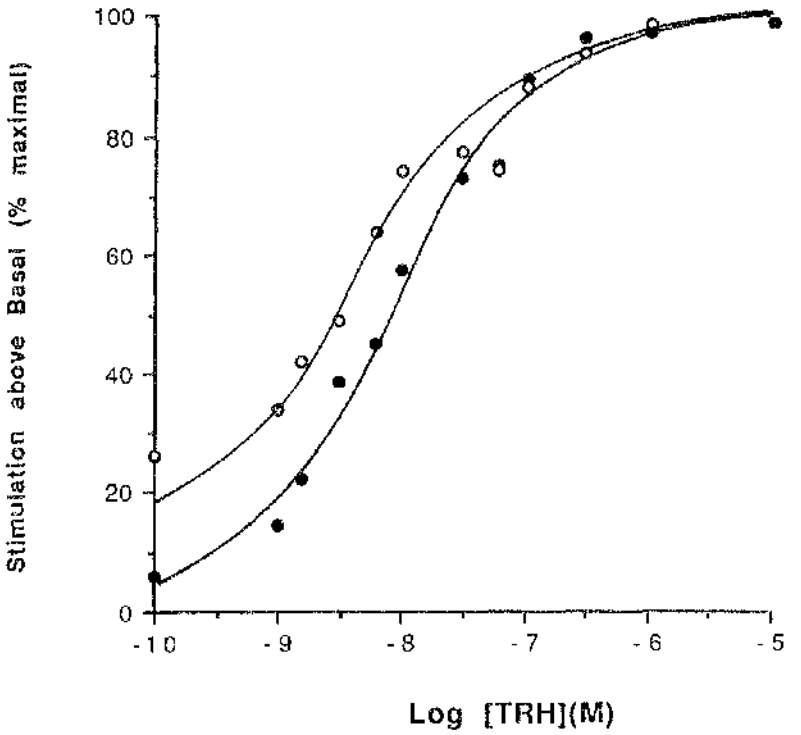
Figure 4.2.4.

The stimulation of inositol phosphate generation by TRH in clone E2 HEK-293 cells.

Panel A demonstrates that TRH stimulated inositol phosphate generation more effectively in GH_3 cells than in E2 HEK-293 cells. The stimulation of inositol phosphate production by varying concentrations of TRH was assessed as in chapter 2.2.17. in both GH_3 (open symbols) and E2 HEK-293 (filled symbols) cells. Results are presented as percent of maximal effect, i.e. that produced by 1 μM TRH.

Panel B demonstrates that TRH stimulation of inositol phosphate generation is attenuated by pretreatment of the cells with TRH. E2 HEK-293 cells were either untreated (open symbols) or treated with TRH (10 μM, 16h) (filled symbols). The cells were washed twice with 0.5 ml of Hanks buffered saline (pH 7.4), containing 1% (w/v) BSA and 10 mM glucose (HBG) and incubated for 10 min with HBG containing 10 mM LiCl prior to analysis of TRH regulation of inositol phosphate generation as described in chapter 2.2.18.

A


Stimulation above Basal (% maximal)
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
Log [TRH](M)

B

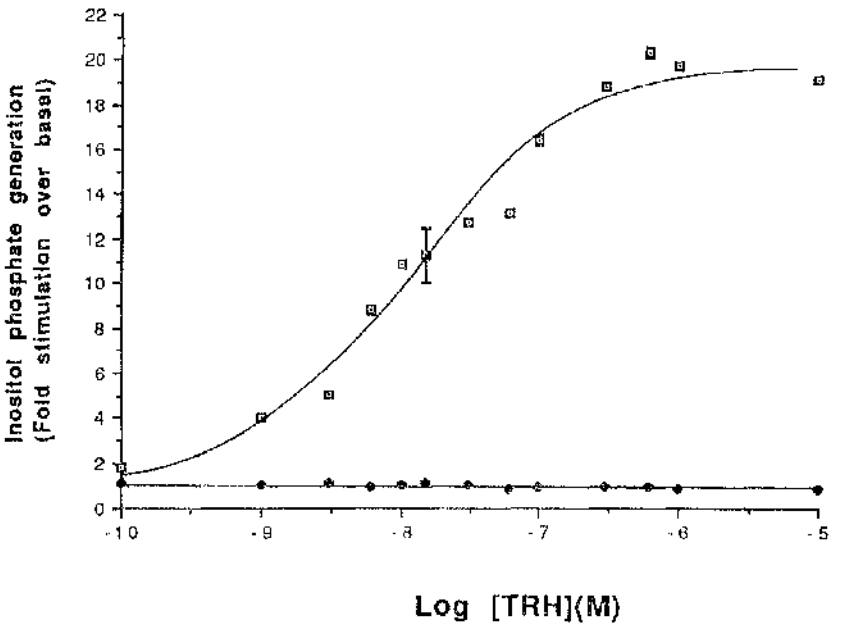

Inositol phosphate generation
(Fold stimulation over basal)
22
20
18
16
14
12
10
8
6
4
2
0
-10
-9
-8
-7
-6
-5
Log [TRH](M)

Figure 4.2.5.

Pertussis toxin treatment of E2 HEK-293 cells causes complete ADP-ribosylation of the population of α subunits of pertussis toxin-sensitive G-proteins.

Membranes (75 μg) derived from E2 HEK-293 cells which were untreated (lanes 1-6) or pretreated with pertussis toxin (25 ng/ml, 16h) (lanes 7-12) were incubated in the absence (lanes 3, 6, 9 and 12) or presence of thiol-activated pertussis toxin (lanes 1, 2, 7 and 8) or cholera toxin (lanes 4, 5, 10 and 11) with [^{32}P]NAD for 2 h as described in 2.2.15. Samples were recovered by precipitation, resolved by SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) and autoradiographed overnight.

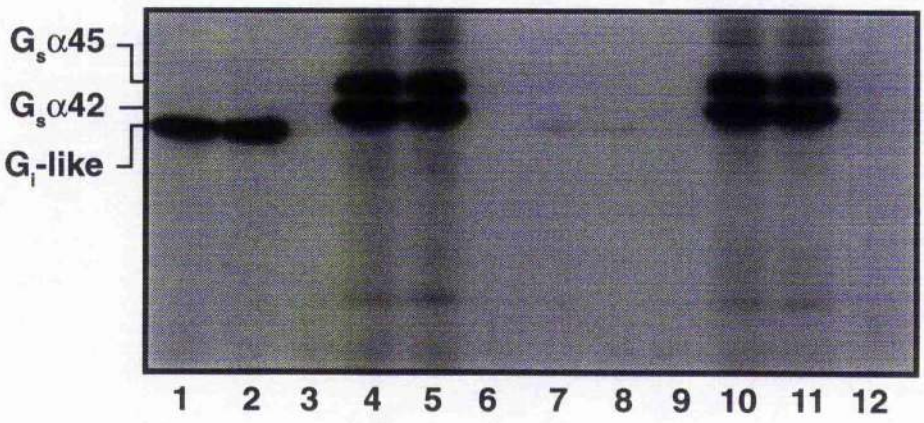
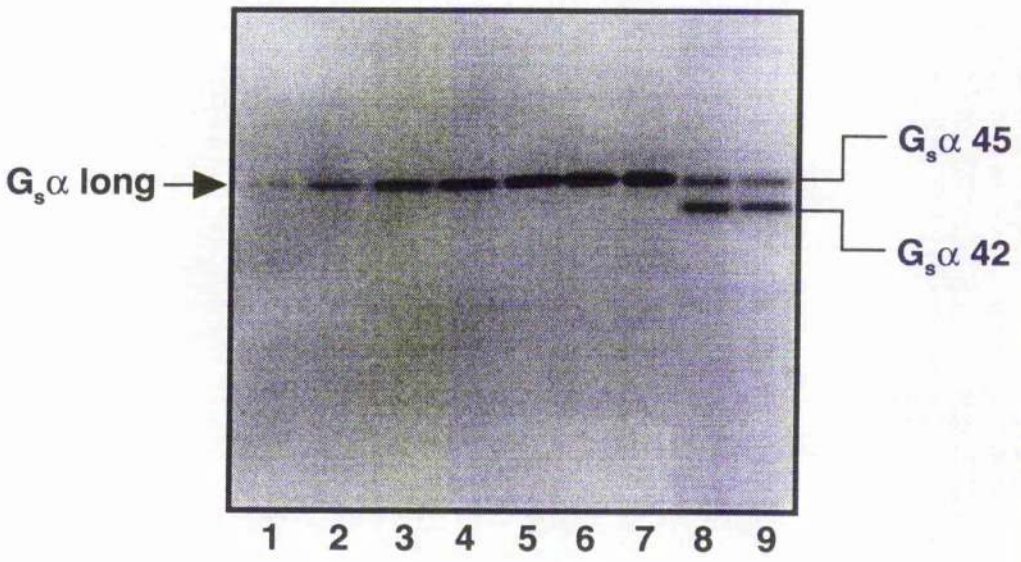

$G_s\alpha45$
$G_s\alpha42$
G_i-like
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4.2.6.

Quantitation of $G_S\alpha$ levels in membranes of E2 HEK-293 cells.

Varying amounts of purified *E. coli* produced recombinant $G_S\alpha$ long form (lane 1, 2.5 ng; lane 2, 5 ng; lane 3, 10 ng; lane 4, 15 ng; lane 5, 20 ng; lane 6, 25 ng; lane 7, 30 ng) were subjected to SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) along with membranes (5 μg) from untreated (lane 8) and TRH-treated (10 μM, 16h) (lane 9) E2 HEK-293 cells. The samples were subsequently immunoblotted for $G_S\alpha$ using antiserum CS (1: 250 dilution). The developed immunoblot (panel A) was scanned to allow construction of a standard curve for $G_S\alpha$ (panel B). Such standard curves were generated routinely for quantitation of $G_S\alpha$ levels. In the example shown $G_S\alpha$ long was calculated to be 11.1 pmol/mg of membranes protein in untreated membranes and 9.7 pmol/mg in TRH-treated cell membranes. $G_S\alpha$ short was 11.9 and 10.1 pmol/mg of membrane protein in membranes of untreated and TRH-treated E2 HEK-293 cells, respectively.

A


$G_s\alpha$ long
$G_s\alpha$ 45
$G_s\alpha$ 42
1 2 3 4 5 6 7 8 9

B

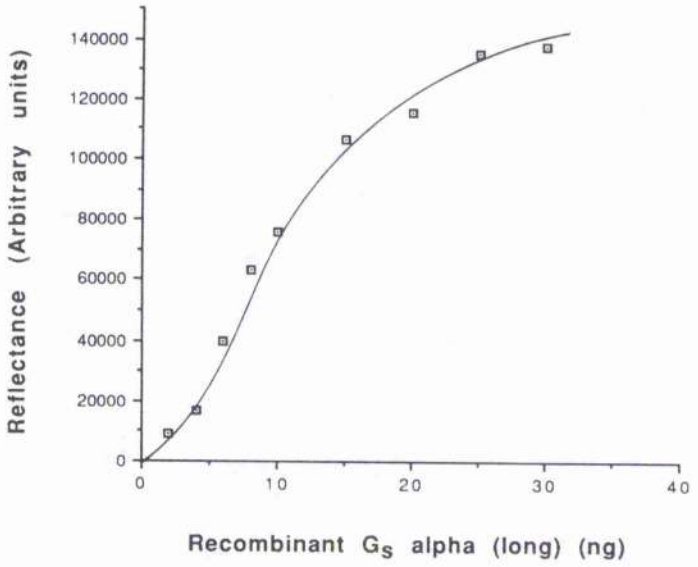
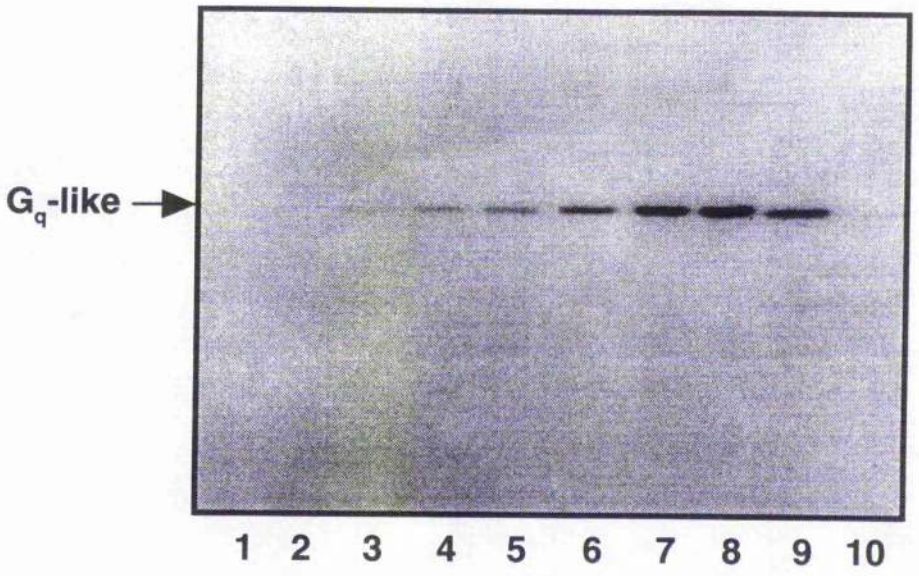

Reflectance (Arbitrary units)
140000
120000
100000
80000
60000
40000
20000
0
0
10
20
30
40
Recombinant G_S alpha (long) (ng)

Figure 4.2.7.

Quantitation of levels of the α subunit of the G_q-like G-proteins in membranes of E2 HEK-293 cells.

Various amounts of purified *E. coli* expressed recombinant $G_q\alpha$ (lane 1, 2 ng; lane 2, 4 ng; lane 3, 6 ng; lane 4, 8 ng; lane 5, 10 ng; lane 6, 15 ng; lane 7, 20 ng; lane 8, 25 ng) were subjected to SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) along with membranes (30 μg) from untreated (lane 9) and TRH-treated (10 μM, 16h) (lane 10) E2 HEK-293 cells. The samples were subsequently immunoblotted for G_q-like G-proteins using antiserum CQ (1: 1000 dilution). Developed immunoblots (panel A) were scanned to allow construction of a standard curve for $G_q\alpha/G_{11}\alpha$ (panel B). Such standard curves were generated routinely for quantitation of $G_q\alpha/G_{11}\alpha$ levels. In the example displayed control membranes expressed 15.5 pmol/mg membrane protein of G_q-like G-proteins and the membranes of TRH treated cells 7.7 pmol/mg of membrane protein.

A


G_q-like
1 2 3 4 5 6 7 8 9 10

B

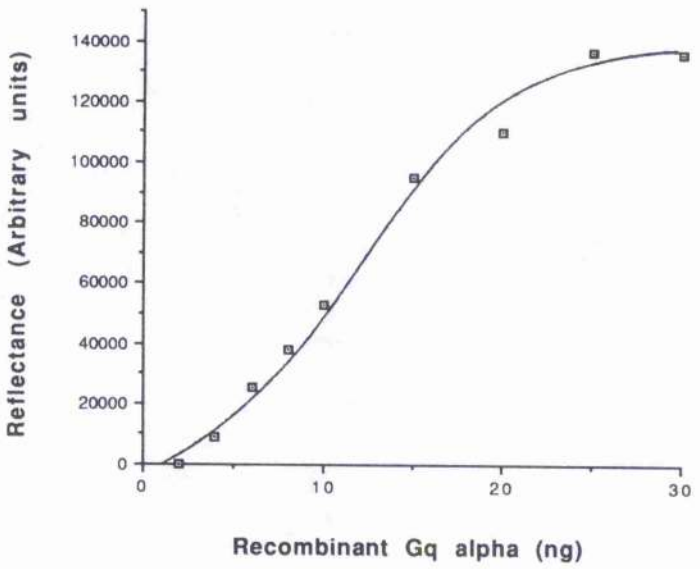

Reflectance (Arbitrary units)
140000
120000
100000
80000
60000
40000
20000
0
0
10
20
30
Recombinant Gq alpha (ng)

Figure 4.2.8.

Sustained exposure of E2 HEK-293 cells to TRH does not alter immunologically detectable levels of $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$ and $G_o\alpha$.

Membranes (Panels A; 60 μg, B; 60 μg and C; 25 μg) of untreated (lane 1) and TRH pretreated (10 μM, 16h) (lane 2) E2 HEK-293 cells were resolved in SDS-PAGE conditions (12.5% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide) able to resolve $G_{i1}\alpha$ and $G_{i2}\alpha$. The samples were then immunoblotted with antisera SG (1: 500 dilution) (anti-$G_{i1}\alpha$ + $G_{i2}\alpha$) (Green *et al.*, 1990) (panel A), I3B (1: 1000 dilution) (anti-$G_{i3}\alpha$) (Green *et al.*, 1990) (panel B) or IM (1: 500 dilution) (anti-$G_o\alpha$) (Mullaney *et al.*, 1990) (panel C) as described in chapter 2.2.6. Examples from a representative experiment are shown. In a range of experiments no significant alteration in membranes levels of any these polypeptides was produced by TRH treatment of the cells (see Table 4.2.4 for quantitative details).

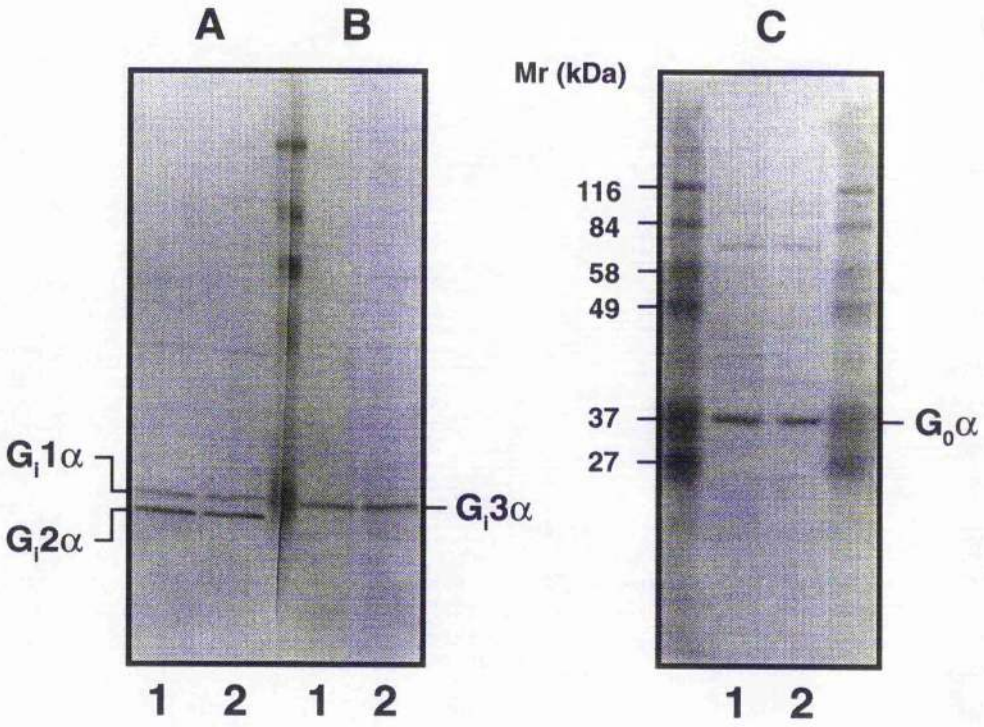

A
B
C
Mr (kDa)
116
84
58
49
37
27
$G_i1\alpha$
$G_i2\alpha$
$G_i3\alpha$
$G_o\alpha$
1 2 1 2
1 2

Figure 4.2.9.

Sustained exposure of E2 HEK-293 cells to TRH does not modify the ability of pertussis or cholera toxin to modify their G-protein substrates.

Membranes (75 μg) from untreated (lanes 1-6) or TRH (10 μM, 16h) treated (lanes 7-12) E2 HEK-293 cells were incubated in the absence (lanes 3, 6, 9 and 12) or presence of thiol-activated pertussis toxin (lanes 1, 2, 7 and 8) or cholera toxin (lanes 4, 5, 10 and 11) with [^{32}P]NAD for 2 h. Samples were recovered by TCA precipitation, resolved by SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) and the dried gel was autoradiographed overnight.

