IDENTIFICATION OF A G-PROTEIN INVOLVED IN MEDIATING AGONIST-STIMULATED PHOSPHOLIPID HYDROLYSIS.

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ABBREVIATIONS.

ADP	Adenosine 5' - diphosphate
AP-1	Activator protein 1
AP-2	Activator protein 2
App[NH]p	Adenylyl 5' - imidodiphosphate
ARF	ADP ribosylation factor
ATP	Adenosine 5' - triphosphate
Bromophenol blue	3', 3", 5', 5" - tetrabromophenolsulfonphthalein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Adenosine 3': 5' - cyclic monophosphate
cDNA	Complimentary DNA
CDP-choline	Cytidine 5' - diphosphocholine
cpm	Counts per minute
C-terminus	Carboxyl terminus
CTx	Cholera toxin
DAG	*sn*-1, 2-diacylglycerol
DG	*sn*-1, 2-diradylglycerol
Dibutryl cAMP	N^6, 2'-o-dibutryladenosine 3': 5' - cyclic monophosphate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethylsulphoxide
DPM	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF-Tu	Bacterial elongation factor - Tu
EGF	Epidermal growth factor
FCS	Foetal calf serum
fMLP	N - formyl - methionyl - leucyl - phenylalanine
GAP	GTPase activating protein
GDP	Guanosine 5' - diphosphate
GDPβS	Guanosine 5' - (2 - *o* - thiodiphosphate)

G_i	Inhibitory G-protein of adenylyl cyclase regulation
GNRP	Guanine nucleotide releasing protein
G_o	G-protein, o indicating "other".
G_p	G-protein, p indicating phospholipase
Gpp[NH]p	Guanylyl 5' - [βγ - imido] diphosphate
G-protein	Guanine nucleotide binding protein
GTP	Guanosine 5' - triphosphate
GTPγS	Guanosine 5' - [3 - *o* - thio] triphosphate
HAT	Hypoxanthine - aminopterin - thymidine
HBG	Hank's buffered saline solution with 1% (w/v) BSA and 10mM glucose
HEPES	N- 2- Hydroxyethylpiperazine - N'- 2- ethane - sulphonic acid
H. p. l. c.	High - performance liquid chromatography
IAP	Islet activating protein (pertussis toxin)
IgG	Immunoglobulin G
$Ins(1,4,5)P_3$	*D - myo* - inositol (1,4,5) trisphosphate
$Ins(1,3,4,5)P_4$	*D - myo* - inositol (1,3,4,5) tetra kisphosphate
K_{cat}	Catalytic rate constant
kDa	Kilodaltons
KLH	Keyhole limpet haemocyanin
K_d	Equilibrium dissociation constant - representing the concentration of a ligand that half - maximally occupies the receptor at equilibrium
LTB_4	Leukotriene B_4
mA	Milliamps
mRNA	Messenger RNA
NAD+	Nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
NFP	N-formylated methionyl peptide
NP - 40	Non-idet P - 40 detergent
N-t-bocMLP	N-t - butoxycarbonyl - methionyl - leucyl - phenylalanine

N-terminus	Amino terminus
p21*ras*	Product from the *ras* family of genes coding for 21kDa GTP-binding proteins
PAF	Platelet activating factor (1 - *o* - Alkyl - 2 - acetyl - *sn* - glyceryl - 3 - phosphorylcholine)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIC	Phosphatidylinositol - specific phospholipase C
PtdIns	Phosphatidylinositol
PtdIns(4,5)P_2	Phosphatidylinositol 4, 5-bisphosphate
PKC	Ca^{2+} and phospholipid-dependent protein kinase
PLA2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMN	polymorphonuclear leukocyte
PMSF	Phenylmethylsulphonyl fluoride
PPH	Phosphatidate phosphohydrolase
PtdCho	Phosphatidylcholine
PtdIns	Phosphatidylinositol
PtdOH	Phosphatidic acid
PTx	Pertussis toxin
SDS	Sodium dodecylsulphate
SDS - PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TCA	Trichloroacetic acid
Td	Transducin
TEMED	N, N, N', N' - tetramethylethylenediamine
TPA	12-*O*-tetradecanoyl-13-acetylphorbol
TRE	TPA-responsive elements
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylenesorbitan monolaurate

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SUMMARY.

G-proteins play a central role in signal transduction, acting to couple transmembrane receptors to a variety of intracellular effector pathways. Of major relevance to the understanding of cellular control is the definition of the specificity with which this family of proteins can perform these functions.

Receptor-mediated activation of the cytosolic enzyme phosphoinositidase C (PIC) results in the cleavage of plasma membrane phosphatidylinositol containing lipids, to generate two second messengers. This bifurcating pathway affects a diversity of both short and long term cellular processes and as such it is of interest to establish how the initial message is transduced.

It is well established that receptors couple to phosphoinositidase C via a G-protein and that this coupling interaction displays pertussis toxin-sensitivity in some systems, but not in others. Several pertussis toxin-sensitive G-proteins have been identified and the work of this thesis was aimed at defining which of these proteins couples to receptors that stimulate phosphoinositidase C activity.

To simplify this objective the choice of a model system was based on the criteria outlined in the research aims. A number of systems were examined with the observations from two of the most suitable systems presented in this thesis.

Cells of the human monocytic cell line, the U937 cell line can be differentiated by a variety of agents to mature phagocytic cells. Concurrent with this maturation process is the onset of responsiveness to the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP). Previous work has established that this peptide stimulates phosphoinositidase C activity in a totally pertussis toxin-sensitive manner in dimethyl sulphoxide (DMSO) -differentiated U937 cells.

Using a panel of specific antipeptide antisera two splice variant forms of $G_s\alpha$ and the pertussis toxin substrates of $G_i2\alpha$ and $G_i3\alpha$ were identified in U937 cell membranes. The other known pertussis toxin substrates, $G_i1\alpha$ and $G_o\alpha$ were not detected. Over a five day DMSO-induced differentiation period, expression of

$G_i3\alpha$ remained unaltered whereas that of both splice variant forms of $G_s\alpha$, $G_i2\alpha$ and the β subunits increased.

Cholera toxin can catalyse the [^{32}P]ADP-ribosylation of the "classical" pertussis toxin substrates under conditions where the α subunit is devoid of guanine nucleotide. This situation arises when an agonist activates its coupling G-protein in the absence of exogenously added guanine nucleotide. The chemotactic peptide stimulated the cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_i2\alpha$, defining receptor interaction with this pertussis toxin substrate, although assay sensitivity may have precluded detection of an interaction with $G_i3\alpha$.

Alternative approaches addressing coupling specificity utilised the fact that the C-terminal region of the G-protein α subunit is important in receptor interaction. Uncoupling of a G-protein from its receptor by perturbation of this interaction lowers the affinity of the receptor for its agonist and inhibits agonist-stimulated GTPase activity. Conditions were established whereby such perturbation was evident even after prolonged preincubation periods with a view towards preincubation of U937 cell membranes with C-terminally directed antipeptide antisera. It was postulated that these would act as definitive uncoupling agents and clarify whether $G_i2\alpha$ and/or $G_i3\alpha$ coupled to phosphoinositidase C in this cell line.

Previous studies of the second model system investigated have established that the α_1-adrenergic receptor in rat white adipocytes couples to phosphoinositidase C via a totally pertussis toxin sensitive G-protein. However, the identity of the pertussis toxin substrates expressed in this tissue has been a source of considerable debate. Using a panel of discriminatory and specific antipeptide antisera the complement of pertussis toxin-sensitive G-proteins was unequivocally identified as that of $G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$ and barely detectable levels of $G_o\alpha$. Thus the potential PIC coupling candidates were identified, although which of these coupled to α_1-adrenergic receptor activation of PIC was not successfully addressed.

CHAPTER 1.

INTRODUCTION.

1.1. SIGNAL TRANSDUCTION - HISTORICAL PERSPECTIVES.

The control of cellular activity is dependent upon the perception of an extracellular signal and its translation into intracellular metabolic alterations. An initial indication of how such "signal transduction" may occur was described by the pioneering work of Sutherland and Rall. They discovered that in liver homogenates, glucagon and adrenaline-stimulated phosphorylase activation was mediated by a heat stable factor, later identified as 3', 5' cyclic-adenosine monophosphate (cAMP). These hormones did not directly activate the phosphorylase suggesting that adenylyl cyclase, the enzyme catalysing the production of cAMP from ATP, could be hormonally regulated (Rall *et al.*, 1956 and 1957). This data formed the basis for proposal of the "second messenger hypothesis", whereby binding of hormone, the primary message, to specific cell surface receptors resulted in the generation of a secondary messenger eg., cAMP, which then altered intracellular metabolism.

The actual mechanism by which ligand binding to a transmembrane receptor exerts an intracellular effect has been the subject of intensive research effort and is currently not fully resolved. An initial insight into the transduction mechanism was provided when Rodbell and colleagues demonstrated that hormonal regulation of adenylyl cyclase in hepatocytes required guanosine triphosphate (GTP), (Rodbell *et al.*, 1971a). Non-hydrolysable analogues of GTP e.g., guanylyl 5'-[βγ-imido]diphosphate (Gpp[NH]p), were shown to persistently activate adenylyl cyclase, in contrast to the transient activation produced by GTP (Londos *et al.*, 1974). Further, agonist association with hormone receptors was shown to increase the rate of adenylyl cyclase stimulation (Salomon *et al.*, 1975).

These studies led to Rodbell's proposals that a specific "receptor", a "transducer" and an "effector" may be involved in signal transduction. He further deduced that the GTP binding "site" may also possess a hydrolytic capability unable

to be utilised in the presence of enzymatically non-hydrolysable GTP analogues. The latter speculation was corroborated by Cassel and Selinger, who first assayed catecholamine-stimulated GTPase activity in turkey erythrocyte membranes (Cassel & Selinger, 1976). They demonstrated that GTP hydrolysis acted as a "switch-off" step in the activation of adenylyl cyclase and that the inactive state was a "GDP-bound" state. Receptor activation was believed to cause the release of bound GDP and its exchange for GTP. This interpretation has proved to be essentially correct and will be discussed in greater detail later (see section 1.5.1).

When Rodbell first suggested a GTP requirement for adenylyl cyclase activation a simultaneous and at the time confusing observation was that GTP actually interfered with glucagon binding to receptors (Rodbell *et al.*, 1971b). Other investigators subsequently found that the effect of guanine nucleotides on receptor binding was agonist specific and reduced agonist affinity for the receptor (Maguire *et al.*, 1976). In the light of the proposal of the GTP regulatory cycle these results could now be interpreted as a further point of "control" in the transduction scheme, with the "transducer" not only regulating its own activity but further influencing the initial message input at the receptor level.

These initial observations and predictions stimulated intensive research effort and the field progressed with individual discoveries reinforcing and broadening the fundamental concepts. Indeed the early appreciation of the role of guanine nucleotides, allowed GTP affinity chromatography steps to be employed which resolved the "transducer" from the effector, adenylyl cyclase. This transducer is now termed G_S, "G" for "guanine nucleotide binding" and "s" for "stimulatory". Such approaches confirmed G_S as a separate entity as opposed to a guanine nucleotide binding site on the catalytic moiety of adenylyl cyclase (Pfeuffer & Helmreich, 1975; Spiegel *et al.*, 1979). The mutant S49 murine lymphoma cell line, cyc^-, provided an ideal functional assay for the purification of G_S. An increase in intracellular cAMP in this cell line is cytocidal, enabling the isolation of

"surviving" mutants defective in cAMP production (Bourne *et al.*, 1975). Initially presumed to be defective in the cyclase component, (termed AC-, then cyc-), it was later shown to be deficient in G_S. This "deficiency" was exploited by Ross and Gilman who restored cyc- cyclase activity using a detergent extract of wild type membranes in which native adenylyl cyclase activity had been inactivated (Ross and Gilman, 1977).

With the evidence that G_S may be a separate entity came initial purification strategies, using rabbit liver as a source and the cyc- cell line as a reconstitutive assay, revealing α subunits of either 52 or 45kDa and a 35kDa β subunit (Northup *et al.*, 1980). G_S was further purified from turkey (Hanski *et al.*, 1981) and human erythrocytes (Hanski & Gilman, 1982), using similar methods, although only the 45kDa form was obtained. Subsequently a low molecular weight γ subunit, previously neglected due to its poor staining properties, was found to be associated with G_S (Hildebrandt *et al*, 1984). Demonstrations of the distinct activities of each subunit came from activation of purified G_S by AMF ($AlCl_3$, $MgCl_2$ and NaF) or GTPγS (a non-hydrolysable analogue of GTP). Such treatments resolved the α subunit, which activated adenylyl cyclase (Northup *et al*, 1983a) and the β subunit which stimulated the rate of G_S deactivation (Northup *et al.*, 1983b).

With the advent of affinity chromatographic techniques purification of labile and low abundance molecules e.g., the β-adrenergic receptor (Shorr *et al.*, 1981) and the effector adenylyl cyclase (Pfeuffer *et al.*, 1985) became possible, further vindicating Rodbells theories.

Early studies had suggested the possibility of hormonal inhibition of adenylyl cyclase (Murad *et al.*, 1962). This was confirmed by demonstration of a biphasic effect of GTP on adenylyl cyclase activity in adipocyte preparations (Rodbell, 1975). In the absence of a stimulatory hormone, GTP, at high concentrations, elicited an inhibition of adenylyl cyclase, which led to the theory of an inhibitory input into regulation of this enzyme (Rodbell, 1980).

The isolation of exotoxins from bacterial cultures has proved incredibly useful for both identification and functional analysis of the G-proteins. Cholera toxin, isolated from *Vibrio cholerae*, caused activation of adenylyl cyclase in pigeon erythrocytes and this correlated with the radiolabelling of a 42kDa polypeptide in the presence of [32P] NAD^+ (Gill & Meren, 1978). Diphtheria toxin had been shown to exert its actions, on a component required for protein synthesis, via an ADP-ribosylation reaction and by analogy cholera toxin was presumed to be catalysing a similar reaction on its substrate (Collier, 1975). The functional consequences of cholera toxin were similar to those obtained with non-hydrolysable analogues of GTP and the toxin was found to attenuate the ability of $G_s\alpha$ to hydrolyse GTP resulting in an irreversibly activated $G_s\alpha$ subunit (Cassel & Selinger, 1977; Birnbaumer *et al.*, 1980).

Pertussis toxin, also termed islet activating protein (IAP), was isolated from *Bordetella pertussis* cultures and was also shown to have effects upon the adenylyl cyclase system. In rat C6 BU1 glioma cells, toxin treatment caused an enhanced GTP activation of adenylyl cyclase concurrent with the incorporation of [32P] label, presumably [32P] ADP-ribose, into a 41kDa membrane-associated protein (Katada & Ui, 1982). Toxin treatment had released a tonic inhibition of adenylyl cyclase activity and provided evidence for the identity of a previously putative inhibitory G-protein of the adenylyl cyclase cascade, now termed G_i, "i" for "inhibitory". In contrast to cholera toxin, pertussis toxin prevents productive coupling between receptor and G-protein, attenuating receptor-mediated inhibition of adenylyl cyclase (Katada & Ui, 1981; Burns *et al.*, 1983).

Concurrent with intensive research on the adenylyl cyclase system, was the realisation that the light activated cGMP phosphodiesterase shared a GTP-dependent regulatory step (Wheeler & Bitensky, 1977). Akin to G_s, the GTP binding "site" in the retinal transduction system was identified as a soluble multimeric protein and termed transducin, Gt (Godchaux & Zimmermann, 1979;

Kuhn, 1980; Fung *et al.*, 1981). The striking resemblance between the two systems was further verified by the similarity between the β subunits of transducin and G_S. As more detailed information about the adenylyl cyclase and phototransduction system emerges it is apparent that many aspects have been conserved.

Such conservation has enabled similar methodologies and assumptions to be applied to other signal transduction pathways such as the agonist-stimulated turnover of membrane phospholipids and ion channel operation. Although producing their own characteristics and complexities, the study of a role for G-proteins in these pathways has been accelerated by the fact that apparently the basic rules have been previously determined.

Further, it is now recognised that a so-called "superfamily" of GTPase proteins exist that bind and hydrolyse GTP (reviewed by Bourne *et al.*, 1990 & 1991). This latter property is central to their ability to act as molecular "on/off" switches in a variety of cellular events e.g., directing ribosomal protein synthesis, translocation of nascent proteins into the endoplasmic reticulum, guiding of vesicular traffic and control of cell differentiation and proliferation. Comparative study of all the members of this superfamily both reinforces and challenges past discoveries, whilst influencing the course of future research. This introduction will concentrate mainly on the classical G-protein "subfamily", now known to mediate the cellular response to 80% of all known hormones, neurotransmitters, autocrine and paracrine factors.

1.2. G-PROTEIN PURIFICATION - Gt and Gi.

1.2.1. Transducin, Gt.

In parallel with studies on the adenylyl cyclase system, work on the visual transduction system in retinal photoreceptor rod cells led to the isolation of

transducin, Gt. As mentioned previously, transducin serves to couple the light receptor rhodopsin to cyclic GMP phosphodiesterase (Baehr *et al.*, 1982). The source of Gt, the rod outer segment membranes, are easily prepared and extraction of this protein in large quantities in aqueous buffers facilitated detailed biochemical studies (Fung, 1983), generation of specific antisera (Gierschik *et al.*, 1984) and amino sequencing of each of the three subunits (Hurley *et al.*, 1984 a and b; Sugimoto *et al.*, 1985; Fong *et al.*, 1986 and Ovchinnikov *et al.*, 1985). In fact the presence of a γ subunit was initially shown for Gt (Kuhn, 1980).

These subunit partial amino acid sequences have been shown to correspond to predicted sequences from cDNA's cloned from bovine retinal libraries. However a cDNA cloned from this library predicts an amino acid sequence not yet found through sequencing of a purified protein, although immunochemical evidence suggests this cDNA encodes a cone photoreceptor cell-specific form of Gtα (Grunwald *et al.*, 1986; Lerea *et al.*, 1986).

1.2.2. Giα.

Gi was initially purified from rabbit liver (Bokoch *et al.*, 1983) and human erythrocytes (Codina *et al.*, 1983) by essentially identical methods to those employed for purification of Gs. In fact an initial purification of Gs had revealed a prominent, approximately 41kDa contaminant, which can now be assigned retrospectively as co-purifying Giα (Sternweis *et al.*, 1981). Gi can be separated from Gs by heptylamine sepharose (Bokoch *et al.*, 1983) or hydroxyapatite column chromatography (Codina *et al.*, 1983) and identified by pertussis toxin-catalysed ADP-ribosylation. The purified rabbit liver α_i subunit was capable of inhibiting cyclase activity in cyc- membranes and a variety of other cell membranes, suggesting the 41kDa polypeptide was responsible for the inhibitory regulation of adenylyl cyclase activity (Katada *et al.*, 1984a, b and c.). However, attempts to purify Gi from brain provided an initial indication that the situation was more complex.

1.3. G-PROTEIN HETEROGENEITY.

1.3.1. Go.

This "complexity" was suggested by the fact that purified preparations of pertussis toxin substrates from bovine brain contained either two (Sternweis & Robishaw, 1984; Milligan & Klee, 1985) or three (Neer *et al.*, 1984) substrate polypeptides in the 39-41kDa range. Further measurements of specific, high affinity guanine nucleotide binding, using [^{35}S] GTPγS, demonstrated that brain contains a greater amount of binding activity than liver (Sternweis & Robishaw, 1984). These studies in conjunction with purification of rat brain substrates (Katada *et al.*, 1986a) led to the identification of two G-proteins with α subunits of 41kDa and 39kDa. The novel 39kDa substrate was highly expressed, comprising approximately 1-2% of the total brain membrane protein. These purified proteins exhibited distinct peptide maps and antibody reactivity, with direct amino acid sequencing confirming α41 as "Gi1" and α39 as G_o, "o" for "other" (Nukada *et al.*, 1986; Itoh *et al.*, 1986).

Further analysis of brain G-protein fractions eluted from a mono-Q column with specific peptide antibodies identified an additional α subunit, which is immunologically indistinguishable from G_o, but precedes it in elution (Goldsmith *et al.*, 1988). This novel "G_o-like" form exhibits a more basic pI than "conventional" G_o, perhaps suggestive of an alternative post-translational modification. More detailed characterisation of these G_o forms has revealed up to four discrete proteins displaying G_o immunoreactivity. Indeed Katada and co-workers recently isolated four such proteins from bovine brain, discounting the possibility that different βγ subunits could account for this heterogeneity by performing the purification under "dissociating" conditions i.e., the presence of GTPγS and Mg^{2+} (Kobayashi *et al.*, 1989). Subsequent peptide mapping and kinetic analysis suggest two subgroups of a pair of similar α subunits (Inanobe *et al.*, 1990). The difference between these subgroups remains to be established but

may be some form of post translational modification.

1.3.2. Gi2α.

Neer and co-workers noted an additional 40kDa Coomassie blue stained band on purification of brain pertussis toxin substrates (Neer *et al.*,1984). Further, immunochemical analysis of human neutrophil plasma membranes using antisera directed against purified transducin and now known to recognise Giα, also indicated the presence of a novel immunoreactive band (Gierschik *et al.*,1986a). By altering the purification protocol, Katada and co-workers succeeded in resolving the porcine brain 40kDa band from Giα and Goα (Katada *et al.*, 1987a). Using similar methodology and peptide specific antibodies, α_{40} from bovine brain was tentatively identified as "Gi-like" and termed "Gi2" (Goldsmith *et al.*, 1988; Mumby *et al.*, 1988). The previously identified α_{41} form was thus regarded as "Gi1".

The predominant pertussis toxin substrate of bovine neutrophils was identified as Gi2, using peptide specific antisera raised against amino acid sequences predicted from cDNA cloning information (Goldsmith *et al.*, 1987). This protein has been purified from rabbit neutrophil membranes (Dickey *et al.*, 1987) and human leukaemic HL-60 cells (Oinuma *et al.*, 1987; Uhing *et al.*, 1987). Gi2 may be expressed ubiquitously (Brann *et al.*, 1987) and its assignment as a distinct Giα has been further confirmed by direct amino acid sequencing (Itoh *et al.*, 1988a).

1.3.3. Gi3 / Gk.

HL-60 cells have also been a source of an α_{41} polypeptide identified as "Gi3" using antipeptide antisera directed against predicted sequence from cDNA information (Goldsmith *et al.*, 1988). Erythrocyte Gi has been found to activate certain membrane potassium channels and hence has been termed "Gk". However, direct amino acid sequencing of Gk identified it as Gi3 (Codina *et al.*, 1988).

1.3.4. Gp.

Numerous studies have implicated a role for G-proteins in coupling receptors to the phospholipid hydrolysing enzyme, phospholipase C (PLC), as will be discussed in more detail in section 1.9. The identity of the G-proteins, termed "Gp", "p" for phospholipase, involved in this process is far from completely resolved (discussed later in section 1.12 and results Chapters). However, recently unique α subunits that are not substrates for pertussis toxin have been purified and current evidence is emerging that these proteins may have a role in mediating pertussis toxin-insensitive PLC activation.

Exton and co-workers demonstrated that cholate-solubilised extracts from bovine liver plasma membranes preincubated with GTPγS displayed enhanced phosphoinositidase C activity. The GTPγS-dependent "activator" was purified by sequential chromatography, on the basis of its ability to activate partially purified phosphoinositidase C. This 42kDa "activator" protein was recognised by two antipeptide antisera raised against regions showing both a partial and a high degree of homology between the "classical" α subunits. Interestingly an antibody directed against a region in the "classical" α subunits, involved in guanine nucleotide binding and hydrolysis, poorly recognised this protein suggesting possible sequence differences in this area (Taylor *et al.*, 1990).

In a second report, novel G-protein α subunits have been purified from rat brain using an immobilised βγ subunit affinity matrix. These 42kDa subunits were not pertussis toxin substrates and bound GTPγS with slow rates. This poor nucleotide binding capability may explain why these proteins had not been detected previously, as GTPγS binding assays are frequently employed as detection methods in standard G"α" purification protocols. Antipeptide antisera raised against unique regions of these proteins indicated their expression at low levels in a variety of tissues, although they were more concentrated in the brain and the lung (Pang & Sternweis, 1990).

1.3.5. Low molecular weight G-proteins.

The best characterised subgroup of the ever expanding group of 20-35kDa, monomeric GTPases are the 21kDa protein products of the *ras* genes. *Ras* genes were initially described as viral oncogenes with the mammalian cellular homologues of these genes, namely the three proto-oncogenes c-Ha-*ras*, c-Ki-*ras* and N-*ras*, encoding proteins that control regulatory pathways critical for normal proliferation and differentiation (Barbacid, 1987). Despite intensive research their precise role in these processes remains elusive. These proteins share the guanine nucleotide binding and GTPase properties common to G-protein α subunits. *Ras* proteins bind guanine nucleotides even after denaturation and electroblotting and this, in combination with cDNA cloning information, has been useful in indicating the existence of a large family of *ras*-related, low molecular weight GTP binding proteins (for more detail see Hall, 1990).

Using similar protocols to those outlined previously for the "classical" G proteins, several other low molecular weight GTP binding proteins have been purified. A protein termed "ARF" for "ADP-ribosylation factor", has been purified from rabbit liver (Kahn & Gilman, 1984a) and bovine brain (Waldo *et al.*, 1987). ARF purification was monitored by its ability to act as a cofactor necessary for cholera toxin-catalysed ADP ribosylation of G_s.

Another low molecular weight G-protein was identified without any functional basis and confusingly termed G_p, where "p" stands for the "placental" source. Gp has been reported to copurify with $\beta\gamma$ complexes indistinguishable from those in heterotrimeric G proteins and is the only example of a low molecular weight GTP binding protein displaying $\beta\gamma$ association (Evans *et al.*, 1986).

1.3.6. β SUBUNITS.

There are at least two similar forms of β subunit, a 36kDa (β_1) and a 35kDa (β_2) protein (Sternweis & Robishaw, 1984; Winslow *et al.*, 1987; Evans *et al.*, 1987). Most tissues express predominantly the 36kDa form, this being the only

form associated with transducin (Woolkalis & Manning, 1987). However the 35kDa form predominates in placenta and has been purified from this source (Evans *et al.*, 1987). More information about heterogeneity of these subunits and γ subunits has been obtained from cloning studies (see section 1.4.3).

1.4. MOLECULAR ASPECTS.

1.4.1. cDNA clones for G-protein α subunits.

Molecular biological approaches have not only confirmed the observations from purification strategies, but also led to the discovery of several novel G-protein subunits. Indeed, cDNA clones have been isolated for all of the α subunits described in the previous section, from a variety of sources. These studies have confirmed the presence of multiple $G_i\alpha$ species (Itoh *et al.*, 1988a & b) and the two transducin subtypes (Lerea *et al.*, 1986).

cDNA clones of $G_o\alpha$ have been isolated from bovine retina (Van Meurs *et al.*, 1987), rat C6BU1 glioma cells (Itoh *et al.*, 1986 and 1988a) and rat olfactory epithelium (Jones & Reed, 1987). Comparisons of the predicted amino acid sequences indicated that these cDNA clones coded for the same $G_o\alpha$ form. However the olfactory cDNA hybridised to two separate mRNA's (Jones & Reed, 1987). This is in agreement with the previously discussed evidence from purification data which demonstrates the occurrence of more than one form of $G_o\alpha$. Recently Yoon and co-workers characterised a *drosophila* gene encoding two proteins each of 354 amino acids and differing in seven residues in the amino terminal region. The deduced amino acid sequences were 81% identical to mammalian $G_o\alpha$ and represented the first evidence of heterogeneity of this protein at the molecular level, with the two proteins being generated by alternate splicing of a single gene (Yoon *et al.*, 1989). More recently $G_o\alpha$ isoforms have been identified in a mammalian system. Screening of a hamster insulin-secreting tumour cell cDNA library revealed not only the presence of a $G_o\alpha$ cDNA equivalent to that

from rat and bovine tissue, but also a novel cDNA clone. This clone codes for a protein that is identical to $G_o\alpha$ in its first two thirds and differs in the remaining carboxyl third of the polypeptide (Hsu *et al.*, 1990).

$G_s\alpha$ cDNA clones have been isolated from the sources that provided the initial $G_o\alpha$ cDNA clone and from others including the mouse macrophage PU-5 cell line and S49 lymphoma cell line (Sullivan *et al.*, 1986; Rall & Harris, 1987). A subtype of G_s, termed G_{olf} has been cloned from olfactory epithelia. Although it may function in a manner analogous to G_s, its sole location is in olfactory neuro-epithelia where it is presumably responsible for olfactant regulation of adenylyl cyclase (Jones & Reed, 1987).

