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STUDIES ON THE ACTIVATION OF G PROTEINS BY OPIOID RECEPTORS AND RECEPTOR-MIMETIC PEPTIDES

by

P. G. Szekeres

A Doctoral thesis submitted in partial fulfilment of the requirements for the award of

Doctor of Philosophy of Loughborough University of Technology

October 1995

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Title: *Studies on the activation of G proteins by opioid receptors and receptor-mimetic peptides*

Philip Graham Szekeres

Key Words: Opioid receptors, G proteins, [^{35}S]GTPγS binding, receptor-mimetic peptides, mastoparan, cloned receptors, SH-SY5Y cells, NG108-15 cells, CHO cells

Abstract

The activation of G proteins by activated-receptor mimics or following agonist occupation of μ- and δ-opioid receptors has been studied in membranes from cultured cells.

In SH-SY5Y cell membranes, the wasp venom peptide mastoparan (70 μM) and mastoparan 7 (25 μM), but not mastoparan 17, activated G proteins as determined by a 3-fold increase in low K_m GTPase activity, though at higher concentrations inhibition of GTPase activity was observed. The μ-opioid peptide DAMGO (1 μM) also caused a 24% stimulation of low Km GTPase activity. In contrast, spermine, benzalkonium chloride, compound 48/80 and octadecyltrimethylammonium bromide were all unable to stimulate low K_m GTPase activity. The results indicate that a native membrane environment confers a degree of selectivity to G protein activation.

An increase in binding of the stable GTP analogue [^{35}S]GTPγS stimulated by agonist occupation of the δ-opioid receptor in NG108-15 membranes has been demonstrated. This effect was critically dependent on the presence of Mg^{2+} and GDP and was improved by Na^+. The stimulation of [^{35}S]GTPγS binding was naloxone-reversible and was completely blocked by pretreatment of cells with pertussis toxin (100 ng/ml).

Stimulation of [^{35}S]GTPγS binding by δ-agonists was determined in CHO cells expressing the mouse δ-opioid receptor. The maximal stimulation produced by δ-opioid agonists was low compared to that seen in NG108-15 membranes. In addition DPDPE caused only a small inhibition of forskolin-stimulated adenylyl cyclase activity. The receptor number (B_{max}) as determined

by [^{3}H]-diprenorphine binding in these cells was 404±23 fmol / mg of membrane protein compared to 183±45 fmol / mg when determined with [^{3}H]-DPDPE. These data suggest many δ-opioid receptors exist in an uncoupled and hence low-affinity form in CHO cells.

Two peptides derived from the third intracellular loop of the human μ-opioid receptor failed to stimulate low K_m GTPase activity in SH-SY5Y membranes. However, these peptides did block both μ-receptor-mediated [^{35}S]GTPγS binding in SH-SY5Y membranes and δ-receptor-mediated [^{35}S]GTPγS binding in NG108-15 membranes, indicating a possible role for this domain of the receptor in G protein-coupling, although the possibility of competitive antagonism at the opioid receptor could not be eliminated. A polyclonal antibody to the C-terminal 35 amino acid residues of the cloned mouse δ-opioid receptor did not block δ-opioid receptor-mediated [^{35}S]GTPγS binding in NG108-15 membranes.

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There are many people whose guidance, support and friendship have been greatly appreciated over the past three years. Firstly I would like to thank Dr John Traynor for his continual encouragement, original scientific ideas and friendly, approachable attitude. I must also thank Prof Frank Wilkinson for understanding why I wished to change my course of study, and for giving me the opportunity to do so.

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Finally, my best and very special friend Helen. For caring, listening, helping, understanding and being there when needed. Especially for your continual love - Thank you.

TABLE OF CONTENTS

Abbreviations

AA	arachidonic acid
ADP	adenosine diphosphate
amino acids	see page iii
AMP-PNP	5'-adenylylimidodiphosphate
ATP	adenosine triphosphate
BAC	benzalkonium chloride
BNTX	7-benzylidenenaltrexone
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	circular dichroism
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CI977	5R-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro-[4,5]dec-8-yl]-4-benzofuranacetamide monohydrochloride
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 [Disulphide bridge: 2-7]
DADLE	[D-Ala^2,D-Leu^5]enkephalin
δC-Ant	polyclonal antibody raised against the C-terminal 35 amino acids of the cloned mouse δ-opioid receptor
DAG	diacylglycerol
DAMGO	[D-Ala^2,MePhe^{4}Gly(ol)^5]enkephalin
DELTI	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
DELT II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulphoxide
DOR-1	cDNA encoding the cloned mouse δ-opioid receptor [Evans *et al*, 1992]
DPDPE	[D-Pen^2,D-Pen^5]enkephalin
DPN	diprenorphine
DSLET	[D-Ser^2,Leu^5,Thr^6]enkephalin
EDTA	ethylenediaminetetraacetic acid
G protein	guanosine triphosphate binding protein
GDP	guanosine diphosphate
GDPβS	guanosine 5'-[β-thio]diphosphate

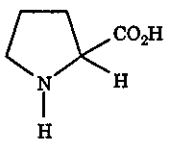
GMP	guanosine monophosphate
GTP	guanosine triphosphate
GTPγS or GTP[S]	guanosine 5'-[γ-thio]triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IBMX	3-isobutyl-1-methylxanthine
ICI 174864	N,N-diallyl-Tyr-Aib-Aib-Phe-Leu (Aib = α-aminoiso-butyric acid)
IP_3	inositol triphosphate
[Leu^5]enkephalin	leucine enkephalin (Tyr-Gly-Gly-Phe-Leu)
MEM	minimum essential medium
[Met^5]enkephalin	methionine enkephalin (Tyr-Gly-Gly-Phe-Met)
$\mu il3_{269-283}$	Gly-Ser-Lys-Glu-Lys-Asp-Arg-Asn-Leu-Arg-Arg-Ile-Thr-Arg-Met
$\mu il3_{277-283}$	Leu-Arg-Arg-Ile-Thr-Arg-Met
NAD	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
NTB	naltriben
OTAB	octadecyltrimethylammonium bromide
PBS	phosphate buffered saline
PD117302	(±)-trans-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzo-[β]thiophene-4-acetamide
PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
P_i	orthophosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
Rh	rhodopsin
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SKF10047	N-allylnormetazocine
TIPP	Tyr-Tic-Phe-Phe (Tic = tetrahydroisoquinoline-3-carboxylic acid)
Tris	tris-[hydroxymethyl]-aminomethane
U69593	(5α,7α,8β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide
UDP	uridine diphosphate

Amino acid structures

Structure and coding of amino acids of the general structure: $H_2N\text{-}CH(R)\text{-}CO_2H$

Amino acid	Three letter symbol	Single letter symbol	-R
Alanine	Ala	A	$-CH_3$
Arginine	Arg	R	$-(CH_2)_3NHC(=NH)NH_2$
Asparagine	Asn	N	$-CH_2CONH_2$
Aspartic acid	Asp	D	$-CH_2CO_2H$
Cysteine	Cys	C	$-CH_2SH$
Glutamine	Gln	Q	$-(CH_2)CONH_2$
Glutamic acid	Glu	E	$-(CH_2)_2CO_2H$
Glycine	Gly	G	-H
Histidine	His	H	$-CH_2$(4-imidazolyl)
Isoleucine	Ile	I	$-CH(CH_3)CH_2CH_3$
Leucine	Leu	L	$-CH_2CH(CH_3)_2$
Lysine	Lys	K	$-(CH_2)_4NH_2$
Methionine	Met	M	$-(CH_2)_2SCH_3$
Phenylalanine	Phe	F	$-CH_2Ph$
Proline	Pro	P	*
Serine	Ser	S	$-CH_2OH$
Threonine	Thr	T	$-CH(CH_3)OH$
Tryptophan	Trp	W	$-CH_2$(3-indolyl)
Tyrosine	Tyr	Y	$-CH_2$(4-hydroxyphenyl)
Valine	Val	V	$-CH(CH_3)_2$

* Proline is an imino acid of the structure:



List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 6

List of Tables

"Everything is a vague to a degree you do not realise
until you have tried to make it precise"

B. Russell

Logic and knowledge:
essays 1901-1950

Chapter 1

GENERAL INTRODUCTION

1.1 Historical perspective.

Pain continues to be one of the most challenging problems facing medical science. In its simplest terms pain can be defined as an unpleasant sensory experience, but it clearly also has emotional elements. It is usually, but not always, caused by intense noxious stimulation which is interpreted as evidence of actual or potential tissue damage. Morphine and related analgesics have the unique ability to selectively relieve the subjective component of pain without affecting the primary sensory modalities, such as touch, vision and hearing. In addition, this class of compounds alleviate the anxiety, tension and fear often associated with intense pain, making them the preferred type of drug for severe pain.

For these reasons, the history of opium predates the written word. However, knowledge of the constituents of opium dates back less than 200 years to 1806, when the active component, named morphine (Fig. 1.1), was isolated by Sertürner from the opium poppy (*Papaver somniferum*).

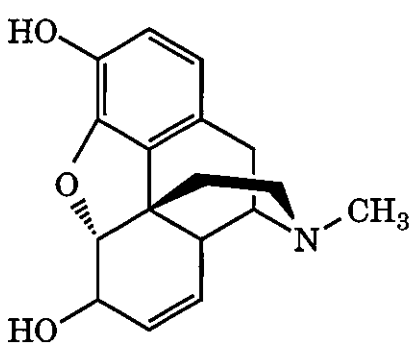


Figure 1.1 Morphine

An understanding of the effects of morphine on a molecular level advanced rapidly in 1973, when several laboratories succeeded almost simultaneously in showing that there were stereospecific opiate alkaloid

binding sites in the central nervous system [Pert and Snyder, 1973; Simon *et al*, 1973; Terenius, 1973].

Since the existence of specific opiate binding sites had been demonstrated, it was reasoned that these proteins might be the targets for endogenous opiate neurotransmitters. In 1975, John Hughes and Hans Kosterlitz [Hughes, 1975; Hughes *et al*, 1975 a,b] isolated two pentapeptides from porcine brain which differed only in the carboxy-terminal amino acid and were named methionine enkephalin ([Met^5]enkephalin) and leucine enkephalin ([Leu^5]enkephalin) (Fig. 1.2). These compounds became known as the endogenous opioids.

(a) Tyr-Gly-Gly-Phe-Met (b) Tyr-Gly-Gly-Phe-Leu

Figure 1.2 (a) [Met^5]enkephalin and (b) [Leu^5]enkephalin.

These peptides interacted selectively with opioid receptors *in vitro*. Furthermore, it soon became apparent that the [Met^5]enkephalin sequence was present on the N-terminus of another endogenous molecule, β-endorphin [Bradbury *et al*, 1976] (Fig. 1.3), a fragment of β-lipotropin that had been isolated several years earlier from pituitary extracts. Like the enkephalins, β-endorphin proved to have a high affinity for brain opioid receptors.

Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-
Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu.

Figure 1.3 β-endorphin (human).

The dynorphins, another group of peptides, which structurally are extensions of [Leu^5]enkephalin, were identified in 1979 [Goldstein *et al*, 1979; Goldstein *et al*, 1981] (Fig. 1.4).

Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln

Figure 1.4 Dynorphin A

The latest family of endogenous opioid peptides to be characterised, the deltorphins, were isolated from the skin of the frogs *Phyllomedusa sauvagei* and *Phyllomedusa bicolor* [Kreil *et al*, 1989; Erspamer *et al*, 1989] (Fig. 1.5).

Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2

Figure 1.5 Deltorphin

The existence of many endogenous ligands supported earlier suggestions that there may be more than one type of opioid receptor. The first conclusive evidence for this was provided by Martin *et al* (1976). These workers examined several opiate drugs in the tolerant chronic spinal dog and looked for "cross-tolerance" among these compounds i.e. the ability of a compound to prevent withdrawal symptoms after removal of a drug from an animal tolerant to its effects. Results from these studies showed that certain compounds did not substitute for each other in the prevention of withdrawal symptoms, and thus suggested the existence of three types of receptor, named after the drugs used in the study: μ (*m*orphine), κ (*k*etocyclazocine) and σ (*S*KF 10,047 or N-allylnormetazocine). However, opioid receptors are defined by their sensitivity towards the antagonist naloxone (Fig. 1.6). Since naloxone has no affinity for the σ-receptor, and the requirement for opiate affinity at the σ-receptor shows the opposite enantioselectivity from opioid receptors, σ-receptors are no longer considered opioid [Walker *et al*, 1990].

Another opioid receptor, the δ-receptor, was proposed by Kosterlitz and colleagues [Lord *et al*, 1977], after comparing the effects of morphine and the enkephalins in inhibiting electrically-induced contractions of the guinea pig ileum and mouse vas deferens. They found that there was no correlation in the rank order of potency in the two bioassay systems. Furthermore, the actions of enkephalins on the vas deferens were relatively insensitive to naloxone (Fig. 1.6).

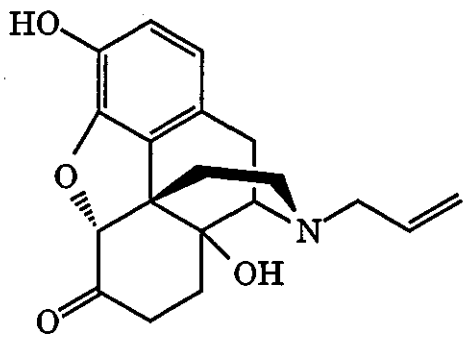


Figure 1.6 Naloxone

Of the endogenous ligands, the dynorphin-related peptides appear to bind principally to κ-receptors [Chavkin *et al*, 1982], whilst the deltorphins, as their name implies, are highly selective δ-receptor agonists [Kreil *et al*, 1989; Erspamer *et al*, 1989]. However, the majority of endogenous opioids are not particularly receptor selective. Therefore, much effort has been put into synthesising opioid receptor specific agonists and antagonists, resulting in additional prototypic ligands for each receptor type (Table 1.1).

In an attempt to understand the pharmacology of opioid receptors on a molecular level, many laboratories set out to purify opioid receptors to homogeneity. However, progress in this area lagged considerably behind that for other cell surface receptors, due perhaps to the relative paucity of opioid receptors in most tissues, and their lability after detergent solubilisation [Loh and Smith, 1990], the conventional first step in most purification schemes. For this reason, the structure of opioid receptors remained unknown until 1992, when two groups published descriptions of the expression cloning of cDNAs encoding the δ-receptor from the neuroblastoma x glioma (NG108-15) cell line [Evans *et al*, 1992; Kieffer *et al*, 1992].

Receptor	Agonist	Antagonist
μ	DAMGO fentanyl morphiceptin	CTOP cyprodime
δ	DPDPE DSLET DELT I & DELT II DADLE	ICI 174864 naltrindole TIPP NTB BNTX
κ	U69593 CI977 PD117302	nor-binaltorphamine

Table 1.1 Opioid receptor ligand specificity (explanations of abbreviations may be found on page (i) at the front of the thesis).

1.2 Molecular cloning of the opioid receptors.

The δ-receptor cloned by the groups of both Evans and Kieffer (DOR-1) [Evans *et al*, 1992; Kieffer *et al*, 1992] is a single polypeptide chain consisting of 372 amino acids. As expected, it binds δ-opioid ligands such as the peptides [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and [D-Ala^2,D-Leu^5]enkephalin (DADLE) as well as other δ-specific ligands with high affinity. It has stereospecific requirements for binding and a marked preference for (-)-naloxone and levorphanol over their enantiomers (+)-naloxone and dextrophan. Furthermore forskolin-stimulated cyclic adenosine monophosphate (cAMP) accumulation in COS cells expressing DOR-1 is inhibited by both the non-selective alkaloid etorphine and the δ-selective peptide DPDPE, illustrating functional coupling of the cloned receptor to the effector enzyme adenylyl cyclase.

The description of a cloned δ-receptor marked the beginning of a race for the characterisation of additional members of the opioid receptor family. Within twelve months of the original reports, the molecular cloning of the rat μ-opioid receptor [Chen et *al*, 1993; Fukuda *et al*, 1993; Wang *et al*, 1993], rat κ-opioid receptor [Chen *et al*, 1993b; Minami *et al*, 1993; Li *et al*, 1993; Meng *et al*, 1993], mouse κ-opioid receptor [Yasuda *et al*, 1993] and rat δ-opioid receptor [Fukuda *et al*, 1993] had been reported. More recently, the amino acid sequences of the human μ- [Wang *et al*, 1994], δ- [Knapp *et al*, 1994; Simonin *et al*, 1994] and κ- [Simonin *et al*, 1995] opioid receptors have been published. The amino acid sequences of the three opioid receptor types are shown in Fig. 1.7.

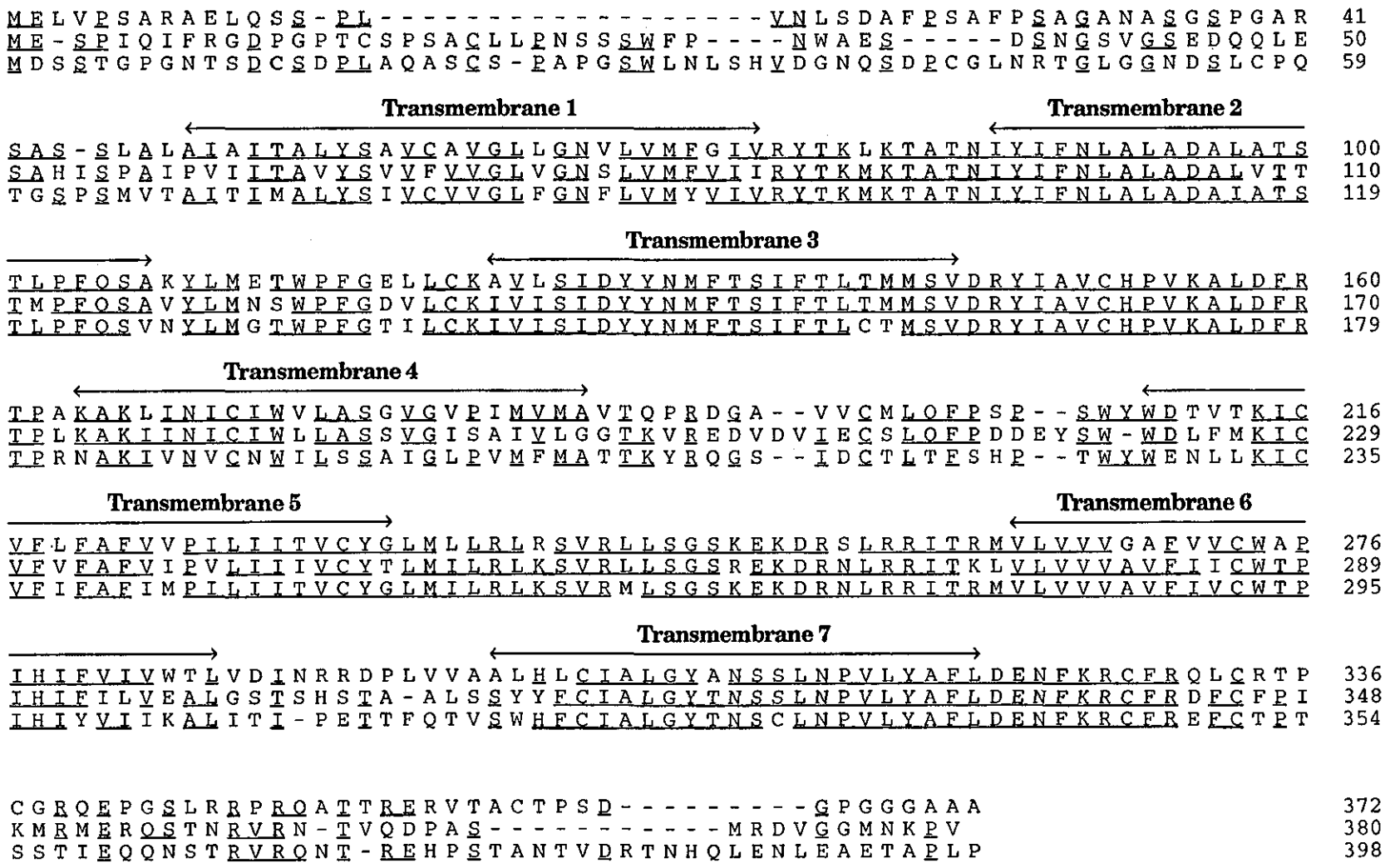


Figure 1.7 : Sequence alignment of the mouse δ [Evans *et al*, 1992], mouse κ [Yasuda *et al*, 1993] and rat μ [Chen *et al*, 1993] opioid receptors. Underlined regions indicate conserved amino acids beween the receptors. (Adapted from Reisine and Bell, 1993).

The cloned mouse δ- and κ- and rat μ-opioid receptors share a high degree of sequence similarity, with approximately 50% of the residues being identical [Reisine and Bell, 1993]. Greatest homology is seen in intracellular loops 1 (90%) and 2 (91%), with the third intracellular loop showing the least sequence homology (78%) of these three regions. The amino acid sequences of the transmembrane domains, with the exception of transmembrane region 4, are also highly conserved. Extracellular loops 2 and 3 show very little sequence homology, whilst extracellular loop 1 is somewhat more conserved. It has been suggested that the divergent structures of the extracellular loops may be vital for ligand binding, and therefore explain ligand selectivity profiles [Meng *et al*, 1994]. The amino- and carboxy-termini show very little sequence or size homology.

Hydropathicity analysis [Kyte and Doolittle, 1982] of the cloned opioid receptors indicates that they belong to the superfamily of G protein-coupled 7-transmembrane domain receptors (Fig. 1.8). Thus, as expected, all three receptors share a distinct set of structural features common to this family of receptors as outlined in more detail in the following section.

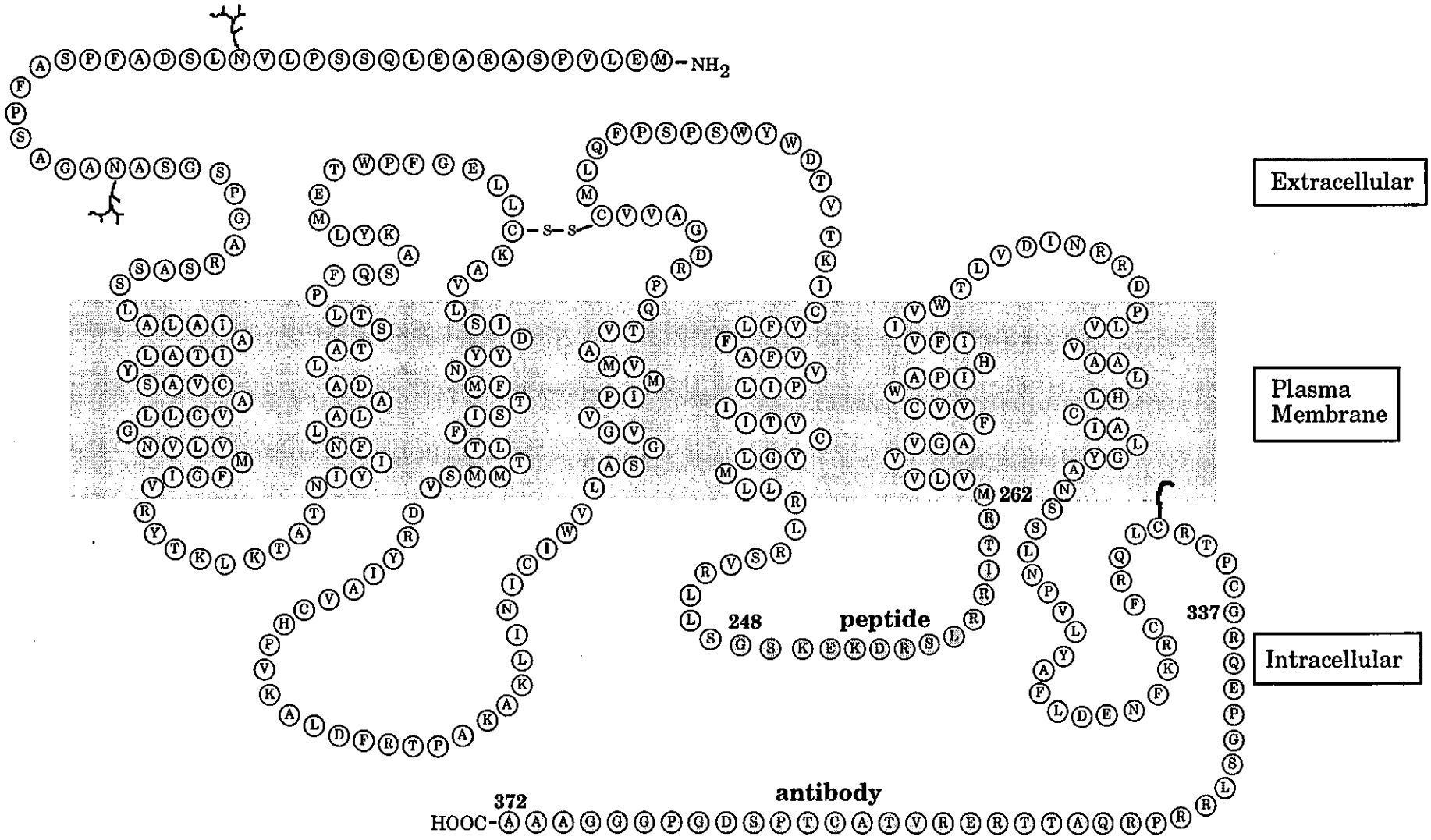


Figure 1.8 Cartoon depicting the proposed seven-transmembrane-spanning domains of the mouse δ-opioid receptor showing predicted sites of glycosylation on the extracellular surface and palmitoylation on the intracellular surface. Shaded amino acids sequences 248-262 and 337-372 represent domains from the receptor investigated in this thesis using peptide and antibody strategies respectively. Amino acid sequence data and proposed membrane topology was obtained from the European Molecular Biology Laboratory, Heidelberg, Germany.

1.3 Structural features of G protein-coupled receptors.

Despite the wide variety of agonists that exist which stimulate diverse second-messenger pathways, G protein-coupled receptors share considerable structural homology, reflecting their common mechanism of action. More than 100 members of this large family have now been cloned. Sequence determination and hydropathicity analysis [Kyte and Doolittle, 1982] shows that they are all characterised by seven hydrophobic stretches of 20-28 amino acids, which by comparison with the known structure of bacteriorhodopsin [Henderson and Unwin, 1975] are predicted to form transmembrane α-helices, connected by alternating extracellular and intracellular loops (shown schematically for the mouse δ-opioid receptor in Fig. 1.8). The amino terminus of the protein chain is always extracellular and the carboxy terminus intracellular.

G protein-coupled receptors are glycoproteins. All of the receptors cloned to date have at least one consensus sequence for N-linked glycosylation (Asn-Xaa-Ser/Thr) contained within their extracellular domains. These putative glycosylation sites are usually located near the N-terminus of the protein, although occasionally there are potential sites in the second extracellular loop. The three opioid receptors shown in Fig. 1.7 have a variable number of possible sites for N-linked oligosaccharide addition. There are two potential sites in the δ- and κ-receptors and five in the μ-receptor. This implies that the receptors could have wide ranging apparent molecular weights as determined on polyacrylamide gels. Furthermore, it is also possible that there may be tissue-specific differences in the glycosylation pattern of these proteins. Prevention of receptor glycosylation by site-directed mutagenesis of the potential glycosylation sites on the receptor, results in a decrease in the level of expression of the β-adrenergic receptor on the cell surface [Rands *et al*, 1990]. However, lack of glycosylation does not seem to have a dramatic effect on the ligand-binding or functional activity of the β-adrenergic [Rands *et al*, 1990] or the neurokinin-1 [Fong *et al*, 1992] receptors.

G protein-linked receptors also contain a number of conserved cysteine residues, some of which appear to play an important role in receptor structure. There are two highly conserved cysteine residues in the first and second extracellular loops of the receptors, (Cys140 and Cys217 in the μ-receptor, Cys121

and Cys198 in the δ-receptor, Cys131 and Cys210 in the κ-receptor (Fig. 1.7). Biochemical analysis of rhodopsin showed that these two cysteine residues are involved in an intramolecular disulphide bridge that links the first and second extracellular loops of the receptor, thus constraining the conformation of the extracellular domain of the protein [Karnik *et al*, 1988]. Substitution of either of these cysteine residues with valine by site-directed mutagenesis results in alterations of the binding characteristics of β-adrenergic receptors [Dixon *et al*, 1987; Dohlman *et al*, 1990], suggesting a role for these residues in maintaining the active binding conformation of the receptor.

The carboxy-terminal tail of many G protein-coupled receptors also contains a highly conserved cysteine residue. This residue has been shown to be palmitoylated in α- and β-adrenergic receptors [O'Dowd *et al*, 1989; Kennedy and Limbird, 1993] and in rhodopsin [Papac *et al*, 1992]. The function of this palmitoyl group is not clear, but it has been speculated that it may anchor a part of the cytoplasmic tail of the receptor to the plasma membrane, thus controlling the tertiary structure of this region of the receptor, as indicated in Fig. 1.8.

Many G protein-coupled receptor ligands, for example acetylcholine, noradrenaline and 5-hydroxytryptamine, contain an amino group. Receptors for such ligands contain conserved aspartate residues in the third transmembrane domain, which provides a counter-ion for the amine group of the ligand [Dohlman *et al*, 1991; Strader *et al*, 1989]. Surratt *et al* (1994) have shown that alanine or asparagine substitution of this residue (Asp147) in the human μ-opioid receptor diminishes both receptor binding and agonist-stimulated inhibition of the effector enzyme adenylyl cyclase. Interestingly, substitution with glutamate causes a decrease in receptor binding, but still allows full inhibition of adenylyl cyclase at high concentrations of agonist.

A highly conserved aspartate residue also exists in transmembrane helix 2. Mutation of this residue (Asp95) to asparagine in the cloned mouse δ-opioid receptor reduces agonist affinity for δ-receptor-selective agonists such as DPDPE and [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET), but does not affect binding of δ-receptor-selective antagonists such as naltrindole or non-selective opioid agonists, such as bremazocine and buprenorphine [Kong *et al*, 1993]. Substitution of this residue also diminishes Na^+ regulation of agonist binding, as found earlier for the α_2-adrenergic receptor [Horstman *et al*, 1990]. Similarly,

alanine replacement of the equivalent aspartate residue (Asp114) in the human μ-opioid receptor causes a reduction in the binding affinity for DAMGO, morphine and naloxone [Surratt *et al*, 1994].

Another highly conserved sequence of residues amongst G protein-coupled receptors is Asp-Arg-Tyr, which occurs at the amino terminus of the second putative cytoplasmic domain [Probst *et al*, 1992]. Whereas the arginine residue in this triplet is invariant, there are a few instances in which the aspartate and tyrosine residues are conservatively substituted. Point mutation of the arginine in this triplet to asparagine results in a decrease of the coupling of m1 muscarinic receptors to activation of phosphoinositide hydrolysis, [Zhu *et al*, 1994]. Mutation of the aspartate in the sequence of the m1 muscarinic receptor [Fraser *et al*, 1989], or the α_{2A}-adrenergic receptor [Wang *et al*, 1991], decreases the potency of agonists in stimulating phosphatidyl inositol hydrolysis or in inhibiting adenylyl cyclase activity respectively, without a change in the maximal response. In contrast, there is an almost complete loss of coupling of β_2-adrenergic receptors to activation of adenylyl cyclase after mutation of the corresponding aspartate residue [Fraser *et al*, 1988]. Thus this conserved triplet of amino acid residues may be of general importance for coupling to G proteins.

1.4 G proteins.

Recognition of an agonist by its receptor is only the first step in a signal transduction cascade which leads to a change in the levels of intracellular second messengers, or membrane conductance, and ultimately a cellular response. More than 100 receptors couple to their associated effector system(s) through approximately 20 distinct G proteins.

The sophistication with which G proteins mediate complex signalling functions derives from the distinct regulatory activities of their subunits. Each G protein is a heterotrimer of a guanine nucleotide binding α-subunit and regulatory β- and γ-subunits. βγ-dimers exist as a tightly associated complex functioning as a single unit, and cannot be separated under non-denaturing conditions. Different G proteins are most readily distinguished by their α-subunits which, by analysis of their amino acid sequences, fall into four major families, namely G_s, G_i, G_q and G_{12}. The functions of G protein α-subunit subtypes are incompletely known, and much current research aims simply to understand to which receptors and effectors they couple (Fig. 1.9).

Originally it was thought that α-GTP was the actual regulator of the target effector enzymes. However, more recently it has become apparent that the βγ-complex can also regulate the activity of effector proteins [Tang and Gilman, 1991; Birnbaumer, 1992; Taussig *et al*, 1993; Sternweis, 1994].

The behaviour of G protein-mediated signalling systems is determined by the kinetics of GTP binding and hydrolysis by the G protein α-subunit, which is illustrated schematically in Fig. 1.10. In the basal state, G proteins exist in their oligomeric form with GDP tightly bound to the α-subunit. Interaction of the G protein with an appropriately liganded receptor stimulates the dissociation of GDP, presumably as a result of a conformational change that results in an "opening" of the guanine nucleotide binding site [Bourne, 1993]. This interaction produces a high affinity state of the receptor for its agonist. In the absence of GTP, the agonist-receptor-G protein complex is relatively stable. However, in the presence of the relatively high cellular concentrations of GTP, the guanine nucleotide binding site is rapidly filled leading to subsequent

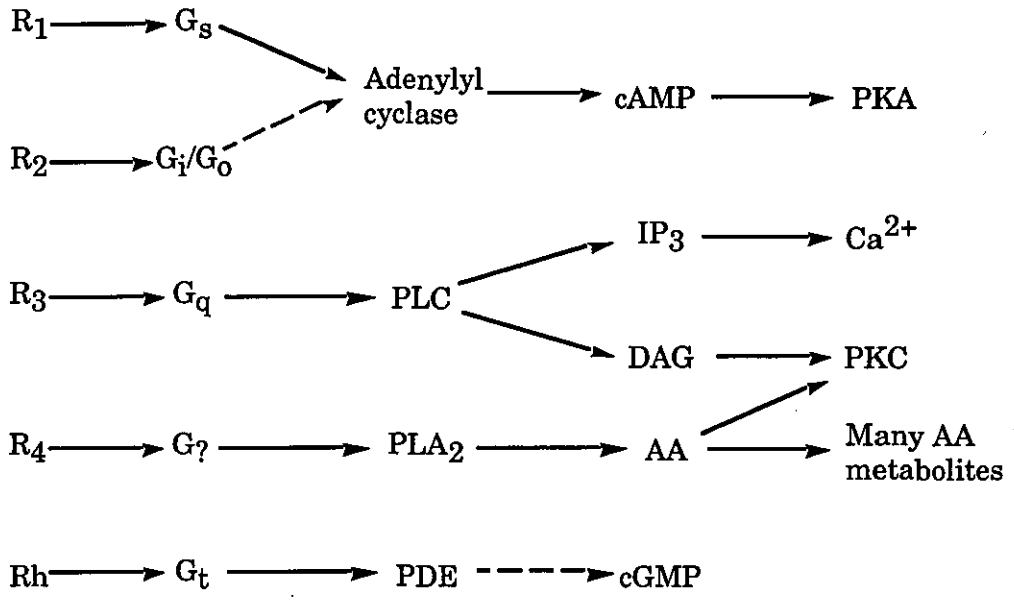


Figure 1.9: The best known second messenger pathways, showing the coupling of G_α subunits to effector systems. Each named macromolecule stands for a family of homologous proteins. Particularly the number of receptors coupling to each pathway is large. Dashed arrows are inhibitory actions. R, receptor; Rh, rhodopsin; PLC, phospholipase C; PLA_2, phospholipase A_2; PDE, phosphodiesterase; IP_3, inositol triphosphate; DAG, diacylglycerol; AA, arachidonic acid; PKC, protein kinase C (Adapted from Hille, 1992).

activation of the G protein. Such activation greatly reduces the affinity of the GTP-liganded α-subunit for the βγ-complex. The resulting dissociation of subunits is an important component of their mechanism, and gives rise to two units which can act on downstream targets. Upon activation, G protein also dissociates from the receptor, thus causing the receptor to return to the low affinity state for agonist.

Deactivation of the G protein is associated with the intrinsic GTPase activity of the α-subunit, which hydrolyses bound GTP to GDP + P_i, resulting in reassociation of the α- and βγ-subunits. Thus G proteins act as switches and timers, with the rate of GTP hydrolysis determining the time spent in the activated state.

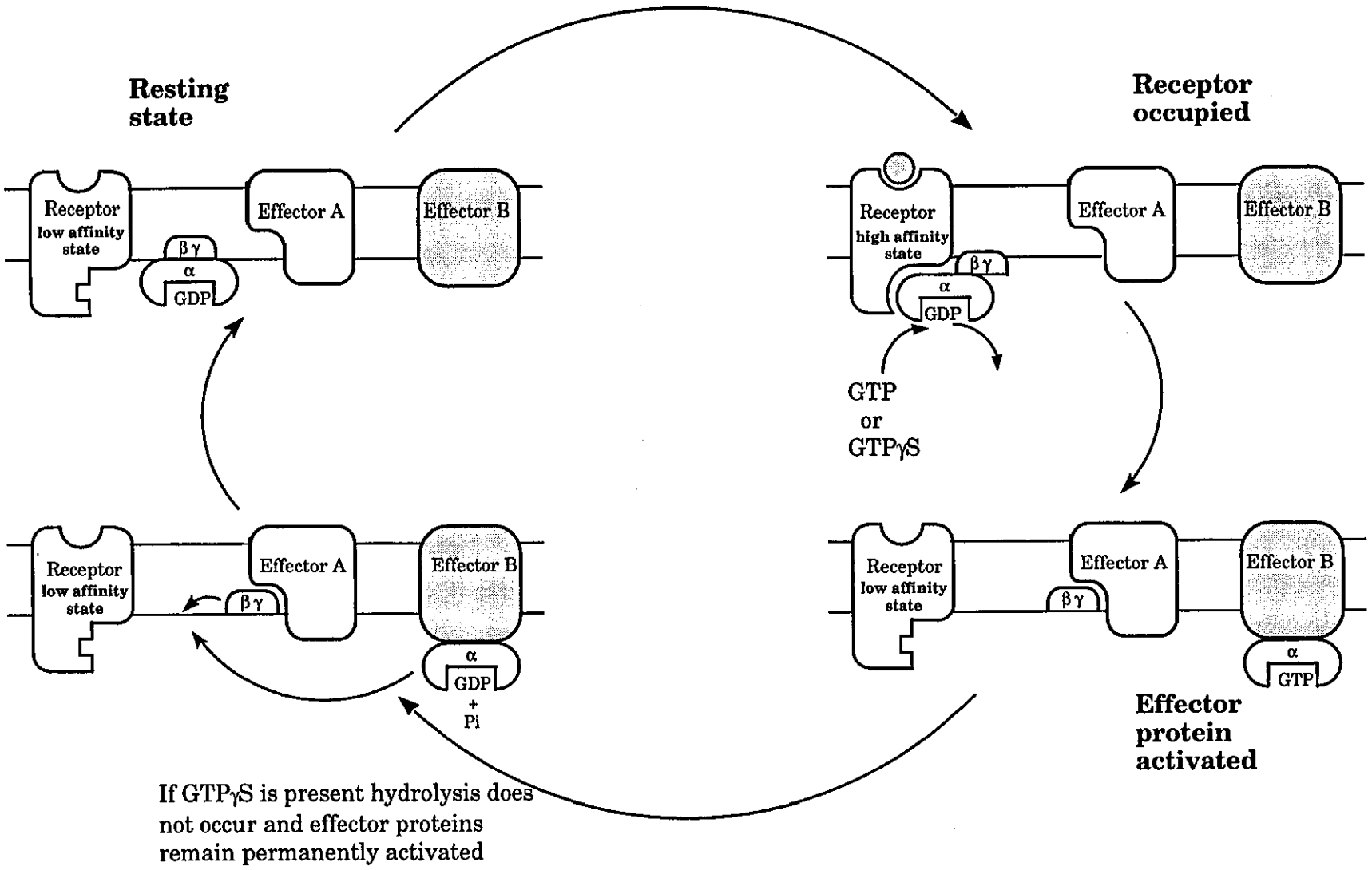


Figure 1.10 Cartoon depicting G protein-mediated transmembrane signalling. A detailed explanation is given in the text (Adapted from Rang and Dale, 1991).

Agonist-bound receptors are thought to act catalytically in this activation cycle, lowering the activation energy barrier which prevents spontaneous transition from inactive α-GDP to active α-GTP, doing so *via* the intermediary formation of the αβγ-trimer [Birnbaumer *et al*, 1990]. Extensive studies have been carried out to determine which of the steps involved in these transitions is rate-limiting, and hence the target of receptor activation. Individual reactions affected by receptors are (a) stimulation of GDP release and facilitation of GTP binding [Cassel and Selinger, 1978] and (b) facilitation of the transition from inactive to active conformation of the GTP-liganded G protein.

The α-subunits of many, but not all G proteins are substrates for bacterial toxins, and this has proven particularly useful in identifying the involvement of G proteins in specific signal transduction pathways. Cholera toxin, isolated from *Vibrio cholera*, catalyses the transfer of an ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) to a specific arginine residue on $G_s\alpha$ and $G_t\alpha$. Modification of these α-subunits by cholera toxin constitutively activates these proteins by inhibiting their GTPase activity. It is intriguing to note that all known G protein α-subunits contain a corresponding arginine residue in their amino acid sequence, although not every α-subunit is a substrate for cholera toxin in the resting state. Interestingly, however, agonist occupation of δ-receptors can promote cholera toxin-catalysed ADP-ribosylation of G_i in NG108-15 membranes [Milligan and McKenzie, 1988], and this has been exploited by Roerig *et al* (1992) to identify exactly which G proteins δ-receptors interact with in this cell line. These results can be explained by suggesting that this conserved arginine residue in $G_i\alpha$ is buried deep within the protein structure in the resting state, but upon activation of the G protein it becomes exposed and hence liable to modification by cholera toxin.

Similarly, pertussis toxin, isolated from *Bordetella pertussis*, ADP-ribosylates those α-subunits which possess a specific cysteine residue four amino acids from the carboxy terminus ($G_i\alpha$, $G_o\alpha$, $G_t\alpha$). This treatment does not produce permanent activation of G protein, but does block receptor-mediated activation of ADP-ribosylated G protein.

G proteins are bound to the inner face of the cytoplasmic membrane. The structurally homologous α-subunits of the G_i subfamily (α_i, α_o and α_z) are modified by myristate, bound in amide linkage at their amino-terminal glycine

residues [Yamane and Fung, 1993]. The transducin α-subunit, α_t, is also heterogeneously modified at the same site by a mixture of fatty acyl groups. Myristoylation increases the apparent affinity of modified α-subunits for βγ and for effector, and may also facilitate association of α with cellular membranes. Some G protein α-subunits, including G_s and G_q, are not myristoylated. However, members of these two G protein families are palmitoylated. The βγ-subunit is also bound strongly to the plasma membrane *via* an isoprenoid group attached to the γ-subunit. Attachment of the α-subunit to the cytoplasmic face may be aided by the βγ-complex [Sternweis, 1986].

A predicted α-helix region at the carboxy-terminus of the Gα-subunit is strongly suspected as a contact site for receptors. Several experimental observations support this:

(a) Synthetic peptides corresponding to the C-terminus of the G protein α-subunit can inhibit the interactions of G proteins with receptors [Hamm *et al*, 1988].

(b) Pertussis toxin catalysed ADP-ribosylation of G_i, G_o and G_t at a cysteine residue four amino acids from the C-terminus prevents receptor-mediated activation.

(c) The unc (uncoupled) mutation in S49 unc mutant cell membranes replaces Arg389 of $G_s\alpha$ by proline at a position six amino acid residues from the C-terminal end [Sullivan *et al*, 1987].

(d) The internal amino acid sequence of arrestin of retinal rod cells resembles the sequence of the C-terminus of $G_t\alpha$. Arrestin competes with $G_t\alpha$ for binding to phosphorylated photorhodopsin.

(e) Mastoparan antagonises the ability of a polyclonal antibody raised against the C-terminus of $G_i\alpha$ to recognise this protein [Weingarten *et al*, 1990].

The N-terminal region of Gα, which is thought to be in close proximity to the C-terminus [Deretic and Hamm, 1987; Mazzoni *et al*, 1991; Higashijima and Ross, 1991], has also been implicated as a region important for receptor coupling for the following reasons:

(a) An N-terminal transducin peptide inhibits interaction of G_t with photorhodopsin [Hamm *et al*, 1988].

(b) A chemically modified mastoparan analogue cross-links to a cysteine residue in the N-terminal region of G_{o2} [Higashijima and Ross, 1991]. It remains possible however, that mastoparan binds only to the C-terminus, but cross-links to the N-terminus due to its close proximity and the availability of a chemically reactive cysteine (Cys3) in the N-terminal region.

(c) In a similar manner, a photoaffinity-labelled α_2-adrenergic receptor third intracellular loop peptide cross links to the N-terminus on $G_o\alpha$. This peptide also cross-links to the $\beta\gamma$-subunits, suggesting a possible functional role of $\beta\gamma$ in the activation of G proteins [Taylor *et al*, 1994].

1.5 Activation of G Proteins by agonist-bound receptors.

One of the fundamental questions concerning G protein-coupled receptor pharmacology is how agonist binding alters the receptor conformation in such a way that nucleotide exchange is catalysed on G protein. At present, little is known of the structural changes that accompany these processes. Activated receptors catalyse release of GDP from the heterotrimer much more efficiently than from α–GDP alone [Florio and Sternweis, 1989], suggesting that the βγ-subunit plays a role in this process. A complete understanding of the mechanisms by which signals are conveyed from receptors through G proteins to effectors awaits elucidation of the three dimensional structure of all the components by X-ray crystallography. Knowledge of the structure of rhodopsin is already available [Schertler *et al*, 1993], although this paper only reported rather limited resolution (9Å). Therefore, many groups are using biochemical and molecular biological techniques to try to determine which areas of the receptor form important binding surfaces for the G protein.

1). Site-Directed Mutagenesis

Site-directed mutagenesis is by far the most commonly used technique to study functionally important areas of G protein-coupled receptors. Residues chosen for mutation are often highly conserved amongst many G protein-coupled receptors and are therefore likely to play an important role in the signal transduction process. Alternatively, amino acids may be replaced on the basis of their functionality and / or physical properties (for example basicity, aromaticity or lipophilicity), or because a similar residue in a related receptor has been shown to play an important role in G protein interaction. Where a mutation is chosen on the basis of its functionality, site-directed mutagenesis has the advantage that the residue which replaces it may either have similar functionality or be completely different, thus providing information on whether the exact chemical nature of the amino acid side chain is important. For example, an intracellular tyrosine residue (Tyr254) in the third cytoplasmic loop of the m3 muscarinic receptor has been shown to be critical for stimulation of phosphatidylinositol hydrolysis [Blüml *et al*, 1994a]. Replacement of Tyr254 with alanine virtually abolishes receptor-mediated effects, whereas substitution by

other aromatic residues such as phenylalanine or tryptophan results in mutant receptors that behave functionally similar to the wild type m3 receptor [Blüml *et al*, 1994b], indicating the importance of an aromatic group in this position. Similarly, a hydrophobic leucine residue (Leu131) found in the second cytoplasmic loop of many seven-transmembrane-domain receptors has been postulated to play an important role in G protein-coupling of the human m1 receptor [Moro *et al*, 1993]. Substitution of Leu131 with polar amino acids or alanine results in strongly defective coupling. However, replacement with phenylalanine results in a m1 receptor which displays normal functional coupling. This has been taken as evidence that a bulky hydrophobic group is necessary at that position in the second cytoplasmic loop for efficient receptor-G protein-coupling.

The substituted amino acid residue can also be chosen in order to confer certain secondary structural characteristics on the altered protein domain. In an elegant example of this, Duerson *et al* (1993) introduced a point mutation into the third intracellular loop of the human m3 receptor which was predicted to disrupt the local putative amphipathic α-helical conformation. Thus replacement of Glu257 with alanine resulted in a mutant receptor which displayed wild type functional characteristics, suggesting that the receptor can accommodate the loss of a negatively charged amino acid at this position. However, replacement of the same glutamate residue with proline, a well known α-helix breaker [Chou and Fasman, 1978], inhibited the m3 receptor response, indicating that the putative α-helical structure of this region of the receptor may be important for a fully functional receptor.

A number of G protein-linked receptors have been shown to regulate multiple effector pathways [Milligan, 1993]. One possible mechanism by which this could occur involves a single receptor species activating multiple G proteins, each of which is able to regulate a separate effector. Suprenant *et al* (1992) have provided evidence for such a signalling pathway for the α_{2a}-adrenergic receptor. Agonist-bound wild type α_{2a}-adrenergic receptors stably expressed in AtT20 mouse pituitary tumour cells inhibit voltage-dependent calcium currents and increase inwardly rectifying potassium currents. However, a mutant receptor (D79N) effectively inhibits Ca^{2+} currents but has no effect on K^+ currents, even in the presence of a 10,000-fold higher concentration of agonist. This suggests that this conserved aspartate residue is necessary to

enable the receptor to communicate with G proteins that mediate potassium channel activation, but is not important for coupling to a distinct set of G proteins which cause calcium channel inhibition.

One limitation in studying structure-function relationships of proteins with mutagenesis is that inferences must be made about the role of a particular amino acid(s) based upon changes in protein function. An implicit assumption in these experiments is that the changes in function are as a *direct* result of the replacement of a specific amino acid. However, modification of a protein sequence by mutagenesis may change its biochemical reactivity or produce an unfavourable structural perturbation, perhaps due to incorrect folding of the peptide sequence. Thus the possibility that a change in protein function is as an indirect consequence of residue mutation is difficult to eliminate and data obtained from mutagenesis studies should therefore be supplemented with results from a more biochemical approach, for example the use of peptide sequences. A further complication to the interpretation of mutagenesis studies is that mutation or deletion from a receptor protein may also alter the level of expression of the receptor [Parker and Ross, 1991]. Interpretation becomes particularly difficult when more than one amino acid is mutated at the same time. Högger *et al* (1995) recently produced a wide range of human m1 muscarinic receptors which contained various mutations in the N- and C-terminal regions of the third cytoplasmic loop. Single point mutations as well as triple point mutations were produced. Surprisingly, single point mutations in two different domains of the receptor were discovered to produce greater disruption to receptor coupling to phosphatidylinositol turnover than corresponding triple point mutations. Therefore, the triple point mutations compensated, at least partially, for the effect of the two single point mutations. Indeed further study of one of the triple point mutations showed that single point mutation of one amino acid caused an inhibition of phosphatidylinositol turnover, whereas mutation of another caused activation of this response.

Lefkowitz and colleagues have also identified mutant adrenergic receptors with enhanced basal activity and hence constitutive activity [Kjelsberg *et al*, 1992; Ren *et al*, 1993; Lefkowitz *et al*, 1993; Samana *et al*, 1993]. In addition to their constitutive activities, these mutated receptors demonstrate a higher affinity for agonists, (even in the absence of G proteins) another primary characteristic of the active conformation of G protein-coupled receptors. In the

case of the β_2-adrenoceptor, the extent of the increase in affinity conferred by the mutation is related to drug activity [Samana *et al*, 1993]. Thus the greater the activity of a drug the greater the increase of its affinity for the constitutively active mutant as opposed to the wild type receptor.

Given that short peptide sequences from G protein-linked receptors can activate G proteins *in vitro* [Cheung *et al*, 1991; Okamoto *et al*, 1991], Lefkowitz and colleagues suggested that the receptor structure must somehow constrain or shield such sequences in the inactive conformation of the receptor. The amino acid substitutions in the constitutively activated mutant receptors hinder this constraining function, allowing the receptors to adopt an active conformation. This must, in some way, mimic the structural changes evoked under physiological circumstances by agonist occupation of the receptor. To account for this an expansion of the ternary complex model was suggested [Lefkowitz *et al*, 1993], which includes an isomerization of receptors from an inactive to an active state which couples to the G protein ("allosteric ternary complex model") (see Fig. 4.23). This isomerisation involves conformational changes which may occur spontaneously, or be induced by agonists or appropriate mutations of the receptors.