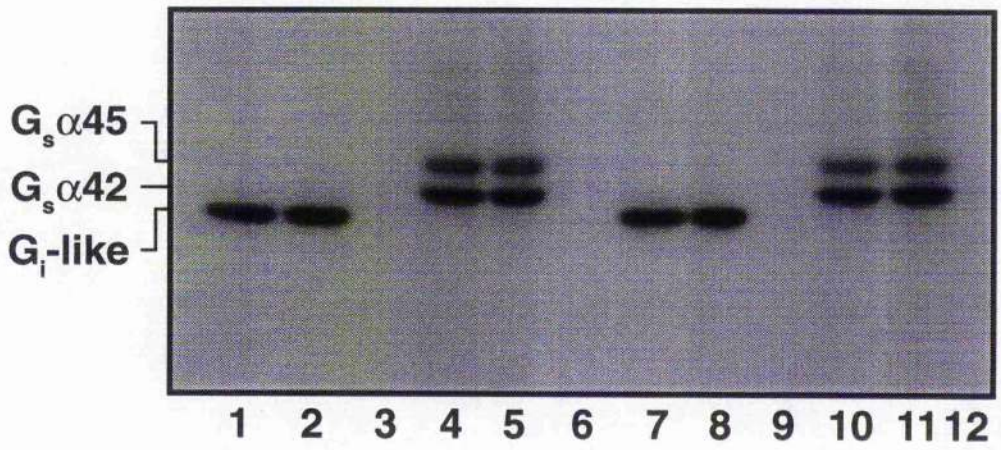

$G_s\alpha45$
$G_s\alpha42$
G_i-like
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4.2.10.

Sustained TRH treatment causes down-regulation of $G_q\alpha/G_{11}\alpha$ but not of $G_S\alpha$ in GH_3 cells.

Membranes (lanes 1 and 2, 10 μg; lanes 3 and 4, 25 μg) from untreated (lanes 1 and 3) and TRH pretreated (1 μM, 16h) (lanes 3 and 4) GH_3 cells were resolved by SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide). Proteins were transferred to nitrocellulose and immunoblotted with antiserum CQ (1: 1000 dilution) (left-hand panel) to detect the presence of $G_q\alpha/G_{11}\alpha$ or CS (1: 250 dilution) (right-hand panel) to detect the isoforms of $G_S\alpha$. The central lane (M) contains molecular weight standard markers (in kDa) which are described in chapter 2.1.1.

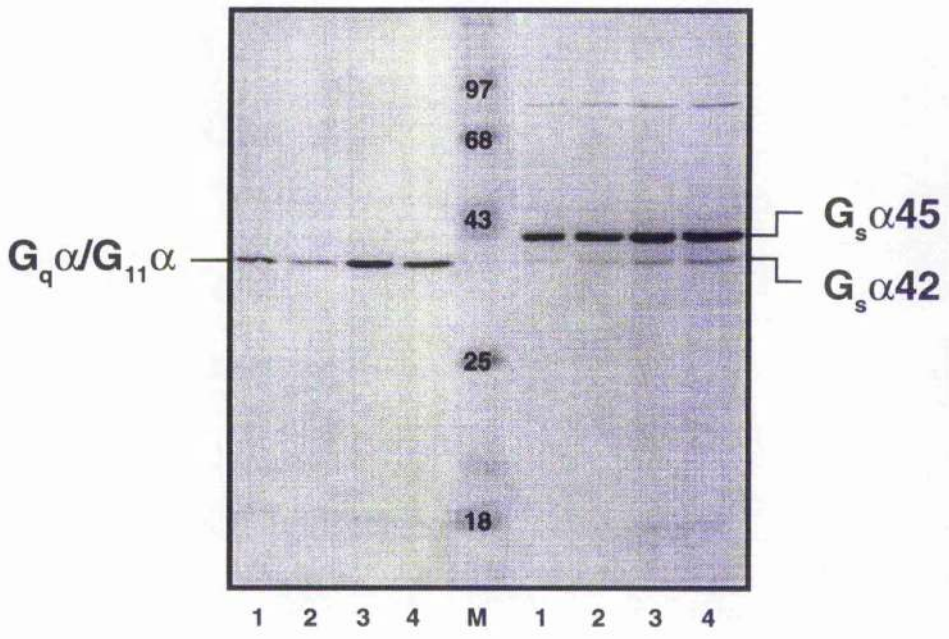
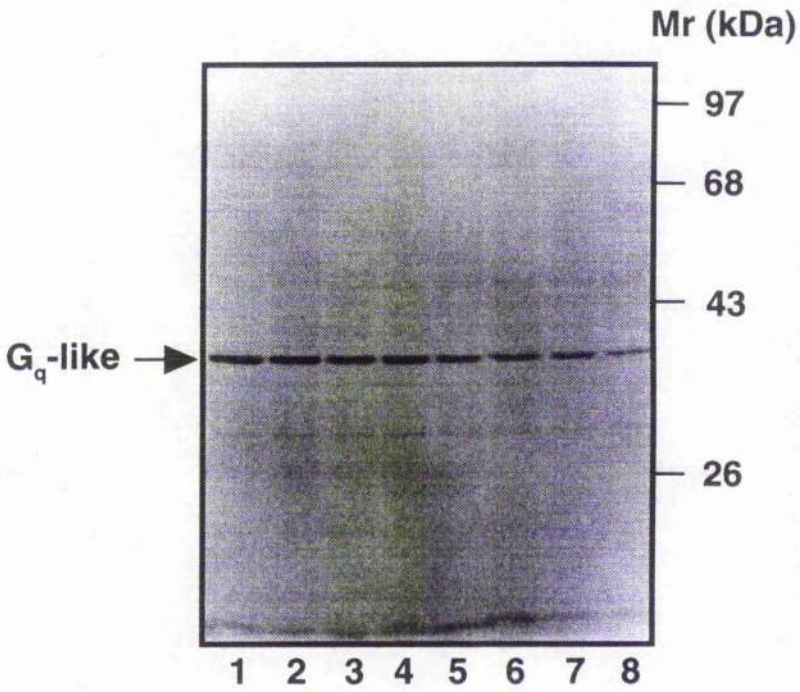


Figure 4.2.11.

TRH induced down-regulation of G_q-like immunoreactivity in E2 HEK-293 cells: Time course.

Membranes (30 μg) of E2 HEK-293 cells which were untreated (lane 1) or exposed to TRH (10 μM) for (lane 2, 2h; lane 3, 4h; lane 4, 6h; lane 5, 8h; lane 6, 10h; lane 7, 12h; lane 8, 16h) were resolved by SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) and immunoblotted with antiserum CQ (1: 1000 dilution) in panel A. This experiment is representative of two others performed with different membrane preparations which gave essentially identical results. Densitometric scanning was used to assess the degree of reduction following exposure to TRH and demonstrated half-maximal reduction at 4 h with a maximal 70% reduction (panel B). Data are displayed as mean ± S.E. (n = 3).

A


Mr (kDa)
97
68
43
26
Gq-like
1 2 3 4 5 6 7 8

B

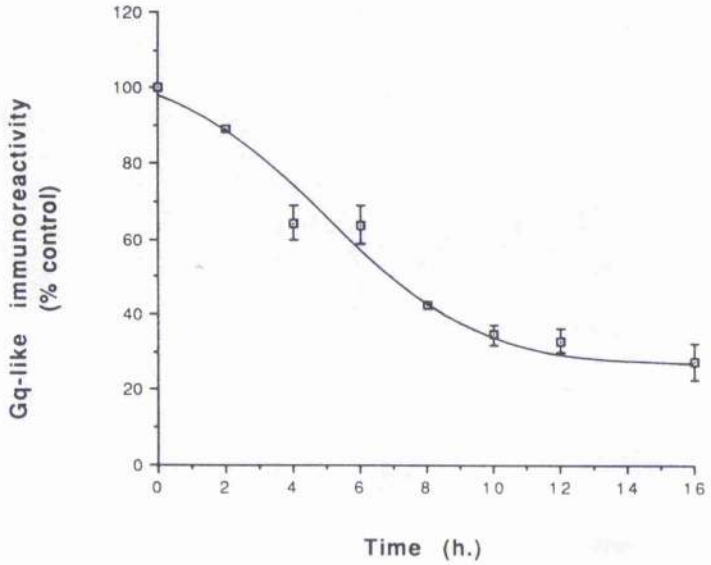

Gq-like immunoreactivity (% control)
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Time (h.)

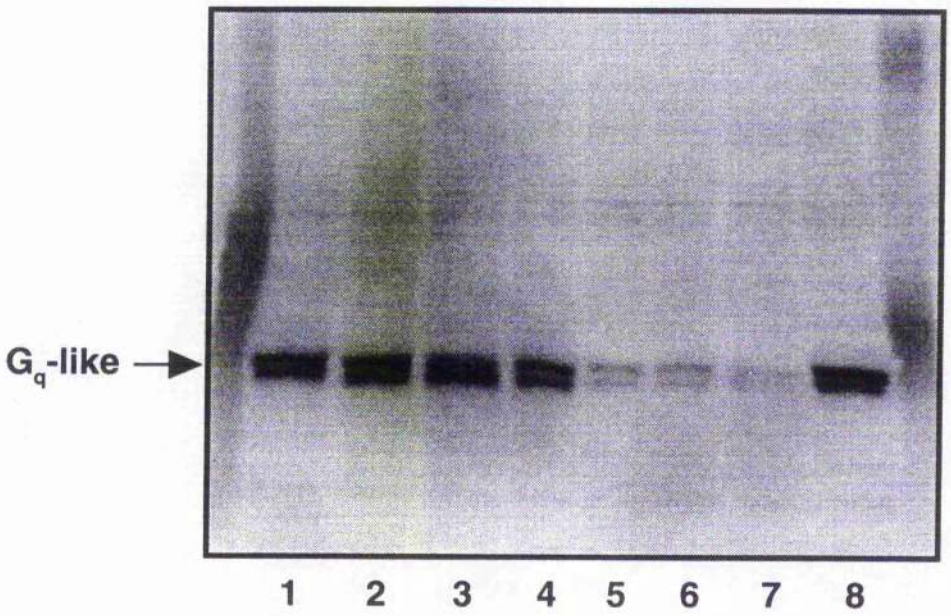
Figure 4.2.12.

TRH down-regulates equally each of the G_q-like G-proteins expressed by E2 HEK-293 cells.

Membranes (60 μg) of E2 HEK-293 cells which were untreated (lanes 1 and 8) or treated for 16h with increasing concentrations of TRH (2) 0.1 nM , (3) 1 nM, (4) 10 nM, (5) 100 nM, (6) 1μM, (7) 10 μM were resolved in the 4-8 M urea gradient gel system described in chapter 2.2.9 and immunoblotted with antiserum CQ (1: 1000 dilution). Both CQ immunoreactive polypeptides were down-regulated equivalently by treatment of the cells with TRH (panel A).

Panel B represents quantitative analysis of the down-regulation of G_q-like G-proteins by TRH treatment. Immunoblots similar to those of panel A were densitometrically scanned and represented graphically. Data for the more rapidly migrating (open symbols) and the less rapidly migrating CQ immunoreactive polypeptides (filled symbols) are plotted as a percent of signal observed without TRH treatment and represent mean ± S.E. (n = 3).

A


G_q-like
1 2 3 4 5 6 7 8

B

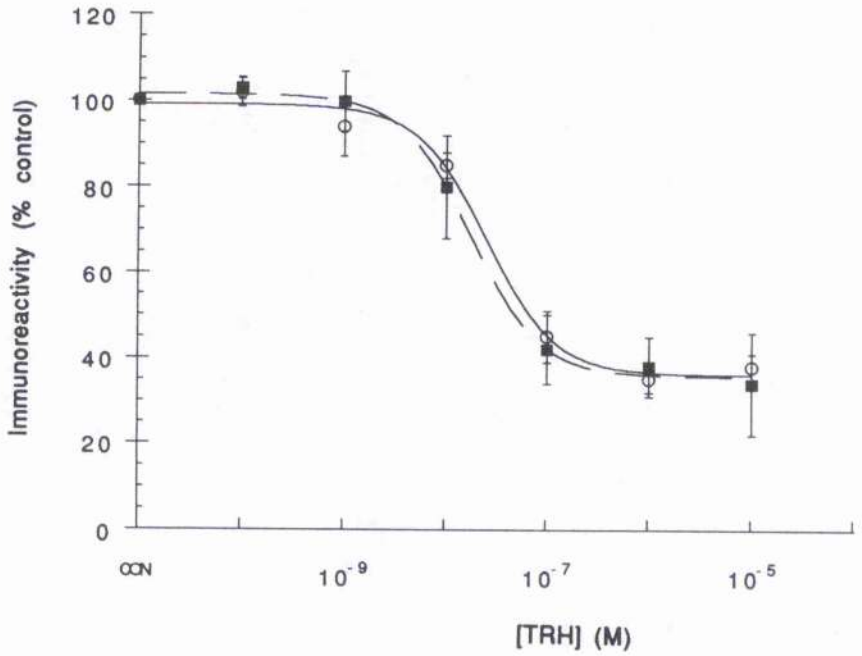

Immunoreactivity (% control)
120
100
80
60
40
20
0
CON
10^{-9}
10^{-7}
10^{-5}
[TRH] (M)

Figure 4.2.13.

Equivalent down-regulation of both $G_q\alpha$ and $G_{11}\alpha$ by TRH treatment of GH_3 cells.

Membranes (lanes 1 and 2, 50 μg; lanes 3 and 4, 75 μg) of GH_3 cells which were either untreated (lanes 1 and 3) or pretreated with TRH (10 μM, 16h) (lanes 2 and 4) were resolved in the 4-8 M urea gradient gel system described in chapter 2.2.9. The left-hand lane demonstrates the migration of prestained lactic dehydrogenase (LDH) in this gel system in which rodent $G_{11}\alpha$ migrates more rapidly than $G_q\alpha$. Identification of the CQ reactive polypeptides in the rodent cells as $G_q\alpha$ and $G_{11}\alpha$ based on their relative mobility in this urea gel system has been described previously (Milligan *et al.*, 1993b) and is described in chapter 4.3. TRH caused equivalent down-regulation of both $G_q\alpha$ (27.9 ± 7.4 %) and $G_{11}\alpha$ (31.2 ± 6.6 %) (mean ± S.E., n = 3).

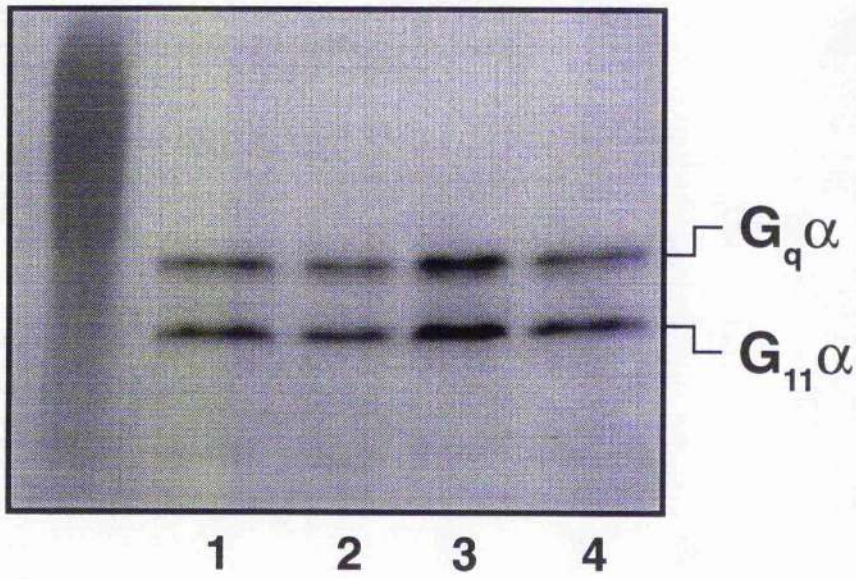

$G_q\alpha$
$G_{11}\alpha$
1
2
3
4

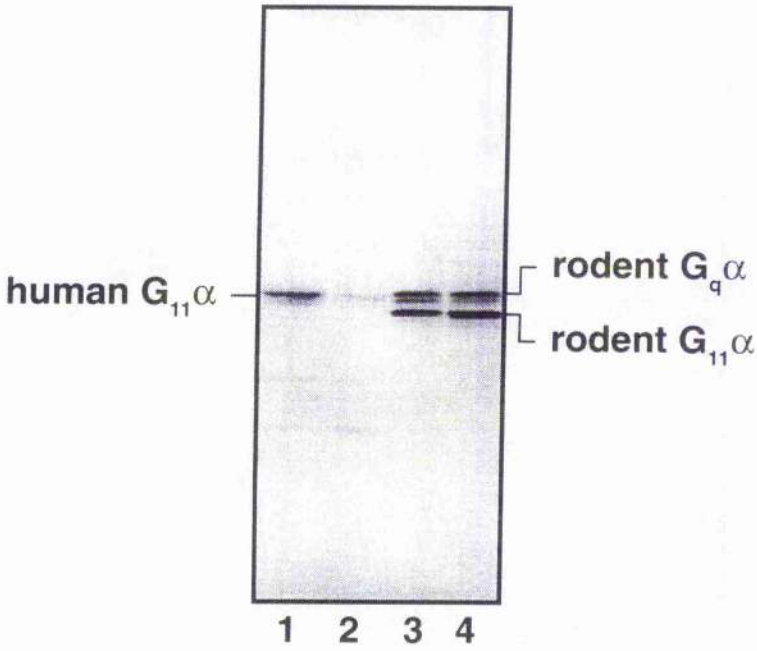
Figure 4.2.14.

Profile of expression of the G_q-like G-proteins by E2 HEK-293 and GH3 cells.

In panel A, membranes (60 μg) from (lane 1) untreated, (lane 2) TRH treated (10 μM, 16h) E2 HEK-293 cells, (lane 3) GH3 cells and (lane 4) neuroblastoma x glioma hybrid NG108-15 cells were resolved in the 6 M urea gel system described in chapter 2.2.10 and immunoblotted with antiserum CQ (1: 1000 dilution) which identifies forms of both $G_q\alpha$ and $G_{11}\alpha$.

Untreated E2 HEK-293 cells were resolved as described for panel A and immunoblotted with either the $G_q\alpha/G_{11}\alpha$ common antiserum CQ (1: 1000 dilution) (lane 1) or the $G_{11}\alpha$ specific antiserum E976 (1: 1000 dilution) (lane 2) (panel B).

A


human $G_{11}\alpha$
rodent $G_q\alpha$
rodent $G_{11}\alpha$
1 2 3 4

B

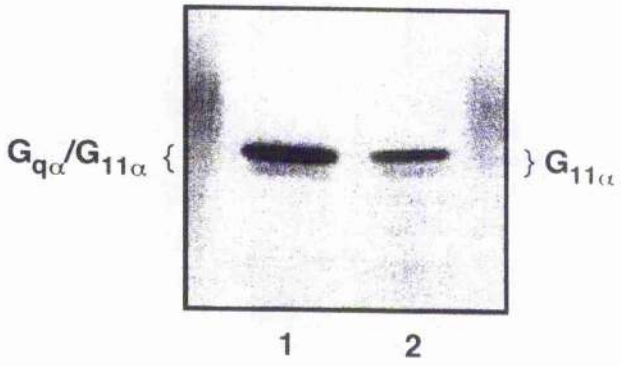

$G_{q\alpha}/G_{11\alpha}$ {
} $G_{11\alpha}$
1 2

Table 4.2.1.

TRH stimulation of inositol phosphate generation is not modified by pertussis toxin pretreatment of E2 HEK-293 cells.

The ability of TRH to stimulate inositol phosphate generation is unaffected by pre-treatment of the cells with pertussis toxin (25 ng/ml for 16h), conditions able to cause ADP-ribosylation of essentially the entire population of pertussis toxin sensitive 'G_i-like' proteins ($G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$ and $G_o\alpha$) in clone E2 HEK-293 cells. Data are presented as mean ± S.D. from triplicate samples derived from a single experiment. A similar pattern of fold stimulation of inositol phosphate levels above basal was recorded in a second independent experiment using a separate passage of the cells.

Condition	Inositol phosphate generation
	Inositol phosphate (DPM) / 10 k DPM in lipid
Basal	48.8 ± 4.4
TRH (10 μM)	934.2 ± 113.9
Pertussis toxin pre-treated + TRH (10 μM)	864.7 ± 129.6

Table 4.2.2.

The long isoform of the rat TRH receptor is unable to regulate adenylyl cyclase activity when expressed in HEK-293 or in a clone of GH_3 cells.