A pertussis toxin-insensitive $G\alpha$ clone has been isolated from rat brain and designated $G_x\alpha$ (Matsuoka *et al.*, 1988). This clone was equivalent to one isolated from human retina and designated, $G_z\alpha$ (Fong *et al.*, 1988). Despite the shared property of pertussis toxin-insensitivity this protein does not appear to be equivalent to the α42kDa proteins previously discussed. The mRNA encoding $G_z\alpha$ appears to be localised to neural tissues and antisera specific to $G_z\alpha$ do not recognise the α42kDa proteins (Matsuoka *et al.*, 1988; Taylor *et al.*, 1990).

Simon and colleagues have recently used the polymerase chain reaction (PCR) to detect additional gene products in mouse brain. PCR can be used to amplify selectively any DNA or RNA segment provided that some sequence information is known. Using, as primers, four sets of mixed oligonucleotides corresponding to highly conserved regions of the known G-protein α subunits, cDNA from total mouse brain and spermatids was amplified and DNA fragments of the expected size cloned. Five sequences, termed α_{10}-α_{14}, were detected. Four of these sequences were novel as α_{10} was found to be equivalent to α_{olf}. The sequence of the α_{11} clone is closely related to that of the α_{14} clone (Strathmann *et al.*, 1989). Strathmann and Simon have more recently obtained a murine cDNA clone, termed α_q, which is 88% identical to α_{11} and have proposed that these

clones represent a third class of α subunits. Interestingly, the amino acid sequences of tryptic fragments from the pertussis toxin-insensitive α_{42} subunits previously described, were identical to the deduced amino acid sequence of α_q. One of the α_{42} peptides also contained a sequence identical to the deduced amino acid sequence of $G\alpha_{11}$. Strathmann and Simon suggest that the "Gq class" of α subunits appeared early in evolution. Indeed a member of this class has been identified in *Drosophila melanogaster* where it is localised to the eye regions. Regulation of phosphoinositidase C in invertebrate eyes is central to the phototransduction cascade (Devary *et al.*, 1987). Such observations strengthened the speculation that the $G\alpha_q$ class represented "Gp-type" proteins, see 1.12.2. for further discussion (Strathmann & Simon, 1990).

Within this newly designated pertussis toxin-insensitive class of α subunits, the Gq class, three other isotypes have been discovered which exhibit tissue specific expression. $G\alpha_{14}$ is found in stromal and epithelial cells, whilst $G\alpha_{15}$ and $G\alpha_{16}$ are found in cells derived from the haematopoietic lineage (Simon *et al.*, 1991; Amatruda *et al.*, 1991).

Simon and co-workers have suggested that the murine cDNA clones, $G\alpha_{12}$ and $G\alpha_{13}$, represent another class of potentially pertussis toxin-resistant α subunits. The translation products are predicted to share less than 45% identity with other α subunits and transcripts have been detected in every tissue examined (Strathmann & Simon, 1991).

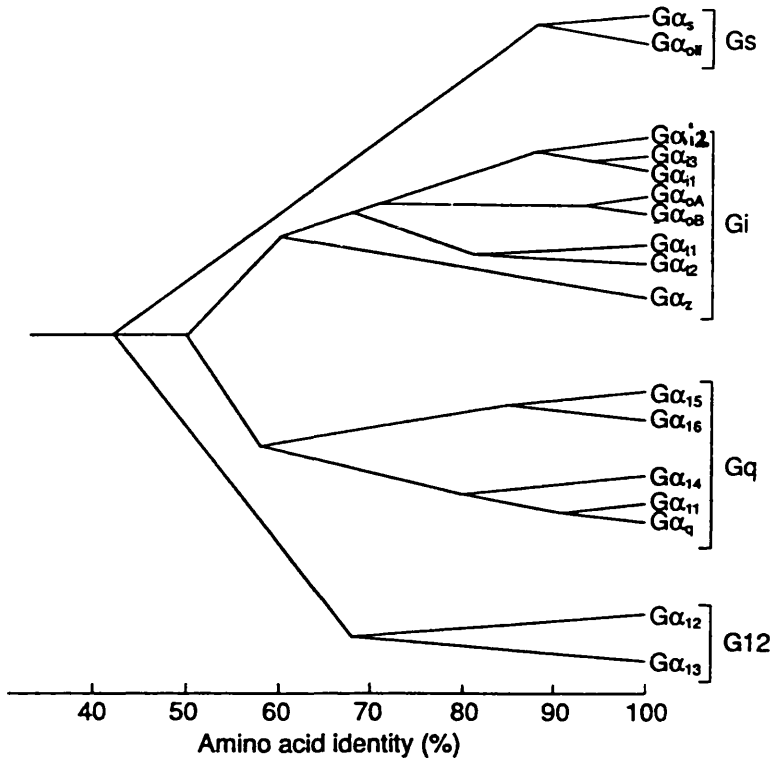
1.4.2. Primary structure conservation between α subunits.

When the predicted or known amino acid sequences of the G-protein α subunits are aligned they exhibit impressive sequence homology, as represented schematically in Figure 1.1, overleaf. Indeed, over 98% identity of amino acid sequence is maintained for $G_i2\alpha$, $G_i3\alpha$, $G_z\alpha$ and $G_o\alpha$ among evolutionary distinct mammalian species, presumably reflecting an evolutionary pressure to preserve the specific physiological function of each G-protein gene product.

Figure 1.1.

RELATIONSHIPS AMONG MAMMALIAN Gα SUBUNITS.

(adapted from Fig.2, Simon *et al.*, 1991).



The α subunits are grouped by amino acid identity which defines four distinct classes of Gα subunit. Branch junctions approximate the values calculated for each pair of sequences. The splice variants of $G_S\alpha$ have been omitted.

1.4.3. Cloning of βγ subunits.

Sequencing of the purified 36kDa β subunit (β_1) correlates with the predicted sequence of a cloned cDNA (Sugimoto *et al.*, 1985; Fong *et al.*, 1986). The 35kDa form of β subunit (β_2) is the product of a distinct cDNA (Fong *et al.*, 1987; Gao *et al.*, 1987). The deduced amino acid sequences of β_1 and β_2 are 90% identical and these sequences are highly conserved in different mammalian species. Recently a third cDNA clone for a β subunit (β_3) has been isolated from a human retinal λgt 10 cDNA library. This protein has a predicted amino acid sequence with approximately 80% identity to β_1 and β_2 and a Mr of 37kDa (Levine *et al.*, 1990).

A cDNA corresponding to the γ subunit, (γ_1), of transducin has been cloned (Hurley *et al.*,1984a). Predicted sequence from this cDNA information suggests a hydrophillic protein of 8.4 kDa, which differs significantly from predicted amino acid sequence of a brain γ subunit, (γ_2) (Robishaw *et al.*, 1989). More recently, PCR technology has been used to isolate a γ_3-subunit cDNA from a bovine brain cDNA library and a γ_4-subunit cDNA from mouse kidney and retina. Antisera specific to each of γ_1, γ_2 and γ_3 reveal variation in the expression of these subunits; γ_2 being ubiquitous; γ_3 specific to a particular tissue and γ_1 cell type-specific (Gautam *et al.*, 1990).

It is apparent from this overview that diversity exists in the structure of all the subunits of the G-protein heterotrimer and with the now routine use of PCR technology that novel G-protein subunits may be awaiting discovery. Indeed recent studies have suggested the daunting prospect that structural and potentially functional diversity of the G-protein family could be increased through preferential associations of the α, β and γ subunits (Gautam *et al.*, 1990).

1.5. MECHANISM OF ACTION OF G-PROTEINS.

1.5.1. The GTPase Cycle.

With the ability to measure hormonal stimulation of high-affinity GTPase activity and knowledge of the "activating" effects of cholera toxin and non-hydrolysable GTP analogues on G_S, Cassel and Selinger proposed a cyclical model of G-protein function (Cassel & Selinger, 1977). A more recent interpretation of this model, is represented in Figure 1.2, overleaf.

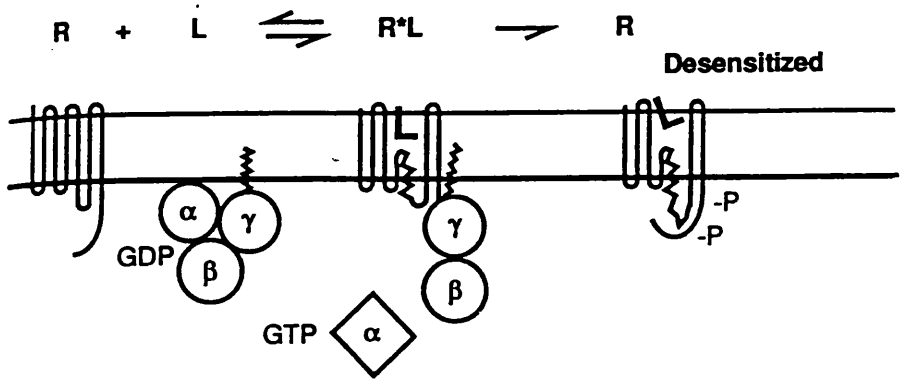
Binding of ligand to its receptor causes the α subunit to lose its bound GDP and bind GTP, in a Mg^{2+}-dependent manner (Brandt & Ross, 1986). The α-GTP dissociates from the βγ subunits, also in a Mg^{2+}-dependent manner. Agonists act to reduce the concentration of Mg^{2+} required for activation e.g., glucagon lowers the magnesium concentration required for GTPγS activation of G_S from 25mM to 10μM. As the intracellular Mg^{2+} is estimated to be of the order of 2mM it can then be utilised (Iyengar & Birnbaumer, 1982). The binding of GTP reduces the affinity of the receptor for the agonist, resulting in dissociation of the receptor-G-protein complex (Maguire *et al.*, 1976). The Gα-GTP is now in an "active" conformation suitable for specific interaction with an effector protein. Several lines of investigation have demonstrated the altered conformation of the α subunit upon activation, e.g., enhanced intrinsic tryptophan fluorescence (Higashijima *et al.*, 1987) and the altered reactivity of cysteine sulphydryls (Winslow *et al.*, 1987). On completion of effector interaction, GTP is hydrolysed to GDP by the intrinsic GTPase activity of the α subunit (Rodbell, 1980). The now inactive GDP-bound α subunit can reassociate with its βγ subunits. The inextricable dependence of this "cycle" upon magnesium is complex and will be discussed again briefly in Chapter 4.

Figure 1.2.

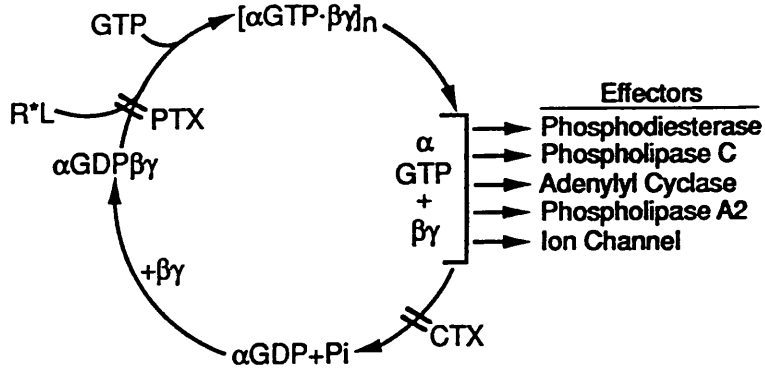
RECEPTOR-G-PROTEIN-MEDIATED SIGNAL TRANSDUCTION.

(adapted from Fig.1, Simon *et al.*, 1991).

A. Receptor - G-protein.



B. The G-protein cycle.



In A, receptor (R) associates with a specific ligand (L), stabilising an activated form of the receptor (R*) which can then initiate the cycle depicted in B and explained in 1.5.1. The βγ heterodimer may remain associated with the membrane through a 20-carbon isoprenyl modification (≷) of the γ subunit. The receptor is desensitised by e.g., specific phosphorylation (-P). Pertussis toxin (PTx) and cholera toxin (CTx) interfere with this "GTPase cycle", as indicated in B and described previously in 1.1.

Necessity for agonist occupancy of the receptor can be circumvented by use of non-hydrolysable analogues of GTP, such as Gpp[NH]p and guanosine 5'-[3-o-thio] triphosphate (GTPγS), although the reaction still requires magnesium (Codina *et al.*, 1983). G-proteins can also be activated by aluminium fluoride (AlF_4^-) which mimics the terminal phosphate of GTP when GDP is in the guanine nucleotide binding site (Bigay *et al.*, 1985).

The "subunit dissociation model" is based in part on its observation in solution, usually in the presence of detergent, after G-protein exposure to non-hydrolysable guanine nucleotides, or AlF_4^- and Mg^{2+}. Further indication of subunit dissociation is suggested by GTPγS induced conformational changes in G_s or G_i, resulting in altered sedimentation coefficients (Codina *et al.*, 1984; Miller *et al.*, 1988). More recently agonist binding to the solubilised somatostatin receptor has been demonstrated to dissociate the β36 subunit from the receptor-$G_i\alpha$ complex (Law *et al.*, 1991). However subunit dissociation has never been demonstrated directly in a membrane system, nor in response to GTP.

1.5.2. Regulation of the GTPase cycle.

Several lines of evidence suggest that the rate of GTP hydrolysis by G-proteins is mainly determined by the dissociation of GDP from the G-protein α subunit. The steady state rate of GTP hydrolysis is low, but when GDP-depleted prior to measurement or reconstituted with agonist-liganded receptor, the G-proteins exhibit a similarly and markedly enhanced rate of GTP hydrolysis (Brandt *et al.*, 1983: Higashijima *et al.*, 1987). Brandt and co-workers demonstrated that the actual rate of GTP hydrolysis was not influenced by agonist-liganded receptors, although several groups have shown that agonist-liganded receptors do increase the dissociation rate of bound GDP (Rodbell, 1980; Schramm & Selinger, 1984). The limiting nature of GDP dissociation is further indicated by the fact that the $k_{cat.GTP}$ is approximately ten-fold higher than the GDP-dissociation rate (Ferguson *et al.*, 1986). Interestingly, recent studies on purified proteins have demonstrated that

$G_i2\alpha$ binds GTPγS and releases GDP more rapidly than $G_i1\alpha$ and $G_i3\alpha$ (Carty *et al.*, 1990).

Two classes of regulatory protein exist that act to increase the intrinsically low rate constants for GDP release and GTP hydrolysis evident for many, but not all, GTPases. The guanine nucleotide release proteins (GNRP's) catalyse release of bound GDP, promoting its replacement with the relatively higher concentrations of cytoplasmic GTP. GTP hydrolysis is increased by the GTPase activating proteins (GAP's).

The GNRP for the signal transducing G proteins can be considered as a contribution from both the ligand-activated receptor and the $\beta\gamma$ subunit. As stated previously the ligand-activated receptor does act to increase the GDP dissociation rate. The receptor binds the $\alpha\beta\gamma$ complex much more tightly than it binds to α alone (Fung, 1983; Fung & Nash, 1983; Florio & Sternweis, 1989). Enhanced receptor association with α could be a consequence of $\beta\gamma$ interaction with the receptor and there is evidence to suggest an interaction of $\beta\gamma$ with the β-adrenergic receptor (Im *et al.*, 1988). Additionally, $\beta\gamma$ exhibits a preference for binding α·GDP and the combination of these properties ensure exchange of GTP for GDP and not *vice versa* (Bourne *et al.*, 1991).

Interestingly no extrinsic activators (GAP'S) of α subunit GTPase have been identified and a current speculative hypothesis is that a "built-in" GAP-like domain of G-protein α subunits maintains constitutive GTPase activity. Compared to the GAP-triggered GTPase activity of $p21^{ras}$ and ribosome-triggered GTPase of elongation factor (EF-Tu), the G-proteins hydrolyse GTP much more slowly, $k_{cat.GTP} = 2\text{-}4\ min^{-1}$ (Freissmuth *et al.*, 1989; Graziano & Gilman, 1989; Landis *et al.*, 1989). However, this intrinsic GTP hydrolytic rate appears to be slow enough to enable signal transduction yet rapid enough to control a turn-off of signal transmission (Bourne *et al.*, 1990 and 1991).

1.5.3. Functional consequences of the GTPase reaction.

The GTPase reaction provides "directionality". Indeed the essentially

irreversible hydrolysis of GTP prevents the "backward" signalling between effector and receptor. Inactivation rate is thus governed by GTPase activity, rather than removal of the activating ligand.

In the adenylyl cyclase system each hormone-receptor complex persists for less than one second, but generates one or more GTP-bound α_S subunits, active for ten seconds or more (Casey & Gilman, 1988). It would appear that the intrinsic rate of GTP hydrolysis in the G-protein has been set to maximise signal amplification.

1.6. G-PROTEIN α SUBUNIT STRUCTURE WITH RESPECT TO FUNCTION.

To date, no Gα subunit has been crystalised although the crystal structure of the bacterial elongation factor, EF-Tu has been resolved (Jurnak, 1985). This crystal structure, in conjunction with the sequence alignment of the predicted sequences of seven G-protein α chains, forms the basis for a model of the GDP-binding domain of a hypothetical "average" α chain, termed α_{avg}, see Figure 1.3., overleaf (Masters *et al.*, 1986).

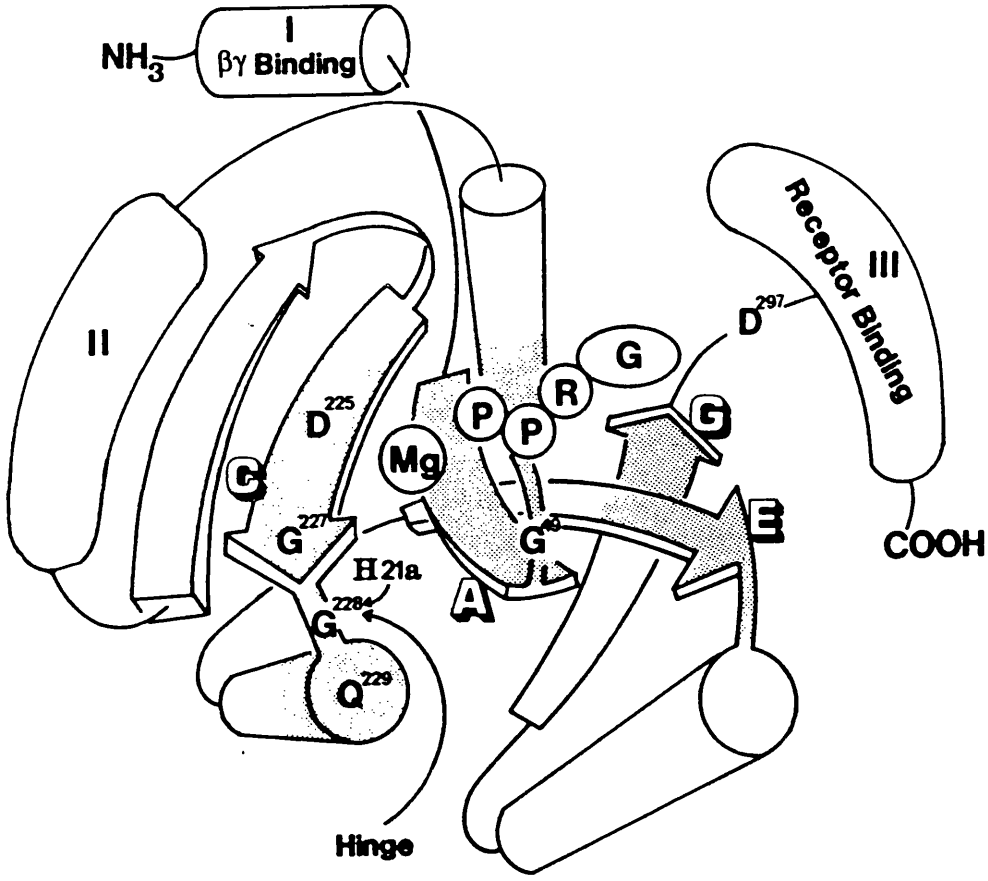
More recently further information, particularly with respect to the GDP binding domain, is available from the elucidation of the crystal structures of the p21^{ras}·GDP complex (deVos *et al.*, 1988) and its comparison with structures of p21^{ras}·GTP (Schlichting *et al.*, 1990) or to the Gpp[NH]p and Gpp[CH_2]p bound forms (Pai *et al.*, 1990; Milburn *et al.*, 1990).

Using information from these predicted structural models, from phenotypes of both natural and man-made mutations in p21^{ras} and Gsα and more recently from analysis of chimeric α subunit constructs, proposed functional regions are beginning to be assigned.

Figure 1.3.

DIAGRAM OF THE PREDICTED GDP-BINDING DOMAIN OF A HYPOTHETICAL AVERAGE α SUBUNIT.

(adapted from Masters *et al.*, 1986).



The GDP molecule (G, guanine ring; R, ribose; P, phosphoryl) nestles into a binding site bounded by turns between β strands and α helices. These turns are in the shaded "Halliday" regions designated A, C, E and G, as described in 1.6.1. Key amino acid residues are numbered as for an α_{avg} with D corresponding to aspartic acid, G to glycine and Q to glutamine. The hinge region and domains I, II and III are explained within 1.6.1.

1.6.1. Guanine nucleotide binding and hydrolysis.

The shaded regions in Figure 1.3., designated A, C, E and G, correspond to four stretches of highly conserved amino acid sequence. Halliday predicted that these regions would form part of the guanine nucleotide binding site and this has since been proved correct (Halliday, 1984).

Studies of p21^{ras} demonstrate that region "A" in Figure 1.3., is important in binding the α and β phosphates of GTP or GDP (Milburn *et al.*, 1990; Pai *et al.*, 1990). In p21^{ras} mutational replacement of Gly12 (equivalent to Gly49 in a_{avg}) by almost any other amino acid produces a protein with decreased GTP hydrolytic activity (Barbacid, 1987) and enhanced transforming capacity (Seeburg *et al.*, 1984).

Region "C", in conjunction with region "A" in the α_{avg} model is important for Mg^{2+} binding and GTP-induced conformational alteration and hydrolysis. An invariant aspartate, Asp57 in p21^{ras}, equivalent to Asp225 in a_{avg}, binds the catalytic Mg^{2+} through an intervening water molecule (Pai *et al.*, 1990). Transforming mutations of p21^{ras} have been identified at positions corresponding to residues 227, 229 and 231 of α_{avg} (Barbacid *et al.*, 1987). GTP-dependent conformational change requires Mg^{2+} and as anticipated regions equivalent to "C" in p21^{ras} do undergo prominent GTP-induced changes. The phenotype of the S49 mutant cell line, H21a results from replacement of glycine by an alanine residue in α_S, equivalent to Gly228 of a_{avg} (Miller *et al.*, 1988). The H21a G_S can bind GTP but cannot stimulate adenylyl cyclase in response to hormones, GTP analogues, AlF_4^- or cholera toxin (Bourne *et al.*, 1981), nor does it dissociate from βγ (Miller *et al.*, 1988). One speculative interpretation of the mutation in the H21a α chain is that glycine 228 may serve as a "hinge" that allows relative movement of separate domains of the protein and that this regions flexibility could be decreased by substitution with an alanine residue.

Studies of p21^{ras} suggest that the GTP hydrolytic mechanism centres around the activation of a water molecule by the carbamoyl oxygen of Gln61,

equivalent to Gln^{227} in α_{avg}, which mounts a nucleophillic attack on the γ phosphorous of GTP. Indeed residues 60-63 in p21*ras* undergo dramatic conformational alterations depending on the nucleotide bound. Further the orientation of Tyr^{32} differs markedly in the GDP and GTP-bound forms of p21*ras*. GTP-binding induces the tyrosine residue to partially block the entrance to the guanine nucleotide binding pocket (Milburn *et al.*, 1990). Tyr^{32} in p21*ras* could be synonymous with Arg^{201} in G-proteins. An arginine cognate to Arg^{201} is conserved among the G-protein α chains and cholera toxin-catalysed attachment of ADP-ribose to this residue slows GTP hydrolysis by α_s (Cassel & Pfeuffer, 1978). This GTPase inhibiting effect is mimicked by oncogenic (*gsp)* mutations of α_s found in pituitary and thyroid tumours, where Arg^{201} is replaced with cysteine or histidine (Landis *et al.*, 1989; Lyons *et al.*, 1990). Mutations in the cognate Arg of Gi2α, termed *gip2*, have been found in tumours of the human adrenal cortex and ovary (Lyons *et al.*, 1990). This arginine residue obviously plays a critical role in the GTPase mechanism of these proteins and from the studies of Tyr^{32} in $p21^{ras}$ it has been speculated that this arginine residue may manoeuvre another residue into a position favourable for nucleophillic attack on the γ phosphorous of GTP (Bourne *et al.*, 1991).

The guanine ring of the nucleotides is thought to be held in a hydrophobic pocket formed by very highly conserved residues from regions E and G in α_{avg}. Substitution of an alanine for an aspartate in $p21^{ras}$, corresponding to Asp^{297} in a_{avg}, lowers the binding affinity for guanine nucleotides and removes discrimination between GDP and the inosine nucleotide, IDP (Sigal *et al.*, 1986).

1.6.2. Receptor interaction site.

Efforts to identify receptor and effector binding domains have concentrated on regions of G-protein α subunits that exhibit divergent amino acid sequence, designated domains I, II and III in Figure.1.3., with the view that if specificity exists it presumably must reflect protein-protein interaction with these areas.

There is evidence to define the carboxy-terminal region of the α chains as one of the sites involved in receptor interaction. The bacterial toxin, pertussis toxin, catalyses ADP-ribosylation of a cysteine residue, located four amino acids from the carboxy terminus of certain G-proteins. Such ADP-ribosylation prevents G_i, G_o and transducin from responding to stimulation from their respective receptors (Ui *et al.*, 1984; Van Dop *et al.*, 1984a).

Uncoupling of G_s from receptors that stimulate adenylyl cyclase is the characteristic phenotype of the *unc* mutation (Haga *et al.*, 1977), in which an arginine is replaced by a proline residue at the extreme C-terminus of α_s (Sullivan *et al.*, 1987; Rall & Harris, 1987).

Construction of recombinant chimeric α chains has produced phenotypes which reinforce the idea of a carboxy-terminal receptor contact region that also confers specificity of interaction. Expression of a chimera, composed of the amino-terminal 60% of α_i2 and the carboxy-terminal 40% of α_s, in S49 cyc^- cells produces a cell in which β-adrenoreceptors stimulate adenylyl cyclase, a function known to be mediated by $G_s\alpha$. The α_i/α_s chimera did not interact with receptors coupled to G_i in these cells (Masters *et al.*, 1988).

Another chimeric α chain, termed α_s-i(38), in which the carboxy-terminal 38 residues of α_s have been replaced with the carboxy-terminal 36 residues of α_i2 has been expressed in Chinese hamster ovary (CHO) cells (Woon *et al.*, 1989). Phenotypically, cells expressing this chimera exhibit a constitutively activated adenylyl cyclase activity even in the absence of receptor agonists. The characteristic temporal lag in activation due to slow dissociation of GDP from α_s is no longer observed. In the wild-type hormone-receptor complexes act to abolish this lag by accelerating the GDP dissociation rate and it is tempting to speculate that the replacement C-terminal 36 residues of $G_i2\alpha$ are mimicking the effect of the hormone-receptor complex on "normal" $G_s\alpha$.

The carboxy-terminal region of α_{avg} is predicted to be an α helix. It has

been suggested that this α helix may suppress release of GDP when the G-protein is in the inactive conformation. Upon receptor contact, the α helix would then be dislodged and GDP released from the activated G-protein. This hypothesis would agree with the idea of receptors as GNRP'S, as discussed previously in 1.5.2. Further, it could account for the phenotype of the previously described chimeric α chain, α_s-i(38), where it is possible that the $\alpha_i 2$ carboxy terminus is a "poor fit" and in effect does not suppress GDP release.

1.6.3. Effector contact site.

The location of the effector contact region of G-protein α chains remains undefined, although from the studies just outlined of the α_s-i(38) chimeric protein it would seem that the site for adenylyl cyclase interaction is not contained within the carboxy-terminal 38 residues of α_s as the chimeric protein constitutively activates adenylyl cyclase.