A particularly clever example of the use of site-directed mutagenesis to probe G protein-coupled structure-activity relationships was reported by Blüml *et al* (1994). A series of five mutant rat m3 muscarinic receptors (m3 (+1A) to m3 (+5A)) were created in which one to five additional alanine residues were inserted between the C-terminal end of the fifth transmembrane domain and the beginning of the third intracellular loop. This particular segment of the cytosolic loop is predicted to form an amphipathic α-helix. Blüml and colleagues speculated that such manipulation should lead to a rotation of the N-terminal segment of this intracellular domain (if it is in fact arranged α-helically), thus producing pronounced effects on receptor-G protein-coupling. Analysis of the various mutant receptors showed that the receptors containing one, three or four alanine inserts retained functional activity, whereas those containing two and five were virtually unable to stimulate phosphatidylinositol hydrolysis. Modelling of this part of the receptor on the basis of an α-helix showed that the active mutants retained a similar side chain orientation as the wild type receptor. In contrast, in the two functionally inactive mutant receptors the hydrophobic and charged faces of the putative α-helices faced a direction

approximately opposite to that found in the wild-type receptor. Thus this study indicated the importance of the relative orientation of amino acid residues in this region of the m3 muscarinic receptor. All five mutant receptors displayed agonist binding affinities which were similar to, or only slightly lower than those obtained with the wild-type receptors, thus differentiating the ability of receptors to activate G proteins from their ability to exhibit high affinity agonist binding.

Deletion mutagenesis has been used considerably less than point mutation techniques, perhaps because complete removal of structural domains of a receptor are more likely to cause difficulties in the correct folding of the receptor than single conservative amino acids replacements. However, using subtypes of the β-adrenergic receptor, which differ in the extent to which they mediate agonist-promoted events such as activation of adenylyl cyclase and receptor sequestration, Green *et al* (1994) studied the role of an unusual proline rich 24-amino acid sequence present in the third intracellular loop of the β_1-adrenergic receptor but not in the β_2-adrenergic receptor. Deletion of this proline rich sequence from the β_1-adrenergic receptor results in an improvement of isoproterenol-stimulated adenylyl cyclase to an EC_{50} value intermediate to that observed in the wild-type β_1- and β_2-receptors. Similarly, this deletion also improves maximal receptor sequestration, more characteristic of the β_2-receptor. In contrast, insertion of the proline rich sequence into the β_2-adrenergic receptor impairs its ability to mediate adenylyl cyclase activity, and results in a receptor which is sequestered to a lesser extent than the wild-type β_2-receptor. Thus the distinct phenotypic patterns observed for these two agonist-promoted events in the β_1- and β_2-receptor subtypes is suggested to be partially due to the conformational effects of this proline rich third intracellular loop sequence.

In conclusion, mutagenesis techniques have been widely used to study regions of 7-transmembrane domain receptors which may be necessary for G protein-coupling. Although many of these studies have concentrated on regions of the third intracellular loop, reports have appeared suggesting important roles for residues in the first and second intracellular loops as well as the C-terminal tail [Chazenbalk *et al*, 1990; Ohyama *et al*, 1992; Josiah *et al*, 1994]. Thus it seems likely that the G protein binding domain of G protein-coupled receptors is composed of several intracellular regions.

2). Chimeric receptors.

One potential disadvantage of using site-directed mutagenesis is that inferences must usually be made about the role of specific domains or residues in receptor-G protein-coupling based on a loss of function. In order to circumvent such problems some groups have constructed chimeric receptors, so that conclusions can be drawn on the basis of the acquisition rather than the destruction of a certain characteristic. Kobilka *et al* (1988) were the first to use this technique to define domains of the β-adrenergic receptor responsible for coupling to G_s. A chimeric receptor of the native α_2-adrenergic receptor with the region from the N-terminal portion of the second extracellular loop to the C-terminal region of the sixth hydrophobic domain replaced by the relevant sequence from the β_2-adrenergic receptor was constructed and displayed an α_2-receptor-like pharmacological binding profile, but stimulated adenylyl cyclase activity, a typical β_2-receptor output. This result indicated that the specificity for coupling to G_s lies within a region of the β_2-receptor extending from the amino-terminal region of the fifth transmembrane domain to the carboxy-terminus of the sixth.

The primary sequence of muscarinic acetylcholine receptors at the N-terminal end of the third cytoplasmic loop is highly conserved among m1, m3, and m5 receptors i.e. those that activate phospholipase C, but is distinct from the corresponding segment of the m2 and m4 receptors which inhibit adenylyl cyclase. However, an m2 chimeric receptor containing a 17 amino acid stretch from the N-terminal end of the third intracellular loop of the m3 receptor triggers an increase in phosphatidylinositol hydrolysis [Wess *et al*, 1989, 1990]. Thus it appears that this 16 to 17 amino acid sequence at the N-terminal end of the third intracellular loop of the m2 and m3 muscarinic acetylcholine receptors may play a key role in determining the ability of these receptors to couple to G protein(s) that activate phospholipase C.

A paper by Ross and colleagues [Wong *et al*, 1990] suggests that the third intracellular loop of the phospholipase C coupled muscarinic receptors is not the only important determinant of G protein-coupling. This group studied chimeras in which the second and / or third cytoplasmic loops of the human m1 receptor were replaced with the analogous sequences derived from the turkey β-adrenergic receptor. Substitution of the entire third intracellular loop with the

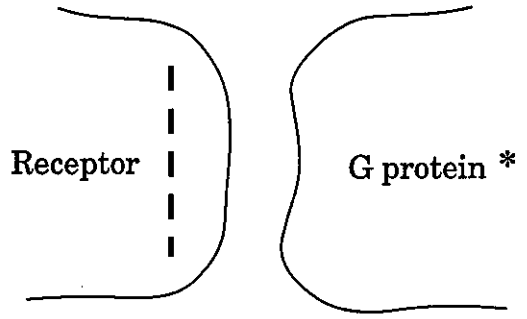
same region of the β-receptor yielded a chimeric receptor which still retained the ability to stimulate inositol phosphate release to the same extent as the wild-type m1 receptor. Replacement of the second intracellular loop of the m1 receptor with the equivalent region from the β-adrenergic receptor also produced a fully functional receptor. However, replacement of both loops together resulted in a 75% reduction in maximal phosphatidyl inositol turnover, suggesting that these loops act in a cooperative manner to determine G protein specificity.

3). Inhibition of G protein-coupled receptor signalling by expression of cytoplasmic domains of the receptor.

A new strategy for examining receptor-G protein interactions was recently introduced by Lefkowitz and coworkers [Luttrell *et al*, 1993; Hawes *et al*, 1994], using minigene constructs to encode small regions of the intracellular domains of receptors. Coexpression of these constructs along with their parent receptors allows the potential role of these small cytoplasmic regions in G protein-coupling to be determined. Thus coexpression of the third intracellular loop of the α_{1B}-adrenergic receptor with its parent receptor inhibits receptor-mediated activation of phospholipase C. This inhibition extends to the closely related α_{1C}-adrenergic receptor subtype, but not to the phospholipase C coupled m1 muscarinic acetylcholine receptor nor the adenylyl cyclase coupled D_{1A} dopamine receptor. Similarly, expression of the third cytoplasmic domain of the D_{1A} dopamine receptor inhibits cAMP production by this receptor, but does not affect α_{1B}-adrenergic receptor-mediated inositol phosphate accumulation [Luttrell *et al*, 1993].

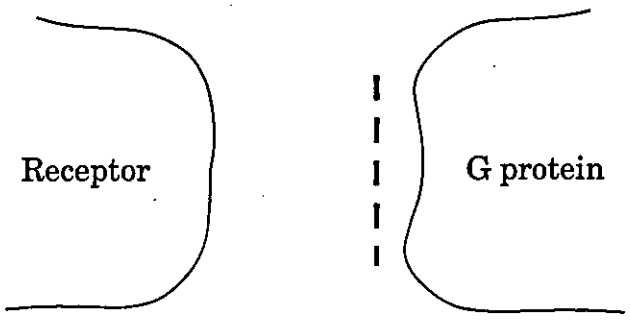
4). Use of synthetic peptides for mapping receptor-G protein interaction.

Synthetic peptides corresponding to interfacial regions of proteins may be competitive inhibitors of the protein-protein interaction, or alternatively they may mimic the biological effect of one protein on the other (Fig. 1.11).



Peptides from the interface can:-

1. Block the interaction



or 2. Activate the G protein

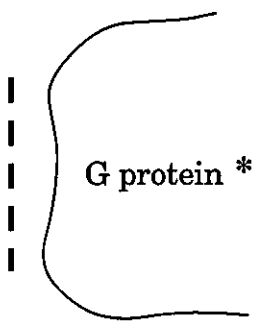


Figure 1.11 Synthetic peptides corresponding to the interfacial regions of interaction with neighbouring proteins can occupy their binding sites on neighbour protein surfaces and thus act as competitive inhibitors of the protein-protein interaction. Some proteins can cause conformational changes on neighbouring proteins. Interfacial peptides can sometimes mimic the effect of the parent proteins binding to the neighbour protein binding site and cause the conformational changes.

Of course, peptides could be taken from either receptor or G protein. Indeed sequences from G proteins have been tested for their biological activity in inhibiting receptor-G protein interaction [Hamm *et al*, 1988, Rasenick *et al*, 1994]. However, for the purposes of this discussion, only studies using receptor-derived peptides will be considered.

Once the interfacial regions have been mapped, analogue peptides with single amino acid substitutions or deletions can provide information on residues critical for protein-protein interaction. An intriguing example of this comes from the work of Nishimoto and colleagues [Okamoto *et al*, 1990] who discovered a 14 amino acid peptide (peptide 14 - RVGLVRGEKARKEK) from the insulin-like growth factor II / mannose-6-phosphate receptor which stimulated [^{35}S]GTPγS binding to purified G_i/G_o proteins. This receptor is of particular interest because it is predicted to span the plasma membrane only once, and as such represents the only non-seven-transmembrane-domain receptor which activates G proteins [Nishimoto *et al*, 1989], although this is still a subject of debate [Körner *et al*, 1995]. Nevertheless peptide 14 certainly activates purified G proteins reconstituted into phospholipid vesicles. Structurally modified peptides suggested that in order to retain activity, the C-terminal motif should be BBXB or BBXXB (B=basic residue, X=non-basic residue) [Okamoto *et al*, 1990]. Substitution of the second arginine residue from the N-terminus suggested that this amino acid was also functionally important. Further studies using a peptide sequence from the α_2-adrenergic receptor with similar structural characteristics led the same group to propose that the C-terminal basic residue could be replaced with an aromatic residue [Ikezu *et al*, 1992]. In fact, Neubig and colleagues have shown, using peptide sequences derived from the same region of the α_2-receptor, that the C-terminal basic residue can be removed altogether [Dalman and Neubig, 1991] or replaced with cysteine [Wade *et al*, 1994] and the resulting peptide still activates purified G_i/G_o proteins. Thus the importance of the whole BBXXB or BBXB sequence must be called into question.

Despite the doubt as to whether basic residues are important, Nishimoto and coworkers have discovered a wide range of G protein-activating peptides derived from various G protein-coupled receptors by searching for such sequences within the cytoplasmic domains of these receptors [Okamoto *et al*, 1991; Okamoto and Nishimoto, 1992]. One such peptide, corresponding to the C-terminal end of the third intracellular loop of the β_2-adrenergic receptor

activates G_s at nanomolar concentrations and only weakly activates G_i [Okamoto *et al*, 1991]. Interestingly, however, phosphorylation of this peptide, which is involved in the desensitisation of the β-adrenergic receptor from G_s [Lefkowitz *et al*, 1990], drastically reduces the effect of the peptide on G_s whilst potentiating its action on G_i, resulting in a total loss of adenylyl cyclase-simulating activity. Thus it seems that this region of the receptor has the potential to activate both G_s and G_i proteins, with the relative selectivity determined by the state of phosphorylation of the peptide.

One advantage of using synthetic peptides as receptor mimics is that structural analysis of the interactions is possible using transferred nuclear Overhauser NMR spectroscopy [Sukumar and Higashijima, 1992; Jung *et al*, 1995]. This can provide dynamic three-dimensional structural information on the biologically active conformation of the peptide when bound to its site on a partner protein, conformational changes in the peptide during biological activity, and point-to-point interactions with the binding site on the partner protein. Circular dichroism spectroscopy has also been used extensively to study the conformation of receptor-mimetic peptides [Voss *et al*, 1993; Shinagawa *et al*, 1994; Jung *et al*, 1995]. This has proven particularly useful because many of the peptides tested adopt a largely α-helical conformation, which is easily detected by this form of spectroscopy. Indeed, the results of one such study led Voss *et al* (1993) to suggest that there is no correlation between the secondary structure of a peptide and its ability to activate G proteins.

In common with mutagenesis techniques, which suggest a role for multiple regions of the cytoplasmic loops in G protein-coupling [O'Dowd *et al*, 1988; Chazenbalk *et al*, 1990; Ohyama *et al*, 1992; Pin *et al*, 1994], there are several pieces of evidence from peptide studies that indicate multiple sequences may be involved in receptor-G protein recognition. For example, peptides from rhodopsin and the β-adrenergic receptor show synergistic effects when sequences from more than one intracellular region are mixed [König *et al*, 1989; Münch *et al*, 1991]. Mastoparan analogues stimulate nucleotide exchange on G_o/G_i with Hill coefficients of 2-4 [Higashijima *et al*, 1990], suggesting that 2-4 molecules of amphiphilic peptide may be required for the productive regulation of G protein. Similarly, covalently linked dimers of α_2-adrenergic receptor peptides show markedly enhanced potency compared with monomeric peptides

to inhibit agonist binding and stimulate purified G_o/G_i GTPase activity in lipid vesicles [Wade *et al*, 1994].

In conclusion, the use of synthetic peptides represents a useful method to probe the molecular mechanisms by which receptors cause G protein activation. Such strategies, when combined with site-directed mutagenesis techniques, should allow the molecular determinants of receptor-G protein selectivity and activation to be resolved.

Studying the molecular determinants of opioid receptor-G protein interaction.

The previous section outlined many studies designed to examine receptor-G protein interaction. The majority of these studies were carried out on the G_s-linked β-adrenergic or the G_t-coupled rhodopsin receptor, though some groups have investigated the G_i/G_o-coupled α_2-adrenergic, m2 / m4 muscarinic acetylcholine and 5-hydroxytryptamine$_{1A}$ receptors. This latter group of 7-transmembrane domain receptors all have a different membrane topology to the opioid receptors, containing very large third intracellular loops. Thus while the cloned opioid receptors have short putative third intracellular loops, consisting of 20-30 amino acids, the equivalent regions in the α_2-adrenergic, m2 / m4 muscarinic acetylcholine and 5-hydroxytryptamine$_{1A}$ receptors typically contain 150-200 residues [Probst *et al*, 1992]. In fact, the opioid receptors form part of a small subset of G protein-coupled receptors which possess a small third intracellular loop of 15-30 amino acids. This group includes receptors for vasoactive intestinal peptide, substance K, bradykinin, interleukin-8 and N-formyl peptides. It is clearly of interest to see if this region of these receptors is important for G protein-coupling.

Using site-directed mutagenesis coupled with studies employing synthetic peptides Ye and colleagues [Prossnitz *et al*, 1993; Schreiber *et al*, 1994] have suggested recently that the third intracellular loop plays no role in coupling to G proteins in the neutrophil N-formyl peptide receptor. Rather, the second intracellular loop and carboxyl-terminal tail were implicated in this respect. The recent cloning of first the δ-, followed by the μ- and κ-opioid

receptors has made it possible to address such questions for this group of receptors.

The aim of the present work was to study opioid receptor-G protein coupling interactions in membranes from cells which endogenously express opioid receptors, in particular μ-receptors in SH-SY5Y cells and δ-receptors in NG108-15 cells. Mechanisms of receptor-mediated G protein activation were investigated by studying:

- Activation of G proteins by receptor-mimetic peptides such as mastoparan analogues.

- Effect of other compounds known to activate purified G proteins.

These experiments were carried out as a prelude to studies using synthetic peptides derived from the third intracellular loop of opioid receptors.

At the onset of the project two biochemical methods were widely used to study activation of opioid receptors in membranes from cultured cells, namely stimulation of GTPase activity and inhibition of forskolin-induced adenylyl cyclase. Stimulation of GTPase activity by opioid compounds is not a very sensitive technique to measure activation of G proteins, and frequently affords only poorly reproducible results [see for example Costa *et al*, 1991]. Whilst opioids produce a robust inhibition of adenylyl cyclase activity using whole cells, the response is more difficult to quantitate using a membrane preparation. Furthermore, this assay only provides an indirect measure of G protein activation, as adenylyl cyclase activity is one step away from G protein activation in the signal transduction cascade. For these reasons, another major aim of this work was to set up a [^{35}S]GTPγS binding assay using NG108-15 membranes, in order to provide a sensitive technique with which to study G protein activation by δ-opioid receptors in a native membrane environment.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Radiochemicals

[^{3}H]-DAMGO ([D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin) (2.22 TBq / mmol; 60 Ci / mmol) was from Amersham, Aylesbury.

[^{3}H]-Diprenorphine (1.11 TBq / mmol; 30 Ci / mmol) was from Amersham, Aylesbury.

[^{3}H]-DPDPE ([D-Pen^2,D-Pen^5]enkephalin) (1.50 TBq / mmol; 40.7 Ci / mmol) was from Dupont, NEN® Research Products, Stevenage.

[^{3}H]-Naltrindole (1.10 TBq / mmol; 29.7 Ci / mmol) was from Dupont, NEN® Research Products, Stevenage.

[^{3}H]-cAMP (cyclic adenosine monophosphate) (1.10 TBq / mmol; 29.7 Ci / mmol) was from Dupont, NEN® Research Products, Stevenage.

[^{35}S]GTPγS (Guanosine 5'-[γ-thio]triphosphate) (46.1 TBq / mmol; 1245 Ci / mmol) was from Dupont, NEN® Research Products, Stevenage.

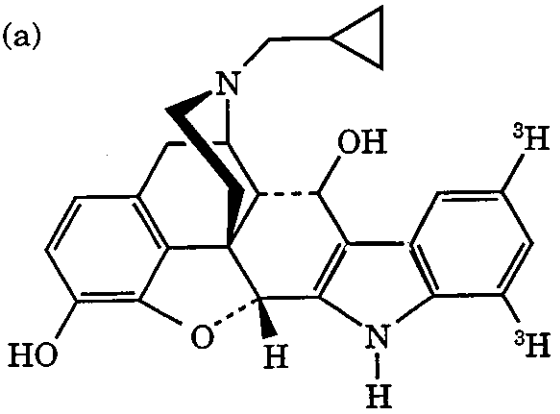
[γ-^{32}P]GTP (Guanosine 5'-[γ-^{32}P]triphosphate) (1.10 TBq / mmol; 30 Ci / mmol) was from Amersham, Aylesbury.

Structures of the radiochemicals are given in Fig. 2.1.

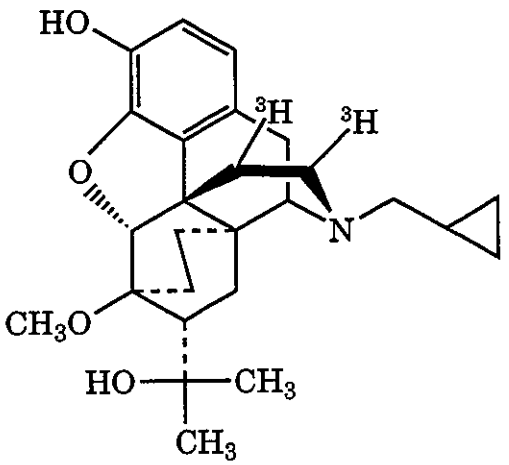
2.1.2 Chemicals

HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), Tris base (Tris[hydroxymethyl]aminomethane), bovine serum albumin (BSA), Folin's reagent, DL-dithiothreitol, phosphocreatine, creatine kinase (type I), 3-isobutyl-1-methylxanthine (IBMX), forskolin, sodium dodecyl sulphate (SDS), bromophenol blue, glycerol, acrylamide, N,N'-methylene-bis-acrylamide,

(a)



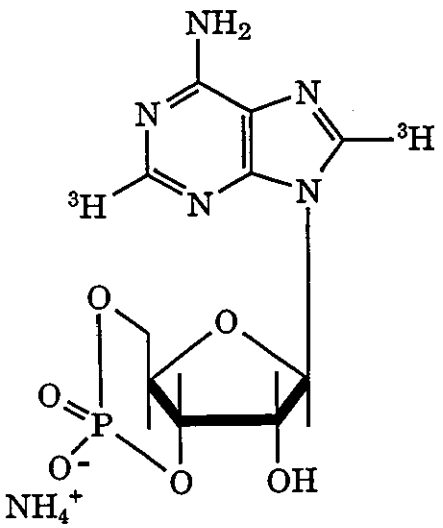
(b)



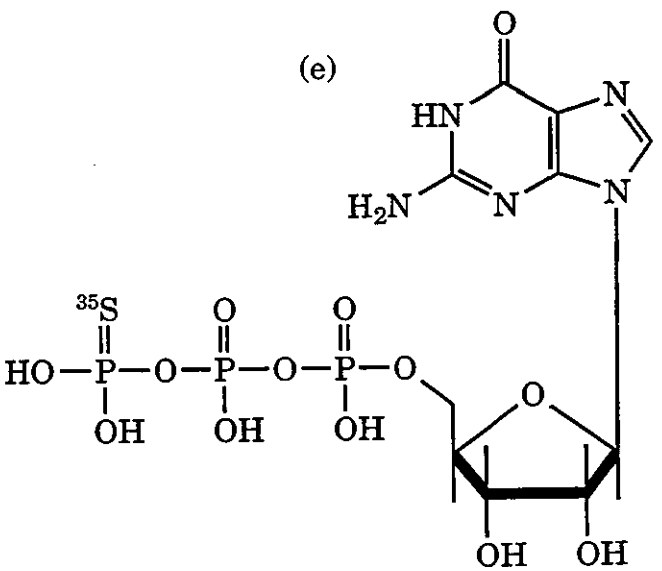
(c)

[3,5,^{3}H]Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$-OH

(d)



(e)



(f)

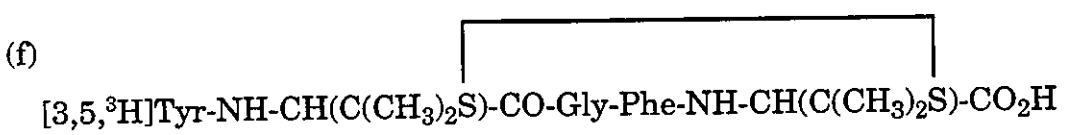


Figure 2.1: (a) [^{3}H]naltrindole, (b) [^{3}H]diprenorphine, (c) [^{3}H]DAMGO, (d) [^{3}H]cAMP, (e) [^{35}S]GTPγS, (f) [^{3}H]DPDPE.

N,N,N',N'-tetramethylethylenediamine, ammonia solution (sp. gr. 0.88) and Norit activated charcoal were from Sigma, Poole.

Sodium hydroxide, sodium carbonate, sodium potassium tartrate, sodium chloride, magnesium chloride, orthophosphoric acid (sp. gr. 1.75), disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, magnesium sulphate, calcium chloride, copper sulphate, potassium chloride, sodium hydrogen carbonate, glucose, ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid (sp. gr. 1.16) were purchased from Fisons, Loughborough.

Ecoscint scintillation fluid was from National Diagnostics, Georgia, USA.

An enhanced chemiluminescence kit for visualising horseradish peroxidase-conjugated antibodies was obtained from Amersham, Aylesbury.

2.1.3 Nucleotides

Guanosine diphosphate (GDP), 5'-adenylylimidodiphosphate (AMP-PNP), adenosine triphosphate (ATP), uridine diphosphate (UDP), adenosine diphosphate (ADP), guanosine monophosphate (GMP), guanosine 5'-[β-thio]diphosphate (GDPβS) and guanosine 5'-[γ-thio]triphosphate (GTPγS) were from Sigma, Poole.

Guanosine triphosphate (GTP) was obtained from Aldrich, Poole.

2.1.4 Bacterial toxins

Pertussis toxin and cholera toxin were purchased from Sigma, Poole.

2.1.5 Drugs and related compounds

Fentanyl citrate, morphine, naloxone, ouabain, benzalkonium chloride (BAC), spermine, compound 48/80, and theophylline were bought from Sigma, Poole.

Etorphine and diprenorphine were donated by Reckitt & Colman, Hull.

7-benzylidenenaltrexone (BNTX), cyprodime and naltriben (NTB) were purchased from Research Biochemicals Inc., SEMAT, St. Albans.

Nalorphine and levallorphan were a generous gift from Zeneca Pharmaceuticals, Alderley Park.

Octadecyltrimethylammonium bromide (OTAB) was from Aldrich, Poole.

2.1.6 Peptides

[D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO), [D-Pen^2,D-Pen^5]enkephalin (DPDPE), [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET), mastoparan, hexa-alanine, hexa-glycine and Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-amide were from Sigma, Poole.

Mastoparan 7, mastoparan 17 and [D-Ala^2,Glu^4]deltorphin II (DELT II) were obtained from Peninsula Laboratories, St. Helens.

N,N-diallyl-Tyr-Aib-Phe-Leu-OH (Aib = α-aminoisobutyric acid) (ICI 174864) was from Research Biochemicals Inc., SEMAT, St. Albans.

Tyr-Tic-Phe-Phe (Tic = tetrahydroisoquinoline-3-carboxylic acid) (TIPP) was kindly donated by Dr. S.J. Paterson, St. Thomas's hospital, London.

Fragments of the human μ-opioid receptor corresponding to amino acid residues 277-283 (μil3$_{277\text{-}283}$; Leu-Arg-Arg-Ile-Thr-Arg-Met) and residues 269-283 (μil3$_{269\text{-}283}$; Gly-Ser-Lys-Glu-Lys-Asp-Arg-Asn-Leu-Arg-Arg-Ile-Thr-Arg-Met) were synthesised by the Central Peptide Facility at Leicester University.

2.1.7 Antibodies and cAMP binding protein

A polyclonal antibody (δC-Ant) raised in rabbits against a glutathione transferase fusion protein containing the C-terminal 35 amino acids of the mouse δ-opioid receptor [Crook *et al*, 1994] was a kind gift from Dr. F. Porreca, Department of Pharmacology, University of Arizona, Tucson.

Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Sigma, Poole.

The cAMP-binding protein prepared from bovine adrenal glands was a generous gift from Dr. D.G. Lambert, Department of Anaesthesia, Leicester University.

2.1.8 Cell culture media

Minimum Essential Medium (with Earle's salts) (MEM), Dulbecco's Modified Eagle Medium (without sodium pyruvate; with 4500mg/l glucose) (DMEM), Dulbecco's Modified Eagle Medium / Nutrient Mix F-12, foetal calf serum, new born calf serum, fungizone®, L-glutamine, penicillin / streptomycin solution, HAT supplement and trypsin / EDTA solution (trypsin (500 μg/ml) and EDTA (200 μg/ml) in modified Puck's saline A) were bought from Gibco Laboratories, Paisley.

Sterile dimethyl sulphoxide (DMSO) was from Sigma, Poole.

2.1.9 Buffers

The composition of the buffer used in GTPase assays was (mM, unless otherwise stated):

NaCl (120), $MgCl_2.6H_2O$ (5), EDTA (0.1), dithiothreitol (2), (AMP-PNP) (1), ouabain (1), ATP (1), phosphocreatine (20), creatine kinase (5U) and Tris (50) adjusted to pH 7.5 with concentrated hydrochloric acid (sp. gr. 1.16).

The hypotonic buffer used in GTPase assays was (mM):

Na_2HPO_4 (0.61), KH_2PO_4 (0.38), $MgSO_4.7H_2O$ (0.2) and dithiothreitol (1) adjusted to pH 7.4 using dilute ammonia solution.

The buffer used for membrane ligand binding studies was Tris (50mM) acidified to pH 7.4 using HCl.

The buffer used in [^{35}S]GTPγS binding studies on CHO cells transfected with the mouse δ-receptor and SH-SY5Y human neuroblastoma cells was (mM) (unless otherwise stated):

NaCl (100), $MgCl_2.6H_2O$ (10) and HEPES (20) adjusted to pH 7.4 using ammonia solution (sp. gr. 0.88).

The buffer used for [^{35}S]GTPγS binding studies in NG108-15 neuroblastoma x glioma cells was (mM) (unless otherwise stated):

NaCl (100), $MgCl_2.6H_2O$ (4) and HEPES (20) adjusted to pH 7.4 using ammonia solution (sp. gr. 0.88).

Krebs / HEPES buffer used for adenylyl cyclase assays contained (mM):

NaCl (118), $NaHCO_3$ (25), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4.7H_2O$ (1.2), $CaCl_2.2H_2O$ (2.5), glucose (2.5) and HEPES (20) adjusted to pH 7.4 using ammonia solution (sp. gr. 0.88).

Adsorption buffer used in adenylyl cyclase assays contained (mM, unless otherwise stated):

EDTA (4), BSA (4 mg/ml), and Tris (50) adjusted to pH 7.4 using concentrated hydrochloric acid (sp. gr. 1.16).

Electrophoresis sample buffer contained (mM, unless otherwise stated):

Tris-HCl (62), glycerol (10% v/v), SDS (2% w/v), DTT (5), bromophenol blue (0.001% w/v) acidified to pH 6.8 using concentrated hydrochloric acid (sp. gr. 1.16).

Phosphate buffered saline (PBS) used for immunoblotting was (mM):

NaCl (137), KCl (2.68), Na_2HPO_4 (10) , KH_2PO_4 (1.75), pH 7.4

2.1.10 Equipment

Ligand binding and radioassays

Brandel cell harvester M-48R, Gaithersburg, USA.
Liquid scintillation counter, either MINAXI TRI-CARB® 4000 series, United Technologies Packard, Pangbourne or 1215 Rackbeta, LKB Wallac, Milton Keynes.
Tissue tearor, Model 985-370, Biospec Products, Bartlesville, OK, USA.
Ultracentrifuge, Optima TL100, Beckman, High Wycombe.
Centrifuge, Universal 1200, Hettich, Tuttlingen, Germany.
Micro-centrifuge, Model 320, Quickfit Instrumentation, Stone.
Whatman GF/B filter strips, Whatman, Maidstone
pH meter, Model PW4909, Philips, Cambridge.

Cell culture

Centrifuge, FP-510, Labsystems Oy, Finland.
Class II Microbiological Safety Cabinet, Walker Safety Cabinets Ltd., Glossop, Derbyshire.
CO_2 Incubator, GC4, Grant Instruments, Cambridge.
Powerpette, Jencons Scientific Ltd., Leighton Buzzard.
Tissue culture plastics, Gibco Laboratories, Paisley.

SDS-PAGE

SDS-PAGE equipment was from Gelman, Northampton, as used in the Department of Cell Physiology and Pharmacology, Leicester University.

2.2 Methods

2.2.1 Cell culture

(a) SH-SY5Y human neuroblastoma cells

The SH-SY5Y cell line was kindly donated by Dr. D.G. Lambert, Department of Anaesthesia, Leicester University. Cells (passage 70-100) were grown in 80 or 175 cm^2 tissue culture flasks in Minimum Essential Medium (MEM) supplemented with 10% foetal calf serum, 2.5μg/ml amphotericin B (fungizone®), 50U/ml penicillin, 50μg/ml streptomycin and 258μg/ml L-glutamine at 37°C in a humidified 5% CO_2 atmosphere. Cells were passaged when confluent using trypsin / EDTA solution and fed thrice weekly. Cells used for experiments were confluent and 7-11 days old.

(b) NG108-15 neuroblastoma x glioma hybrid cells

NG108-15 cells were kindly provided by Dr. M. Keen, Department of Pharmacology, University of Birmingham. Cells were grown in 80 or 175 cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal calf serum and HAT (hypoxanthine (13.6μg/ml), aminopterin (0.176μg/ml) and thymidine (3.88μg/ml)) at 37°C in a 5% CO_2 atmosphere. Cells were passaged when confluent (by gentle mechanical agitation) and fed every day, with the exception of day 1 after sub-culture.

(c) Chinese Hamster Ovary (CHO) cells, transfected with the mouse δ-opioid receptor (CHOδ)

CHO cells transfected with the mouse δ-opioid receptor were a kind gift from Dr. C.J. Evans, Department of Psychiatry, UCLA, Los Angeles, California. Cells were grown in 80 or 175 cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium / F-12 Nutrient Mixture supplemented with 5% foetal calf serum, 2.5μg/ml amphotericin B (fungizone®), 50U/ml penicillin, 50μg/ml streptomycin and 258μg/ml L-glutamine at 37°C in a 5% CO_2 atmosphere. Cells

were passaged when confluent using trypsin / EDTA solution and fed every day, with the exception of day 1 after sub-culture.

2.2.1.1 Treatment of cells with bacterial toxins

Cells were treated with pertussis (100 ng/ml) or cholera (10 μg/ml) toxin for 20-24h before harvesting. Control cells were treated with the appropriate vehicle for the same length of time.

2.2.1.2 Long term storage of cells

Cells were rinsed once and harvested using trypsin / EDTA solution by gentle agitation. The cell pellet obtained after centrifugation (250*g*, 2 min, room temperature) was suspended in foetal calf serum : DMSO (9:1 v/v) at a protein concentration of approximately 10 mg/ml and this mixture was frozen slowly by placing it in a freezer (-20°C) for 3h followed by dry ice for 3h. Stock cell cultures were then placed in liquid nitrogen for long term storage.

2.2.2 Membrane preparation for ligand binding and [^{35}S]GTPγS binding assays

(a) SH-SY5Y human neuroblastoma cells

Confluent monolayers were rinsed once and harvested by gentle agitation in HEPES (20mM, pH 7.4) buffered saline containing 0.02% (w/v) EDTA. Centrifugation (250*g*, 2 min, room temperature) produced a cell pellet which was resuspended in the appropriate buffer (Tris-HCl (pH 7.4) for ligand-binding assays or [^{35}S]GTPγS binding buffer for [^{35}S]GTPγS binding experiments). Membranes were prepared by treatment of cells with a tissue tearor (2x5s, 30,000rpm) and collected by centrifugation (50,000*g*, 15 min, 4°C). Membrane pellets were resuspended in the appropriate buffer using the tissue tearor (2x1s, 5,000rpm), and then recentrifuged as before. The resulting pellet was resuspended in Tris-HCl (pH 7.4) or [^{35}S]GTPγS binding buffer by homogenisation (2x5s, 30,000rpm) at a protein concentration of approximately 0.25mg/ml. All procedures were performed at 0-4°C.

(b) NG108-15 neuroblastoma x glioma hybrid cells

NG108-15 cells were harvested by gentle agitation in DMEM. Membranes were then prepared as described above.

(c) CHO cells transfected with the mouse δ-opioid receptor (CHOδ)

Confluent flasks of cells were harvested into DMEM / F-12 Nutrient Mixture by gently scraping the surface of the flask with a rubber policeman. Membranes were then prepared as described above.

2.2.3 Membrane preparation for [γ-^{32}P]GTPase assays in SH-SY5Y cells

Surface growing confluent monolayers were rinsed once and harvested in HEPES (20mM, pH 7.4) buffered saline containing 0.02% (w/v) EDTA by gentle agitation. Centrifugation (250*g*, 2 min, room temperature) produced a cell pellet which was resuspended in hypotonic buffer (pH 7.4). The pellet was gently dispersed and membranes were prepared by treatment with a Dounce ground glass homogeniser (30 strokes). The resulting homogenate was centrifuged (500g, 2 min, room temperature) to remove nuclear debris and unbroken cells, and cell membranes were collected by centrifugation of the supernatant (50,000*g*, 15 min, 4°C). The membrane pellet was resuspended in hypotonic buffer using the ground glass homogeniser and recentrifuged as above three times. The final membrane pellet was suspended in hypotonic buffer at a protein concentration of approximately 1mg/ml. All procedures were performed at 0-4°C.

2.2.4 Ligand Binding assays

Membrane protein (100-400μg) was incubated in Tris-HCl (pH 7.4) with the appropriate tritiated ligand (and competing drug if required) in a final volume of 1ml in triplicate for 1 hour at 25°C. The mixture was rapidly vacuum-filtered to separate bound from free ligand, and the filters were rinsed three times in 3ml of ice-cold buffer (Tris-HCl, pH 7.4). Filters were dispersed in

Ecoscint scintillation fluid, and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

Non-specific binding was defined as the binding remaining in the presence of 10μM naloxone; specific binding was calculated by subtraction of non-specific binding from total binding. Specific binding was typically >80% of total binding at the radioligand K_d.

In experiments to determine the effect of peptide fragments of the human μ-opioid receptor $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$, or control peptides, on ligand binding, the peptides were incubated with the membrane preparation on ice for 60 min before addition of the radioligand.

2.2.5 [^{35}S]GTPγS binding assays

Membrane protein (50-400μg) was incubated in [^{35}S]GTPγS binding buffer containing 100pM [^{35}S]GTPγS (approximately 5kBq) and GDP (SH-SY5Y or CHOδ cell membranes - 3 μM; NG108-15 cell membranes - 100 μM) in triplicate for 1 hour at 30°C (unless otherwise stated) in the presence of varying concentration of drug. The mixture was then rapidly vacuum-filtered and washed three times with 3ml of ice-cold [^{35}S]GTPγS binding buffer. The filter papers were placed in Ecoscint scintillation fluid, and bound radioactivity was determined by liquid scintillation spectrometry in the carbon-14 channel of a liquid scintillation counter.

Non-specific binding was defined in the presence of 10 μM unlabelled GTPγS; specific binding was calculated by subtraction of non-specific binding from total binding. Specific binding was typically 90-95% of total binding in the absence of agonist.

In experiments to determine the effect of peptide fragments of the human μ-opioid receptor $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$, or control peptides, or δC-Ant, on [^{35}S]GTPγS binding, the peptides or antibody were incubated with the membrane preparation on ice for 60 min before addition of the radioligand and drugs.

2.2.6 [γ^{32}P]GTPase assays

A modification of the method of Cassel and Selinger (1976) was used to measure low K_m GTPase activity in membranes from SH-SY5Y human neuroblastoma cells. Membrane protein (10-30μg) was equilibrated at 37°C in [γ-^{32}P]GTPase buffer containing various concentrations of agonist for 15 min. The reaction was initiated by the addition of [γ-^{32}P]GTP (0.5μM; approximately 2.5kBq) (final volume = 100μl; triplicate samples), allowed to proceed at 37°C for 15 min, and terminated by the addition of 1ml of ice-cold 10% (w/v) activated charcoal suspended in 20mM phosphoric acid (pH 2.3). After standing on ice for 30 min, the tubes were centrifuged (7000*g*, 2 min, room temperature or 1500*g*, 20 min, room temperature) and 600μl of supernatant was added to 5ml of water and analysed for liberated $^{32}P_i$ using Cerenkow radiation, with a measured counting efficiency of 37%. Basal activity in the absence of test substance and hydrolysis of [γ-^{32}P]GTP in the absence of membranes were routinely measured in each assay.

High K_m GTPase activity, which accounted for 25-50% of the total observed hydrolysis, was measured in the presence of GTP (50 μM).

Inhibition of non-specific nucleoside triphosphatases was achieved by the inclusion of AMP-PNP (1mM) in the reaction buffer. In addition, an ATP regenerating system was present (phosphocreatine (20mM) and creatine kinase (5U)) to suppress the transfer of γ-$^{32}P_i$ liberated from [γ-^{32}P]GTP to ADP [Cassel and Selinger, 1976]. Further reduction in the non-specific hydrolysis of [γ-^{32}P]GTP by ATPase enzymes was obtained by the inclusion of ouabain (1mM) to inhibit Na^+ / K^+ ATPase.

2.2.7 Adenylyl cyclase assays

Inhibition of forskolin (10 μM)-stimulated adenylyl cyclase activity by opioid agonists was measured using a competitive binding protein assay [Brown *et al*, 1971] to determine the levels of cAMP formed in a cell suspension.

Cells were harvested in HEPES (20mM, pH 7.4) buffered saline containing 0.02% (w/v) EDTA by gentle agitation. Centrifugation (250*g*, 2 min, room temperature) produced a cell pellet which was gently resuspended in Krebs / HEPES buffer at a protein concentration of approximately 2 mg/ml. For measurement of adenylyl cyclase activity, intact cells (300-400µg protein) were incubated in triplicate in Krebs / HEPES buffer in a final volume of 300µl at 37°C for 15 min in the presence of forskolin (10µM), IBMX (1 mM) and various concentrations of opioid agonist. The assay was terminated by the addition of HCl (10M, 20µl), followed by NaOH (10M, 20µl) and Tris-HCl (1M, pH 7.4, 180µl) to neutralize the reaction mixture.

cAMP was quantified on the basis of competition between [^{3}H]-cAMP and unlabelled cAMP formed in the reaction mixture for a crude cAMP binding protein prepared from bovine adrenal glands. Typically, 50µl of reaction mixture (or a dilution thereof) or standard cAMP solution was incubated with 2 pmol [^{3}H]-cAMP (ca. 2.2 kBq) and binding protein at 4°C in the dark overnight. Non-specific binding of [^{3}H]-cAMP was determined using a 125-fold excess of unlabelled cAMP. Free [^{3}H]-cAMP was adsorbed by activated charcoal (10 mg/ml) suspended in adsorption buffer and removed by centrifugation (7000*g*, 2 min, room temperature). [^{3}H]-cAMP bound to the soluble binding protein was determined by counting an aliquot of the supernatant in the tritium channel of a liquid scintillation counter. A typical standard curve is shown in Figure 2.2.

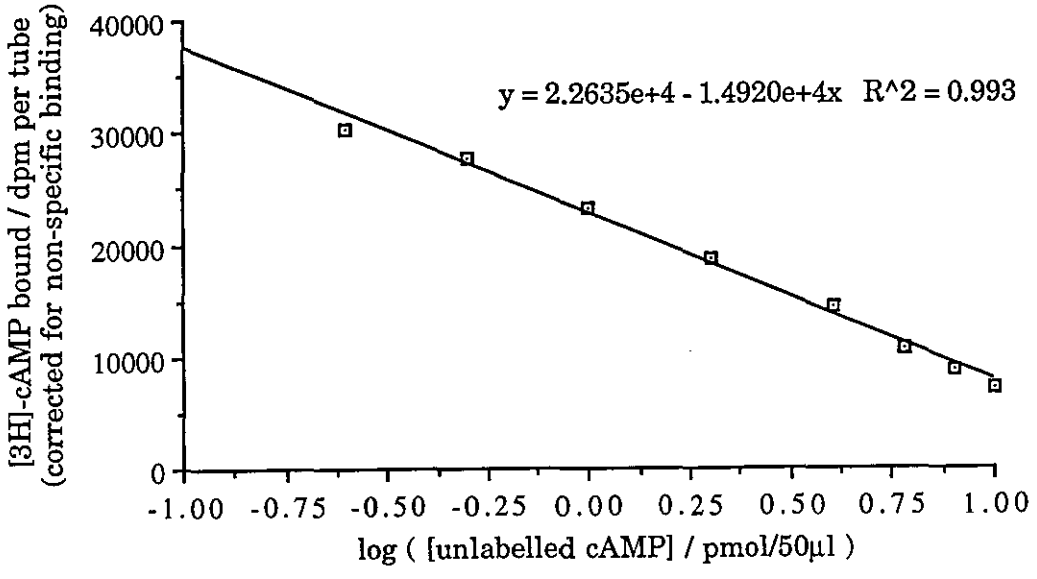


Figure 2.2 A representative standard curve as used for adenylyl cyclase assays.

2.2.8 Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Separation and identification of the δ-opioid receptor in a membrane fraction from NG108-15 cells was accomplished with the discontinuous system described by Laemmli [Laemmli, 1970] followed by Western blot analysis. A crude membrane preparation was prepared by treatment of a cell suspension containing approximately 5 mg of protein / ml in Tris-HCl (50mM, pH 7.4) with a tissue tearor (2x5s; 30,000rpm). The resultant membrane preparation was diluted into electrophoresis sample buffer (final protein concentration approximately 0.5mg/ml) and boiled for 90s in a water bath. Proteins (1-10μg per lane) were resolved by SDS-PAGE (8% (w/v) polyacrylamide, 0.22% (w/v) bisacrylamide) and subsequently transferred to a nitrocellulose membrane by electrophoretic elution. The blots were blocked overnight at 4°C in a 5% (w/v) Marvel® solution in phosphate buffered saline (PBS). Primary antibodies were added and incubated for 1.5h at room temperature. After three washes with PBS, the filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution with PBS) for 1.5h. Antibody complexes were visualised using an enhanced chemiluminescence kit (Amersham, Aylesbury), following the manufacturers instructions, with subsequent exposure to photographic film (15 min).

2.2.9 Molecular modelling

Peptide chains were assembled from the supplied L-amino acid library and modelled on an Evans and Sutherland workstation using SYBYL (version 6.1, Tripos Associates, St. Louis, USA).

2.2.10 Determination of protein

The method of Lowry *et al* (1951) was used, with bovine serum albumin as standard.

2.2.11 Data analysis

(a) Radioligand binding data

Radioligand binding data was analysed with the computer programme LIGAND [Munson and Rodbard, 1980], which provided estimates for the total receptor number (B_{max}), binding affinity (K_d or K_i) and Hill slope of the binding isotherms. Where appropriate, the data was fit to both one-site and two-site models. To determine if the two-site model produced a statistically better fit of the data than the one-site model, the variance ratio test (F-test) facility of LIGAND was used, where $P < 0.05$ was considered significant.

All graphical representations of the data reported in this thesis show specific binding.

(b) [γ-^{32}P]GTPase and [^{35}S]GTPγS binding assays

Maximal stimulation of GTPase activity caused by analogues of mastoparan was calculated manually from concentration-effect curves of the data.

The stimulation of [^{35}S]GTPγS binding obtained at various drug concentrations was fit, using the nonlinear least squares curve fitting programme GraphPad Prism (ISI), to the sigmoidal function

$$Y = Y_{min} + ([Y_{max} - Y_{min}] / [1 + 10^{(\log EC50 - X).n}])$$

where
- Y is the response
- Y_{min} is the minimum response
- Y_{max} is the maximum response
- X is the logarithm of the drug concentration
- EC_{50} is the concentration of drug that produces a half-maximal effect
- n is the Hill coefficient or slope factor.

Antagonist affinity (K_e) values were calculated from [^{35}S]GTPγS binding assay dose-response curves in the absence or presence of a single concentration of antagonist using the equation:

$$K_e = [\text{antagonist}] / (DR-1)$$

where DR = (EC_{50} in the presence of antagonist) / (EC_{50} in the absence of antagonist) [Leslie, 1987].

All graphical representations of [^{35}S]GTPγS binding data reported in this thesis represent specific binding.

(c) Adenylyl cyclase assays

Standard cAMP solutions were used to determine the amount of [^{3}H]-cAMP bound to the binding protein in the presence of varying amounts of unlabelled cAMP.This data was then used to plot a calibration graph. cAMP content of the unknown samples was determined by interpolation from this graph. Maximal inhibition of forskolin-stimulated adenylyl cyclase activity caused by opioid agonists, and concentrations of agonist which caused 50% of the maximal response (IC_{50}) were calculated manually from concentration-effect curves of the data.

(d) Statistics

Statistical comparisons between groups of data were made by Student's t-test, where $P<0.05$ was considered significant.

Chapter 3

LOW K_m GTPase ACTIVITY IN SH-SY5Y MEMBRANES

3.1 Introduction

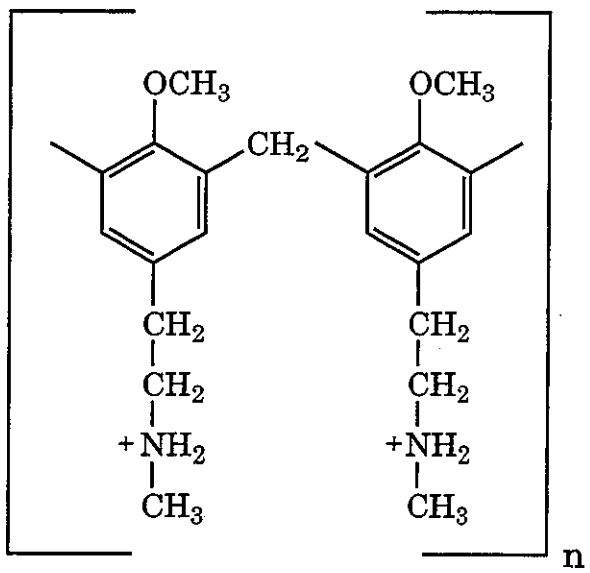
G protein-coupled receptors share a large degree of structural, functional and regulatory homology. The area of the receptor that binds and regulates G proteins is presumed to lie on the cationic, cytoplasmic face of the receptor, because the GTP-binding α-subunits of G proteins are located at the cytoplasmic surface of the plasma membrane. Various experimental techniques have confirmed that intracellular regions from these receptors are important for correct coupling to G proteins, as discussed in Chapter 1 (pages 20-31). However, despite the vast amount of reports describing which areas of particular receptors are important for G protein-coupling, the exact molecular determinants of G protein-coupling specificity and activation remain to be elucidated. Indeed, the lack of primary sequence homology amongst different receptors which couple to the same G protein(s) has led to the hypothesis that it is the secondary structure, and more specifically the amphipathic, α-helical nature of these regions which is important in determining their G protein-activating function [Higashijima *et al*, 1988; Cheung *et al*, 1991].

Mastoparan [Higashijima *et al*, 1988 and 1990] (Fig. 3.1) and a variety of other cationic, amphiphilic peptides, for example Substance P [Mousli *et al*, 1990] and bradykinin [Mousli *et al*, 1990a], directly stimulate purified G proteins reconstituted into phospholipid vesicles. Mastoparan forms an amphipathic α-helix when bound to phospholipid membranes [Wakamatsu *et al*, 1983] and G proteins [Sukumar and Higashijima, 1992], and a cysteine-containing analogue of mastoparan cross-links with G_o at the amino-terminus of the G protein [Higashijima and Ross, 1991]. Mastoparan is thus thought to mimic part of the active conformation of G protein-coupled receptors. Certainly the stimulation of purified G proteins by mastoparan and the receptor-mediated stimulation show many similarities, for example dependence on Mg^{2+} ions and sensitivity to pertussis toxin [Higashijima *et al*, 1988]. Consequently mastoparan represents a useful tool to probe the molecular mechanisms by which receptors interact with G proteins. However, several non-peptide hydrophobic amines, including natural polyamines such as spermine [Bueb *et al*, 1992] as well as benzalkonium chloride [Higashijima *et al*, 1990], octadecyltrimethylammonium chloride [Higashijima *et al*, 1990] and compound 48/80 [Mousli *et al*, 1990;

Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2 MP
Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-**Ala**-**Leu**-Leu-NH_2 MP 7
Ile-Asn-Leu-Lys-Ala-**Lys**-Ala-Ala-Leu-Ala-Lys-Lys-**Leu**-Leu-NH_2 MP 17

Figure 3.1 Amino acid sequence for mastoparan (MP), mastoparan 7 (MP 7) and mastoparan 17 (MP 17). Differences from mastoparan are shown in bold.