Adenylyl cyclase activity was measured as described in chapter 2.2.17 in membranes (10 μg) from clone E2 HEK-293 cells and GH_3 cells either basally or in the presence of TRH (10 μM), forskolin (100 μM) or a combination of these two agents. Results are presented as mean ± S.E.M. from triplicate samples derived from a single experiment on each cell line. A similar pattern was recorded in a second independent experiment using membranes derived from separate passages of the cells.

Condition	**Adenylyl cyclase activity**	
	E2 HEK-293	**GH_3**
	(pmol/min/mg membrane protein)	
Basal	82 ± 2	79.5 ± 10.9
Forskolin (100 μM)	1052 ± 11	1135.7 ± 21.6
TRH (10 μM)	73 ± 1	80.1 ± 6.6
Forskolin (100 μM) + TRH (10μM)	1166 ± 2	1053.6 ± 14.2

Table 4.2.3.

Levels of expression of $G_s\alpha$ and the G_q-like G-proteins in GH_3 and E2 HEK-293 cells.

Data were derived by quantitative immunoblotting using cellular membranes and *E. coli* expressed recombinant G-protein α subunits as described in chapter 2.2.14. In the case of E2 HEK-293 cells data are presented as mean ± S.E.M., n = 6. In the case of GH_3 cells, n = 3.

G-proteins	GH_3	E2 HEK-293
	(pmol/mg membrane protein)	
$G_s\alpha$ (total)	101.8 ± 4.3	21.6 ± 1.7
$G_s\alpha$ (long)	97.8 ± 4.4	10.3 ± 1.3
$G_s\alpha$ (short)	4.0 ± 0.1	11.3 ± 1.1
$G_q\alpha$ like ($G_q\alpha$+$G_{11}\alpha$)	14.4 ± 0.5	12.2 ± 1.2

Table 4.2.4.

Regulation of G-protein α subunits in E2 HEK-293 cells by sustained TRH treatment.

Quantitative immunoblotting using *E. coli* recombinantly expressed standard G-proteins was used to measure absolute levels of $G_s\alpha$ and $G_q\alpha/G_{11}\alpha$ as described in Figures 4.2.6 and 4.2.7. In the absence of such standards, only relative intensity immunoblotting could be performed for the G_i-like G-proteins. Data for $G_s\alpha$ and $G_q\alpha/G_{11}\alpha$ are presented as mean ± S.E.M., n = 6 and for the other G-proteins as mean ± range, n = 2.

G-proteins	TRH treatment (10 μM, 16h)	
	(-)	(+)
	(pmol/mg membrane protein)	
$G_s\alpha$	21.6 ± 1.7	24.6 ± 2.6
$G_q\alpha/G_{11}\alpha$	12.2 ± 1.2	5.7 ± 0.8
	G-protein levels following TRH treatment	
	(% of untreated)	
$G_{i1}\alpha$	105 ± 3	
$G_{i2}\alpha$	97 ± 8	
$G_{i3}\alpha$	110 ± 3	
$G_o\alpha$	88 ± 4	

4.2.2. Discussion.

TRH has been reported to cause activation of both phosphoinositidase C (Imai *et al.*, 1987) and adenylyl cyclase activities (Paulssen *et al.*, 1992) and to regulate Ca^{2+} channel function (Gollasch *et al.*, 1993) in pituitary GH_3 cells. These effects have been shown directly to be produced by activation of, respectively, a G_q-like G-protein(s) (Hsieh *et al.*, 1992; Aragay *et al.*, 1992), by $G_s\alpha$ (Paulssen *et al.*, 1992) and by $G_{i2}\alpha$ (Gollasch *et al.*, 1993). Recent data on splice variants of both the EP3 receptor (Namba *et al.*, 1993) and the pituitary adenylyl cyclase activating polypeptide receptor (Spengler *et al.*, 1993) have provided strong evidence for the regulation of different G-proteins and signalling cascades by individual receptor splice variants. Therefore, as GH_3 cells have been shown to express at least two distinct isoforms of the TRH receptor which arise by differential splicing mechanisms (de la Pena *et al.*, 1992b) and there are currently no pharmacological agonists which are likely to be able to selectively activate the individual receptor isoforms it is difficult to assess in the cells which co-express these two distinct variants of the TRH receptor if each variant is able to activate all of these signalling cascades and hence act as a multifunctional receptor (Milligan *et al.*, 1993b) or whether the individual isoforms have the ability to differentially regulate signalling cascades by having differing abilities to activate different G-proteins.

In order to address some of these questions, HEK-293 cells which do not endogenously express a TRH receptor were stably transfected with the long isoform of the rat TRH receptor and the ability of this receptor to regulate both phosphoinositidase C and adenylyl cyclase activity and to interact with and regulate levels of the G-protein population of these cells was subsequently assessed.

Clone E2 HEK-293 which expresses high levels of the TRH receptor was selected on the assumption that the levels of expression of the splice variant form of the receptor and of the G_q-like G-proteins and $G_s\alpha$ are likely to determine the exact nature of the signal generated in response to TRH in receptor-mediated activation of cell signalling cascades. Furthermore, reverse transcriptase-PCR analysis confirmed

that transfection with cDNA encoding the long isoform of the TRH receptor resulted in the production of mRNA encoding the long isoform of the receptor only with no detectable contribution from the short isoform (Figure 4.2.2).

Addition of TRH to these cells produced a large activation of inositol phosphate generation but was unable to regulate adenylyl cyclase activity in membranes from these E2 HEK-293 cells (Table 4.2.2). It was also unable to produce regulation of adenylyl cyclase activity in the clone of GH_3 cells used in these studies (Table 4.2.2). However I also failed to note any obvious expression of the short TRH receptor isoform in this clone (Figure 4.2.2) although this variant of the receptor was isolated from a GH_3 cell library (de la Pena *et al.*, 1992b). It seems reasonable to assume that this reflects clonal variation between GH_3 cell lines.

Dose response curves for TRH stimulation of inositol phosphate generation indicated that TRH was substantially less potent in clone E2 HEK-293 cells than in GH_3 cells (EC_{50} = 20 ± 10 nM in clone E2 cells vs. 3.0 ± 1.2 nM in GH_3 cells) (Figure 4.2.4A). This was somewhat surprising as although receptor reserve is not normally a phenomenon associated with receptor regulation of phosphoinositidase C activation the high level expression of the long isoform of the TRH receptor in clone E2 (B_{max} = 17.6 ± 0.8 pmol/mg membrane protein) was not anticipated to result in an alteration in the concentration-effect curve to the right in comparison to GH_3 (B_{max} = 4.4 ± 0.3 pmol/mg membrane protein) cells (Scatchard plot not shown). This may reflect a number of features including receptor availability and the differences in activity of rodent vs. human G_q-like G-proteins. However, the total population of CQ immunoreactive G-protein (and as such the G_q-like G-proteins) was not substantially different (per mg membrane protein) in the two cell lines (clone E2 = 12.2 ± 1.2 pmol/mg membrane protein, GH_3 cells = 14.4 ± 0.5 pmol/mg membrane protein). It may also reflect the expression of only the long isoform of the TRH receptor in clone E2 cells and in the clone of GH_3 cells used in these studies as opposed to the co-expression of the long and short isoforms of the receptor in GH_3 cells reported by others (de la Pena *et al.*, 1992b).

The inability of the long isoform of the rat TRH receptor to stimulate adenylyl cyclase activity in membranes of cells of clone E2 indicates a lack of significant interaction with $G_s\alpha$. Again, as noted above, this may reflect the expression of only the long isoform of the receptor in clone E2 cells but may also reflect the absolute and relative levels of expression of $G_s\alpha$ and the G_q-like G-proteins in clone E2 vs. GH_3 cells. Clone E2 cells express somewhat lower total levels of G_q-like G-proteins ($G_q\alpha$ = 12.2 ± 1.2 pmol/mg membrane protein) than of $G_s\alpha$ (total (45 kDa + 42 kDa) $G_s\alpha$ = 21.6 ± 1.7 pmol/mg membrane protein) (Table 4.2.3) whereas both the relative levels of expression of $G_s\alpha$ vs. the G_q-like G-proteins (some 8: 1) in GH_3 cells and the absolute levels of expression of $G_s\alpha$ in GH_3 vs. clone E2 cells (101 ± 4.3 pmol/mg membrane protein vs. 21.6 ± 1.7 pmol/mg membrane protein) are considerably higher in GH_3 cells than in E2 HEK-293 cells. If the long isoform of the rat TRH receptor indeed has some affinity for interaction with $G_s\alpha$ then both the absolute and relative levels of G_s vs. G_q-like G-proteins in GH_3 cells vs. E2 HEK cells would favour interaction with $G_s\alpha$ and hence stimulation of adenylyl cyclase in GH_3 rather than E2 HEK-293 cells. It should be noted in this context that even in GH_3 cells the degree of adenylyl cyclase stimulation by TRH is relatively weak.

In 10% acrylamide SDS-PAGE, $G_q\alpha$ and $G_{11}\alpha$, which appear to be co-expressed in the majority of cells and tissues (Simon *et al.*, 1991; Amatruda *et al.*, 1991), co-migrate and thus immunological detection with an antiserum directed to the C-terminal decapeptide, which is completely conserved between these two proteins, can only provide a composite signal for these two polypeptides (Figure 4.2.12A). Immunoblotting E2 HEK-293 membranes resolved in urea-containing SDS-polyacrylamide gels (also described in chapter 4.3) with the G_q-like G-protein antiserum CQ was able to demonstrate the presence of two polypeptides migrating close together which represent the human versions of $G_{11}\alpha$ and $G_q\alpha$. In addition, an antiserum E976 (Taylor *et al.*, 1991a) specific for $G_{11}\alpha$ clearly demonstrated the expression of human $G_{11}\alpha$ in these cells but that the human version of this polypeptide migrated in a different position to rodent $G_{11}\alpha$, close to the position

observed for rodent $G_q\alpha$ (Figure 4.2.14B). It has previously been demonstrated that treatment of CHO cells transfected to express the human muscarinic M1 acetylcholine receptor with varying amounts of the cholinergic agonist carbachol results in an equivalent degree of down-regulation of $G_q\alpha$ and $G_{11}\alpha$ at each concentration of the agonist and this has been interpreted to indicated that the M1 muscarinic receptor does not functionally select between these two G-proteins (Mullaney *et al.*, 1993a). In these studies, TRH acting on the long isoform of the TRH receptor to down-regulate each of the G_q-like polypeptides expressed in E2 HEK-293 cells and caused the down-regulation of both of these G_q-like polypeptides with identical concentration-effect curves (Figure 4.2.12B). TRH-mediated down-regulation of G_q-like G-proteins was also observed in our clone of GH_3 cells (Figure 4.2.13) and this agonist-induced down-regulation was restricted to $G_q\alpha$ and $G_{11}\alpha$. As agonist-induced down-regulation of G-proteins appears to be restricted to the G-protein(s) which are activated by a receptor (Milligan *et al.*, 1993b), these observations, in parallel with the lack of stimulation or inhibition of adenylyl cyclase by TRH in both E2 HEK-293 and our clone of GH_3 cells indicate that the G_q family of G-proteins are the only ones which are activated to a significant effect by the long isoform of the TRH receptor.

The results presented in these studies demonstrate a highly selective coupling of the long isoform of the rat TRH receptor with the G-proteins responsible for phosphoinositidase C regulation. Reported regulation of both adenylyl cyclase by activation of $G_s\alpha$ (Paulssen *et al.*, 1992) and of Ca^{2+} channel function by activation by $G_{i2}\alpha$ (Gollasch *et al.*, 1993) in GH_3 cells may thus be a reflection of the activity of other splice variants of the TRH receptor but if so this would suggest that different clones of GH_3 cells would have to show different patterns of relative expression of TRH receptor isoforms.

4.3. Stimulation of the long splice variant of the rat TRH receptor induces subcellular redistribution and differential down-regulation of co-expressed $G_{11}\alpha$ species variants.

4.3.1. Results.

Resolution of membranes from frontal cortex of human, baboon, pig, guinea pig, rat and mouse in SDS-PAGE (10% (w/v) acrylamide 0.25% (w/v) bisacrylamide) followed by transfer to nitrocellulose and immunoblotting using antiserum CQ as primary reagent led to the detection of an apparently single polypeptide of 42 kDa in all species (Figure 4.3.1A). Immunoblotting equivalent gels using antiserum IQB which was generated against a synthetic peptide corresponding to amino acids 119-134 of murine $G_q\alpha$, as described in chapter 2.2.6, as primary reagent produced similar results (Figure 4.3.1B).

When membranes from frontal cortex from the various species were resolved in SDS-PAGE (10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide) which additionally incorporated 6 M urea a much more complex pattern of CQ immunoreactivity was recorded (Figure 4.3.2). Rat frontal cortex demonstrated the immunological detection of three prominent CQ reactive polypeptides and the presence of a fourth polypeptide which was detected more weakly by the antibody. Previous studies have shown that the most rapidly migrating CQ reactive polypeptide in such urea containing gels is $G_{11}\alpha$ based on comigration in a range of gel systems with purified $G_{11}\alpha$ from bovine liver (Mullaney *et al.*, 1993a). This was confirmed by immunoblotting membranes separated in the urea containing gels with the $G_{11}\alpha$ specific antiserum E976 (Taylor *et al.*, 1991a) (Figure 4.3.3). The staining intensity of the polypeptides migrating in the position equivalent to $G_q\alpha$ was considerable greater than that for $G_{11}\alpha$ in rat frontal cortex (Figure 4.3.2) in confirmation of previous results using SDS-PAGE system in which the relative mobilities ($G_q\alpha < G_{11}\alpha$) are similar to those reported herein (Milligan *et al.*, 1993a). A weakly staining CQ reactive polypeptide migrated between $G_q\alpha$ and $G_{11}\alpha$. The pattern of immunostaining of CQ

reactive polypeptides was similar in mouse frontal cortex except that the relative levels of expression of $G_q\alpha$ to $G_{11}\alpha$ was close to 1: 1. Pig had a similar general pattern of expression of CQ reactive polypeptides to mouse and rat but very different patterns of expression of CQ reactive polypeptides were noted in the frontal cortex of the other species examined. Guinea pig exhibited very weak expression of a polypeptide migrating in the position of rat and mouse $G_{11}\alpha$ but similar levels and patterns of the two polypeptides migrating with rat and mouse $G_q\alpha$. The most striking feature in guinea pig however, was high level immunostaining of the polypeptide migrating between $G_q\alpha$ and $G_{11}\alpha$ (Figure 4.3.2). This polypeptide was shown to be the predominant guinea pig form of $G_{11}\alpha$ as this polypeptide was identified by antiserum E976 (Figure 4.3.3). Membranes from frontal cortex of baboon and human were again different with no detectable immunostaining in a position corresponding to rat and mouse $G_{11}\alpha$ (Figure 4.3.2). Immunoblotting of human and baboon membranes with antiserum E976 demonstrated that the predominant form of $G_{11}\alpha$ in these species migrated more slowly than in rat and mouse in a position very close to $G_q\alpha$ (Figure 4.3.3).

In order to examine further the differences in mobility of immunodetectable $G_{11}\alpha$ in primate frontal cortex compared to rodent species membranes from a range of both human and rodent neuroblastoma cell lines were resolved in the urea containing SDS-PAGE system (Figure 4.3.4). Both mouse neuroblastoma x rat glioma hybrid NG108-15 cells as demonstrated previously (Milligan *et al.*, 1993d) and mouse neuroblastoma x hamster embryonic brain cell NCB20 cells demonstrated the expression of a CQ immunoreactive polypeptide in the position anticipated for rodent $G_{11}\alpha$ and which was confirmed to be $G_{11}\alpha$ by immunoblotting with antiserum E976 (Figure 4.3.4B). However, in membranes from the human neuroblastoma cell line, IMR-32 no polypeptide comigrating with rodent $G_{11}\alpha$ was detected by antiserum CQ (Figure 4.3.4A, lane 1) while antiserum E976 demonstrated the expression of a $G_{11}\alpha$ in these cells which migrated in a position close to $G_q\alpha$ (Figure 4.3.4B, lane 1).

To assess if both primate and rodent $G_{11}\alpha$ could be detected unambiguously and concurrently in membranes of a single cell by a difference in mobility of primate and rodent $G_{11}\alpha$, a murine $G_{11}\alpha$ cDNA subcloned into plasmid pCMV was transfected transiently into monkey kidney COS-1 cells. 72 h after transfection the cells were harvested and membranes prepared from mock-transfected and murine $G_{11}\alpha$ transfected cells. These membranes were separated in the resolving 6 M urea containing SDS-PAGE (10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide) and immunoblotted with either antiserum CQ or E976 (Figure 4.3.5). Using either antiserum an immunoreactive polypeptide was detected in the position anticipated for rodent $G_{11}\alpha$ in membranes of the cells transfected with the murine $G_{11}\alpha$ cDNA, but not in the mock transfected cells, which was well resolved from either the combination of endogenously expressed monkey $G_q\alpha$ and $G_{11}\alpha$ (when immunoblotting was performed with antiserum CQ) (Figure 4.3.5A) or from monkey $G_{11}\alpha$ (when the immunoblots were performed with antiserum E976) (Figure 4.3.5B).

The generation and analysis of clone E2 HEK-293 cells, which expresses high levels of the long splice variant of the rat TRH receptor, and the interaction of this receptor with the cell signalling machinery to cause stimulation of phosphoinositidase C activity has previously been described in detail (chapter 4.2). On the basis of concurrent immunological detection of both primate and rodent forms of $G_{11}\alpha$ following their co-expression in a single cell (Figure 4.3.5) E2 HEK-293 cells were further co-transfected with the plasmids pCMV (10 µg) encoding murine $G_{11}\alpha$ cDNA (Strathmann *et al.*, 1990) and the plasmid pBABE hygro (1 µg), which allows expression of resistance to the antibiotic hygromycin B. Clones were selected on the basis of resistance to hygromycin B (200 µg/ml) and the continued expression of the TRH receptor and novel expression of murine $G_{11}\alpha$ were examined (Figure 4.3.7). Membranes derived from a number of individual clones were examined for their ability to bind [^{3}H]TRH specifically. A clone designated E2M11 was selected and expanded for detailed analysis. Clone E2M11 bound [^{3}H]TRH with both high capacity and high affinity (Figure 4.3.8) and with values for both parameters similar to those reported

previously for clone E2. Furthermore, following labelling of these cells with myo-[^{3}H]inositol a large stimulation of accumulation of [^{3}H]inosotol phosphates was produced by addition of TRH with similar EC_{50} as noted in clone E2 HEK-293 cells (data not shown).

Resolution of membranes from both clone E2 and clone E2M11 cells by SDS-PAGE in a gel containing 6 M urea as previously mentioned followed by transfer to nitrocellulose and immunoblotting with an antiserum (E976) (Taylor *et al.*, 1991a) specific for $G_{11}\alpha$ demonstrated endogenous expression of human $G_{11}\alpha$ in both clones and the expression of murine $G_{11}\alpha$ in clone E2M11 (Figure 4.3.9). As previously demonstrated rodent (murine) forms of $G_{11}\alpha$ migrate more rapidly through such gels than primate (human) versions of this polypeptide (Figure 4.3.5). A similar conclusion was reached following immunoblotting membrane samples from these cells with the $G_{11}\alpha/G_q\alpha$ common antiserum CQ (Figure 4.3.9).

Sustained incubation of clone E2 cells with TRH (10 μM, 16h) resulted in a large decrease in the level of human $G_{11}\alpha$ in a crude membrane fraction (Figure 4.3.10A) as previously described in chapter 4.2. Equivalent incubation of clone E2M11 cells with TRH (10 μM, 16h) resulted in a reduction in membrane levels of both the human and murine isoforms of $G_{11}\alpha$ (Figure 4.3.10). Surprisingly, the degree of reduction of murine $G_{11}\alpha$ caused by TRH treatment was much less than of the human $G_{11}\alpha$ subunit. The human isoform was diminished to very low levels (15%) when compared with controls while the murine $G_{11}\alpha$ was decreased to only 65% when compared with untreated cells.