Indeed Masters and co-workers have proposed that domain II in Fig.1.3., may mediate effector interactions (Masters *et al.*, 1986). However if this is correct then the 60:40 α_i/α_s chimera described previously would not be expected to stimulate adenylyl cyclase, as domain II is derived from $\alpha_i 2$. This chimera does mediate stimulation of adenylyl cyclase by receptors that stimulate this enzyme in normal cells. These observations suggest that domain II does not specify effector interaction in this instance and raises the possibility of a role for domain III. Further experimentation is required to address this question and it may be that residues in both domains II and III contribute to effector interaction.

1.6.4. Contact with βγ subunits.

Proteolytic removal of the amino-terminal 21 amino acids of the α chain of the rod cell transducin destroys its ability to bind to βγ (Navon & Fung, 1987). The truncated α_t can no longer bind to rhodopsin or exchange guanine nucleotide. An interpretation of these effects is that βγ binds to the extreme amino terminus of the α chain. This region (domain I) is not highly conserved in amino acid sequence

although a predicted α helical secondary structure is conserved among the α chains. It has been suggested that the α helical nature of this region, rather than strict conservation of amino acid sequence, may specify interaction with βγ. Definition of the site of interaction of α-βγ assumes more importance as the critical functional role of the βγ complex is becoming more apparent.

1.7. βγ SUBUNITS.

β and γ subunits, although not covalently linked to each other, cannot be dissociated without denaturation and form a single functional unit rendering the G-protein as a functional dimer.

1.7.1. Membrane association of βγ subunits.

It has been suggested that βγ complexes may serve as a hydrophobic anchor holding hydrophillic α subunits at the membrane. This hypothesis is based on the failure of purified α subunits to associate with artificial phospholipid vesicles unless βγ complexes were also present (Sternweis, 1986). The C-terminal region of γ subunits have been shown to be isoprenylated. The rod photoreceptor γ subunit is farnesylated, whereas brain γ subunits have a geranyl-geranyl modification (Fukada *et al.*, 1990; Mumby *et al.*, 1990a). However a number of studies have demonstrated that α subunits can associate with the membrane independently of βγ complexes and that N-terminal myristoylation of certain α subunits is critical to membrane association (Mumby *et al.*, 1990b). Lipid modification of G-protein subunits and the molecular basis of membrane association is currently being elucidated (reviewed by Spiegel *et al.*, 1991).

1.7.2. βγ subunit - α subunit interactions.

The true pertussis toxin substrate is the αβγ heterotrimer (Tsai *et al.*, 1984; Katada *et al.*, 1986 b; Mattera *et al.*, 1987). However, using the ability of βγ to influence toxin catalysed ADP-ribosylation of the α subunit, it has been observed that α_o has an apparent three fold lower affinity for βγ than of α_i (Huff & Neer, 1986; Katada *et al.*, 1986b). In the light of recent developments this may reflect

preferential associations between different G-protein subunits rather than altered affinities of interaction with "shared" βγ subunits (Gautam *et al.*, 1990).

βγ subunits affect the guanine nucleotide binding properties of the α subunit, as discussed previously (see 1.5.2). The affinity of α_o for GDP is increased by association with βγ from a Kd of 40nM to a Kd of 0.1nM, an effect diminished by Mg^{2+}. As expected at low Mg^{2+} concentrations, βγ inhibits the GTPase activity of α_o. It should be noted that these α and βγ subunit reconstitution studies are performed in the absence of hormone receptor (Higashijima *et al.*, 1987). This ability of βγ to stabilize α·GDP may ensure reduction of spontaneous, receptor-independent release of GDP. This property may be considered as an additional potential advantage of incorporating βγ into the GTPase as well as the previously mentioned promotion of nucleotide exchange (Bourne *et al.*, 1991). Interestingly, farnesylation of the γ_1 subunit has recently been shown to be essential for the GTP binding activity of transducin (Fukada *et al.*, 1990).

1.7.3. βγ Interaction with adenylyl cyclase.

Northup and co-workers demonstrated that by increasing the βγ concentration the activation of adenylyl cyclase by GTPγS-liganded α_s is reversed (Northup *et al.*, 1983a and b). This work led to the first assay for "free" βγ subunit function and using this approach it has been demonstrated that βγ subunits from various different sources were all able to deactivate α_s, although there were differences in the concentration dependence (Cerione *et al.*, 1987). These studies suggested the possibility that βγ released from activation of Giα could "mop up" and inhibit the α_s subunit.

However, the fact that somatostatin can inhibit adenylyl cyclase activity in the S49 cyc- mutant cell (which lacks Gsα) suggests an alternative inhibitory mechanism (Hildebrandt *et al.*, 1982). A possibility is that α_i directly inhibits the catalytic unit of adenylyl cyclase, but this has proved difficult to demonstrate, with

inhibition by GTPγS-liganded α_i requiring initial Gsα stimulation (Katada *et al.*, 1986b). However, a drawback of these reconstitution systems is that α_i subunit effects may be artificially influenced by subunit stoichiometry.

Katada and co-workers have proposed that the βγ subunit may inhibit the cyclase catalytic unit by association of βγ with calmodulin. The brain adenylyl cyclase catalytic unit is a form sensitive to activation by Ca^{2+}-calmodulin (Salter *et al.*, 1981). Relatively low βγ subunit concentrations inhibited the activity of this adenylyl cyclase form, inhibition being attributable to the association of βγ with calmodulin (Katada *et al.*, 1987b). However recent work has demonstrated that this proposed mechanism is incorrect. Indeed two groups have discovered that in the presence of Gsα, the different forms of adenylyl cyclase display differential sensitivities to stimulation by βγ subunits, with one form inhibited, some activated and others not affected (Tang & Gilman, 1991; Federman *et al.*, 1992). The implications of these studies will be discussed further in Chapter 7.

1.7.4. βγ Interaction with phospholipases and ion channels.

Jelesma and Axelrod demonstrated that addition of transducin α inhibited βγ-induced stimulation of phospholipase A_2 (PLA_2) in dark-adapted, "transducin-depleted" rod outer segments. This inhibition appears to be dependent upon subunit reassociation as the GTPγS-liganded α subunit did not exert an inhibitory effect (Jelsema & Axelrod, 1987).

In the *Xenopus* oocyte, muscarinic acetylcholine receptor activation of phosphoinositidase C results in an inositol(1,4,5)trisphosphate activated Cl^- current. This current can be almost totally inhibited by injection of human erythrocyte or bovine brain βγ, but not α subunits (Moriarty *et al.*, 1988). βγ subunits also modified AlF_4^- and P_{2Y}-purinergic receptor-stimulated PLC activity in turkey erythrocyte membranes (Boyer *et al.*, 1989). Importantly,these studies did suggest that a heterotrimeric G-protein was involved in PLC coupling.

Akin to the recent observations with certain forms of adenylyl cyclase,

cardiac potassium channels can be activated by both α and βγ subunits (Logothetis *et al.*, 1988, Yatani *et al.*, 1988). However, in this case βγ effects may be mediated by PLA_2 (Kim *et al.*, 1989).

1.8. G-PROTEIN LINKED RECEPTORS.

Since the purification of the β-adrenergic receptor by Shorr and co-workers, intensive research effort has been aimed at deciphering many aspects of receptor structure and function (Shorr *et al.*, 1981).

1.8.1. Identification of a G-protein linked receptor.

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

As described previously, ligand binding assays initially demonstrated the possibility of a G-protein link to a rat liver glucagon receptor, by the decreased affinity of glucagon binding in the presence of guanine nucleotides (Rodbell *et al.*, 1971b). Maguire and co-workers later verified that this effect was peculiar to agonists and not antagonists (Maguire *et al.*, 1976). Analysis of competition curves from further studies on β-adrenergic receptors suggested the existence of two receptor affinity states for the agonist as opposed to one affinity state for the antagonist. It was proposed that in the presence of guanine nucleotides receptors adopted a low affinity state, whereas a high affinity state for agonist persisted in the absence of guanine nucleotide (Delean *et al.*, 1980). These workers hypothesised that in the basal state, the inactive G-protein α subunit may interact with a receptor

leading to the agonist-promoted formation of a "high affinity ternary complex". Upon agonist promoted guanine nucleotide exchange, the ternary complex is destabilised and dissociated. The agonist and G-protein can dissociate from the now low affinity receptor. This "ternary complex model" was originally based on the observations of agonist-induced stimulation of adenylyl cyclase, however receptors linked to the "inhibitory arm" of this transduction pathway were also found to fit this interpretation (U'Prichard *et al.*, 1978; Koski *et al.*, 1981). These observations led to the general conclusion that guanine nucleotide-induced agonist affinity alterations implicate a G-protein coupled receptor. Recent methodological approaches to assign coupling specificity using these properties as a basis will be described later (see Chapter 5).

Interestingly, guanine nucleotides have been shown recently to modulate receptors that possess an intrinsic tyrosine kinase activity, which perhaps questions the view that these receptors interact directly with effector molecules and as such constitute a class of receptors distinct from G-protein coupled receptors (reviewed by Ives, 1991).

Another property characteristic of a G-protein linked receptor is agonist-stimulated GTPase activity. The details of this process have been described previously and although GTPase activity was first identified as G_S mediated, it has only been possible to measure G_S GTPase activity in a limited number of systems, namely the turkey erythrocyte (Cassel & Selinger, 1976) and the human platelet (Houslay *et al.*, 1986). In contrast, stimulation of GTPase activity by receptors which interact with pertussis toxin-sensitive G-proteins has been reported for a wide variety of membrane systems, in particular those mediating inhibition of adenylyl cyclase (McClue *et al.*, 1992).

Agonist-stimulated GTPase activity has only recently been demonstrated for a potential pertussis toxin-insensitive "Gp". Thromboxane A_2 stimulated a small, yet significant, increase in GTPase activity in human platelet membranes mediated via the Gq protein (Shenker *et al.*, 1991). The low level of stimulated GTPase

activity may explain the lack of success of previous attempts to measure this activity upon stimulation of receptors that couple to a pertussis toxin-insensitive "Gp". It is becoming evident that the intrinsic GTP hydrolytic properties of the different Gα subtypes may differ. Indeed, the intrinsic GTPase activity of purified Gsα is lower than that of purified Giα (Gilman, 1987) and purification data suggests that these Gq/G11α proteins do bind GTPγS with slow rates (as discussed in 1.3.4). Thus, with these caveats, receptor-stimulated high affinity GTPase activity is a useful indication of a G-protein linked receptor.

Using these approaches, among others, has led to the identification of approximately 80 distinct G-protein coupled receptors that recognise at least 40 hormones and neurotransmitters and at present couple to approximately 15 effector actions (tabulated in Birnbaumer *et al.*, 1990). Birnbaumer and coworkers have predicted that the total number of distinct G-protein coupled receptors could be of the order of 100-150 (Birnbaumer *et al.*, 1990).

As previously mentioned these receptors mediate regulation of the activity of adenylyl cyclase (Krupinski *et al.*, 1989; Levitzki, 1986) and cGMP phosphodiesterase (Stryer, 1988). Further they mediate agonist-stimulation of phosphoinositidase C (Litosch *et al.*, 1985), phospholipase A_2 (Okajima & Ui, 1984) and possibly phospholipase D (Bokkino *et al.*, 1987). G-protein coupled receptors also mediate the inhibition of voltage operated Ca^{2+} channels (Yatani *et al.*, 1987b; Rosenthal *et al.*, 1988) and the stimulation of certain voltage operated K^+ channels (Yatani *et al.*, 1987a; Brown & Birnbaumer, 1988).

1.8.2. Structural features of G-protein coupled receptors.

Although these receptor-mediated functions are diverse, the cloning and sequencing of several G-protein linked receptors has demonstrated strong structural homology. The list of mammalian receptors which have been purified and cloned is increasing constantly and includes four opsins mediating visual transduction, adrenergic and muscarinic receptor subtypes, substance K and dopamine receptors

and serotonin subtypes (for references see Birnbaumer *et al.*, 1990). The adrenergic receptors are the most well studied G-protein coupled receptors, with current research aimed at establishing if this family are representative.

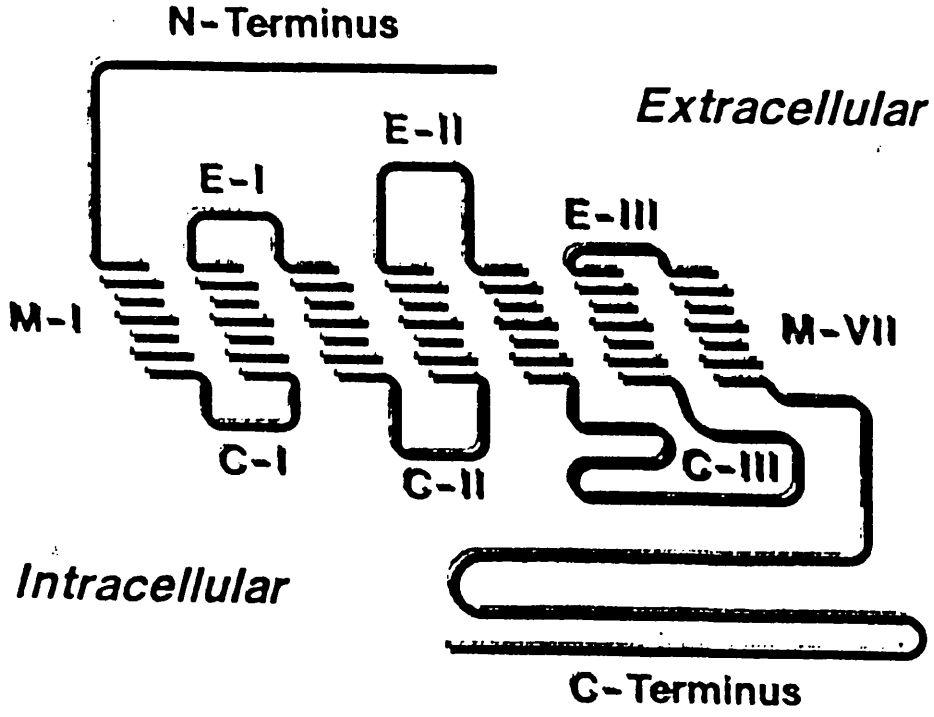
All the G-protein coupled receptors are integral membrane proteins that require detergents for their solubilisation. At present most structural information is based on analogy with the major membrane protein of *Halobacterium halobium*, bacteriorhodopsin. Electron diffraction studies have revealed a bundle of seven membrane-spanning α-helices, a structure verified by study of vertebrate rhodopsin (reviewed in Applebury & Hargrave, 1986). Hydropathy analysis of the amino acid sequences of the other G-protein coupled receptors has revealed seven hydrophobic, putative membrane spanning regions each composed of 19-24 amino acids, connected by six alternating intra- and extracellular loops (Strader *et al.*, 1989), represented schematically in Figure 1.4, overleaf. Studies of the hamster lung β2-adrenergic receptor using techniques of limited proteolysis have verified various features of the general receptor model and identified two amino-terminal sites of N-glycosylation and intracellular carboxyl terminal sites for *in vitro* phosphorylation (Dohlman *et al.*, 1987a).

Although there appears to be strong structural homology when predicted amino acid sequences of the cloned mammalian receptors are compared only 19 amino acids of the 450 to 600 are conserved (Birnbaumer *et al.*, 1990). Greater sequence homology is evident among receptor subtypes, particularly in transmembrane spanning regions, and this has been useful in the design of oligonucleotide probes to isolate cDNA's encoding other G-protein coupled receptors (Libert *et al.*, 1989; Zeng *et al.*, 1990; Parmentier *et al.*, 1989). The first two cytoplasmic loops are reasonably conserved between different receptors, but the extracellular domains, the third cytoplasmic loop and the carboxyl-terminal tail are all quite divergent, possibly reflecting a molecular basis for variable ligand and G-protein specificity.

Figure 1.4.

SCHEMATIC REPRESENTATION OF G-PROTEIN COUPLED RECEPTOR PROTEINS WITHIN THE PLASMA MEMBRANE.

(adapted from Fig. 1, page 298, Caron *et al.*, in *G-proteins*, Iyengar, R and Birnbaumer, L. eds, Academic Press Inc, 1990).



Segments M-I to M-VI are proposed to span the lipid bilayer. The amino terminus and the connecting loops E-I, E-II and E-III are postulated to be extracellular. Connecting loops C-I, C-II and C-III and the carboxyl terminus are proposed to be exposed to the cytoplasmic side of the plasma membrane.

1.8.3. Ligand binding domains.

Current data suggests that ligand binds to receptor within the plane of the lipid bilayer (Findlay & Pappin, 1986; Dixon *et al.*, 1986). Mutants of the hamster β_2-adrenergic receptor with deletions midway through the seventh or after the fifth putative membrane-spanning regions of the protein do not bind ligands (Kobilka *et al.*, 1987). Further single amino acid changes of the highly conserved residues present in membrane-spanning domains II, III or VII lead to alterations in the ligand binding properties of the β-adrenergic receptor (Dixon *et al.*, 1987; Strader *et al.*, 1987a). Although the general area for ligand binding is beginning to be mapped, the residues involved in determining ligand specificity and receptor subtype ligand selectivity remain to be defined.

1.8.4. G-Protein binding domains.

From theoretical considerations of the transient and specific nature of receptor - G-protein interactions, it has been proposed that they must occur at the inner surface of the plasma membrane (Chabre, 1987). The cytoplasmic loops C-I and C-II, in Figure 1.4, generally show significant conservation in size and sequence among the hormone receptors (Dohlman *et al.*, 1987b). However loop C-III displays remarkable variability being relatively small in opsin and large in the α_2-adrenergic and muscarinic receptors, leading to speculation of its involvement in G-protein binding. Several deletional mutants in this loop, including those at the amino terminal end of the loop, impair the ability of the hamster β_2-adrenergic receptor to mediate stimulation of adenylyl cyclase (Strader *et al.*, 1987b). However further studies have indicated that the residues at the amino-terminal end of this loop are not crucially involved in regulating the specificity of G-protein binding. When this portion of the human β-adrenergic receptor loop was replaced by the sequence from the human platelet α_2-adrenergic receptor, which mediates inhibition of adenylyl cyclase *in vivo*, only a slight loss of receptor-stimulated adenylyl cyclase activity was observed (O'Dowd *et al.*, 1988). Expression of a

series of α_2/β_2-adrenergic receptor chimerae in *Xenopus* oocytes has defined minimal regions of the β_2-adrenergic receptor which confer on the α_2-adrenergic receptor the ability to mediate stimulation of adenylyl cyclase. These involve the regions extending from the amino-terminal region of the fifth membrane-spanning domain through the third cytoplasmic loop to the carboxy-terminal region of the sixth membrane-spanning domain (Kobilka *et al.*, 1988).

Conversely, limited tryptic digestion of the turkey erythrocyte β-adrenergic receptor implies that most of its third cytoplasmic loop and the carboxy-terminal region are not necessary for agonist-stimulated regulation of G_S (Rubenstein *et al.*, 1987). Studies are at present addressing the reason for these contradictory observations.

1.8.5. Regulation of receptor function.

The tight control of hormonal responsiveness is reflected at the level of the receptor. One major form of agonist-dependent regulation is desensitisation. This is a process by which the response to a stimulus decreases with time despite the presence of a constant stimulus. In homologous desensitisation responsiveness to only the specific desensitising hormone is attenuated, whereas in heterologous desensitisation there is also a decrease in responsiveness to multiple agonists operating through distinct receptors. Evidence suggests that multiple mechanisms aimed at each component of the "signalling machinery" are involved in these desensitisation processes (reviewed by Nathanson, 1987). Indeed the processes associated with the desensitisation of the β-adrenergic receptor are rapid uncoupling (mediated by phosphorylation), sequestration and down-regulation.

Briefly, receptor phosphorylation appears to be involved in both forms of desensitisation, with evidence for cAMP-independent and cAMP-dependent processes being involved in homologous and heterologous desensitisation, respectively. Indeed from extensive study of the β-adrenergic receptor the possibility of receptor regulation by three different kinases each exhibiting a

differential requirement for agonist occupancy has emerged (Caron *et al.*, 1990). Whether these processes occur for all G-protein linked receptors remains to be elucidated.

Down-regulation is defined as the loss of receptor binding sites in the cell with an accompanying loss of effector stimulation and occurs following prolonged stimulation. Current knowledge about the molecular mechanisms underlying this process with respect to adrenergic receptors has been reviewed by Collins and co-workers (Collins *et al.*, 1991).

Thus the G-protein family and the receptors with which they interact have been introduced. The effectors that these "transducers" interact with have been investigated just as intensively, with recent appreciation not only of their individual complexity but also their interdependence upon one another. The remainder of this introduction will concentrate on a brief account of the phospholipase enzymes. Studies of these enzymes and the metabolic pathways that they are involved in are fields of intensive research in themselves. As such only an overview will be provided.

1.9. INOSITOL PHOSPHOLIPID HYDROLYSIS.

1.9.1. HISTORICAL OVERVIEW.

Agonist-stimulated inositide metabolism was discovered when it was demonstrated that a variety of stimuli including acetylcholine, pancreozymin and catecholamines enhanced the metabolic turnover of phosphatidylinositol (PtdIns) and its precursor phosphatidate (PtdOH) in many secretory and nervous tissues. It was proposed that the metabolism of this membrane phospholipid functioned to regulate membrane properties such as transmembrane ion transport or exocytotic secretion (Hokin & Hokin, 1953).

Despite these early observations it was between 1969 and 1979 that the concept of inositol-containing lipids being involved in novel receptor-coupled signalling mechanisms evolved. The Hokins had suggested that the initial receptor mediated enzymatic step in inositol lipid metabolism might be the hydrolysis of PtdIns to DG and inositol 1-phosphate by an phosphatidylinositol lipid-specific phospholipase C (Hokin, 1968). However Durell and co-workers appreciated that although such a phospholipase C-catalysed hydrolytic mechanism was a central reaction , its substrate had not been clearly defined, raising the possibility that phosphorylated derivatives of PtdIns could fulfil this role (Durell *et al.*, 1969).

A series of experimental observations lent indirect credence to the concept of a role for inositol lipid breakdown in receptor-mediated calcium mobilisation. Studies of the adrenal medulla, platelet and parotid gland suggested that the receptor-triggered rise in cytosolic calcium was not the cellular activator of inositol lipid metabolism. These observations stimulated the hypothesis that calcium mobilization may be resultant from receptor-activated inositol lipid metabolism (Michell, 1975; Jones & Michell, 1978). A prerequisite for inositol lipid breakdown as a "signalling" reaction was that it was a rapid event and this was observed (Michell *et al.*, 1977). In further agreement with the concept of a receptor-coupled reaction, agonist-stimulated inositol lipid breakdown occurs in a dose-dependent manner corresponding to that of agonist occupancy of the receptor (Michell *et al.*, 1976; Michell *et al.*, 1977; Jones & Michell, 1978). Another fundamental observation was that increased inositol lipid metabolism appeared to correlate with proliferation of normal and transformed cells (Michell, 1979).

A major advance was the demonstration that a receptor that stimulated calcium mobilisation could be desensitised upon prolonged agonist exposure and that resensitisation was facilitated by an inositol-containing medium. This was observed with 5-hydroxytryptamine stimulation of calcium mobilisation in blow fly salivary glands and demonstrated the essential requirement for some inositol derivative in this receptor controlled calcium flux (Fain & Berridge, 1979).

1.9.2. The phosphatidylinositol cycle.

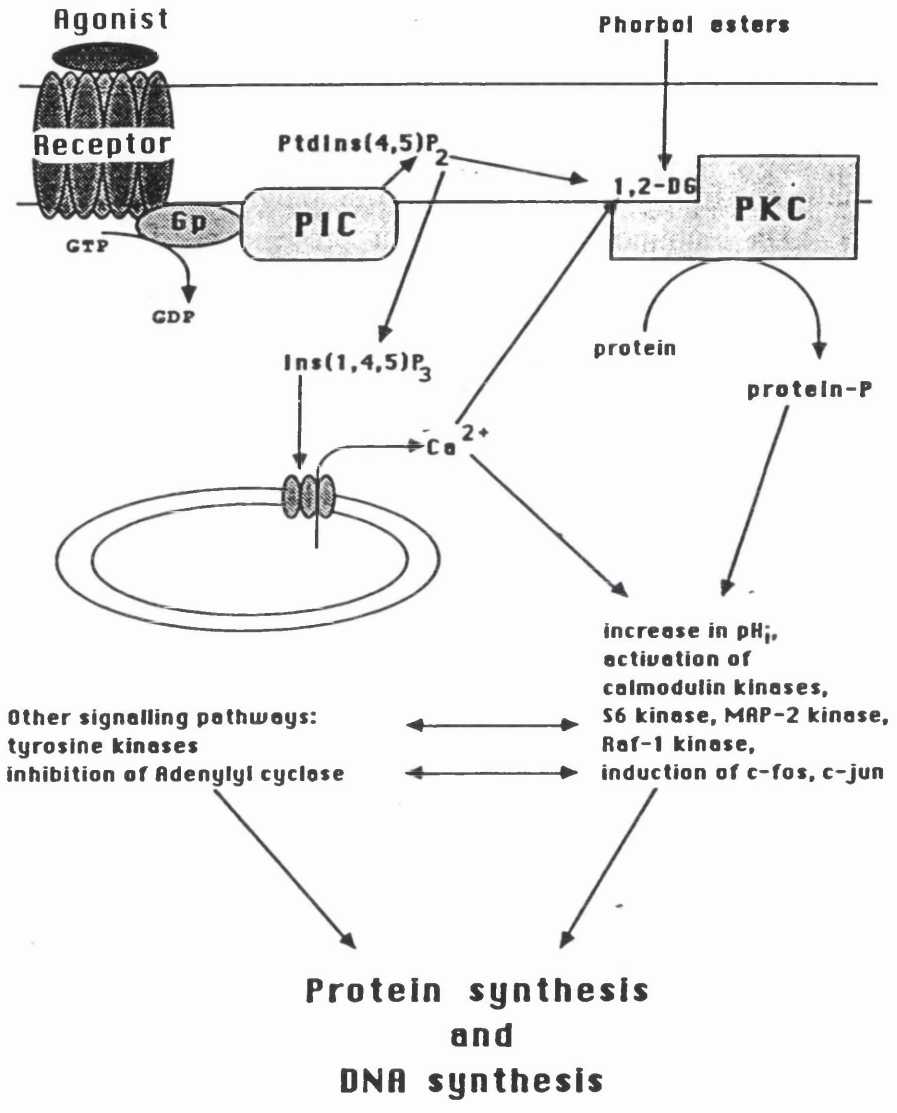
Thus the current view of receptor-controlled inositol lipid breakdown has been formed from these and other principle observations although the intensity of the research is continuously altering and refining the model. It is now appreciated that phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2) rather than phosphatidylinositol (PtdIns) is the initial lipid hydrolysed upon receptor activation (Berridge, 1984; Berridge & Irvine, 1984; Downes & Michell, 1982). PtdIns(4,5)P_2 constitutes only a few percent of the inositol phospholipids implying a necessity for rapid metabolic turnover in both stimulated and unstimulated cells. Indeed, in unstimulated hepatocytes, at metabolic steady state, PtdIns(4,5)P_2 and PtdIns(4)P have a turnover time of a few minutes (Thomas *et al.*, 1983). Evidence from a variety of studies now implies that inositol lipids exist in at least two metabolically distinguishable pools, with only the smaller pool subject to rapid degradation on hormone addition (Monaco & Woods, 1983).

Agonist-stimulated hydrolysis of PtdIns(4,5)P_2, catalysed by phosphoinositidase C (PIC), generates two second messengers, namely *sn*-1, 2-diradylglycerol (DG) and *D* - *myo* - inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3), as outlined simplistically in Figure 1.5, overleaf. The term diradylglycerol has been suggested to avoid inferring the form of the linkage of the fatty acid chains to the glycerol backbone, although diacylglycerol is more commonly used. Diacylglycerol activates a calcium and phospholipid-dependent protein kinase, protein kinase C (Takai *et al.*, 1979; Kishimoto *et al.*, 1980; Nishizuka, 1984). Ins(1,4,5)P_3 stimulates the release of calcium from an intracellular, non-mitochondrial pool. The first indirect evidence of this process came from studies using permeabilised pancreatic cells, where a calcium electrode monitored the uptake of calcium into the endoplasmic reticulum (ER) and its subsequent release following the addition of Ins(1,4,5)P_3 (Streb *et al.*, 1983).

Figure 1.5.

AGONIST-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS.

(Adapted from Fig. 1, Cook & Wakelam, 1992).