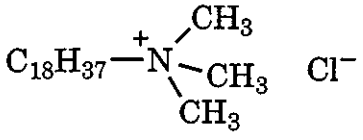
(a)



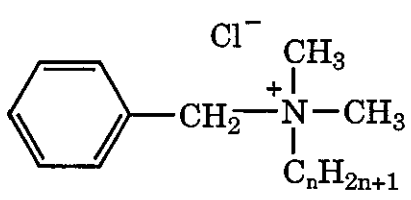
(b)

H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2

(c)



(d)



(n=12-18)

(e)

D-Pro-Gln-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2

Figure 3.2 Structures of (a) Compound 48/80, (b) Spermine, (c) Octadecyltrimethylammonium chloride, (d) Benzalkonium chloride (BAC), (e) [D-Pro^4,D-$Trp^{7,9,10}$]-Substance P fragment 4-11

Tomita *et al*, 1991] are also able to stimulate purified G proteins directly (see Fig. 3.2 for structures), so the specificity and usefulness of using short fragments of receptor sequences as mimics to study receptor-G protein interaction is called into question.

This study was designed to compare the ability of mastoparan and related peptides with various amphiphilic, cationic amines to stimulate G proteins in a native membrane environment, as a prelude to studies using peptide sequences from intracellular loop regions of opioid receptors. G protein stimulation was determined as an increase in the low K_m GTPase activity in membranes from human neuroblastoma SH-SY5Y cells.

3.2 Results

3.2.1 Determination of incubation conditions

The stimulation of GTPase activity by opioids in membranes from cell lines containing μ- or δ-opioid receptors has been reported previously [Koski and Klee, 1981; Costa *et al*, 1991; Carter and Medzihradsky, 1992; Selley and Bidlack, 1992]. However, reports of GTPase assays in the SH-SY5Y cell line suggested that variability between experiments may be high [Costa *et al*, 1991; Carter and Medzihradsky, 1992]. Therefore preliminary experiments were designed to ensure that an opioid-mediated stimulation could be reproduced in our laboratory.

1. Dependence of basal low K_m GTPase activity on protein concentration

Variation of basal low K_m GTPase activity with the amount of membrane protein added to the assay is shown in Figure 3.3. In a single experiment, low K_m GTPase activity was linear over only a limited protein concentration. Therefore, in all subsequent experiments, 10-30 μg protein per assay tube was used. This is similar to the protein concentration of SH-SY5Y membranes used in previous reports [Costa *et al*, 1991; Carter and Medzihradsky, 1992].

2. Time and temperature of incubation

Figures 3.4 and 3.5 show the variation of basal and DAMGO (1 μM)-stimulated low K_m GTPase activity with the time and temperature of incubation. Optimal stimulation is seen at incubation times of 15-20 min; however, because the assay becomes non-linear at times greater than 20 min, a 15 min incubation was chosen for subsequent experiments. An incubation temperature of 37°C was chosen to obtain a maximal stimulation window.

The μ-opioid peptide DAMGO (1μM) caused a small (24.2±1.7%, n=3), but significant stimulation of low Km GTPase activity in membranes from SH-SY5Y cells under the optimal conditions chosen, in agreement with previous work [Costa *et al*, 1991; Carter and Medzihradsky, 1992].

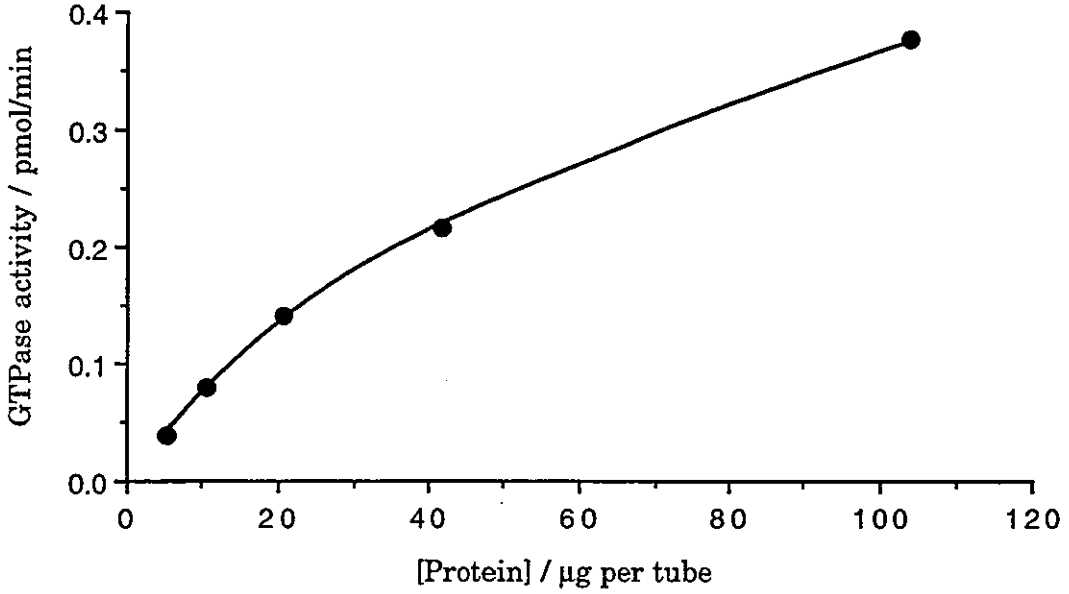


Figure 3.3 Effect of protein concentration on low K_m GTPase activity in SH-SY5Y membranes (n=1). Experimental conditions were as described in Materials and Methods, except the incubation was for 10 min at 37°C.

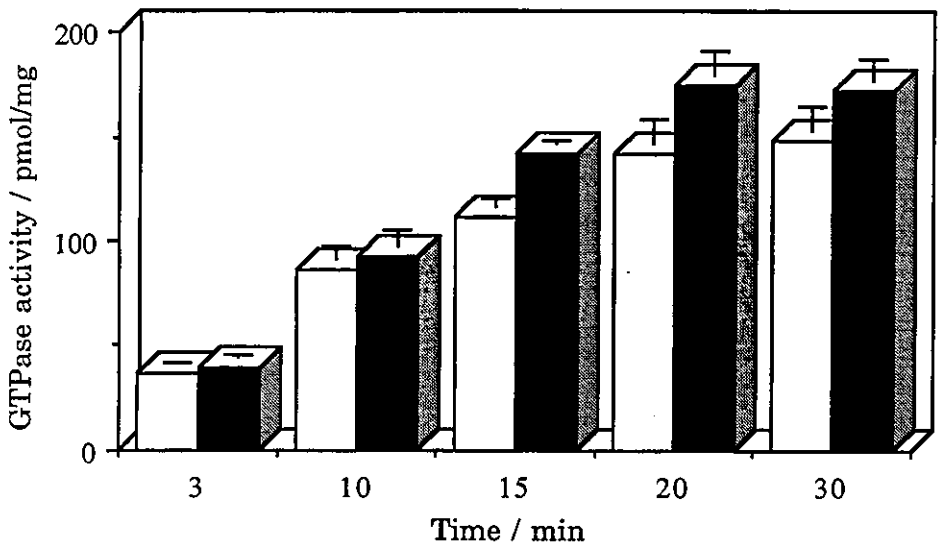


Figure 3.4 Effect of incubation time on basal (white) and stimulated (1 µM DAMGO; black) GTPase activity in SH-SY5Y membranes. Incubation temperature was 37°C. Values represent means ± range (n=2).

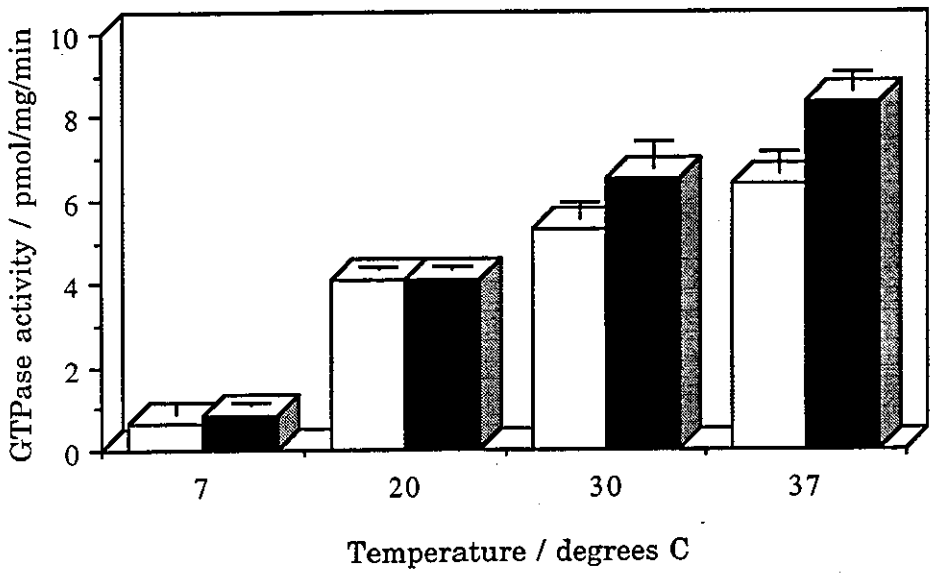


Figure 3.5 Effect of temperature on basal (white) and stimulated (1 μM DAMGO; black) GTPase activity in SH-SY5Y membranes. Assay incubation time was 15 min. Values represent means±range (n=2).

3.2.2 *Effect of mastoparan analogues on low Km GTPase activity in membranes from SH-SY5Y cells.*

In membranes from SH-SY5Y human neuroblastoma cells, mastoparan stimulated low K_m GTPase activity three-fold (Fig. 3.6). Mastoparan 7 produced a similar maximal stimulation, but was more potent than mastoparan (Fig. 3.6). The concentration-effect curve for mastoparan 7 was steep, producing a maximal effect at 25 μM, whereas the curve for mastoparan was shallower and produced a maximal effect at 70 μM. The dose-effect curves for both compounds were bell-shaped, and returned to basal values at 85 μM for mastoparan 7 and 250 μM for mastoparan. Higher concentrations than this resulted in an inhibition of the basal GTPase activity (Fig. 3.6). In contrast, no effect of mastoparan 17 (Fig. 3.1), either stimulatory or inhibitory, was observed at concentrations up to 100 μM (Fig. 3.6).

In addition mastoparan 17 (10-100 μM) did not block the stimulation of low K_m GTPase activity caused by mastoparan (50 μM) (Fig. 3.7).

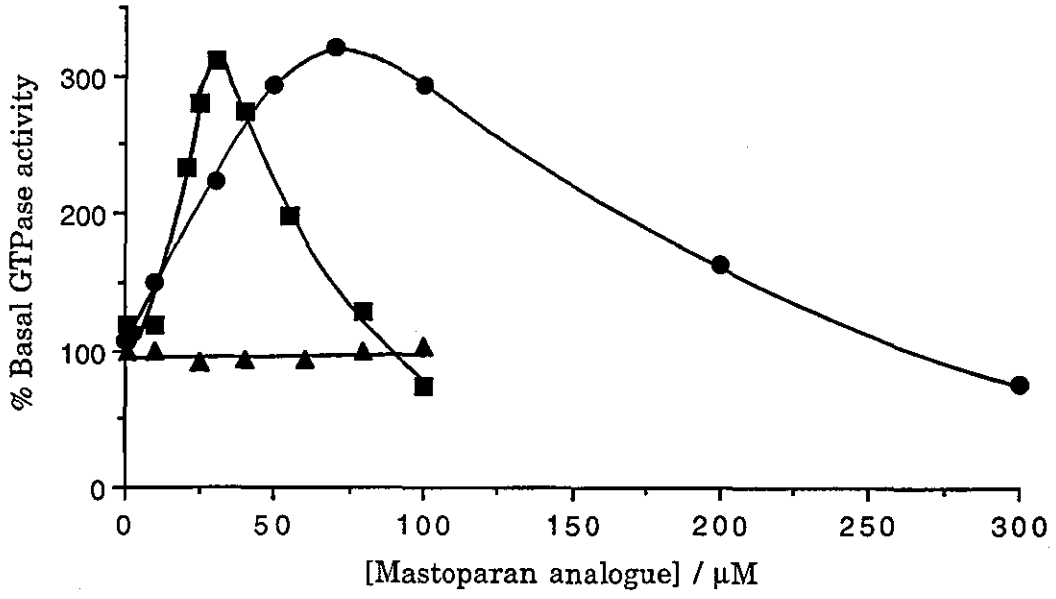


Figure 3.6 A representative graph showing the effect of mastoparan (●), mastoparan 7 (■) and mastoparan 17 (▲) on GTPase activity in SH-SY5Y membranes (n≥3 for mastoparan and mastoparan 7; n=1 for mastoparan 17). Basal GTPase activity was 5.41±0.56 pmol/mg/min (n=13).

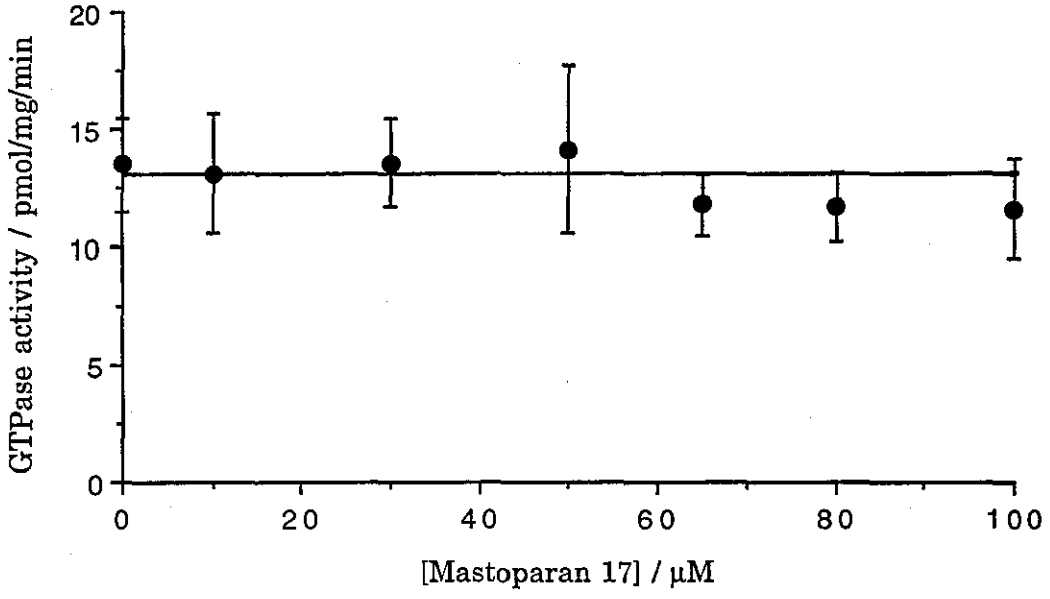


Figure 3.7 Effect of mastoparan 17 on mastoparan (50 μM)-stimulated GTPase activity in SH-SY5Y membranes. GTPase activity was 4.88±1.94 pmol/mg/min and 13.63±1.99 pmol/mg/min in the absence and presence of mastoparan (50 μM) respectively. Values are means±range (n=2).

3.2.3 Effect of mastoparan analogues on opioid agonist and antagonist binding

According to the ternary complex model for G protein-coupled receptors proposed by De Lean *et al* (1980), high affinity agonist, but not antagonist binding is dependent on the interaction of receptor with G protein. Therefore, mastoparan analogues might be expected to alter opioid agonist binding affinity.

The effect of mastoparan on the binding of the opioid agonist [^{3}H]-DAMGO in SH-SY5Y membranes is shown in Figure 3.8. Mastoparan decreased [^{3}H]-DAMGO binding by approximately 20%, reaching a plateau at 30 μM such that no further inhibition was observed. However, mastoparan (70 μM) reduced [^{3}H]-diprenorphine and [^{3}H]-DAMGO binding to a similar degree (Fig. 3.9). Furthermore, the inactive mastoparan analogue, mastoparan 17, was also effective in inhibiting [^{3}H]-DAMGO binding (Fig. 3.9).

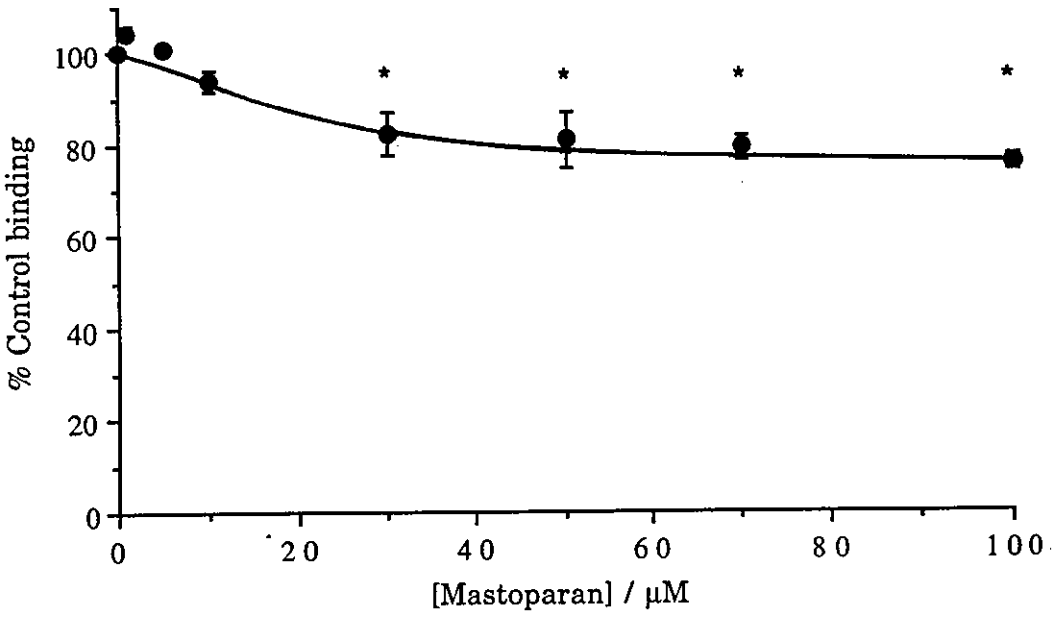


Figure 3.8 Effect of mastoparan on [^{3}H]-DAMGO (1.25 nM) binding in membranes from SH-SY5Y cells. Values are means±sem (n≥3). * : P < 0.01 versus control.

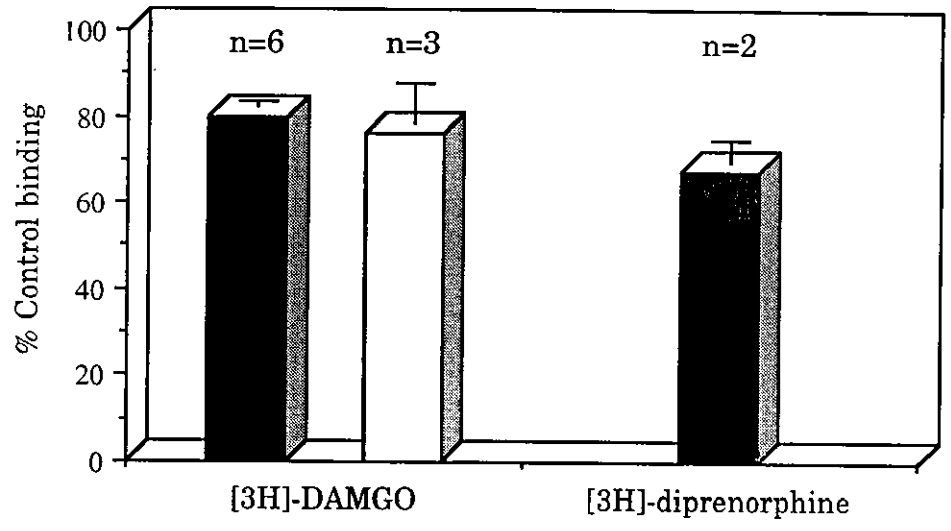


Figure 3.9 Comparison of the effects of mastoparan (70 µM; black) and mastoparan 17 (70µM; white) on specific [^{3}H]-DAMGO (1.25 nM) and [^{3}H]-diprenorphine (0.40 nM) binding in SH-SY5Y membranes. Values represent means±sem ([^{3}H]-DAMGO) or means±range ([^{3}H]-diprenorphine).

3.2.4 *Effect of amphiphilic cationic amines on low K_m GTPase activity in membranes from SH-SY5Y cells*

The effect of benzalkonium chloride (BAC), spermine, compound 48/80 and octadecyltrimethylammonium bromide (OTAB) on low K_m GTPase activity in membranes from SH-SY5Y cells is shown in Fig. 3.10.

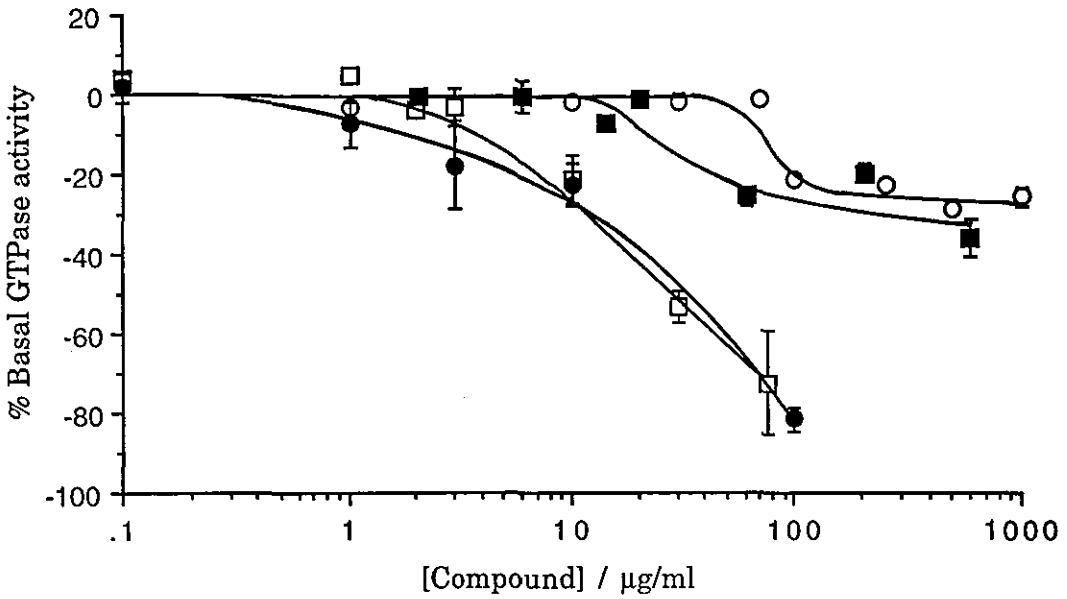


Figure 3.10 Effect of benzalkonium chloride (□), spermine (■), compound 48/80 (○) and octadecyltrimethylammonium bromide (●) on low K_m GTPase activity in SH-SY5Y membranes. Values represent means±sem (n≥3). Basal activity was 8.19±0.95 pmol/mg/min (n=15).

None of the amines caused any observable stimulation of low K_m GTPase activity in membranes from SH-SY5Y cells at any concentration tested. Conversely, at high concentrations all of the amines caused an inhibition of basal GTPase activity, with BAC and OTAB causing a larger inhibition of activity than compound 48/80 and spermine (100µM mastoparan is equivalent to 147.9µg/ml).

BAC and a novel Substance P analogue, [D-Pro^4,D-Trp^{7,9,10}]-Substance P fragment 4-11 (Fig. 3.2) have both been reported to antagonise receptor and / or

mastoparan mediated G protein activation [Mukai *et al*, 1992; Higashijima *et al*, 1990]. The ability of [D-Pro^4,D-Trp^{7,9,10}]-Substance P fragment 4-11 and benzalkonium chloride to antagonise mastoparan (70 μM)-stimulated GTPase activation in SH-SY5Y membranes is shown in Figs. 3.11 and 3.12.

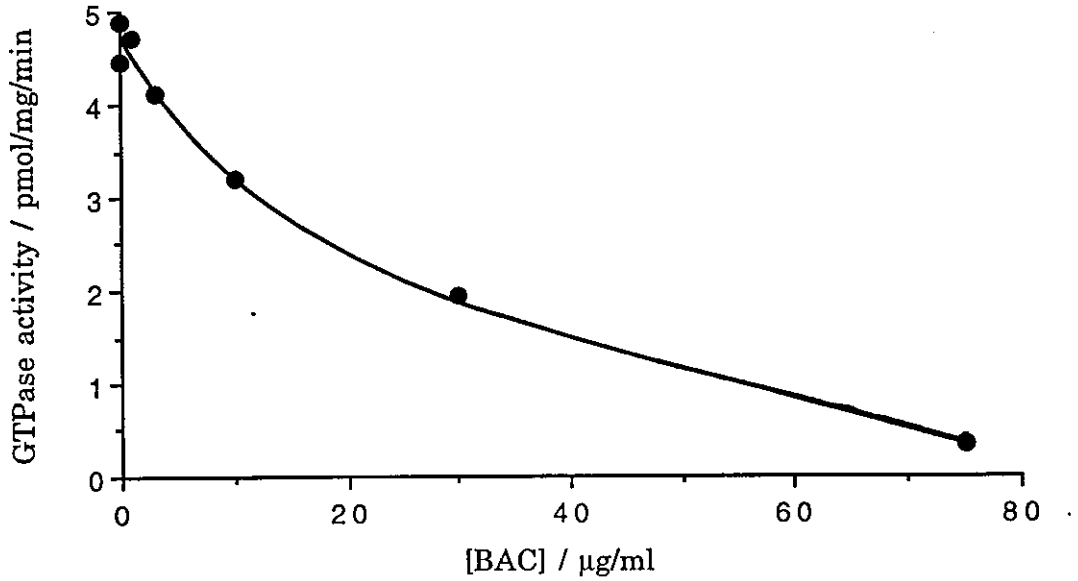


Figure 3.11 Effect of benzalkonium chloride on mastoparan (70μM)-stimulated GTPase activity in SH-SY5Y membranes (n=1). GTPase activity was 2.20 pmol/mg/min and 4.47 pmol/mg/min in the absence and presence of mastoparan (70 μM) respectively.

Benzalkonium chloride reduced mastoparan (70μM)-stimulated GTPase activity in SH-SY5Y membranes. However, this compound had an identical effect on basal GTPase activity (Fig. 3.10), and so it is likely that the reduction in mastoparan stimulated GTPase activity does not occur as a result of competition between mastoparan and BAC for the G protein, but rather as a result of a non-specific inhibition of GTPase activity by BAC. In a single experiment the potential G protein antagonist [D-Pro^4,D-Trp^{7,9,10}]-Substance P fragment 4-11 had no effect on mastoparan (70 μM)-stimulated GTP hydrolysis in membranes from SH-SY5Y cells (Fig. 3.12).

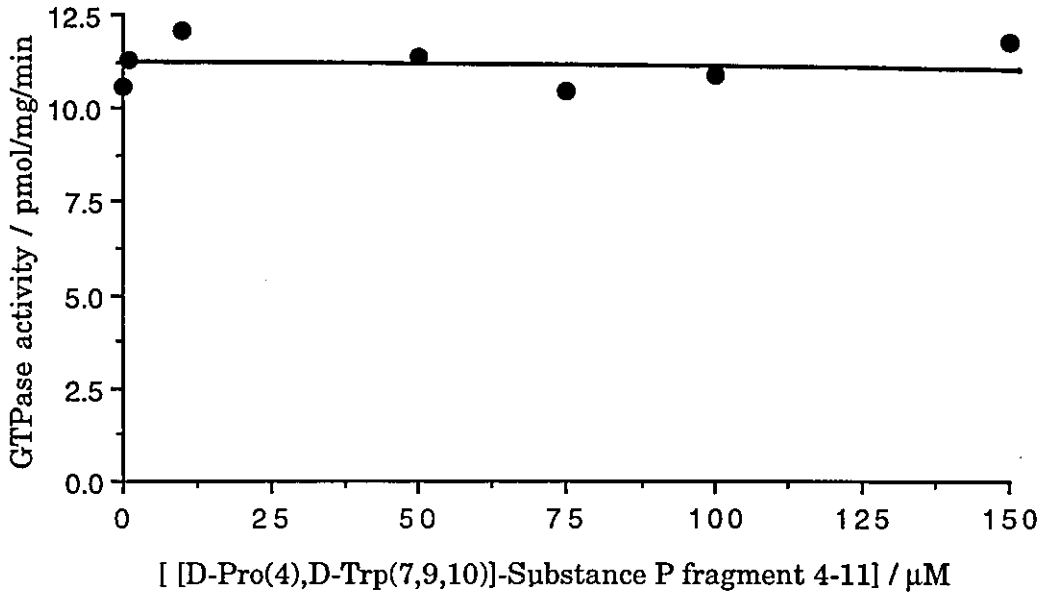


Figure 3.12 Lack of effect of [D-Pro^4,D-Trp^{7,9,10}]-Substance P fragment 4-11 on mastoparan (70µM)-stimulated GTPase activity in SH-SY5Y membranes (n=1). GTPase activity was 4.62 pmol/mg/min and 10.59 pmol/mg/min in the absence and presence of mastoparan (70 µM) respectively.

To test if benzalkonium chloride causes a reduction in GTPase activity by denaturing the G protein, the reversibility of the inhibition was studied (Table 3.1).

[BAC] present in initial incubation / (µg/ml)	Basal GTPase activity (pmol/mg/min)
0	6.93
50	5.72
100	4.71
200	4.31

Table 3.1 Membranes from SH-SY5Y cells were incubated for one hour on ice in the presence of varying concentrations of BAC, after which membranes were pelleted, washed and repelleted three times. GTPase activity of the membranes was then assayed (n=1).

Incubation with successively higher concentrations of BAC progressively lowered the basal GTPase activity of the membrane preparation. However, by comparison with Fig. 3.10, it would appear that the inhibitory mechanism is rather complex; a large proportion of the inhibition can be 'washed out'.

3.3 Discussion

A low K_m GTPase assay has been investigated as a potential method to study G protein activation in membranes from SH-SY5Y cells by opioid receptors and small molecule peptide and non-peptide potential receptor mimics. Preliminary experiments were designed to confirm that μ-opioid agonists could reproduce the stimulation of low K_m GTPase activity in membranes from these cells reported by others [Costa *et al*, 1991; Carter and Medzihradsky, 1992]. Under optimal conditions, DAMGO (1 μM) caused a 24.2±1.7% increase in basal low K_m GTPase activity. This stimulation was significant at the 95% confidence limit, but in terms of actual numbers, this represented no more than an increase of approximately 2,000 dpm on a background level of between 4,000-10,000 dpm. The relatively modest stimulation compared to background signal could possibly be because the [γ-^{32}P]GTP was used as received (> 95% radiochemical purity) without further purification. The presence of tiny amounts (1% or less) of impurities (e.g. orthophosphate or pyrophosphate) may cause substantial increases in the assay background [Ross and Higashijima, 1994]. In addition, the variability both within and between experiments was often large. There are several potential reasons for this:

(a) High K_m GTPase activity (which is insensitive to the presence of opioids) must be subtracted from each sample. In individual experiments this accounted for 25-50% of the total GTP hydrolysis, as found by Selley and Bidlack (1992). Consequently, the measured basal low K_m GTPase activity varied considerably.

(b) The total reaction volume of only 100 μl caused any slight pipetting inaccuracies to be magnified.

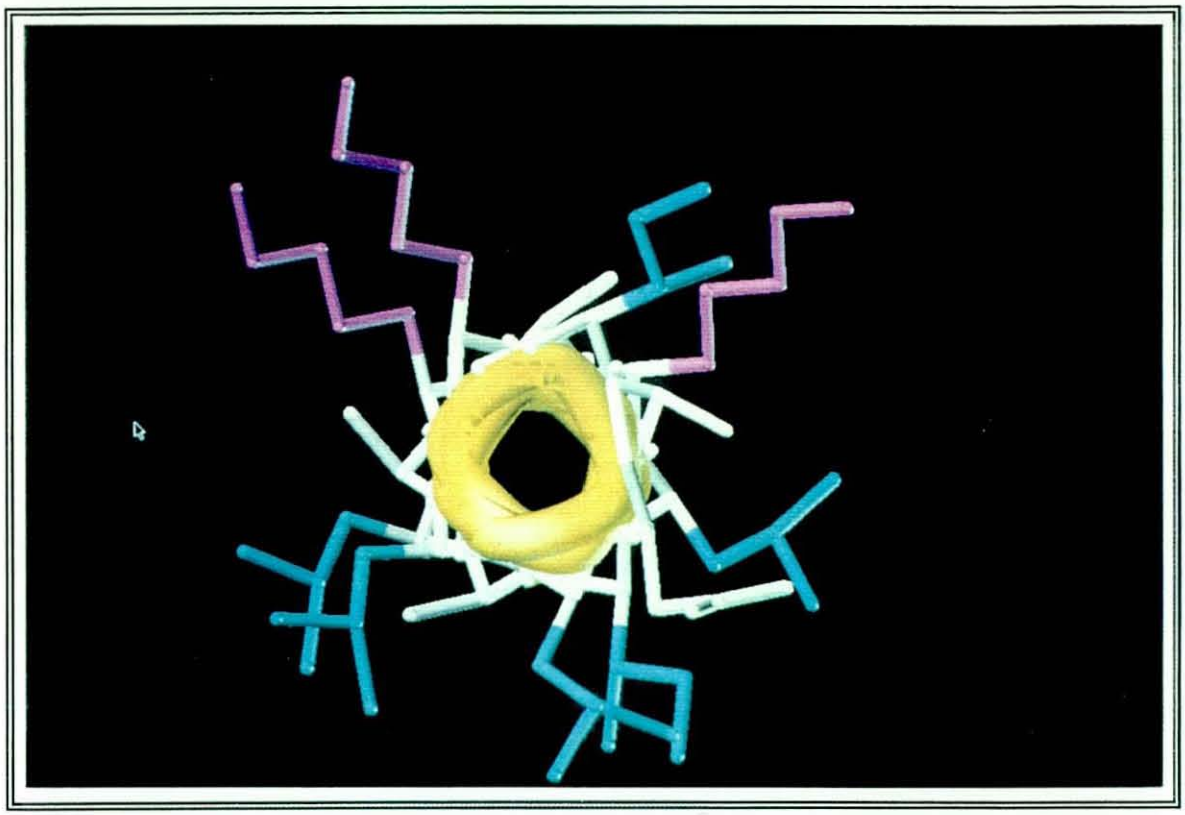
(c) Separation of [γ-^{32}P]GTP from [$^{32}P_i$] was carried out using activated charcoal. To pellet the charcoal tubes were centrifuged at room temperature, due to technical constraints. However, this step is best carried out at 4°C, as heating the tubes to room temperature encourages charcoal to stick to the side of the Eppendorf tube, and makes it more difficult to remove the supernatant cleanly [McKenzie, 1992]. Small particles of charcoal with

adsorbed [γ-^{32}P]GTP present in the supernatant, which is removed for scintillation counting, will clearly cause variability within individual replicates.

The stimulation of 2,000 dpm caused by DAMGO (1 μM) was considered too small to be of use for further functional characterisation of the activation of G proteins by μ-opioid receptors in SH-SY5Y membranes. However, despite this lack of sensitivity low K_m GTPase activity in SH-SY5Y membranes was stimulated 3-fold by the wasp venom peptide mastoparan (70 μM). Direct activation of purified G_o and / or G_i proteins by mastoparan is well documented [Higashijima *et al*, 1988, 1990; Oppi *et al*, 1992]. GTPase activation was also observed with the mastoparan analogue mastoparan 7, but not with mastoparan 17. Previous studies using purified G proteins reconstituted into phospholipid vesicles have shown mastoparan 7 to be an active analogue of native mastoparan, but mastoparan 17 to be inactive [Higashijima *et al*, 1990].

One possible explanation for this differential activity of the various mastoparan analogues could be the relative positioning of basic and hydrophobic amino acid residues in the peptide chain, as described by Oppi *et al* (1992) for peptides representing sequence permutations of mastoparan. It is known that mastoparan-X, a functionally active analogue of mastoparan, forms an α-helix when bound to phospholipid membranes [Wakamatsu *et al*, 1983] and G proteins [Sukumar and Higashijima, 1992]. Molecular models of mastoparan, mastoparan 7 and mastoparan 17 based on a perfect α-helix are shown in Figs. 3.13 - 3.15. When modelled in this way it can be seen that both active mastoparan analogues, mastoparan and mastoparan 7, possess one face of the helix which is positively charged containing basic amino acids, and one face which is hydrophobic, consisting predominantly of amino acid residues which have hydrocarbon side-chains. Higashijima *et al* (1990) have suggested that the hydrophobic moment formed by such charge separation on the α-helical backbone plays an important role in the regulatory activity of these peptides. Although transferred nuclear Overhauser NMR spectroscopy is able to determine that the G protein-bound conformation of mastoparan analogues is α-helical, unfortunately it provides no information on which side of the helix is

(a)



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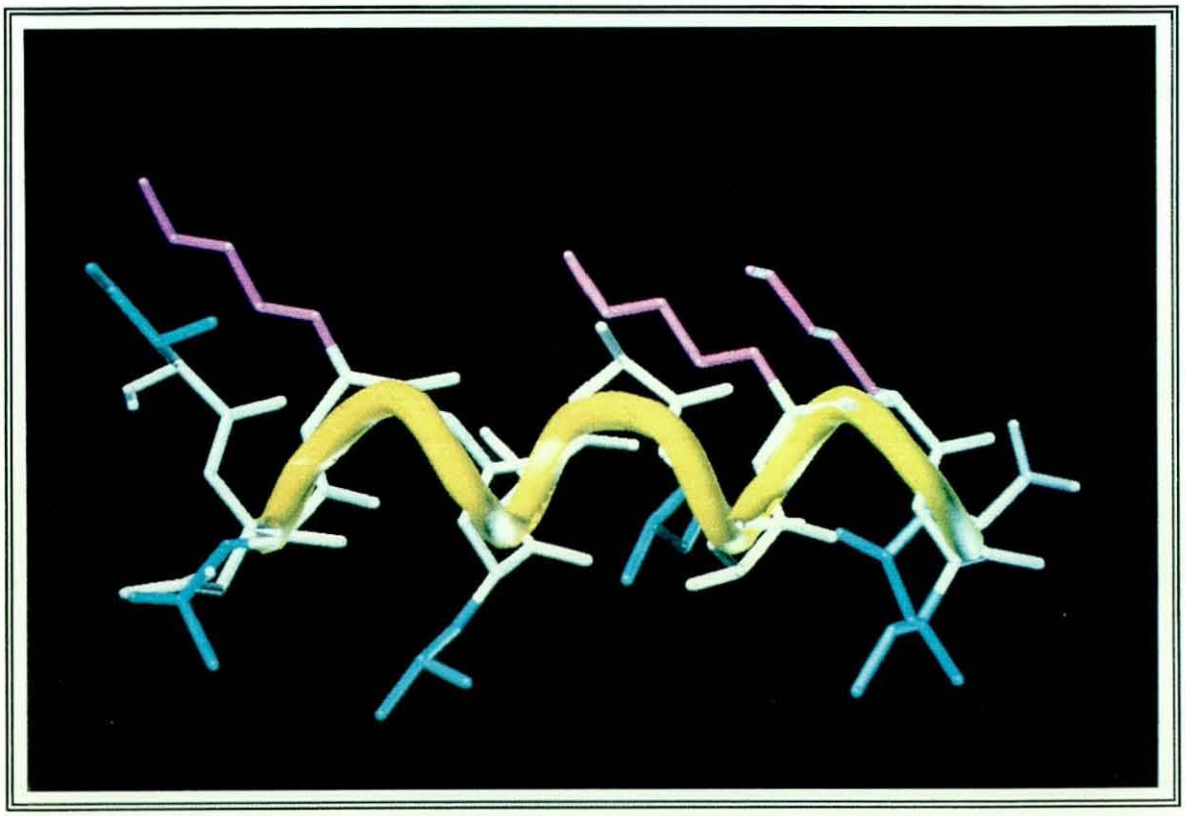


Figure 3.13 Mastoparan modelled as an α-helix viewed (a) from above the helix (N-terminus coming out of the page) and (b) from the side of the helix (N-terminus to the left). Basic and hydrophobic residues are shown in magenta and cyan respectively. The α-helix backbone is shown in yellow.

(a)

(b)

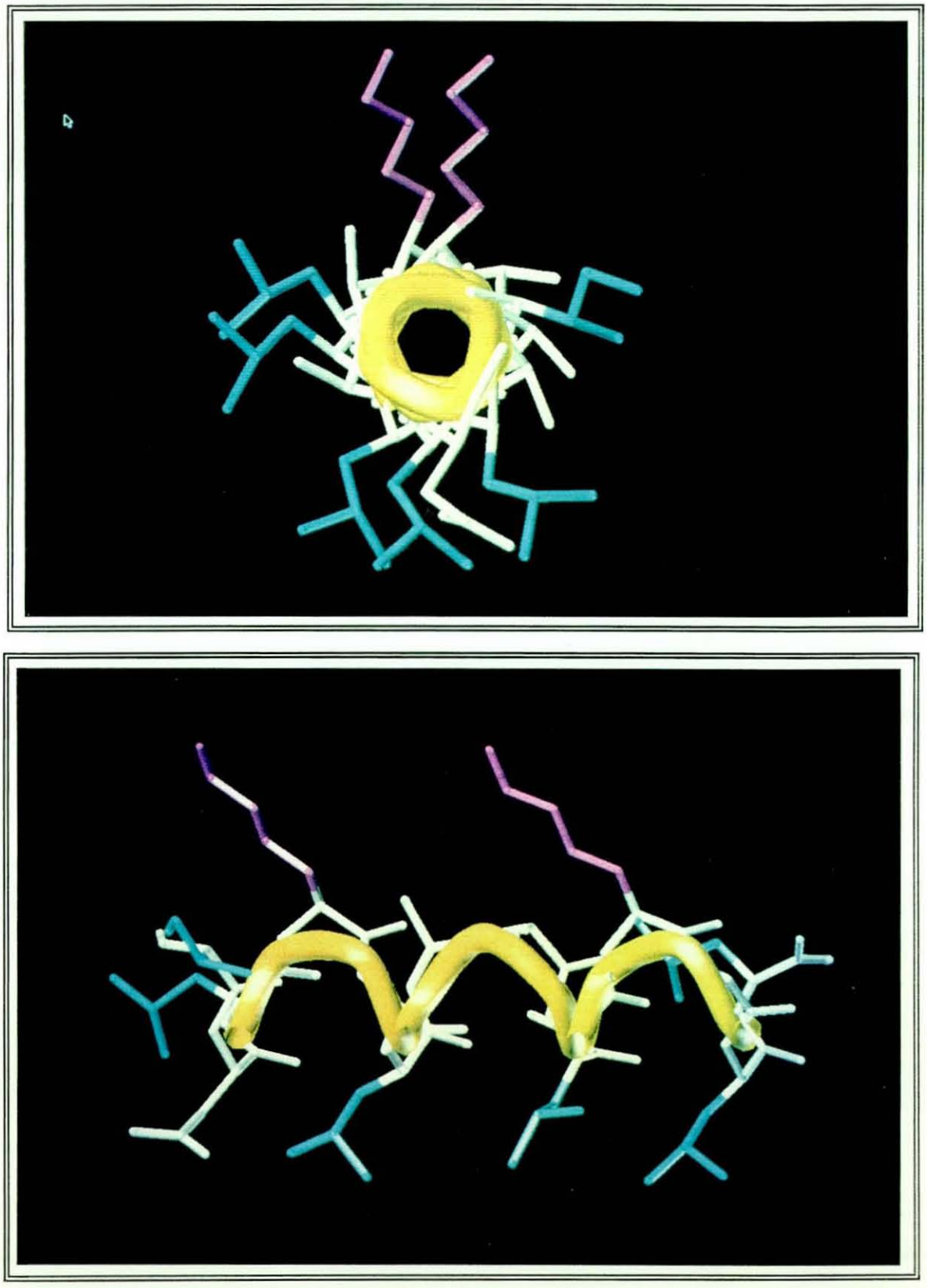


Figure 3.14 Mastoparan 7 modelled as an α-helix viewed (a) from above the helix (N-terminus coming out of the page) and (b) from the side of the helix (N-terminus to the left). Basic and hydrophobic residues are shown in magenta and cyan respectively. The α-helix backbone is shown in yellow.

(a)

(b)

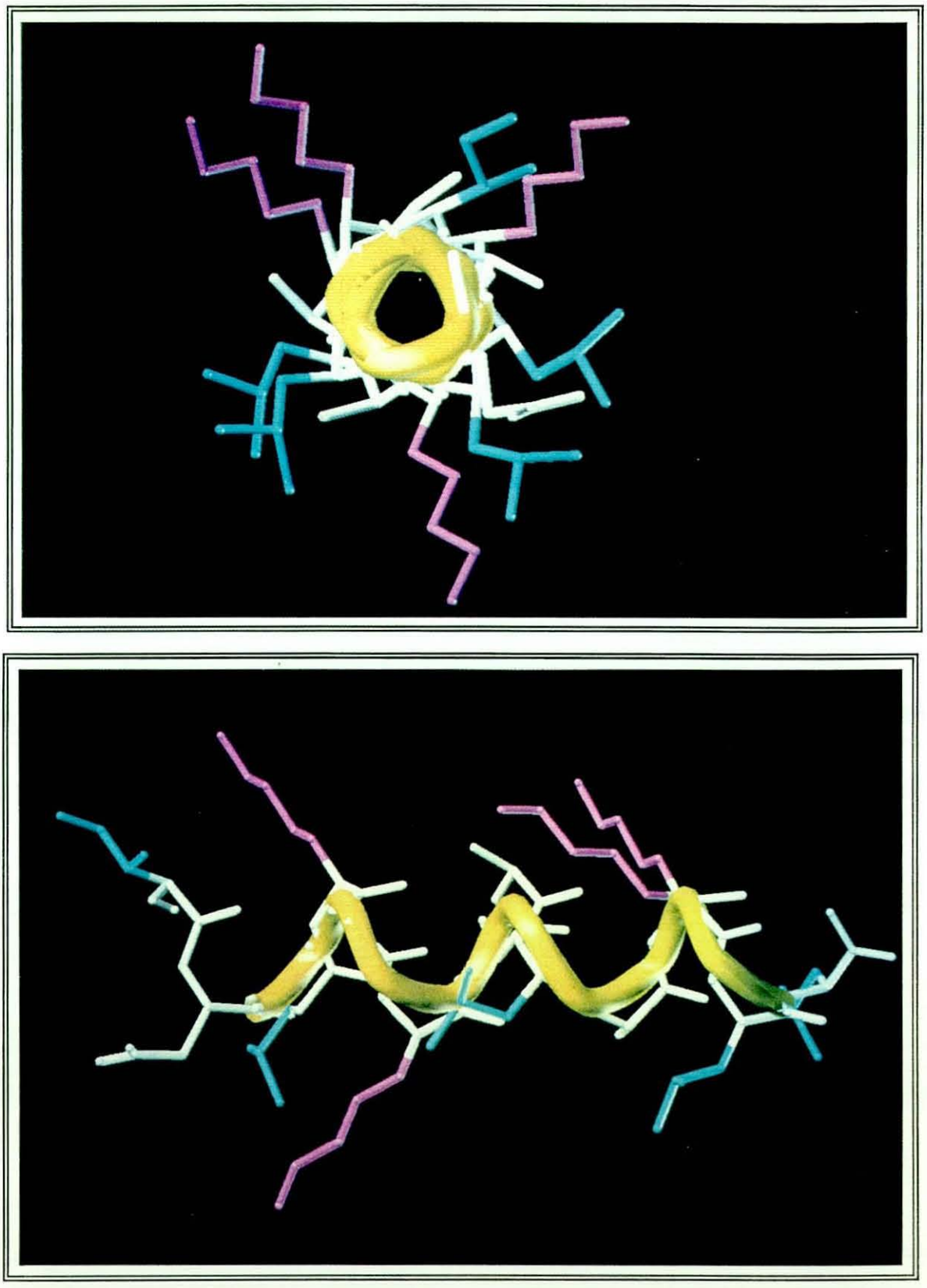


Figure 3.15 Mastoparan 17 modelled as an α-helix viewed (a) from above the helix (N-terminus coming out of the page) and (b) from the side of the helix (N-terminus to the left). Basic and hydrophobic residues are shown in magenta and cyan respectively. The α-helix backbone is shown in yellow.

actually in contact with the G protein [Sukumar and Higashijima, 1992]. Structure-activity relationships of mastoparan analogues show that positively charged residues at N- and C-terminal ends of the molecule are crucial for activity [Higashijima *et al*, 1990]. Thus it is reasonable to suppose that the polar face of the amphiphilic mastoparan molecule is involved in G protein binding. Indeed, based on the results of studies using mast cell degranulating peptide and its exact optical isomer (synthesised using D-amino acids instead of the L-forms) it has been suggested that a cluster of cationic residues on one side of the α-helical surface are more important in direct activation of G proteins than a specific α-helical structure [Tomita *et al*, 1991a; Fujimoto *et al*, 1991].

In contrast to mastoparan and mastoparan 7, the inactive analogue, mastoparan 17, has charge oriented towards what would otherwise be the hydrophobic face of the helix (Fig. 3.15). It is tempting to speculate that this disruption in the amphiphilicity of the peptide causes the molecule to be inactive. Such a hypothesis suggests that the hydrophobic face of the helix does indeed play an important role in determining the activity of mastoparan analogues, perhaps by anchoring the peptide to the cell membrane. It is known that the two amino acid substitutions in mastoparan 17 compared to mastoparan cause this peptide to have a lower fractional α-helical content [Higashijima *et al*, 1990], and it is also possible that this underlies the inability of mastoparan 17 to activate G proteins.

It has been suggested that mastoparan may interact with a nucleoside diphosphate kinase [Klinker *et al*, 1994; Kowluru *et al*, 1995]. This ubiquitous family of enzymes catalyse phosphorylation of nucleoside 5'-diphosphates *via* the formation of high-energy phosphohistidine intermediates. Thus activation of nucleoside diphosphate kinase could cause an indirect activation of G proteins by increasing the local concentration of GTP synthesised from free GDP [Seifert *et al*, 1988; Wieland *et al*, 1991; Lacombe and Jakobs, 1992]. However, mastoparan 17 also activates nucleoside diphosphate kinase at similar concentrations to mastoparan [Kowluru *et al*, 1995]. Since mastoparan 17 has no stimulatory activity in SH-SY5Y membranes, it seems likely that mastoparan and mastoparan 7 stimulate GTP hydrolysis through a direct interaction with G proteins in SH-SY5Y cell membranes, rather than as a result of their interaction with nucleoside diphosphate kinase.

The stimulation afforded by both mastoparan and mastoparan 7 reversed at higher concentrations, resulting in bell-shaped concentration-effect curves and even inhibition of basal GTPase activity at higher concentrations. Such biphasic effects of mastoparan on GTP hydrolysis have been reported previously in membranes from HL-60 cells [Klinker *et al*, 1994]. This effect may be the result of a combination of a specific action on G proteins and a well documented membrane-perturbing action [Mellor and Sansom, 1990; Tanimura *et al*, 1991; Danilenko *et al*, 1993]. Alternatively, the inhibition at high mastoparan concentrations may be a result of G protein denaturation caused by mastoparan [Higashijima *et al*, 1990]. Such denaturation, which may reflect the instability of nucleotide-free G protein [Ross and Higashijima, 1994], could cause an underestimation of maximal stimulation, as denaturation of G protein begins to outweigh increasing GTPase activity.

In contrast to mastoparan and mastoparan 7, compound 48/80, BAC, spermine and OTAB were unable to stimulate the low K_m GTPase activity of SH-SY5Y cell membranes. In comparison, these compounds (or in the case of OTAB the closely related octadecyltrimethylammonium chloride) have been reported to activate purified G proteins reconstituted into artificial phospholipid vesicles [Higashijima *et al*, 1990; Mousli *et al*, 1990; Tomita *et al*, 1991; Bueb *et al*, 1992]. This apparent discrepancy may suggest that a native membrane environment can confer a degree of selectivity on G protein activation which is not apparent when using purified G proteins reconstituted into artificial membranes. Thus, cell membrane preparations may represent an improved model over purified G proteins for probing the activation of receptor-coupled G proteins. Another potential explanation could be that amphiphilic hydrophobic amines may induce G protein activation in purified preparations reconstituted into phospholipid vesicles non-specifically, by, for example, presenting a row of positively charged amine groups to the G protein. This may not represent the physiological mechanism by which G proteins become activated, and therefore does not occur in a cell membrane preparation. In this context, it is important to note that many cationic compounds, even NH_4^+ [Ferguson *et al*, 1986], can accelerate nucleotide exchange on G proteins to some extent and many cationic peptides can stimulate exchange by 50-100% [Higashijima *et al*, 1990], a level that the authors considered insignificant in comparison to the large and specific effects seen with mastoparan and its analogues [Ross and Higashijima, 1994].