To get information about the total cellular content and distribution of $G_{11}\alpha$ proteins in E2M11 cells, homogenates prepared from untreated and TRH-treated (10 μM, 16h) cells were centrifuged for 2 h at 200, 000 x *gav* and crude membrane (MB) and cytosol (CYT) fractions prepared and examined (Figure 4.3.10B). Figure 4.3.10A shows the decrease of both human and murine $G_{11}\alpha$ in the crude membrane fractions was clear. However, a substantial amount of immunodetectable murine $G_{11}\alpha$ and a clearly detectable amount of human $G_{11}\alpha$ was also noted in the cytosol

fractions of both control and TRH-treated cells and this was greater in cells which had been treated with TRH. The cytosolic content of $G_{11}\alpha$ of control samples was calculated to represent 7.5 ± 6.4 % (mean ± S.D., n = 4) of the total cellular pool of a combination of human and murine $G_{11}\alpha$. By contrast, cytosol from the TRH-treated cells contained 28.8 ± 6.1 % (mean ± S.D., n = 4) of the remaining total cellular content of these proteins (Table 4.3.1). However, the net increase in cytosol located $G_{11}\alpha$ proteins in E2M11cells i.e. from 24 to 145 arbitrary units in the example displayed in Table 4.3.1 (expressed per the same number of cells) was much less than the amount of $G_{11}\alpha$ removed from the total cellular membrane fraction by treatment with TRH, i.e. from 1153 to 476 arbitrary units as shown in Table 4.3.1. As such, the amount of $G_{11}\alpha$ no longer associated with the total membrane fraction following TRH treatment of E2M11 cells could not be quantitatively recovered in the cytosolic fraction. Calculation of the total cellular amount of $G_{11}\alpha$ proteins (membranes plus cytosol) indicated some 47% down-regulation in response to TRH when compared with controls (Table 4.3.1).

E2M11 cells were incubated with 10 μM TRH for increasing lengths of time between 10 min and 16 h to examine the time course of TRH induced redistribution of $G_{11}\alpha$ proteins. Murine and human variants of $G_{11}\alpha$ were resolved by 6 M urea containing SDS-PAGE as mentioned previously and the developed immunoblots were densitometrically scanned. As is demonstrated in Figure 4.3.10 the decrease of both $G_{11}\alpha$ species variants in the crude membrane fraction was accompanied by an increase in these polypeptides in the cytosol. The redistribution of $G_{11}\alpha$ from membrane to cytosol as well as down-regulation (decrease of total cellular amount) (Figure 4.3.11 and Table 4.3.2), was considerably more pronounced for the human variant than for the murine $G_{11}\alpha$. The cytosolic human $G_{11}\alpha$ of untreated E2M11 cells, which represented 4% of the total cellular pool increased to 82% of the total immunodetectable pool after 8 h of TRH (10 μM) treatment whereas the corresponding change of murine $G_{11}\alpha$ was from 14% in untreated to 28% in TRH-treated samples (Figure 4.3.12B and Table 4.3.2). The total cellular amount (sum of

membrane plus cytosol pools) of $G_{11}\alpha$ proteins was reduced by TRH to 23% and 68% for human and murine $G_{11}\alpha$ respectively compared to control (Table 4.3.2 and Figure 4.3.12B).

The TRH induced changes in subcellular localization of the co-expressed $G_{11}\alpha$ species variants were further analysed using sucrose density gradient centrifugation as described in chapter 2.2.3 to allow separation of plasma membranes from other membrane structures. Application of 19, 23, 27, 31, 35, and 43% (w/v) sucrose density gradients, which have been used previously for subcellular fractionation of S49 lymphoma cells (Svoboda *et al.*, 1992), CHO cell lines (Svoboda *et al.*, 1994) and for brown adipose tissue (Svoboda *et al.*, 1993), to homogenates of clone E2M11 cells, indicated that the cellular distribution profile of human and murine versions of $G_{11}\alpha$ were identical (Figure 4.3.13A) and demonstrated a high enrichment of the $G_{11}\alpha$ proteins in untreated E2M11 cells in fractions (centred on fractions 4 and 5) previously characterised to contain plasma membranes (Svoboda *et al.*, 1992) (Figure 4.3.13A). The specific content (density of the immunoblot signal divided by the total protein content) of $G_{11}\alpha$ proteins in fraction 4 was nearly 100 fold higher than in faction 1S (cytosol) and some 20 fold higher than in fraction 8 (nucleus plus cell debris), the fraction which continued the majority (some 85%) of the total cellular proteins (Figures 4.3.14 and 15).

TRH (10 μM, 16h) treatment of clone E2M11 cells (Figures 4.3.13B and 15) resulted in a dramatic shift of the $G_{11}\alpha$ polypeptides from high and medium-density (fraction 4-7) to lower-density regions of the gradient (fractions 1S, 1P, 2-3). This was achieved without an overall alteration in the protein profile of the gradient (Figure 4.3.14). The greatest effect of TRH treatment was observed in cytosol fraction 1S where the specific content of $G_{11}\alpha$ increased some 30 fold i.e. from 11.7 to 309.4 arbitrary units (defined as density of immunoblot signal divided by the amount of protein applied per lane) (Figure 4.3.15(I)) or from 1.2% to 29.2% of total cellular $G_{11}\alpha$ (Figure 4.3.15(II)). These values agree well with those obtained above using the simple crude membranes versus cytosol distribution studied by differential

centrifugation and represented an unparalleled finding (Figure 4.3.10 and Table 4.3.1).

A direct comparison of the redistribution of human and murine $G_{11}\alpha$ in key fraction of the sucrose density gradients is shown in Figure 4.3.16. The plasma membrane containing fractions (fractions 3, 4, 5) displayed a substantial reduction in immunodetectable levels of both human and murine $G_{11}\alpha$ in response to TRH with virtual complete removal of the human variants and somewhat lower reductions in levels of the murine form. By contrast, elevated levels of both species variants of $G_{11}\alpha$ were noted in the cytosol following treatment with TRH.

A potential explanation for the detailed quantitative differences in TRH receptor regulation of the human and murine variants of $G_{11}\alpha$ in these cells might have reflected a difference in the physical state of endogenously expressed human $G_{11}\alpha$ compared to the exogenously introduced murine $G_{11}\alpha$, perhaps representing a non-physiological aggregation of the introduced proteins. However, treatment of membranes of E2M11 cells with the detergent sodium cholate (1% (w/v), 1 h, 4 °C) resulted in equivalent and essentially compete solubilization of both human and murine versions of $G_{11}\alpha$ (Figure 4.3.17).

To examine potential changes in the distribution and levels of G protein β-subunits in response to TRH, parallel samples of gradient fractions analysed in Figure 4.3.13, were tested immunologically for their content of β-subunit (Figure 4.3.18). There was little if any detectable β-subunit in the cytosolic fraction 1S prior to treatment with TRH and this was unchanged by TRH-treatment. However, similarly to the results presented for the $G_{11}\alpha$ subunits a major, dramatic, plasma membrane to low density membrane redistribution occurred with TRH treatment (Figure 4.3.18). Resolution of crude membrane and cytosol fractions from control and TRH treated E2M11 cells also indicated a down-regulation of the total cellular amount of β-subunits in TRH-treated cells to 67% of control levels (Table 4.3.3). Although both α and β-subunits of G-proteins seem to follow similar intermembrane redistribution pathway from plasma membrane to low-density membranes fraction (light-vesicles,

endosomes, endoplasmic reticullum), movement to the cytosol is specific for α-subunits only.

Figure 4.3.1.

Frontal cortex membranes from various mammalian species all express a G_q-like G-protein(s).

Membranes (panel A, 30 μg; panel B, 35 μg) from frontal cortex of human (H), baboon (B), pig (P), guinea pig (G-P), rat (R) and mouse (M) were resolved by SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide) and following transfer to nitrocellulose immunoblotted using antiserum CQ (panel A), antiserum IQB (panel B). Both of the primary antipeptide antisera were used at dilution of 1: 1000. All samples displayed an apparently single polypeptide of some 42 kDa.

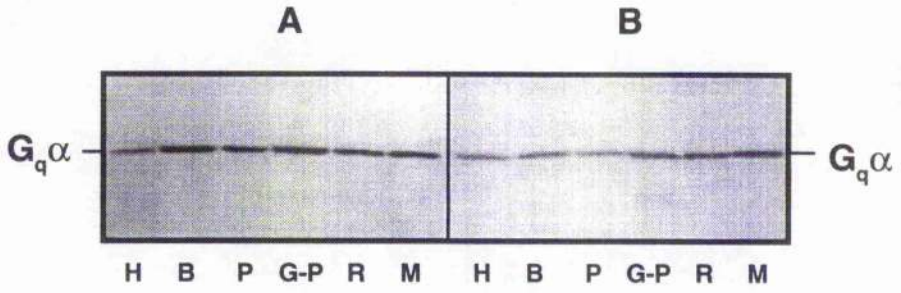


Figure 4.3.2.

Resolution of multiple G_q-like polypeptides in mammalian frontal cortex in SDS-PAGE containing 6 M urea.

Membranes (60 μg) from frontal cortex of human (H), baboon (B), pig (P), guinea pig (G-P), rat (R) and mouse (M) were resolved by SDS-PAGE (10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide containing 6 M urea) and following transfer to nitrocellulose immunoblotted using antiserum CQ (1: 1000 dilution).

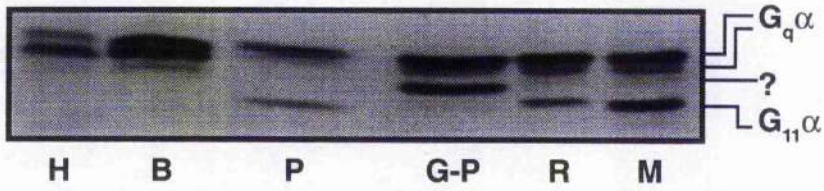

$G_q\alpha$
?
$G_{11}\alpha$
H
B
P
G-P
R
M

Figure 4.3.3.

Differences in mobility of immunodetectable $G_{11}\alpha$ between species.

Membranes (70 µg) from frontal cortex as used in Figure 4.3.2 were resolved in the 6 M urea containing gels detailed in chapter 2.2.10 and immunoblotted with the $G_{11}\alpha$-specific antiserum E976 (1: 500 dilution).

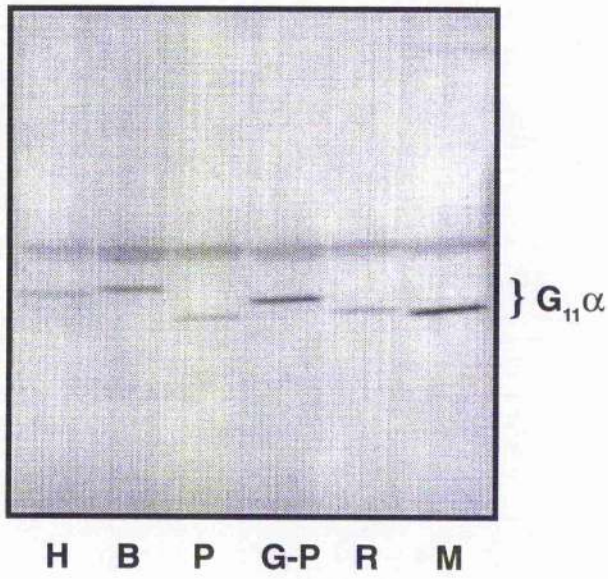

} $G_{11}\alpha$
H
B
P
G-P
R
M

Figure 4.3.4.

Differences in electrophoretic mobility between immunodetectable human and rodent $G_{11}\alpha$ in neuroblastoma cell lines.

Membranes (60 µg) from the human neuroblastoma cell line IMR 32 (lane 1) or the rodent neuroblastoma hybrids NCB20 (lane 2) or NG108-15 (lane 3) were resolved in the 6 M urea gel system described in chapter 2.2.10, and subsequently immunoblotted with antiserum either CQ (1: 1000 dilution) (panel A) or E976 (1: 500 dilution) (panel B).

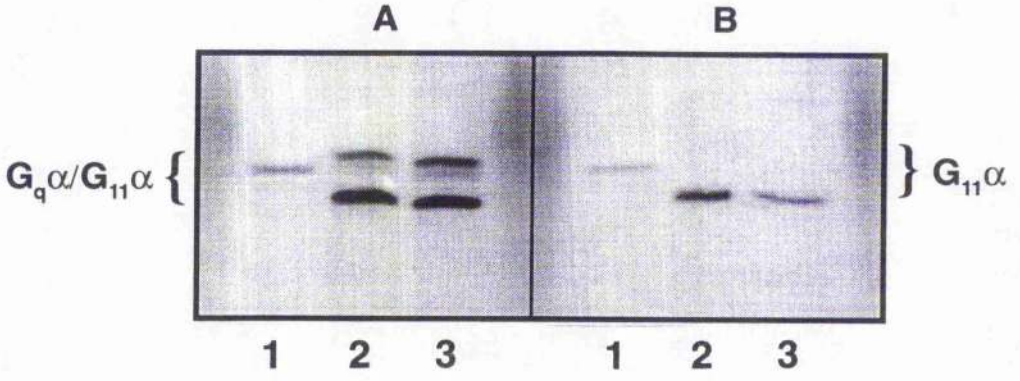

A
B
$G_q\alpha/G_{11}\alpha$
$G_{11}\alpha$
1 2 3 1 2 3

Figure 4.3.5.

Co-expression and simultaneous detection of monkey and mouse $G_{11}\alpha$ following transfection of COS-1 cells with a mouse $G_{11}\alpha$ cDNA.

Monkey COS-1 cells were either mock transfected (lane 1) or transfected with plasmid pCMV (100 μg) containing a full-length mouse cDNA (lane 2) using DOTAP reagent according to the manufacturer's instructions as described in chapter 2.2.22. 72h later cells were harvested and membranes prepared from these cells. Membranes (panel A, 55 μg; panel B, 100 μg) from transiently transfected monkey COS-1 cells were resolved in 6 M urea containing SDS-PAGE and immunoblotted with either antiserum CQ (1: 1000 dilution) (panel A) or antiserum E976 (1: 500 dilution) (panel B). Following transfection with murine $G_{11}\alpha$, a specific polypeptide which was substantially resolved from monkey $G_{11}\alpha$ and $G_q\alpha$ was detected by both antisera.

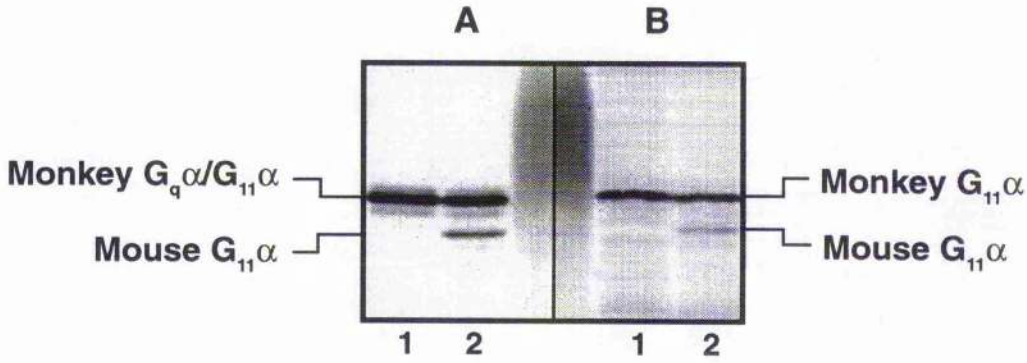

A
B
Monkey $G_q\alpha/G_{11}\alpha$
Mouse $G_{11}\alpha$
Monkey $G_{11}\alpha$
Mouse $G_{11}\alpha$
1
2
1
2

Figure 4.3.6.

A. Comparison of species variations in sequences of the G_q-like G-proteins in the regions used to generate antisera CQ, IQB and E976.

Antiserum CQ.

mouse $G_{q\alpha}$	350	QLNLKEYNLV	359
mouse $G_{11\alpha}$		QLNLKEYNLV	
human $G_{11\alpha}$		QLNLKEYNLV	
bovine $G_{11\alpha}$		QLNLKEYNLV	
mouse $G_{14\alpha}$		QLNLREFNLV	
bovine $G_{14\alpha}$		QLNLREFNLV	

Antiserum IQB.

mouse $G_{q\alpha}$	119	EKVSAFENPYVDAIKS	134
mouse $G_{11\alpha}$		EKVTTFEHQYVNAIKT	
human $G_{11\alpha}$		EKVTTFEHQYVSAIKT	
bovine $G_{11\alpha}$		EKVTTFEHRYVSAIKT	
mouse $G_{14\alpha}$		DKVTALSRDQVAAIKQ	
bovine $G_{14\alpha}$		DKVSTLSRDQVEAIKQ	

Antiserum E976.

mouse $G_{11\alpha}$	160	YLTDVDRIATVGY	172
human $G_{11\alpha}$		YLTDVDRIATLGY	
bovine $G_{11\alpha}$		YLTDVDRIATSGY	
mouse $G_{q\alpha}$		YLNDLDRVADPSY	
mouse $G_{14\alpha}$		YLTDIERIAMPSF	
bovine $G_{14\alpha}$		YLTDIDRIAMPAF	

Figure 4.3.6.

B. Cross-comparison of amino acid mismatches in the regions of the G_q-like G-proteins used to generate antisera CQ, IQB and E976.

CQ

	Mq	M11	H11	B11	M14	B14
Mq		0	0	0	2	2
M11	0		0	0	2	2
H11	0	0		0	2	2
B11	0	0	0		2	2
M14	2	2	2	2		0
B14	2	2	2	2	0	

IQB

	Mq	M11	H11	B11	M14	B14
Mq		6	6	6	9	9
M11	6		1	2	9	9
H11	6	1		1	9	9
B11	6	2	1		9	9
M14	9	9	9	9		3
B14	9	9	9	9	3	

E976

	M11	Mq	H11	B11	M14	B14
M11		6	1	1	6	5
Mq	6		6	6	6	6
H11	1	6		1	6	5
B11	1	6	1		6	5
M14	6	6	6	6		2
B14	5	6	5	5	2	

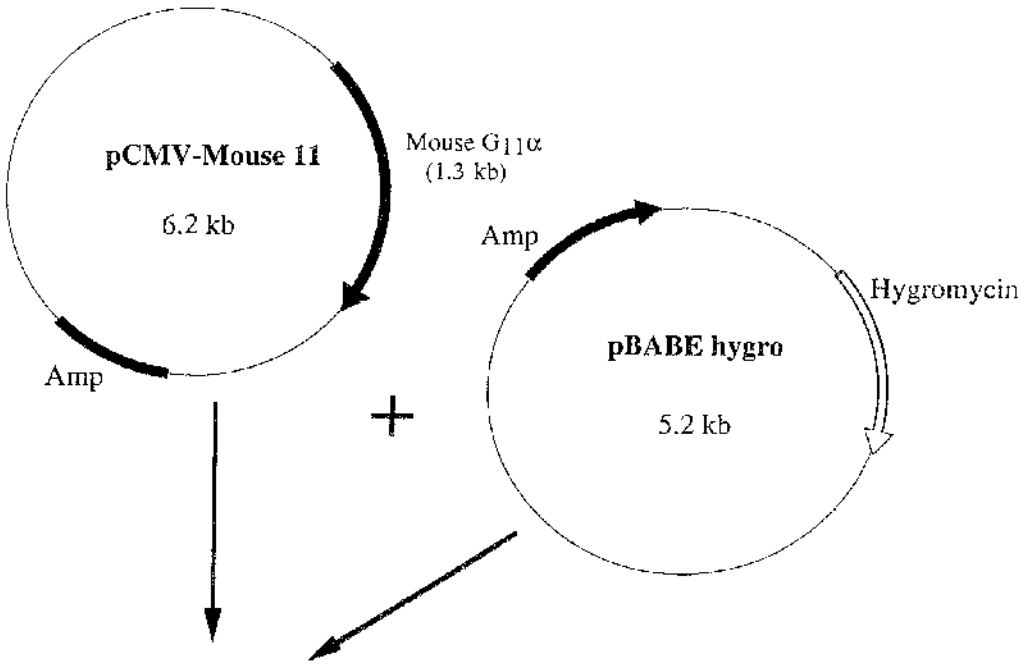
M = mouse, H = human, B = bovine, q = $G_q\alpha$, 11 = $G_{11}\alpha$ and 14 = $G_{14}\alpha$.

Antiserum CQ was raised against a C-terminal 10 amino acid peptide, IQB against a 16 amino acid peptide (119-134 of mouse $G_q\alpha$) and E976 against a 13 amino acid peptide (160-172 of mouse $G_{11}\alpha$).

Figure 4.3.7.