This highly simplified diagram outlines some of the consequences of receptor-stimulated PtdIns(4,5)P_2 hydrolysis by phosphoinositidase C, with particular emphasis on the pathways that may lead to activation of mitogenesis.

These two second messengers can be metabolised via two separate pathways. Briefly, DG can be phosphorylated to phosphatidic acid or hydrolysed by a lipase to form monoacylglycerol, which is further hydrolysed to release arachidonic acid (Berridge, 1987). Inositol (1,4,5) trisphosphate can dephosphorylated to free inositol or it can be phosphorylated to form other inositol polyphosphates (reviewed in Shears, 1989).

This bifurcating pathway provides an extremely versatile signalling mechanism, which can control short-term cellular responses such as secretion, contraction and metabolism and longer term processes of cell growth and differentiation. The evidence for G-protein-mediated activation of PIC will be discussed in 1.12.1.

1.9.3. Interconversion of the inositol-containing phospholipids.

Rigorous chemical degradation studies of bovine brain lipids, formed the basis for the positional assignment of the monoesterified phosphates in PtdIns(4)P and PtdIns(4,5)P_2 (reviewed by Hawthorne & Kemp, 1964). Two futile cycles, reflecting the balance between kinase and phosphatase activities, determine the levels of phosphoinositides.

More recently, 3-phosphate containing lipids have been found in very small amounts in transformed, normal and non-growing cells (reviewed in Bansal & Majerus, 1990). The only known pathway for their degradation is via a 3-phosphatase and they are poor substrates for phosphoinositidase C isoforms which have been tested (Serunian *et al.*, 1989), rendering it unlikely that they are precursors of inositol phosphate messenger molecules. However agonists that stimulate inositol phospholipid turnover have been shown to increase labelling of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Traynor-Kaplan *et al.*, 1989). The exact pathway for formation of these phospholipids is uncertain, although a PtdIns 3-kinase has been isolated (Carpenter *et al.*, 1990). Interestingly, it has been demonstrated that the rise and fall of PtdIns(3,4,5)P_3 in neutrophils parallels actin

polymerisation and depolymerisation in response to N-formyl peptide stimulation (Eberle *et al.*, 1990). Whether 3-phosphate containing phospholipids mediate cytoskeletal interactions and/or other functions is currently under investigation (reviewed by Downes & Carter, 1991).

1.9.4. Phosphoinositidases C.

PIC can also be termed phosphodiesterase or phospholipase C. However use of the general term phosphoinositidase C has been suggested by Downes & Michell (1985). Whilst indicating hydrolysis of inositol-containing lipids it avoids inferring specificity for any particular inositol lipid, with the "C" defining the site of enzymic attack. The general reaction catalysed by these enzymes is outline in Figure 1.6, overleaf.

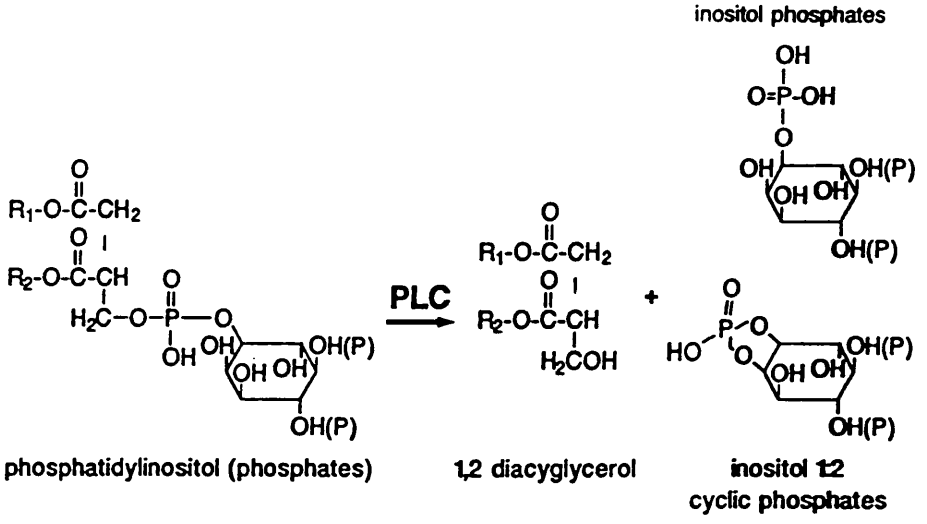
Soluble PIC activity was initially detected in the pancreas and the liver (Dawson, 1959; Kemp *et al.*, 1961). PIC activity is found predominantly in the cytosol of mammalian cells, with initial claims of distinct membrane bound forms being rejected (Irvine & Dawson, 1978). Reversible membrane binding is an attractive, although as yet unproven, hypothesis to explain how these enzymes contact their phospholipid substrates and indeed the transducing G-protein(s).

It is now known that multiple PIC isoforms exist in mammalian tissues as deduced from direct protein isolation and molecular cloning studies. A single tissue may contain multiple, immunologically distinct PIC forms and two such cytosolic forms, termed I and II were isolated from sheep seminal vesicles (Hofmann & Majerus, 1982). These forms were similar with respect to substrate specificity and enzyme kinetics, but differed in apparent molecular weight. Antibodies against PIC-I inhibited 50% of the activity from cultured human skin fibroblasts, implying that a single cell contained multiple PIC forms.

Figure 1.6.

THE GENERAL REACTION CATALYSED BY PHOSPHOINOSITIDASE C.

(adapted from Figure 1, Majerus *et al.*, 1990).



Inositol containing phospholipids are hydrolysed to yield six water soluble inositol phosphates corresponding to cyclic 1,2-phosphates and 1-phosphates of inositol mono-, bis- and trisphosphates. (P) denotes that substrates may or may not have phosphate at those positions.

Nine isoforms of PIC have been categorised into four groups, designated α, β, δ and γ (reviewed in Meldrum *et al.*, 1991). These four isozymes actually exhibit a surprising degree of heterogeneity in both molecular weight and sequence, although two domains of homology have been identified, designated "I" and "II". PIC-α represents one of the forms originally isolated from sheep seminal vesicles and it is actually the most divergent isoenzyme. Three isoforms of PIC-β have been cloned, displaying between 65%-85% homology in regions I and II and a C-terminal domain that distinguishes them from δ and γ (Kriz *et al.*, 1990). Two isoforms of PIC-γ have been isolated which are abundant in brain but also present in many other tissues and cell types, including lymphocytes and fibroblasts (Emori *et al.*, 1989; Ohta *et al.*, 1988). PIC-δ has three isoforms of lower molecular weight than the β and γ forms, that are expressed in brain, seminal vesicles and fibroblasts (Rhee *et al.*, 1989; Kriz *et al.*, 1990). More recently the expression of multiple isoforms of PIC in one cell or tissue type has been confirmed using monoclonal and polyclonal antibodies specific to PIC isoforms. Interestingly, these studies reveal quite diverse patterns and levels of expression between different tissues and cells (Rhee *et al.*, 1991).

Interestingly PIC-γ_1 and γ_2 differ from the other isoforms in that domains I and II are separated by a region referred to as the *src*-homology or SH region. An SH_2 domain has been found in other non receptor tyrosine kinases, viral oncogenes, GAP and PI-3 kinase and appears to function in binding to tyrosine phosphorylated sequences (Koch *et al.*, 1991). Indeed PIC-γ_1 is unique among the PIC isoforms in that it associates with and is a substrate for growth factor receptor tyrosine kinases. Tyrosine phosphorylation increases the catalytic activity of this isoform (Nishibe *et al.*, 1990). It has been proposed that autophosphorylation of receptor tyrosine kinases may serve to "recruit" effector enzymes via their SH_2 domains (Ullrich & Schlessinger, 1990). Interestingly the SH_3 region present in the PIC-γ isoforms has been proposed to have an inhibitory role, as removal of this region from c-*src* enhances oncogenicity (Potts *et al.*,

1988). The soluble actin-binding protein, profilin, has actually been shown to exert a tonic inhibition of PIC-γ_1, which is relieved by EGF receptor-mediated phosphorylation of the PIC (Goldschmidt-Clermont *et al.*, 1991). Whether the SH_3 domain is involved in this process remains to be demonstrated. Indeed, whether such mechanisms of regulation extend to other agonists and indeed other PIC isoforms is not clear as yet, but generally domains I and II are required for catalytic activity with the intervening non conserved region proposed to act as a "hinge" region (Kriz *et al.*, 1990).

The purpose of the PIC isoform multiplicity is unknown, although the co-expression of two or more isoforms in a single cell suggests some functional advantage. As illustrated with PIC-γ_1, it may be that the different PIC forms are regulated by separate factors increasing responsive versatility (discussed in Chapter 7). The *in vitro* properties of these PIC isoforms also differ, although it should be noted that *in vitro* conditions may artificially accentuate any small differences between various PIC isoforms and how or indeed if these differences exist in the *in vivo* situation is as yet not clear.

The isoform, PIC-β_1, displays an inherent catalytic specificity for polyphosphoinositides (Katan & Parker, 1987). More recently this has also been demonstrated for PIC-β_2 implying that within a family the different isoforms have a common substrate specificity (Kriz *et al.*, 1990). PIC-γ_1 displays a similar specific activity towards PtdIns(4,5)P_2 and PtdIns at physiological calcium concentrations (Rhu *et al.*, 1987). Thus the γ class of PIC isoforms appear to display less of a specificity towards hydrolysis of PtdIns(4,5)P_2 than the β class. The δ class are more similar to the β class, but unlike PIC-β_1 will hydrolyse PtdIns at millimolar calcium concentrations (Meldrum *et al.*, 1989).

Interestingly, the different isoforms will form cyclic and non-cyclic inositol phosphates at different ratios. Some groups believe that cyclic inositol phosphates may act as "long-term" calcium mobilisers, perhaps increasing the significance of

this observation (discussed in Meldrum *et al.*, 1991).

The selective expression of a particular PIC isoform may also influence the mode of regulation of its activity. Elevated cAMP levels can in some systems inhibit and in others augment PIC activation implying that a feed forward or feedback effect may be influenced by the particular PIC isoforms expressed, perhaps increasing responsive versatility (Meldrum *et al.*, 1991).

It would appear that PIC-γ does have a role in cell proliferation and anti-PIC-γ antibodies, but not antibodies against PIC-β, do block serum and *ras*-stimulated DNA synthesis in NIH-3T3 cells (Smith *et al.*, 1990).

Recently, the concept that different PIC isoforms may be involved in coupling to different types of receptors mediating functions other than cell growth has evolved. Indeed in *Drosophila*, the *norp A* gene encodes a PIC-β homologue expressed in the head region. Decreased PIC activity and blindness result from mutations in this gene (Bloomquist *et al.*, 1988). Certainly in leukocytic cell types it is evident that PIC activation mediates functional responses of these cells other than cell growth (see Chapter 3).

1.9.5. Ins(1,4,5)P3 and calcium flux.

Cells respond to calcium-mobilising agonists, using both intra- and extracellular calcium sources. Typically, the initial response is mobilisation of calcium from intracellular stores followed by a sustained response that requires calcium entry from the extracellular space. Ins(1,4,5)P_3 binds to its own intracellular receptor and releases calcium from a non-mitochondrial pool thought to be in the membranes of a fraction of the ER (Streb *et al.*, 1984). The fact that Ins(1,4,5)P_3 does not release all the calcium sequestered by this pool suggests the existence of an Ins(1,4,5)P_3-insensitive pool.

The purification of the Ins(1,4,5)P_3 receptor has enabled its immunocytochemical localisation to the nuclear envelope and on parts of the ER near the nucleus (Supattapone *et al.*, 1988; Ross *et al.*, 1989). Both Ins(1,4,5)P_3

binding and calcium release exhibit similar response profiles to inhibitors, Ins(1,4,5)P_3 derivatives and enantiomers suggesting the receptor itself and the calcium channel may be the same protein. Indeed the action of Ins(1,4,5)P_3 seems highly cooperative, channel opening requiring three molecules of Ins(1,4,5)P_3 (Meyer *et al.*, 1988).

The calciosome is a small membrane vesicle containing calcium pumps and the calcium binding protein, calsequestrin and is a candidate for Ins(1,4,5)P_3-sensitive and/or insensitive pools (Volpe *et al.*, 1988). Recently an Ins(1,4,5)P_3-binding, calireticulin-containing intracellular compartment has been purified from the human myeloid HL-60 cell line. Calireticulin is a putative calcium storage protein (Van Delden *et al.*, 1992).

Many agonists promote an influx of external calcium, in addition to mobilising internal calcium via Ins(1,4,5)P_3. The mode of calcium entry is a poorly understood process. There have been several proposed mechanisms by which inositol phosphates control calcium entry into cells, including an Ins(1,4,5)P_3-controlled calcium channel in the plasma membrane (Kuno & Gardner, 1987). Putney has proposed a "capacitative calcium entry" model whereby the Ins(1,4,5)P_3-mediated emptying of internal calcium stores provides the signal stimulating calcium entry, with calcium moving between the plasma membranes and stores via a "pathway" avoiding most of the cytoplasm (Putney, 1986). Experiments in a variety of systems support this basic model and although it is now apparent that no "pathway" exists, exactly how the state of the internal stores is communicated to the plasma membrane remains to be clarified. In some tissues, Ins(1,4,5)P_3 alone, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 together, or even calcium itself stimulate calcium entry, but these properties are neither universal nor obligatory controls. It may be that in some systems Ins(1,3,4,5)P_4 has a role in controlling the transfer of calcium between intracellular pools, further extending the capacitative model (Irvine *et al.*, 1988).

Indeed the idea of a well-ordered system of inter-communicating calcium pools operating via a "calcium-induced calcium release" process has been suggested as a possible mechanism to explain calcium waves and oscillations propagated in an identical manner in a variety of cells (reviewed by Berridge & Irvine, 1989).

Inositol (1,4,5) trisphosphate, through its calcium releasing properties, plays a role in influencing numerous divergent physiological processes by virtue of several modes of calcium action (reviewed by Exton, 1986). The major intracellular target for calcium is the calcium-dependent, regulatory protein, calmodulin. Calmodulin is ubiquitously expressed and binds calcium with a high affinity. From a functional viewpoint, the major enzymes affected by calcium-calmodulin are the isoenzymes of the multifunctional calmodulin-dependent protein kinase. These enzymes are present in many tissues and affect the activity of proteins such as glycogen synthase and tyrosine hydroxylase. More specific calmodulin-dependent protein kinases are stimulated by calcium, such as phosphorylase kinase, which contains calmodulin as its δ subunit. Other targets for the calcium-calmodulin complex include phosphatase 2B, brain adenylyl cyclase and Ins(1,4,5)P_3 kinase. These targets illustrate how calcium can not only feedback regulate its own production, but how it can influence other transduction routes.

Further submicromolar calcium concentrations can mediate effects upon proteins that do not appear to require or contain calmodulin. Targets include major metabolic enzymes such as pyruvate dehydrogenase phosphatase and glycerol 3-P dehydrogenase and proteins controlling actin filament assembly and disassembly.

Thus calcium can influence numerous processes including muscle contraction, cell shape changes, exocytotic secretion, metabolic alterations, early events of fertilisation and longer term changes such as cell growth and differentiation and information storage in the nervous system.

1.9.6. Diacylglycerol and protein kinase C activation.

Protein kinase C, (PKC), was identified by Nishizuka and co-workers as a

novel and widespread serine and threonine-directed phosphokinase. They demonstrated that this enzyme required calcium, phospholipid, particularly phosphatidylserine and *sn*-1,2-diacylglycerol for maximum activity. Diacylglycerol dramatically increased the apparent affinity of PKC for calcium to the extent that the enzyme can become fully active at cytosolic calcium concentrations (Takai *et al.*, 1979). Protein kinase C can be activated by a variety of DG analogues, but this is a highly specific process (reviewed in Bell & Burns, 1991). Tumour-promoting phorbol esters, such as 12-*O*-tetradecanoyl-13-acetylphorbol (TPA) also activate PKC. Kinetic analysis demonstrated that TPA greatly increased the enzymes calcium sensitivity (Castagna *et al.*, 1982). Activation of the cytosolic protein kinase C by DG or TPA, which intercalate into the plasma membrane, is proposed to involve translocation of protein kinase C from the cytosol to the plasma membranes (Nishizuka, 1988).

However, it is now apparent that several discrete subspecies of PKC exist, termed α, β, γ, δ, ε, and ζ, derived from both multiple genes and from alternative splicing of a single mRNA transcript (reviewed by Kikkawa *et al.*, 1989). These enzymes are composed of single polypeptide chains, ranging from 68kDa-84kDa, with a regulatory amino-terminal domain and a carboxy-terminal protein kinase domain. The subtypes display differences in their expression, sensitivity to activators and catalytic activity towards endogenous substrates.

The physiological effects of the activation of C-kinase are thought to be mediated via phosphorylation of specific proteins and a large number of proteins act as substrates *in vitro*. However it has proved difficult to identify the function of the smaller number of proteins phosphorylated in intact cells. Although it is not yet possible to assign biological roles to individual PKC subspecies, it is apparent that PKC subspecies do show preferences for substrate proteins. The EGF receptor in the A431 epidermoid carcinoma cell line can be rapidly phosphorylated by the ubiquitous α-subspecies of PKC, but is more slowly phosphorylated by the brain-

specific γ-subspecies (Ido *et al.*, 1987).

The steady state level of diacylglycerol for activation of PKC is a function of its phosphorylation and hydrolysis, as discussed previously. Interestingly ,the γ subspecies of PKC is significantly activated by arachidonic acid, a process that does not require calcium and is independent of phospholipid and diacylglycerol (Naor *et al.*, 1988). This raises the interesting possibility that some PKC subspecies may be activated at different phases of cellular responses by a series of phospholipid metabolites that appear subsequent to receptor stimulation.

Several physiological functions have been assigned to PKC, including involvement in secretion and exocytosis, modulation of ion conductance, receptor modulation, gene expression and cell proliferation (reviewed in Kikkawa & Nishizuka, 1986).

1.9.7. Interaction between calcium and PKC activation.

It is now well recognised that synergistic interaction between PKC and calcium pathways underlies a variety of cellular responses to external stimuli. In platelets, serotonin release is dependent upon protein phosphorylation mediated by protein kinase C and calcium-calmodulin. Neither phosphorylation on its own is sufficient to promote a secretory response (Yamanishi *et al.*, 1983). Such synergism is evident in a variety of other processes, but is not universal as calcium alone controls potassium efflux in parotid acinar cells (Putney *et al.*, 1984).

Protein kinase C also influences various steps of cell signalling processes, with both positive feedforward and negative feedback control. A major role of PKC is to decrease elevated levels of calcium and in various cell types it has been shown to activate the Ca^{2+}-transport ATPase and the Na^{+}/Ca^{2+} exchange protein (Lagast *et al.*, 1984). PKC often blocks receptor-mediated hydrolysis of inositol phospholipids and various studies have implicated the site of inhibition at the level of the receptor and Ins(1,4,5)P_3 phosphatase (Leeb-Lundberg *et al.*, 1985; Connolly *et al.*, 1986). The Gp protein(s) may serve as substrates for PKC,

although this has not yet been demonstrated *in vivo*. A negative feedback role of PKC is not confined to short term responses and the long term activation and down regulation of PKC by tumour promoting phorbol esters may relieve the cell from the feedback inactivation of growth factor receptors, enabling uncontrolled cell proliferation.

There is also a positive feedforward action of the enzyme with evidence of a role for PKC in gene expression, such as protooncogene activation (reviewed in Kikkawa *et al.*, 1989).

Protein kinase C can exert heterologous modulatory roles, influencing other signal pathways, particularly the cAMP system. There are numerous examples where activation of inositol lipid hydrolysis potentiates receptors that form cAMP. Protein kinase C may facilitate the interaction between G_S and adenylyl cyclase, (Bell *et al.*, 1985), or phosphorylate and reduce the activity of the inhibitory $G_{i\alpha}$ (Katada *et al.*, 1985). The cyclic nucleotides, cAMP and cGMP can exert reciprocal inhibitory effects upon inositol lipid metabolism, as discussed in 1.9.4. Further the DG/C-kinase pathway can exert both positive and negative modulatory roles upon voltage-dependent calcium channels (Rane & Dunlap, 1990).

Thus the "phosphatidylinositol pathway" performs modulatory functions, via a highly integrated and finely tuned signalling system capable of controlling a diversity of physiological processes.

1.10. PHOSPHATIDYLCHOLINE HYDROLYSIS.

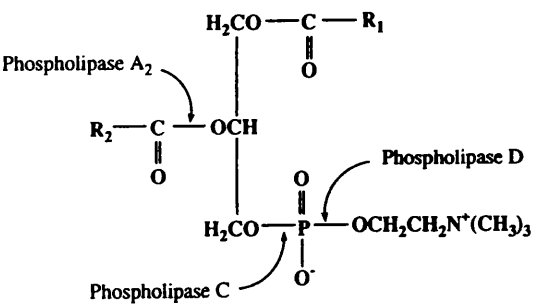
It is now apparent that many agonists that stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate also stimulate the breakdown of phosphatidylcholine (PtdCho). There is evidence for the hydrolysis of this phospholipid by phospholipases C, A_2 and D to yield a diverse array of potentially important physiological products, outlined in Figure 1.7, overleaf.

Figure 1.7.

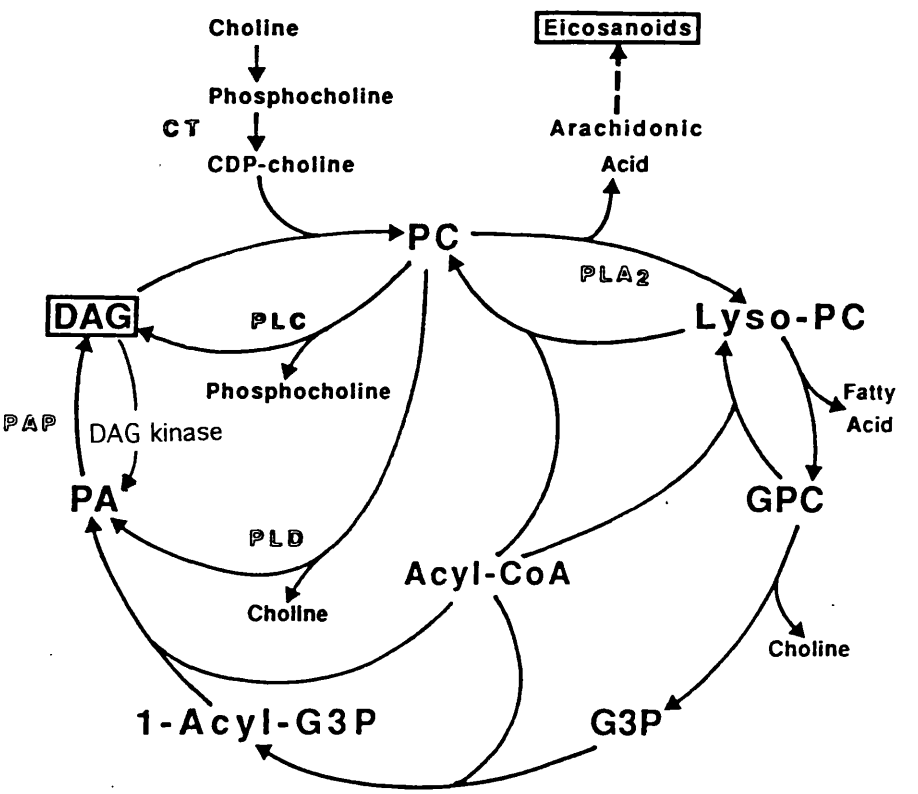
THE ENZYMATIC HYDROLYSIS OF PHOSPHATIDYLCHOLINE.

(Panel A was provided by Dr. M. J. O. Wakelam. Panel B was adapted from Fig. 1, Pelech & Vance, 1989).

A. Sites of enzymatic attack.



B. Phosphatidylcholine cycles.



Phosphatidylcholine cycles for generation of second messenger diacylglycerol and eicosanoids. The diacylglycerols (DAG) can be generated directly via phospholipase C (PLC) or by the action of phospholipase D (PLD) to yield phosphatidic acid (PA), which is cleaved to DAG by PA phosphohydrolase (PAP). Alternatively, the phospholipase A_2 (PLA_2)-catalysed hydrolysis of PC provides for the production of lyso-PC and arachidonic acid ($C_{20:4}$), an immediate precursor of eicosanoids. The lyso-PC may be re-esterified to PC or catabolized to glycerophosphocholine (GPC), which can be further degraded to glycerol 3-phosphate (G3P) and choline. G3P can be converted back to DAG via PA synthesis. DAG can react with CDP-choline to complete another PC turnover cycle.

The involvement of phospholipase C enzymes and the fact that agonists regarded previously as phosphoinositide-specific phospholipase C activators may be stimulating PtdCho hydrolysis via other phospholipase enzymes merits a brief discussion of this pathway (reviewed by Billah & Anthes, 1990; Exton, 1990).

1.10.1. Phosphatidylcholine.

Phosphatidylcholine is the major phospholipid class in mammalian tissues accounting for up to 50% of the total cellular phospholipid content. Mass measurements demonstrate that 3-5% of this pool can be phosphodiesterically cleaved ensuring product formation without drastic alterations in membrane integrity (Billah *et al.*, 1989a). Further the variable fatty acid composition of PtdCho can result in the production of multiple molecular species of the products of phosphodiesteratic cleavage.

1.10.2. PtdCho hydrolysis by phospholipases C and D.

PtdCho-specific phospholipase C activity catalyses the hydrolysis of PtdCho with the production of DG and choline phosphate, as outlined in Fig. 1.7. Phospholipases C that utilise PtdCho as a substrate and not phosphatidylinositol have been partially purified from a variety of sources e.g., the human monocytic cell line, U937 (Clark *et al.*, 1986).

Phospholipase D (PLD) catalyses a unique transphosphatidylation reaction, where the phosphatidyl moiety of the phospholipid substrate is transferred to primary alcohols. When the acceptor is water, the resulting hydrolysis reaction yields phosphatidic acid (PtdOH) and choline. Mammalian phospholipase D acting on PtdCho was first detected in a microsomal rat brain preparation (Saito *et al.*, 1975). Subsequent studies have demonstrated PtdCho-preferring phospholipase D activity in a variety of tissues and an enzyme has been partially purified from human eosinophils (Kater *et al.*, 1976). Interestingly, multiple PLD isoforms may exist, as PLD activities vary in their Ca^{2+}, fatty acid and detergent dependency (Billah & Anthes, 1990).

1.10.3. Relative contribution of phospholipases C and D to PtdOH and DG generation.

Although the products of phospholipase C and D-mediated hydrolysis of PtdCho are not equivalent, they can be rapidly interconverted by specific kinases and phosphatases resulting in ambiguity as to which phospholipase was responsible for a particular agonist-stimulated cleavage of PtdCho, evident from Figure 1.7.

However recent developments in assay techniques have begun to resolve these problems. The transphosphatidylation reaction is specific to PLD and using a primary alcohol as the acceptor, e.g., ethanol, the formation of the relatively metabolically stable phosphatidylethanol is indicative of a phospholipase D activity (Billah *et al.*, 1989b).

The relative contribution of the PLD and PLC/DG kinase pathways to PtdOH production can be assessed by dual labelling PtdCho with [^{32}P] and [^{3}H], the [^{3}H] being in the acyl chain. Upon stimulation [^{32}P]PtdOH is formed as a result of hydrolysis by PLD, whereas [^{3}H]PtdOH could be formed by both PLD and PLC/DG kinase routes. By comparing the ratio of the phospholipase D-mediated production of [^{32}P]PtdOh with the total [^{3}H]PtdOH, it is possible to assess the contribution of each pathway. In cytochalasin B-primed neutrophils stimulated with chemotactic peptide, this approach identifies an exclusive phospholipase D-mediated PtdOH production in the early phase of stimulation. However, the phospholipase C/DG kinase pathway made no contribution towards the increase in PtdOH (Billah *et al.*, 1989a).

PtdOH formed by phospholipase D can be degraded by PtdOH phosphohydrolase to produce DG, as outlined in Figure 1.7. PtdOH phosphohydrolase is more commonly abbreviated to "PPH", but in Figure 1.7 "PAP" has been used. In the "dual labelling" study just outlined, use of the PPH inhibitor, propranalol, identified the major route to DG formation as the sequential action of phospholipase D and PPH (Billah *et al.*, 1989a). This pathway is

believed to contribute significantly to DG formation in various cell types, although in certain cultured cells the phospholipase C route may predominate (Grillone *et al.*, 1988).