Alternatively, it may be that SH-SY5Y cell membrane preparations are not sensitive enough tools with which to detect activation of G proteins by hydrophobic amines. The stimulation of GTPase activity by hydrophobic amines is frequently smaller than that produced by mastoparan [Higashijima *et al*, 1990; Tomita *et al*, 1991; Mousli *et al*, 1990], although the compounds tested in these studies always produced at least 50% of the response caused by mastoparan (100 μM), with the exception of spermine which was not compared directly to mastoparan. Since, in the present results, mastoparan caused a 3-fold increase in basal low K_m GTPase activity in SH-SY5Y cell membranes, one would have expected to see at least a small stimulatory response to the hydrophobic amines. However, none of the compounds caused any stimulation of GTPase activity at any concentration tested.

At high concentrations compound 48/80, BAC, spermine and OTAB all caused an inhibition of basal low K_m GTPase activity in membranes from SH-SY5Y cells. An inhibitory action of BAC and octadecyltrimethylammonium chloride (100 μg/ml) on purified G_i GTPase activity has been reported previously [Higashijima *et al*, 1990], which was attributed to the destabilising effect which high concentrations of hydrophobic amines have on G proteins. Since all of the compounds tested caused an inhibition of GTPase activity at high concentrations, a non-specific action such as G protein denaturation seems a reasonable explanation for these effects.

In a single experiment to test the reversibility of BAC inhibition of GTPase activity, by washing BAC from the membrane preparation before assaying for GTP hydrolysis, it was shown that removal of the BAC caused only a partial recovery of membrane GTPase activity. Therefore, if chemical denaturation of the G protein is responsible for the inhibition of GTPase activity elicited by BAC, it would seem that this effect is partly reversible on removal of BAC.

An unexplained anomaly is the effect of mastoparan analogues on opioid agonist and antagonist binding in SH-SY5Y membranes. All three peptides, both active and inactive with respect to stimulation of GTPase activity, caused a reduction in both agonist and antagonist binding. If a reduction in only agonist binding were observed, then a possible explanation might be that the peptides

were causing a disruption to receptor-G protein-coupling. However, since antagonist binding is also affected, it seems more likely that the reduction in binding is due to an unknown non-specific effect.

Chapter 4

[^{35}S]GTPγS BINDING IN MEMBRANES FROM NG108-15 CELLS

4.1 Introduction

In order to understand the molecular mechanisms which underlie opioid receptor activation, and to develop more clinically useful opiates, a sensitive and reliable functional assay to detect the activated state of the receptor is necessary. The first methods devised to test for opioid receptor activity involved the use of whole animal models, such as the antinociceptive effects elicited by opioids in the mouse tail flick test, or the use of opioid receptor selective animal tissues, such as the guinea pig ileum or mouse vas deferens [Casey and Parfitt, 1985]. Whilst these methods are suitable for screening novel compounds for opioid activity, they lack the sensitivity required to further our understanding of the molecular basis of opioid action, and also suffer the drawback of requiring a large amount of animals or tissue. In recent years therefore, there has been an increasing trend towards the use of immortalised cell lines to study the mechanisms of drug action. The success of such an approach depends on the development of reliable, reproducible and meaningful assays to study drug-receptor interactions.

The NG108-15 mouse neuroblastoma x rat glioma hybrid cell line provides a useful model system to study the δ-opioid receptor because it contains a homogenous population of δ-opioid receptors [Chang and Cuatrecasas, 1979; Law *et al*, 1983]. In neuronal cells activated δ-opioid receptors inhibit adenylyl cyclase [Law *et al*, 1982; Kurose *et al*, 1983] and voltage-dependent Ca^{2+} channels [Heschler *et al*, 1987]. Activation of δ-opioid receptors can additionally lead to an increase in the conductance of an inwardly rectifying K^+ channel [North, 1986; North *et al*, 1987] and regulation of cAMP phosphodiesterase [Law and Loh, 1993] and phospholipase C [Jin *et al*, 1992, 1994] activities. Until now, the most widely used biochemical method of detecting δ-opioid receptor activation in NG108-15 cells has been *via* the inhibition of adenylyl cyclase activity, measured as a decrease in the levels of cAMP formed in whole cells or membrane homogenates. However, this assay is relatively time consuming, insensitive and suffers from the disadvantage that the adenylyl cyclase activity is only loosely coupled to the δ-opioid receptor [Costa *et al*, 1988], meaning that small changes in the activation state of the receptor may be difficult to observe.

One of the first biochemical events after agonist occupation of G protein-linked receptors is guanine nucleotide exchange [Gilman, 1987; Birnbaumer *et*

al, 1990]. Thus, agonist occupation of the receptor leads to the opening of the nucleotide binding site, dissociation of bound GDP and replacement with GTP, with subsequent activation of the G protein. Deactivation of the G protein is associated with the intrinsic GTPase activity of the G protein, as discussed in section 1.4. The GTPase activity of G proteins has been used to study the activation of δ-opioid receptors [Koski and Klee 1981; Burns *et al*, 1983; Selley and Bidlack, 1992]. However, this assay is not very sensitive and suffers from the important complication that the stimulated low K_m GTPase activity has to be distinguished from the high K_m enzymatic activity, which contributes significantly to the overall [^{32}P]GTP hydrolysis.

More recently, G protein activation has been studied *via* receptor stimulated photolabelling of G protein α-subunits with the photoreactive GTP analogue [α-^{32}P]GTP-azidoanilide [Offermanns *et al*, 1991; Laugwitz *et al*, 1993; Prather *et al*, 1994; Offermanns *et al*, 1994]. A similar technique which uses [α-^{32}P]NAD^+ to label activated G proteins with [α-^{32}P]ADP-ribose takes advantage of the fact that agonist-bound receptors which usually couple to pertussis toxin-sensitive G proteins promote cholera toxin catalysed ADP-ribosylation of these pertussis toxin substrates [Milligan and McKenzie, 1988; Roerig *et al*, 1992]. These two methods share the significant advantage that the molecular identity of the particular G protein species which becomes activated is identifiable. However, once again these methods are rather time consuming and expensive to be used for the routine screening of opioid activity, and do not lend themselves readily to measuring subtle quantitative changes in G protein activation.

In the presence of the relatively hydrolysis resistant GTP analogue GTPγS, G proteins become permanently activated [Higashijima *et al*, 1987]. In the presence of Mg^{2+}, this GTP analogue dissociates slowly from the G protein, and therefore accumulates in membranes or vesicles containing G proteins. The use of the radiolabelled GTP analogue, [^{35}S]GTPγS, thus allows the measurement of the rate and extent of GTPγS binding to a membrane preparation. This assay has been successfully used to study the activation of pertussis toxin sensitive G proteins by muscarinic [Hilf *et al*, 1989; Lazareno *et al*, 1993], formyl peptide [Gierschik *et al*, 1991], adenosine A_1 [Lorenzen *et al*, 1993], α_2-adrenergic [Tian *et al*, 1994] and μ-opioid [Traynor and Nahorski, 1995]

receptors, and provides a convenient, rapid and accurate assay system to study the activation of G proteins.

The present work was designed to develop a [^{35}S]GTPγS binding assay for the δ-opioid receptor in membranes from NG108-15 cells. Optimisation of this assay could potentially provide a useful and sensitive method of determining the efficacy and potency of δ-opioid compounds.

4.2 Results

4.2.1 Ligand binding assays in membranes from NG108-15 cells

Initially, ligand binding assays were performed on membranes from NG108-15 cells to confirm that these cells had essentially the same δ-opioid receptor profile as those used in other laboratories.

The presence of a homogenous population of δ-opioid receptors was established by the displacement of the non-selective antagonist [^{3}H]-diprenorphine with the δ-selective ligand DPDPE [Corbett *et al*, 1984; Cotton *et al*, 1985], the μ-selective ligand DAMGO [Corbett *et al*, 1984] and the κ-selective ligand PD117302 [Leighton *et al*, 1987] (Fig. 4.1). DPDPE displacement of [^{3}H]-diprenorphine (0.75 nM) from membranes of NG108-15 cells was monophasic (Hill coefficient = 0.95±0.06) yielding a K_i of 1.64±0.07 nM (n=3). This value is in good agreement with a previously published K_d of 1.24 nM for this ligand in membranes from NG108-15 cells [Akiyama *et al*, 1985]. Displacement of [^{3}H]-diprenorphine by the μ-selective agonist DAMGO, and the κ-preferring

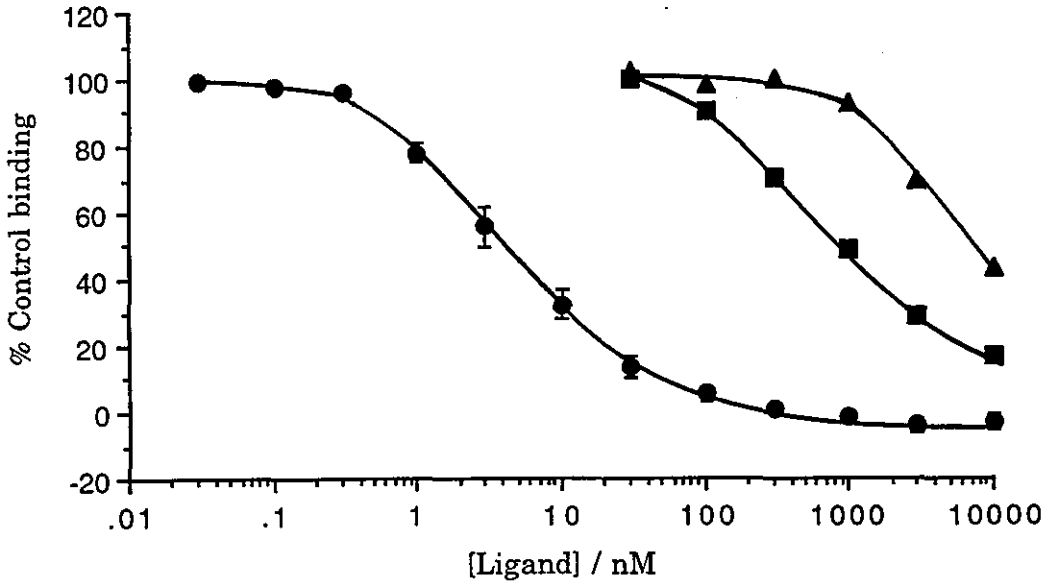


Figure 4.1 Displacement of [^{3}H]-diprenorphine (0.75 nM) by DPDPE (●) (n=3), DAMGO (■) (n=1) and PD 117302 (▲) (n=1) in NG108-15 cell membranes. Values for DPDPE data represent means±sem.

agonist PD117302, only occurred at concentrations of the displacing ligand ≥100 nM as expected, affording IC_{50} values ≥1000 nM, confirming the lack of μ- and κ-sites.

The maximum number of δ-opioid binding sites (B_{max}) in membranes from NG108-15 cells was determined by saturation binding analysis using [^{3}H]-diprenorphine (Fig. 4.2) and [^{3}H]-DPDPE (Fig. 4.3). Labelling of the δ-opioid receptors with [^{3}H]-diprenorphine gave a receptor number (B_{max}) of 559±61 fmol/mg, in good agreement with a previously published value of 570 fmol/mg [Prather *et al, 1994*], and an affinity (K_d) for [^{3}H]-diprenorphine of 0.32±0.01 nM. Similarly, [^{3}H]-DPDPE gave a B_{max} value of 501±94 fmol/mg and an affinity for [^{3}H]-DPDPE of 1.68±0.51 nM. Thus the opioid receptor profile in these NG108-15 cells is similar to that in cells used in other laboratories.

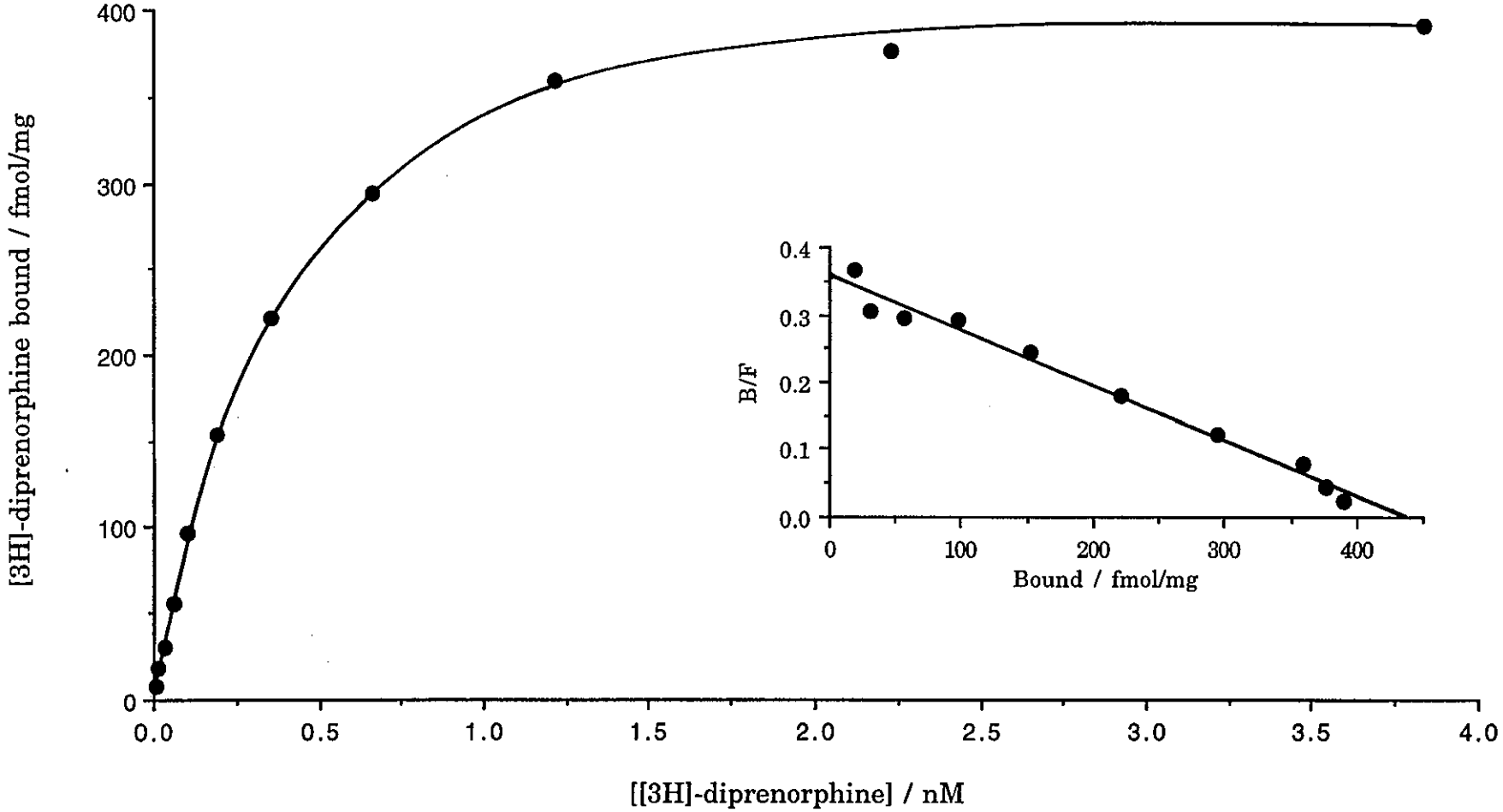


Figure 4.2 A representative graph showing saturation [^{3}H]-diprenorphine binding to NG108-15 membranes in Tris-HCl buffer (pH 7.4). Inset is the corresponding Scatchard plot.

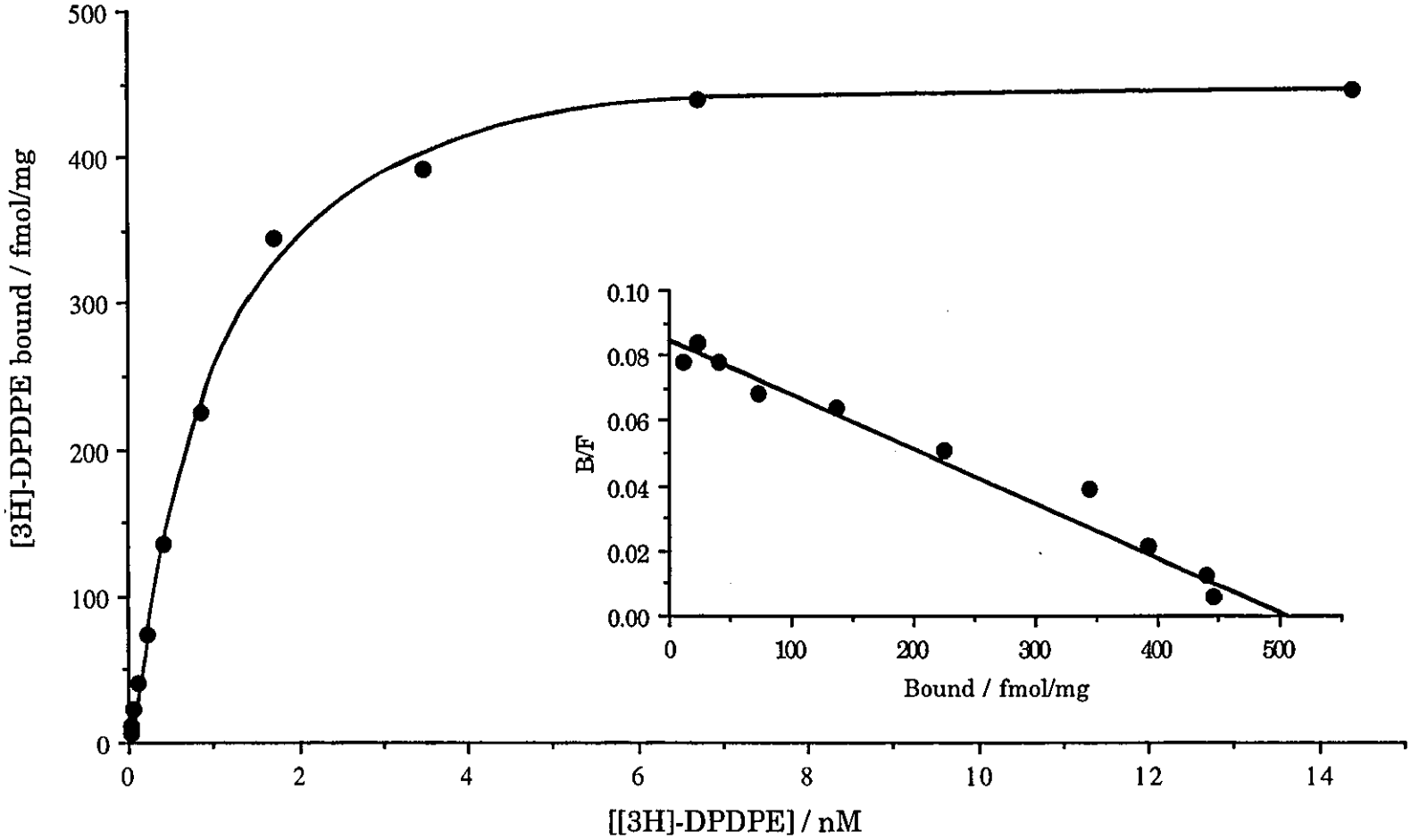


Figure 4.3 A representative graph showing saturation [^{3}H]-DPDPE binding to NG108-15 membranes in Tris-HCl buffer (pH 7.4). Inset is the corresponding Scatchard plot.

4.2.2 Opioid stimulated [^{35}S]GTPγS binding in membranes from NG108-15 cells

4.2.2.1 Optimisation of reaction conditions

(a) Effect of nucleotides

Previous studies on the stimulation of [^{35}S]GTPγS binding by adenosine A_1 receptors in bovine brain membranes [Lorenzen *et al*, 1993], muscarinic receptors in porcine cardiac membranes [Hilf *et al*, 1989], N-formyl peptide receptors in HL-60 cell membranes [Gierschik *et al*, 1991] and μ-opioid receptors in membranes from SH-SY5Y cells [Traynor and Nahorski, 1995], but not by α_2-adrenergic receptors in PC-12 cell membranes [Tian *et al*, 1994], have shown that it is necessary to include GDP in the reaction buffer in order to observe receptor-mediated stimulation of [^{35}S]GTPγS binding. Stimulation of [^{35}S]GTPγS binding by the δ-opioid selective agonist DPDPE in membranes from NG108-15 cells at various GDP concentrations is shown in figures 4.4 and 4.5.

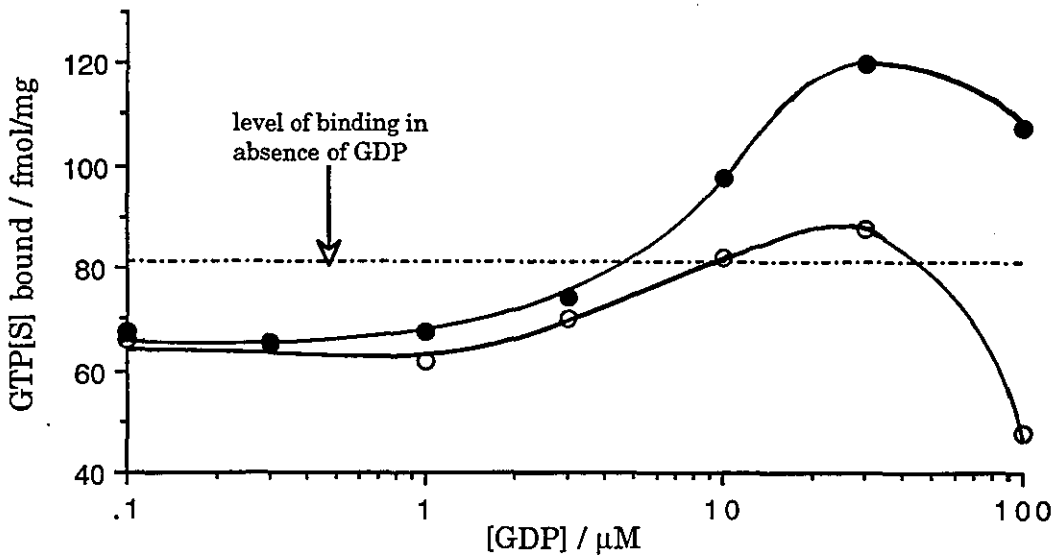


Figure 4.4 Effect of varying the concentration of GDP in the absence (○) or presence (●) of DPDPE (1 μM) on [^{35}S]GTPγS binding in NG108-15 membranes. Experimental conditions were as described in Materials and Methods, except the magnesium concentration was 10 mM. Results from a typical experiment are shown, representative of three performed.

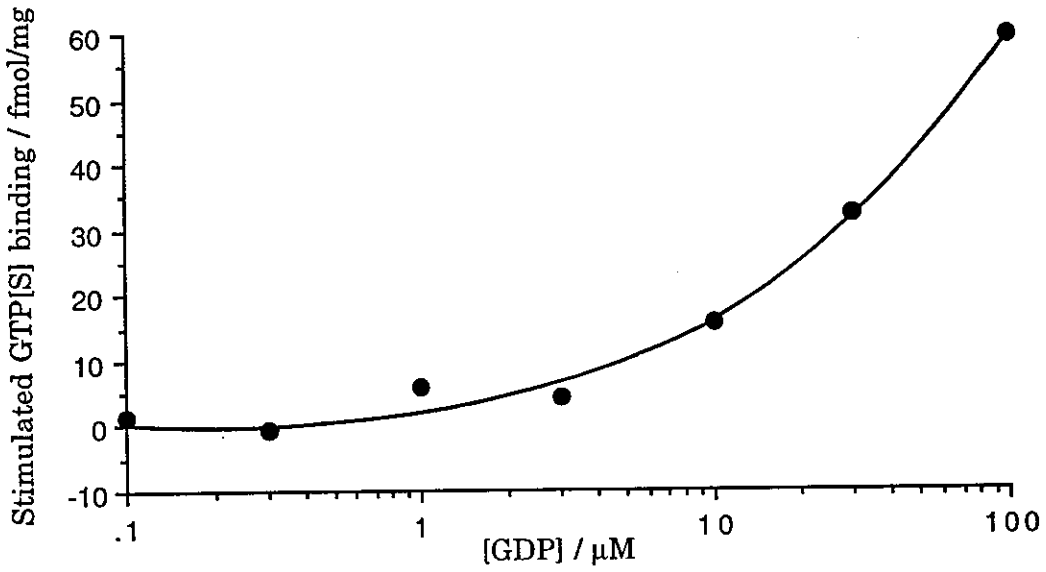


Figure 4.5 Stimulation of [^{35}S]GTPγS binding in NG108-15 membranes by DPDPE (1 μM) at various GDP concentrations. Results shown are from the same experiment as that shown in Fig. 4.4, representative of three performed.

Agonist stimulated binding of [^{35}S]GTPγS was increased in the presence of increasing concentrations of GDP (Fig. 4.5). The effect of GDP on [^{35}S]GTPγS binding showed a complex concentration dependence. Low concentrations of GDP (0.1-1 μM) decreased the level of binding of the labelled nucleotide. However, as the concentration of GDP was increased (3-30 μM), enhanced binding of [^{35}S]GTPγS (both basal and agonist-stimulated) was observed, which reached a maximum at 30 μM GDP and began to decrease again by 100 μM GDP. To investigate the mechanism by which GDP unmasks agonist stimulated binding of [^{35}S]GTPγS, the effect of several nucleotides on basal and agonist stimulated [^{35}S]GTPγS binding was studied. The observed results for GDP were replicated by the poorly hydrolyzable GDP analogue, GDPβS (Figs. 4.6 and 4.7). However, the maximal opioid-mediated stimulation of [^{35}S]GTPγS binding seen in the presence of this nucleotide was not as large as that seen with GDP. Neither ADP, UDP nor GMP could substitute for GDP, in that no stimulation of [^{35}S]GTPγS binding was seen in the presence of DPDPE at any concentration of

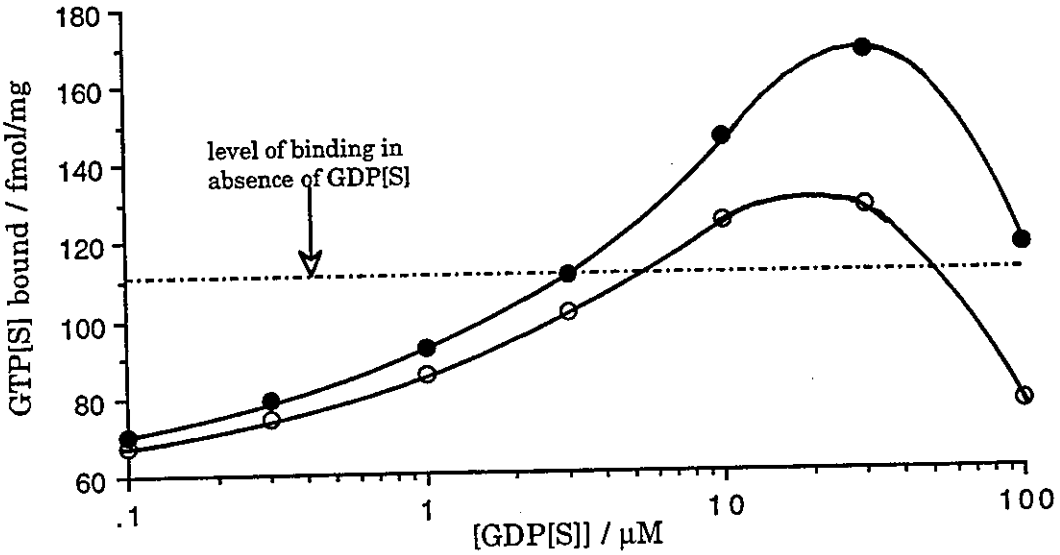


Figure 4.6 Effect of varying the concentration of GDPβS in the absence (○) or presence (●) of DPDPE (1 μM) on [^{35}S]GTPγS binding in NG108-15 membranes. Experimental conditions were as described in Materials and Methods, except the magnesium concentration was 10 mM. Results from a typical experiment are shown, representative of three performed.

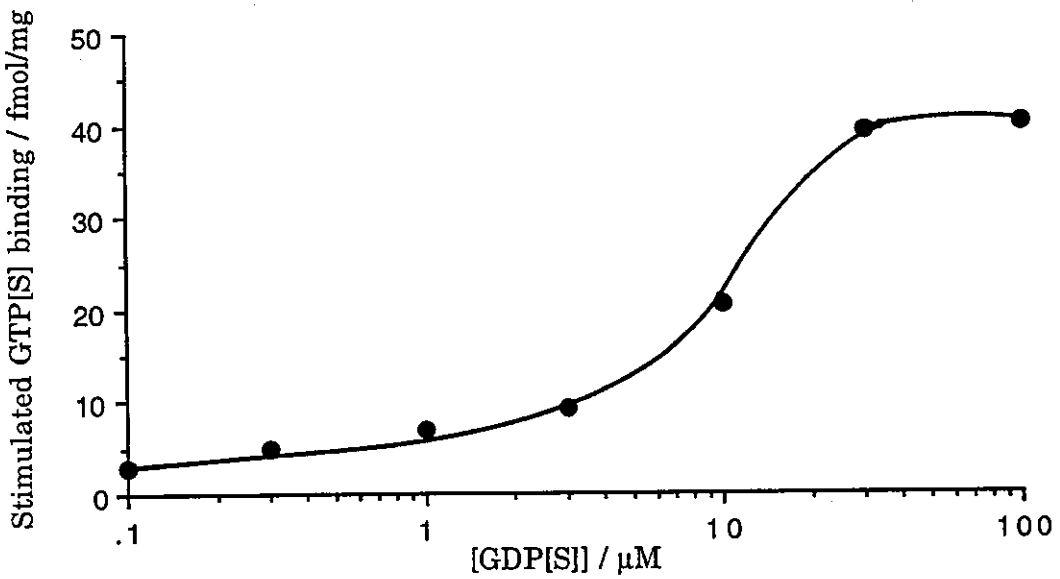


Figure 4.7 Stimulation of [^{35}S]GTPγS binding in NG108-15 membranes by DPDPE (1 μM) at various GDPβS concentrations. Results shown are from the same experiment as that shown in Fig. 4.6, representative of three performed.

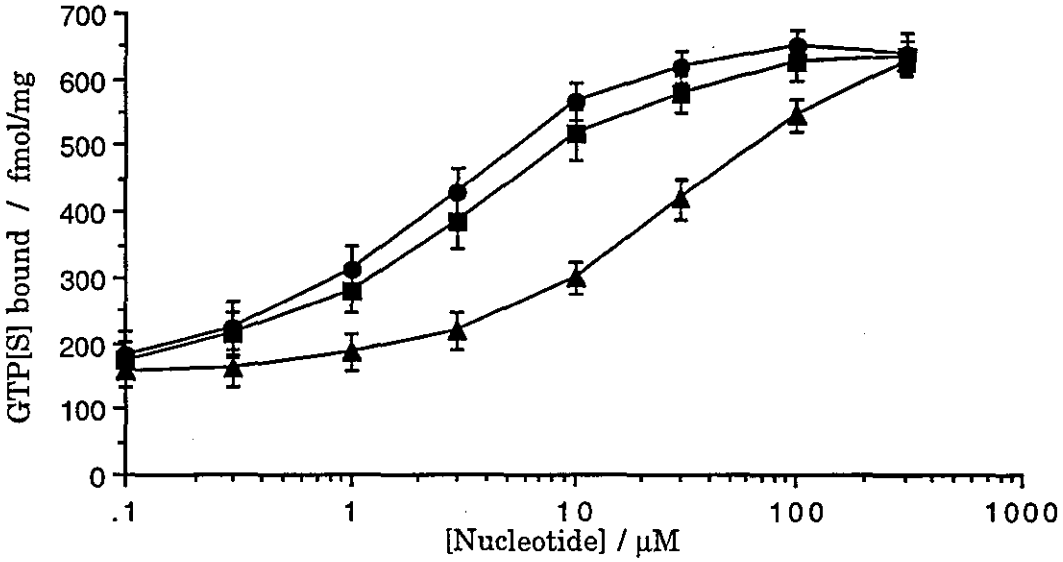


Figure 4.8 Effect of the nucleotides ADP (●), GMP (■) and UDP (▲) on [^{35}S]GTPγS binding in membranes from NG108-15 cells. Experimental conditions were as described in Materials and Methods, except the magnesium concentration was 10 mM. Values represent means±sem (n=3).

the nucleotides tested (0.1-300 μM; data not shown). Nevertheless, all three nucleotides produced a dose-dependent increase in the amount of [^{35}S]GTPγS bound to the NG108-15 membranes (Fig. 4.8). The maximal amount of radioactivity incorporated into the membranes was the same for all three nucleotides; however, the potency of the two purine nucleotides, ADP and GMP was significantly higher than that of the pyrimidine nucleotide, UDP.

(b) Variation of [^{35}S]GTPγS binding with protein concentration

Maximal stimulation of [^{35}S]GTPγS binding was seen at protein concentrations of 150-250 μg per tube, and stimulation was linear up to approximately 150 μg per tube (Fig. 4.9). Therefore, in order to obtain a large stimulation of binding, whilst working on the linear part of the curve, a protein concentration of 80-150 μg per tube was chosen for all subsequent experiments.

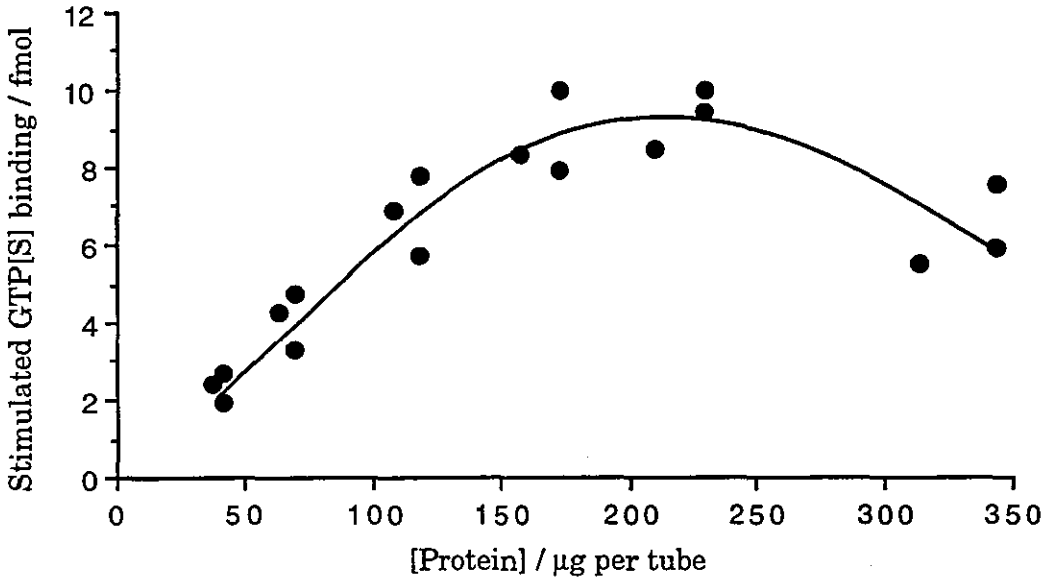


Figure 4.9 Effect of protein concentration on the stimulation of [^{35}S]GTPγS binding in NG108-15 membranes by DPDPE (1 µM). Experimental conditions were as described in Materials and Methods, except the magnesium concentration was 10 mM. Results from three separate experiments are shown.

(c) Effect of Na^+ and Mg^{2+} on [^{35}S]GTPγS binding

Na^+ and Mg^{2+} are known to modulate both binding [Pert and Snyder, 1974; Wüster *et al*, 1984; Puttfarcken *et al*, 1986; Rodriguez *et al*, 1992; Standifer *et al*, 1993] and functional response [Costa *et al*, 1990; Horstman *et al*, 1990; Tian *et al*, 1994] of G protein-coupled receptors. Therefore the effect of varying the concentration of these two cations in the reaction buffer was studied (Figs. 4.10 and 4.11).

The stimulation of [^{35}S]GTPγS binding by agonists required the presence of Mg^{2+}, which showed a biphasic effect on both basal and DPDPE (1 µM)-stimulated binding. Increasing the concentration of Mg^{2+} from 10 µM to 3 mM caused a 3-fold increase in basal levels of [^{35}S]GTPγS binding, whereas binding in the presence of DPDPE (1 µM) was increased 6.5-fold over the same range

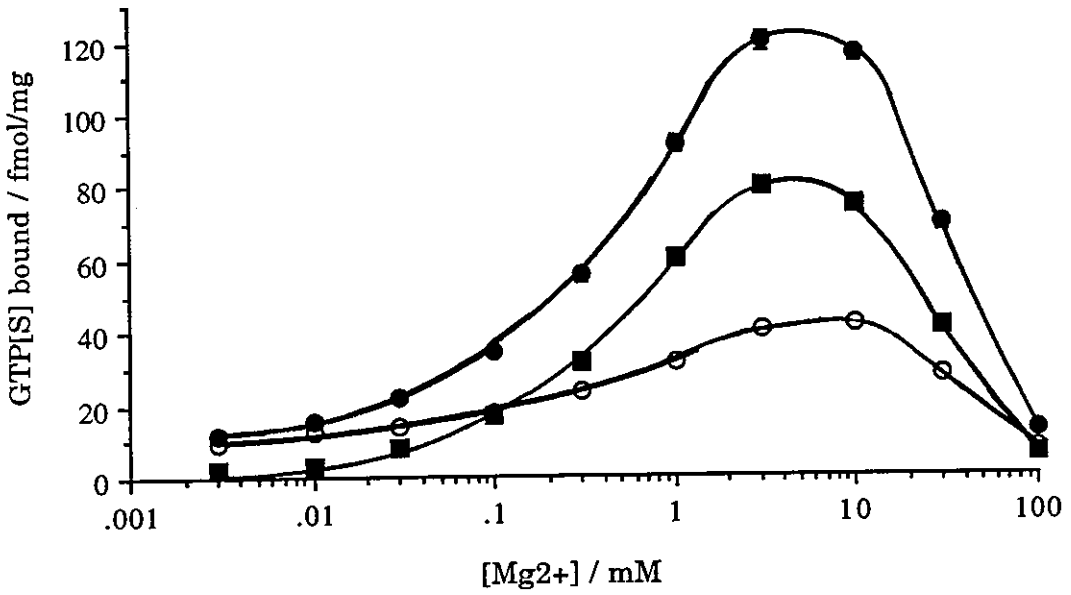


Figure 4.10 Effect of Mg^{2+} on basal (○) and DPDPE (1 μM)-stimulated(●) [35S]GTPγS binding in NG108-15 membranes. The difference between these two values is also shown (■). Values are means±sem (n=3).

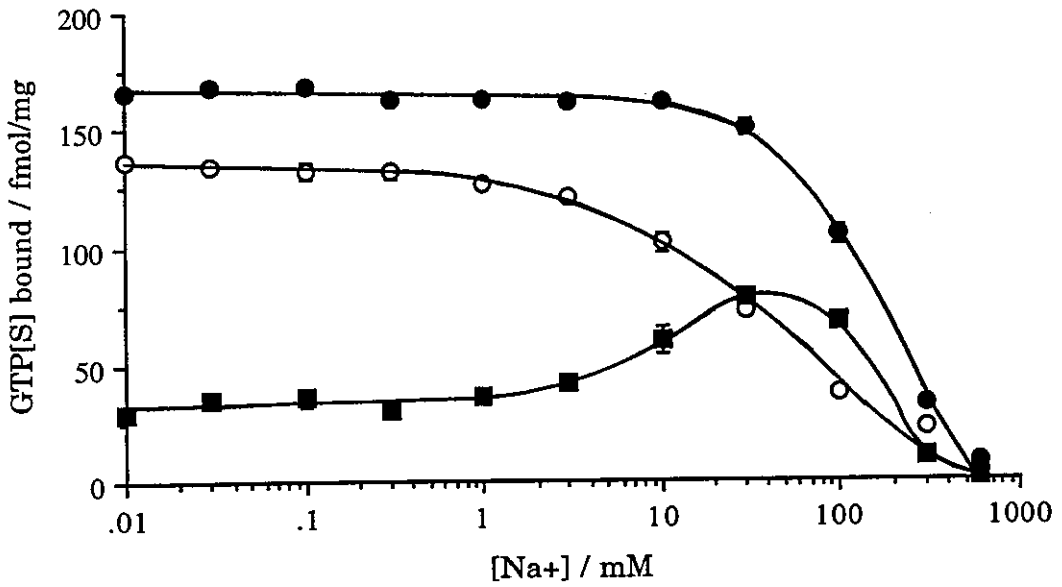


Figure 4.11 Effect of Na^{+} on basal (○) and DPDPE (1 μM)-stimulated (●) [35S]GTPγS binding in NG108-15 membranes. The difference between these two values is also shown (■). Values represent means±sem (n=3).

(Fig. 4.10). Further increase in the concentration of Mg^{2+} caused a decline in both basal and stimulated [^{35}S]GTPγS binding. Based upon these results, a value of 4 mM Mg^{2+} was used in subsequent studies.

In NG108-15 membranes, Na^+ (3-300 mM) progressively decreased basal [^{35}S]GTPγS binding (Fig. 4.11). However, in the presence of DPDPE (1 μM), the effect of Na^+ was shifted to higher concentrations, such that the stimulation of binding caused by DPDPE (1 μM) increased from 36.4±1.4 fmol/mg at 1 mM Na^+ to 78.0±2.0 fmol/mg at 30 mM Na^+. Basal and agonist stimulated [^{35}S]GTPγS binding were not affected by Na^+ concentrations less than 0.3 mM. It is interesting to note that unlike Mg^{2+}, Na^+ was not required to observe DPDPE stimulated [^{35}S]GTPγS binding in NG108-15 membranes *per se*, but this ion merely enhanced the stimulation of binding seen in its absence. For further experiments, a Na^+ concentration of 100 mM was chosen.

(d) Optimisation of the time and temperature of incubation

The optimal time and temperature for incubation of the reaction mixture was also studied (Figs. 4.12 and 4.13). At 20°C and 25°C, a 'lag phase' in both stimulated and basal [^{35}S]GTPγS binding could be seen, which was not evident at higher temperatures. DPDPE (1 μM) caused a stimulation of both the rate of [^{35}S]GTPγS binding and the total amount of binding at times up to 345 min. At short times of incubation, higher temperatures were required to see optimal agonist-stimulated [^{35}S]GTPγS binding, whereas at longer incubation times lower temperatures became favourable. Maximal stimulated binding was observed at 20°C after a 4-5 hour incubation period. However, for the sake of convenience a 60 min incubation at 30°C was routinely used.

Thus the optimal conditions for performing [^{35}S]GTPγS binding experiments using NG108-15 cells had been established. In all future experiments using the NG108-15 cell line, [^{35}S]GTPγS binding was performed using 80-150 μg membrane protein per tube in the presence of 100 mM NaCl, 4 mM $MgCl_2.6H_2O$ and 100 μM GDP for 1 hour at 30°C.

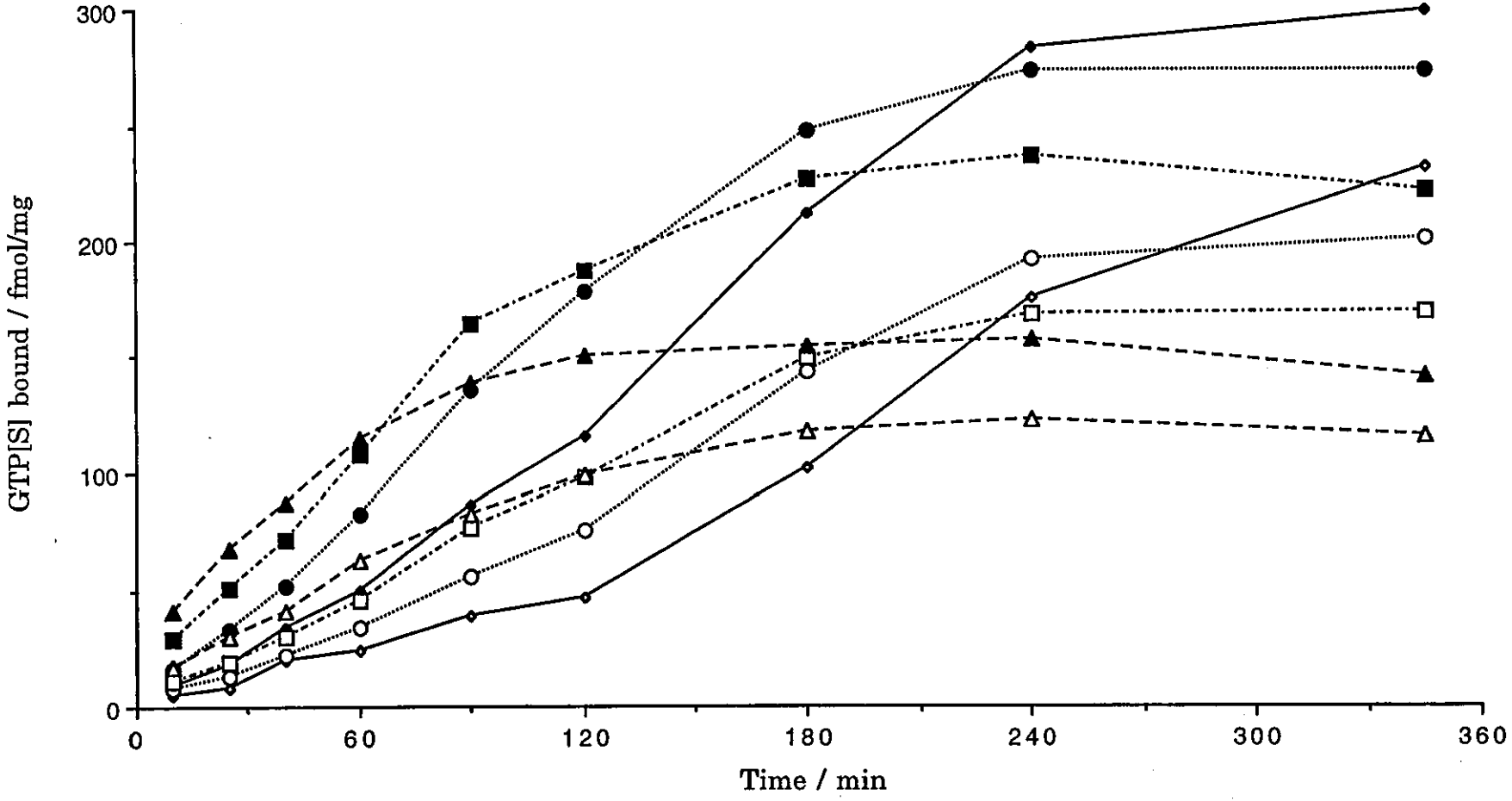


Figure 4.12 Effect of the temperature (◇,◆; 20°C: ○,●; 25°C: □,■; 30°C: △,▲; 37°C) and time of incubation on [35S]GTPγS binding in NG108-15 membranes in the absence (◇,○,□,△) or presence (◆,●,■,▲) of DPDPE (1 μM). Values from a single experiment are shown. Another experiment gave similar results.

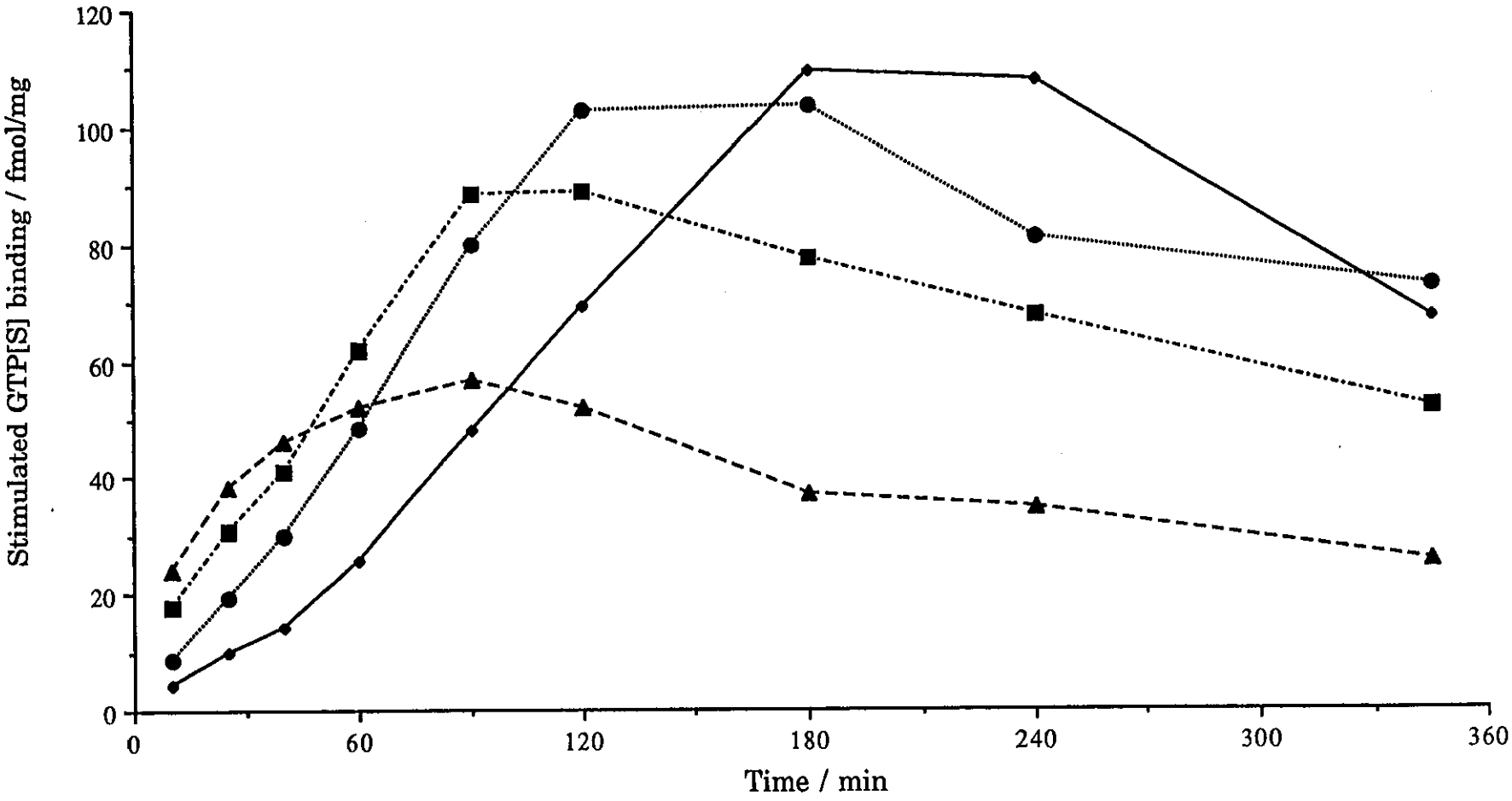


Figure 4.13 Effect of the temperature (◆,20°C; ●,25°C; ■,30°C: ▲,37°C) and time of incubation on the stimulation of [35S]GTPγS binding by DPDPE (1 μM). Results are from the same experiment as that shown in figure 4.12.