Generation and isolation of clones of E2M11 HEK-293 cells which express the long splice variant of the TRH receptor and both human and mouse forms of $G_{11}\alpha$.

Monolayer cultures of clone E2 HEK-293 cells described in chapter 4.2, were co-transfected with the eukaryotic expression vector pCMV into which a cDNA encoding mouse $G_{11}\alpha$, was inserted at the Cla I/Xho I sites, and the plasmid pBABEhygro, which allows expression of resistance to the antibiotic hygromycin B, in a 10: 1 ratio by using DOTAP reagent as suggested by the manufacturer. Clones were selected by resistance to hygromycin B (200 μg/ml) and the continued expression of the TRH receptor and novel expression of mouse $G_{11}\alpha$ were examined. Clone E2M11 was selected and expanded for detail analysis.



Clone E2 expressing the long isoform of the rat TRH receptor was co-transfected.

Transfectants were selected with geneticin sulphate (800 µg/ml) and hygromycin B (200 µg/ml).

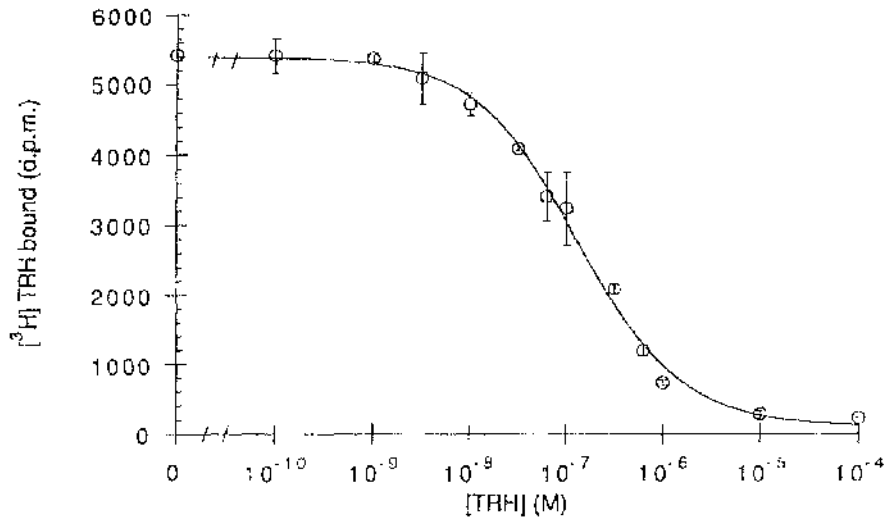
Clone E2M11 was selected, expanded and assayed for the expression of the TRH receptor and the mouse $G_{11}\alpha$.

Figure 4.3.8.

[^{3}H]TRH binds with high affinity and capacity to membranes of E2M11 cells.

Membranes (25 μg) from clone E2M11 cells were incubated with [^{3}H]TRH (10.3 nM) in the absence and presence of varying concentrations of TRH as described in Figure 4.2.3. Displacement of [^{3}H]TRH under these conditions by cold TRH was achieved with estimated IC_{50} of 98 nM when the Hill coefficient was restrained to 1.0. The data from this self-competition curve (Figure 4.3.8A) were subjected to the formalisms of DeBlasi *et al.*, 1989. The estimated B_{max} was 26 pmol/mg membrane protein and the K_d for [^{3}H]TRH at this receptor binding site, under these conditions, was 120 nM. The data was also converted to a Scatchard plot (Figure 4.3.8B), analysis of which produced binding parameters of B_{max} = 31 pmol/mg membrane protein and K_d for the binding of TRH to the low-affinity state of the long splice variant of the receptor was 140 nM. Two other identical experiments produced similar results in different membrane preparations.

A


6000
5000
4000
3000
2000
1000
0
[^{3}H] TRH bound (d.p.m.)
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[TRH] (M)

B

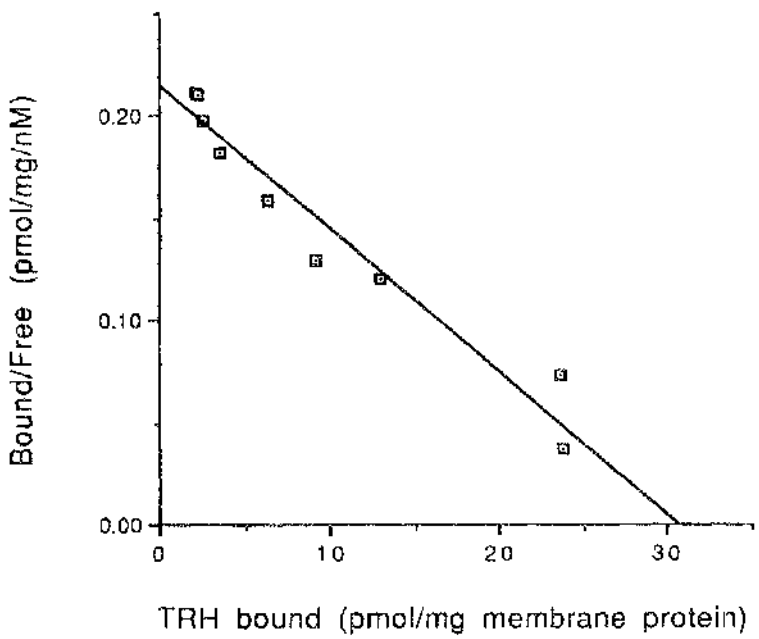

Bound/Free (pmol/mg/nM)
0.20
0.10
0.00
0
10
20
30
TRH bound (pmol/mg membrane protein)

Figure 4.3.9.

Membranes from clone E2M11 but not clone E2 cells co-express human and murine $G_{11}\alpha$.

Membranes (60 μg) derived from either clone E2 (lane 1) clone E2M11 (lane 2) were resolved in the urea-gradient SDS-PAGE system mentioned in chapter 2.2.10. Proteins were transferred to nitrocellulose and immunoblotted specifically for the presence of variants of $G_{11}\alpha$ (panel A) by use of the $G_{11}\alpha$ specific antiserum E976 (1: 500 dilution). E2 cells endogenously express human $G_{11}\alpha$ whereas E2M11 cells express a second form of $G_{11}\alpha$, the murine variant. Confirmation of the identity of the extra polypeptide expressed in E2M11 cells as murine $G_{11}\alpha$ was achieved by immunoblotting equivalent gels with antiserum CQ (1: 1000 dilution) (panel B).

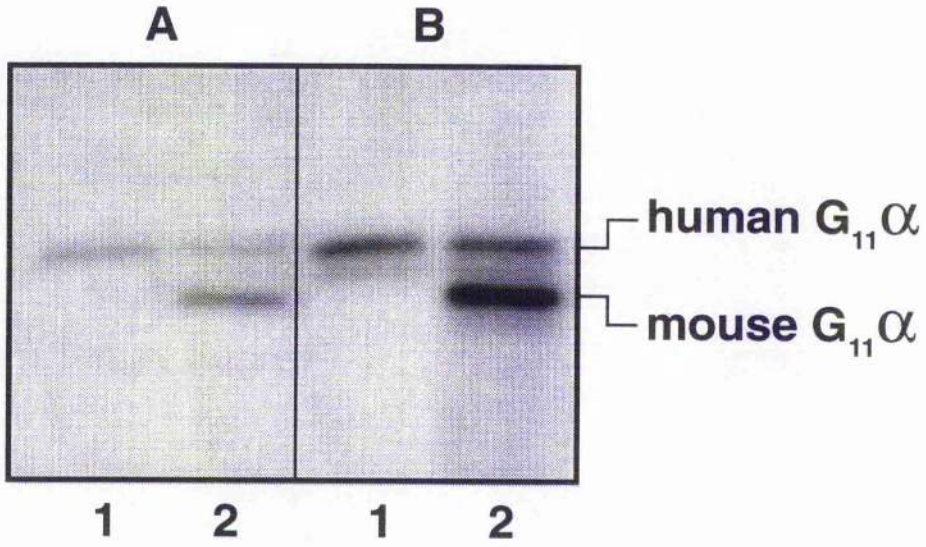

A
B
human $G_{11}\alpha$
mouse $G_{11}\alpha$
1
2
1
2

Figure 4.3.10.

The effect of TRH on the content of $G_{11}\alpha$ G-proteins in crude membrane and cytosol fractions of E2 and E2M11 cells.

75 cm^2 tissue culture flasks of either E2 (panel A, left hand side) or E2M11 cells (panel A, right hand side and panel B), grown to 70% confluency, were incubated for 16 h with (T) or without (C) 10 μM TRH. Crude membrane (MB) and cytosol (CYT) fractions were prepared as described in chapter 2.2.2. The $G_{11}\alpha$ proteins in membranes and cytosol were resolved by 6 M urea SDS-PAGE as mentioned previously using 60 μg (panel A) or 150 μg (panel B) of protein per lane and identified by immunoblotting with antiserum CQ (1: 1000 dilution). Results are from a representative experiment.

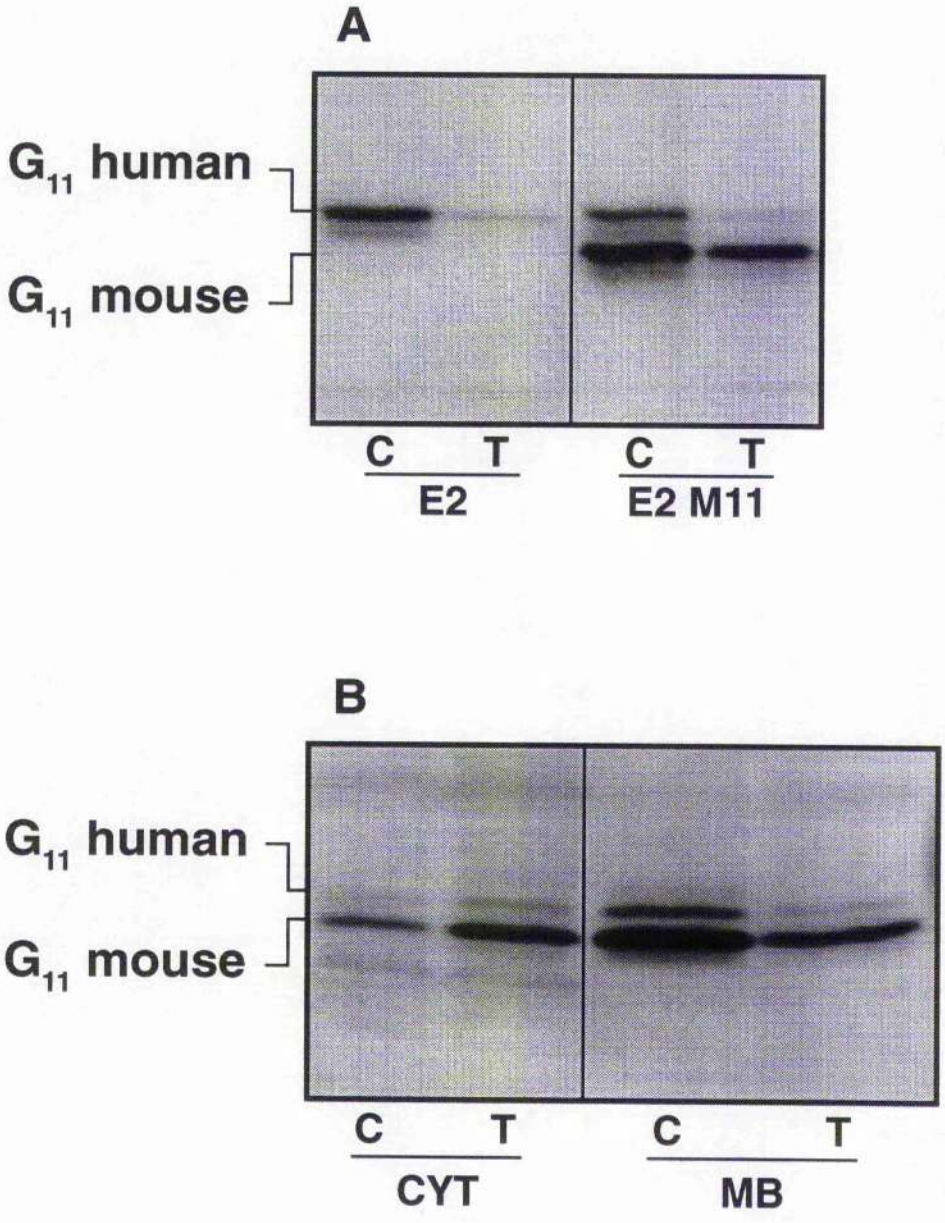

A
G_{11} human
G_{11} mouse
C
T
C
T
E2
E2 M11
B
G_{11} human
G_{11} mouse
C
T
C
T
CYT
MB

Figure 4.3.11.

Time course of membrane removal and cytosolic accumulation of variants of $G_{11}\alpha$.

E2M11 cells were untreated (C) or treated with TRH (10 μM) for varying times and crude membrane and cytosolic fractions subsequently prepared as in chapter 2.2.2. Immunoblotting with antiserum CQ was performed following resolution of the fractions (crude membranes, 60 μg/lane; cytosol, 150 μg/lane) in 6 M urea containing SDS-PAGE as in Figure 4.3.10

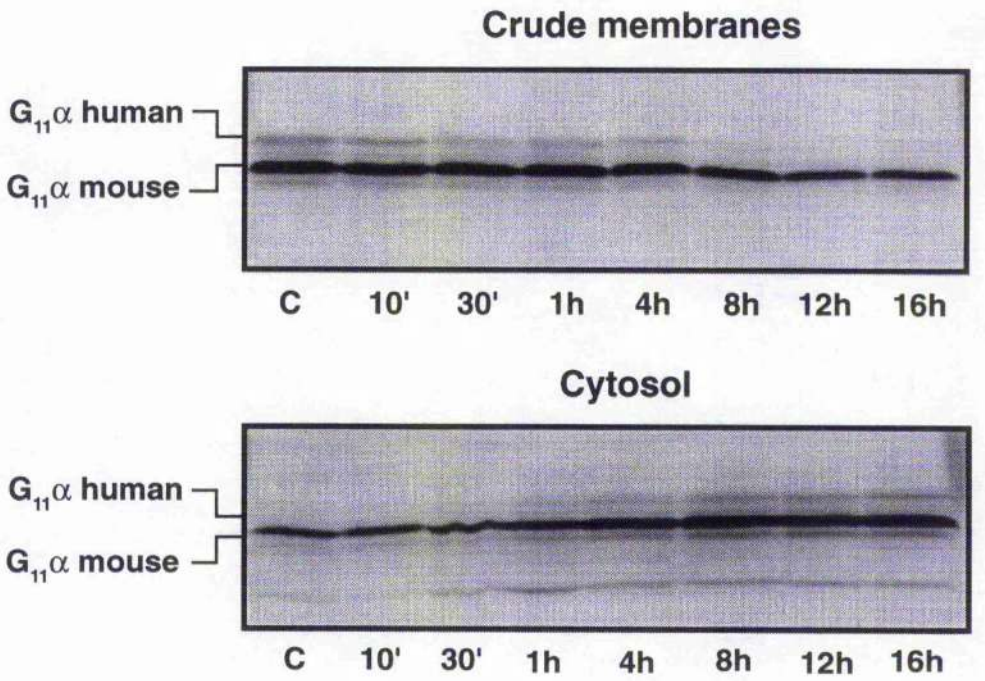
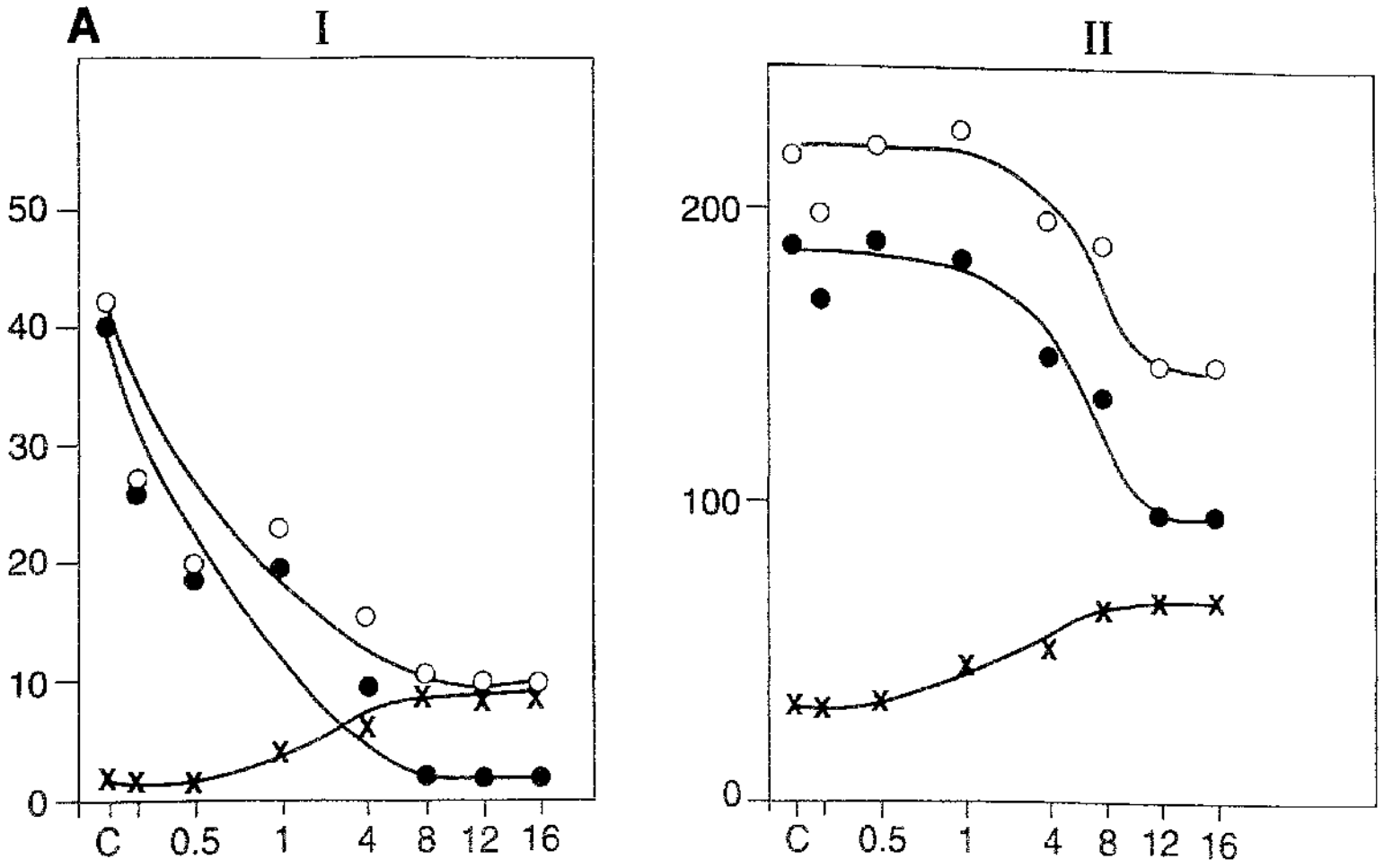

Crude membranes
$G_{11}\alpha$ human
$G_{11}\alpha$ mouse
C
10'
30'
1h
4h
8h
12h
16h
Cytosol
$G_{11}\alpha$ human
$G_{11}\alpha$ mouse
C
10'
30'
1h
4h
8h
12h
16h

Figure 4.3.12.

TRH induced cellular redistribution and down-regulation of human and murine variants of $G_{11}\alpha$ (Quantitative analysis).

Panel A represents the immunological signal of total (open circles), membrane bound (closed circles) and cytosolic (crosses) forms of human (I) and murine (II) variants of $G_{11}\alpha$ as expressed in arbitrary units for each time interval.

In panel B, the relative amount of human (I) and murine (II) $G_{11}\alpha$ subunits in membrane and cytosol fractions was expressed as % of total signal (membranes + cytosol) for each time interval. The down-regulation (III) of human (open circles) and murine (closed circles) $G_{11}\alpha$ was expressed as the decrease of total immunological signal with time.