1.10.4. Regulation of cellular levels of PtdOH and DG.

Many cells produce DG in a biphasic manner, the initial phase peaking within 30 seconds and correlating with Ins(1,4,5)P_3 production. The delayed, quantitatively larger phase reaches a maximum within 2-15 minutes of stimulation and is long-lasting. The kinetics of the sustained DG and the fact that its formation can be induced by agents that do not provoke phosphoinositide hydrolysis, have stimulated debate as to whether this DG is derived from PtdCho hydrolysis (Billah & Anthes, 1990). DG derived from phosphoinositides is rich in arachidonic acid, whereas DG species from PtdCho contain little arachidonic acid (Pessin & Raben, 1989). Recently it has been shown that the membrane-bound form of DG kinase preferentially uses arachidonyl-DG as a substrate, although no specificity is apparent for the cytosolic forms (MacDonald *et al.*, 1988). It has been suggested that this specificity may govern the temporally different DG phases, in that activation of the cytosolic kinase and translocation to the membrane will facilitate the conversion of the initial, phosphatidylinositol lipid-derived DG whilst the reduced levels of cytosolic activity facilitate a sustained PtdCho derived-DG response (Billah & Anthes, 1990).

Regulation of receptor-linked activation of PtdCho hydrolysis may involve multiple factors, including calcium, DG, PKC and G-proteins. The role of G-proteins in mediating coupling reactions will be discussed later (see section 1.12.).

Receptor-mediated calcium influx at the plasma membrane does appear to be necessary for enhanced PtdCho hydrolysis (Truett *et al.*, 1988). Studies in cell-free preparations from several tissues suggest that the calcium requirement may vary among cell types.

Phorbol esters appear to be universally effective in inducing PtdCho

hydrolysis, by both phospholipase C and D. Although the mechanism is thought to be via protein kinase C activation, its inhibition can only partially block phospholipase activities in some cells, suggesting possible PKC-independent modes of action (Billah *et al.*, 1989b). Addition of TPA to HL-60 cell membrane preparations stimulates phospholipase D activity, implying possibilities of a direct interaction (Kiss & Anderson, 1989).

Evidence currently suggests that PtdOH phosphohydrolase, DG kinase and cytidyltransferase are important in the regulation of PtdOH and DG. Studies with TPA suggest that DG and PtdOH are metabolised through the CDP-choline pathway, as outlined in Figure 1.7 (Muir & Murray, 1987).

1.10.5. Role of agonist-induced PtdCho hydrolysis.

It is generally accepted that the primary role of DG is to activate PKC, but sustained DG generation from PtdCho activation may be important for long term responses such as proliferation. DG subspecies generated from PtdCho and phosphoinositides contain differing fatty acids and it is possible that these DG subspecies affect different PKC isoforms (Billah & Anthes, 1990). Indeed, α_1-adrenergic stimulation in MDCK cells activates PKC when the hydrolysis of phosphoinositides is inhibited (Slivka *et al.*, 1988).

Although PtdOH is degraded to produce DG, it may also function in a second messenger capacity. The exogenous addition of PtdOH and its more potent lyso-derivative (lyso-PtdOH) to cells results in the increased hydrolysis of polyphosphoinositides, activation of PKC, inhibition of adenylyl cyclase and stimulation of DNA synthesis (Moolenaar *et al.*, 1986; Murayama & Ui, 1987; Van Corven *et al.*, 1989). However, it is now appreciated that some of the reported effects of PtdOH may have been due to lyso-PtdOH contamination of commercial preparations (Jalink *et al.*, 1990). Interestingly, recent reports have suggested that PtdOH may activate a kinase distinct from PKC (Bokkino & Exton, 1990). As such there is currently much interest in PtdOH as a potential second messenger.

1.11. PHOSPHOLIPASE A2: FUNCTION AND REGULATION.

1.11.1. Phospholipase A2.

Phospholipases A_2 are a diverse family of enzymes that hydrolyse the *sn*-2 acyl bond of arachidonic acid-containing phospholipids to generate equimolar amounts of lysophospholipids and arachidonic acid (AA). However, at least two other separate mechanisms can liberate arachidonic acid, evident from Figure 1.7, namely the combined action of a phosphoinositidase C and diacylglyceride lipase or a phosphatidic acid specific phospholipase A_2 (Bell *et al.*, 1979; Billah *et al.*, 1981).

The secretory PLA_2 from human platelets resembles the well characterised pancreatic PLA_2's and those from snake venoms (Kramer *et al.*, 1989). However evidence now suggests that these enzymes may not represent the intracellular cytosolic PLA_2's involved in receptor-mediated liberation of arachidonic acid (Kramer *et al.*, 1990). Such a cytosolic enzyme has been characterised from various sources and exhibits an increased molecular weight and enhanced calcium sensitivity relative to the secretory PLA_2 enzymes (Diez & Mong, 1990). Discrepancies do exist between individual studies as to the molecular weight and identity of this intracellular PLA_2. Recently, however, a cytosolic PLA_2 has been purified from human monocytic U937 cells which responds to physiological calcium concentrations and is a 100kDa protein (Kramer *et al.*, 1991).

1.11.2. Arachidonic acid metabolism and function.

Three pathways of arachidonic acid metabolism have been described in animal tissues, the cyclooxygenase pathway, the lipoxygenase pathway and the cytochrome P-450 or epoxygenase pathway (Piomelli & Greengard, 1990). Through these metabolic routes arachidonic acid acts as the precursor for a number of important signal molecules such as prostaglandins, prostacyclin, thromboxanes and leukotrienes. Acetylation of lysoplatelet-activating factor results in the

generation of platelet-activating factor (PAF), a lipid mediator which is produced by and activates a range of inflammatory cells (reviewed by Braquet *et al.*, 1987). Indeed arachidonic acid metabolites as well as PAF are potent mediators of allergic and inflammatory reactions (reviewed by Needleman *et al.*, 1986). Certain metabolites such as thromboxane A_2 may function as calcium ionophores (Volpi *et al.*, 1980). Prostaglandins may act as local hormones and recent work has suggested that lipoxygenase metabolites may participate in the regulation of neurotransmitter release (reviewed by Piomelli & Greengard, 1990).

More recently it has been suggested that AA may itself act as a second messenger (reviewed by Naor, 1991). A particular subspecies of PKC, namely the γ-type enzyme, is activated at low concentrations of AA (~10μM). This is independent of phosphatidylserine, calcium and DG (Shearman *et al.*, 1989). AA has also been reported to activate adenylyl cyclase (Poon *et al.*, 1981), activate PIC (Zeitler & Handwerger, 1985) and mobilise cellular calcium (Wolf *et al.*, 1986). Whether these effects are mediated by PKC activation is not yet clear, but these observations would suggest the possibility that an agonist may not have to stimulate PtdIns(4,5)P_2 hydrolysis to activate PKC and mobilise calcium.

1.11.3. Regulation of phospholipase A2.

The regulation of phospholipase A_2 is complex and several factors modulate its activity. The calcium ionophore, A23187, is a potent inducer of PLA_2 and in cell free preparations calcium is required for activity (Billah *et al.*, 1986). This effect may be mediated in part through calmodulin as antagonists to this protein have been shown to inhibit AA release (Lapetina *et al.*, 1982). However, in HL-60 cell homogenates a phospholipase A_2 degrades phosphatidylcholine in the absence of free calcium, although whether this enzyme is involved in agonist-stimulated or possibly only basal phospholipid turnover remains to be established (Billah *et al.*, 1986).

There would appear to be a synergistic interaction between diacylglycerols

and calcium in the activation of phospholipase A_2. In neutrophils stimulated with chemotactic peptide, cytochalasin B causes accumulation of diacylglycerols and prolongs the increase in intracellular calcium concentration (Honeycut & Niedel, 1986; Treves *et al.*, 1987). Further, cytochalasin B greatly enhances AA release by chemotactic peptides, although is inactive itself. Interestingly, the 5-lipoxygenase metabolites are also capable of amplifying the phospholipase A_2 response initiated by calcium (Billah *et al.*, 1985).

Glucocorticoids are potent antiinflammatory drugs, a property attributed in part to the inhibition of phospholipase A_2 activities. A family of inhibitory proteins, termed lipocortins, are secreted when cells are treated with glucocorticoids (Flower *et al.*, 1984). The lipocortins share sequence homology with the calpactins, the predominant protein substrates of growth factor and transforming specific protein kinases. It has been speculated that the calpactins might function by linking membrane phospholipids to cytoskeletal elements (Brugge, 1986). Whether lipocortins, by binding phospholipids, limit the accessibility of membrane lipids to PLA_2 remains speculative. Protein kinase C phosphorylates lipocortin *in vivo* (Gould *et al.*, 1986), which may serve to decrease its inhibitory action upon PLA_2 by altering phospholipid-protein interactions. Indeed this regulatory scheme may be relevant to other enzymes dependent on the availability of lipid substrates.

Thus the phospholipase enzymes represent critical "effectors" that influence diverse physiological functions through highly complex and interdependent metabolic pathways.

1.12. G-PROTEIN COUPLING TO PHOSPHOLIPASES.

Over the past decade there has been intensive research effort aimed towards the identification of the G-proteins involved in agonist-stimulated phospholipid turnover. One of the initial indications that these proteins may mediate such

coupling was the observation that non-hydrolysable GTP analogues, introduced into the cytosol of mast cells caused histamine secretion in response to addition of extracellular calcium (Gomperts, 1983). In his discussion of these observations Gomperts suggested extending the concepts of G-protein coupling, which existed at that time, to include a potential role in coupling to Ca^{2+}-mobilising receptors.

Rather than describe numerous studies on this topic only a selection of examples will be discussed. However, these should illustrate some of the present concepts on the evidence for and identity of the G-protein(s) involved in coupling to phospholipases. The recent discovery of potential Gp proteins has generated great excitement in this area of research and will be discussed briefly.

1.12.1. Evidence for a G-protein coupling to PIC.

Guanine nucleotides, their analogues and aluminium fluoride have been invaluable "tools" to implicate a G-protein in receptor and effector coupling in general and have been fundamental in the study of Gp(s). As discussed in 1.8.1, ligand binding that is modulated by guanine nucleotides is indicative of a G-protein coupled receptor. As Gomperts commented in the study just described, this property applied to agonists that activated PIC and was really the first indication of the existence of Gp. Cantau and coworkers observed that GTP regulated [3H] vasopressin binding to isolated rat hepatocytes and liver membranes (Cantau *et al.*, 1980). This finding has been corroborated by a number of other studies including those of the oligopeptide chemoattractant receptor in human polymorphonuclear leukocytes, as will be discussed again later in Chapter 5 (Koo *et al.*, 1983).

Numerous "cell-free" systems i.e., permeabilised cells and membranes, have been employed to study the regulation of PIC by guanine nucleotides, fluoride and agonists (for an extensive list see Table II, Meldrum *et al.*, 1991). Study of PIC activation in membranes includes the use of endogenous substrate, where the radioactively prelabelled substrate is provided by the membrane, or exogenously supplied substrate, which involves adding radioactively labelled substrate to an unlabelled membrane. Phosphoinositidase C activity is monitored by measuring the

production of labelled water-soluble products.

Using permeabilised platelets, GTPγS enhanced thrombin-stimulated phosphoinositidase C activity (Haslam & Davidson, 1984). Cockcroft and Gomperts reported that in plasma membrane preparations from human neutrophils the breakdown of prelabelled polyphosphoinositides was stimulated specifically by non-hydrolysable guanine nucleotide analogues. This occurred in the presence of calcium concentrations that pertain in the "resting" cell (Cockcroft & Gomperts, 1985). In membranes prepared from [^{32}P]-labelled turkey erythrocytes, both fluoride and GTPγS stimulated phosphoinositide breakdown (Harden *et al.*, 1987). Fluoride stimulates polyphosphoinositide breakdown in a number of other systems and in prelabelled liver membranes this action is inhibited by GDPβS (Cockcroft & Taylor, 1987). Studies in prelabelled blowfly salivary gland membranes demonstrated that guanine nucleotides were essential to promote agonist activation of phosphoinositidase C activity, exhibiting a rank order of potency of GTPγS > Gpp[NH]p > GTP (Litosch *et al.*, 1985). Using this system, but adding exogenous soluble [^{3}H]PtdIns(4,5)P_2, serotonin (5-HT) increased the [^{3}H]PtdIns(4,5)P_2 breakdown in a guanine nucleotide-dependent manner (Litosch & Fain, 1985). These represent only a few of the many cell-free systems that lend further credence to the view that a "stimulatory" G-protein couples to the phosphoinositidase C system.

However, the use of pertussis toxin in definition of a potential Gp(s) has created complexities as opposed to clarifying the situation. Study of a variety of systems with a selection of different agonists that are known to activate PIC and in some cases also PLA_2 and PLD, have indicated that "Gp" is pertussis toxin-sensitive in some systems but not in others (for an extensive list see Table III, Meldrum *et al.*, 1991). Indeed some systems exhibit a partially pertussis toxin-sensitive activation of PIC. This suggests the existence of, at the very least, two different "Gp-type" G-proteins.

In systems which exhibit a partial pertussis toxin sensitivity it is difficult to conceive that a single G-protein could act as only a partial pertussis toxin substrate. Indeed it may be that the PIC enzyme is under the "additive" influence of both a toxin-insensitive and toxin-sensitive G-protein. The toxin would interfere with one mediatory input causing a reduced but not totally inhibited response. From the previous account of the phospholipase enzymes it is evident that they exhibit calcium sensitivity. Although only briefly mentioned previously G-proteins do mediate ion channel coupling (Hescheler *et al.*, 1990). The regulation of Ca^{2+} channels is complex, involving multiple G-protein controlled mechanisms. However if one of the G-proteins in the "additive" scenario is coupling to a Ca^{2+} channel, then Ca^{2+}-mediated stimulation of PIC could either be inhibited or maintain a reduced level of PIC activation depending on the toxin sensitivity of the channel coupling G-protein.

Totally pertussis toxin-sensitive coupling to PIC has been demonstrated in a number of different blood cell types, namely neutrophils (Bradford & Rubin, 1985; Ohta *et al.*, 1985), leukocytes (Verghese *et al.*, 1985a) and HL-60 leukaemic cells (Brandt *et al.*, 1985). Using the HL-60 cell line it has been demonstrated that under certain conditions these "Gp" proteins are also substrates for cholera toxin. In fact this sensitivity to cholera toxin was utilised in the identification of these toxin-sensitive proteins as $G_i2\alpha$ and $G_i3\alpha$ (Gierschik *et al.*, 1989a). It would appear that the $G_i\alpha$ proteins actually act in a "stimulatory" manner in this situation. These systems are discussed in greater detail in Chapters 3, 4 and 5.

Another system exhibiting a totally pertussis toxin-sensitive Gp coupling is that of α_1-adrenergic receptor activation in rat white adipocytes (Rapiejko *et al.*, 1986). Again, this system is discussed further in Chapter 6. In contrast, pertussis toxin does not prevent α_1-adrenergic stimulated breakdown of phosphoinositides in brown adipocytes (Schimmel & Elliot, 1986). If the same subtype of the receptor is being activated in both cell types then this implies that a receptor, or at least one

receptor family, may use different Gp's depending on the cell type.

Evidence would also suggest that different types of Gp can selectively couple to different receptors expressed in the same cell. In platelets, thrombin operates via a pertussis toxin-sensitive Gp, while thromboxane A_2 operates via an apparently pertussis toxin-insensitive protein (Brass *et al.*, 1987).

There are few reports of the involvement of a cholera toxin substrate in coupling to phosphoinositidase C, although this toxin has been found to activate phosphoinositide hydrolysis in flow 9000 cloned pituitary cells (Lo & Hughes, 1987). This toxin can also exert effects in systems that use a pertussis toxin-sensitive Gp, as will be discussed later (see Chapter 4).

It has been suggested that a G-protein-mediated inhibition of phosphoinositidase C may exist. As previously described in 1.7.4, $\beta\gamma$ subunits can exert inhibitory effects upon a phosphoinositidase C-mediated chloride current. Further, dopamine prevents the formation of inositol phosphates in pituitary cells stimulated by thyrotropin releasing hormone in a pertussis toxin-sensitive manner (Journot *et al.*, 1987).

1.12.2. Identification of a pertussis toxin-insensitive Gp.

The purification and cloning of two new "classes" of pertussis toxin-insensitive G-proteins has been described in 1.3.4 and 1.4.1. The pertussis toxin-insensitivity of these proteins immediately suggested their potential as toxin-insensitive Gp's. Indeed as mentioned previously, one of these proteins, now termed Gq, was purified on the basis of its ability to activate partially purified PIC (Taylor *et al.*, 1990). In a series of investigations there is mounting evidence that Gq does serve as a Gp. Using a mixed phospholipid reconstitution system purified αq from bovine brain activated a partially purified, bovine brain PIC. This stimulation was markedly increased by AlF_4^-. Interestingly neither purified $\beta\gamma$ nor several pertussis toxin-sensitive G-proteins affected the PIC activity. The β, but not the δ or the γ isoforms of PIC were detected in the PIC preparation used. In

addition to stimulating the maximal activity of the PIC the G-protein lowered the calcium requirement for activation of the enzyme (Smrcka *et al.*, 1991). This latter observation is in agreement with previous studies in a diversity of systems, including those utilising a pertussis toxin-sensitive Gp (Smith *et al.*, 1986). Using this reconstitutive approach it has been demonstrated that Gq activated β_1, but not γ_1 or δ_1 isoforms of PIC (Taylor *et al.*, 1991). This would imply the experimentally daunting possibility that the different isoforms of PIC may exhibit specificity in their choice of Gp.

1.12.3. Evidence for a G-protein coupling to phospholipase A2.

Phospholipase A2 activity has been shown to be pertussis toxin-sensitive in a number of systems e.g., neutrophils and fibroblasts (Okajima *et al.*, 1985; Murayama & Ui, 1985). GTPγS stimulates inositol phosphate formation and arachidonic acid release in FRTL5 rat thyroid cells. It was confirmed that the noradrenaline-stimulated arachidonic acid was released as a result of a pertussis toxin-sensitive PLA2 activity, although noradrenaline-stimulated inositol phosphate formation was pertussis toxin-insensitive (Burch *et al.*, 1986). It would appear that in this system the G-proteins coupling to PIC and PLA2 are different proteins.

The most direct evidence to demonstrate a G-protein coupling to PLA2 was the activation of this enzyme by light in the rod outer segments of bovine retina. In this case transducin is mediating coupling to PLA2 as well as mediating coupling to a cyclic GMP phosphodiesterase enzyme. Transducin is a substrate for both pertussis and cholera toxin and both toxins inhibit activation of PLA2 by light (Jelesma, 1987). As outlined briefly in section 1.7.4, βγ subunits have been found to stimulate phospholipase A2 activity in rod outer segments, indicating that potentially not only the α subunit has a role in modulating phospholipase A2 activity (Jelsema & Axelrod, 1987).

1.12.4. Evidence for a G-protein coupling to phospholipase D.

The regulation of PLD and phosphatidylcholine-specific phospholipase C is currently under investigation, with more information emerging about PLD

regulation. As yet a direct receptor-coupled PLD activity has not been conclusively demonstrated, with studies indicating that the onset of PLD activity is kinetically downstream of agonist-stimulated Ins(1,4,5)P_3 generation (Cook *et al.*, 1990). As mentioned previously in 1.10.4, phorbol esters stimulate PLD activity in a variety of cell types, further suggesting PLD activation could be a secondary consequence of PIC activation. Thus any indications of G-protein involvement could simply reflect a coupling to PIC.

Interestingly recent reports are suggestive of alternative routes to PLD activation. Indeed, epidermal growth factor-stimulated PLD activity occurs in the absence of inositol lipid hydrolysis in fibroblasts (Wright *et al.*, 1990). In Rat-1 fibroblasts transfected with the platelet α_{2A}-adrenergic receptor, α_2-adrenergic agonists stimulated PLD activity but not inositol lipid hydrolysis. PLD activity was totally abolished by pertussis toxin pretreatment of these cells (MacNulty *et al.*, 1992). Whether this system reflects the *in vivo* situation is debatable, but these observations would implicate a role for a pertussis toxin-sensitive G-protein in PLD coupling. GTPγS can activate PLD activity in a variety of systems e.g., rat liver plasma membranes and HL-60 homogenates (Hurst *et al.*, 1990; Anthes *et al.*, 1991). Indeed, recent approaches are beginning to dissociate GTPγS activated PLD from GTPγS activated PIC and do implicate a G-protein in PLD coupling (discussed further in Chapter 7).

1.12.5. Hypothetical models for G-protein activation of phospholipases.

The most obvious functional mechanism is that Gp may act in an analogous manner to Gs, in that the GTP-liganded α subunit could serve as an activator of the phospholipase. The results from systems in which phospholipase activity is pertussis toxin-sensitive indicate that, contrary to the adenylyl cyclase pertussis toxin substrate, this toxin substrate acts to positively regulate phospholipase activity.

An alternative possibility is that phospholipases are complexed with their G-protein, the G-protein acting as an inhibitor of the enzyme. Receptor activation could induce derepression of the phospholipase activity by dissociating the G-protein subunits from the enzyme.

It is conceivable that the phospholipases could contain inhibitory subunits or associated proteins and that the activation of a G-protein may serve to prevent these inhibitory influences. There is a precedent for this idea, in that activation of transducin by rhodopsin leads to the GTP-liganded transducin α subunit activating cGMP phosphodiesterase by binding to its inhibitory γ subunit (Stryer, 1986).

Thus despite intensive research effort there are many aspects of G-protein modulated regulation of phospholipase activity to be defined, not least the identification of the proteins involved.

1.13. SPECIFICITY OF RECEPTOR - G-PROTEIN - EFFECTOR INTERACTION.

From the previous discussion it can be appreciated that a vast amount of information on receptors, G-proteins and effector systems exists, with current research providing greater detail on the complexity of the signal transduction pathways. However a major current goal is to define the specificity of interaction between these proteins. A selection of the approaches used to address this issue, mainly with respect to the pertussis toxin substrates, are discussed briefly.

1.13.1. Identification and resolution of the G-proteins.

As discussed previously G-proteins can be identified by a toxin-catalysed mono-ADP ribosylation of the α subunits. However the specificity of these reactions is limited. Indeed the ability of cholera toxin to catalyse ADP-ribosylation of the "classical" pertussis toxin substrates, under certain conditions, has emerged as a method to define receptor - G-protein coupling specificity and will be discussed in Chapter 4. Perhaps more restricting was the realisation that a multiplicity of

pertussis toxin-sensitive G-proteins existed, as described previously in section 1.3. These properties prevented definitive study of a particular G-protein and stimulated the production of immunological probes.

Polyclonal antisera raised against purified α subunits were useful in indicating G-protein heterogeneity, as previously described in sections 1.2 and 1.3. They were further instrumental in indicating that cells could co-express different G-proteins and regulate their levels independently (Gierschik *et al.*, 1986b; Watkins *et al.*, 1987). Ironically these discoveries actually limited the use of these antisera, raised against "pure" α subunits, as it became apparent that a homogeneous purification of a single G-protein may contain other G-protein variants.

Thus an alternative strategy has been to generate anti-peptide antisera (Milligan, 1990). Using predicted amino acid sequence information anti-peptide antisera have been raised that will specifically recognise most members of the G-protein family, with those targetted against conserved sequences recognising multiple substrates. These antisera have been particularly useful in defining the molecular nature of the various pertussis toxin-sensitive G-proteins present in a single cell or tissue type (see Chapters 3 and 6).

1.13.2. Cellular distribution of G-proteins as an indicator of function.

In certain cells the expression of a particular G-protein may be indicative of its functional properties and thus imply possible effector specificity. Using immunocytochemical techniques, $G_{o}\alpha$, unlike transducin, is found in inner and outer plexiform layers rather than rod outer segments (Lad *et al.*, 1987). This localisation is suggestive of a role for $G_{o}\alpha$ in synaptic transmission and not photon transduction. In agreement with this idea, $G_{o}\alpha$ is the most abundant G-protein in brain, being concentrated in the forebrain regions (Asano *et al.*, 1987). More rigorous approaches aimed at definition of this proteins specificity in receptor and effector coupling would appear to agree with a role for G_{o} in nervous system

transduction routes (see 1.13.3).

Interestingly, within the Gq class of α subunits, the Gα14, Gα15 and Gα16 isotypes all show restricted patterns of tissue specific expression which may reflect their specific transducing role (Simon *et al.*, 1991).

The different forms of Giα vary in their tissue distribution, with $G_i2\alpha$ being found in all tissues examined (Brann *et al.*, 1987). As such the tissue expression of Giα subtypes provides less clues about possible effector coupling roles than Goα, although alterations in the level of expression of these proteins coincidental with functional adaptations may prove to be informative (see Chapter 4).

1.13.3. The use of antipeptide antisera to assign coupling specificity.

There is a considerable amount of evidence to implicate the G-protein C-terminus as a site of receptor contact and antipeptide antisera raised against this region have been successfully used as "specific uncoupling agents", defining receptor - G-protein interaction in a number of systems. G-protein - effector interaction can also be elucidated in systems where the effector can be assayed in the membrane e.g., adenylyl cyclase activity. Such approaches have defined $G_i2\alpha$ as coupling receptors to inhibition of adenylyl cyclase in certain systems (described in more detail in Chapter 5). Recently, an affinity purified antibody raised against a C-terminal peptide common to both Gqα and $G_{11}\alpha$ attenuated stimulation of PtdIns(4,5)P_2 hydrolysis by different agonists in different cell and tissue membranes (Gutowski *et al.*, 1991). Indeed with the knowledge of the predicted amino acid sequences of a number of newly cloned α subunits which are predicted to be insensitive to pertussis toxin it may be possible to raise and use specific antipeptide antisera to define possible coupling roles and specificities of this new class of G-proteins.

In whole cell studies, injection of anti-Goα antipeptide antibodies into prostaglandin E1-differentiated NG108-15 cells prevented α_2-adrenergic mediated

inhibition of voltage-operated calcium channels. The ability of noradrenaline to block these calcium currents was not impaired by antipeptide antisera that recognise the other pertussis toxin-sensitive G-proteins expressed in this cell line, implying a specific role for $G_o\alpha$ in this process (McFadzean *et al.*, 1989). This cell is particularly suited to microinjection, although the development of alternative strategies to introduce antipeptide antisera into whole cells will obviously be an important advance in assigning receptor - G-protein - effector coupling specificity.

1.13.4. The use of antisense DNA in assigning coupling specificity.

The use of antisense DNA is designed to perturb functional coupling at the level of the G-protein. It makes use of the fact that often there is greater distinction between the α subunits at the nucleic acid level rather than in the translated protein. This is the case for the recently discovered subtypes of $G_o\alpha$, generated by alternate splicing of a single transcript, described previously in 1.4.1. The recent work of Kleuss and co-workers demonstrates that injection of antisense oligonucleotides into the nuclei of rat pituitary GH_3 cells inhibits the expression of G-protein subunits. Oligonucleotides complimentary to mRNA's encoding the two α_o subunits remove the inhibition of voltage-dependent calcium currents. The "α_o1" and "α_o2" subunits differ only in their C-terminal regions, indicative of possible selectivity in receptor coupling. The results of this study agree with this concept as G_o1 and G_o2 were found to mediate calcium channel inhibition through muscarinic and somatostatin receptors, respectively (Kleuss *et al.*, 1991). The authors propose that the use of antisense oligonucleotides could be used to study a variety of ion channel activities and suggest that this method is suitable for determining the function of any protein provided that assay of its activity is possible in a single cell.

1.13.5. Reconstitution studies as an approach to defining coupling specificity.

As described previously, it was the availability of the cyc^- mutant of the S49 lymphoma cell line and its use as a functional reconstitution system which enabled

detection of exogenous $G_s\alpha$. A similar mutant in $G_i\alpha$ is not available. However, the fact that pertussis toxin pretreatment attenuates coupling has been utilised in creating systems devoid of functional $G_i\alpha$ and hence suitable for testing the role of exogenous $G_i\alpha$ forms in coupling activities. Some of the major drawbacks to reconstitution methods are ensuring homogeneity of the reconstituted components and that the *in vivo* conditions are mimicked. Despite these problems reconstitution has been used to address the G-protein regulation of ion channel activity. Cell-free membrane patches have enabled control of an ion channels environment with addition of magnesium, GTP and its analogues and ligands all implicating the existence of agonist-activated potassium channels in cardiac myocytes. As discussed in 1.3, addition of a G_i-type protein (G_k) reconstituted agonist activation of inwardly rectifying potassium current in atrial myocytes (Yatani *et al.*, 1987a). More recent analysis by this group has demonstrated that all the $G_i\alpha$ subtypes, both the native and recombinant proteins can functionally reconstitute potassium current activity in atrial myocytes (Yatani *et al.*, 1988). These observations challenge the idea of fidelity of a receptor for one G-protein. More recent "reconstitution-type" approaches, introduced in 1.12.4., include the transfection of a single receptor subtype into a cell devoid of that receptor. This eliminates problems derived from the expression of multiple receptor subtypes in a particular cell and is beginning to provide information on coupling interactions (MacNulty *et al.*, 1992).