4.2.2.2 Pharmacology of the δ-opioid-mediated increase in [^{35}S]GTPγS binding

Having optimised the conditions to observe δ-opioid receptor-mediated stimulation of [^{35}S]GTPγS binding in membranes from NG108-15 cells, the next objective was to validate this technique pharmacologically. The δ-opioid peptide DPDPE stimulated [^{35}S]GTPγS binding in a dose-dependent manner, affording an EC_{50} of 29±4 nM (Fig. 4.14). This stimulation was antagonised by naloxone (300 nM), which shifted the concentration-effect curve to the right by some 13-fold (Fig. 4.14), to give an apparent pA_2 for naloxone of 7.65, indicative of δ-opioid receptor involvement [Leslie, 1987].

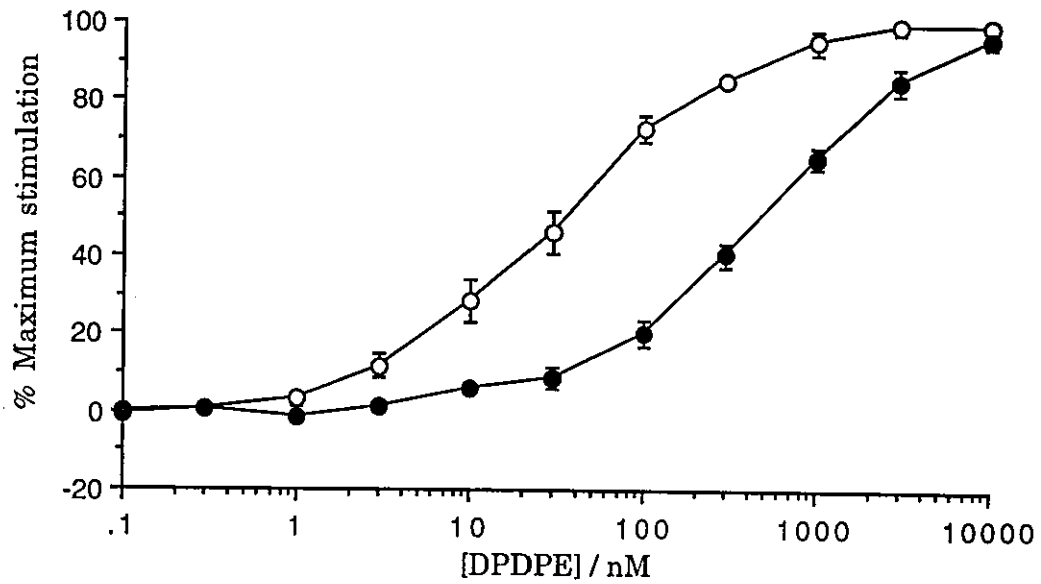


Figure 4.14 Stimulation of [^{35}S]GTPγS binding to membranes of NG108-15 cells by DPDPE in the absence (○) or presence (●) of naloxone (300 nM). Values represent means±sem (n=3). Binding of [^{35}S]GTPγS was 39.1±5.3 fmol/mg of protein in the absence of DPDPE and 107.9±9.2 fmol/mg of protein in the presence of DPDPE (10 μM).

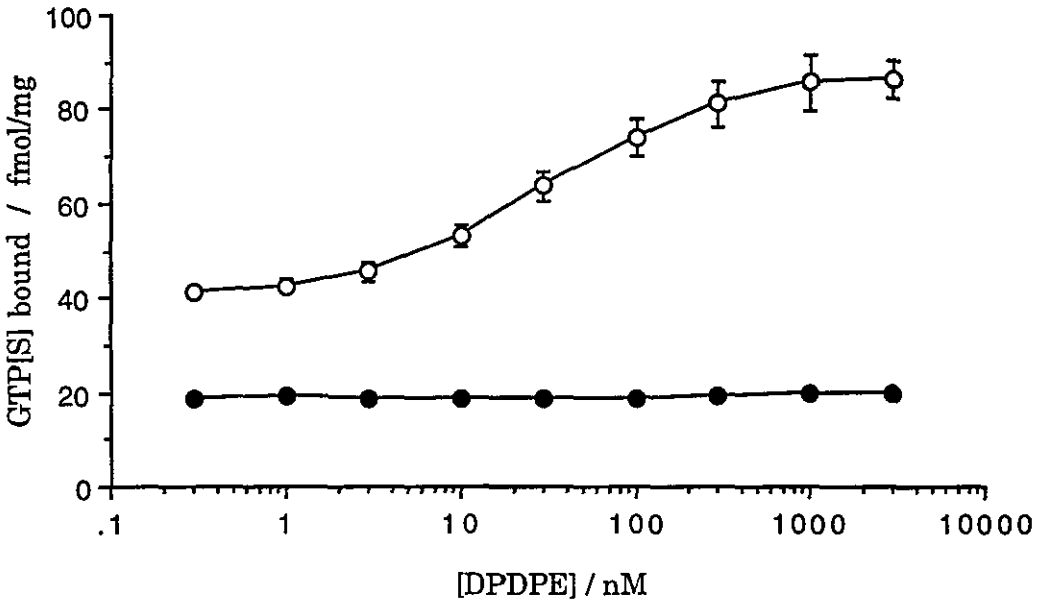


Figure 4.15 Stimulation of [^{35}S]GTPγS binding to NG108-15 membranes prepared from control (○) and pertussis toxin (100 ng/ml, 24 h) treated (●) cells by DPDPE. Values are means±sem (n=3).

To assess the involvement of G_i or G_o proteins in the δ-opioid receptor-mediated stimulation of [^{35}S]GTPγS binding to NG108-15 membranes, confluent monolayers of cells were treated for 24h with pertussis toxin (100 ng/ml) before membrane preparation. The stimulation of [^{35}S]GTPγS binding by the δ-opioid agonist DPDPE was completely abolished by this pretreatment (Fig 4.15), confirming that the event is mediated entirely through pertussis toxin-sensitive G proteins. Indeed, pertussis toxin pretreatment of cells reduced the control binding in subsequently prepared membranes by 60±4 %.

There is considerable evidence from both *in vivo* studies (Jiang *et al*, 1991; Mattia *et al*, 1991; Sofuoglu *et al*, 1991) and ligand binding assays (Negri *et al*, 1991; Sofuoglu *et al*, 1992; Portoghese *et al*, 1992) to support the existence of δ-opioid receptor subtypes (for review see Traynor and Elliott, 1993). To determine whether δ-opioid receptor subtypes were present in NG108-15 membranes and involved in mediating the stimulation of [^{35}S]GTPγS binding, DPDPE dose-response curves were constructed in the presence of the δ_2-preferring opioid antagonist, NTB (0.2 nM) and the δ_1-preferring antagonist, BNTX (10 nM). Both

compounds antagonised the stimulation of [^{35}S]GTPγS binding caused by DPDPE (Fig. 4.16). However, NTB was significantly more potent in this action yielding an apparent affinity (K_e) of 78±10 pM, compared to BNTX which had an affinity of 1.57 ± 0.39 nM, implicating the possible involvement of δ_2-type opioid receptors in the stimulation of [^{35}S]GTPγS binding in membranes from NG108-15 cells.

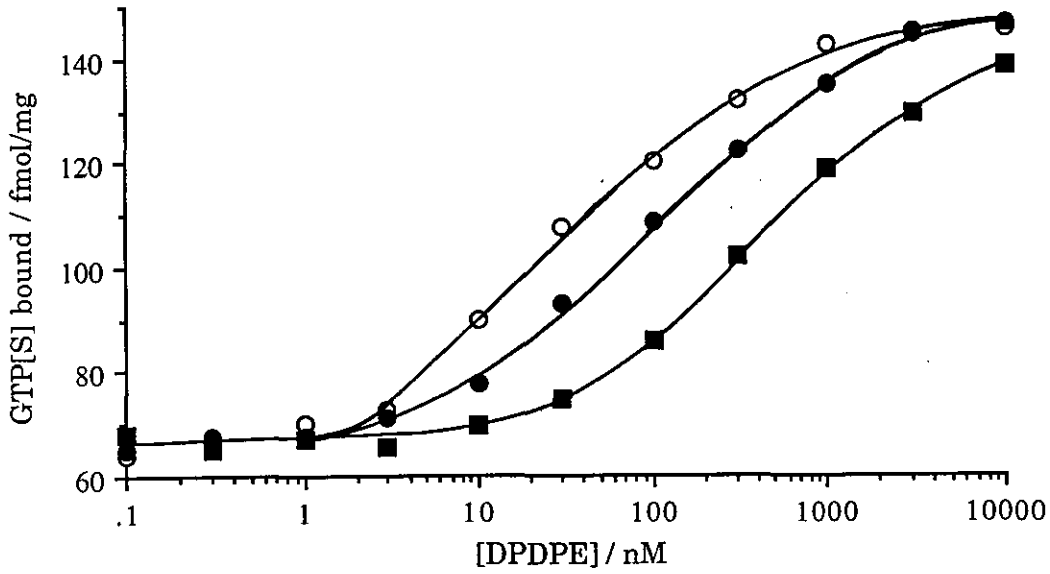


Figure 4.16 Stimulation of [^{35}S]GTPγS binding to membranes from NG108-15 cells by DPDPE in the absence (○) or presence of NTB (0.2 nM, ●) or BNTX (10 nM, ■). Results from a typical experiment are shown, representative of three performed.

In order to characterise the δ-opioid receptor in NG108-15 membranes further, displacement of [^{3}H]-diprenorphine (0.50 nM) binding by these two ligands was studied (Fig. 4.17). Surprisingly, both compounds afforded very shallow displacement curves for the displacement of [^{3}H]-diprenorphine, giving Hill coefficients of 0.45±0.02 and 0.51±0.05 for BNTX and NTB respectively. Such shallow slopes suggest the presence of receptor heterogeneity, and indeed computer modelling of the data revealed that a two-site model gave a significantly better fit of the data than a single-site model ($P < 0.01$). The results of this computer analysis are shown in Table 4.1. As expected from the

[^{35}S]GTPγS binding results, the affinity of NTB was higher at each respective site than that of BNTX.

δ-Ligand	Affinity (K_i) / nM		% of high affinity sites
	High affinity site	Low affinity site	
BNTX	0.091±0.027	9.8±2.1	45±7
NTB	0.0012±0.0009	0.47±0.05	38±9

Table 4.1 Binding potencies of BNTX and NTB at the δ-opioid receptor in NG108-15 membranes.

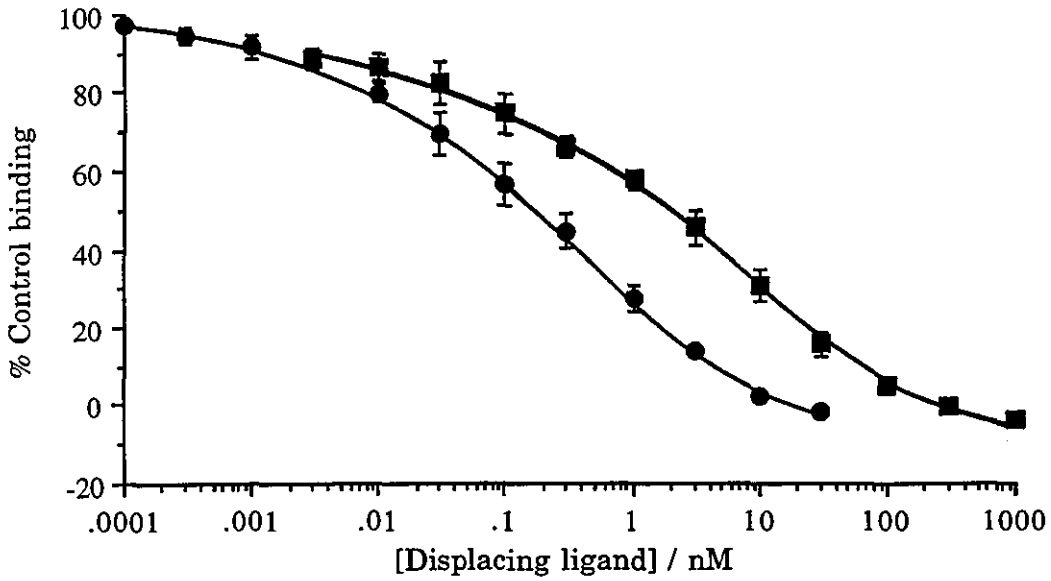


Figure 4.17 Displacement of [^{3}H]-diprenorphine (0.5 nM) specific binding by NTB (●) and BNTX (■) in membranes from NG108-15 cells. Values represent means±sem (n=4).

4.2.2.3 Relative intrinsic activities of δ-opioid ligands

The ability of the [^{35}S]GTPγS binding assay to discriminate between full and partial agonists, and also to show a response to an 'inverse agonist' [Costa and Herz, 1989; Costa *et al*, 1992; Milligan *et al*, 1995] was tested. Concentrations of each drug were chosen that were expected to elicit a maximal response, either as a result of previous work, or from a knowledge of their binding

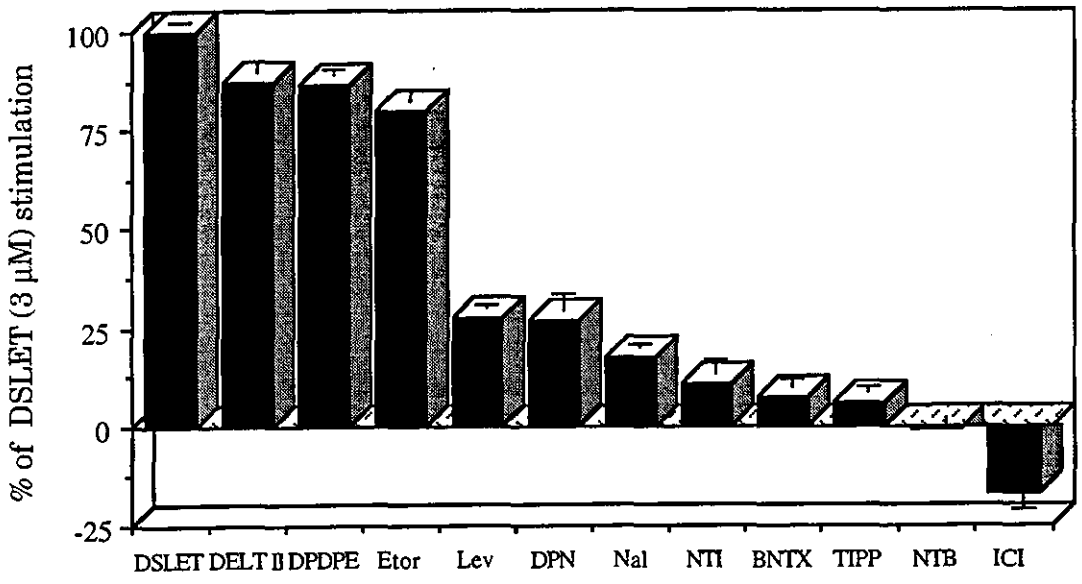


Figure 4.18 Relative intrinsic activity of various opioid ligands to stimulate [^{35}S]GTPγS binding in NG108-15 membranes, normalised to the effect elicited by DSLET (3 μM). Values represent means±sem (n≥3).DSLET = DSLET (3 μM); DELT II = DELT II (3 μM); DPDPE = DPDPE (3 μM); Etor = etorphine (10 μM); Lev = levallorphan (10 μM); DPN = diprenorphine (10 μM); Nal = nalorphine (10 μM); NTI = naltrindole (10 μM); BNTX = BNTX (1 μM); TIPP = TIPP (5 μM); NTB = NTB (10 μM); ICI = ICI 174864 (10 μM). Stimulated [^{35}S]GTPγS binding by DSLET was 37.6±2.9 fmol/mg of protein.

affinity. Figure 4.18 shows the effect of various δ-opioid ligands on [^{35}S]GTPγS binding in NG108-15 membranes, normalised against the stimulation afforded by DSLET (3 μM). DSLET, a putative δ_2-agonist, caused the greatest stimulation of all of the agonists tested, producing a significantly greater effect ($P < 0.05$)

than either DELT II (putative δ_2-agonist), DPDPE (putative δ_1-agonist) or etorphine (non-selective between δ-opioid subtypes). As expected, levallorphan, diprenorphine and nalorphine were all partial agonists in this assay, similar to their effect on adenylyl cyclase [Law *et al*, 1983], though the relative activity of diprenorphine compared to etorphine was smaller than reported by Law *et al* (1983). ICI 174864 was able to inhibit the basal levels of [^{35}S]GTPγS binding and acted as an inverse agonist in this system, confirming previous reports of a similar effect on GTPase activity in NG108-15 cell membranes [Costa and Herz, 1989].

It has been suggested that the relative intrinsic activity of opioid agonists and inverse agonists to stimulate or inhibit GTPase activity is dependent on the type of cation(s) present in the reaction mixture [Costa and Herz, 1989]. In accordance with this, replacement of Na^+ with K^+ (at constant [Cl^-]) in the reaction buffer had a pronounced effect on both basal and DSLET-stimulated [^{35}S]GTPγS binding. An increase in basal tone was observed, with a corresponding decrease in the net activity stimulated by the full agonist DSLET (3 μM). However, the net stimulation afforded by diprenorphine was not decreased by replacement of Na^+ with K^+, thereby making the difference observed between DSLET and diprenorphine smaller (Fig. 4.19).

Replacement of Na^+ with K^+ also affected the inhibition of [^{35}S]GTPγS binding observed with the δ-opioid inverse agonist ICI 174864 (Fig. 4.20). Substitution of Na^+ with K^+ in the reaction buffer afforded a much larger inhibition of [^{35}S]GTPγS binding by ICI 174864, yielding an IC_{50} of 77.2±27.0 nM.

Naloxone (10 μM) reversed the inhibition of [^{35}S]GTPγS binding seen with ICI 174864 (3 μM), confirming that this effect was indeed a result of δ-opioid receptor occupation (Fig. 4.21).

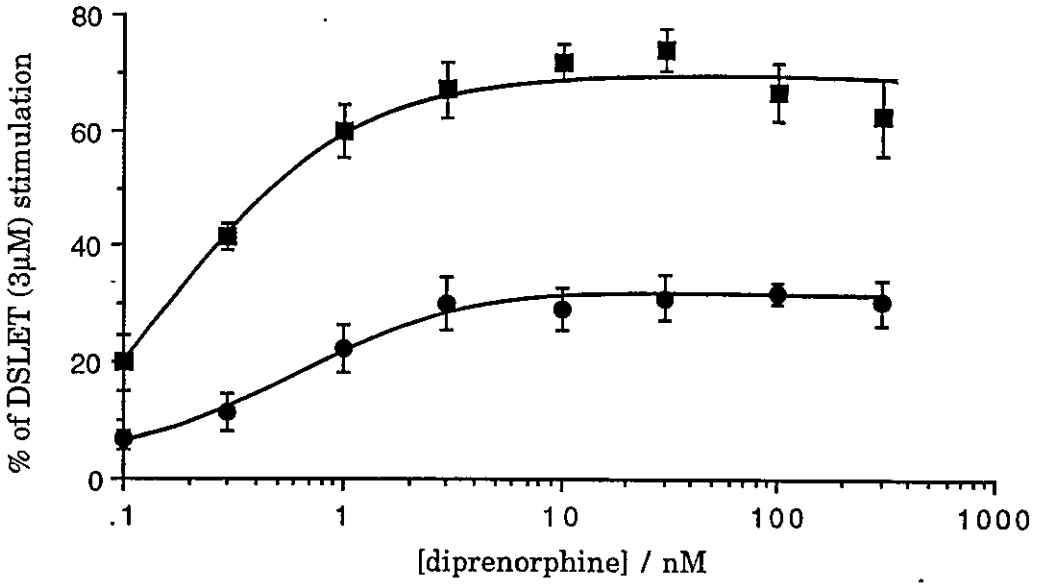


Figure 4.19 Comparison of diprenorphine stimulation of [35S]GTPγS binding in NG108-15 membranes relative to that afforded by DSLET (3 μM) in Na^+- (●) or K^+- (■) containing buffer (100 mM). Basal [35S]GTPγS binding was 53.2±6.8 fmol/mg (Na^+ buffer) and 78.5±12.0 fmol/mg (K^+ buffer). [35S]GTPγS binding in the presence of DSLET (3 μM) was 102.8±1.1 fmol/mg (Na^+ buffer) and 112.6±13.6 fmol/mg (K^+ buffer). Calculated EC_{50} values were 0.46±0.15 nM (Na^+ buffer) and 0.23±0.10 nM (K^+ buffer).Values are means±sem (n=3).

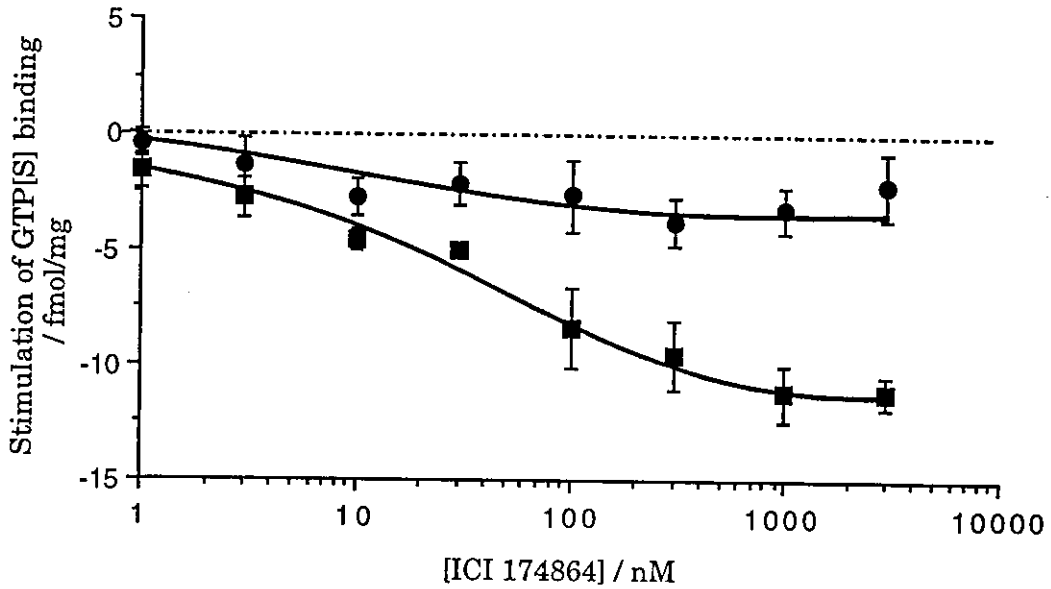


Figure 4.20 Effect of ICI 174864 on [35S]GTPγS binding in NG108-15 membranes in Na^+- (●) or K^+- (■) containing buffer (100 mM). Values represent means±sem (n=3). Basal [35S]GTPγS binding was 76.2±3.2 fmol/mg (Na^+ buffer) and 102.4±9.8 fmol/mg (K^+ buffer).

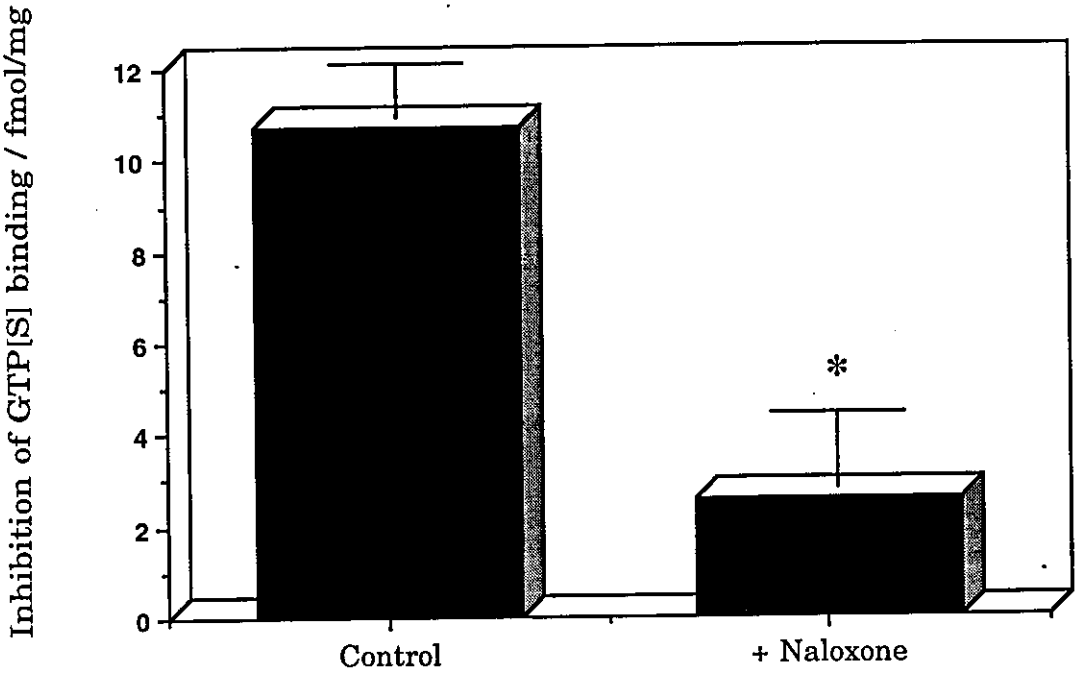


Figure 4.21 Inhibition of [^{35}S]GTPγS binding in NG108-15 membranes by ICI 174864 (3 μM) in the absence (control) or presence of naloxone (10 μM) (n=3). Experimental conditions were as described in Materials and Methods, except that 100 mM NaCl was replaced by 100 mM KCl in the reaction buffer.
*: Significantly different from control ($P < 0.01$).

4.3 Discussion

δ-Opioid receptors stimulated [^{35}S]GTPγS binding in membranes from NG108-15 cells. Variation of the amount of membrane protein added per assay tube showed that the stimulation of binding caused by δ-opioid agonists was linear up to 150 μg of membrane protein. Routinely, 80-150 μg protein per assay tube was used. Stimulated binding was sensitive to the temperature and time of incubation, with the maximal effect of opioid agonists occurring at lower temperatures (for example 20°C) and extended incubation times (for example 3-4 hours). However, δ-opioid agonists caused enhancement of [^{35}S]GTPγS binding at all temperatures tested (20-37 °C) and over a wide range of incubation times. For the sake of convenience a 60 min incubation at 30°C was used.

δ-Opioid-mediated stimulation of [^{35}S]GTPγS binding required the presence of GDP (or the hydrolysis resistant analogue, GDPβS). This dependence on GDP has also been observed in studies on the stimulation of [^{35}S]GTPγS binding by μ-opioid agonists in SH-SY5Y cell membranes [Traynor and Nahorski, 1995] and also by adenosine A_1 agonists in bovine brain [Lorenzen *et al*, 1993], muscarinic agonists in porcine cardiac membranes [Hilf *et al*, 1989] and by the chemotactic peptide fMet-Leu-Phe in membranes from HL-60 cells [Gierschik *et al*, 1991]. In contrast however, Tian *et al* (1994) found that epinephrine occupation of α_{2D}-adrenergic receptors stimulates [^{35}S]GTPγS binding to membranes from PC-12 cells to approximately the same extent at all GDP levels tested, including in its absence. Cloned muscarinic receptors stably expressed in CHO cells show an even more complex dependence on GDP. Increasing concentrations of this nucleotide cause an increase in the absolute agonist response at m2 and m4 muscarinic receptors, which like δ-opioid receptors are coupled to inhibition of adenylyl cyclase, but only an increase in the ratio of response to basal activity at m1 and m3 receptors coupled to phospholipase C [Lazareno *et al*, 1993].

The effect of GDP on [^{35}S]GTPγS binding has been suggested to result from the ability of GDP to bind to empty guanine nucleotide binding sites on the G protein and hence reduce the basal level of [^{35}S]GTPγS binding [Wieland *et al*, 1992]. The requirement for GDP when studying fMet-Leu-Phe-mediated stimulation of [^{35}S]GTPγS binding in HL-60 cell membranes shows a marked

temperature dependence. Agonist activation of N-formyl peptide receptors increases binding of [^{35}S]GTPγS to membrane G proteins at 30°C only in the presence of added GDP, which reduces the level of binding in the absence of agonist. However, at 0°C fMet-Leu-Phe stimulates the binding of this radiolabelled GTP analogue to G proteins maximally without addition of GDP. Parallel studies on [^{3}H]-GDP release showed that at the lower temperature membrane G proteins release their bound GDP only slowly, whereas the rate of this release is very much enhanced at 30°C. This observation led Wieland *et al* (1992) to suggest that no effect of agonist is observed at 30°C in the absence of GDP because many of the nucleotide binding sites on G proteins are vacant at this temperature, allowing a large receptor-independent basal level of [^{35}S]GTPγS binding. Such high levels of basal binding effectively mask the small agonist stimulation of binding. Furthermore, under conditions resulting in degradation of membrane-bound GDP, by inclusion of alkaline phosphatase in the reaction buffer, control binding of [^{35}S]GTPγS measured at 0°C was significantly increased, whereas the extent of agonist-stimulated binding was reduced, lending further support to the idea that G proteins must exist in the GDP-bound form in order to observe an agonist-mediated signal.

Under the conditions used in the present experiments, δ-opioid-induced [^{35}S]GTPγS binding to NG108-15 membranes was observed only in the presence of guanine dinucleotides; neither GMP, ADP nor UDP could substitute for GDP in the reaction buffer. GDP and GDPβS affected both basal and agonist-stimulated [^{35}S]GTPγS binding in a biphasic manner. This biphasic effect of guanine dinucleotides on [^{35}S]GTPγS binding has not been reported previously for any receptor system. The decrease in [^{35}S]GTPγS binding observed at GDP concentrations greater than 30μM presumably results from a competition between GDP and [^{35}S]GTPγS for the guanine nucleotide binding site on the G protein(s). However, the reason for the initial increase in both basal and agonist-stimulated binding at GDP concentrations between 1-30 μM is unknown. One feasible explanation for this effect is that the presence of a second nucleotide protects [^{35}S]GTPγS from degradation by non-specific nucleotidases and phosphatases.

Although δ-opioid-mediated stimulation of [^{35}S]GTPγS binding is not observed if GDP is replaced by GMP, ADP or UDP, all three nucleotides alone

stimulate [^{35}S]GTPγS binding. A number of explanations are possible for this result:

1. In the presence of large concentrations of a second nucleotide, [^{35}S]thiophosphate-transfer from [^{35}S]GTPγS may be catalysed by enzymes such as nucleoside diphosphate kinase [Seifert *et al*, 1988; Wieland *et al*, 1991]. This would result in the radioactivity being 'scrambled' amongst the various nucleotides present. If this second nucleotide bound to the cell membrane, then a corresponding dose-related increase in bound radioactivity would be observed. However, this hypothesis does not explain why an increase in bound radioactivity is seen with GDPβS, since this nucleotide is not expected to be a substrate for transphosphorylating enzymes [Goody *et al*, 1972; Wieland *et al*, 1991].

2. Various nucleotidases and phosphatases may be present in the membrane preparation which could cause degradation of guanine nucleotides (see for example Wieland *et al*, 1992). In the absence of a second nucleotide, [^{35}S]GTPγS may be degraded, with a consequent loss of bound radioactivity. The presence of a second nucleotide would act as a protective agent, blocking this non-specific degradation of labelled [^{35}S]GTPγS.

3. ADP, UDP and GMP may be stimulating the binding of [^{35}S]GTPγS *via* a G protein-coupled 'nucleotide' receptor. There is compelling evidence for a nucleotide receptor that responds to both the pyrimidine UTP and the purines [O'Connor *et al*, 1991; Murrin and Boarder, 1992; Wilkinson *et al*, 1993]. Furthermore, the existence of a new subtype of purinoceptor in NG108-15 cells has recently been suggested [Matsuoka *et al*, 1995]. However, this possibility seems unlikely as there are no reports in the literature of a receptor which responds to UDP and GMP. Furthermore, ADP, UDP and GMP also cause an increase in the amount of radioactivity retained by the membranes of CHOδ cells in [^{35}S]GTPγS binding assays (basal level of [^{35}S]GTPγS binding in the absence of nucleotides was 223.0±4.6 fmol/mg of membrane protein, which was increased to 309.2±9.7 fmol/mg, 331.6±9.6 fmol/mg and 288.2±4.8 fmol/mg in the presence of ADP (300 μM) UDP (300 μM) and GMP (300 μM) respectively). This cell line has not been reported to express purinergic or 'nucleotide' receptors, so overall it seems more likely

that this increase in [^{35}S]GTPγS binding occurs as a result of a non-specific mechanism, such as those in (1) and (2) above.

The ionic environment in the reaction buffer is also a very important consideration in a [^{35}S]GTPγS binding assay. Agonist-stimulated [^{35}S]GTPγS binding required the presence of Mg^{2+} in membranes from NG108-15 cells. Mg^{2+} is known to exert a wide range of effects on G protein-coupled receptor systems:

1. Low concentrations of Mg^{2+} (10-100 nM) are required to enable G_s and G_i to hydrolyse GTP [Sunyer *et al*, 1984; Brandt and Ross, 1986]. Mg^{2+} is thought to stabilise the negative charge developed in the pentacoordinated phosphorus transition state as a result of the attack of a water molecule during the hydrolysis reaction, and hence leads to enhanced catalysis through transition state stabilisation (Fig. 4.22) [Noel *et al*, 1993].

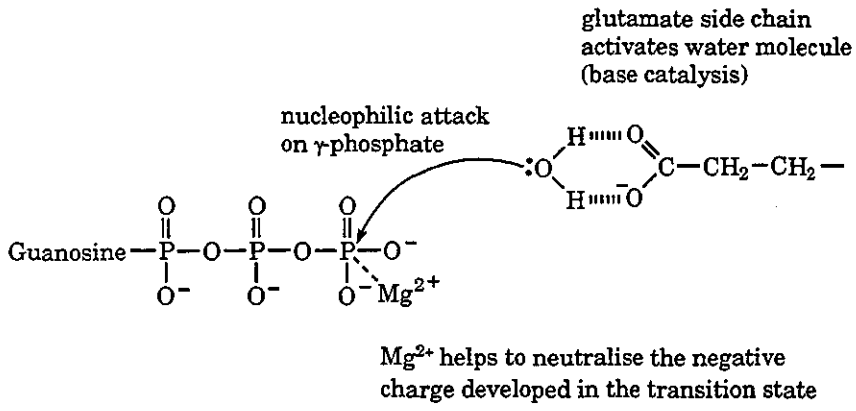


Figure 4.22 Mg^{2+} serves to neutralise the negative charge developed in the pentacoordinated transition state as a water molecule attacks the γ-phosphate group in the GTP hydrolysis reaction.

2. The rate of activation of purified G_s by GTPγS, as measured by the ability of G_s to reconstitute adenylyl cyclase activity in membranes from cyc$^-$ S49 lymphoma cells (which lack functional G_s), is increased by high

concentrations of Mg^{2+} (5-100 mM) These concentrations of Mg^{2+} increase the rate of binding of GTPγS to G_s and G_i [Sternweis *et al*, 1981; Bokoch *et al*, 1984].

3. High concentrations of Mg^{2+} (millimolar) promote the dissociation of GDP from heterotrimeric G proteins [Higashijima *et al*, 1987]. Furthermore, in the presence of Mg^{2+}, bound GTPγS dissociates very slowly from G proteins [Higashijima *et al*, 1987].

4. Mg^{2+} (millimolar) increases μ-opioid binding, apparently by promoting the formation of a slowly dissociating, very high affinity receptor [Standifer *et al*, 1993].

5. Mg^{2+} (0.1-10 mM) increases the binding of agonists and antagonists to all three opioid receptor types in homogenates of guinea pig brain [Rodriguez *et al*, 1992]. The effect on opioid antagonist binding suggests that Mg^{2+} may be exerting its effect by an action at the receptor binding site, rather than by promoting receptor-G protein coupling.

Since Mg^{2+} shows such a broad spectrum of biological effects on G protein-coupled receptor systems, it is difficult to conclude with any certainty the exact mechanism by which this ion is causing its effects in the [^{35}S]GTPγS binding assay. It seems probable that a combination of these individual effects is responsible for the overall effect. The initial increase in both basal and agonist-stimulated [^{35}S]GTPγS binding seen at 0.01-5 mM Mg^{2+} may be accounted for by the increased rate of dissociation of GDP and increased rate of association of [^{35}S]GTPγS expected at these Mg^{2+} concentrations. Furthermore, δ-opioid binding would be enhanced at these Mg^{2+} levels, resulting in a differential increase in agonist-stimulated over basal [^{35}S]GTPγS binding. The reason for the sharp decline in both basal and agonist-stimulated [^{35}S]GTPγS binding at Mg^{2+} concentrations greater than 5 mM is not known, but may reflect a non-specific inhibition of nucleotide binding to G protein at increasing ionic strengths.

The effect of Na^+ on [^{35}S]GTPγS binding in NG108-15 membranes was apparently not as complex as that of Mg^{2+}. Na^+ (3-300 mM) decreased both basal and agonist-stimulated [^{35}S]GTPγS binding. However, in the presence of DPDPE (1 μM) the effect of Na^+ shifted to higher concentrations, leading to an increased stimulation window at Na^+ concentrations between 10-100 mM.

The original model proposed to explain the effect of Na^+ on opioid receptors suggested that the receptor could exist in one of two conformations, one agonist-preferring, and one antagonist-preferring [Pert and Snyder, 1974; Pasternak and Snyder, 1975; Burgisser *et al*, 1982]. Increasing concentrations of Na^+ would shift the equilibrium between these two receptor conformations towards the antagonist-preferring form, hence leading to a reduced affinity for agonists and an increased affinity for antagonists. However, more recently the effect of Na^+ on G protein-coupled receptors has been interpreted as reflecting a modulation of the affinity of 'empty' receptors for G protein [Costa *et al*, 1990; Jagadeesh *et al*, 1990; Costa *et al*, 1992]. Thus, G protein-coupled receptors are thought to be able to activate G protein even in the absence of agonist [Lefkowitz *et al*, 1993] causing constitutive activity of the receptor. Na^+ is thought to regulate this interaction. Indeed, the amino acid residue in G protein-coupled receptors involved in sodium regulation has been identified by site-directed mutagenesis of the α_2-adrenergic receptor gene [Horstman *et al*, 1990]. Mutation of a highly conserved aspartate residue in the second transmembrane helix (Asp-79 of the α_2-adrenergic receptor) entirely eliminates the allosteric regulation of ligand binding by Na^+ without perturbing the selectivity of adrenergic binding. Mutation of the equivalent aspartate (Asp-95) to asparagine in the δ-opioid receptor also yields a receptor which is insensitive to regulation by Na^+ ions [Kong *et al*, 1993]. However, this mutation also causes a large rightward shift in affinity for several δ-opioid agonists. Interestingly, only agonists which are selective for the δ-receptor, such as DPDPE, DSLET and BW373U86 are affected. Non-selective compounds such as bremazocine and buprenorphine are unaffected by this mutation, as are δ-receptor selective antagonists, suggesting that selective agonists and antagonists bind differently to the δ-receptor.

The effect of Na^+ on basal [^{35}S]GTPγS binding in NG108-15 membranes may be explained by suggesting that the constitutive activity of opiate and

possibly other G protein-coupled receptors is reduced in the presence of Na^+. This inhibition is reversed by agonist-bound receptors, leading to an enhanced stimulation of [^{35}S]GTPγS binding at concentrations of Na^+ between 10-100 mM. Interestingly, Tian *et al* (1994) have shown recently that the major influence of receptor interaction with G protein appears to involve the induction of apparent high affinity binding for [^{35}S]GTPγS by the G protein. In the presence of high Na^+ concentrations only low affinity binding of [^{35}S]GTPγS is observed in membranes from PC-12 cells. However, in the absence of Na^+ 'empty' receptors can promote high affinity binding of [^{35}S]GTPγS (hence constitutive activity), and agonist can further increase the apparent [^{35}S]GTPγS affinity.

δ-Opioid receptors are known to couple to G proteins of the G_i/G_o family [Offermanns *et al*, 1991; Roerig *et al*, 1992; Laugwitz *et al*, 1993; Prather *et al*, 1994]. The involvement of G_i/G_o proteins in the δ-opioid-mediated increase in [^{35}S]GTPγS binding in NG108-15 membranes was assessed using pertussis toxin. Treatment of cells with pertussis toxin (100 ng/ml; 24h) prior to harvesting and membrane preparation completely abolished δ-opioid receptor-mediated stimulation of [^{35}S]GTPγS binding, as found previously for μ-opioid [Traynor and Nahorski, 1995] and α_2-adrenergic [Tian *et al*, 1994] receptors. Furthermore, toxin treatment reduced control [^{35}S]GTPγS binding by 60±4 %. Since pertussis toxin only uncouples G proteins from receptors and does not alter other properties of G proteins, such as their ability to bind GTP and interact functionally with adenylyl cyclase [Katada *et al*, 1986], this provides further evidence for the presence of constitutively active receptors (though not necessarily of the δ-opioid type) in membrane preparations of NG108-15 cells. The effect on [^{35}S]GTPγS binding caused by pertussis toxin agrees with previous work on δ-opioid receptors in NG108-15 membranes using GTPase assays to measure G protein activation. Pertussis toxin treatment of cells renders GTPase activity completely insensitive to opioid agonists [Burns *et al*, 1983; Costa *et al*, 1988; Costa *et al*, 1990; Selley *et al*, 1994]. However the effect of pertussis toxin on basal GTPase activity in NG108-15 membranes is not clear, with conflicting reports suggesting that it is either unaffected [Burns *et al*, 1983] or significantly reduced [Costa *et al*, 1988, 1990; Selley *et al*, 1994].

ICI 174864, a δ-opioid ligand with negative intrinsic activity [Costa and Herz, 1989], reduced the constitutive activity of δ-opioid receptors in NG108-15

membranes, as shown by the reduction in control [^{35}S]GTPγS binding. This effect was much more pronounced in buffer containing potassium instead of sodium, similar to the effect of ICI 174864 on GTPase activity [Costa and Herz, 1989]. The effects of sodium and ICI 174864 may be rationalised by a modification of the ternary complex model first proposed by Lefkowitz and colleagues [Samana *et al*, 1993; Lefkowitz *et al*, 1993], and shown in figure 4.23.

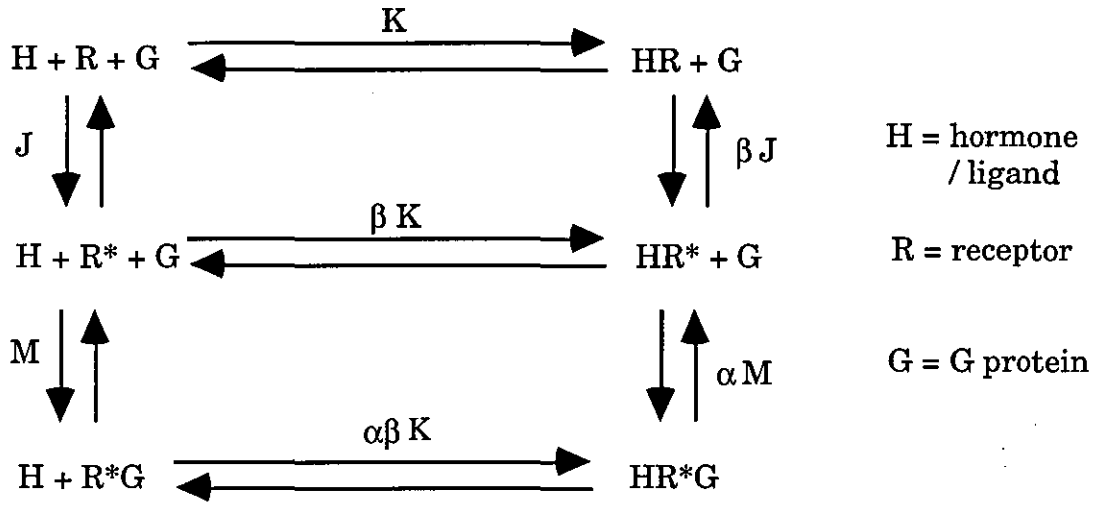


Figure 4.23 The extended version of the ternary complex model or 'allosteric ternary complex model'. In this version, **R** undergoes an allosteric transition with an equilibrium constant **J**, which leads to the formation of an **R*** intermediate. The latter, in turn, interacts with the other components , **H** and **G**, in a fashion similar to **R** in the classical form of the model. Adapted from Lefkowitz *et al* (1993).

The overall reaction scheme for a receptor to change from the inactive (R) form to the active ternary complex (HR*G) is described by the equilibria shown in figure 4.23. The equilibrium between the inactive form (R) and the active form (R*) is shown in the top ring. This step may be regarded as an isomerisation reaction, governed by an equilibrium constant (J). The allosteric effect, exerted by the ligand (H) on this equilibrium is given by β. The lower ring indicates the interaction between 'active' receptor (R*) and G protein (G), defined by the

equilibrium binding constant (M). The allosteric constant (α) accounts for the effect exerted by the ligand on this interaction.

It is apparent from this model that anything which can exert an effect on the equilibrium between R and R* i.e. J, will affect the interaction of receptor with G protein. Thus, it may be hypothesised that Na^+ decreases the value of J, and thereby leads to a smaller population of receptors in the R* conformation. Since it is these receptors which are responsible for constitutive activity, Na^+ would thereby lead to a decrease in G protein activity, which is manifested as a reduction in 'basal' [^{35}S]GTPγS binding.

Ligands with negative intrinsic activity would have $\beta < 1$ according to this model. Therefore ICI 174864, an inverse agonist at the δ-opioid receptor would be expected to show a similar effect to Na^+ in that a smaller population of R* would be anticipated in the presence of ICI 174864, despite the fact that Na^+ and ICI 174864 are acting through different mechanisms to achieve their effect. Evidently, the presence of Na^+ would mask the effect of ICI 174864, since both bring about the same result, namely a reduction in R* with a concomitant rise in R.

A large amount of evidence supports the existence of δ-opioid receptor subtypes (for review see Traynor and Elliott, 1993). Studies using the subtype-selective δ-opioid antagonists naltriben (NTB, δ_2) and 7-benzylidenenaltrexone (BNTX, δ_1) suggested that the receptor in NG108-15 membranes may be of the δ_2-type, since NTB was much more potent than BNTX in antagonising DPDPE-mediated [^{35}S]GTPγS binding, affording K_e values of 78±10 pM and 1.57±0.39 nM respectively. The cloned mouse δ-opioid receptor displays a pharmacological profile consistent with that of the δ_2 receptor [Raynor *et al*, 1994], since putative δ_2 ligands such as naltriben (NTB), [D-Ala^2]deltorphin II (DELT II) and [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) have higher affinity than putative δ_1 ligands such as [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and BNTX, as assessed by radioligand binding assay. Since this δ-opioid receptor was cloned from NG108-15 cells (Evans *et al*, 1992; Kieffer *et al*, 1992], then it is logical to assume that the receptor in this cell line is of the δ_2-type, although this does not preclude the possibility of there being more than one type of δ-receptor in these cells.

Radioligand displacement assays of [^{3}H]-diprenorphine using these two antagonists also revealed that the affinity of NTB for δ-opioid receptors in membranes from NG108-15 cells was higher than that of BNTX. However, for both NTB and BNTX the displacement of [^{3}H]-diprenorphine was biphasic, and computer modelling of the data revealed that a two site model gave a significantly better fit than a one site model. Comparison of the affinity values (K_i) generated from the displacement curves for these two antagonists, with K_e values calculated from antagonism of DPDPE-mediated stimulation of [^{35}S]GTPγS binding, showed that for both compounds the affinity (K_e) derived from the [^{35}S]GTPγS binding experiments was midway between the two K_i values. This suggests that a combination of the high and low affinity sites are involved in antagonising DPDPE-mediated stimulation of [^{35}S]GTPγS binding in NG108-15 membranes.

The biphasic nature of the curves obtained for the displacement of [^{3}H]-diprenorphine for these two δ-receptor antagonists was unexpected, particularly in light of the monophasic displacement of [^{3}H]-diprenorphine by the δ-opioid agonist DPDPE under identical conditions. One possible explanation for this is that δ-opioid receptor subtypes exist in NG108-15 cells, which are recognised by NTB and BNTX but not by DPDPE. However, this seems unlikely as displacement of [^{3}H]-diprenorphine by NTB and BNTX at the cloned mouse δ-opioid receptor expressed in CHO cells resulted in similarly shallow curves (Hill slopes were 0.54 and 0.36 for NTB and BNTX respectively (n=1)), although it is possible that the difference between these two subtypes of δ-opioid receptor occurs as a result of post-translational modification which may take place in both CHO and NG108-15 cells. Another possibility is that multiple conformations of the δ-opioid receptor exist in NG108-15 membranes, which can be discriminated by NTB and BNTX but not by DPDPE. These differing states could be caused, for example, by coupling of the δ-receptor to multiple G protein subtypes. This also seems somewhat unlikely as NTB and BNTX are both antagonists and would therefore not be expected to discriminate between G protein-coupled and free receptor. A third potential reason for the anomalous effects shown by NTB and BNTX is that the displacement by these ligands may not be fully competitive, for example these ligands may not be acting at the [^{3}H]-diprenorphine binding site, but rather at an allosteric binding site which affects the binding of [^{3}H]-diprenorphine. Finally both of these antagonists may require more than one hour at 25°C (the conditions of the binding assay) to reach

equilibrium; binding data from experiments which have not been allowed to reach equilibrium are clearly not valid [Bennett and Yamamura, 1985]. However, it should be noted that Sofuoglu *et al* (1992) obtained monophasic displacement of three different δ-opioid agonists in mouse brain with NTB after a 20 min incubation at 37°C.

It has been suggested that the relative intrinsic activity of opioid agonists and inverse agonists is dependent on the type of cation(s) present in the reaction mixture [Costa and Herz, 1989]. In the presence of 100 mM Na^+, diprenorphine only produced approximately 30% of the maximal stimulation of [^{35}S]GTPγS binding produced by DSLET. However, when Na^+ was replaced by K^+ in the reaction buffer, diprenorphine produced 70% of the DSLET response. These results correlate well with similar experiments measuring low K_m GTPase activity [Costa and Herz, 1989], where diprenorphine is a weaker partial agonist (30-40% of the maximum effect produced by DADLE) in Na^+-containing buffer than in K^+-containing buffer (maximal effect 70-80% of that of DADLE).

In conclusion, δ-opioid agonists have been shown to stimulate [^{35}S]GTPγS binding in membranes from NG108-15 cells in a naloxone-reversible manner. Optimal conditions for this assay have been established. This assay should provide a rapid, sensitive and reproducible method to measure the potency and intrinsic activity of G protein activation by δ-opioid agonists, and will also be useful in studying molecular mechanisms of G protein activation by δ-opioid receptors, because of its superior sensitivity compared to GTPase and adenylyl cyclase assays.

Chapter 5

CLONED MOUSE δ-RECEPTOR EXPRESSED IN CHO CELLS: FUNCTIONAL COUPLING TO G PROTEINS

5.1 Introduction

Cloning of the opioid receptors is leading to rapid advances in our understanding of their action at the molecular level. The availability of cDNAs encoding the three opioid receptor types allows the use of many new experimental approaches to study the molecular determinants of receptor function. For example, antisense oligodeoxynucleotide strategies can be used to inhibit translation of receptor proteins [Adams *et al*, 1994; Lai *et al*, 1994]. Regulation and expression of receptor mRNA levels under various conditions may be studied using *in situ* hybridization [Anton *et al*, 1994] or alternatively by competitive polymerase chain reaction assay [Búzás and Cox, 1994]. Furthermore, the ability to mutate receptor genes and express them at will in appropriate cell lines enables ideas about receptor structure / function relationships to be tested [Kong *et al*, 1993, 1994; Meng *et al*, 1994; Xue *et al*, 1994], and should ultimately afford information useful for the rational design of potent and selective opioid agonists and antagonists.

The expression of cloned receptors in appropriate cell lines also provides useful models for more traditional pharmacological approaches, such as ligand binding assays. The availability of cell lines expressing cloned opioid receptors allows each receptor subtype to be examined independently of the others. One of the major difficulties in studying the pharmacology of the opioid receptors is that most tissues coexpress several types of opioid receptors. This has confounded the analysis of binding studies, since many of the compounds used are not completely selective for one subtype of opioid receptor, and therefore cross-react with other receptors present to varying degrees. The ability to control the genotype of a cell overcomes the problems associated with interpreting results from experiments using tissues or cells which express multiple opioid receptor subtypes.