A
I
II
50
40
30
20
10
0
200
100
0
C
0.5
1
4
8
12
16

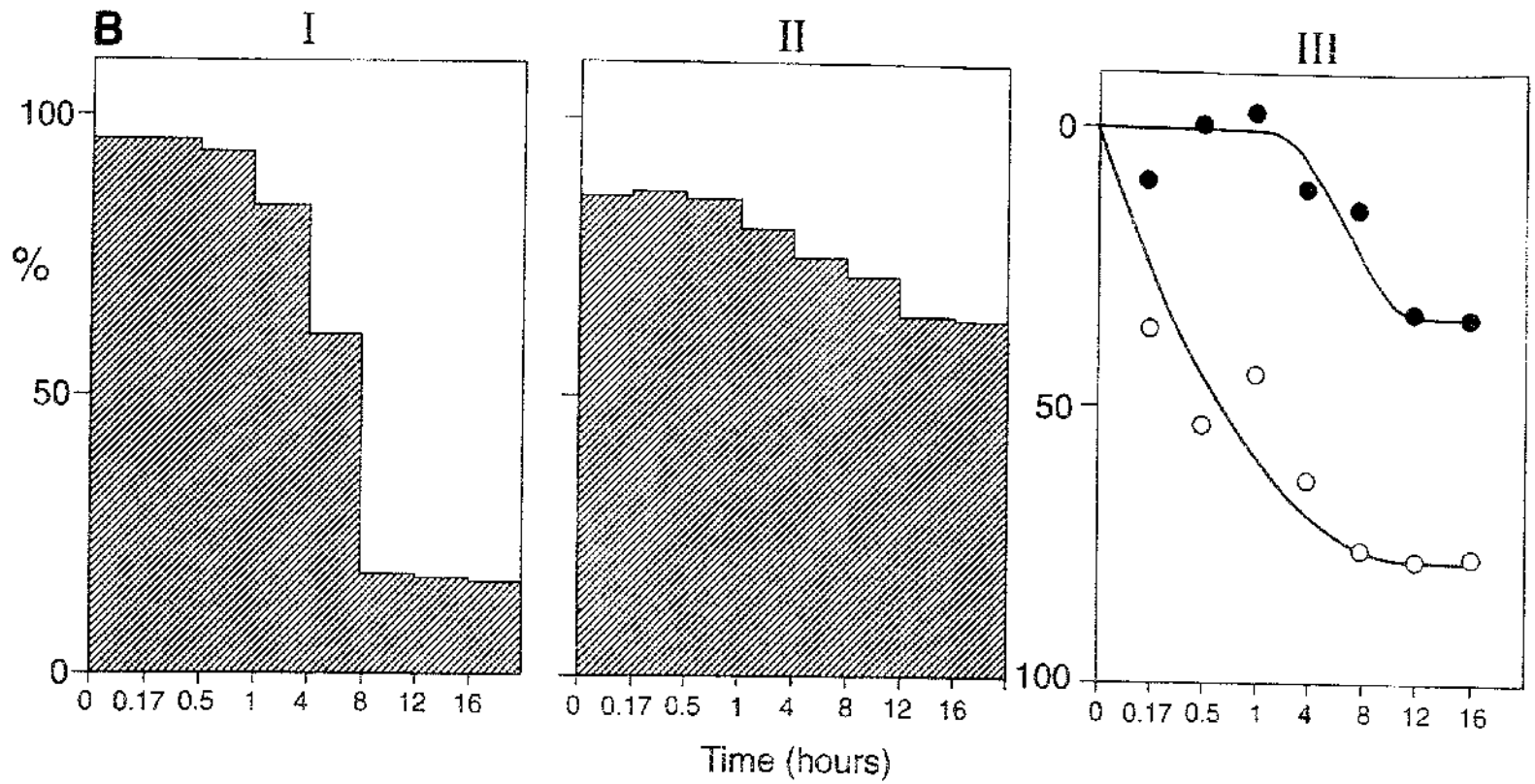

B
I
II
III
100
%
50
0
0 0.17 0.5 1 4 8 12 16
0 0.17 0.5 1 4 8 12 16
0
50
100
0 0.17 0.5 1 4 8 12 16
Time (hours)

Figure 4.3.13.

TRH treatment of E2M11 cells results in a transfer of $G_{11}\alpha$ variants from the plasma membrane to low-density membrane fractions (Immunoblot studies).

Homogenates prepared from the same amount (each derived from 6 x 75 cm^2 tissue culture flasks) of untreated (control) (panel A and panel B) or TRH (10 μM, 16h) treated cells (panel B) were fractionated on discontinuous sucrose density gradients as described in chapter 2.2.3. and the plasma membrane fraction (centred on fraction 4) was separated from cytosol (fraction 1S), light vesicle (1P), low-density (microsomes, endoplasmic reticulum, fractions 2 and 3) and high-density (mitochondria, fractions 6 and 7) membranes as well as the gradient pellet (fraction 8).

Panel A displays the identical cellular distributions of both endogenous human $G_{11}\alpha$ and exogenously introduced murine $G_{11}\alpha$. 0.75 ml of sucrose density fractions 1-8 of the homogenate from untreated E2M11 cells were TCA precipitated (6% final concentration, 1 h, 0 °C) and resolved in 6 M urea-containing SDS-PAGE as in Figure 4.3.10 to detect the presence of the human and murine variants of $G_{11}\alpha$.

Panel B represents TRH induced redistribution of cellular $G_{11}\alpha$ from the plasma membrane to low-density membrane fractions. Homogenates from control and TRH treated E2M11 cells were subjected to centrifugation on discontinuous sucrose density gradients, proteins were resolved by 6M urea containing SDS-PAGE (the gels were shown in Figure 4.3.14.) and immunoblotted with antiserum CQ at a dilution of 1: 1000. Data are taken from a representative experiment.

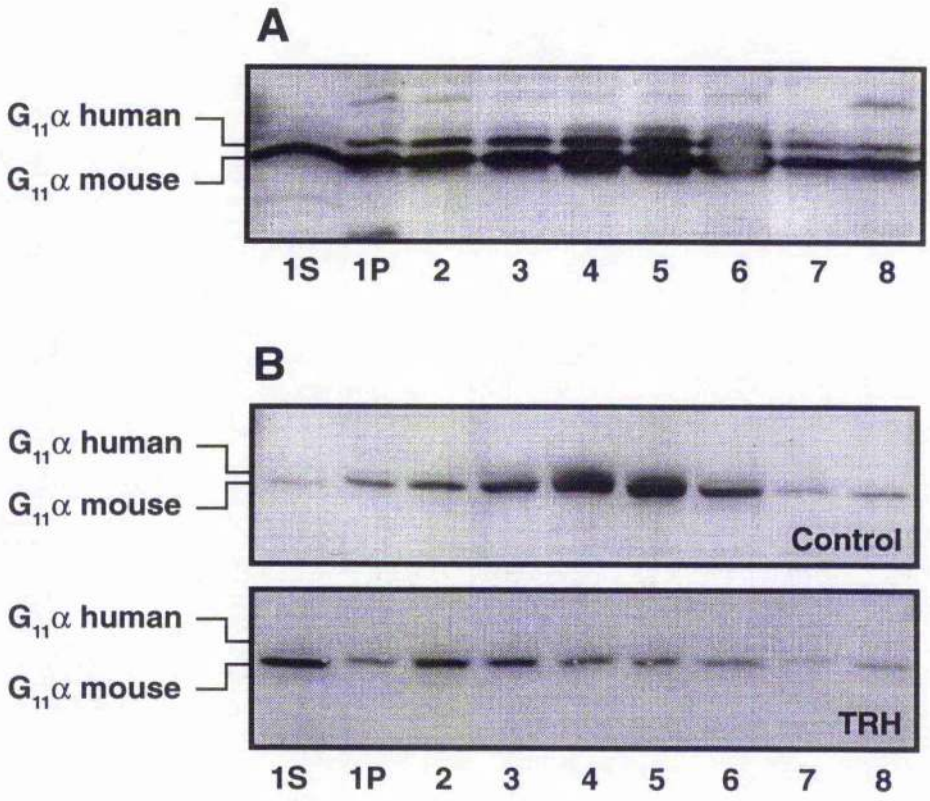

A
$G_{11}\alpha$ human
$G_{11}\alpha$ mouse
1S 1P 2 3 4 5 6 7 8
B
$G_{11}\alpha$ human
$G_{11}\alpha$ mouse
Control
$G_{11}\alpha$ human
$G_{11}\alpha$ mouse
TRH
1S 1P 2 3 4 5 6 7 8

Figure 4.3.14.

TRH treatment of E2M11 cells does not result in a gross redistribution of cellular polypeptides.

Homogenates of control and TRH-treated (10 μM, 16h) E2M11 cells were subjected to centrifugation on discontinuous sucrose gradients. 0.75 ml of sucrose density fractions 1-8 were precipitated with trichloracetic acid (6% final concentration, 1 h, 0 °C) and the resulting proteins resolved by 6 M urea containing SDS-PAGE (10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide). The gels were stained with Coomassie brilliant blue R-250 as described in chapter 2.2.9..

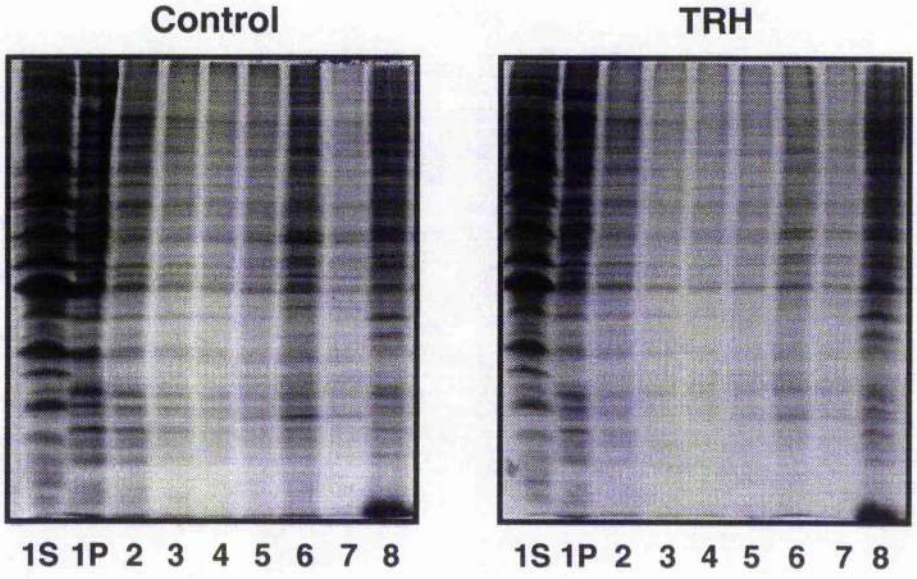

Control
TRH
1S 1P 2 3 4 5 6 7 8
1S 1P 2 3 4 5 6 7 8

Figure 4.3.15.

Specific content and redistribution of $G_{11}\alpha$ proteins in control and TRH treated E2M11 cells along the sucrose density gradient.

In panel (I) the specific content of $G_{11}\alpha$ (both variants) present in gradient fractions 1S, 1P and 2-8 of control (open symbols) and TRH-treated (10 μM, 16h) (filled symbols) E2M11 cells was calculated by dividing the immunological signal by the amount of protein applied to the gel. The immunological signals (arbitrary units) corresponding to both human and murine variants of $G_{11}\alpha$ shown in Figure 4.3.13. were analysed by densitometric scanning and the amount of protein applied was determined in parallel samples TCA precipitated from 0.75 ml aliquots of the sucrose density gradient fractions.

The relative amount of $G_{11}\alpha$ present in a given fraction from homogenates of both control (open bars) and TRH treated (filled bars) E2M11 cells was expressed in panel (II) as a percentage over the entire gradient.

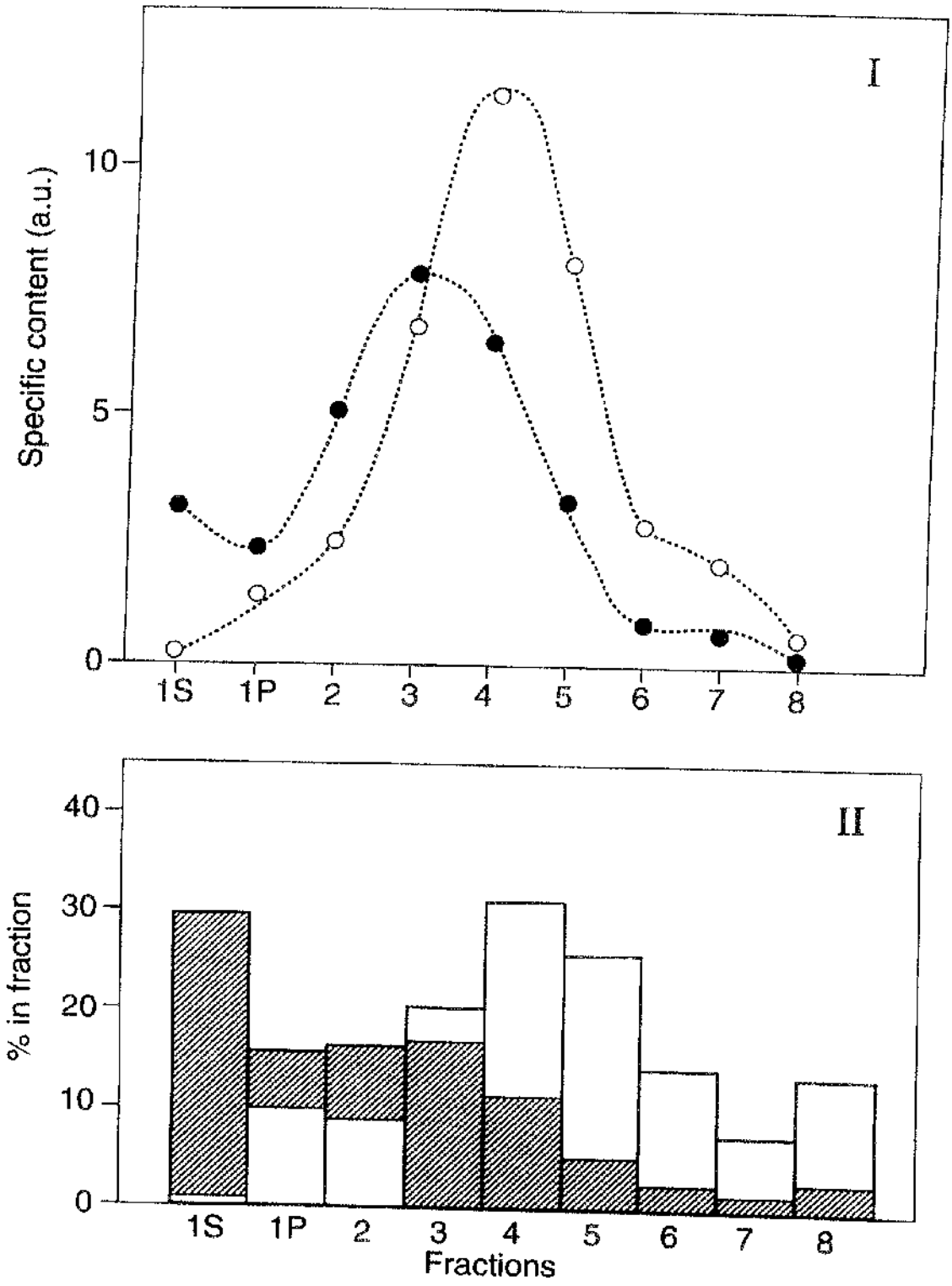

I
Specific content (a.u.)
10
5
0
1S
1P
2
3
4
5
6
7
8
II
% in fraction
40
30
20
10
0
1S
1P
2
3
4
5
6
7
8
Fractions

Figure 4.3.16.

TRH-treatment of E2M11 cells results in a greater cellular redistribution and down-regulation of human $G_{11}\alpha$ than of murine $G_{11}\alpha$.

Fractions 1S (320 μg), 1P (170 μg), 3 (40 μg), 4 (35 μg) and 5 (40 μg) (the protein content of 0.35 ml of each fraction) from centrifugation on a discontinuous sucrose density gradient of control (C) and TRH-treated (T) (10 μM, 16h) E2M11 cells were resolved in 6 M urea-containing SDS-PAGE and immunoblotted using antiserum CQ (1: 1000 dilution)

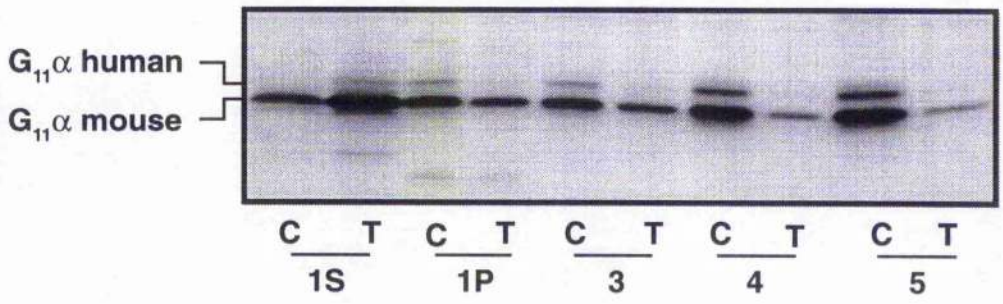

$G_{11}\alpha$ human
$G_{11}\alpha$ mouse
C T C T C T C T C T
1S 1P 3 4 5

Figure 4.3.17.

Both human and murine $G_{11}\alpha$ are efficiently solubilized from membranes of E2M11 cells by sodium cholate.

Crude membranes (60 μg) of E2M11 cells were either untreated (lane 1) or treated with 1% (w/v) sodium cholate (1 h, 4 °C) (lanes 2 and 3). The sodium cholate treated samples were then resolved into remaining particulate (lane 2) and solubilized (lane 3) fractions by centrifugation (200, 000 x *gav* for 30 min at 4 °C). Samples were resolved by 6 M urea-containing SDS-PAGE and immunoblotted using antiserum CQ. If the transfected polypeptide of murine $G_{11}\alpha$ had formed an aggregated mass, then after detergent solubilization as detailed in chapter 2.2.4, they would still be found in the particulate fraction whereas G-protein α subunits which were membrane associated should be released into the solubilized supernatant fraction.

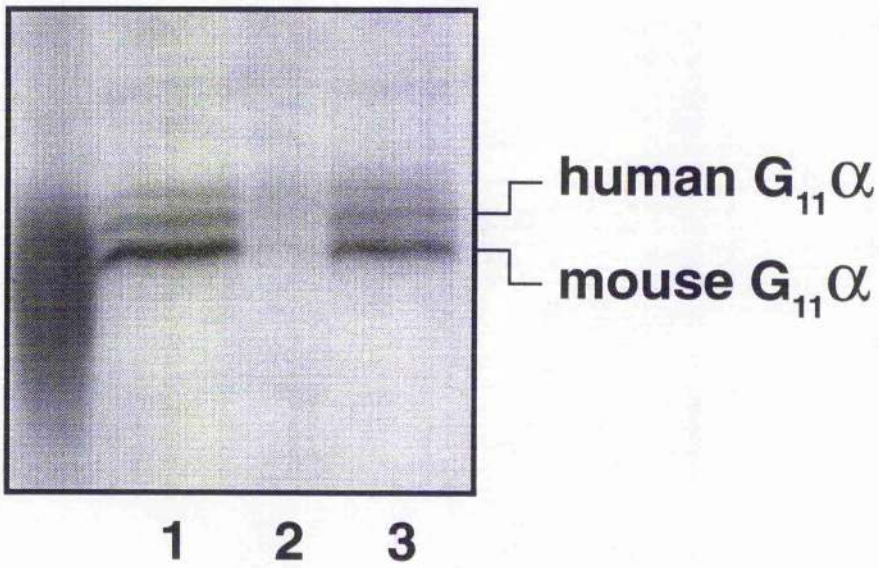

human $G_{11}\alpha$
mouse $G_{11}\alpha$
1
2
3

Figure 4.3.18.

The cellular distribution of G-protein β-subunit is altered by treatment of E2M11 cells with TRH.

Homogenates of control (panel A) and TRH-treated (10 μM, 16h) (panel B) E2M11 cells were centrifuged on discontinuous sucrose density gradients as described in the legend to Figure 4.3.13. 0.75 ml of sucrose density gradient fractions 1-8 used in Figure 4.3.13 were precipitated with TCA (final conc.: 6%, 1 h, 0 °C) and then resolved in SDS-PAGE (10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide). β-subunit was subsequently detected by immunoblotting with antiserum βN (1: 200 dilution).