Thus, the use of these and other methodologies has elucidated G-protein - effector links (Table 1.1., overleaf). Whether these constitute the exclusive or indeed the universal functions of these proteins is currently under investigation.

Table 1.1. EFFECTOR REGULATION BY G-PROTEIN α SUBUNITS.

Gα subunit	Effector function	Reference
G_s	Adenylyl cyclase stimulation	Simonds *et al.*, (1989a)
Golf	Adenylyl cyclase stimulation	Jones & Reed, (1989)
G_{i1}	K^+ channel	Yatani *et al.*, (1988)
G_{i2}	Adenylyl cyclase inhibition	McKenzie & Milligan, (1990)
	?Pertussis toxin-sensitive Gp	Gierschik *et al.*, (1989a)
	?K^+ channel	Yatani *et al.*, (1988)
G_{i3}	K^+ channel	Yatani *et al.*, (1988)
	?Pertussis toxin-sensitive Gp	Gierschik *et al.*, (1989a)
G_o	Ca^{2+} channel	McFadzean *et al.*, (1989)
		Hescheler *et al.*, (1990)
G_z	?inhibition of cAMP accumulation	Wong *et al.*, (1992)
G_q/G_{11}	Pertussis toxin-insensitive Gp	
	coupling to PICβ1	Gutowski *et al.*, (1991)
$G_{12\text{-}16}$	unknown	Simon *et al.*, (1991)
T_{d1}/T_{d2}	cGMP phosphodiesterase	Fung *et al.*, (1981)
T_{d1}	PLA_2	Jelsema, (1987).

? indicates that the assignment of function is not definitive.

RESEARCH AIMS.

The objective of the work of this thesis was to identify the pertussis toxin-sensitive G-protein(s) that couples to receptors that stimulate phosphoinositidase C (PIC) activity.

From the previous introduction it can be appreciated that this objective was somewhat complicated by the fact that individual cell types may express more than one receptor subtype, multiple PIC isoenzymes and multiple pertussis toxin substrates. Further, individual agonists that stimulate PIC activity can activate other effector enzymes including phospholipase activities, in some cases also in a pertussis toxin-sensitive manner.

Thus to simplify the objective of this thesis an initial approach was to attempt to choose a system in which there was a totally pertussis toxin-sensitive activation of phosphoinositidase C by an agonist that was specific for this effector system. Ideally the system of choice would express a minimal number of different pertussis toxin-sensitive G-proteins, reducing the number of potential coupling candidates.

Upon finding a system that best fit these criteria the aim was to use antipeptide antisera that recognised the known pertussis toxin substrates not only in identification of the potential coupling candidates but also to develop assays to define receptor - G-protein coupling specificity.

CHAPTER 2.

MATERIALS AND METHODS.

2.1. MATERIALS.

All reagents employed during the course of this research were of the highest quality available and were obtained from the following suppliers;

GENERAL REAGENTS.

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

App[NH]p, Creatine phosphate, Creatine phosphokinase, Dithiothreitol, GDP, Gpp[NH]P, GTP, GTPγS, Thymidine, Triethanolamine hydrochloride, Tris.

B.R.L. Paisley, Scotland.

Prestained molecular weight markers. The biotinylated prestained proteins employed were myosin H-chain (200kDa), phosphorylase *b* (97.4kDa), bovine serum albumin (68kDa), ovalbumin (43kDa), carbonic anhydrase (29kDa) or α-chymotrypsinogen (25kDa) depending on the particular standards supplied, β-lactoglobulin (18.4kDa) and lysozyme (14.3kDa).

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

D-glucose, sodium hydrogen carbonate.

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

Acetic acid (glacial), Acrylamide, Citric acid, Folin and Ciocalteu's phenol reagent, Hydrogen peroxide, N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid, N, N'-methylenebisacrylamide.

Genetic Research Instrumentation Ltd., Essex, England.

Fuji RX X-ray film.

Johnson Matthey Materials Technology, Herts., England.

Silver nitrate.

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

DMSO, magnesium sulphate, sodium potassium tartrate.

Kodak Ltd., Hemel Hempstead, England.

Developer (LX-24), Fixer (FX-40).

May & Baker, Dagenham, England.

Ammonium persulphate.

National Diagnostics, Aylesbury, Buckinghamshire, England.

"Ecoscint" scintillation fluid.

Porton Products, Porton Down, Salisbury, Wiltshire, England.

Pertussis toxin (200μg/ml in 50% glycerol, 50% 0.05M phosphate buffer, pH 7.2. which is 0.5M in sodium chloride).

Schleicher and Scheull, Dassel, W. Germany.

Nitrocellulose (0.45μM).

Scottish Antibody Production Unit, Carluke, Lanarkshire, Scotland.

Peroxidase-conjugated goat anti-rabbit IgG.

Sigma Chemical Company Ltd., Fancy Rd., Poole, England.

Antipain, Aprotinin, Arginine hydrochloride, ATP disodium salt, Benzamidine, Bovine Serum Albumin, Bromophenol Blue, Bovine Serum Albumin, Cholera

toxin (1mg/ml in 0.05M Tris-HCL, 0.2M NaCl, 3mM NaN_3, 0.1mM Na_2EDTA, pH 7.5), Coomassie blue R-250, Freund's complete adjuvant, Freund's incomplete adjuvant, Gelatin (Type A), Keyhole limpet Haemocyanin, Nicotinamide adenine dinucleotide, N-ethylmaleimide, N-formyl-methionyl-leucyl-phenylalanine, Non-idet P-40 detergent, Norit-A charcoal, Ortho-dianisidine dihydrochloride, Ouabain, Pepstatin A, PMSF, Protein-A Sepharose 4B, Protein-A Agarose, Soybean trypsin inhibitor, N-t-butoxycarbonyl-methionyl-leucyl-phenylalanine, N, N, N', N'-Tetramethylethylenediamine, Thimerosal, Trypsin, Tween 20.

Whatman International Ltd., Maidstone, England.

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

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

RADIOCHEMICALS.

Amersham plc.,Buckinghamshire, England.

Guanosine 5'-[γ-^{32}P] triphosphate, triethylammonium salt (specific activity 10Ci/mmol).

[2-^{3}H]D-*myo*-inositol (1,4,5) trisphosphate (specific activity 20-60Ci/mmol).

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

[Adenylate-^{32}P]-Nicotinamide adenine dinucleotide, di(triethylammonium) salt (specific activity, 33Ci/mmol).

[^{3}H]-N-formyl-methionyl-leucyl phenylalanine (specific activity, 50Ci/mmol).

[^{125}I]-Goat Anti-Rabbit IgG (2-10μCi/μg).

CELL CULTURE MEDIA.

Gibco Life Technologies, Paisley, Scotland.

Dulbecco's modification of Eagle's medium (DMEM 10X), RPMI 1640 medium, Gentamicin (10mg/ml), Glutamine (200mM, 100X), HAT (50X): Hypoxanthine (5mM), Aminopterin (0.02mM), Thymidine (0.8mM), Penicillin (10000 I.U./ml, 100X), Sodium Bicarbonate (7.5%), Streptomycin (10000 μg/ml, 100X).

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

Foetal Calf Serum.

TISSUE CULTURE PLASTICWARE.

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

75cm^2 tissue culture flasks, 150cm^2 tissue culture flasks.

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

Biofreez vials.

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

50ml centrifuge tubes.

STANDARD BUFFER COMPOSITION.

Hanks buffered saline (Hanks).

1.26mM calcium chloride hexahydrate, 0.5mM magnesium chloride hexahydrate, 0.4mM magnesium sulphate heptahydrate, 5.37mM potassium chloride, 137mM sodium chloride, 4.2mM sodium hydrogen carbonate,

0.35mM sodium di-hydrogen phosphate.

When freshly prepared the pH was between 7.2-7.4.

Hanks buffered saline with glucose and BSA (HBG).

Buffer was prepared as above but contained 10mM D-glucose and 1% (w/v) BSA (fraction V) and the pH adjusted to 7.4.

Phosphate Buffered Saline (PBS).

2.7mM potassium chloride, 137mM sodium chloride, 1.5mM potassium di-hydrogen orthophosphate, 8mM of disodium hydrogen orthophosphate heptahydrate.

When freshly prepared PBS was between pH 7.3-7.5.

Sterile trypsin solution for cell passage.

Trypsin was prepared as a solution of 0.1% (w/v) trypsin, 0.025% (w/v) EDTA and 10mM glucose in PBS at pH 7.4. The solution was filtered through a sterile 0.22μM membrane (Flow pore D), prior to aliquoting into sterile tubes and storing at -20°C. The trypsin solution was used after only one freeze/thaw cycle.

Tris Buffered Saline (TBS).

500mM sodium chloride, 20mM Tris-HCL.

When freshly prepared TBS was pH 7.5.

Tris Buffered Saline with Tween 20 (TTBS).

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

When freshly prepared TBS was pH 7.5.

All buffers and solutions were prepared in distilled, deionised water.

EXPERIMENTAL METHODS.

2.2. CELL CULTURE.

2.2.1. CELL GROWTH.

NG108-15 neuroblastoma x glioma hybrid cells were a kind gift from Dr. W. Klee (N.I.H., Bethesda, M.D. USA). These adherent cells were cultured in 75cm^2 tissue culture flasks in 0.0375% (w/v) sodium bicarbonate buffered Dulbecco's modification of Eagle's Medium (DMEM), containing 10% (v/v) foetal calf serum (FCS) which had been heat inactivated at 56°C for 90 minutes. The medium was supplemented with glutamine (2mM) and hypoxanthine (0.1mM), aminopterin (0.4μM) and thymidine (16μM) i.e., HAT. To prevent the selection of resistant strains of bacteria, a penicillin (100 units/ml)/streptomycin (100μg/ml) combination was used in rotation with gentamicin (50μg/ml). 10-12ml of medium was routinely used in the flasks. The growth media will henceforth be termed DMEM/10% (v/v) FCS.

C6 BU1 cells are an adherent cell line. They were cultured as for the NG 108-15 cell line except that HAT was omitted from the growth medium.

U937 cells were a kind gift from Dr. K. Pollock (ICI Pharmaceuticals, Alderley Park, England). They were cultured in 75cm^2 or 150cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum (FCS) which had been heat inactivated at 56°C for 90 minutes. The medium was supplemented with glutamine (2mM). To prevent the selection of resistant strains of bacteria, a penicillin (100 units/ml)/streptomycin (100μg/ml) combination was used in rotation with gentamicin (50μg/ml). The 75cm^2 flasks routinely contained 28ml of cells in suspension culture, whereas 56ml was cultured in the 150cm^2 flasks.

All cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C.

2.2.2. CELL SUBCULTURE.

Confluent adherent cells (typically 10^7 cells per $75cm^2$ flask) were passaged using a trypsin solution under sterile conditions. Growth media was removed from the cells and 3mls of trypsin solution added. When the cells had been removed from the surface of the flask, trypsin activity was inhibited by the addition of two volumes of growth medium. This cell suspension was centrifuged at 800 x g_{av} in a MSE centaur for three minutes to pellet the cells. The cell pellet was resuspended in growth medium and the cells harvested after a repeat 800 x g_{av}, three minute "wash" spin. The cell pellet was resuspended in growth medium and cells aliquoted into sterile flasks, typically 1ml of cell suspension and 11ml of fresh medium/$75cm^2$ flask. Flasks were rocked gently to ensure an even distribution of cells across the flask and returned to the incubator. Cells were not cultured beyond ten passages from the original frozen stocks.

U937 cells grow as a suspension culture and as such were routinely reseeded at less than 10^5 cells/ml by dilution in fresh medium. The flasks were regularly rocked to ensure an even suspension of the cells. The cells grew to a maximum density of 10^6 cells/ml. Cells were not cultured beyond ten dilutions of the original frozen stocks.

2.2.3. U937 CELL DIFFERENTIATION.

Differentiation of the U937 cells was achieved by addition of dimethyl sulphoxide, 1.25% (v/v) final concentration, to the culture medium for the required period of time i.e., minimally 24 hours and maximally 120 hours. The medium was as outlined previously except for a reduction in the foetal calf serum concentration to 2.5% (v/v). Cell densities were adjusted to ensure that an equivalent number of cells were harvested/differentiation time point and that cell density did not exceed 10^6 cells/ml at harvest.

2.2.4. CRYOGENIC PRESERVATION OF CELL LINES.

Adherent cells were removed from the surface of the flask by trypsinisation as described above. U937 cells were pelleted by centrifugation at 800 x g_{av} for five minutes. The cells were then resuspended in freezing medium, which consisted of 8% (v/v final) DMSO in FCS, at approximately 10^6 cells/ml for both cell lines. The suspension was aliquoted (0.5ml volumes) into sterile freezing vials. Cells were slow frozen, by packing in cotton wool, for 24 hours at -80°C prior to transfer to liquid nitrogen for storage.

To bring cells from liquid nitrogen storage, vials were thawed rapidly at 37°C, resuspended in 10mls of the appropriate growth medium and centrifuged at 800 x g_{av} in a MSE centaur for three minutes to pellet the cells. As the U937 cells were treated with DMSO to differentiate them it was extremely important to thoroughly remove any residual DMSO from the freezing medium. This was achieved by repeating this resuspension and centrifugation step, typically using 40ml of growth medium. The cell pellets were resuspended in fresh growth medium and plated out in a final volume of 12mls or 28mls, depending on the cell type, in a 75 cm^2 flask. Adherent cells were left overnight to attach and the following day the medium was replaced, to remove any cell debris.

2.2.5. TOXIN TREATMENT OF CELLS.

When cells were at the required density bacterial toxins were added to the cell growth medium, whether "normal" or DMSO-containing growth medium, with pertussis toxin used at a final concentration of 50ng/ml and cholera toxin at a final concentration of 100ng/ml. A parallel set of control flasks were treated with an equal volume of each toxins vehicle. After 16 hours treatment, cells were harvested as described overleaf.

2.2.6. CELL HARVESTING.

A. When adherent cells were confluent growth medium was removed from the cell culture flask and 10mls of ice-cold PBS added. Cells were gently washed from the surface of the flask, collected in a 50ml conical tube and centrifuged at 800 x g in a Beckman TJ6 centrifuge for 10 minutes at 4°C. The resulting cell pellet was washed with ice-cold PBS and re-centrifuged. The final pellet was stored for maximally one week at -80°C until use. The U937 cells were harvested by centrifugation as for the adherent cells and the pellet washed twice in ice-cold PBS buffer and stored as for the adherent cells.

B. When the high proteolytic activity of these cells was discovered some changes to the harvesting procedure were introduced. The PBS buffer was altered to include a "cocktail" of protease inhibitors, namely benzamidine (3mM final), leupeptin (10μM final), phenyl methionyl sulphonyl fluoride, (1mM final) and soybean trypsin inhibitor (0.1μM final). The buffer pH remained unaltered with the addition of these inhibitors but was always rechecked. The U937 cells were maintained at 4°C throughout the procedure which was as described in A, although plasma membranes were prepared immediately after cell harvesting, as outlined below.

C. The procedure described in B was modified to include an additional three protease inhibitors in the ice-cold PBS buffer, namely antipain dihydrochloride (1.5μM final), aprotinin (0.2μM final) and pepstatin A (1.5μM final).

2.3. PLASMA MEMBRANE PREPARATION.

A. Membranes were prepared according to Koski & Klee (1981). Frozen cell pellets, obtained by the method described in 2.2.6.A, were thawed and suspended in 5 volumes of ice-cold 10mM Tris-HCL, 0.1mM EDTA, pH 7.5 and

hand-homogenised in a Potter-Elvejhem homogenisor with 15 up/down strokes of a teflon pestle, on ice. The homogenate was centrifuged at 500 x g_{av} for 10 minutes at 4°C, in a Beckman L5-50B centrifuge with a Ti50 rotor, to remove unbroken cells and nuclei. Crude plasma membranes were obtained by centrifugation of the supernatant fluid at 45,000 x g_{av} for 10 minutes at 4°C, resuspension of the resulting pellet in 10 volumes of the ice-cold 10mM Tris-HCL, 0.1mM EDTA, pH 7.5 buffer and a repeat centrifugation at 45,000 x g_{av} for 10 minutes at 4°C. The pellet was then resuspended in the same buffer to a final protein concentration of between 1-4 mg/ml, aliquoted and stored at -80°C until required. This preparation was used for membranes of the NG108-15 and C6BU1 glioma cell lines and the initial preparations of the U937 cell membranes. It was also the method used to prepare rat cortical membranes after dissection of the tissue from male Sprague Dawley rats.

B. To combat problems of proteolytic degradation of the membrane G-proteins in the U937 cell line, the procedure described in A was modified. The cell pellet was obtained using the procedure outlined in 2.2.6.B. The equivalent protease inhibitors, at identical final concentrations, were also included in the ice-cold 10mM Tris-HCL, 0.1mM EDTA, pH 7.5 preparation buffer. Again the buffer pH was checked and adjusted to pH 7.5, if necessary. The initial centrifugation step was altered to 1000 x g_{av} for 10 minutes at 4°C. The procedure in A was then followed and the crude plasma membranes were assayed for protein concentration and experiments performed on them immediately after preparation, without storage at -80°C. The average yield of crude plasma membrane protein was consistently 1mg/10^8 U937 cells.

C. The membranes were prepared as in B except that the cell pellet was obtained using the procedure outlined in 2.2.6.C. The additional protease inhibitors described in 2.2.6.C were also included in the ice-cold 10mM Tris-HCL, 0.1mM EDTA, pH 7.5 buffer. These additional protease inhibitors were used at

the same final concentration as described in 2.2.6.C.

D. Plasma membranes prepared from rat white adipocytes were a kind gift from Dr. E. David Saggerson and were prepared as outlined in Chohan *et al.*, (1984).

Plasma membranes prepared from HL-60 cells differentiated to neutrophil-like cells, by inclusion of dibutryl cAMP (300μM) in the growth media for 2 days, were a kind gift from Dr. S. Cockcroft and were prepared as described in Bennett *et al.*, (1982).

2.4. PROTEIN CONCENTRATION DETERMINATION.

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

Stock Solutions:

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

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

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

Just prior to use, the stock solutions were mixed in the following ratio, A:B:C, 100:1:1, to produce solution D. Protein standards were prepared using a 1mg/ml bovine serum albumin (fraction V), and a standard curve constructed for 0, 2, 5, 10, 15, 20, 25 and 30μg of BSA, in duplicate. Unknowns were assayed in 2, 4 and 8μl volumes, in duplicate. 1ml of solution D was added to each sample, mixed and left to stand for 10 minutes. 100μl of Folin's Ciocalteau reagent (diluted 1:1 with H_2O) was added to each sample, mixed and allowed to stand for a further 20 minutes. The absorbance of light by each sample was assessed spectrophotometrically at 750nm in a LKB Ultrospec 2.

2.5. PRODUCTION OF ANTIPEPTIDE ANTISERA.

This section is included for information purposes, as I did not perform this technique.

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

3mg of the appropriate peptide and 10mg of keyhole limpet haemocyanin were dissolved slowly in 1ml of 0.1M phosphate buffer pH 7.0. 0.5ml of gluteraldehyde 21mM, also in 0.1M phosphate buffer pH 7.0, was then added dropwise with stirring and the combined 1.5ml incubated overnight at room temperature. The 1.5ml solution was mixed with an equal volume of complete Freund's adjuvant and briefly sonicated with a Branson 'soniprobe' (Type 7532B). 1ml aliquots of the resulting emulsion were injected in multiple subcutaneous sites in New Zealand white rabbits. Four weeks later each animal received a booster immunisation with material identically prepared, except one half as much keyhole limpet haemocyanin and peptide were injected in incomplete Freund's adjuvant.

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

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

TABLE 2.1. ANTI G-PROTEIN ANTIPEPTIDE ANTISERA.

Antiserum	Peptide Used	G-protein sequence	Antiserum Identifies
AS7	KENLKDCGLF	Tdα 341-350	Tdα, $G_{i}1\alpha$, $G_{i}2\alpha$.
SG1	KENLKDCGLF	Tdα 341-350	Tdα, $G_{i}1\alpha$, $G_{i}2\alpha$.
I3B	KNNLKECGLY	Gi3α 345-354	$G_{i}3\alpha$.
OC1	ANNLRGCGLY	Goα 345-354	$G_{o}\alpha$.
IM1	NLKEDGISAAKDVK	Goα 22-35	$G_{o}\alpha$
ON1	GCTLSAEERAALERSK	Goα 1-16	Goα
CS1	RMHLRQYELL	Gsα 385-394	$G_{s}\alpha$.
LE2/3	LERIAQSDYI	Gi2α 160-169	$G_{i}2\alpha$.
I1C	LDRIAQPNYI	Gi1α 159-168	$G_{i}1\alpha$.
BN1	MSELDQLRQE	Gβ. 1-10	β1, β2.

Amino acids are represented using the one letter code.

2.6. AFFINITY PURIFICATION OF ANTIPEPTIDE ANTISERA.

Crude antisera were chromatographed on a 1.5cm x 2 cm column of protein-A-sepharose 4B. A 5ml volume of antiserum in glycine buffer (1.5M Glycine, 3M NaCl, pH 8.9) was added to the column and allowed to equilibrate. The column was washed with glycine buffer until the eluate had an A_{280} of zero and was then eluted with 100mM citric acid, pH 4.0 into 2M Tris/HCL, pH 7.5. The eluted IgG fractions were dialysed overnight against 1000 volumes of 10mM Tris-HCl, 0.1mM EDTA (pH 7.5), lyophilised and stored at -80°C. Just prior to use, samples were reconstituted to the required dilution in the 10mM Tris-HCl, 0.1mM EDTA (pH 7.5)buffer.

2.7. GEL ELECTROPHORESIS.

Protein separation was achieved using a discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) system as originally described by Laemmli, (1970) and modified by Milligan & Klee, (1985).

2.7.1. Resolving Gel Preparation.

Stock solutions:

(filtered through Whatman No.1 filter paper prior to use and stored at 4°C).

Solution A	1.5M Tris, 0.4% (w/v) SDS, adjusted to pH 8.8 with HCL.
Solution B	0.5M Tris, 0.4% (w/v) SDS, adjusted to pH 6.8 with HCL.
Solution C	30% (w/v) Acrylamide, 0.8% (w/v) N, N'-methylene bisacrylamide.
Solution D	30% (w/v) Acrylamide, 0.15% (w/v) N, N'-methylene bisacrylamide.
Solution E	50% (v/v) Glycerol.
Solution F	10% (w/v) Ammonium persulphate (freshly prepared).
Solution G	N, N, N', N'-Tetramethylenediamine (Temed).

10% (w/v) Acrylamide/0.25% (w/v) N, N'-methylenebisacrylamide gels were prepared from stock solutions in the following proportions:

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

To a final volume of 24ml with distilled water.

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

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

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

To a final volume of 36ml with distilled water.

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

2.7.2. Stacking gel preparation.

Stacking gels were prepared from the stock solutions as follows:

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

To a final volume of 15ml with distilled water.

The solution was mixed, layered on top of the resolving gel and allowed to polymerise around a 15 well teflon plate for approximately one hour.

2.7.3. Preparation of samples for electrophoretic separation.

A. Protein precipitation.

Membrane samples were prepared for gel electrophoresis by sodium deoxycholate/ trichloroacetic acid precipitation. This was initiated by addition of 6.25μl of 2% (w/v) sodium deoxycholate to the required amount of membrane protein, followed by 750μl of distilled water and then 250μl of 24% (w/v) trichloroacetic acid. Samples were centrifuged in a Hettich Mikro Rapid/K at 12000 x g_{av} for 10 minutes at 4^{o}C. The supernatants were then removed and the pellets dissolved in 20μl of 1M Tris base followed by 20μl of Laemmli buffer which consisted of 5M Urea, 0.17M SDS, 0.4M DTT, 50mM Tris-HCl pH 8.0, 0.01% Bromophenol Blue.

B. Protein Alkylation.

In order to increase the resolution of the pertussis toxin-sensitive G-proteins, samples to be run on 12.5% (w/v) Acrylamide, 0.0625% (w/v) N, N'-methylenebisacrylamide gels were firstly alkylated by treatment with N-ethylmaleimide (NEM) a modification of the method described by Sternweis & Robishaw, (1984).

Samples were centrifuged in a Hettich Mikro Rapid/K at 12000 x g_{av}, the supernatants discarded and the pellets resuspended in 20μl of 10mM Tris-HCL, 1mM EDTA, pH 7.5. After the addition of 10μl of 5% (w/v) SDS, 50mM DTT to each sample they were then vortex mixed and placed in a boiling water bath for 5-10 minutes. Samples were then cooled to room temperature and 10μl of 100mM NEM added. After alkylation for at least 15 minutes, at room temperature, sample

preparation was completed by addition of 20μl of Laemmli buffer to each sample. This 15 minute alkylation period was demonstrated to be sufficient to separate $G_i1\alpha$ and $G_i2\alpha$ into two well resolved, immunologically distinguishable polypeptides.

2.7.4. Operating conditions.

Samples were loaded into the wells of the stacking gel using a well rinsed Hamilton microsyringe. For reference, prestained molecular weight markers were similarly loaded onto every gel.

The gel tank was immersed in reservoir buffer (0.025M Tris, 0.192M Glycine, 0.1% (w/v) SDS, pH 7.5) and electrophoresis performed overnight at either 50V/20mA when using the Bio-rad system, or 100V/35mA when using the (Protean II system).

2.8. STAINING OF GELS FOR PROTEIN.

2.8.1. Coomassie blue staining.

After electrophoresis, the gel was covered in stain solution which consisted of 0.1% (w/v) Coomassie blue in 50% (v/v) distilled water, 40 % (v/v) methanol, 10% (v/v) glacial acetic acid and shaken gently for 1 hour. The stain solution was then discarded and the gel soaked in destain solution (identical to stain solution, but lacking Coomassie blue) until proteins were apparent on the gel as discrete bands.

2.8.2. Silver staining.

Gels were silver stained according to the method of Morrissey, (1981). It should be noted that care was taken throughout the staining procedure to minimise even gloved contact with the gel. After electrophoresis, the gel was prefixed for 30 minutes in a 50% (v/v) methanol, 10% (v/v) acetic acid solution. This was followed by a 30 minute incubation in a 5% (v/v) methanol, 7% (v/v) acetic acid solution. The gel was then placed in 10% (v/v) gluteraldehyde for 30 minutes, then rinsed in distilled water and left to soak for a minimum of two hour in a large

volume of distilled water. The gel was then soaked in 5μg/ml dithiothreitol for 30 minutes. This solution was discarded and without rinsing 0.1% (w/v) silver nitrate was added and left for 30 minutes after which the gel was rinsed rapidly with a small volume of distilled water (typically 30-50ml was sufficient to cover the gel if a plastic tub or petri dish was used). The gel was then rinsed in a small amount of developing solution, 50μl of 37% (v/v) formaldehyde in 100ml of a 3% (v/v) sodium carbonate solution and finally left to soak in this solution until the required level of staining was achieved. Development was stopped by addition of 2.3M citric acid and agitation for 10 minutes. The gel was then rinsed in distilled water.

2.9. AUTORADIOGRAPHY.

After staining, gels were dried down onto Whatman 3mm chromatography paper at 60°C for 1 hour, under vacuum. After drying the prestained molecular weight markers were marked with a 0.5μl spot of "^{14}C-spiked" ink solution. The gel was then covered in cling film and placed adjacent to Fuji X-ray film in a cassette containing intensification screens for various periods, as indicated in the figure legends, at -80°C. Films were developed both by hand using Kodak LX-24 developer and FX-40 fixer following exactly the instructions issued with these products and using the automated X-Omat developing system.

Analysis of incorporation of radioactivity into polypeptides of interest was performed by scanning the autoradiographs with a Bio-rad scanner driven by an Olivetti M24 personal computer. Absorption was measured in arbitrary units.