Molecular expression strategies can have limitations, however, particularly in terms of the genetic background of the cells selected for expression. The host cells (generally fibroblasts) are often selected for ease of transfection and rapidity of isolation of clones, rather than as a control for the physiological functioning of the receptor. The levels of expression of each of the G protein subtypes and other cellular machinery which may be necessary for a

functional response are often not considered, leading to results which may be of little physiological relevance. Even when expressed in very similar cell types cloned receptors may show variable functional responses. This has been demonstrated recently for cloned human somatostatin receptors [Patel *et al*, 1994], which have been shown to inhibit adenylyl cyclase activity when expressed in CHO-K1 cells but not when expressed in CHO-DG44 cells.

When a new clonal cell line is generated, functional responses must be analysed carefully in order to confirm that the cloned receptor behaves in a manner consistent with the endogenously expressed receptor. This study was designed to establish a [^{35}S]GTPγS binding assay to study the activation of G proteins by opioid agonists in chinese hamster ovary (CHO) cells expressing the mouse δ-opioid receptor (CHOδ cells).

5.2 Results

5.2.1 Determination of δ-opioid receptor density in CHOδ cells

An initial experiment was performed to confirm that the CHO cells were expressing opioid receptors. Saturation analysis of membranes from CHO cells using the δ-receptor partial agonist [^{3}H]-diprenorphine afforded a receptor number (B_{max}) of 404±23 fmol/mg and an affinity (K_d) for [^{3}H]-diprenorphine of 0.20±0.01 nM (Fig 5.1).

By comparison, saturation binding studies on membranes from the same batch of cells using the δ-opioid agonist [^{3}H]-DPDPE afforded a receptor number (B_{max}) of 183±45 fmol/mg of membrane protein, and an affinity (K_d) of 1.34±0.15 nM (Fig. 5.2). Therefore, only 45% of the receptors labelled by [^{3}H]-diprenorphine in CHOδ membranes were labelled by [^{3}H]-DPDPE.

5.2.2 Optimisation of a [^{35}S]GTPγS binding assay

When performing [^{35}S]GTPγS binding assays it is often necessary that a predetermined concentration of GDP is present to optimise the increase in binding caused by agonists. The level of basal and DPDPE (1 μM)-stimulated [^{35}S]GTPγS binding in the presence of varying GDP concentrations is shown in Figs. 5.3 and 5.4. Increasing concentrations of GDP caused a decrease in both basal and agonist-stimulated [^{35}S]GTPγS binding and also increased reproducibility within experiments. Basal [^{35}S]GTPγS binding was affected at lower concentrations of GDP than agonist-stimulated binding. Optimal DPDPE (1 μM)-mediated [^{35}S]GTPγS binding was observed in the presence of 3 μM GDP, and this concentration of GDP was chosen for further experiments.

To optimise the assay in this cell line further, the concentration of membrane protein was varied in the absence or presence of DPDPE (1 μM) (Figs. 5.5 and 5.6). Agonist-stimulated binding was linear up to 150 μg/ml, and reached a maximum at 200-250 μg/ml. For further experiments a protein

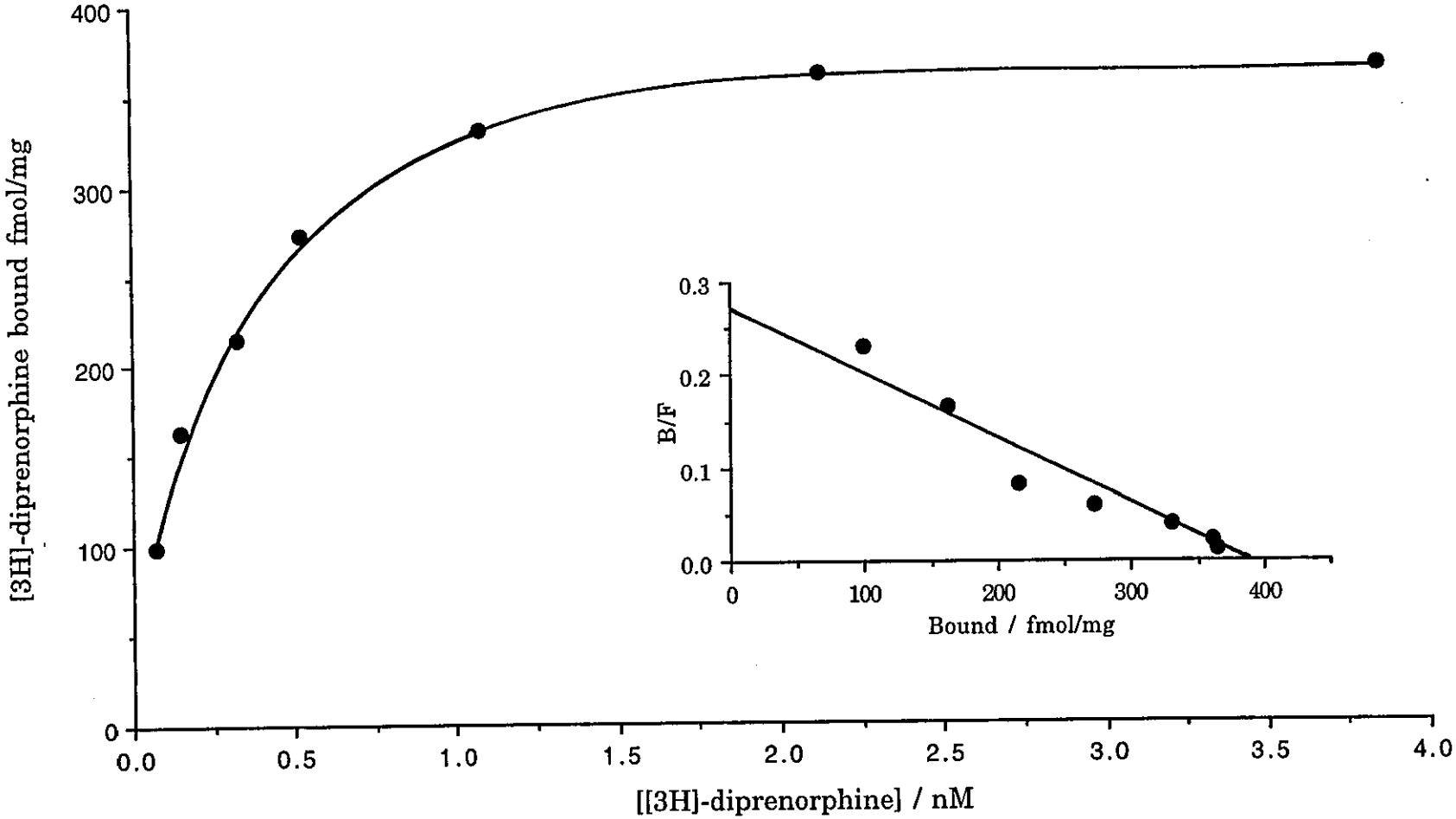


Figure 5.1 A representative graph showing saturation [^{3}H]-diprenorphine binding to CHOδ membranes in Tris-HCl buffer (pH 7.4). Inset is the corresponding Scatchard plot.

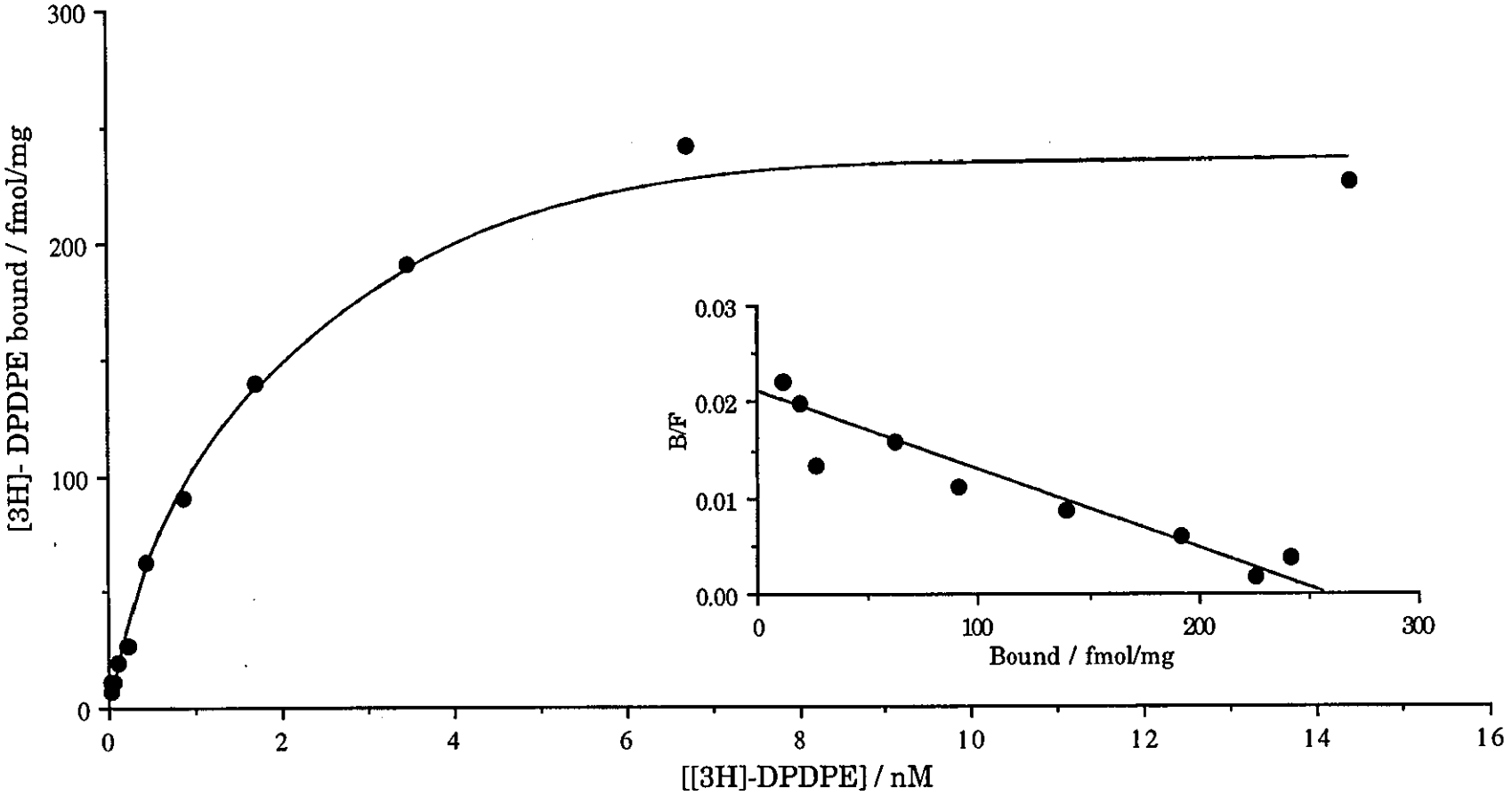


Figure 5.2 A representative graph showing saturation [^{3}H]-DPDPE binding to CHOδ membranes in Tris-HCl buffer (pH 7.4). Inset is the corresponding Scatchard plot.

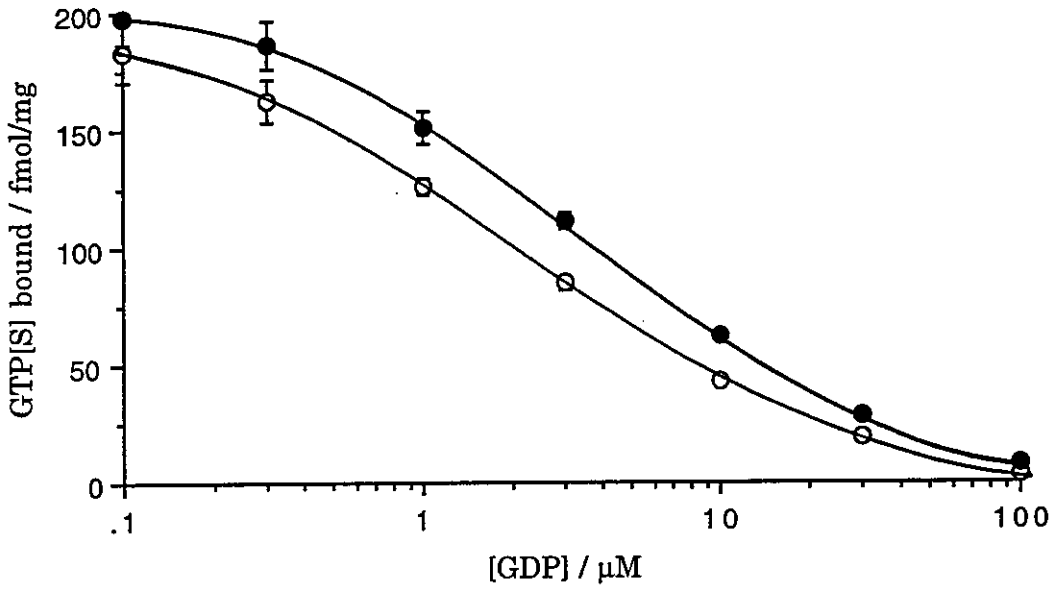


Figure 5.3 Effect of varying the concentration of GDP in the absence (○) or presence (●) of DPDPE (1 μM) on [^{35}S]GTPγS binding in CHOδ membranes. Experimental conditions were as described in Chapter 2, except [$MgCl_2.6H_20$] was 4 mM. Values represent means±sem (n=3).

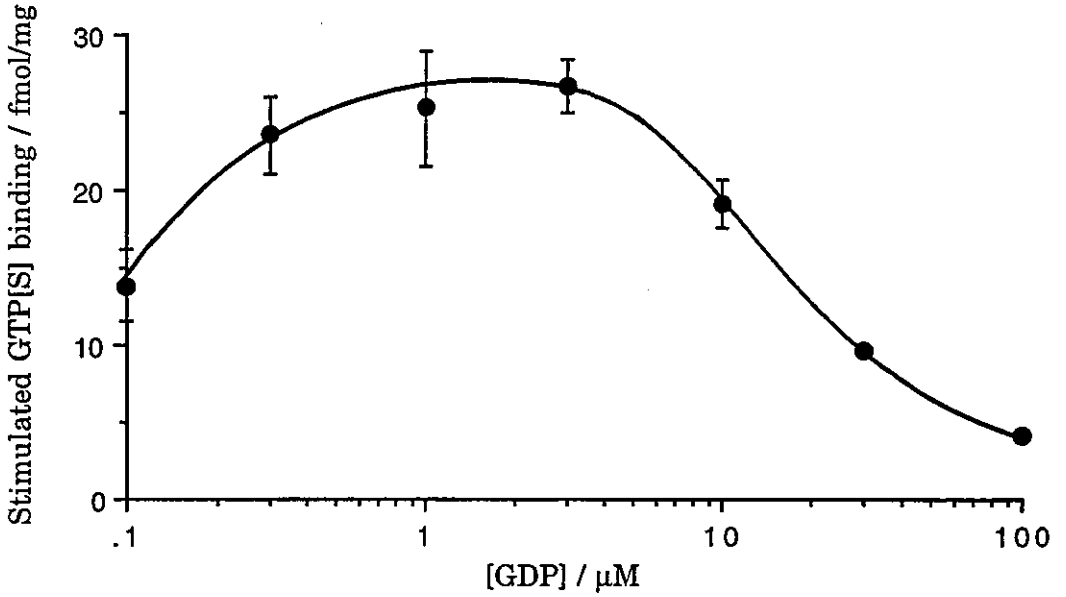


Figure 5.4 Stimulation of [^{35}S]GTPγS binding by DPDPE (1 μM) in CHOδ membranes. Results are the difference between control and DPDPE data from the experiment shown in Figure 5.3. Experimental conditions were as described in Chapter 2, except [$MgCl_2.6H_20$] was 4 mM. Values represent means±sem (n=3).

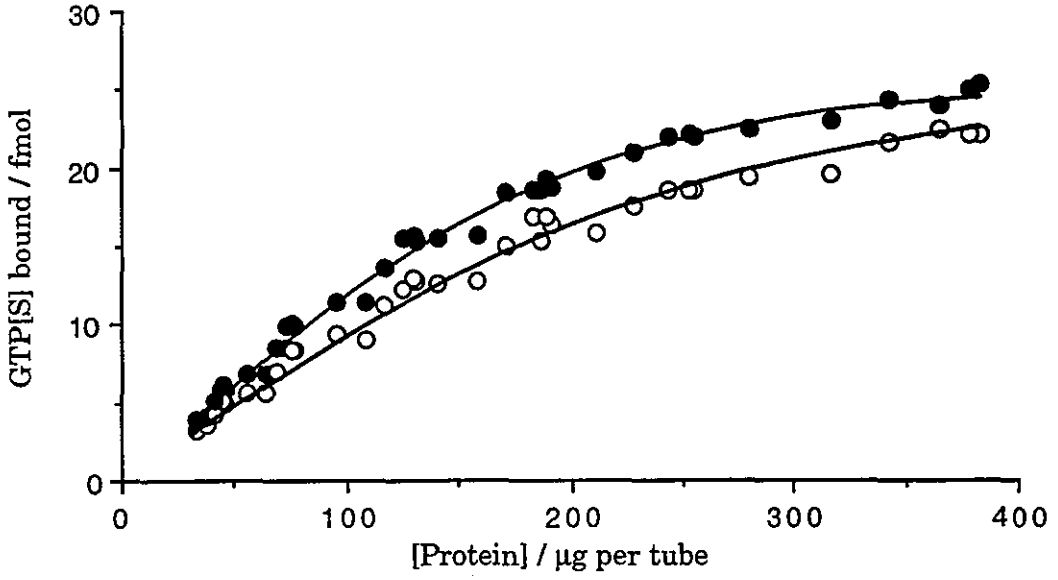


Figure 5.5 Effect of CHOδ cell membrane protein concentration on [^{35}S]GTPγS binding in the absence (○) or presence (●) of DPDPE (1µM). Experimental conditions were as described in Chapter 2, except [$MgCl_2.6H_20$] was 4 mM. Results from 6 separate experiments are shown.

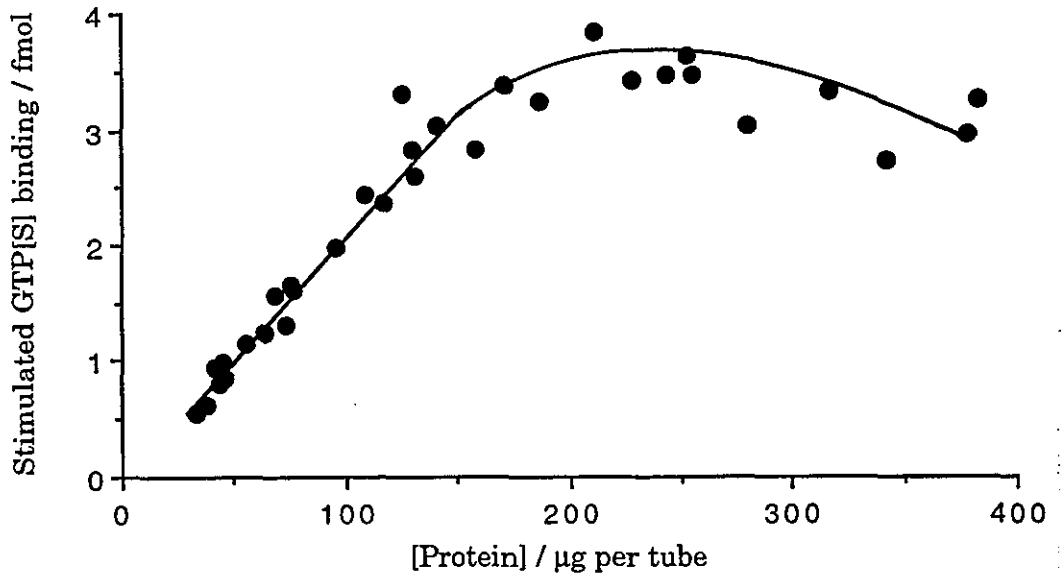


Figure 5.6 Effect of CHOδ cell membrane protein concentration on the stimulation of [^{35}S]GTPγS binding by DPDPE (1 µM). Results are the difference between control and DPDPE data from the experiments shown in Fig. 5.5. Experimental conditions were as described in Chapter 2, except [$MgCl_2.6H_20$] was 4 mM.

concentration of approximately 150 μg/ml was chosen, in order to obtain the maximum possible stimulation whilst remaining on the linear part of the curve.

The optimal Mg^{2+} concentration was also investigated (Figs. 5.7 and 5.8). Mg^{2+} (1-10 mM) increased both basal and DPDPE (1 μM)-stimulated [^{35}S]GTPγS binding. However, both curves reached a maximum at 10 mM Mg^{2+}, and higher concentrations than this caused a decrease in [^{35}S]GTPγS binding. Maximal stimulation of [^{35}S]GTPγS binding over basal was achieved in the presence of 1-30 mM Mg^{2+}. For subsequent experiments 10 mM Mg^{2+} was used.

Increasing concentrations of Na^+ (10-300 mM) caused a decrease in both basal and DPDPE (1 μM)-stimulated [^{35}S]GTPγS binding (Figs. 5.9 and 5.10). The stimulation window produced by the agonist was very small. DPDPE (1 μM) only produced an increase in [^{35}S]GTPγS binding at Na^+ concentrations ≥ 100 mM. Even at these high concentrations, the stimulation observed was very weak, representing less than a 20% enhancement of the binding seen in the absence of agonist.

To attempt to improve the signal-to-noise ratio for the assay, the effect of temperature and time of incubation was investigated (Figs. 5.11 and 5.12). In this set of experiments, the δ-opioid agonist DSLET (1 μM) was used, because previous experiments using NG108-15 membranes had shown that DSLET produces a larger maximal stimulation of [^{35}S]GTPγS binding than DPDPE (Fig. 4.18). At temperatures of 20°C or more, basal and agonist-stimulated [^{35}S]GTPγS binding reached maximum levels within 2h. Interestingly the maximum level of [^{35}S]GTPγS binding increased with increasing temperature. Thus at 20°C the maximal binding of [^{35}S]GTPγS in the absence of agonist corresponded to approximately 100 fmol/mg of membrane protein which was increased to 140 fmol/mg at 37°C. Figure 5.12 shows that irrespective of the time and temperature used for incubation of the samples, the response to DSLET (1 μM) was never greatly improved as compared to a 60 min incubation at 30°C, which had been used for all of the prior experiments. Thus, no manipulation of experimental conditions made it possible to observe greater than 25% stimulation of [^{35}S]GTPγS binding over basal levels.

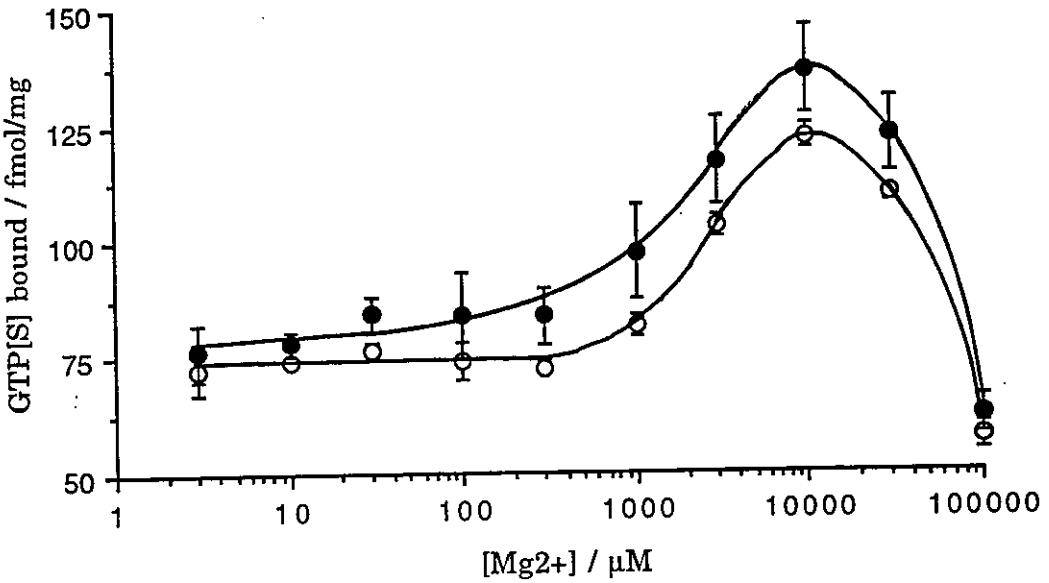


Figure 5.7 Effect of Mg^{2+} on [^{35}S]GTPγS binding in CHOδ membranes in the absence (○) or presence (●) of DPDPE (1 μM). Values represent means±sem (n=3).

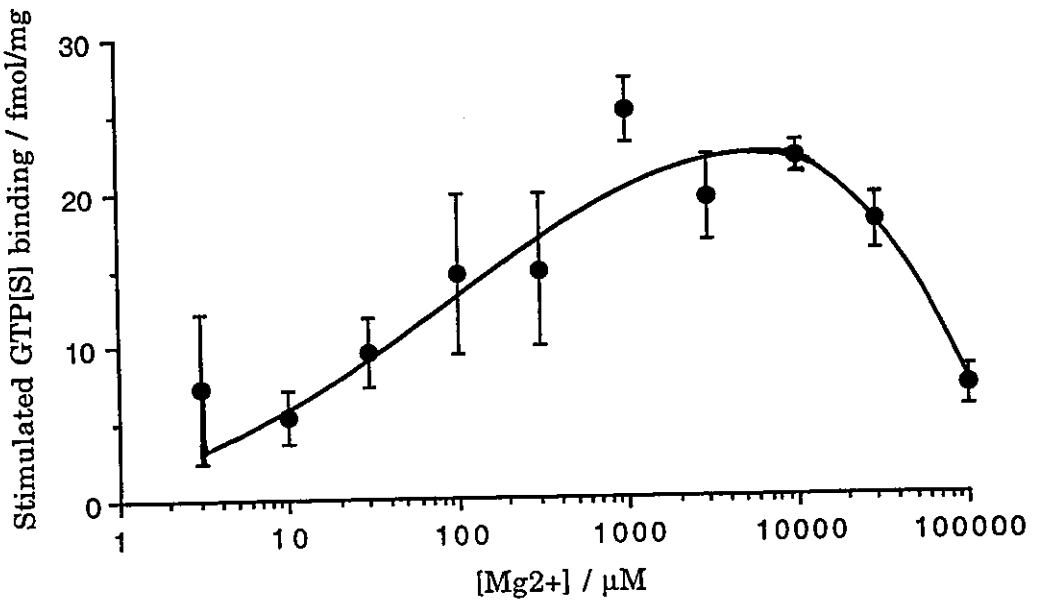


Figure 5.8 Effect of Mg^{2+} on the stimulation of [^{35}S]GTPγS binding by DPDPE (1 μM) in CHOδ membranes. Results are the difference between control and DPDPE data from the experiments shown in Fig. 5.7. Values are means±sem (n=3).

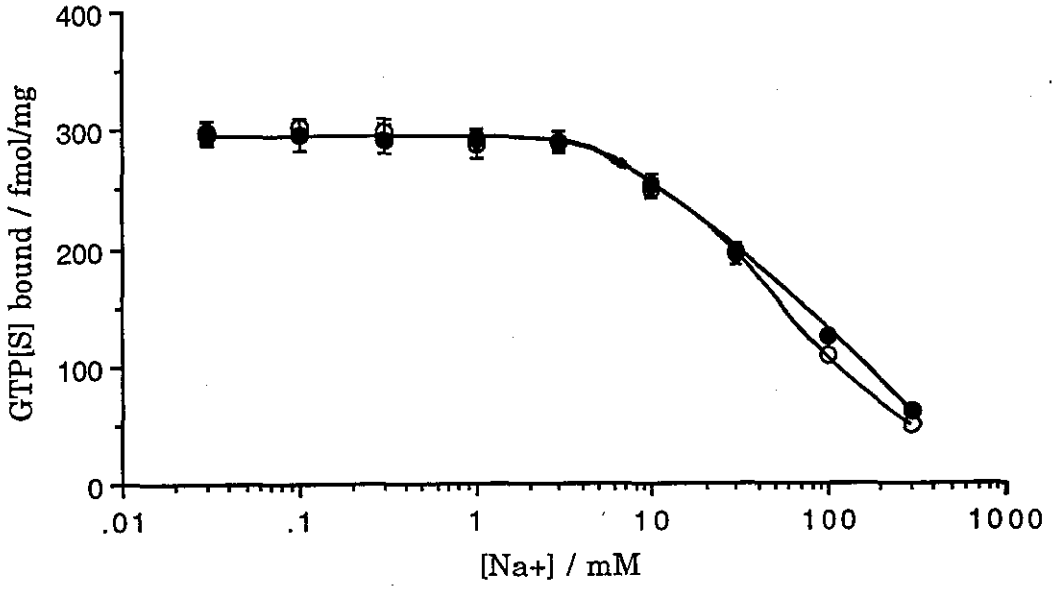


Figure 5.9 Effect of Na^+ on [^{35}S]GTPγS binding in CHOδ membranes in the absence (○) or presence (●) of DPDPE (1 μM). Values represent means±sem (n=3).

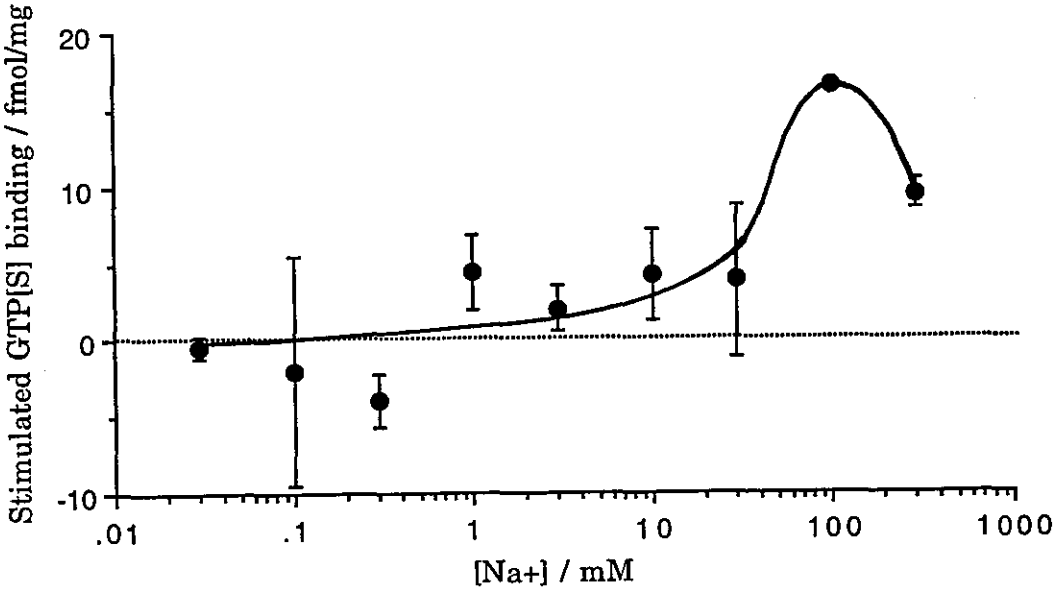


Figure 5.10 Effect of Na^+ on the stimulation of [^{35}S]GTPγS binding by DPDPE (1 μM) in CHOδ membranes. Results are the difference between control and DPDPE data from the experiments shown in Fig. 5.9. Values are means±sem (n=3).

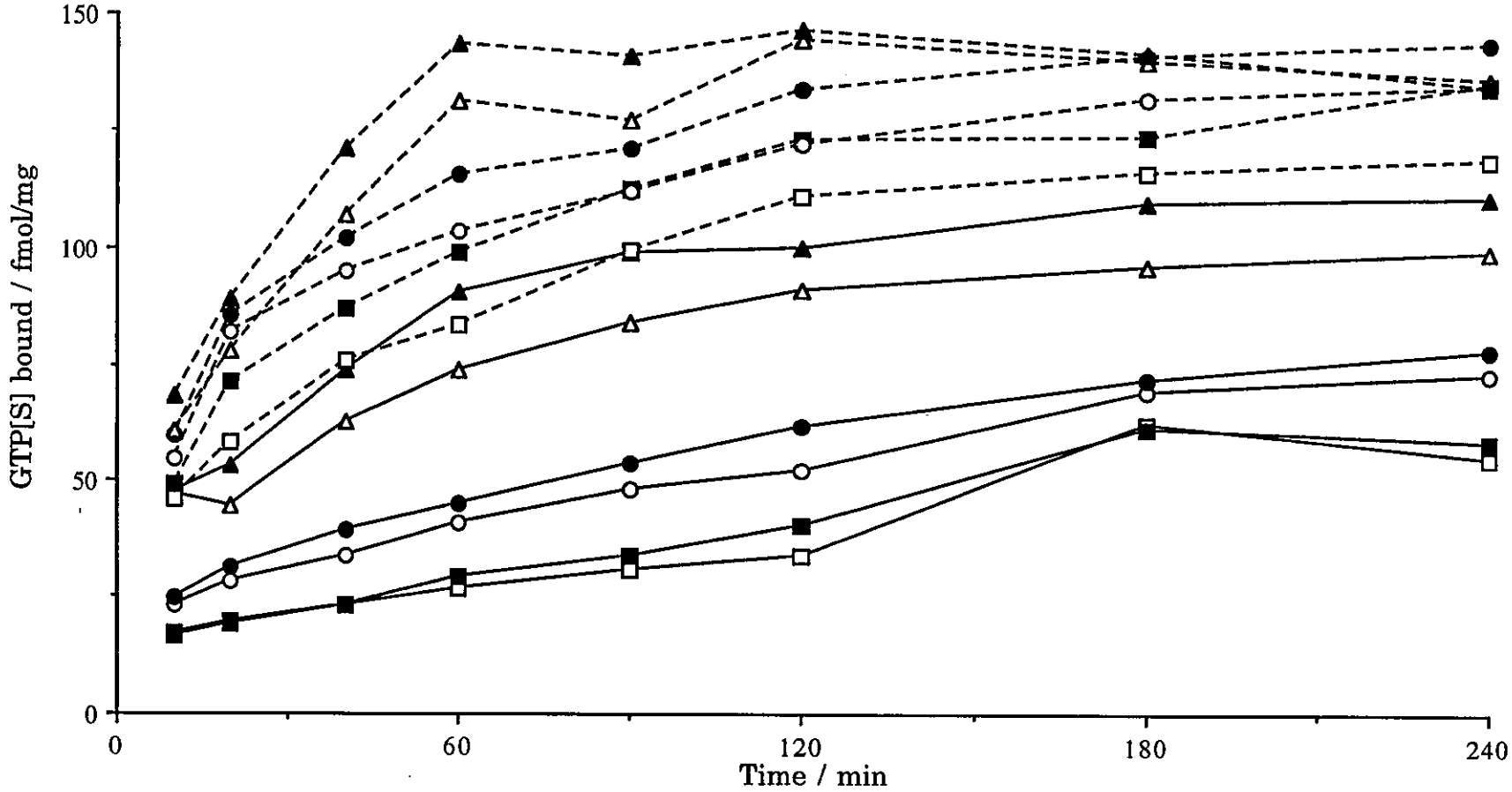


Figure 5.11 Basal (□,○,△) and DSLET (1 μM)-stimulated (■,●,▲) [^{35}S]GTPγS binding in CHOδ membranes at 4°C (-□- , -■-), 10°C (-○- , -●-), 20°C (-△- , -▲-), 25°C (-□·· , -■··), 30°C (-○·· , -●··) and 37°C (-△·- , -▲·-) (n=1).

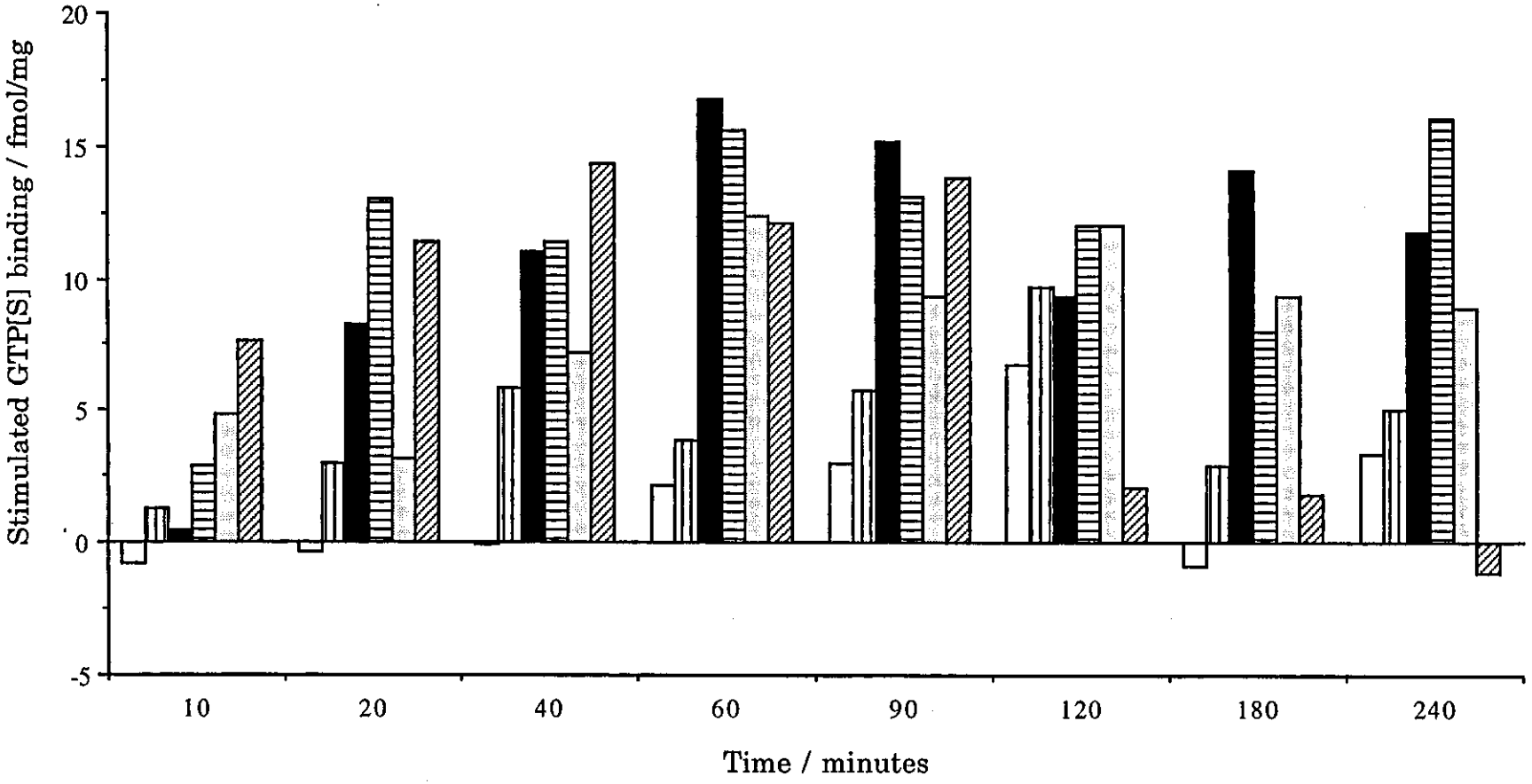


Figure 5.12 Stimulation of [^{35}S]GTPγS binding in CHOδ membranes by DSLET (1 μM) at 4°C (white), 10°C (vertical stripes), 20°C (black), 25°C (horizontal stripes), 30°C (dots) and 37°C (diagonal stripes). Values are the differences between control and DSLET (1 μM) data shown in Figure 5.11 (n=1).

The results thus far indicate that it may not be possible to obtain a good δ-opioid receptor-mediated increase in [^{35}S]GTPγS binding using membranes from CHOδ cells. One explanation could be that the receptors in this cell line are not tightly coupled to relevant G proteins. In order to test this hypothesis, the inhibition of adenylyl cyclase activity by the δ-opioid agonist DPDPE was studied in this cell line, and compared to that observed in NG108-15 cells (Fig 5.13).

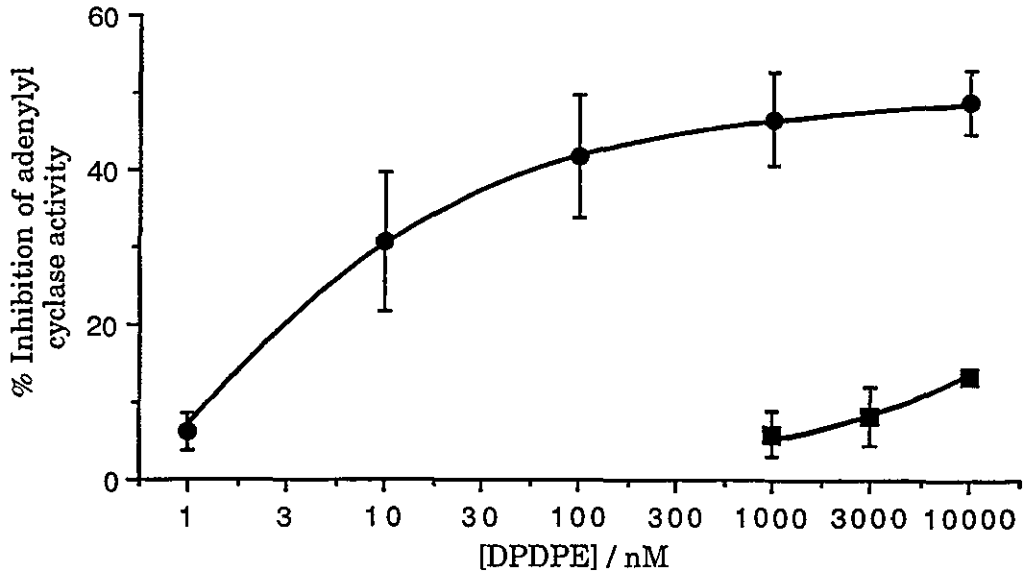


Figure 5.13 Inhibition of forskolin (10 μM)-stimulated adenylyl cyclase activity by DPDPE in NG108-15 (●) and CHOδ (■) cells. Forskolin-stimulated adenylyl cyclase activity was 99.0±15.5 pmol/min/mg of protein (NG108-15 cells) and 57.9±3.0 pmol/min/mg of protein (CHOδ cells). Values represent means±sem (n≥3).

The potent δ-opioid agonist DPDPE inhibited adenylyl cyclase activity in NG108-15 cells affording an IC_{50} value of 6.2±2.7 nM and a maximal inhibition of 49.0±4.0 % (Fig. 5.13). By comparison, the inhibition of adenylyl cyclase activity in CHOδ cells was very weak. DPDPE caused only a 13.3±0.7 % decrease in adenylyl cyclase activity at a concentration of 10 μM.

5.3 Discussion

Clonal cell lines containing an homogenous, well-defined receptor population offer many advantages to study signal transduction pathways, and as such are likely to be used increasingly for this purpose. One of the most common host cell lines used for the transfection of 'foreign' DNA is the chinese hamster ovary (CHO) cell line, probably because of the relative ease of such procedures using these cells. The possibility of using CHO cells expressing the cloned mouse δ-opioid receptor (CHOδ cells) as a model system to study δ-opioid receptor-G protein coupling was investigated by attempting to set up a [^{35}S]GTPγS binding assay in this cell line.

In membranes from CHOδ cells the selective δ-opioid agonist DPDPE (1 μM) stimulated the binding of [^{35}S]GTPγS. This effect was dependent on the presence of GDP, with optimal enhancement of binding occurring at 3 μM GDP. This result can be compared to the situation in NG108-15 cell membranes, where the level of stimulation of [^{35}S]GTPγS binding by the δ-opioid agonist DPDPE (1 μM) increased with increasing GDP concentrations up to 100 μM GDP (Fig. 4.5). Therefore it would seem that the optimal concentration of GDP required to observe δ-opioid receptor-mediated stimulation of [^{35}S]GTPγS binding is cell-type specific.

δ-Opioid receptors are known to couple to G proteins of the G_i/G_o family [Offermanns *et al*, 1991; Roerig *et al*, 1992; Laugwitz *et al*, 1993; Prather *et al*, 1994]. Differential G protein expression in NG108-15 cells and the CHO cells used in this study may account for the different GDP concentrations required to observe optimal stimulation of [^{35}S]GTPγS binding by δ-opioid agonists in membranes from the two cell lines, as different G protein α-subunits may be expected to have varying relative affinities for GDP and GTPγS. However other explanations for this difference are also possible. It is worth noting that [^{35}S]GTPγS binding assays have been described for many 7-transmembrane domain receptors in membranes from a variety of cell lines, and the optimal GDP concentration required to observe agonist-mediated stimulation of binding usually lies in the range 0.1-3 μM [Hilf *et al*, 1989; Gierschik *et al*, 1991; Lazareno *et al*, 1993; Tian *et al*, 1994; Traynor *et al*, 1995]. Thus it is possible that

NG108-15 cells differ from most other cell lines in some way. One possibility is the presence of an exceptionally high level of nucleotidase activity in membranes from this cell line, which makes it necessary to carry out the [^{35}S]GTPγS binding assay using membranes from these cells in the presence of a particularly high concentration of GDP.

Using CHOδ membranes, stimulation of [^{35}S]GTPγS binding by δ-opioid agonists was linear up to 150 μg protein per assay tube. Ideally, a protein concentration of 80-100 μg of protein per assay tube should have been used to ensure that experiments were carried out using a linear part of the 'stimulation window' versus protein concentration curve. However, because the response to δ-opioid agonists was weak in CHOδ cells, a protein concentration of approximately 150 μg per assay tube was chosen for further experiments, in order to achieve the maximum stimulation window possible whilst remaining on the linear part of the curve.

Initial experiments were carried out using a 60 min incubation at 30°C. Previous work using the NG108-15 cell line suggested that an improved response to δ-opioid agonists may have been possible by varying the incubation conditions. However, in a single set of experiments, no manipulation of either the time or temperature of incubation caused a noticeable improvement in the stimulation of [^{35}S]GTPγS binding by DSLET (1 μM).

The ionic composition of the medium was also varied to try to improve the δ-opioid-mediated increase in [^{35}S]GTPγS binding. The effect of Mg^{2+} on basal and δ-opioid-stimulated [^{35}S]GTPγS binding in CHOδ membranes was very similar to the effect of this ion using NG108-15 membranes. Mg^{2+} had a biphasic effect on both basal and δ-receptor-stimulated [^{35}S]GTPγS binding in CHOδ membranes, causing both to increase at concentrations from 1-10 mM. Higher concentrations than this caused a decrease in both basal and δ-receptor-stimulated binding. The presence of 1-30 mM Mg^{2+} resulted in optimal stimulation of [^{35}S]GTPγS binding by the δ-opioid agonist DPDPE. A discussion of the mechanisms by which Mg^{2+} may cause these effects is provided in section 4.3.

Na^+ also caused a similar effect on [^{35}S]GTPγS binding in CHOδ and NG108-15 membranes. Increasing concentrations of Na^+ resulted in decreased levels of [^{35}S]GTPγS binding in CHOδ membranes, both in the absence and presence of DPDPE (1 μM). However, in CHOδ membranes both basal and agonist-stimulated [^{35}S]GTPγS binding were equally affected by the presence of Na^+, and therefore the addition of millimolar concentrations of Na^+ to the medium did not result in a large stimulation of [^{35}S]GTPγS binding by DPDPE (1 μM). This can be compared to identical experiments performed using membranes from NG108-15 cells, in which Na^+ produced less of an effect on [^{35}S]GTPγS binding in the presence of DPDPE (1 μM) compared to in its absence (Fig. 4.11). In fact, significant stimulation of binding by DPDPE in CHOδ membranes was only seen at the two highest concentrations of Na^+ tested (100 mM and 300 mM).

Even though conditions for the [^{35}S]GTPγS binding assay using CHOδ membranes had been optimised, the degree of stimulation afforded by δ-receptor agonists was very weak compared to that seen in NG108-15 membranes. This low level of G protein activation in CHOδ cells was supported by experiments comparing inhibition of adenylyl cyclase activity observed in these cells with that seen using NG108-15 cells. In NG108-15 cells, the selective δ-opioid agonist DPDPE caused a maximal 50% inhibition of adenylyl cyclase activity, affording an IC_{50} of 6.2±2.7 nM. By comparison the effect of DPDPE in CHOδ cells was very weak - DPDPE caused only a 13% inhibition of adenylyl cyclase activity in CHOδ cells, even at a concentration of 10 μM. Thus both the potency and maximal effect observed in this cell line were much reduced compared to that seen in NG108-15 cells, even though both cell lines expressed a similar number of δ-receptors.

Saturation binding studies using [^{3}H]-diprenorphine and [^{3}H]-DPDPE provided further evidence that the majority of δ-opioid receptors in CHOδ membranes are uncoupled from G protein. High affinity agonist binding is only expected if receptor is functionally coupled to G protein [Birnbaumer *et al*, 1990]. Therefore, if much of the δ-opioid receptor population in CHOδ cells exists in an uncoupled form, then this fraction of the receptor population would be expected to exhibit only low affinity binding for an agonist and would not be detected in a saturation binding assay using an agonist such as [^{3}H]-DPDPE. However, since

antagonist binding is insensitive to the presence or absence of G protein [Birnbaumer *et al*, 1990], one would expect the receptor number (B_{max}) determined using an antagonist to be significantly higher than that measured with an agonist. As expected, the receptor number determined using the full agonist [^{3}H]-DPDPE was only 45% of that obtained when [^{3}H]-diprenorphine was used to label the receptors. It should be noted that diprenorphine was shown to be a partial agonist at the δ-receptor in NG108-15 cell membranes (Figs. 4.18 and 4.19), and it is possible that the total number of δ-opioid binding sites in CHOδ membranes may therefore have been underestimated using [^{3}H]-diprenorphine. As an alternative a 'purer' antagonist, such as [^{3}H]-naloxone, could have been used to determine the total δ-opioid receptor density in these cells.

Babey *et al* (1995) have obtained similar results using saturation binding analysis to study CHO cells stably expressing the mouse δ-opioid receptor. Saturation binding studies using radiolabelled naltrindole, DPDPE, DADLE and DELT II demonstrated that high affinity agonist binding for each of the tritiated agonists did not account for more than approximately one third of the total [^{3}H]-naltrindole binding. In addition, the affinity constants of all ligands in saturation studies were comparable to those seen in NG108-15 membranes, but the K_i values as competitors of [^{3}H]-naltrindole binding were almost 10-fold greater than expected. Furthermore, addition of the non-hydrolyzable guanine nucleotide analogue, guanosine 5'-[β,γ-imido]triphosphate (GPPNHP), did not shift the agonist competition curves. These results also led Babey *et al* (1995) to suggest that CHO cells stably expressing the mouse δ-opioid receptor appear to contain a high proportion of uncoupled receptors.

The results demonstrate that δ-opioid receptors expressed in CHO cells used in the present study cause only a small activation of G protein in the presence of δ-opioid agonists, as measured by an increase in the binding of the hydrolysis resistant GTP analogue [^{35}S]GTPγS. Furthermore, this poor activation of G protein also results in a very weak inhibition of adenylyl cyclase activity. In comparison, however, several groups have reported that δ-opioid receptors expressed in CHO cells can produce a large (up to 87%) inhibition of adenylyl cyclase activity [Prather *et al*, 1994a; Cvejic *et al*, 1995].