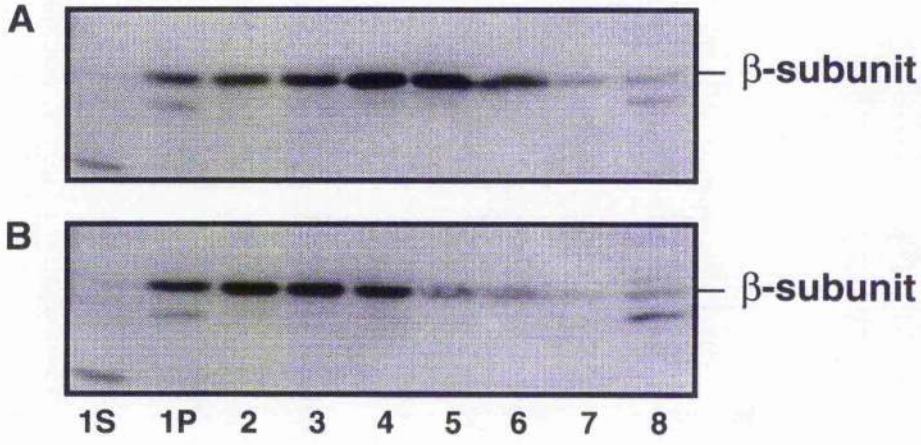

A
β-subunit
B
β-subunit
1S
1P
2
3
4
5
6
7
8

Table 4.3.1.

The distribution of $G_{11}\alpha$ G-proteins in clone E2M11 cells and the effect of TRH.

The immunological signals (arbitrary units) corresponding to both human and murine variants of $G_{11}\alpha$ proteins shown in Figure 4.3.10 (panel B) were analysed by densitometric scanning and related to the amount of protein present in membrane and cytosol fractions prepared from the same number of cells (1 x 75 cm^2 flask of cells). Down-regulation by TRH (1 μM, 16h) was expressed as a percentage of total signal (membranes plus cytosol) in untreated cells. Similar results were obtained in 3 other experiments.

A. Control.

	$G_{11}\alpha$ (%)	**Down-regulation (%)**
Membrane	1152.9 (98.0%)	
Cytosol	23.8 (2.0%)	
Total	1176.7 (100.0%)	0

B. TRH-treated (10 μM, 16h).

	$G_{11}\alpha$ (%)	**Down-regulation (%)**
Membrane	476.2 (76.6%)	
Cytosol	145.2 (23.3%)	
Total	621.4 (100.0%)	47.2

Table 4.3.2.

Time course of TRH-induced alterations in membrane to cytosol distribution and down-regulation of human and murine $G_{11}\alpha$ G-proteins in E2M11 cells.

The immunological signal (arbitrary units) corresponding to both species variants of $G_{11}\alpha$ shown in Figure 4.3.11 was analysed by densitometric scanning and related to the amount of protein present in membrane and cytosol fractions. Down-regulation by TRH (10 μM, 16h) was expressed as percentage of total signal (membranes plus cytosol) in untreated cells. Results are representative of 4 experiments performed.

Human $G_{11}\alpha$

	Membrane	Cytocol	Total	Down-regulation
Control	40.3 (96%)	1.7 (4%)	42.0 (100%)	0 %
10 min	26.0 (96%)	1.0 (4%)	27.0 (100%)	36%
30 min	18.4 (94%)	1.2 (6%)	19.6 (100%)	53%
1h	19.5 (84%)	3.8 (16%)	23.3 (100%)	44%
4h	9.3 (61%)	6.0 (39%)	15.3 (100%)	63%
8h	1.8 (18%)	8.4 (82%)	10.2 (100%)	76%
12h	1.6 (17%)	7.8 (83%)	9.4 (100%)	78%
16h	1.6 (17%)	8.0 (83%)	9.6 (100%)	77%

Mouse $G_{11}\alpha$.

	Membrane	Cytocol	Total	Down-regulation
Control	187.6 (86%)	30.8 (14%)	218.4 (100%)	0%
10 min	169.1 (85%)	29.5 (15%)	198.6 (100%)	9%
30 min	188.9 (86%)	32.1 (15%)	221.0 (100%)	-1%
1h	182.2 (81%)	43.8 (19%)	226.0 (100%)	-3%
4h	149.0 (75%)	48.5 (25%)	197.5 (100%)	10%
8h	136.2 (72%)	52.4 (28%)	188.6 (100%)	14%
12h	95.3 (65%)	52.0 (35%)	147.3 (100%)	33%
16h	95.6 (65%)	51.8 (35%)	147.4 (100%)	32%

Table 4.3.3.

The regulation of G-protein β-subunits in E2M11 cells by treatment with TRH.

E2M11 cells (1 x 75 cm^2 flask), which were either control or treated with TRH (10 μM, 16h), were homogenized in 0.5 ml of TME buffer and centrifuged for 2 h at 50, 000 rpm. The sediment was suspended with re-homogenization in 0.5 ml of TME buffer (crude membranes) and the supernatant represents the cytosol fraction. The immunodetectable signal corresponding to G-protein β-subunits (arbitrary units) was normalized to the amount of protein in these fractions. Down-regulation by TRH was expressed to compared to untreated cells. Results represent mean ± S.D. of 3 experiments.

A. Control.

	β subunit (%)	**Down-regulation (%)**
Membrane	437.0 ± 46.9 (99.0%)	
Cytosol	6.6 ± 3.3 (1.0%)	
Total	443.6 (100.0%)	0

B. TRH-treated (10 μM, 16h).

	β subunit (%)	**Down-regulation (%)**
Membrane	294.2 ± 39.5 (99.0%)	
Cytosol	2.4 ± 0.9 (1.0%)	
Total	296.6 (100.0%)	32.2

4.3.2. Discussion.

Many receptor expression studies are performed in heterologous systems, not only in terms of the cell type used for expression but also in terms of the species origin of the cell. Little attention is given to the possibility that there may be differences either at a qualitative or quantitative level in the interactions of a receptor with G-proteins derived from another mammalian species although, at the receptor level, there are many examples of how pharmacological profiles of receptor-ligand interactions may differ between species homologous based on as little as a single amino acid substitution (Hall *et al.*, 1993; Link *et al.*, 1993).

The extreme C-terminal region of G-protein α subunits plays a key role in receptor recognition. Mutation within this region, i.e., in the *unc* phenotype of S49 lymphoma cells in which a single base substitution leads to the alteration of Arg to Pro six amino acids from the C-terminus of $G_s\alpha$, prevents interaction of receptors with the mutant G-protein (Sullivan *et al.*, 1987). Furthermore, pertussis toxin functions to prevent receptor interaction with G proteins of the $G_i\alpha$ family by catalyzing ADP-ribosylation of the conserved Cys residue which is located four amino acids from the C-terminus of all of these G-proteins (Milligan *et al.*, 1988). It is also known that designed mutagenesis in this region of a G-protein can regulate the nature of receptors which interact with the G-protein (Conklin *et al.*, 1993a). G-protein α subunits are in general highly conserved between mammalian species (Kaziro *et al.*, 1990) and the C-terminal region is predicted to be virtually identical in $G_{11}\alpha$ in all of the mammalian species. In the case of $G_{11}\alpha$, clones corresponding to which have been isolated from mouse (Strathmann *et al.*, 1990), bovine (Nakamura *et al.*, 1991) and human (Jiang *et al.*, 1991) cDNA libraries, there is complete identity within this region (Figure 4.3.6) and the clones corresponding to $G_{11}\alpha$ predict some 97-98% overall amino acid identity between these three species. This high degree of conservation allows immunological probes generated against an amino acid sequence within the G-protein from one species to be useful in the detection of the same G-protein in cells and tissues from a range of species but poses a substantial limitation in attempts to define the

species from which a form of a G-protein might be derived. However, the system which I have developed in this study offers a suitable means by which to measure the functional importance of any designed mutation as the functionality of modified form of murine $G_{11}\alpha$ expressed in a primate cell line can be measured using the function of the endogenously expressed primate $G_{11}\alpha$ as control (McCallum *et al.*, 1995).

The results reported herein demonstrate that a considerable number of G_q-like polypeptides are expressed in mammalian frontal cortex and that the profile of expression and mobility of these polypeptides varies markedly between species. I have also shown an ability to express species variants of the same G-protein in a single cell and to detect concurrently and unambiguously both of these polypeptides. The approach used in this study has relied on immunodetection using primarily an antiserum generated against a synthetic peptide corresponding to the sequence predicted to the C-terminal decapeptide of both mouse $G_q\alpha$ and $G_{11}\alpha$ as mentioned above.

Resolution of frontal cortex from a number of mammalian species in 10% (w/v) acrylamide, 0.25% (w/v) bisacrylamide SDS-PAGE demonstrated not only no ability to resolve $G_{11}\alpha$ forms from different species but also that $G_{11}\alpha$ was not resolved from the closely related G-protein $G_q\alpha$ (Figure 4.3.1). Such results provide no information, however, on whether $G_q\alpha$ and $G_{11}\alpha$ are co-expressed in these tissues and if they are about the relative levels of expression of these two peptides. To address this question it has been reported that an imidazole buffer system in a 13% acrylamide gel can provide separation of $G_q\alpha$ and $G_{11}\alpha$ (Blank *et al.*, 1991). However, in my hands this method achieves a lesser degree of separation and is not as reproducible as the 4-8 M urea gradient method described in Milligan *et al.*, 1993d. The disadvantage of the 4-8 M urea gradient method that it also was not routinely reproducible in separating $G_q\alpha$ and $G_{11}\alpha$. Therefore, a new gel system was developed in this study to demonstrate that $G_{11}\alpha$ from different species can be separated from each other and from $G_q\alpha$. This gel system applied a 10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide matrix into which 6 M urea has been

incorporated. It is technically easier than the urea gradient method previously reported and allowed me to examine a member of the G_q family from different species. Resolution of frontal cortex from a number of mammalian species under 10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide SDS-PAGE conditions which incorporated 6 M urea into the gel demonstrated the presence of a number of G_q-like polypeptides in mammalian frontal cortex and that the profile of expression and electrophoretic mobility of these polypeptides varied markedly between species (Figure 4.3.2).

Detection of these polypetides was based principally on the use of two antisera. The first of these (CQ) (Mitchell *et al.*, 1991) was generated against a synthetic peptide corresponding to the sequence predicted to the C-terminal decapeptide of both mouse $G_q\alpha$ and $G_{11}\alpha$ (Figure 4.3.6). The second antiserum used (E976) (Taylor *et al.*, 1991a) was generated against a peptide predicted to correspond to amino acids 160-172 in mouse $G_{11}\alpha$ (Figure 4.3.6). This sequence differs in but a single amino acid between the mouse (170 Valine), human (170 Leucine) and bovine (170 Serine) forms but varies markedly from the equivalent sequences of $G_q\alpha$ and $G_{14}\alpha$ and thus provides a specific probe for $G_{11}\alpha$ which will identify rodent and primate forms of $G_{11}\alpha$ similarly.

In this study I was able to detect a polypeptide migrating in the position anticipated for $G_{11}\alpha$, based upon the previously established mobility of rat $G_{11}\alpha$ (Milligan *et al.*, 1993d), in membranes of frontal cortex from human, baboon or guinea pig. Confirmation of the presence of immunodetectable $G_{11}\alpha$ was produced in immunoblots using the $G_{11}\alpha$ specific antiserum E976. The position of migration of primate $G_{11}\alpha$ in these resolving gels was substantially different from rodent $G_{11}\alpha$, migrating more slowly and in a position very close to $G_q\alpha$. Immunoreactivity corresponding to $G_{11}\alpha$ in guinea pig was shown to migrate in a different position to either rat and mouse or the primates (Figure 4.3.3) and to correspond to the heavily CQ immunostained polypeptide migrating between rat and mouse $G_q\alpha$ and $G_{11}\alpha$ (Figure 4.3.2). Antiserum CQ would also be anticipated to cross react with $G_{14}\alpha$. Indeed in ELISA assays this antiserum reacts strongly with a peptide which

corresponds to the C-terminal decapeptide of $G_{14}\alpha$ (Milligan *et al.*, 1992). This is because although this region of $G_{14}\alpha$ has two amino acid differences to that of $G_q\alpha$ and $G_{11}\alpha$ (Figure 4.3.6.B) they are both conservative substitutions (Figure 4.3.6A). As such it is possible that one of the polypeptides identified by antiserum CQ is $G_{14}\alpha$. However, conservation between $G_q\alpha$, $G_{11}\alpha$ and $G_{14}\alpha$ is weak in the regions used to generate antisera IQB and E976 (Figure 4.3.6) and it is thus unlikely that these antisera would identify $G_{14}\alpha$. It has also been indicated that the tissue distribution of $G_{14}\alpha$ is very restricted (Wilkie *et al.*, 1991). It was also a potential concern that some of the complexity of the CQ immunoreactive pattern uncovered by the urea containing gels might represent a series of proteolytic fragmentations. However, inclusion in the membrane preparation protocol of a cocktail of inhibitors of proteolysis had no ability to alter the immunoreactive pattern (data not shown).

It has been shown that a human muscarinic M1 acetylcholine receptor is able to activate and regulate cellular levels of both rat $G_q\alpha$ and $G_{11}\alpha$ equally (Mullaney *et al.*, 1993a). By contrast, there has been some limited evidence to support the concept that $G_q\alpha$ and $G_{11}\alpha$ function differently and that receptors can select between them (Lipinsky *et al.*, 1992). Therefore, the ability to coexpress and subsequently independently detect species isoforms of $G_{11}\alpha$ now offered the possibility to measure whether a receptor interacts equally or selectively with G-proteins derived from different species. The stable co-expression of a receptor and murine $G_{11}\alpha$ in a human cell line allowed measurement of the relative interaction of the receptor with each of mouse and human $G_{11}\alpha$ for example. As a means to begin to address questions of this nature mouse $G_{11}\alpha$ was expressed transiently in monkey COS-1 cells (which express endogenously the monkey form of the protein) and membranes derived from these cells was resolved by including 6 M urea in 10% (w/v) acrylamide, 0.0625% (w/v) bisacrylamide SDS-PAGE which is able to separate excellently between the mouse and monkey forms (Figure 4.3.5).

On the basis of transient expression of mouse $G_{11}\alpha$ in COS-1 cells, the stable co-expression of a receptor and murine $G_{11}\alpha$ in a human cell line was developed. The

system used in this study is a clonal cell line derived from the human embryonic kidney cell line HEK-293 by two consecutive stable transfections. In the first stage the cells were transfected to express high levels of the long isoform of the rat TRH receptor and subsequently a clone from this transfection (E2) was transfected to express the murine form of $G_{11}\alpha$ (clone E2M11) (Figure 4.3.7). The requirement for high levels of a receptor was based on our previous observations that to record agonist mediated down-regulation of a G-protein, high levels of receptors are required (Adie *et al.*, 1994a, b; Milligan *et al.*, 1995b) as a significant fraction of the cellular population of the relevant G-protein must be activated by the receptor. Clone E2M11 allowed me to address a further question which has not been previously examined in any systematic manner i.e. can a single receptor type utilize species variants of the same G-protein equivalently?

As previously noted, rodent and primate variants of $G_{11}\alpha$ migrate in markedly different positions in urea-containing SDS-PAGE. In this study I demonstrated conclusively that clone E2M11 but not clone E2 expressed murine $G_{11}\alpha$ while both of these clones express human $G_{11}\alpha$ endogenously due to the genetic background of the HEK-293 cells. In Figures 4.3.9 and 10, murine $G_{11}\alpha$ is expressed at some 2-3 fold higher levels than the human variant in E2M11 cells (this ratio varied somewhat in individual passages of the cells, a feature previously well appreciated for a variety of polypeptides in many transfected cell lines). Importantly, before these studies could progress usefully, it was necessary to demonstrate that the cellular distribution of the endogenous human $G_{11}\alpha$ and the introduced murine $G_{11}\alpha$ were identical (Figure 4. 3.13A) as judged by their co-distribution in various membrane fractions prepared by centrifugation of cellular homogenates on discontinuous sucrose density gradients. Furthermore, both human and murine $G_{11}\alpha$ were effectively solubilized from a crude membrane fraction by exposure to sodium cholate (Figure 4.3.17). I have used this as an indication that the immunodetectable mouse $G_{11}\alpha$ did not reflect either incorrect folding or aggregation of the expressed protein..

As previously shown in chapter 4.2 for clone E2, sustained exposure of E2M11 cells to a maximally effective concentration of TRH resulted in a marked reduction in $G_{11}\alpha$ levels in a crude membrane fraction of these cells, confirming that TRH treatment of these cells can result in down-regulation of a substantial fraction of the expressed $G_{11}\alpha$. However, a key observation over a large number of experiments was that down-regulation of human $G_{11}\alpha$ was quantitatively more effective than down-regulation of murine $G_{11}\alpha$. These data provide the first indication that a receptor may not interact identically with the same G-protein from two separate species when presented with a concurrent choice between them and when it is clear that the two G-proteins are identically presented and distributed in a cell. This was surprising as the two species variants are some 97% identical in primary sequence (Strathmann *et al.*, 1990) and are very highly conserved in regions believed to play important roles in interactions with receptors. Furthermore, in a range of studies little has been observed to suggest that receptors can functionally select between the α subunits of endogenously expressed $G_{11}\alpha$ and $G_q\alpha$ (Mullaney *et al.*, 1993a) the prevalent and widely expressed phosphoinositidase C-linked G-proteins which, although derived from distinct genes, are highly homologous (Strathmann *et al.*, 1990). It should be noted, however, that difference in the interaction of the mammalian neuromedin B receptor with *Xenopus* forms of $G_q\alpha$ and $G_{11}\alpha$ have been recorded using an antisense based approach (Shapira *et al.*, 1994) and between murine $G_q\alpha$ and $G_{11}\alpha$ with the murine TRH receptor following their expression by injection of cRNA into *Xenopus* oocytes (Lipinsky *et al.*, 1992).

By simple fractionation of E2M11 cell homogenates into crude membrane and cytosolic fractions it was noted that in the unchallenged cells only some 4% of the total cellular content of the $G_{11}\alpha$ species variants could be found in the soluble fraction of the cell. However, upon challenge of the cells with TRH an increase in the soluble content of $G_{11}\alpha$ was observed (Figure 4.3.10B and Figure 4.3.11). This could be observed for both the human and murine variants of $G_{11}\alpha$ and was also observed to increase over an 8 h period. When presented as a fraction of the total cellular content

of $G_{i}1\alpha$ at any time period, the cytosolic fraction increased to some 20-25% of the total, but it must be remembered that this value is made apparently more impressive as the total cellular content of $G_{i}1\alpha$ falls (it is down-regulated). The increase in cytosolic content of $G_{i}1\alpha$ was insufficient to account for the bulk loss of $G_{i}1\alpha$ from the particulate fraction, confirming a true cellular reduction in $G_{i}1\alpha$ levels. A number of other studies have reported an accumulation of cytosolic $G_s\alpha$ following agonist challenge of cells (Ransnas *et al.*, 1989; Levis *et al.*, 1992; Negishi *et al.*, 1992). Recently there has been considerable emphasis of examining the interaction of G-protein α subunits with membranes as all of the widely expressed α subunits have been shown to be palmitoylated close to their N-terminus (Parenti *et al.*, 1993; Linder *et al.*, 1993; Degtyarev *et al.*, 1993a; Wedegaertner *et al.*, 1993; Grassie *et al.*, 1994). Studies indicate that the palmitoylation status (of at least $G_s\alpha$) is dynamic and to be regulated by the activation status of the G-protein (Degtyarev *et al.*, 1993a; Wedgaertner *et al.*, 1994; Mumby *et al.*, 1994). Furthermore, cytosolic G-protein α subunits have been reported to be devoid of palmitate. It will thus be of interest to examine, in time, the relative palmitoylation status of $G_{i}1\alpha$ in the various cellular compartments during and following TRH-treatment of E2M11 cells.