2.10. IMMUNOBLOTTING.

Proteins were separated under appropriate resolving conditions on SDS/polyacrylamide gels and then transferred to a nitrocellulose sheet and detected using a modification of the method of Towbin *et al.*, (1979).

2.10.1. Transfer of proteins to nitrocellulose.

After removal of the stacking gel the resolving gel was allowed to soak in transfer buffer (0.192M Glycine, 25mM Tris, 20% (v/v) methanol) for a few minutes. A blotting "sandwich" was constructed in a plastic holder such that the gel was in contact with the nitrocellulose sheet, with a sheet of Whatman 3mm chromatography paper and an outer piece of foam on either side. This sandwich was then placed into a LKB Transblot tank and completely immersed in transfer buffer. A LKB Transphor powerpack was connected such that the negatively charged proteins transferred to the nitrocellulose sheet and run at maximum voltage (1.5A) for 1.5 hours.

2.10.2. Immunodetection of the G-protein subunits.

After transfer the nitrocellulose sheet was incubated for 2 hours at 30°C in a solution of 5% (w/v) gelatin in Tris buffered saline (TBS), prepared as outlined in 2.1. This incubation was included to block potential non-specific antiserum binding sites. The sheet was then washed free of excess gelatin with several rinses of distilled water and the nitrocellulose sheet incubated overnight at 30°C with the appropriate dilution of antipeptide antiserum, diluted in 1% (w/v) gelatin in TBS. The primary antiserum was removed and the sheet subjected to several washes initially in TTBS, prepared as outlined in 2.1, and then in TBS for 15 minutes with each buffer. The immunoblot was then incubated with a second antibody (peroxidase-conjugated goat anti-rabbit IgG) for 2 hours at 30°C. This second antibody was used at a 1:200 dilution in all experiments and prepared as described for the primary antipeptide antiserum. The second antibody was removed and the sheet subjected to another series of washes in TTBS and TBS as described previously. After removal of the TBS the immunoblot was then rehydrated in 40mls of 10mM Tris pH 7.5. The immunoblot was developed by addition of

0.025% (w/v) ortho-dianisidine and 10μl of 30% (w/v) hydrogen peroxide solution. Development of the resultant brown stain was arrested by extensive washing of the blot in distilled water.

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

This general procedure was employed in all the immunoblots represented although slight alterations in the buffers, blocking agent and detergent were used in some cases. This is indicated in the figure legends by the following notation:

A. replacing gelatin with Marvel throughout the above procedure.

B. using 5% (w/v) gelatin in phosphate buffered saline (PBS) prepared as outlined previously in 2.1. The antisera were prepared in 1% gelatin (w/v) in PBS containing 0.2% (v/v) non-ionic detergent NP-40. The TTBS wash was replaced with PBS containing 0.2% (v/v) NP-40 and then followed with PBS rinses. However, the immunoblots were developed as in A, although the reaction was arrested using a 1% (w/v) sodium azide solution in distilled water.

2.10.3. Quantification of the primary immunoreactive signal with an [125I]-labelled secondary antibody.

After development of the immunoblot with a non-radioactive secondary antiserum an [^{125}I]-labelled Goat Anti-rabbit IgG (0.05-0.1μCi/ml) was prepared as described for the primary antipeptide antiserum in 2.10.2 in a small conical flask and the immunoblot then rehydrated in this antibody solution. Plastic containers were used for only this purpose and were maintained in good condition to ensure that no radioactivity bound to them. The immunoblot was incubated at 30°C for a minimum of two hours, the antibody discarded and the nitrocellulose washed as outlined in 2.10.2. During the washing procedure the immunoblot was monitored

to assess the background levels of iodination and the wash conditions altered accordingly to achieve a low background signal. The immunoblot was then dried and autoradiography performed as described in 2.9. Identically sized "chips" were cut from the immunoblot. These were from the peroxidase-stained G-protein subunit and from "background" areas of the nitrocellulose i.e., both outwith where the samples were loaded and from every sample lane. These chips were then Cerenkov counted in a LKB 1275 minigamma counter, the background level of iodination subtracted and counts associated with the G-protein subunits evaluated.

2.11. BACTERIAL TOXIN-CATALYSED ADP-RIBOSYLATION.

The *in vitro* mono ADP-ribosylation of membrane G-proteins was assayed with a modified version of that described by Hudson & Johnson, (1980).

The appropriate bacterial toxin was added at a final concentration of 10μg/ml (pertussis toxin) or 50μg/ml (cholera toxin). Both toxins were activated prior to use by preincubating with an equal volume of freshly prepared 100mM dithiothreitol (DTT) for 1 hour at room temperature.When a no toxin control was performed 50mM DTT was included in the reaction.

Membranes to be ADP-ribosylated were diluted in 10mM Tris-HCL, 0.1mM EDTA pH 7.5, to an appropriate protein concentration and 20μl aliquots were assayed in a final volume of 50μl containing the following:

	+GTP	-GTP
Toxin	5μl	5μl
Agonist/vehicle	—	5μl
Membrane protein	20μl	20μl
Reaction mix	25μl	20μl

The "-GTP" conditions were employed when agonist-stimulated cholera toxin-catalysed [32P]ADP-ribosylation was investigated, as indicated in the appropriate

figure legends. When using these conditions the membranes were "washed" by pelleting the membranes in a Hettich Mikro Rapid/K at 12000 x g_{av} for 10 minutes at 4°C and then resuspending in 10mM Tris-HCL, 0.1mM EDTA pH 7.5 buffer and this procedure repeated. The agonist concentration and that of a vehicle control are also indicated in the figure legends, where relevant. The reaction mix contained the following components:

250mM potassium phosphate buffer, pH 7.0,

[^{32}P] NAD$^+$ (2μCi/sample),

20mM Thymidine,

1mM ATP, pH 7.5,

100μM GTP, pH 7.5 (when the presence of GTP was required),

20mM Arginine/HCL.

The NAD$^+$ concentration in the assay was initially dependent upon the [^{32}P]NAD$^+$ added and was generally 1μM final. Laterally a constant NAD$^+$ concentration of 0.7μM final was employed, achieved by the addition of suitable concentrations of unlabelled NAD$^+$.

The components of the ribosylation assay were added to each other upon ice in the descending order indicated above, vortex mixed and the reaction initiated by incubation in a water bath at 37°C.

The toxin was actually added directly to the mix in most experiments to prevent any possibility of variation in incorporation of [^{32}P]ADP-ribose into the G-protein α subunits being attributable to inaccuracy in the 5μl toxin additions. Obviously the volume of "mix + toxin" was increased accordingly and reaction mix added to the "- toxin" samples prior to "toxin spiking".

The "cocktail" of protease inhibitors outlined in 2.2.6 C was included, at equivalent final concentrations, in the ribosylation reaction where indicated.

Assays proceeded for between 1-2 hours (or as indicated in the figure legend) and were terminated by removal to ice followed by sodium

deoxycholate/trichloroacetic acid precipitation as detailed in 2.7.3.A. Samples were then resolved under appropriate SDS-PAGE conditions, as outlined in 2.7 and the [^{32}P]ADP-ribosylated proteins visualised by autoradiography of the dried gel as outlined in 2.9.

2.12. IMMUNOPRECIPITATION OF G-PROTEIN α SUBUNITS.

This method was adapted from that of Rothenburg & Kahn, (1988).

When an immunoprecipitation was performed on previously toxin ribosylated proteins then the reaction was stopped with 500μl of ice cold buffer, 10mM Tris/0.1mM EDTA (pH 7.5). Samples were then centrifuged in a Hettich Mikro Rapid/K (12000 x g_{av}, 10 mins., 4°C) and the supernatants discarded. The membrane pellets were resuspended in 50μl of 1% SDS and boiled for approximately 3 minutes. After cooling, 950μl of Kahn solubilisation buffer (1% Triton X-100, 10mM EDTA, 100mM NaH_2PO_4, 10mM NaF, 100μM Na_3VO_4, 50mM Hepes, pH 7.2 at 4°C) containing the equivalent final concentrations of the protease inhibitors outlined previously in 2.2.6.B and C was added. The samples were incubated on ice for a minimum period of 1 hour and then respun in the Hettich Mikro Rapid/K as previously outlined to pellet any non-solubilised material. The supernatant was removed and whole serum antipeptide antiserum added at an appropriate concentration, as indicated in the relevant figure legend. Pre-immune whole serum, at an equivalent concentration was added to one sample to serve as a control. The samples were incubated overnight at 4°C.

A 1:1 suspension of protein-A-agarose (50μl) was then added to each sample. The solution was mixed "end over end" on a slowly rotating wheel, for a minimum incubation period of two hours at 4°C. The immune complex was pelleted as outlined previously, reducing the spin duration to 5 minutes. The supernatant was then carefully removed and the proteins TCA precipitated, as detailed in 2.7.3.A. The immune complex was washed (three 1ml washes) by a process of resuspending in Kahn wash buffer and then spinning as previously

outlined, again reducing the spin duration to 5 minutes. Kahn wash buffer (1% Triton X-100, 100mM NaCl, 100mM NaF, 50mM NaH_2PO_4, 50mM Hepes, pH 7.2 at 4°C) was prepared as a stock without SDS, which was then added to a final concentration of 0.1%. The immune complex pellet was then resuspended in 30μl of Laemmli buffer (2.7.3.A) and the samples were boiled for approximately five minutes, with gentle resuspension of the agarose during the boiling process. The samples were then centrifuged as previously indicated and the supernatant loaded directly onto a resolving gel (as in 2.7).

2.13. ASSAY OF HIGH AFFINITY GTPase ACTIVITY.

The assay monitors the release of ^{32}Pi from $\gamma[^{32}P]GTP$ and was essentially performed using the method of Koski and Klee, (1981). This is a modification of the original method described by Cassel and Selinger, with the concentration of 5' adenyl-imidodiphosphate (App[NH]p) being lowered to 0.1mM (Cassel & Selinger, 1976).

The reaction mixture contained 0.5μM $\gamma[^{32}P]GTP$ (~50,000 c.p.m.), 0.5μM GTP, 0.1mM App[NH]p, 1mM ATP, 1mM ouabain, 10mM creatine phosphate, 5 units creatine phosphokinase, 100mM sodium chloride, 5mM magnesium chloride, 2mM dithiothreitol, 0.1 mM EDTA, 20 mM Tris-HCl, pH 7.5.

Aliquots of the reaction mixture (50μl) were added to tubes on ice containing membrane protein (3-10μg as indicated in the relevant figure legends) and the appropriate ligand in a final volume of 100μl. Low affinity hydrolysis of $\gamma[^{32}P]GTP$ was assessed in the presence of GTP (100μM final) and blank values were determined by the replacement of membrane protein with the buffer they were suspended in i.e., 10mM Tris-HCL, 0.1mM EDTA pH 7.5. Hydrolysis of $\gamma[^{32}P]GTP$ at 0°C was negligible.

The reaction was initiated by transferring the tubes to a 37°C water bath.

After 20 minutes, the tubes were immersed in an ice bath and 900μl aliquots of 20mM phosphoric acid (pH 2.3) containing 5% (w/v) activated charcoal were then added. After centrifugation for 20 minutes at 12000 x g_{av} in a MSE microcentaur, 500μl of the supernatant fluid was carefully removed with the minimum displacement of the charcoal and radioactivity in the sample assessed by Cerenkov counting in a rackbeta scintillation counter set to the tritium counting channel.

In some experiments, membrane protein was preincubated under various conditions (as indicated in the relevant figure legends) in the presence of the GTPase assay reagents, with the exception of γ[^{32}P]GTP and receptor ligands. After this preincubation period, both γ[^{32}P]GTP and suitable ligands were added, and the assay conducted as just outlined.

2.14. RADIOLIGAND BINDING ASSAY.

Radiolabelled chemotactic peptide binding assays were performed using a modification of the method described by Herrmann *et al.*, (1989).

The binding buffer consisted of 50mM Tris-HCL, 1mM EDTA, 20mM magnesium chloride, pH 7.3 (at both temperatures used). The protease inhibitors detailed in 2.2.6 B were included in the assay buffer in certain experiments , whereas the additional inhibitors outlined in 2.2.6 C were present in the assay buffer in later experiments. Again these different assay conditions are indicated in the relevant figure legends. The binding reaction contained 100-300μg of membrane protein (as indicated in the relevant figure legend), binding buffer and [^{3}H]fMLP (at the concentrations indicated in the figure legends), in a final volume of 250μl. Non-specific binding was determined as the amount of radioligand bound in the presence of 100μM fMLP. This non-specific binding increased linearly with increasing concentrations of radioligand and was between 5-10% and 20-30% of the total binding at the "high" and "low" affinity sites, respectively. Blank values were determined by replacement of membrane protein with buffer.

The assay was initiated by transferral of tubes to a 25°C water bath for 30 minutes, after which time the tubes were removed to ice and a 200µl volume rapidly filtered through Whatman GF/C glassfibre filters, which had been presoaked in ice-cold assay buffer, followed with three 5ml washes of the filter with ice-cold buffer. Filters were soaked overnight in ecoscint scintillation fluid, prior to counting in a rackbeta scintillation counter.

In some assays, membranes were pre-incubated under various conditions. The non hydrolysable analogue of GTP, Gpp[NH]p (100μM final) was preincubated for five minutes with U937 cell membranes prior to a binding assay being performed, as was the fMLP antagonist N-t-butoxycarbonyl-Met-Leu-Phe (100μM final). In some assays, membranes were preincubated with an IgG fraction purified from either preimmune rabbit serum or an anti-G-protein antipeptide antiserum (as detailed in section 2.6) for 5 hours on ice, prior to the binding assay. In some instances, data were manipulated according to Scatchard, (1949).

2.15. MASS MEASUREMENT OF STIMULATED INS(1,4,5)P3 GENERATION.

2.15.1. Preparation of samples for measurement of Ins(1,4,5)P3 mass.

U937 cells were differentiated for 72 hours in DMSO (1.25% v/v) as described in 2.2.3. Cells were harvested by pouring the cell suspension into sterile centrifuge tubes and a centrifugation spin performed as described in 2.2.6. A, but at 37°C. The cells were then washed twice with Hanks at 37°C (pH 7.4), prepared as in 2.1. The cell pellet was then resuspended in an appropriate volume of HBG (pH 7.4), prepared as described in 2.1 and incubated at 37°C for 20 minutes. The cells were gently resuspended by swirling the tube during this incubation period. This

"recovery" period was assessed in an extensive series of experiments with a variety of cell types and harvesting procedures and was found to be necessary to prevent the artificially high basal Ins(1,4,5)P3 levels recorded if cells were assayed immediately after cell harvesting.

Aliquots (50μl) containing the required cell number (as indicated in the relevant figure legend) were stimulated with 5μl of agonist (fMLP at the concentration indicated in the relevant figure legend) at 37°C in plastic polytubes. The reactions were terminated by the addition of 25μl of ice-cold 10% (v/v) perchloric acid. The samples were extracted for 20 minutes on ice and neutralised with 1.5M KOH/60mM Hepes containing Universal indicator. The cell debris and precipitated potassium perchlorate were removed by centrifugation at 14,000 x g_{av} for 3 minutes at 4°C. A 25μl aliquot of the supernatant was removed for assay of Ins(1,4,5)P3 mass.

2.15.2. Mass Ins(1,4,5)P3 assay.

Samples were prepared as just described and assayed for Ins(1,4,5)P3 mass using the competitive binding assay developed by Palmer and co-workers (Palmer *et al.*, 1989).

The assay employs a bovine adrenocortical microsomal preparation which possesses a single population of specific high affinity binding sites for Ins(1,4,5)P3. Bovine adrenals were defatted and the medulla region removed, on ice. The tissue was blended in a Waring blender (10 seconds at low speed and 1 minute at high speed) in homogenisation buffer consisting of 20mM $NaHCO_3$, 1mM DTT, pH 7.5 at 4°C, 2.5ml of buffer/g wet weight of adrenal tissue. The blended material was then homogenised using a rotating pestle (setting 4) for 3 up/down strokes. The homogenate was centrifuged at 500 x g_{av} for 10 minutes at 4°C. A microsomal preparation was obtained by centrifugation of the supernatant fluid at 35,000 x g_{av} for 20 minutes at 4°C, resuspension of the resulting pellet in the homogenisation buffer and a repeat centrifugation again at 35,000 x g_{av} for 20

minutes at 4°C. The final pellet was then resuspended in the same buffer to a final protein concentration of approximately 20mg/ml and stored as 1ml aliquots at -80°C until required. A typical yield was 3g of "binding protein" from 20 adrenal glands.

Aliquots (50μl) of adrenal cortex preparation (20mg/ml of "binding"protein) were incubated, on ice for 20 minutes, with 150μl of binding buffer (100mM Tris, 4mM EDTA, 4mg/ml BSA, pH 8.5), 100μl of sample or Ins(1,4,5)P_3 standard (usually 25-50μl of sample made up to volume with binding buffer or 25μl of standard similarly made up to volume) and 100μl of [^{3}H] Ins(1,4,5)P_3 ($\sim$ 3,000 c.p.m. i.e., 25μl made up to volume with binding buffer) in a total volume of 400μl. Non-specific binding was determined in the presence of 1μM Ins(1,4,5)P_3. Incubations were terminated by centrifugation in a Hettich Mikro Rapid/K at 12,000 x g_{av}, for 3 minutes at 4°C and the supernatant then aspirated. The radioactivity bound to the pellet was determined by liquid scintillation counting after solubilisation in ecoscint. In every assay conducted the use of a standard curve, in which displacement of [^{3}H]Ins(1,4,5)P_3 by known concentrations of unlabelled Ins(1,4,5)P_3, enabled estimation of Ins(1,4,5)P_3 mass in the cell extracts. The unlabelled Ins(1,4,5)P_3 was generously provided by Dr. R. Irvine of the A.F.R.C. unit, Cambridge, England.

2.16. DATA ANALYSIS.

When two mean values, each with a deviation, were subtracted from or divided by each other a "compounded" error was calculated using the appropriate formulae.

Where appropriate, data were analysed for statistical significance using the either the paired or unpaired Student's *t*-distribution test with "n" numbers representing the degrees of freedom, as indicated. In the paired test this "n" value was calculated from the number of experiments (n') performed -1, whereas in the unpaired test it was calculated from $n_1' + n_2' - 1$.

CHAPTER 3.

THE U937 CELL LINE - G-PROTEIN α AND β SUBUNIT PROFILE AND ALTERATIONS IN EXPRESSION OF THESE PROTEINS UPON DMSO-INDUCED DIFFERENTIATION.

3.1. INTRODUCTION.

Differentiation can be defined as a phase of growth during which unspecialised cells become specialised for particular functions. This process underlies the production of the many types of blood cells, with functions which range from oxygen transport to antibody production. These different types of terminally differentiated blood cells all originate from pluripotent, haematopoietic stem cells in a complex manner (summarised in Diagram 3.1).

Myeloid stem cells form the colony-forming unit for granulocytes-monocytes (CFU-GM) and leave the proliferative pool (Sieff, 1987). They then begin monocytic differentiation, a process taking approximately six days in the bone marrow. After migration into different tissues monocytes terminally differentiate into macrophages, with morphological and in some cases functional characteristics typical for a particular tissue (Auwerx, 1991). Macrophages are essential for host resistance to infecting organisms, neoplastically transformed cells and denatured macromolecules. Further they secrete products which display varied biological activities ranging from induction of cell growth to provocation of cell death (Nathan, 1987). Macrophages can also interact with lymphocytes, to which they present antigen. These lymphocytes release regulatory proteins, termed lymphokines, which activate macrophages resulting in microbicidal activity (Adams & Hamilton, 1984).

Indeed the multifunctional nature of the monocyte implies that this cell has to undergo a very complex differentiation process involving finely tuned changes in gene expression. This complex process is sustained through a family of glycoprotein hormonal growth factors including various colony stimulating factors (CSFs), erythropoietin, interleukins and interferons (Sieff, 1987). The active vitamin D compound, 1α, 25-dihydroxyvitamin D_3, γ interferon and tumour necrosis factor (TNF) are more strictly limited to macrophage differentiation and activation (Auwerx, 1991). It is believed that all these factors are interacting in a

complex network, although the details of their individual signal transduction routes and degree of "crosstalk" between them is at present unclear.

Limited amounts of human monocytes are available and the study of their differentiation has been accelerated by the generation of several lines of human leukaemic cells which are blocked at certain steps of differentiation. The different maturation stages can thus be analysed in a relatively homogeneous population of cells which can be readily cultured and manipulated in sufficient quantities for biochemical characterisation. The human HL-60 cell line can be differentiated along both monocytic and granulocytic lineages, depending on the differentiating agent employed (Huberman & Callaham, 1979; Chaplinski & Niedel, 1982). Cells from the human cell line, U937, represent the neoplastic derivatives of committed progenitors of monocytes. The U937 cell line was derived from a patient with a generalised histiocytic lymphoma (Sundström & Nilsson, 1976). Some of the initially characterised properties of this cell line are summarised in Table 3.1. Subsequent research demonstrated that this cell line would differentiate into "macrophage-like" cells when exposed to supernatants from lectin-stimulated lymphocytes. These supernatants were termed "conditioned medium" (Koren *et al.*, 1979). Since these studies a variety of well defined compounds, such as dimethylsulphoxide (DMSO) and N^6, O^{2-} dibutryladenosine 3': 5'-cyclic monophosphate (dibutryl cAMP), have been found to induce differentiation of U937 cells. With the exception of the superoxide anion response, DMSO was as effective as conditioned medium for inducing maturation associated biological responses (Kay *et al.*, 1983).

A large range of both artificial and natural agents induce differentiation of these cell lines. These agents modulate a vast array of constitutive and inducible histiochemical and biochemical properties that are characteristic of differentiation. A selection of these responses, evident in the U937 cell line, are summarised in Table 3.2 (reviewed by Harris & Ralph, 1985).

It is apparent from the examples provided in Table 3.2 that gene expression alters significantly upon differentiation. The primary control of gene expression occurs at the level of transcription. An increasing number of factors are being discovered which have the ability to bind to DNA in a sequence specific manner and then interact with other factors or with the RNA polymerase itself, influencing transcription positively or negatively (reviewed by Latchman, 1990). A transcription activity designated activator protein 1 (AP-1) has been shown to bind specifically to a sequence located within enhancer elements of genes. This short core sequence motif was common to several TPA-responsive elements (TRE). Indeed, AP-1 is thought to be involved in regulating both basal level activation of transcription and a specific response to induction by the phorbol ester, 12-*o*-tetradecanoylphorbol 13-acetate (TPA), (Lee *et al.*, 1987). TPA is a commonly used differentiating agent inducing both HL-60 and U937 cell lines into monocytoid development. Another enhancer binding protein has been identified and designated activator protein 2 (AP-2). In common with AP-1, AP-2 binds to the control elements of a large number of genes, although it appears to have a more limited tissue distribution. The binding site for AP-2 forms a cell-type-specific enhancer element whose activity is increased in response to treatment of cells with phorbol esters and agents that elevate cAMP levels (Imagawa *et al.*, 1987). Interestingly a variety of agents that increase intracellular cAMP levels induce differentiated characteristics in the human myeloid cell lines.

Differentiation of monocytes or myeloid cell lines into macrophages has been associated with altered expression of various oncogenes (Collins, 1987). The protein products of two proto-oncogenes, *fos* and *jun* bind to AP-1 sites (Angel *et al.*, 1988; Chiu *et al.*, 1988). Although the jun protein can bind as a homeodimer, the fos protein can only bind in heterodimeric form with jun (Chiu *et al.*, 1988). In the HL-60 cell line TPA treatment induced predominantly c-*fos* mRNA with limited effect on c-*jun* mRNA (Auwerx *et al.*, 1990). Such alterations in fos/jun heterodimeric transcription complexes may be involved in

increasing the diversity of cellular responses to differentiating agents. However, there are oncogenes whose expression is unchanged or decreased upon cell differentiation and as such it remains to be established whether these effects are causal or only an associated phenomenon of differentiation.

The altered expression of numerous proteins manifests itself in the development of "maturation-specific" biological responses. Indeed the increased expression of receptors for chemoattractants e.g., the N-formyl peptide receptor, enables these agents to elicit a vast array of intracellular biochemical alterations including morphological polarisation and chemotaxis. Exposure of phagocytes to doses of chemotactic factors generally at least ten-fold greater than those required to initiate chemotaxis, stimulates the secretion of lysosomal enzymes and the production of superoxide anion via the "respiratory burst" reaction. The stimulus-response coupling for these diverse and complex properties is beginning to be defined in biochemical terms and what is becoming apparent is that phospholipid-derived messengers are of key importance.

There is evidence to suggest that activation of phospholipase C may be necessary for chemotaxis and in generation of a respiratory burst reaction. Chemotaxis can be defined as a directed cellular migration along a concentration gradient of a chemoattractant (reviewed by Devreotes & Zigmond, 1988). One consequence of PKC-catalysed phosphorylation is the activation of an amiloride-sensitive Na^+-H^+ exchanger, which leads to an increase in intracellular pH (Simchowitz, 1985). Elevation of cytoplasmic pH is reported to correlate with chemotaxis (Simchowitz & Cragoe, 1986). The respiratory burst results from the activation of a membrane bound enzyme system, NADPH:O_2-oxidoreductase, which transfers electrons from cytosolic NADPH to extracellular oxygen, producing superoxide (O^{2-}), (reviewed by Rossi, 1986, Baggiolini & Wymann, 1990). Superoxide is released at the outer surface of the plasma membrane into the extracellular space or into phagocytic vacuoles. The oxidase is inactive in resting

phagocytes and is turned on rapidly upon chemotactic stimulation or phagocytosis. A rigorously controlled signalling system is suggested by the rapidity of the onset of the response (approximately 2 seconds), and its immediate cessation upon displacement of the activating stimulus. In human neutrophils, there is evidence to suggest that both Ca^{2+}-dependent and Ca^{2+}-independent signal transduction sequences are important in mediating superoxide anion generation (von Tscharner *et al.*, 1986; Wymann *et al.*, 1987). It has been demonstrated that both are required for functional activation of the respiratory burst response (Dewald *et al.*, 1988).

As introduced in 1.11, PLA_2 activation results in the release of arachidonic which is the precursor for two classes of potent inflammatory mediators, prostaglandins and leukotrienes. The other product of the PLA_2 reaction, the lysophospholipid, can serve as a precursor to the inflammatory mediator, platelet activating factor (PAF). Furthermore, some of these metabolites e.g., leukotriene B_4 (LTB_4) and PAF have chemoattractant properties, potentially initiating a positive feedback loop that amplifies the initial stimulus. As discussed previously, at least part of the antiinflammatory action of glucocorticoids can be attributed to their ability to inhibit PLA_2 activity via lipocortins. Lipocortin has been found to block neutrophil chemotaxis and the cytotoxic action of peripheral blood lymphocytes, again indicating a role for PLA_2 activity in mediating these functions (Hirata, 1985).

The process of exocytotic secretion is highly complex, with different cell types exhibiting different control mechanisms (reviewed by Gomperts, 1990). Recent investigation has demonstrated that induction of receptor-regulated PLD activity, during granulocytic differentiation of HL-60 cells, could be correlated with the induction of receptor-regulated myeloperoxidase release from azurophilic granules (Xie *et al.*, 1991). Other studies suggest that phospholipase D activation is functionally linked to superoxide production in the human neutrophil (Bonser *et*

al., 1989). However, Kessels and co-workers have demonstrated that the respiratory burst can be induced by fMLP, albeit with a much longer lag time, in human neutrophils in which PLD activation has been inhibited by calcium chelation (Kessels *et al.*, 1991). The implication from this latter study is that PLD activation is important, although perhaps not absolutely necessary, for this maturation-related property of these cells.