The reason for the poor level of G protein activation seen in the present study is not completely understood. It is possible that various strains of CHO cells (with corresponding variations in the type and amount of G protein expressed) used by different laboratories for the transfection of δ-receptors could cause results to vary between laboratories. Another possible explanation for the poor G protein activation seen in the present study may be that although all relevant G proteins are expressed by the CHO cell line, they are not expressed at the same level as in NG108-15 cells or alternatively are not tightly functionally coupled to δ-opioid receptors in this particular clone. Prather *et al* (1994a) studied the inhibition of adenylyl cyclase in CHO cells expressing ten different levels of δ-opioid receptors (30 to 2610 fmol/mg of membrane protein), and observed maximal inhibition of adenylyl cyclase which varied between 33-87% for the individual clones. This variation in maximal inhibition showed no correlation with the receptor density expressed by these clonal cell lines. In the same study, DADLE-induced increases of the incorporation of [α^{32}P]-azidoanilido-GTP into G protein α-subunits did show a correlation between receptor density and maximal effect, with the exception of the two clonal cell lines which expressed the highest receptor density. The authors interpreted these results as showing that there is no apparent correlation between the amount of G protein activated and the maximal effect of an agonist on adenylyl cyclase activity, although in a later paper by the same group [Law *et al*, 1994] results from 3 of these 10 clones were used to suggest that there may be a relationship between the maximal inhibition of adenylyl cyclase and receptor density.

Another possible interpretation of the results from Prather *et al* (1994a) is that transfection of CHO cells with plasmids containing δ-opioid receptor cDNA may damage part of the cellular machinery responsible for causing an inhibition of adenylyl cyclase, and the resultant individual clones therefore inhibit adenylyl cyclase activity to various degrees in a random manner. Alternatively, the variable responses may be a function of the cellular levels of components of the signal transduction pathway or second messengers found in the individual clones. Toll and Polgar (1995) studied the influence of receptor number on agonist ability to inhibit adenylyl cyclase in a single clonal CHO cell line expressing μ-opioid receptors using β-funaltrexamine to progressively block μ receptors. These studies showed that the ability of μ agonists to inhibit

adenylyl cyclase activity was dependent on receptor number - both agonist potency (IC_{50} value) and maximal activity decreased upon increasing the β-funaltrexamine concentration used to block receptors, as expected. Thus it seems plausible that maximal inhibition of adenylyl cyclase activity may vary in an unpredictable manner in CHO cells from clone to clone. Indeed results utilising several different CHO clonal cell lines all transfected with opioid receptor cDNA using an identical protocol have suggested that this may be the case (Toll, L. and Babey, A.M., personal communications). Therefore, it is possible that the individual CHO clone used in this study may possess an intrinsically poorly coupled δ receptor-G protein-adenylyl cyclase system.

In conclusion the results demonstrate that [^{35}S]GTPγS binding may be used to assay G protein activation in membranes from CHO cells expressing the mouse δ-opioid receptor. However, the efficiency of the assay, and thus of the response, is strongly dependent on tissue variables such as the type and relative amounts of expressed G protein.

Chapter 6

THE ROLE OF INTRACELLULAR REGIONS OF μ- AND δ-OPIOID RECEPTORS IN G PROTEIN-COUPLING IN SH-SY5Y AND NG108-15 CELL MEMBRANES

6.1 Introduction

The similar mechanisms by which all G protein-coupled receptors convert an extracellular signal into a cellular response suggest that it may not be unreasonable to assume that as this receptor family has evolved, a common set of structural features relating to these shared functions has been kept. Therefore, within the limits of the fact that receptors of the gene family bind different ligands and interact with different G proteins, one would hope to define a set of general principles that govern how ligands are bound, how agonists activate receptor, how receptors interact with G proteins and how receptor functions are regulated by post-translational modifications.

The cloning of first the δ- [Evans *et al*, 1992; Kieffer *et al*, 1992; Yasuda *et al*, 1993] and then the μ- [Fukuda *et al*, 1993; Wang *et al*, 1993; Chen *et al*, 1993; Wang *et al*, 1994] and κ- [Chen *et al*, 1993b; Minami *et al*, 1993; Li *et al*, 1993; Meng *et al*, 1993; Yasuda *et al*, 1993] opioid receptors has allowed such questions to be addressed at the molecular level for this sub-family of G protein-coupled receptors. One might intuitively expect that the putative cytoplasmic domains of G protein-coupled receptors would form the site(s) for receptor-G protein interaction, since G proteins are attached to the cytoplasmic face of the plasma membrane [Gilman, 1987; Birnbaumer *et al*, 1990]. Indeed, mutagenesis [Kubo *et al*, 1988; O'Dowd *et al*, 1988; Savarese and Fraser, 1992; Ostrowski *et al*, 1992] and receptor-derived peptide [König *et al*, 1989; Cheung *et al*, 1991; Dalman and Neubig, 1991; Münch *et al*, 1991] studies of other G protein-coupled receptors have revealed that several cytosolic domains are involved in the functional coupling of receptors and G proteins, as discussed in section 1.5. Many reports suggest that the third intracellular loop of G protein-coupled receptors participates in interactions with G proteins [Strader *et al*, 1989; Cotecchia *et al*, 1990; Wong *et al*, 1990; Malek *et al*, 1993; Dell'Acqua *et al*, 1993], particularly the N- and C-terminal ends of this loop, which are in close proximity to the plasma membrane. Thus it is of interest to determine whether or not opioid receptors use similar G protein-coupling mechanisms to other cloned receptors.

Although specific domains involved in receptor-mediated activation of G proteins have been identified, the mechanism by which activation is accomplished has not been determined. Insight into this mechanism has

perhaps been provided from studies of the receptor for insulin-like growth factor II [Okamoto *et al*, 1990; Okamoto and Nishimoto, 1991], and by the use of mastoparan analogues [Higashijima *et al*, 1988; Higashijima *et al*, 1990; Oppi *et al*, 1992] to study structure-activity relationships for G protein interaction. These studies suggest that short peptide sequences from a receptor, which are predicted to form amphipathic α-helices with one face charged and the other hydrophobic, can directly activate G proteins. When a receptor is in the resting state, these domains may be masked from the G protein. Conformational changes induced by agonist binding will probably make these regions accessible to the G protein.

Peptide competition studies, in which short synthetic peptides competitively bind to G proteins but do not activate them, have been useful in mapping regions of G protein-coupled receptors that are likely to contact the G protein [Palm *et al*, 1989; Dalman and Neubig, 1991; Nishimoto *et al*, 1991]. In a similar manner, antipeptide antibodies may also block receptor-mediated stimulation of G proteins, by physically interfering with the G protein-coupling site on the receptor. Site-directed antibodies have been used previously to study the interaction of rhodopsin and transducin [Weiss *et al*, 1988].

This study was designed to test whether short peptide sequences from the third intracellular loop of opioid receptors are capable of interacting with and activating G proteins. Unlike many other G protein-coupled receptor families, the opioid receptors share a remarkably conserved third intracellular loop [Probst *et al*, 1992; Reisine and Bell, 1993] (Fig. 6.1).

--R	L	K	S	V	R	**M**	L	S	G	S	K	E	K	D	R	N	L	R	R	I	T	R--	μ
--R	L	**R**	S	V	R	L	L	S	G	S	K	E	K	D	R	**S**	L	R	R	I	T	R--	δ
--R	L	K	S	V	R	L	L	S	G	S	**R**	E	K	D	R	N	L	R	R	I	T	**K**--	κ

Figure 6.1 Sequence alignment of the putative third intracellular loops of the cloned rat μ-, δ- and κ-opioid receptors. Amino acids which are not conserved across all three receptors are shown in bold. Sequences were obtained as follows: μ - Chen *et al*, 1993; δ - Fukuda *et al*, 1993; κ - Chen *et al*, 1993b.

It can be seen that there is only a single amino acid which is not conserved in the C-terminal region of the third intracellular loops of the cloned rat μ- and δ-opioid receptors (asparagine-274 of the μ-receptor is replaced with serine in the δ-opioid receptor). Thus, although peptides μil3$_{277\text{-}283}$ (LRRITRM) and μil3$_{269\text{-}283}$ (GSKEKDRNLRRITRM) were originally synthesised from the known sequence of the μ-opioid receptor, it is not unreasonable to suspect that they may also be active with respect to uncoupling of the δ-opioid receptor. Therefore, the ability of μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$ to block both δ-receptor- and μ-receptor-mediated activation of G proteins was investigated.

The role of the C-terminal 35 amino acid residues of the δ-opioid receptor in G protein interaction was also investigated using a site-directed polyclonal antibody to this region of the receptor (δC-Ant). Whilst the third intracellular loops of the three opioid receptor subtypes are almost identical, the C-terminal tails of these three receptors contain divergent structures, both in terms of amino acid composition and overall residue length (Fig. 1.7). The C-terminal 35 amino acids of the μ-, δ- and κ-opioid receptors show almost no sequence similarity at all, and this region of the receptor therefore represents the most likely coupling domain if selective G protein-coupling for each opioid receptor subtype is to be expected, as has been suggested by Laugwitz *et al* (1993).

6.2 Results

6.2.1. Studies on the μ-opioid receptor

The effect of μil3$_{277-283}$ and μil3$_{269-283}$ on low K_m GTPase activity of SH-SY5Y membranes was compared with that observed in the presence of mastoparan (Fig. 6.2).

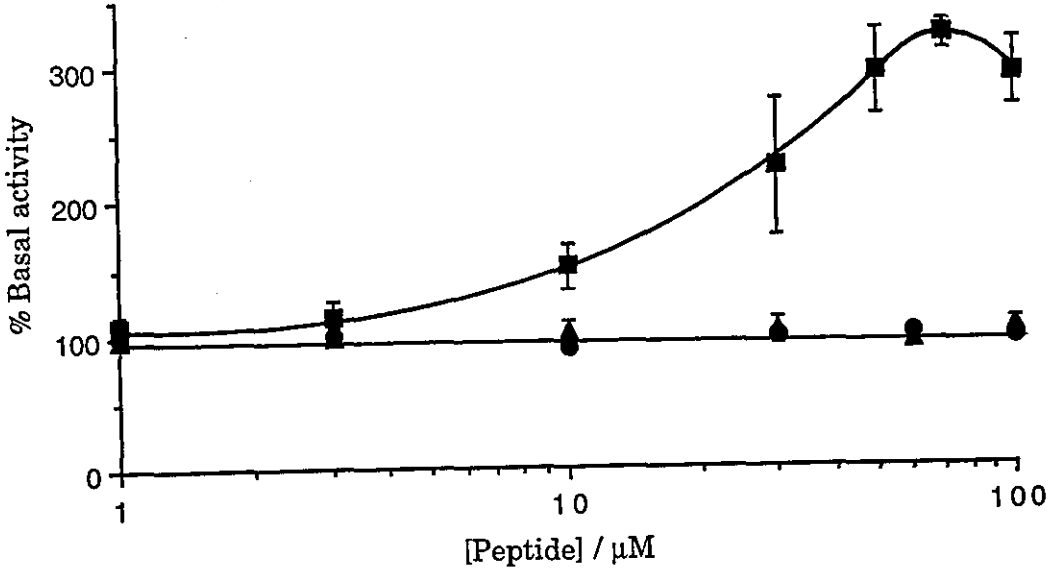


Figure 6.2 Comparison of the effect of mastoparan (■) with μil3$_{277-283}$ (▲) and μil3$_{269-283}$ (●) on low K_m GTPase activity of membranes from SH-SY5Y cells. Values are means±sem (n ≥ 3). Basal activity was 7.95±1.10 pmol/mg/min (n=12).

Unlike mastoparan, peptides μil3$_{277-283}$ and μil3$_{269-283}$ caused no stimulation of GTPase activity in SH-SY5Y membranes at any concentration tested up to 100 μM.

Since no activation of GTPase activity was seen in the presence of μil3$_{277-283}$ and μil3$_{269-283}$, the ability of these peptides to inhibit fentanyl-mediated stimulation of [^{35}S]GTPγS binding in SH-SY5Y cells was examined (Fig. 6.3). An approximate EC_{50} dose of fentanyl (Fig. 6.4) was used in order to test the

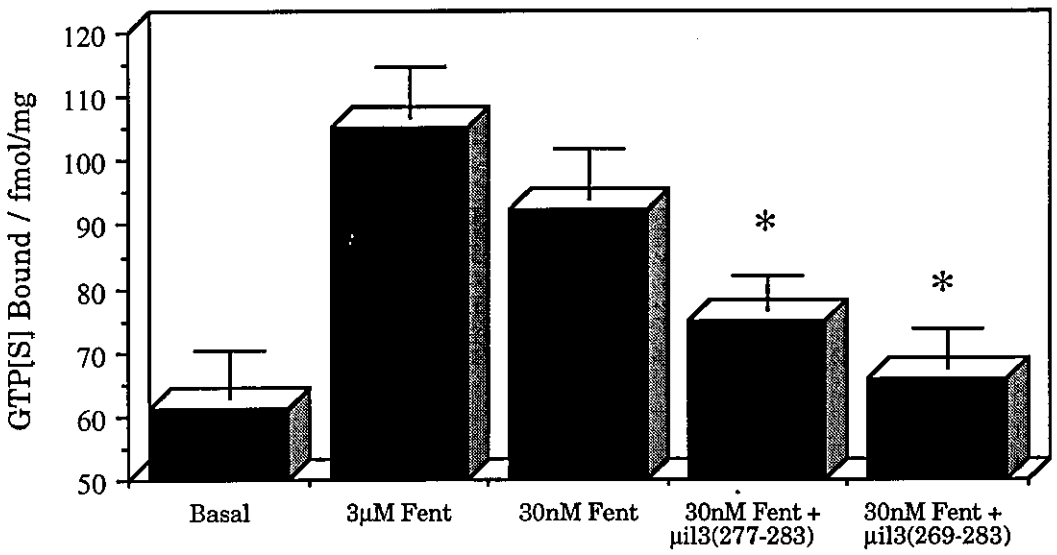


Figure 6.3 Effect of peptides $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ on fentanyl-stimulated [^{35}S]GTPγS binding in membranes from SH-SY5Y cells. Values represent means±sem (n=3). *: Significantly different from 30 nM fentanyl alone ($P < 0.05$).

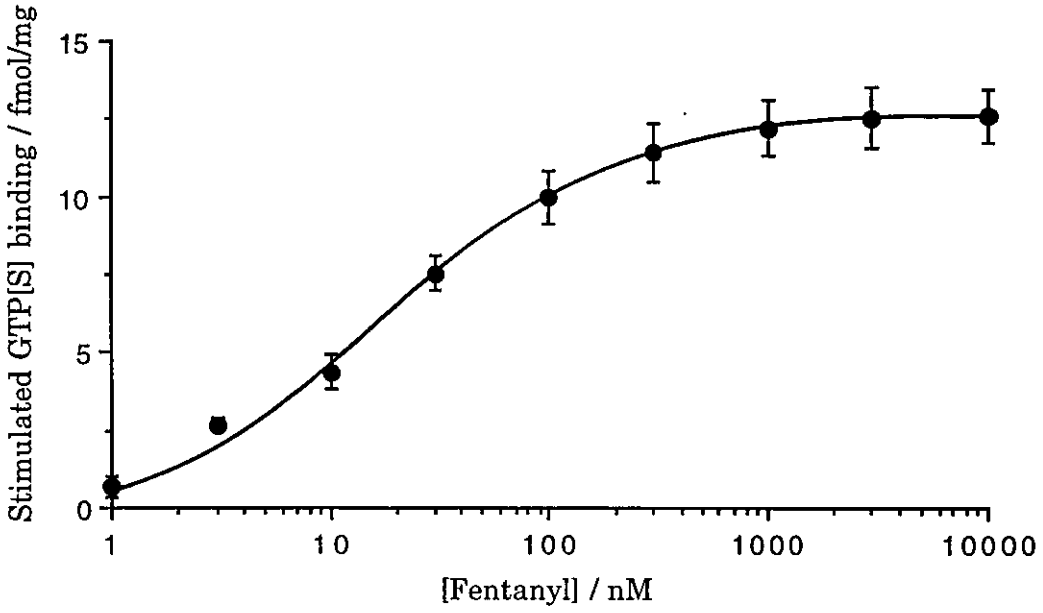


Figure 6.4 Stimulation of [^{35}S]GTPγS binding in membranes of SH-SY5Y cells by fentanyl. Values shown are means±sem (n=3). Basal [^{35}S]GTPγS binding was 26.78±5.91 fmol/mg. Calculated EC_{50} for fentanyl was 23.0±2.1 nM.

effect of the intracellular loop peptides. Both peptides μil3$_{277-283}$ (100 μM) and μil3$_{269-283}$ (100 μM) inhibited the stimulation of [^{35}S]GTPγS binding caused by fentanyl (30 nM) in membranes from SH-SY5Y cells. The inhibition of [^{35}S]GTPγS binding caused by μil3$_{269-283}$ was significantly larger ($P < 0.05$) than that caused by μil3$_{277-283}$.

Peptide μil3$_{269-283}$ (100 μM) also caused a reduction in basal [^{35}S]GTPγS binding (Fig. 6.5), although the magnitude of this reduction was smaller than its effect on fentanyl (30 nM)-stimulated binding (Fig. 6.3).

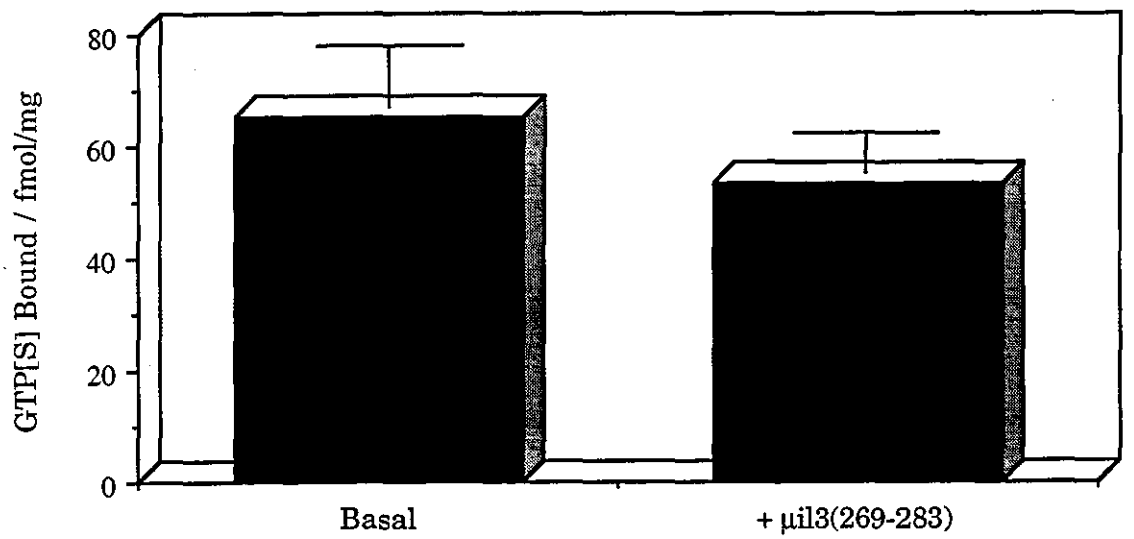


Figure 6.5 Effect of μil3$_{269-283}$ (100 μM) on basal [^{35}S]GTPγS binding in membranes from SH-SY5Y cells. Values represent means±range (n=2).

To further understand the effects caused by these intracellular loop peptides their effect on receptor binding was studied. Specific [^{3}H]-DAMGO (1.7 nM) binding was reduced by both peptides μil3$_{277-283}$ and μil3$_{269-283}$ (Fig. 6.6.), although only at concentrations ≥ 100 μM. This made it difficult to draw any firm conclusions from this experiment since it was not possible to construct a complete concentration-effect curve at high peptide concentrations due to economic constraints.

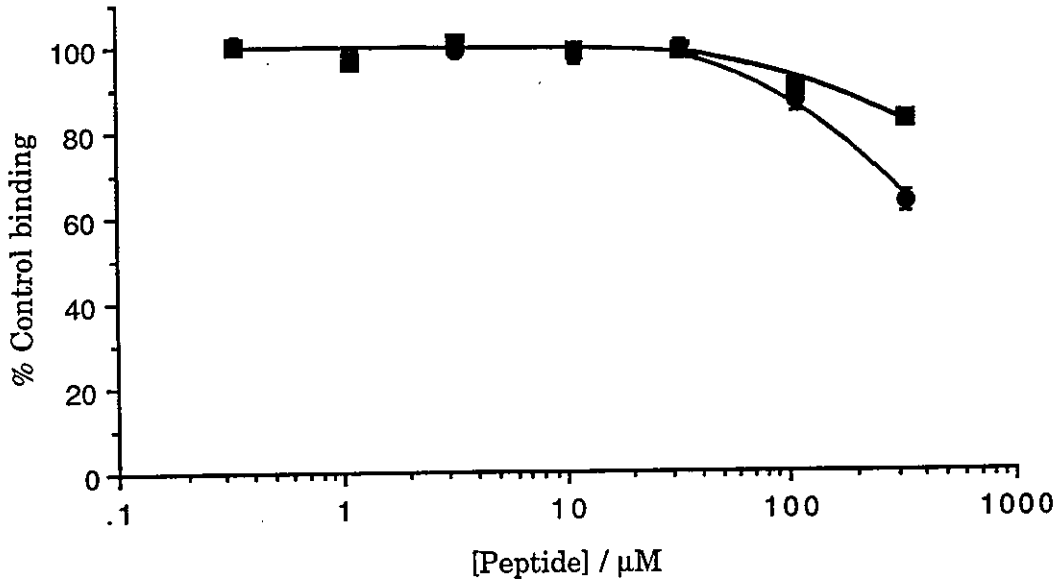


Figure 6.6 Effect of peptides μil3$_{277\text{-}283}$ (●) and μil3$_{269\text{-}283}$ (■) on [^{3}H]-DAMGO (1.7 nM) binding in SH-SY5Y membranes. Experimental conditions were as described in Materials and Methods, except that the reaction volume was 230 μl. Values are means±sem (n=3 for all points except those at 300 μM peptide where n=2).

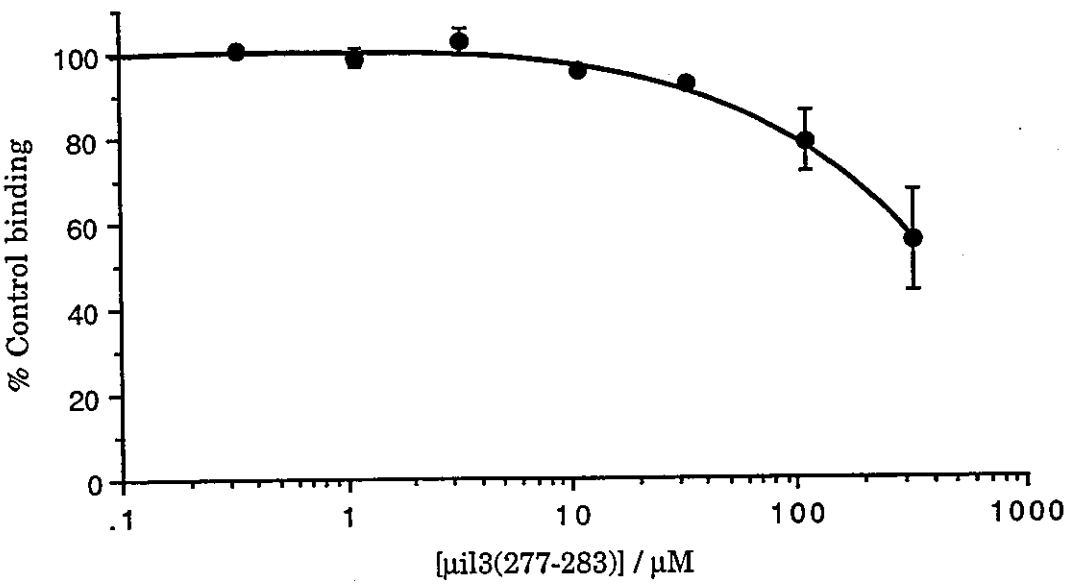


Figure 6.7 Effect of μil3$_{277\text{-}283}$ on [^{3}H]-diprenorphine (0.5 nM) binding in membranes from SH-SY5Y cells. Experimental conditions were as described in Materials and Methods, except that the reaction volume was 230 μl. Values are means±range (n=2).

The effect of $\mu il3_{277-283}$ on the binding of the μ-opioid antagonist [^{3}H]-diprenorphine was also examined (Fig. 6.7). $\mu il3_{277-283}$ caused an inhibition of both opioid agonist ([^{3}H]-DAMGO) and antagonist ([^{3}H]-diprenorphine) binding, with a similar concentration-effect relationship. To study the mechanism of this inhibitory action further, cells were pre-treated with pertussis toxin to uncouple receptor from G protein, prior to examining the effect of $\mu il3_{277-283}$ and $\mu il3_{269-283}$ on ligand binding.

Saturation binding analysis of membranes from SH-SY5Y cells which had been treated with pertussis toxin (100 ng/ml, 24h; Figs. 6.8 and 6.9) , using the computer programme LIGAND [Munson and Rodbard, 1980], suggested that pertussis toxin treatment led to a decrease in the total amount of binding sites (B_{max}) for [^{3}H]-DAMGO (82±13 fmol/mg in control membranes compared to 44±9 fmol/mg in membranes prepared from pertussis toxin-treated cells; $P < 0.05$) with no significant difference in binding affinity (K_d) (0.45±0.20 nM in control membranes compared to 0.62±0.06 nM in membranes from pertussis toxin-treated cells). However, inspection of the data by eye suggested that pertussis toxin treatment resulted in complex [^{3}H]-DAMGO binding. When the data was re-analysed manually using Scatchard plots to estimate binding affinities and capacities different results were obtained. In this case, a small reduction in B_{max} was observed (98±6 fmol/mg for control membranes compared with 75±7 fmol/mg for membranes from toxin-treated cells; $P < 0.05$), with a 2.2-fold shift in K_d (0.55±0.15 nM in control membranes compared with 1.21±0.25 nM in membranes from toxin-treated cells; $P < 0.05$).

In contrast, toxin treatment of SH-SY5Y cells led to no significant change in either the binding affinity or capacity of [^{3}H]-diprenorphine labelled sites in membranes from these cells (Fig. 6.9). Both manual Scatchard analysis and LIGAND gave very similar results in this case. Thus, LIGAND analysis of the saturation binding isotherms resulted in a B_{max} for [^{3}H]-diprenorphine of 205±16 fmol/mg for control membranes compared to 187±4 fmol/mg for membranes from toxin treated cells with affinities (K_d) of 0.17±0.03 and 0.15±0.01 nM respectively.

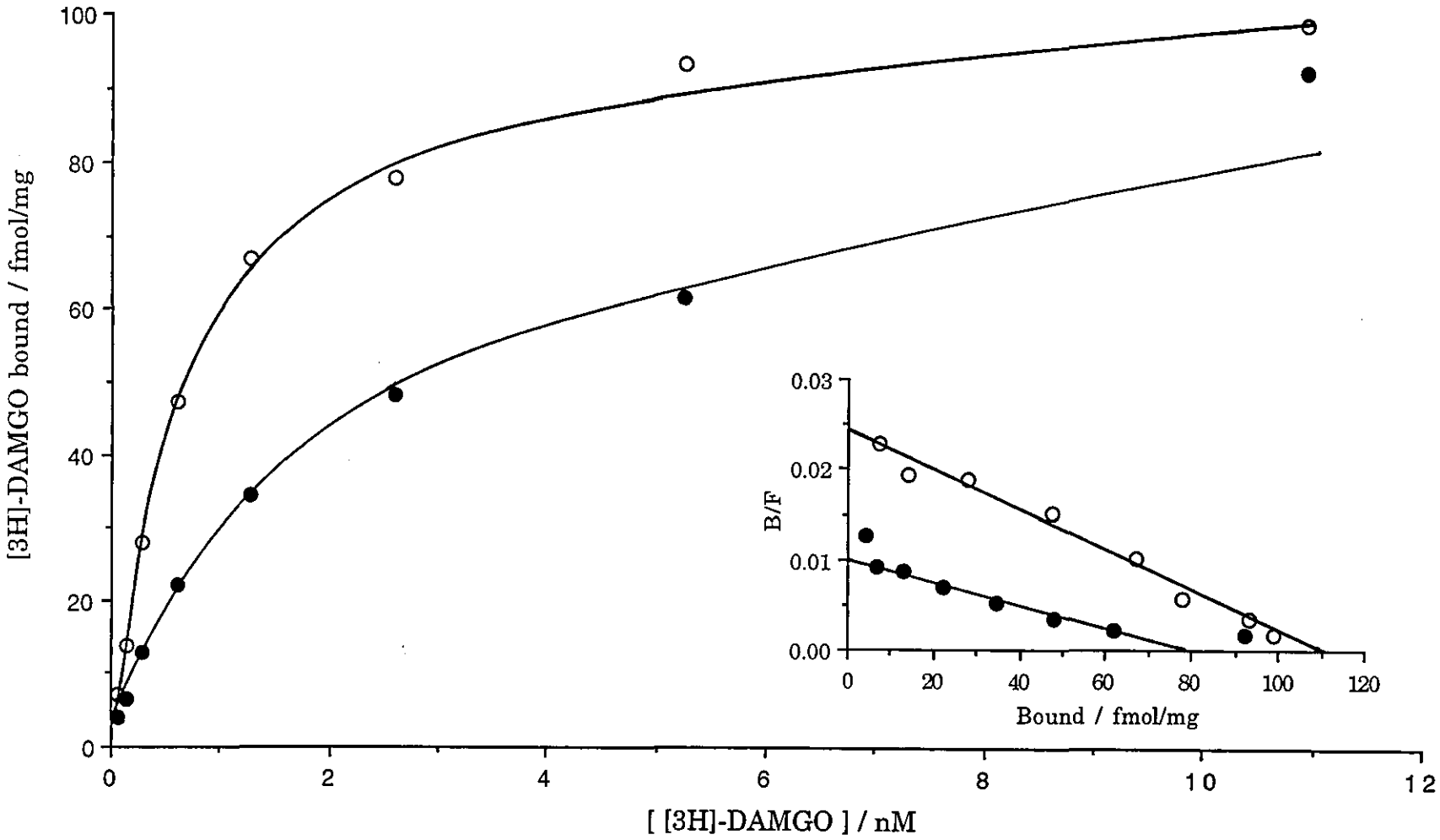


Figure 6.8 Representative graphs showing saturation [^{3}H]-DAMGO binding to SH-SY5Y membranes in Tris-HCl (pH 7.4) prepared from control (○) and pertussis toxin treated (100 ng/ml, 24h) (●) cells (n=3). Inset are the corresponding Scatchard plots.

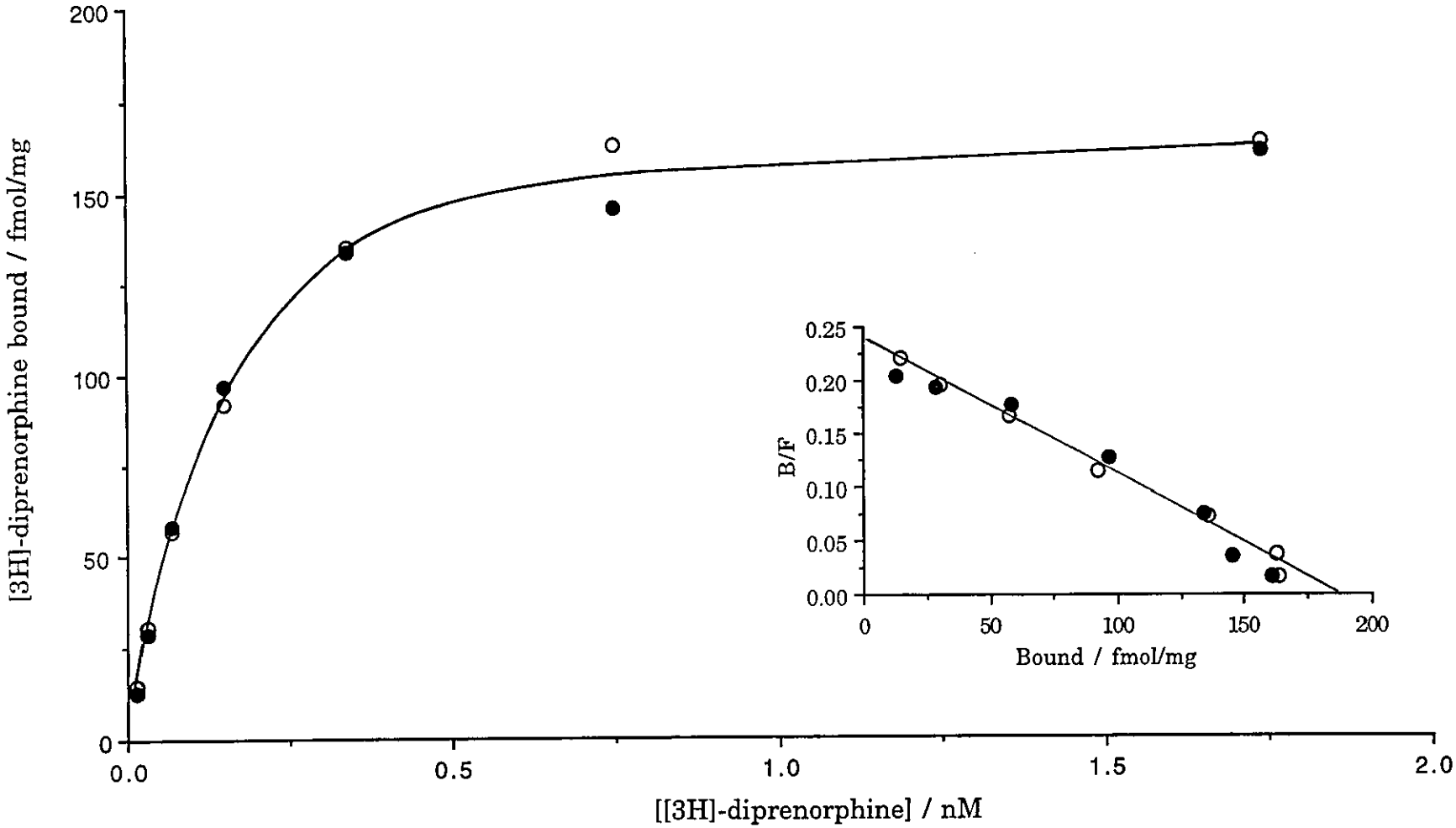


Figure 6.9 Representative graphs showing saturation [^{3}H]-diprenorphine binding to SH-SY5Y membranes in Tris-HCl (pH 7.4) prepared from control (○) and pertussis toxin treated (100 ng/ml, 24h) (●) cells (n=3). Inset are the corresponding Scatchard plots.

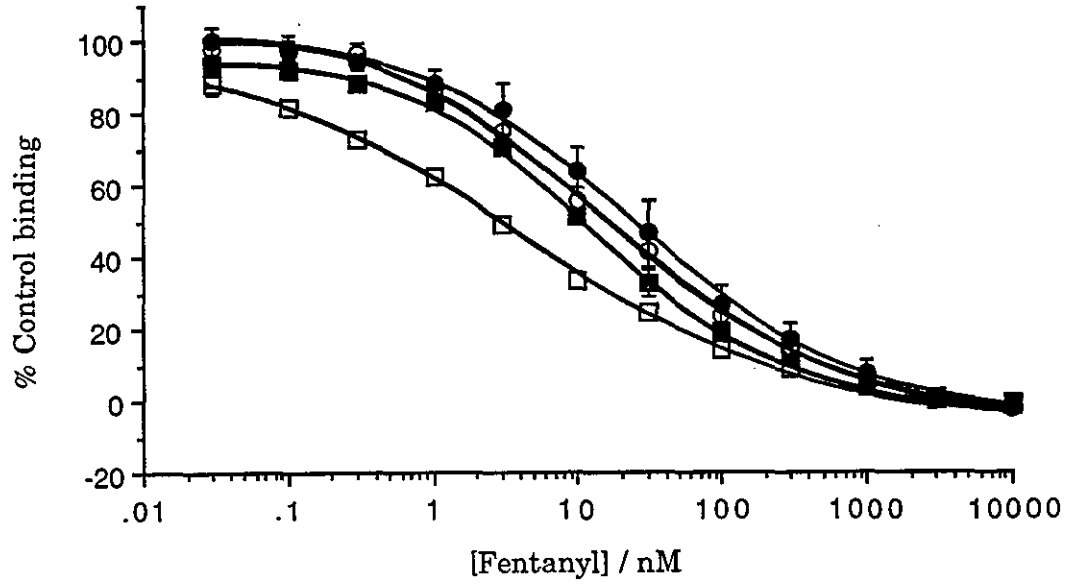


Figure 6.10 Displacement of [^{3}H]-diprenorphine (0.30 nM) from membranes of naive (□,■) and pertussis toxin-treated (100 ng/ml, 24h) (○,●) SH-SY5Y cells by fentanyl in the absence (□,○) or presence (■,●) of GTPγS (50 μM). Values represent means±sem (n=3).

	- GTPγS			+ GTPγS		
	K_i(high)/ nM	K_i(low)/ nM	% of high affinity sites	K_i(high)/ nM	K_i(low)/ nM	% of high affinity sites
Naive	0.61 ± 0.10	73 ± 18	73 ± 2	2.90 ± 0.24†	188 ± 19†	81 ± 1
PTX treated	3.00 ± 0.46†	184 ± 33†	73 ± 3	5.41 ± 1.68*	196 ± 39*	75 ± 2

Table 6.1 Binding parameters for the experiment represented in Fig. 6.10 obtained using LIGAND. †: $P < 0.05$ compared to naive cells in the absence of GTPγS; *: Not significantly different to pertussis toxin-treated cells in the absence of GTPγS.

The displacement of [^{3}H]-diprenorphine by the μ-opioid agonist fentanyl was also studied in membranes from naive and pertussis toxin-treated cells (Table 6.1 and Fig. 6.10). Displacement of [^{3}H]-diprenorphine by fentanyl was biphasic, with approximately 75% of sites in the high affinity state, under all conditions studied. In membranes prepared from naive cells, GTPγS (50μM) caused the displacement curve to shift to the right, resulting in a 4.8- and 2.6-fold shift in the K_d of the high and low affinity sites respectively. Treatment with pertussis toxin caused an identical shift in the displacement curves in the absence of GTPγS, confirming the results obtained earlier with saturation studies. GTPγS (50 μM) had no further effect on the displacement curve in membranes from cells treated with pertussis toxin, suggesting that both GTPγS and pertussis toxin cause an identical effect by uncoupling the μ-opioid receptor from its cognate G protein(s).

μil3$_{277\text{-}283}$ inhibited [^{3}H]-DAMGO and [^{3}H]-diprenorphine binding even when the experiment was performed using SH-SY5Y membranes which had been prepared from pertussis toxin-treated cells (Fig. 6.11). Surprisingly, the results suggest that μil3$_{277\text{-}283}$ has a greater effect on [^{3}H]-DAMGO binding in cells treated with pertussis toxin compared to control cells (compare Figs. 6.6 and 6.11), though this effect did not attain statistical significance because the experiments were only performed twice.

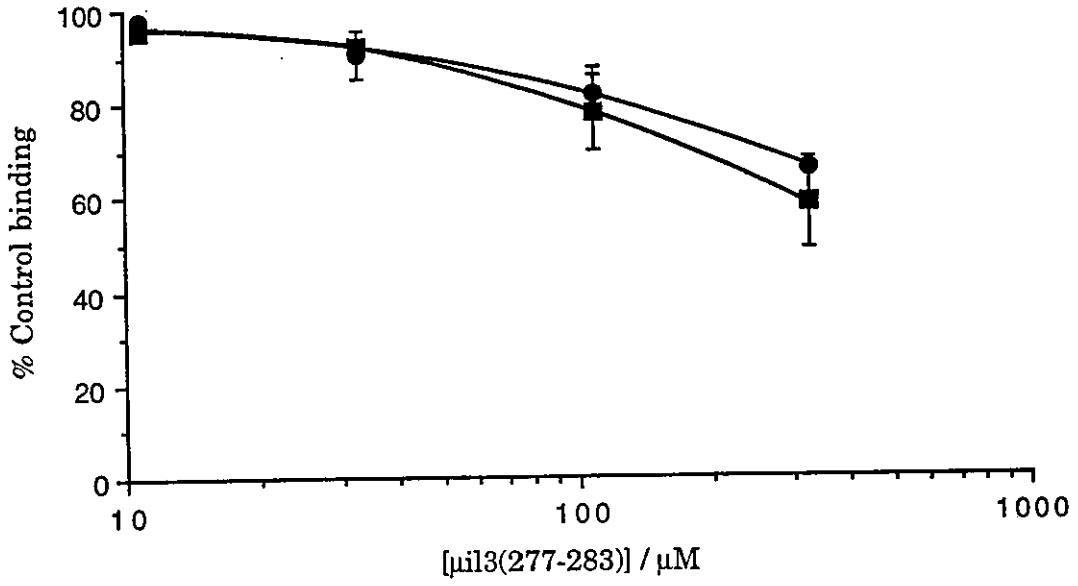


Figure 6.11 Effect of $\mu il3_{277\text{-}283}$ on [^{3}H]-DAMGO (1.50 nM, ■) and [^{3}H]-diprenorphine (0.40 nM, ●) binding in membranes from SH-SY5Y cells which had been pre-treated with pertussis toxin (100 ng/ml, 24h). Experimental conditions were as described in Materials and Methods, except that the reaction volume was 230 μl. Values represent means±range (n=2).

6.2.2 Studies on the δ-opioid receptor

6.2.2.1 Effect of $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ on δ-opioid receptor-G protein-coupling

Both $\mu il3_{269\text{-}283}$ and $\mu il3_{277\text{-}283}$ caused a dose-dependent inhibition of DPDPE (50 nM)-stimulated [^{35}S]GTPγS binding (Fig. 6.12). At the maximum concentration tested (100 μM), $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ both decreased the stimulation of [^{35}S]GTPγS binding caused by DPDPE by approximately 60%. Higher concentrations than this were not tested in order to conserve peptide.

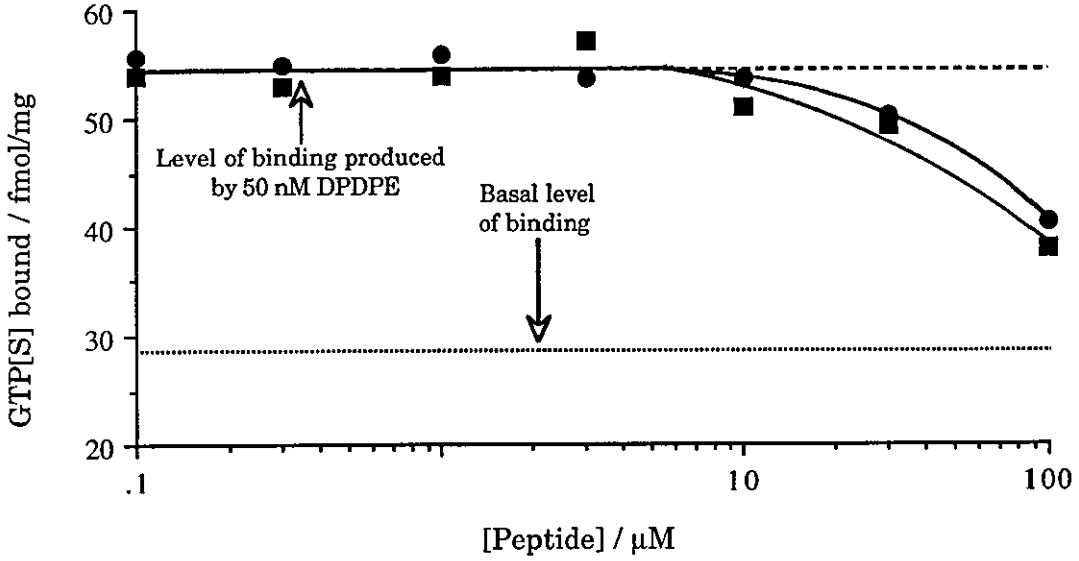


Figure 6.12 Effect of $\mu il3_{269-283}$ (■) and $\mu il3_{277-283}$ (●) on DPDPE (50 nM)-stimulated [^{35}S]GTPγS binding in membranes from NG108-15 cells. A typical experiment is shown, representative of three performed.

Experimental conditions	GTPγS bound / fmol/mg
Basal activity	40.84 ± 8.51
+ 100 μM $\mu il3_{277-283}$	33.71 ± 6.76
+ 100 μM $\mu il3_{269-283}$	32.06 ± 6.57

Table 6.2 Effect of $\mu il3_{277-283}$ and $\mu il3_{269-283}$ on basal [^{35}S]GTPγS binding in membranes from NG108-15 cells. Values are means±sem (n=3).

Basal [^{35}S]GTPγS binding in NG108-15 membranes was affected by the intracellular loop peptides in a similar manner, as shown in Table 6.2. The mean decrease in [^{35}S]GTPγS binding elicited by $\mu il3_{277-283}$ and $\mu il3_{269-283}$ failed to reach statistical significance as a result of variation in the basal [^{35}S]GTPγS binding between experiments. However, in each of the three experiments

performed, a reproducible, robust inhibition of the basal level of binding by approximately 20% was observed in the presence of either $\mu il3_{277-283}$ or $\mu il3_{269-283}$.

The effect of $\mu il3_{277-283}$ and $\mu il3_{269-283}$ (100 μM each) on the binding of δ-opioid radioligands to NG108-15 membranes was also studied (Fig. 6.13). In general, the effect of the peptides was small, causing < 25% of the radiolabelled ligand to be displaced.

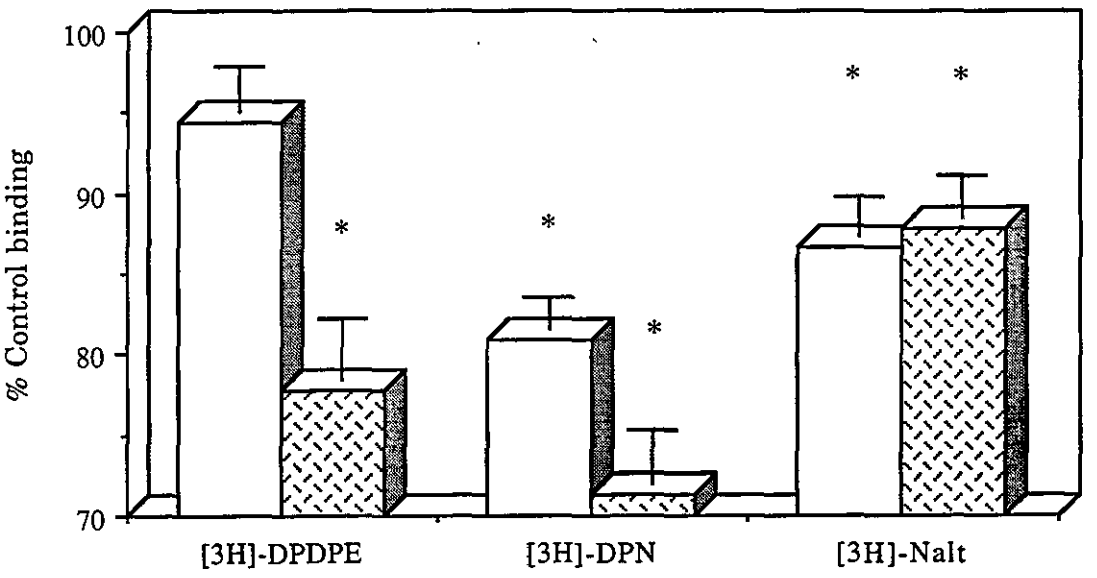


Figure 6.13 Effect of $\mu il3_{277-283}$ (open bars) and $\mu il3_{269-283}$ (hatched bars) on the binding of [3H]-DPDPE (1.0 nM), [3H]-diprenorphine ([3H]-DPN, 0.5 nM) and [3H]-naltrindole ([3H]-Nalt, 0.25 nM) to NG108-15 membranes. Values represent means±sem ($n \geq 3$). *: Peptide has a significant effect on binding ($P < 0.05$).

6.2.2.2 Effect of control peptides on δ-opioid receptor-G protein-coupling

The effect of several control peptides on DPDPE (50 nM)-stimulated [^{35}S]GTPγS binding in NG108-15 membranes was also tested (Fig. 6.14). Hexa-Gly (100 μM) had no effect on the stimulation of [^{35}S]GTPγS binding by DPDPE (50 nM). In contrast hexa-Ala (100 μM) completely abolished the stimulatory

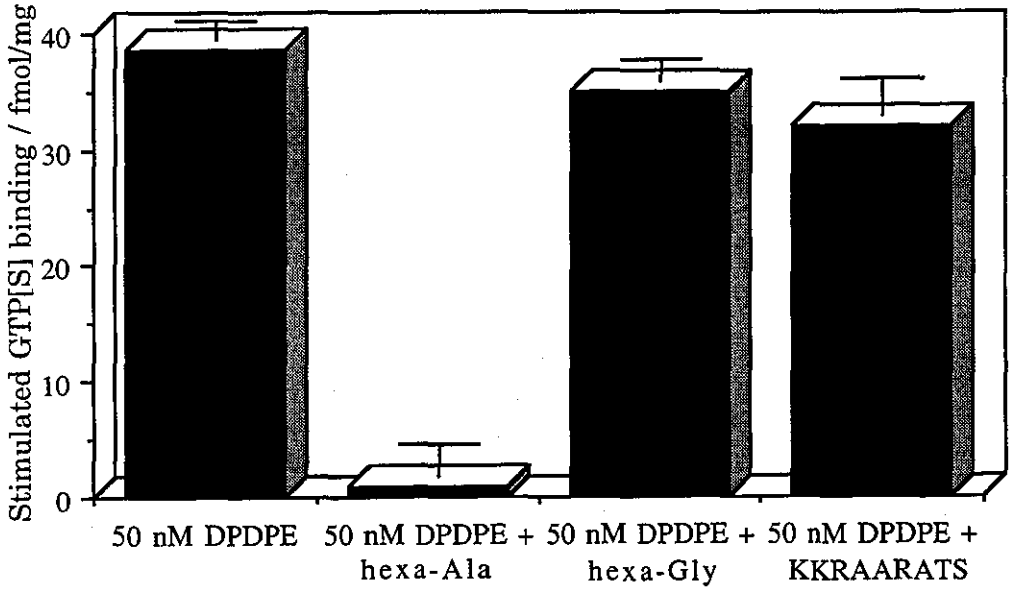


Figure 6.14 Effect of hexa-Ala (100 μM), hexa-Gly (100 μM) and KKRAARATS-NH_2 (100 μM) on DPDPE (50 nM)-stimulated [^{35}S]GTPγS binding in NG108-15 membranes. Values represent means±sem (n=3), except data for KKRAARATS-NH_2 where values are means±range (n=2).