Cellular redistribution of the α subunit of the stimulatory G-protein $G_s\alpha$ from membrane to cytosol has been recorded in a number of studies following agonist-occupation of a receptor which links to this G-protein (Ransnas *et al.*, 1988, 1989; Levis *et al.*, 1992; Negishi *et al.*, 1992). However, the quantitative details relating to the fraction of the G-protein moved and the time course of this effect have either been somewhat variable or have not been examined in detail. Furthermore, there has been little previous attempt to analyse if the activated G-protein might redistribute within membrane fractions upon agonist activation of a receptor. It has recently been reported that sustained agonist occupation of a human muscarinic M1 acetylcholine receptor stably transfected into CHO cells results in a cellular redistribution of the α subunits of the phosphoinositidase C linked G-protein $G_q\alpha$ and $G_{i}1\alpha$ (Svoboda *et al.*, 1994). Strong evidence also indicates that maintained exposure of a cell to an agonist can

results in a reduction in cellular levels of the G-protein(s) activated by the receptor for that agonist (Mullaney *et al.*, 1993a, b; McKenzie *et al.*, 1990b; Green *et al.*, 1992), an effect which appears to reflect accelerated degradation of the activated G-proteins (Shah *et al.*, 1995; Mitchell *et al.*, 1993). There are thus clearly a variety of dynamic processes in which cellular G-proteins are participants following receptor activation of a cell. The establishment of a suitable cellular system in this study has allowed me to examine concurrently both the qualitative and quantitative details of many of these processes.

Separation of the total particulate fraction of untreated and TRH-treated E2M11 cells into a series of membrane fractions by centrifugation of cellular homogenates on discontinuous sucrose density gradients (Svoboda *et al.*, 1994) demonstrated a further clear and novel cellular redistribution of $G_{11}\alpha$ in response to TRH. While the distribution of $G_{11}\alpha$ in the untreated cells indicated that a large preponderance of the G-protein was in the plasma membrane, as might be expected given its role, TRH-treatment produced a profound membrane redistribution such that the bulk of the remaining immunodetectable $G_{11}\alpha$ was now located in lower-density membrane fractions (Figure 4.3.13B where the main peak of $G_{11}\alpha$ immunoreactivity is in fractions 2 and 3 following TRH treatment compared to fractions 4 and 5 in the untreated samples). This redistribution of $G_{11}\alpha$ was achieved without a bulk redistribution of protein as the gross pattern of protein expression in the various sucrose density gradient fractions was not altered by TRH-treatment of the cells (Figure 4.3.14). This is reminiscent of the redistribution towards lower-density membranes observed for both $G_q/G_{11}\alpha$ and the muscarinic M1 acetylcholine receptor following challenge with carbachol of CHO cells transfected to express this receptor (Svoboda *et al.*, 1994).

Somewhat to my surprise, the membrane redistribution of G-protein subunits upon TRH-treatment was not restricted to $G_{11}\alpha$. Equivalent analysis of the particulate distribution of G-protein β-subunit indicated that there was also a substantial movement of immuno-detectable β-subunit from the plasma membrane containing

fractions to light membrane fractions (Figure 4.3.18). In contrast to $G_{11}\alpha$, however, there was no increase in detection of cytosolic β-subunit upon TRH treatment and this remained as insignificant fraction. Thus, the hormone-induced movement of $G_{11}\alpha$ subunits to the cytosol induced by TRH stimulation of E2M11 cells must be associated with physical dissociation from β-subunits which remain in the particulate fraction.

The physical processes involved in alteration in the membrane fraction distribution profile of $G_{11}\alpha$ and β-subunits upon sustained TRH stimulation remain undefined but these may contribute to the process of desensitization. Furthermore, these may represent part of the process of membrane to cytosol transfer of $G_{11}\alpha$. The low-density membranes represent a heterogeneous mixture of membrane structures (endosome, endoplasmic reticulum, golgi) which has previously been suggested to contribute to internalization of hormone receptors following agonist challenge (Harden *et al.*, 1980; Waldo *et al.*, 1983; Strader *et al.*, 1984; Kassis *et al.*, 1986), again as part of mechanisms designed to regulate cellular sensitivity to agonists (Hausdorff *et al.*, 1990; Kobilka *et al.*, 1992; Zastrow *et al.*, 1992).

The results presented in this study demonstrate a complex pattern of cellular G-protein redistribution following agonist-induced activation of receptors which may be a complex as that of the G-protein-linked receptors themselves. They also provide the prospect that species variants of G-proteins may display subtle differences in their regulation by receptors, a concept which may indicate that greater care should be afforded the choice of genetic background for detailed analysis of cell signalling properties of transfected receptor. The detailed mechanisms responsible for agonist-induced cellular G-protein redistribution will be considered as the basis of future studies.

Chapter 5.

Conclusions.

This chapter summarise the major observations of this thesis.

G-protein-linked transmembrane cellular signalling cascades include a receptor, G-protein and an effector. The studies presented in this thesis focus on agonist-induced signal transduction via G-proteins which result in the stimulation of adenylyl cyclase and stimulation of phospholipase C activity.

Although the basic characteristics of agonist-mediated second messenger generation has been widely studied little was known about the levels of expression of each polypeptide component in individual cells, their cellular disposition or the stoichiometry of their interactions. In chapter 3, neuroblastoma x glioma hybrid, NG108-15, cells (Hamprecht *et al.*, 1985) have been studied as a model system to analyse signal transduction by the adenylyl cyclase cascade as these cells express a considerable range of receptors which act in both a stimulatory and inhibitory manner on the generation of cyclic AMP (Milligan *et al.*, 1990). In these cells, reduction in the levels of $G_s\alpha$ produced by treatment of the cells with the IP prostanoid receptor agonist, iloprost (McKenzie *et al.*, 1990) results in a heterologous desensitization of receptor-mediated generation of cyclic AMP. Amounts of the IP prostanoid receptor, $G_s\alpha$ and the functional complex of $G_s\alpha$ with adenylyl cyclase, which acts as the cyclic AMP generator, were measured in membranes of NG108-15 cells. As measured by the specific binding of [^{3}H]prostaglandin E1, the IP prostanoid receptor was present at some 100,000 copies/cell. $G_s\alpha$, assessed by quantitative immunoblotting with recombinantly expressed protein, was shown to be present in considerably higher levels (1,250,000 copies/cell). The stimulation of specific high-affinity binding of [^{3}H]forskolin as produced by either NaF or Gpp(NH)p to total membranes of and intact NG108-15 cells was used to attempt to define the maximal number of GSAC complexes that can be formed in these cells. However, the maximal formation of a complex of $G_s\alpha$ and adenylyl cyclase represented only some 17,500 copies/cell. The previous established 8:1 stoichiometry of concurrent down-regulation of $G_s\alpha$ and IP prostanoid receptor in these cells (Adie *et al.*, 1992) indicates that full-agonist occupation of the receptor should be able to activate some 65% of the expressed $G_s\alpha$.

Despite the potential 70 fold excess of $G_s\alpha$ over the $G_s\alpha$/adenylyl cyclase complex, IP prostanoid receptor agonist-mediated reduction of $G_s\alpha$ levels by 35% resulted in a 25% reduction in the maximal formation of $G_s\alpha$/adenylyl cyclase complex. Alousi *et al.*, 1991 have also presented a similar ratio of each component in S49 lymphoma cells, the absolute numbers of β_2-adrenoceptor: $G_s\alpha$: adenylyl cyclase being 1,500: 100,000: 3,500. In both NG108-15 and S49 lymphoma cells the measured levels of the adenylyl cyclase catalytic subunit are substantially lower than those of the corresponding G-protein, suggests that much of the cellular $G_s\alpha$ may not have access to adenylyl cyclase, which is likely to be the limiting component for information transfer.

I then utilized both wild type NG108-15 cells (Hamprecht *et al.*, 1985), which express endogenously a $G_s\alpha$-linked IP prostanoid receptor (Adie *et al.*, 1992), and clones derived from these cells transfected to express differing levels of the human β_2-adrenoceptor (Adie *et al.*, 1993; 1994 a, b), to examine agonist regulation of the interaction of $G_s\alpha$ and adenylyl cyclase.

Addition of the IP prostanoid receptor agonist iloprost to intact NG108-15 cells resulted in a dose-dependent increase in formation of the complex of $G_s\alpha$ and adenylyl cyclase (GSAC) as measured by the specific, high affinity binding of [^{3}H]forskolin. NG108-15 cells which were transfected to express either relatively high (clone βN22) or low (clone βN17) levels of the human β_2-adrenoceptor both showed dose-dependent increases in specific [^{3}H]forskolin binding in response to the β-adrenoceptor agonist isoprenaline and, maximally effective concentrations of isoprenaline resulted in the generation of close to the theoretically maximal number of GSAC complexes in both clones. The dose-effect curve in clone βN22, however, was some 15 fold to the left of that produced in clone βN17, similar to that noted for isoprenaline-mediated stimulation of adenylyl cyclase activity (Adie *et al.*, 1994b). In contrast, dose-effect curves for iloprost-stimulation of [^{3}H]forskolin binding were not different in clones βN22 and βN17.

Basal specific [^{3}H]forskolin binding, in the absence of agonist, was significantly greater in cells of clone βN22 than clone βN17. This was not a reflection of higher immunological levels of adenylyl cyclase in this clone, thus indicating that the elevated basal formation of GSAC is likely to be a reflection of empty receptor activation of $G_s\alpha$. This higher basal specific [^{3}H]forskolin binding was partially reversed by the β-adrenoceptor antagonist propranolol.

To assess whether IP prostanoid receptor-mediated stimulation of high affinity [^{3}H]forskolin binding in NG108-15 cells would be compromised by activation of a receptor that mediates inhibition of adenylyl cyclase in these cells, I observed that the δ opioid peptide DADLE, which mediates inhibition of adenylyl cyclase via the activation of $G_{i2}\alpha$ (McKenzie *et al.*, 1990a), was unable to reduce iloprost-stimulated [^{3}H]forskolin binding in clone βN22 cells.

Sustained exposure of clone βN22 cells to isoprenaline or iloprost resulted in a substantial and selective down-regulation of $G_s\alpha$. Agonist-mediated selective down-regulation of the G-protein(s) activated upon receptor occupancy is a well established phenomenon (Milligan *et al.*, 1993). Treatment of βN22 cells with the irreversible β-adrenoceptor antagonist bromoacetyl alprenolol menthane (BAAM) diminished both the potency and the maximal ability of isoprenaline but not of iloprost to cause $G_s\alpha$ down-regulation. These results demonstrate that the extent of agonist-mediated $G_s\alpha$ down-regulation is dependent upon the availability of receptor to agonist.

Receptor theory anticipates that the observed intrinsic activity and potency of agonist will also depend on the level of receptor expression and receptor reserve, and potentially on the level at which the transmitted signal is measured (Kenakin *et al.*, 1989). Whether measuring agonist-stimulation of adenylyl cyclase activity in membranes of these cells or agonist-stimulation of the formation of the complex of $G_s\alpha$ and adenylyl cyclase which acts as the high affinity binding site for [^{3}H]forskolin in whole cells, a series of β-adrenoceptor agonists, including dichloro-isoprenaline, ephedrine, dobutamine and salbutamol, displayed higher intrinsic activity and showed concentration-response curves which had substantially lower EC_{50} values in clone

βN22 compared to clone βN17. Furthermore, following elimination of smaller fractions of the receptor population by treatment of the cells with the irreversible antagonist BAAM, the agonist properties of drugs with lower intrinsic activity are reduced more than other drugs with greater intrinsic activity. In clone βN22 cells and membranes, reduction in the observed intrinsic activity for ephedrine required elimination of a smaller fraction of the β_2-adrenoceptor reserve than for salbutamol and reduction in the effect of the full agonists isoprenaline and epinephrine was only noted with high fractional elimination of the receptor pool. The effect of isoprenaline was substantially reduced, however, by BAAM treatment of clone βN17 cells, where β_2-adrenoceptor number was approaching extremely low levels.

In NG108-15 cells transfected to express varying levels of the human β_2-adrenoceptor, that maximal adenylyl cyclase activity can be achieved by occupation by isoprenaline of only some 200 fmol/mg membrane protein of this receptor. Expression of higher levels of the receptor do not result in greater adenylyl cyclase activity but only in a progressive shift in the agonist concentration-response curve to lower concentrations (Adie *et al.*, 1994b), indicative of the presence of spare receptors.

Increased expression of $G_s\alpha$ in this genetic backgound, as anticipated from the basal stoichiometries noted above, does not result in any significant alteration in the maximal effectiveness of the adenylyl cyclase cascade even though receptors can access and activate the introduced $G_s\alpha$ as effectively as the endogenous G-protein pool (Mullaney *et al.*, 1994).

To further extend such analyses, I isolated stable clonal cell lines following transfection of adenylyl cyclase type 2 (Feinstein *et al.*, 1991; Lustig *et al.*, 1993) into this genetic background and examined how this alters the effectivness of signal transduction from both endogenously expressed and stably transfected $G_s\alpha$-coupled receptors in clones in which the total adenylyl cyclase content of the cell was increased by up to ten fold as assessed by guanine nucleotide-stimulated specific high affinity binding of [^{3}H]forskolin to cellular membranes.

Basal adenylyl cyclase activity was markedly elevated compared to a clone expressing similar levels of the β_2-adrenoceptor in the absence of adenylyl cyclase type 2. Each of NaF, forskolin and the poorly hydrolysed analogue of GTP, Gpp(NH)p, produced substantially higher levels of adenylyl cyclase activity in membranes of the clones positive for expression of adenylyl cyclase type 2 than achieved with the parental cells. Both isoprenaline, acting at the introduced β_2-adrenoceptor, and iloprost, acting at the endogenously expressed IP prostanoid receptor, stimulated adenylyl cyclase activity to much higher levels in the clones expressing adenylyl cyclase type 2 compared to the clone lacking this adenylyl cyclase; however, the concentration-effect curves for adenylyl cyclase stimulation to these two agonists were not different between parental and adenylyl cyclase type 2 expressing clones. A maximally effective concentration of the β-adrenoceptor partial agonist ephedrine displayed similar efficacy and potency to stimulate adenylyl cyclase in membranes of clones with and without adenylyl cyclase type 2. Both secretin and NECA (acting at an endogenous A_2 adenosine receptor) were also able to produce substantially greater maximal activations of adenylyl cyclase in the clones expressing excess adenylyl cyclase type 2 without alterations in agonist efficacy or potency. These results demonstrate that the maximal output of the stimulatory arm of the adenylyl cyclase cascade can be increased by increasing total levels of adenylyl cyclase in the genetic background of NG108-15 cells. This is in contrast to increasing levels of receptor expression which results in increases in potency but not maximal effectiveness of full agonist ligands (Adie *et al.*, 1994b).

Agonist-induced specific down-regulation of the pertussis-toxin-insensitive phospholipase C-linked G-proteins $G_q\alpha$ and $G_{11}\alpha$ were observed in human embryonic kidney (HEK-293) cells which express stably the long splice variant form of the rat thyrotropin releasing hormone (TRH) receptor.

The established mechanism of action of TRH is via activation of a phospholipase C to generate inositol 1,4,5 trisphosphate and hence release Ca^{2+} from intracellular stores to raise the cytoplasmic concentrations of this ion. It has been noted

however, at least in pituitary GH_3 cells which are one of the most popular model systems for the study of TRH receptor action, that TRH is also able to activate adenylyl cyclase (Paulssen *et al.*, 1992). It has been realised that GH_3 cells express at least two separate isoforms of the TRH receptor which appear to arise via differential splicing of pre-mRNA transcribed from a single gene (de la Pena *et al.*, 1992a). The modifications occur in the C-terminal tail of the receptor after the 7th transmembrane helix. Whilst the third intracellular loop of G-protein-coupled receptors is a key determinant for contacts with G-proteins, both the C-terminal region and the second intracellular loop have been implicated in contributing to the specificity of such interactions. It is thus possible that the multiple signalling effects of TRH noted in pituitary GH_3 cells are actually produced by pharmacologically similar but genetically distinct receptors.

In addition, previous studies have not been able to assess whether both $G_q\alpha$ and $G_{11}\alpha$ are involved in this process and whether different splice variants of the receptor selectively interact with different G-proteins to regulate different signal transduction cascades as has recently been indicated for both the EP3 prostanoid receptor where, like the TRH receptor, the splice vairants occur in the C-terminal tail (Namba *et al.*, 1993) and the PACAP receptor (Spengler *et al.*, 1993).

In chapter 4, I have demonstrated that activation of the long isoform of the TRH receptor caused a large stimulation of production of inositol phosphates in a manner which was insensitive to pretreatment of the cells with pertussis toxin. In contrast, TRH did not produce either activation of basal adenylyl cyclase or inhibition of forskolin-amplified adenylyl cyclase activity despite the fact that TRH has been reported also to stimulate adenylyl cyclase activity via activation of $G_s\alpha$ and to stimulate L-type Ca^{2+} channels via activation of $G_{i2}\alpha$. The effect of TRH on inositol phosphate production in E2 HEK-293 cells was some 10 fold less potent than in GH_3 cells and was completely attenuated by pretreatment of the cells with TRH.

Sustained exposure of E2 HEK-293 cells to TRH resulted in a substantial reduction in cellular levels of $G_q\alpha$-like immunoreactivity from some 12 to 5 pmol/mg

of membrane protein without significant alterations in cellular levels of the α subunits of $G_s\alpha$ (22 pmol/mg membrane protein) and $G_{i2}\alpha$, the G-proteins involved respectively in stimulatory and inhibitory regulation of adenylyl cyclase, or $G_{i1}\alpha$, $G_{i3}\alpha$ and $G_o\alpha$. Dose-response curves indicated that half-maximal effects on cellular G_q-like immunoreactivity were produced by 50 nM TRH and time courses demonstrated that half-maximal loss in response to a maximally effective dose of TRH (10 μM) was produced by 3-4 h. Separation of clone E2 HEK-293 cell membranes in SDS-PAGE conditions able to resolve individual members of the G_q family demonstrated the presence of two related G-proteins. In membranes of E2 HEK-293 cells treated with TRH both of the expressed G_q-like G-proteins were observed to be down-regulated in parallel. The similarity of dose-response curves and time courses for loss of the two G-proteins indicates that the long isoform of the rat TRH receptor does not functionally select between these two transducer proteins. The differences in adenylyl cyclase and phospholipase C regulation in response to TRH in GH_3 cells and in E2 HEK-293 cells may reflect the co-expression of both long and short splice variants of the rat TRH receptor in GH_3 cells or the high steady-state levels (100 pmol/mg membrane protein) of $G_s\alpha$ and ratio of expression of G_s/G_q-like G-proteins in GH_3 cells (8: 1) in comparison to E2 HEK-293 cells (2: 1).

The isolation of cDNA species corresponding to $G_q\alpha$ and $G_{11}\alpha$ from a murine cDNA library has demonstrated these two G-proteins to be some 89% identical at the amino acid level (Strathmann *et al.*, 1990). In the case of $G_{11}\alpha$, cDNA clones from murine, human and bovine have 97% identity in amino acid sequence between these forms. Particularly high sequence conservation is noted in the C-terminal portion of these G-proteins which, at least by analogy with other G-proteins, provides the sites for interaction of the G-protein with both receptor and effector polypeptides (Wilkie *et al.*, 1991; Amatruda *et al.*, 1991; Kaziro *et al.*, 1990).

Both because of this high degree of sequence identity and because heterologous expression of G-protein-linked receptors into cell lines derived from different species regularly results in the expression of functionally coupled receptors,

there has been little consideration as to whether individual receptors might display a degree of selectivity of interaction if concurrently offered the choice between species variants of the same G-protein. Furthermore, experimental designs to approach this question have been difficult to develop. In chapter 4.3, however, I have developed a 6M urea containing SDS-PAGE system which allowed the concurrent detection and unambiguous assignment of identity to species isoforms of the phospholipase C-linked G-protein $G_{11}\alpha$.

In an attempt to address whether a single receptor type can utilise species variants of the same G-protein equivalently, clone E2M11 cells which express the long isoform of the rat TRH receptor and stably co-express the endogenous human and exogenously introduced murine $G_{11}\alpha$ were generated. The cellular distribution in unstimulated clone E2M11 cells of human and murine $G_{11}\alpha$ was identical and the introduced murine $G_{11}\alpha$ displayed the same ability as the human $G_{11}\alpha$ to be solublized by treatment with sodium cholate. However, although treatment of E2M11 cells with TRH results in a qualitatively similar pattern of cellular redistribution and down-regulation of the $G_{11}\alpha$ isoforms, quantitative analysis indicated that the exogenous murine isoform of $G_{11}\alpha$ was less effectively regulated by the long isoform of the rat TRH receptor than the endogenous human form of $G_{11}\alpha$. A redistribution of both α and β-subunits into low-density membrane fractions with transfer of α subunits only to the cytosol and a reduction in total cellular levels of these polypeptides was observed in response to TRH.

Chapter 6.

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