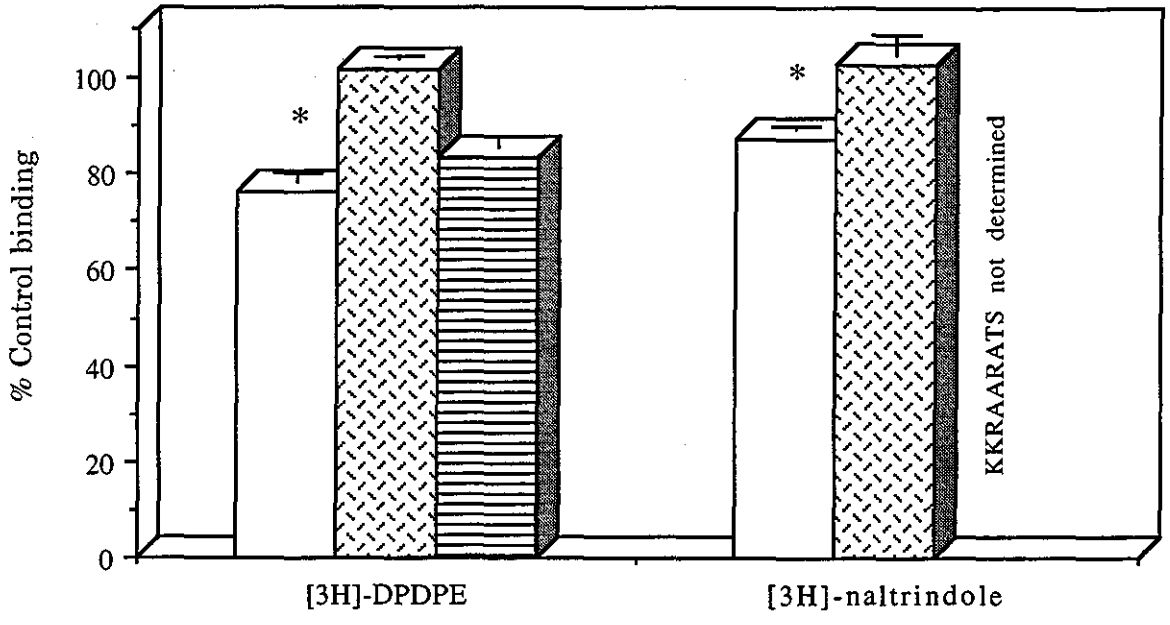


Figure 6.15 Effect of hexa-Ala (100 μM, white bars), hexa-Gly (100 μM, stippled bars) and KKRAARATS-NH_2 (100 μM, striped bar) on [^{3}H]-DPDPE (1.00 nM) and [^{3}H]-naltrindole (0.30 nM) binding in NG108-15 membranes. Values represent means±sem (n=3), except data for KKRAARATS-NH_2 where values are means±range (n=2).
*: $P < 0.05$ compared to control.

effect of the δ-opioid receptor agonist. The multicationic peptide KKRAARATS-NH_2 (100 μM) caused a small (approximately 15%) reduction in DPDPE (50 nM)-stimulated binding.

These control peptides were also tested for their effect on [^{3}H]-DPDPE and [^{3}H]-naltrindole binding in NG108-15 membranes (Fig. 6.15). Hexa-Gly (100 μM) had no effect on either [^{3}H]-DPDPE (1.00 nM) or [^{3}H]-naltrindole (0.30 nM) binding. However, hexa-Ala (100 μM) inhibited the binding of [^{3}H]-DPDPE and [^{3}H]-naltrindole by 23.8±1.7% and 12.7±0.5% respectively. KKRAARATS-NH_2 reduced [^{3}H]-DPDPE binding by 16.4±2.0%.

6.2.2.3 *Effect of a polyclonal antibody raised against the C-terminal 35 amino acids of the mouse δ-opioid receptor (δC-Ant) on δ-opioid receptor-mediated stimulation of [^{35}S]GTPγS binding*

In order to confirm that δC-Ant recognises the δ-opioid receptor from NG108-15 membranes, this antibody was used to immunoblot the δ-receptor after separation of membrane proteins on 8% (w/v) SDS-polyacrylamide gels containing low concentrations of bisacrylamide. δC-Ant labelled a protein band which migrated with an apparent molecular mass of 72 kDa (Figs. 6.16 and 6.17). Two minor bands of apparent molecular mass 48.5 kDa and 51.0 kDa were also identified by δC-Ant. These proteins possibly represent proteolytic breakdown products or non-glycosylated forms of the δ-opioid receptor.

The effect of δC-Ant (1:100 dilution) on DPDPE (50 nM)-mediated stimulation of [^{35}S]GTPγS binding to membranes from NG108-15 cells was studied (Fig. 6.18). δC-Ant (1:100) caused a small, but not significant, increase in the level of [^{35}S]GTPγS binding. The addition of non-immune rabbit serum (1:100), used as a control in these experiments, also resulted in a similar increase in [^{35}S]GTPγS binding, indicating that the observed effect may be the result of a slightly higher level of non-specific binding in the presence of serum.

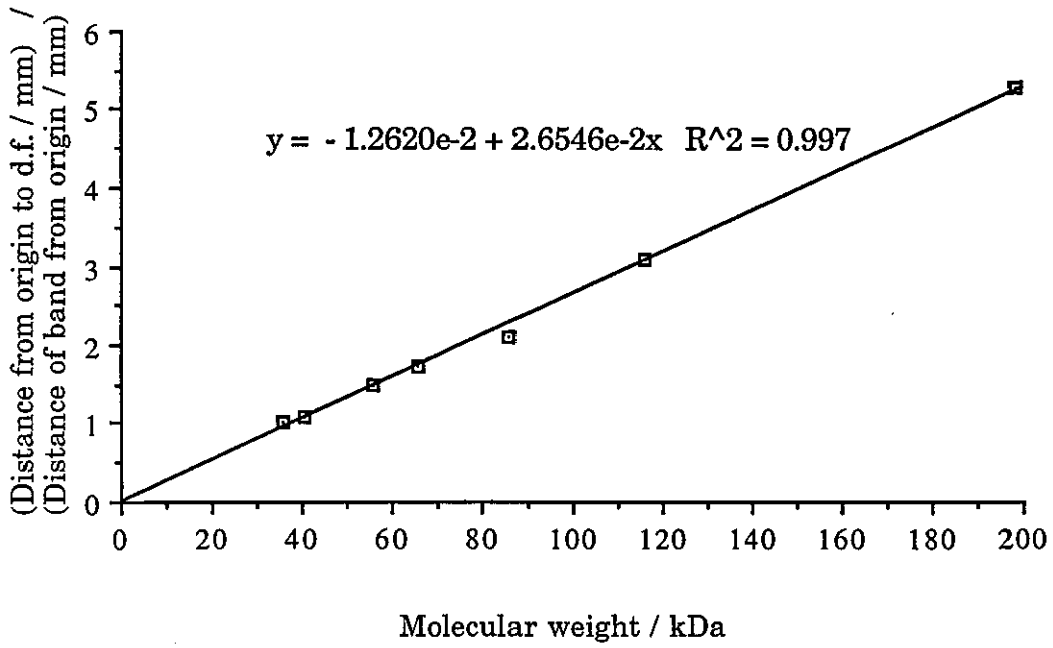


Figure 6.16 Calibration graph for the molecular weight standards used in Western blot analysis of the δ-opioid receptor from NG108-15 cells using δC-Ant. Prestained standards were (kDa): 36.0 - triosephosphate isomerase; 40.5 - lactic dehydrogenase; 56.0 - fumarase; 66.0 - pyruvate kinase; 86.0 fructose-6-phosphate kinase; 116.0 - β-galactosidase; 198.0 - α_2-macroglobulin.

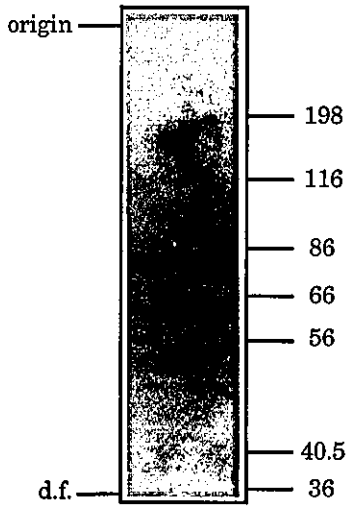


Figure 6.17 Immunostaining of the δ-opioid receptor in membranes from NG108-15 cells using δC-Ant. Numbers to the right of the blot indicate the positions of molecular weight standards (kDa) run in parallel with the membrane preparation; d.f. = dye front.

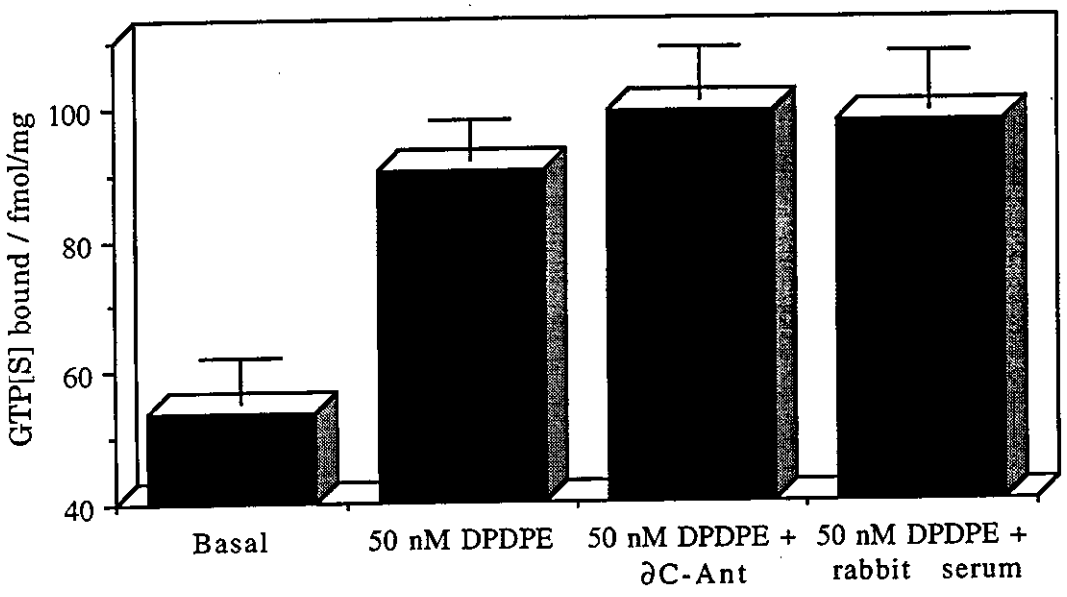


Figure 6.18 Effect of δC-Ant (1:100) and non-immune rabbit serum (1:100) on DPDPE (50 nM)-stimulated [^{35}S]GTPγS binding in membranes from NG108-15 cells. Values represent means±sem (n≥3).

6.3 Discussion

The role of the third intracellular loop of μ- and δ-opioid receptors in G protein-coupling has been studied using synthetic peptides derived from this region. Peptides μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$ were tested for their ability to activate G proteins by examining their effect on low K_m GTPase activity in SH-SY5Y membranes. Neither peptide caused any stimulation of low K_m GTPase activity at any concentration tested up to 100 μM. In comparison, the known G protein activator, mastoparan (70 μM), caused an approximate 3-fold increase in GTPase activity over basal. Whilst interpretation is difficult due to the small degree of stimulation seen with the potent agonist DAMGO acting at the native μ-opioid receptor in this cell line (Figs. 3.4 and 3.5), these results do not suggest a role for the C-terminal region of the third intracellular loop of the μ-opioid receptor in G protein activation.

The effect of μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$ on basal [^{35}S]GTPγS binding in membranes from both SH-SY5Y and NG108-15 cells also indicated that these peptides alone could not mimic the activation of G proteins caused by opioid receptors. Indeed, both peptides actually caused the opposite effect, that is they blocked opioid receptor-mediated stimulation of G proteins, as measured by [^{35}S]GTPγS binding.

The inability of these peptides to activate G proteins was unexpected, since μil3$_{269\text{-}283}$ contains two basic residues at its N-terminal side and the amino acid sequence BBXXB (B = basic residue, X = non-basic residue) at its C-terminal end, which has been suggested as necessary for a potential G protein-activator peptide [Okamoto *et al*, 1990; Okamoto *et al*, 1991; Ikezu *et al*, 1992; Okamoto and Nishimoto, 1992]. However, it should be noted that Okamoto and Nishimoto (1992) found many such potential G protein activator regions within G protein-coupled receptors based on these criteria, though when synthetic peptides corresponding to these sequences were tested for their ability to stimulate [^{35}S]GTPγS binding to purified G proteins, some, but not all of these peptides were active. In addition, peptides have been reported which activate G proteins but do not contain these two structural features [Cheung *et al*, 1991; Voss *et al*, 1993; Malek *et al*, 1993]. Clearly then, the presence of a defined set of positive charges alone is not enough to specify G protein activating function -

some other property of the peptide is also necessary. Indeed, as a result of mutagenesis studies, several groups [Cheung *et al*, 1992; Arden *et al*, 1992; Blüml *et al*, 1994] have suggested that hydrophobic rather than hydrophilic residues in the intracellular loops of G protein-coupled receptors play the major role in determining G protein interaction.

The lack of primary sequence homology amongst different receptors which couple to the same G proteins has led to the hypothesis that it is the secondary structure, and more specifically the amphipathic, α-helical nature of the intracellular regions, which is important in determining their G protein activating function [Higashijima *et al*, 1988; Cheung *et al*, 1991; Duerson *et al*, 1993]. Possible conformations of $\mu il3_{269\text{-}283}$ were analysed using the method of Chou and Fasman (1978). The C-terminal region of the third intracellular loop of the δ-opioid receptor has previously been reported as possessing an α-helical conformation [Evans *et al*, 1992]. However, Chou-Fasman analysis of $\mu il3_{269\text{-}283}$ showed that this peptide actually has a low probability of forming an α-helix (Fig. 6.19). Indeed, the C-terminal six residues of $\mu il3_{269\text{-}283}$ would actually be expected to exist in a β-sheet conformation. In contrast the same analysis of mastoparan showed that this peptide would be expected to be α-helical along its entire sequence (Fig. 6.20). In fact, the α-helical nature of active mastoparan analogues when bound to phospholipid vesicles or purified G proteins has been confirmed by NMR and CD spectroscopy [Wakamatsu *et al*, 1983,1992; Higashijima *et al*, 1990; Sukumar and Higashijima, 1992].

Similarly, computer-aided secondary structure predictions for $\mu il3_{269\text{-}283}$ using the molecular modelling programme SYBYL confirmed that this peptide has little potential to form an α-helix, whilst mastoparan was predicted to adopt a largely α-helical conformation by this method. However, on a cautionary note, it should be added that the exact conformation predicted for $\mu il3_{269\text{-}283}$ depended to some extent on the method chosen to predict secondary structure. Furthermore, modelling of small peptides can clearly not take into account long range interactions which may be present between different domains of the receptor polypeptide, neither can it allow for possible interactions between the plasma membrane and residues within the amino acid sequence. Therefore, in the absence of corroborating experimental evidence, the results of such molecular modelling should be treated with caution.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Residue Number
i_β	b_β	b_β	B_β	b_β	i_β	i_β	b_β	h_β	i_β	i_β	H_β	h_β	i_β	H_β	β-Sheet Assignment*
B_α	i_α	I_α	H_α	I_α	i_α	i_α	b_α	H_α	i_α	i_α	I_α	i_α	i_α	h_α	α-Helical Assignment†
Gly	Ser	Lys	Glu	Lys	Asp	Arg	Asn	Leu	Arg	Arg	Ile	Thr	Arg	Met	Amino Acid
0.53	0.79	1.07	1.53	1.07	0.98	0.79	0.73	1.34	0.79	0.79	1.00	0.82	0.79	1.20	P_α#
0.81	0.72	0.74	0.26	0.74	0.80	0.90	0.65	1.22	0.90	0.90	1.60	1.20	0.90	1.67	P_β#

Residue Numbers	1-6	2-7	3-8	4-9	5-10	6-11	7-12	8-13	9-14	10-15
$<P_\alpha>$	1.00	1.04	1.03	1.07	0.95	0.90	0.91	0.91	0.92	0.90
$<P_\beta>$	0.68	0.69	0.68	0.76	0.87	0.90	1.03	1.08	1.12	1.20

Figure 6.19 Prediction of the secondary structure of $\mu il3_{269\text{-}283}$ by the method of Chou and Fasman (1978), using a segment length of six residues. # P_α and $P\beta$ are the helix and β-sheet conformational parameters respectively. † Helical assignments: H_α (strong helix former), h_α (helix former), I_α (weak helix former), i_α (helix indifferent), b_α (helix breaker), B_α (strong helix breaker). * β-Sheet assignments: H_β (strong β-former), h_β (β-former), I_β (weak β-former), i_β (β-indifferent), b_β (β-breaker), B_β (strong β-breaker).

1	2	3	4	5	6	7	8	9	10	11	12	13	14	Residue Number
H_β	b_β	h_β	b_β	I_β	h_β	I_β	I_β	h_β	I_β	b_β	b_β	H_β	h_β	β-Sheet Assignment*
I_α	b_α	H_α	I_α	H_α	H_α	H_α	H_α	H_α	H_α	I_α	I_α	I_α	H_α	α-Helical Assignment†
Ile	Asn	Leu	Lys	Ala	Leu	Ala	Ala	Leu	Ala	Lys	Lys	Ile	Leu	Amino Acid
1.00	0.73	1.34	1.07	1.45	1.34	1.45	1.45	1.34	1.45	1.07	1.07	1.00	1.34	P_α#
1.60	0.65	1.22	0.74	0.97	1.22	0.97	0.97	1.22	0.97	0.74	0.74	1.60	1.22	P_β#

Residue Numbers	1-6	2-7	3-8	4-9	5-10	6-11	7-12	8-13	9-14
$<P_\alpha>$	1.16	1.23	1.35	1.35	1.41	1.35	1.31	1.23	1.21
$<P_\beta>$	1.07	0.96	1.02	1.02	1.05	1.02	0.94	1.04	1.08

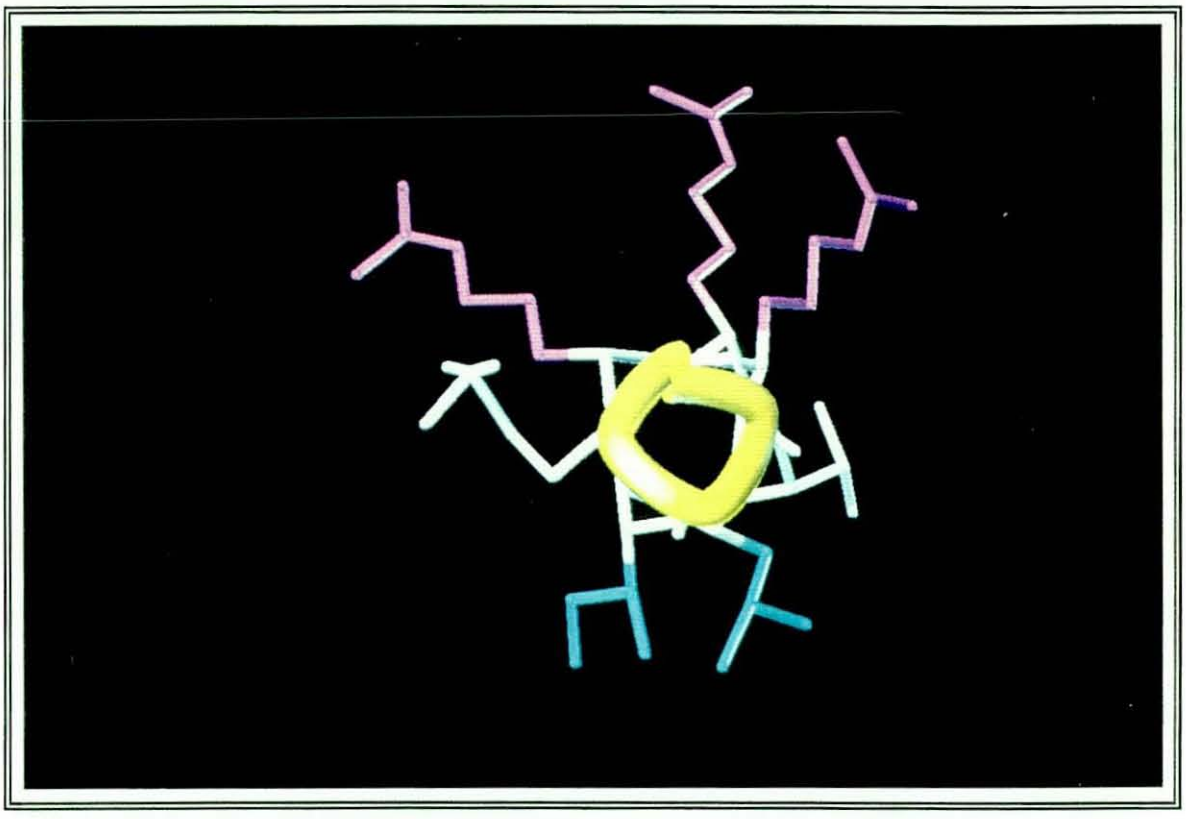
Figure 6.20 Prediction of the secondary structure of mastoparan by the method of Chou and Fasman (1978), using a segment length of six residues. # P_α and P_β are the helix and β-sheet conformational parameters respectively. † Helical assignments: H_α (strong helix former), h_α (helix former), I_α (weak helix former), i_α (helix indifferent), b_α (helix breaker), B_α (strong helix breaker). * β-Sheet assignments: H_β (strong β-former), h_β (β-former), I_β (weak β-former), i_β (β-indifferent), b_β (β-breaker), B_β (strong β-breaker).

Although secondary structure prediction algorithms suggest that $\mu il3_{269\text{-}283}$ would not be expected to form an α-helix, it should be remembered that peptide structures within a receptor protein have a certain amount of conformational flexibility. Indeed current thinking suggests that it is a conformational change in the intracellular regions of a receptor which may lead to activation of the receptor and subsequent G protein-coupling. It is possible that interaction with G protein results in an "induced fit", of parts of the receptor into a binding pocket on the G protein, such that the conformation of the receptor in the ground state is quite different to that in the activated G protein-coupled state. Therefore, whilst molecular modelling of receptor-derived peptides may be useful in determining predicted or lowest energy conformations, one must bear in mind that these may not necessarily be the "active" conformations.

Molecular models of $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ based on an α-helix are shown in Figs. 6.21 and 6.22. It can be seen that if the region corresponding to amino acid residues 269-283 of the μ-opioid receptor were to form a perfect α-helix, amphiphilicity would not be maintained, as one positively charged side chain (Lys-273) is positioned on the hydrophobic face of the helix. Indeed, this peptide resembles mastoparan 17 (Fig. 3.15), the inactive analogue of mastoparan, in having a positively charged amino acid residue on an otherwise hydrophobic face of the helix. In an elegant study Oppi *et al* (1992) showed that sequence permutations of mastoparan which result in a non-amphipathic peptide do not activate G proteins, and may even inhibit basal activity. This provides a possible explanation why $\mu il3_{269\text{-}283}$ does not activate G proteins. Indeed, it is interesting to note that $\mu il3_{269\text{-}283}$ had a small inhibitory effect on [^{35}S]GTPγS binding in membranes from both SH-SY5Y and NG108-15 cells, similar to the effect of some of the peptides studied by Oppi *et al* (1992).

Reisine *et al* (1994) have suggested that the 3rd intracellular loop of the δ-receptor is necessary for inhibition of adenylyl cyclase. Subcloning of the 3rd intracellular loop of the δ-opioid receptor into the corresponding region of the somatostatin receptor (SSTR1), and expression in CHO-DG44 cells, produces a chimeric receptor capable of inhibiting adenylyl cyclase, whereas the wild-type SSTR1 does not couple to inhibition of adenylyl cyclase in this cell line. Furthermore, a δ-opioid receptor chimera containing only the 3rd intracellular

(a)



(b)

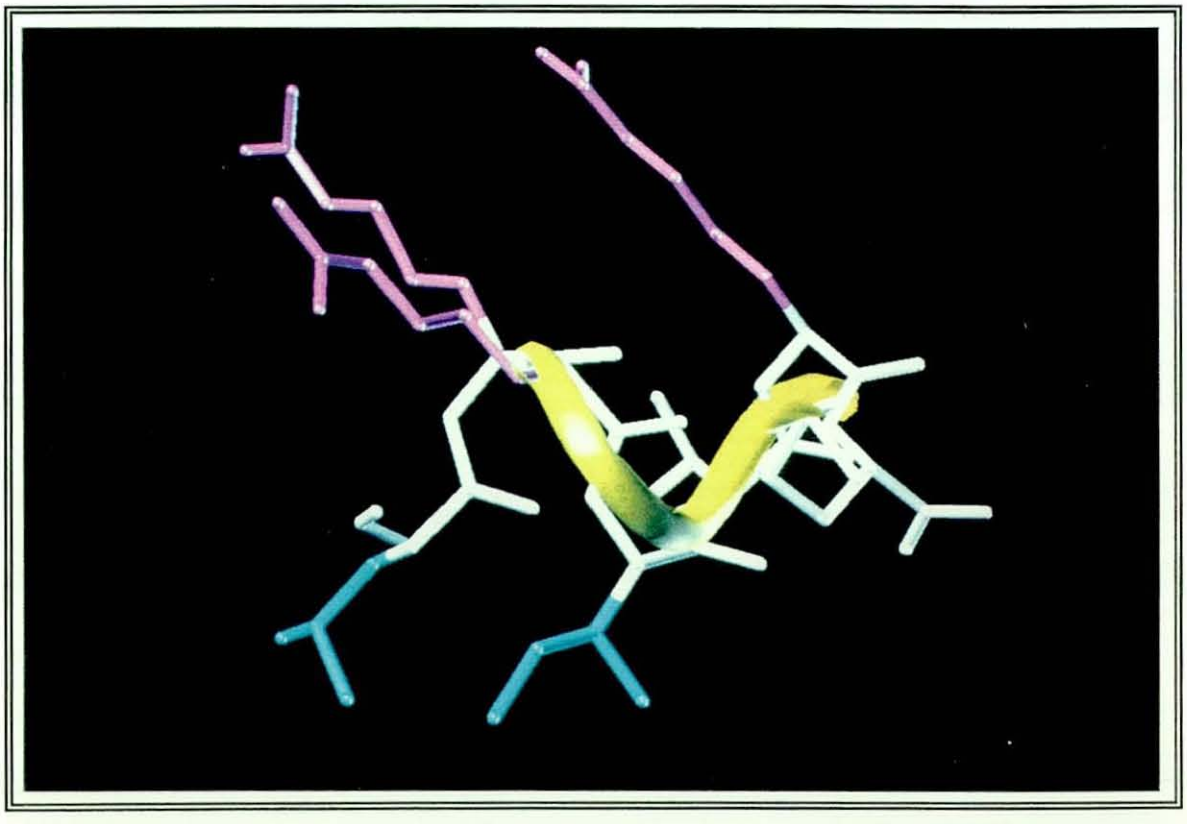
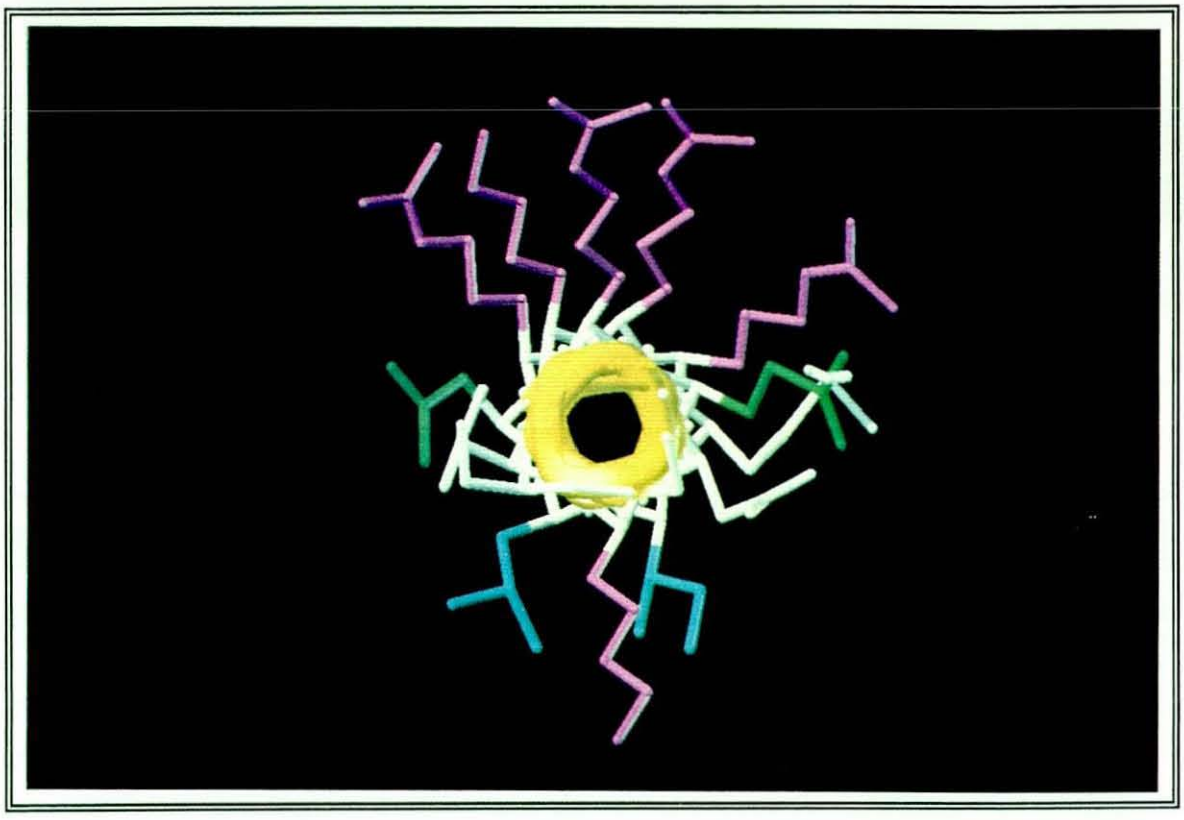


Figure 6.21 μil3$_{277\text{-}283}$ modelled as an α-helix viewed (a) from above the helix (N-terminus coming out of the page) and (b) from the side of the helix (N-terminus to the left). Basic and hydrophobic residues are shown in magenta and cyan respectively. The α-helix backbone is shown in yellow.

(a)



(b)

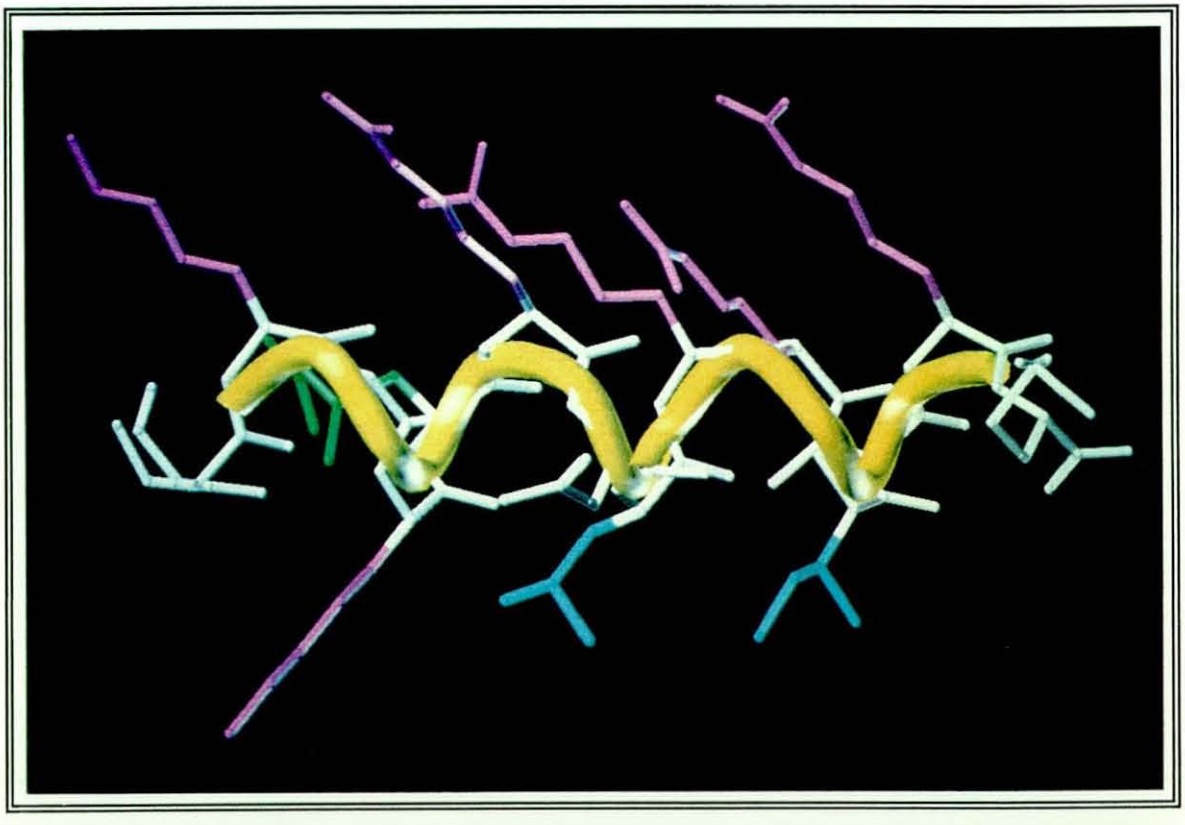


Figure 6.22 $\mu il3_{269\text{-}283}$ modelled as an α-helix viewed (a) from above the helix (N-terminus coming out of the page) and (b) from the side of the helix (N-terminus to the left). Basic, hydrophobic and acidic residues are shown in magenta, cyan and green respectively. The α-helix backbone is shown in yellow.

loop of SSTR1 is unable to decrease adenylyl cyclase activity. Thus it may be that the C-terminal region of the 3rd intracellular loop of the δ-opioid receptor alone cannot activate G proteins, but the whole 3rd intracellular loop does contain the structural information necessary to cause G protein activation. The results of Reisine and colleagues must be treated with a certain degree of caution however, as Patel *et al* (1994) have recently shown that the wild-type SSTR1 can cause an 80% inhibition of adenylyl cyclase activity when transfected into CHO-K1 cells.

Although $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ did not stimulate the low K_m GTPase activity of SH-SY5Y membranes, both peptides inhibited the stimulation of GTPγS binding to G proteins caused by agonist-bound μ- and δ-opioid receptors. Thus it seems possible that although this region of the receptor alone is not sufficient to cause G protein activation, it may nevertheless form part of the G protein binding site. In this way, peptides $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ compete for the site on the G protein which binds activated receptor, and thereby inhibit receptor-G protein-coupling. Relatively high concentrations ($\geq$ 30 μM) of peptide were required to achieve this effect, but this is in line with other studies which have used receptor-mimetic peptides to study receptor-G protein interaction [Dalman and Neubig, 1991; Schreiber *et al*, 1994; Varrault *et al*, 1994; McClue *et al*, 1994].

Peptides $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ also caused a small inhibition of basal [^{35}S]GTPγS binding in membranes from both SH-SY5Y and NG108-15 cells. These results might suggest the possibility that μ- and δ-opioid receptors cause a constitutive activation of G protein in membranes from these cells, which can be inhibited by $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$. Constitutive activation of G protein by δ-opioid receptors in NG108-15 membranes was suggested from results in chapter 4. In addition, Traynor and Nahorski (1995) have provided evidence for constitutive activation of G protein in membranes from SH-SY5Y cells.

The effect of hexa-alanine, hexa-glycine and the cationic peptide KKRAARATS-NH_2 on δ-agonist-mediated [^{35}S]GTPγS binding in NG108-15 membranes may provide a potential explanation of the molecular mechanisms by which $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$ inhibit receptor-G protein interaction. Hexa-glycine did not affect the stimulation of [^{35}S]GTPγS binding by δ-opioid agonists, and KKRAARATS-NH_2 caused only a small inhibition of binding, suggesting

that the effects of μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$ on opioid receptor-stimulated [^{35}S]GTPγS binding are not due simply to non-specific effects of (cationic) peptides. However, hexa-alanine completely blocked δ-opioid receptor-stimulated binding of [^{35}S]GTPγS. These differential effects of hexa-alanine and hexa-glycine may be the result of differences in the chemical nature of their side chains (CH_3- and H- respectively). However, it is also possible that some other property of hexa-alanine may be responsible for its ability to block δ-opioid receptor-mediated stimulation of [^{35}S]GTPγS binding. In light of the well known fact that alanine is one of the most abundant residues found in α-helical domains of protein structures [Chou and Fasman, 1978], it is tempting to speculate that hexa-alanine forms an α-helix in solution and this structural feature allows it to block G protein activation by δ-opioid receptors. However, according to Zimm-Bragg helix-coil transition theory [Zimm and Bragg, 1959], in the absence of specific stabilising interactions short α-helices (6-20 residues) are unstable in aqueous solution. In addition, conformational energy calculations for a 16-residue alanine homopolymer indicate that a very low fractional helical content (0.06) is expected for this peptide in water [Vila *et al*, 1992], in agreement with the near zero helix content measured for a 10-residue alanine block copolymer [Ingwall *et al*, 1968]. However, others suggest that short alanine-based peptides do have an intrinsic tendency to exist in the α-helical conformation in aqueous solution [Marqusee *et al*, 1989].

Clearly the conformation of hexa-alanine is a matter of some speculation. The peptide may be more likely to adopt a helical conformation in the presence of a plasma membrane or in the presence of the high salt concentrations employed for the [^{35}S]GTPγS binding assay [Ingwall *et al*, 1968]. Without determining the conformation of the peptide under the [^{35}S]GTPγS binding assay conditions, it is difficult to be certain of the exact structure of this peptide. Nevertheless, whether or not the secondary structure of hexa-alanine explains its biological activity, the use of small model peptides containing only one type of amino acid represents an interesting approach to the investigation of the molecular determinants of receptor-G protein interaction.

To examine the role of the C-terminal region of μ- and δ-opioid receptors in G protein recognition further, opioid-ligand binding studies were undertaken in the presence of μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$. In general, both peptides exerted only a small effect, displacing ≤ 25% of the specific opioid binding. However,

surprisingly for both μ-opioid binding in SH-SY5Y membranes and δ-opioid binding in NG108-15 membranes, [^{3}H]-agonist and [^{3}H]-antagonist binding were equally affected. Antagonist binding at G protein-coupled receptors is generally unaffected by GTPγS and pertussis toxin [Birnbaumer *et al*, 1990], and it is therefore assumed that antagonists are insensitive to the signalling state of G proteins. Thus, the effects of μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$ on opioid binding might, at first, seem difficult to explain in terms of an inhibition of receptor-G protein-coupling. However, it is possible that the binding of an intracellular loop peptide between receptor and G protein in some way distorts the conformation of the receptor, which could equally well affect agonist as well as antagonist binding, particularly if the relative positions of critical residues for antagonist binding are disturbed. Effects of intracellular loop peptides on antagonist binding of G protein-coupled receptors have been observed previously [Dalman and Neubig, 1991]. A peptide from the carboxy-terminal region of the 3rd intracellular loop of the α_{2A}-adrenergic receptor inhibits both agonist and antagonist binding. However, in this case the peptide is approximately 10-fold more potent in inhibiting agonist than antagonist binding.

A possible interpretation of these binding results is that both μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$ are low affinity, non-selective opioid peptides. Indeed, this possibility cannot be ruled out from the results of opioid ligand binding studies. Alternatively the peptides may be binding to an alternative site on the receptor which allosterically modifies the binding properties of the receptor.

In order to try to understand the mechanisms by which μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$ were causing their effect on opioid ligand binding, cells were treated with pertussis toxin to uncouple opioid receptors from G_i/G_o proteins. Pertussis toxin pretreatment of SH-SY5Y cells caused a slight rightward shift in affinity of the μ-opioid receptor for agonist, as assessed by saturation binding analysis of SH-SY5Y membranes using [^{3}H]-DAMGO, and displacement of [^{3}H]-diprenorphine by fentanyl from membranes of toxin-treated and control cells. These results are similar to those of McKenzie and Milligan (1990). These authors found that pertussis toxin treatment of NG108-15 cells led to a 2.3-fold reduction of binding affinity for [^{3}H]-DADLE at the δ-opioid receptor with a small but non-significant reduction in total binding sites. However, the measured number of [^{3}H]-DAMGO binding sites remaining in SH-SY5Y cells

after pertussis toxin treatment in this study depended on the method of data analysis. Thus the computer programme LIGAND only identified receptor sites with high affinity, indicating that 54% of [^{3}H]-DAMGO binding sites remained after toxin treatment. In contrast manual manipulation of the data using Scatchard plots suggested that at high concentrations [^{3}H]-DAMGO binding could reach the same level as that seen in control cells. Clearly, these results indicate that a certain amount of caution should be exercised in the analysis of binding data.

[^{3}H]-μ-Opioid ligand binding in SH-SY5Y membranes from cells that had been pre-treated with pertussis toxin was still inhibited by peptides $\mu il3_{277\text{-}283}$ and $\mu il3_{269\text{-}283}$. Peptides derived from intracellular regions of other G_i/G_o protein-coupled receptors have been shown to elicit pertussis toxin-insensitive effects. For example, two peptides derived from the 5-hydroxytryptamine$_{1a}$ receptor inhibit forskolin-stimulated adenylyl cyclase activity [Varrault *et al*, 1994], but this inhibition is completely insensitive to the effects of pertussis toxin. In addition, an insulin-like growth factor-II receptor-related peptide activates pertussis toxin-ADP-ribosylated G_i [Okamoto *et al*, 1991a], whilst another peptide from the same receptor is only partially sensitive to the effects of pertussis toxin [Okamoto *et al*, 1990]. Furthermore, Weingarten *et al* (1990) have shown that mastoparan can still bind to the carboxyl-terminus of G_i, even after pertussis toxin-catalysed ADP-ribosylation of the G protein, showing that physical interaction between peptides and G proteins is possible after pertussis toxin modification. It is known that pertussis toxin treatment of G_i/G_o proteins inhibits receptor mediated activation; however, it remains to be determined as to whether or not there is still any physical contact between receptor and G protein in this "uncoupled" state. If there is, then clearly pertussis toxin treatment of SH-SY5Y cells may not abolish the effect of the peptides on μ-opioid binding. Evidence that receptors which are uncoupled from their effector may still be sensitive to G protein comes from the work of Fraser *et al* (1988) who produced a mutant β-adrenergic receptor (Asp130Asn) which was unable to stimulate adenylyl cyclase activity. However, agonist binding to the mutant receptor was still sensitive to the presence of guanine nucleotides.

Opioid ligand binding studies using the cationic 'control' peptide KKRAARATS-NH_2, which caused a 16.4±2.0% reduction in [^{3}H]-DPDPE

binding, suggest that opiate binding may be inherently sensitive to inhibition by small positively charged peptides via a non-specific mechanism. Indeed, every cationic peptide tested (KKRAARATS-NH_2 , mastoparan (chapter 3), mastoparan 17 (chapter 3), $\mu il3_{277-283}$, and $\mu il3_{269-283}$) decreased both agonist and antagonist binding at micromolar concentrations. However, although KKRAARATS-NH_2, $\mu il3_{277-283}$ and $\mu il3_{269-283}$ all had a similar effect on the binding of [^{3}H]-DPDPE in NG108-15 membranes, the inhibitory effect of $\mu il3_{277-283}$ and $\mu il3_{269-283}$ on DPDPE-mediated [^{35}S]GTPγS binding was much larger than that of KKRAARATS-NH_2, suggesting that the effects of $\mu il3_{277-283}$ and $\mu il3_{269-283}$ on [^{35}S]GTPγS binding are not caused by a non-specific inhibition of agonist binding.

The use of synthetic peptides suggested a role for the third intracellular loop of the μ- and δ-opioid receptors in G protein-coupling. Potential interactions of the C-terminal region of the δ-opioid receptor with G proteins were also studied using a polyclonal antibody to the C-terminal 35 amino acid residues of this receptor. SDS-PAGE followed by immunoblot analysis of a crude membrane fraction from NG108-15 cells using δC-Ant detected a protein band of 72 kDa. This is in good agreement with the results of Svingos *et al* (1994), who generated several antipeptide antibodies against the cloned mouse δ-opioid receptor which labelled a band at approximately 70 kDa from NG108-15 cells and rat olfactory bulb, and those of Crook *et al* (1994) who used δC-Ant to label a protein band of 67 kDa from NG108-15 cells. Therefore the results indicate that δC-Ant recognises the δ-opioid receptor from NG108-15 cells. However, binding of δC-Ant to the δ-opioid receptor caused no functional effects, compared to rabbit serum, as assessed by its effect on DPDPE-mediated stimulation of [^{35}S]GTPγS binding in membranes from these cells, suggesting that this region of the receptor does not play a role in transducing the agonist signal to G protein. This agrees with results on opioid ligand binding studies performed in NG108-15 membranes, where δC-Ant is also without effect (Crook, T., personal communication).

One potential explanation for the lack of effect of δC-Ant in the [^{35}S]GTPγS binding assays could be that access of the antibody to its epitope on the δ-receptor is restricted in a native membrane environment, whereas boiling the membrane preparation prior to gel electrophoresis disrupts the membrane structure and eliminates this restriction. However, δC-Ant has been used

successfully in immunofluorescence staining of δ-opioid receptors in intact NG108-15 cells [Crook *et al*, 1994].

Using a molecular biological approach, Cvejic *et al* (1995) have produced results suggesting that the C-terminal 37 amino acids of the δ-opioid receptor are not critical for G protein activation. A mutant of the δ-opioid receptor was generated which had these residues deleted. This mutant receptor couples to adenylyl cyclase activation giving similar responses to the wild-type receptor, indicating that this region of the receptor is not necessary for inhibition of adenylyl cyclase activity.

Interestingly, Uhl and coworkers [Surratt *et al*, 1994, 1994b], have shown recently that C-terminal tail deletion of the μ-opiate receptor may have agonist-specific effects. Thus deletion of 33 C-terminal amino acids from the human μ-opiate receptor yields a receptor at which morphine, but not the substituted enkephalin DAMGO, inhibits adenylyl cyclase. Such results suggest the possibility that δC-Ant may be effective at blocking δ-opioid receptor activation by some, but not all δ-opioid agonists.

The C-terminal region of the rat μ-opioid receptor has been implicated as an important region involved in μ-receptor desensitization [Zimprich *et al*, 1995]. This group expressed two isoforms of the rat μ-opioid receptor in CHO-K1 cells, which differed only in length and amino acid composition at the C terminal 12 amino acid residues. Both μ-opioid receptor isoforms show similar affinities for opioid compounds and are equally effective in the inhibition of forskolin-induced cAMP accumulation, indicating a functional coupling to G protein. However, studies measuring the ability of the potent μ-opioid agonist to inhibit adenylyl cyclase in cells that been exposed to DAMGO (1 μM) for varying periods of time indicated that the two isoforms show different kinetics of receptor desensitization.

In conclusion, a potential role for the C-terminal half of the 3rd intracellular loop of the μ- and δ-opioid receptors in G protein-coupling has been demonstrated. The C-terminal tail of opioid receptors is highly divergent, and as such represents a potential site for directing specificity of G protein-coupling.

However, this study provides no evidence that the C-terminal 35 amino acid residues of the δ-opioid receptor are involved in G protein interaction.

Chapter 7

OVERVIEW

One of the fundamental unanswered questions of G protein-coupled receptor pharmacology is how agonist binding to the core of the receptor exposes or alters the G protein binding site such that it catalyses guanine nucleotide exchange on the G protein. An important step in elucidating the molecular mechanisms by which 7-transmembrane domain receptors transduce a signal across the plasma membrane is the determination of the particular region(s) of the receptor that form the G protein binding surface. Small molecules which are able to activate G proteins represent useful tools with which to study the mechanisms of G protein activation.

A range of compounds reported to activate purified G proteins reconstituted into phospholipid vesicles were tested for their ability to increase GTPase activity in SH-SY5Y membranes. Whilst the wasp venom peptide mastoparan and its potent analogue, mastoparan 7, both produced a 3-fold increase in the basal rate of GTP hydrolysis, benzalkonium chloride, spermine, compound 48/80 and octadecyltrimethylammonium bromide all failed to stimulate GTPase activity in SH-SY5Y membranes. Indeed all four compounds produced an inhibition of GTPase activity at high concentrations. This is interpreted as providing evidence that a native membrane environment may provide a degree of selectivity to G protein activation which is not apparent when studying purified G proteins reconstituted into phospholipid vesicles.

To further these studies, a [^{35}S]GTPγS binding assay was developed to study activation of heterotrimeric G protein by δ-opioid receptors using membranes from NG108-15 cells. Under optimal conditions, the δ-opioid agonist DPDPE caused a 3-fold increase in the level of [^{35}S]GTPγS binding seen in membranes from these cells. This assay provides a sensitive method of determining the relative intrinsic activities and potencies of δ-opioid ligands. Furthermore ICI 174864, a putative inverse agonist at the δ-opioid receptor, was able to inhibit the basal level of [^{35}S]GTPγS binding in NG108-15 membranes. This technique will therefore prove useful for further investigation of this novel class of compounds.

Molecular cloning of the μ-, δ- and κ-opioid receptors is undoubtedly leading to rapid advances in our understanding of the mechanisms of opioid receptor activation. A clonal cell line stably expressing the mouse δ-opioid receptor has been investigated as a potential model for studying δ-receptor-G

protein interactions. Agonist occupation of the δ-opioid receptor caused a small (approximately 25%) stimulation of [^{35}S]GTPγS binding over basal values. This can be compared to the situation in NG108-15 membranes, where δ-opioid agonists routinely caused a doubling of the basal level of [^{35}S]GTPγS binding. Thus, in this study, δ-opioid receptors expressed in CHO cells seemed to be poorly coupled to G protein. This low level of coupling to G protein was also reflected in the ability of δ-opioid agonists to inhibit forskolin-stimulated adenylyl cyclase activity in this cell line. DPDPE (10 μM) inhibited forskolin-induced adenylyl cyclase by only 13%. In comparison, DPDPE afforded a 49% inhibition of adenylyl cyclase activity in NG108-15 cells, with an IC_{50} of some 6 nM. Saturation binding analysis of the δ-opioid receptor population in membranes from CHO cells showed that the selective δ-opioid agonist [^{3}H]-DPDPE labelled only 45% of the binding sites recognised by the δ-receptor partial agonist [^{3}H]-diprenorphine, lending further support to the concept that a high proportion of the expressed δ-receptors appear to be uncoupled from G proteins.

Knowledge of the primary amino acid sequences of the opioid receptor types is allowing ideas about structure / function relationships of various critical residues to be tested. Two peptides derived from the C-terminal end of the third intracellular loop of the μ-opioid receptor, μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$, have been shown to block both μ- and δ-opioid mediated stimulation of [^{35}S]GTPγS binding in SH-SY5Y and NG108-15 membranes respectively. This effect is suggested to result from the ability of these peptides to inhibit interaction between opioid-receptor and G protein. Neither of the peptides caused an activation of low K_m GTPase activity in SH-SY5Y membranes, suggesting that whilst the C-terminal region of the third intracellular loop may be an important area of contact between receptor and G protein, this region alone does not possess the ability to activate heterotrimeric G protein. Studies with two model peptides, hexa-alanine and hexa-glycine, showed that whilst hexa-alanine completely blocked δ-receptor mediated stimulation of [^{35}S]GTPγS binding in NG108-15 membranes, hexa-glycine was without effect. It is possible that this difference is a result of differing secondary structures of the two peptides. Study of other simple oligopeptides containing only one type of amino acid may help to elucidate the structural characteristics required to inhibit opioid receptor-G protein interaction. In particular, elucidation of the secondary structure of these peptides along with that of μil3$_{277\text{-}283}$ and μil3$_{269\text{-}283}$, using a

combination of CD and NMR spectroscopy together with computational structure prediction methods, may suggest a correlation between secondary structure and functional activity, and represents an important goal for future work. Although a possible role for the third intracellular loop of the μ- and δ-opioid receptors in G protein coupling has been demonstrated, investigation of the C-terminal 35 amino acids of the δ-opioid receptor using a polyclonal antibody to this region of the receptor was unable to provide any evidence to suggest that this domain is also required for functional interaction with G protein.

In conclusion, a [^{35}S]GTPγS binding assay has been set up in membranes from NG108-15 cells. This assay has been used to show that the C-terminal region of the third intracellular loop of μ- and δ-opioid receptors may be an important domain of the receptor involved in G protein-coupling in NG108-15 and SH-SY5Y membranes. Adaptation of the [^{35}S]GTPγS binding assay for use with CHO cell membranes expressing mouse δ-opioid receptors suggested that a high proportion of receptors in these cells exist uncoupled from G protein. Such variation between the 'same' receptor expressed in different systems may be explained in terms of differences in the levels of intracellular machinery required for functional activity, such as G proteins. Other explanations, such as the level of coupling / uncoupling between the various proteins, or differences in their post-translational processing are also possible. Understanding these processes at the molecular level, using assays such as those described in this thesis, represents a significant challenge for future signal transduction research.

Chapter 8

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