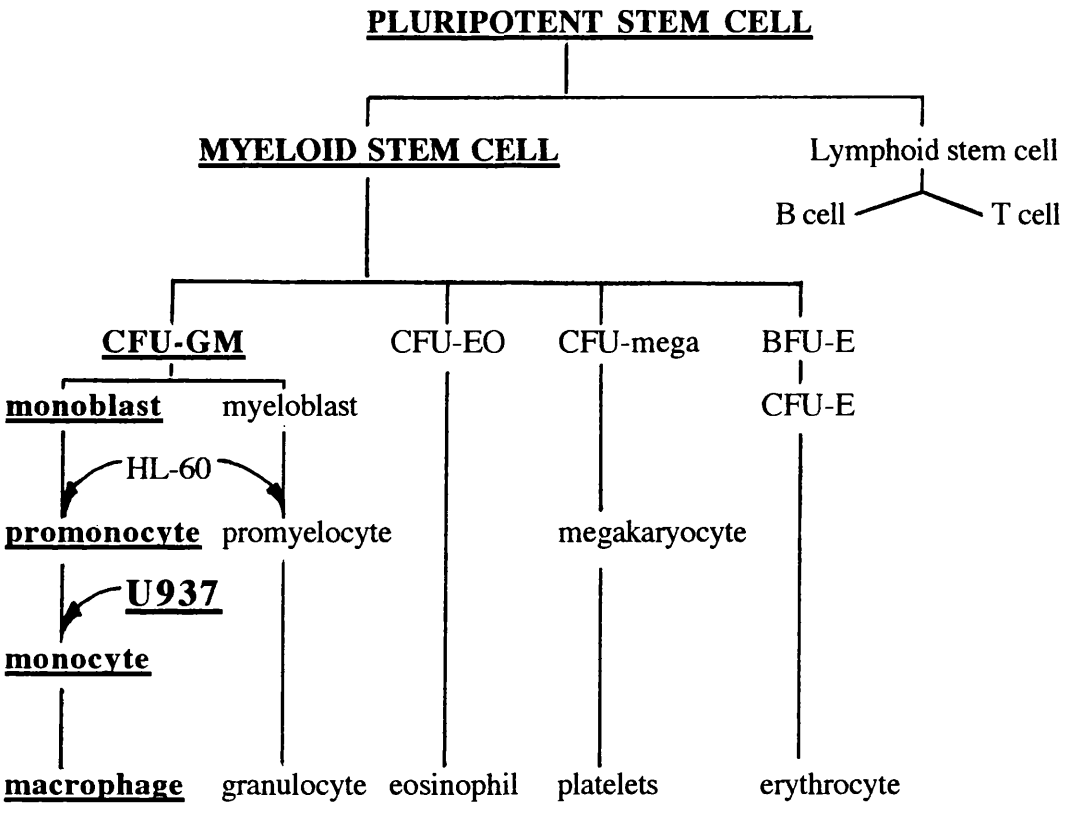
As introduced previously, a range of blood cell types exhibit N-formyl peptide-stimulated GTPase and phospholipase activities that are inhibited by pertussis toxin, as are the respiratory burst and chemotactic responses (Okajima *et al.*, 1985; Verghese *et al.*, 1985a; Becker *et al.*, 1986, Xie *et al.*, 1991). Thus blood cells represent systems in which a pertussis toxin-sensitive protein(s) has a fundamental role in the mediation of agonist-stimulated phospholipid turnover and also in the processes dependent on such metabolism.

It has previously been demonstrated that upon 72 hour DMSO treatment of U937 cells, $G_i2\alpha$ expression is increased and N-formyl-methionyl-leucyl-phenylalanine (fMLP) couples to PIC in a totally pertussis toxin-sensitive manner (Pollock *et al.*, 1990). As mentioned previously, U937 cells have also been a source of phosphatidylcholine-preferring phospholipase C and phospholipase A_2 isoforms (Clark *et al.*, 1990; Kramer *et al.*, 1991). Thus it appeared that the U937 cell line offered a potential system in which to attempt to define the pertussis toxin-sensitive G-protein(s) involved in coupling to fMLP-stimulated phospholipid metabolism.

Prior to undertaking such studies an initial aim was to identify the repertoire of G-proteins expressed, particularly the pertussis toxin substrates, in both non-DMSO-differentiated (control) and DMSO-differentiated U937 cell membranes. As DMSO-induced differentiation of the U937 cells was a prerequisite for fMLP responsiveness, the expression of these G-protein subunits was investigated over a five day DMSO-induced differentiation time course.

Diagram 3.1. HAEMATOPOIETIC CELL DIFFERENTIATION.

(adapted from Figure 1, Auwerx, 1991).



BFU = blast forming unit.

CFU = colony forming unit.

CFU-GM = colony forming unit for granulocytes-monocytes.

CFU-E = colony forming unit for erythrocytes.

CFU-EO = colony forming unit for eosinophils.

CFU-mega = colony forming unit for megakaryocytes.

Relevant cell lines are included with the arrows indicating their differentiation potential.

Table 3.1.

CHARACTERISTICS OF THE HUMAN HISTIOCYTIC LYMPHOMA CELL LINE, U-937. (adapted from Table V, Sundström & Nilsson, 1976).

MORPHOLOGY:

Monocytoid, cell diameter ranging from 8-17μ, round-ovoid in shape, villi present on most cells, irregular, lobated nucleus, large nucleolus, high nucleo-cytoplasmic ratio, sparse endoplasmic reticulum, well developed Golgi apparatus, abundant vesicles.

CYTOCHEMISTRY:

Strong esterase activity, β-glucuronidase present in moderate amounts.

SURFACE RECEPTORS:

C3, Fc.

SECRETORY ACTIVITY:

Lysozyme production (0.7μg/10^5 cells/24 hours), β_2-microglobulin production production (200ng/5 x 10^5 cells/ 65 hours).

GROWTH PROPERTIES:

Population doubling time of approximately 95 hours, suspension culture with a maximum cell density of 0.9-1.1 x 10^5 cells/ml.

CLONALITY:

Monoclonal.

KARYOTYPE:

Aneuploid.

Table 3.2.

DIFFERENTIATION-ASSOCIATED ALTERATIONS IN THE PROPERTIES OF THE U937 CELL LINE.

PROPERTY	EXAMPLES	REFERENCE
Morphological.	increases in cell size.	
	nucleus becomes lobated.	
	vacuoles replace cytoplasmic granules.	(**1**).
Increased enzyme activity.	increased phosphatase, esterase and	
	β-glucuronidase activity.	(**2**).
Induction of hydrogen peroxide production.		(**2**).
Increased cytokine release.	interleukin-1.	
	complement component, C2.	(**3**).
Induction of receptor and membrane antigen expression.	IgG1, Mac-1 and Mac-3 antigens.	(**4**, **5**).
	N-formyl-methionyl-leucyl phenylalanine	
	receptors.	(**6**).

References:

1. (Harris & Ralph, 1985).
2. (Harris *et al.*, 1985).
3. (Palacios *et al.*, 1982).
4. (Guyre *et al.*, 1983).
5. (Ralph *et al.*, 1983).
6. (Pike *et al.*, 1980).

3.2. RESULTS.

A. G-PROTEIN SUBUNIT EXPRESSION.

The treatment of both control and differentiated U937 cell membranes with thiol-activated pertussis toxin and [^{32}P] NAD^+ led to incorporation of radioactivity into an apparent single band of approximately 40kDa, presumably representing "$G_i\alpha$" (lanes 3 and 4, Fig. 3.1). When these membranes were treated with thiol-activated cholera toxin in the presence of [^{32}P]NAD^+, incorporation of radioactivity into a polypeptide with a molecular mass of approximately 42kDa was observed, presumably representing one of the four possible splice variant forms of $G_s\alpha$ (lanes 5 and 6, Fig. 3.1). An approximately 44kDa molecular weight form of $G_s\alpha$ is expressed by these cells, detectable both by altering the immunoblotting and ADP-ribosylation conditions as will be described later in this chapter and in Chapter 4 . Increased expression of both pertussis and cholera toxin substrates was evident in membranes prepared from 72 hour DMSO-differentiated U937 cells relative to non-differentiated U937 cell membranes, as will be discussed later in greater detail. Incorporation of radioactivity into other proteins, predominately of approximately 20-25kDa molecular weight, occurred in a toxin-independent manner (lanes 1 and 2, Fig. 3.1). However, cholera toxin-catalysed [^{32}P]ADP-ribosylation of an approximately 18kDa protein in both control and differentiated U937 cell membranes. This protein possibly represents the A_1 subunit of cholera toxin itself which has been reported to be a substrate for auto-ADP-ribosylation (lanes 5 and 6, Fig. 3.1).

In differentiated U937 cell membranes a GTP-dependent, pertussis toxin-catalysed [^{32}P]ADP-ribosylated doublet of polypeptides was evident, under SDS-PAGE conditions which cannot resolve the pertussis toxin substrates (lane 2 as compared to lane 4, Fig. 3.2). Such doublet formation was observed, to varying degrees, in eight differentiated U937 cell membrane preparations examined (results not shown). In an attempt to identify this more rapidly migrating protein, an *in vitro* pertussis toxin-catalysed [^{32}P]ADP-ribosylation reaction was performed and

the [32P]-labelled membrane samples immunoblotted and probed with a combination of antipeptide antisera capable of recognising $G_{i}1\alpha$, $G_{i}2\alpha$ and $G_{i}3\alpha$ subunits. This immunoblot was then subjected to autoradiography. Membranes from the neuroblastoma x glioma cell line, NG108-15 served as a control. Under these SDS-PAGE conditions, which should not resolve the individual pertussis toxin-sensitive α subunits, only one [32P]ADP-ribosylated and immunoreactive band would be anticipated and indeed was observed for the NG108-15 cell membranes (lanes 13-15, panels A and B, Fig. 3.3). However, in both control and differentiated U937 cell membranes an immunoreactive and [32P]ADP-ribosylated doublet was observed, appearing in a GTP-dependent manner (lanes 2, 5, 8 and 11, panels A and B, Fig. 3.3). Further, the relative amount of immunoreactive substrate expressed in U937 cell membrane preparations was reduced after the 37°C incubations, compared to the sample precipitated on ice. This reduction in immunoreactivity was apparent to varying degrees between different membrane preparations, with one of the control preparations displaying substantial loss of immunoreactive substrate (lanes 4 and 5 relative to lane 6, panel A, Fig. 3.3). Indeed in this preparation only the more rapidly migrating polypeptide was [32P]ADP-ribosylated (lanes 4-6, panel B, Fig. 3.3). The level of incorporation of [32P]ADP-ribose was also much lower in membranes from the U937 cell compared with those of the NG108-15 cell line, despite similar levels of immunoreactivity (compare lanes 7 and 14, panels A and B, Fig. 3.3). Subsequent experiments, using different membrane preparations and selective antipeptide antisera, demonstrated that even when membrane samples were precipitated on ice, more rapidly migrating immunoreactive polypeptides than both $G_{i}2\alpha$ and $G_{i}3\alpha$ were detected. Further, this also occurred in a GTP-independent manner (results not shown).

These observations suggested the possibility that proteolytic degradation of these $G_{i}\alpha$ subunits was occurring. The membrane preparation was thus modified

to include protease inhibitors throughout the cell harvesting and preparation stages (see procedure B in 2.2.6 and 2.3). In two different preparations of control cell membranes an apparently single immunoreactive polypeptide, presumably comprising of both $G_i2\alpha$ and $G_i3\alpha$, was observed as opposed to a "doublet" of immunoreactive proteins detected in differentiated membranes prepared in the absence of the protease inhibitors (lanes 1-8 compared to lanes 9 and 10, Fig. 3.4). In contrast to membranes prepared in the presence of protease inhibitors, in membranes prepared in the absence of these inhibitors their inclusion throughout the incubation did prevent some $G_i2\alpha/G_i3\alpha$ degradation (lanes 13 and 14 relative to lane 15, Fig. 3.4). Using this alternative method of preparation similarly prevented $G_i\alpha$ degradation in 72 hour DMSO-differentiated U937 cell membranes. The other G-protein subunits expressed in U937 cell membranes were also maintained (results not included as this is evident from later figures).

In an attempt to identify the G-proteins expressed by both control and differentiated U937 cells, a series of immunoblots were performed using the antipeptide antisera described in Table 2.1. With the exception of rat brain, other tissues were not used in a comparative approach as the antisera specificity has been demonstrated by a variety of studies (see discussion), including the work defining the G-protein α subunits expressed in rat white adipocyte membranes to be discussed later (see Chapter 6). Antiserum I1C recognises $G_i1\alpha$ and recognised this polypeptide in membranes derived from rat brain, but not from either control or 72 hour DMSO-differentiated U937 cell membranes (lanes 1-3, panel A, Fig. 3.5). An immunoreactive protein, of higher molecular weight than $G_i1\alpha$, was detected in the U937 cell membranes, but its identity was not investigated further (lanes 2 and 3, panel A, Fig. 3.5). Altering the SDS-PAGE conditions and probing with a mixture of antipeptide antisera specific for each of $G_i2\alpha$ and $G_i3\alpha$, identified the expression of both these G-protein α subunits in control and 72 hour DMSO-differentiated U937 cell membranes. Indeed, it appeared that $G_i2\alpha$ expression

increased upon differentiation, whereas that of $G_i3\alpha$ remained unaltered (lanes 2 and 3, panel B, Fig. 3.5). Such alterations in expression will be discussed in greater detail later.

Control and differentiated U937 cell membranes express both the 44kDa and the 42kDa molecular weight forms of $G_s\alpha$, the less mobile form only detectable upon applying the immunoblotting procedure "B" detailed in 2.10.2. Immunoreactive proteins, predominantly of higher molecular weight than the $G_s\alpha$ forms, were detected in the U937 cell membranes, although their identity was not investigated further (lanes 2 and 3, panel C, Fig. 3.5).

However, neither control nor differentiated membranes expressed $G_o\alpha$, clearly expressed and identified in rat brain by the antipeptide antiserum IM1. (lanes 1-3, panel D, Fig. 3.5). Again, immunoreactive proteins, of higher molecular weight than $G_o\alpha$, were detected in the U937 cell membranes, but their identity was not investigated further (lanes 2 and 3, panel D, Fig. 3.5). To further ensure that this pertussis toxin substrate was not expressed in U937 cell membranes antipeptide antisera directed against both the N- and C-terminal amino acid residues of $G_o\alpha$ were employed. Gel electrophoretic conditions were such that the individual pertussis toxin substrates could be resolved. IM1 again failed to detect any polypeptide in the U937 cell membranes (lanes 14 and 15, panel E, Fig. 3.6). The antipeptide antiserum, SG1, is capable of recognising both $G_i1\alpha$ and $G_i2\alpha$. The absence of $G_i1\alpha$ in the U937 cells is again demonstrated by the lack of an immunoreactive polypeptide that co-migrated with the higher molecular weight polypeptide in rat brain (lanes 11 and 12 compared to lane 13, respectively, panel D, Fig. 3.6). Using both the C-and N-terminally directed antipeptide antisera directed against $G_o\alpha$, immunoreactive polypeptides were evident in the U937 cell membranes (lanes 2 and 3, panel A and 8 and 9, panel C, respectively, Fig. 3.6). However these cross reacting proteins co-migrated with both the $G_i3\alpha$ and $G_i2\alpha$ polypeptides expressed in these cells (lanes 5 and 6, panel B and lanes 11 and 12,

panel D, respectively, Fig. 3.6) and not the rat brain Goα form (lane 1, panel A and lane 7, panel C, Fig. 3.6).

The antipeptide antiserum, BN1, was raised against a sequence common to both the 35kDa and the 36kDa molecular weight forms of the β subunit. However under the resolving conditions employed only one immunoreactive polypeptide was apparent and was expressed in both control and DMSO-differentiated U937 cell membranes (lanes 1-4, Fig. 3.7).

B. DIFFERENTIATION INDUCED ALTERATIONS IN G-PROTEIN SUBUNIT EXPRESSION.

Differentiation-induced alterations in the expression of the G-protein α and β subunits in U937 cell membranes were assessed by analysis of changes in both the toxin-catalysed [^{32}P]ADP-ribosylation signals and specific immunoreactivity, over a five day time course. The area of detection most sensitive to any alteration in G-protein subunit expression was assessed by varying the membrane concentration whilst maintaining a constant toxin or antipeptide antiserum concentration. The 72 hour DMSO-treated cell membranes were chosen as representative of the "differentiated state". Any alterations in subunit expression were tested for significance above a control value of unity using the paired Student's *t*-distribution test. The "n" value quoted represents the "degrees of freedom", unless otherwise stated.

1) Altered expression of pertussis toxin-sensitive G-proteins.

Pertussis toxin-catalysed [^{32}P]ADP-ribosylation was found to be in the linear range between 0-10μg of both control and differentiated membranes (panels A-C, Fig. 3.8).

Quantification of an immunoreactive signal detected with increasing concentrations of control and differentiated membrane protein was achieved by probing the peroxidase stained immunoblot with an iodinated second antibody. Each immunoreactive band was then excised from the blot and counted in a gamma counter (see section 2.10.3). The results were plotted correcting for the

background signal at every membrane concentration. Using this approach for the antipeptide antiserum SG1, specific for $G_{i}2\alpha$ in these cells, linearity was found to be in the range of 0-25μg of membrane protein for both control and differentiated U937 cell membranes (panels A-C, Fig. 3.9 and panel A, Fig. 3.10, respectively). This range of protein concentration was also that most sensitive to change as detected by the $G_{i}3\alpha$ specific antipeptide antiserum, I3B, for both control and differentiated membranes (panel B, Fig. 3.10).

The expression of the pertussis toxin substrates in these cells increased upon differentiation to an estimated level of 3.4 ± 0.4 fold ($p < 0.01$, $n = 3$) over control cell levels after 120 hours DMSO treatment (panels A and D, Fig. 3.11). This increase did not apparently reflect an increase in the expression of $G_{i}3\alpha$. Indeed an apparent decreased expression of this α subunit was suggested although this did not prove significant when tested (panels C and E, Fig. 3.11). The expression of the only other known pertussis toxin substrate $G_{i}2\alpha$ did increase significantly, with a 2.6 ± 0.1 ($p < 0.01$, $n = 2$) fold increase over control levels, after 120 hours exposure to DMSO (panels B and D, Fig. 3.11). However, it is evident from the comparative plot of the quantification of the toxin-catalysed [^{32}P]ADP-ribosylation and immunoblotting data that, although there is an upward trend in the level of expression of the pertussis toxin substrates and $G_{i}2\alpha$, there appears to be a slight, yet significant, difference in the onset of this effect (panel D, Fig. 3.11).

2) Alterations in expression of $G_{s}\alpha$.

Cholera toxin-catalysed [^{32}P]ADP-ribosylation resulted in incorporation of radioactivity predominantly into a 42kDa form of $G_{s}\alpha$. This incorporation was linear in the range, 0-75μg for both control and differentiated cell membranes (panels A-C, Fig.3.12). A low level of [^{32}P]ADP-ribose incorporation into the 44kDa form of $G_{s}\alpha$ was observed in the differentiated cell membranes (panel B, Fig. 3.12), but was not detectable in the control cell membranes (panel A, Fig.

3.12). Indeed due to the low relative level of [32P]ADP-ribose incorporation it was only possible to qualitatively assess any alterations in expression of the 44kDa form of Gsα. Incorporation of radioactivity into other proteins at the maximal membrane concentrations occurred in a toxin-independent manner (lane 10 in panels A and B, Fig. 3.12). To further assess any alterations in Gsα subunit expression the antipeptide antiserum (CS1) capable of recognising both the 42kDa and 44kDa splice variant forms of Gsα was employed. Quantification of immunoreactive signals was only performed for the α42kDa form of Gsα and proved less successful than with the other antipeptide antisera due to a high background level of iodination. However, at the antipeptide antiserum concentration detailed, the protein concentration most sensitive to alteration was between 0-5μg of both control and differentiated membrane protein (Fig. 3.13).

Over the time course of differentiation there was an increased level of expression of both forms of Gsα present in the U937 cell line (lanes 1-6, panels A and B, Fig. 3.14). Indeed from the ribosylation data the expression of the α42kDa form of Gsα, after 120 hours of DMSO treatment, increased 1.9±0.3 ($p < 0.1$, $n = 3$) fold over control levels. After 120 hours DMSO treatment of the U937 cells a 2.3±0.5 ($p < 0.1$, $n = 3$) fold increase in the expression of Gsα42kDa, relative to control levels, was observed when the immunoblots were quantified. Further the trend of this α subunits increased expression with increased exposure to DMSO did not significantly differ when both methods of detection were quantified and represented graphically (panel C, Fig.3.14). To ensure that any altered expression of G-protein subunits was not simply a reflection of unequal "loading" of membrane protein the SDS-PAGE gels from the immunoblots and the ADP-ribosylations were routinely stained for protein. The Coomassie Brilliant blue R stained SDS-PAGE gel of the cholera toxin-catalysed [32P]ADP-ribosylation represented in panel A is shown in panel D, Fig. 3.14. The majority of the major membrane proteins have been stained to equal intensity.

3) Altered expression of the β subunit.

Titration of the antipeptide antiserum (BN1), which recognises both the 35kDa and 36kDa forms of the β subunit, displayed sensitivity to alteration in substrate concentration in the range of 0-200μg for both control and differentiated membranes (Fig. 3.15). Expression of the β subunit increased over the time course of differentiation to 2.6 ± 0.6 fold ($p < 0.1$, $n = 4$) above control levels after 120 hours DMSO exposure (panels A and B, Fig. 3.16). Again to ensure that these alterations were not explicable by incorrect membrane protein loading a silver stain of the gel performed in panel A is represented in panel C, Fig. 3.16. The majority of proteins have been stained to equal intensity across the differentiation time course, with in this experiment perhaps a slight excess of control membrane protein. However, it was appreciated that the altered expression of different membrane proteins concurrent with the onset of cell maturation does make unequal sample loading difficult to assess.

Figure 3.1.

TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF CONTROL AND DIFFERENTIATED U937 CELL MEMBRANES.

U937 cell membranes (50μg), prepared as in 2.3.A, from either 72 hour DMSO (1.25% v/v)-differentiated cells (lanes 2, 4 and 6), or control, non-treated cells (lanes 1, 3 and 5), were ADP-ribosylated with thiol-preactivated bacterial toxins and [32P]NAD+ as described in section 2.11. Cells were treated with DMSO as outlined in 2.2.3. Lanes 1 and 2 represent [32P]ADP-ribosylation reactions performed in the absence of bacterial toxins. Lanes 3 and 4 represent pertussis toxin-catalysed [32P]ADP-ribosylation reactions, whereas cholera toxin-catalysed the [32P]ADP-ribosylation reactions represented in lanes 5 and 6 The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as described in section 2.7. The dried gel was autoradiographed for 72 hours as described in section 2.9.

This experiment is representative of four others, performed using different membrane preparations, which gave essentially identical results.

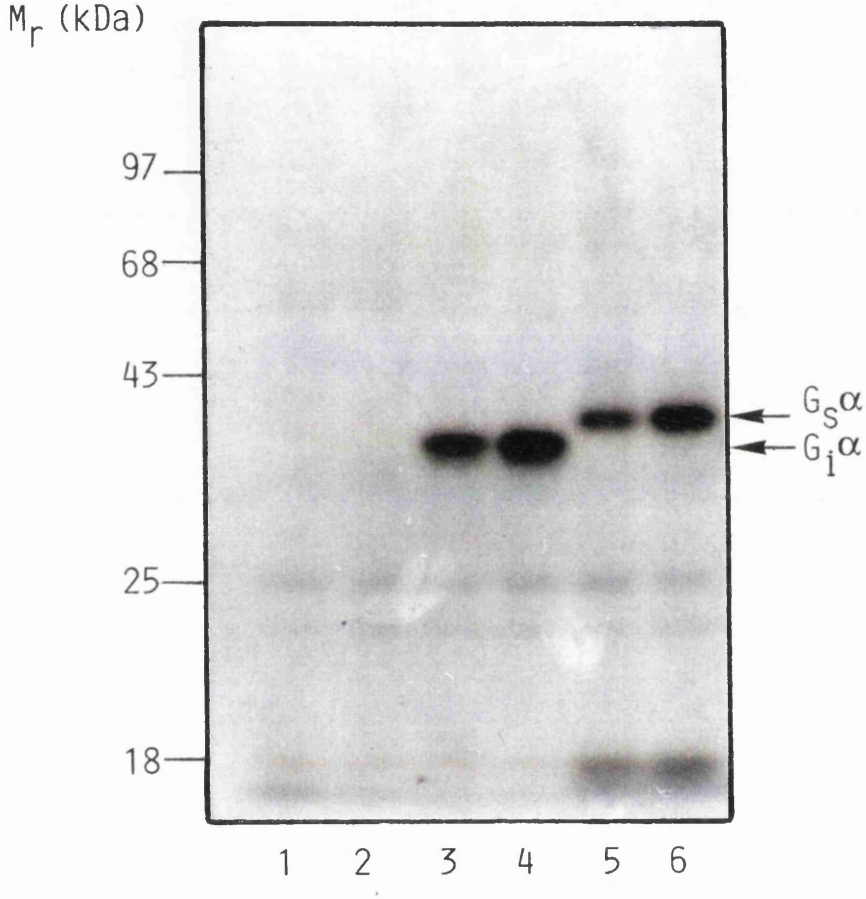

M_r (kDa)
97
68
43
25
18
$G_s\alpha$
$G_i\alpha$
1
2
3
4
5
6

Figure 3.2.

PERTUSSIS TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF DIFFERENTIATED U937 CELL MEMBRANES - GTP-DEPENDENT, DOUBLET FORMATION.

72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg) were ADP-ribosylated with thiol-preactivated toxins and [32P]NAD+ as described in section 2.11. Cells were treated with DMSO as outlined in 2.2.3 and membranes prepared as in 2.3.A. Lanes 1-3 represent [32P]ADP-ribosylation reactions performed in the presence of GTP, whereas GTP was omitted from the reaction in lane 4. Lane 1 represents the reaction performed in the absence of toxin. Lanes 2 and 4 represent [32P]ADP-ribosylations performed in the presence of pertussis toxin. Lane 3 represents a cholera toxin-catalysed [32P]ADP-ribosylation reaction. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as described in 2.7. The dried gel was autoradiographed for 120 hours as described in 2.9.

This experiment is representative of two others, performed with different membrane preparations, which gave essentially identical results.

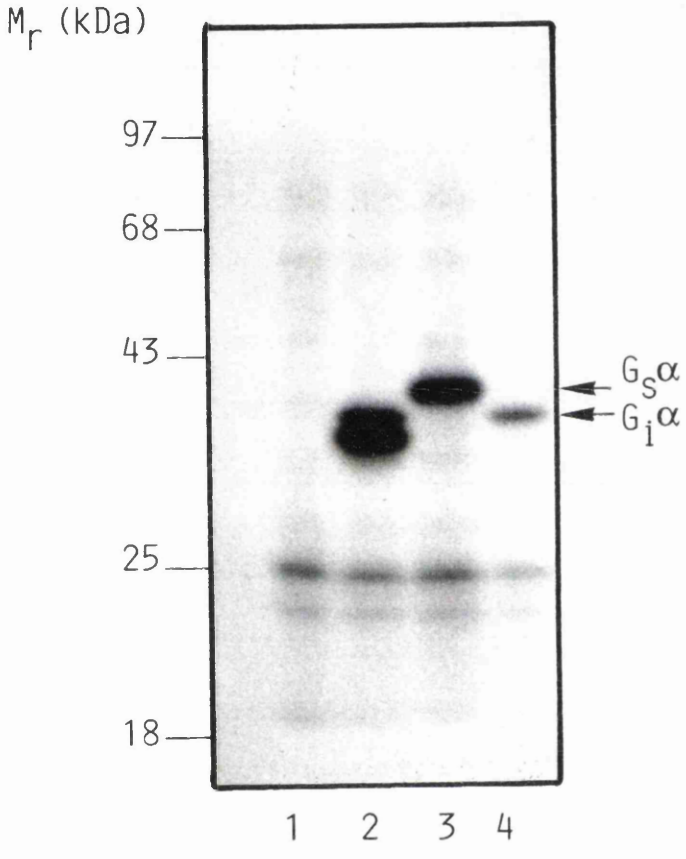

M_r (kDa)
97
68
43
25
18
$G_S\alpha$
$G_i\alpha$
1
2
3
4

Figure 3.3.

PERTUSSIS TOXIN CATALYSED [32P]ADP-RIBOSYLATION OF CONTROL AND DIFFERENTIATED U937 CELL MEMBRANES AND IMMUNOLOGICAL IDENTIFICATION OF THE TOXIN SUBSTRATES.

Panel A represents an immunoblot of [^{32}P]ADP-ribosylated Giα subunits and panel B represents the corresponding autoradiograph of this immunoblot.

The membranes (50μg/lane), were prepared as in 2.3.A., from two different cultures of 72 hour DMSO-differentiated U937 cells (denoted by "D") in lanes 1-3 and 7-9 and two different cultures of control U937 cells (denoted by "C") in lanes 4-6 and 10-12. Cells were treated with DMSO as outlined in 2.2.3. Membranes (50μg/lane) from NG108-15 cells (denoted by "NG") were prepared as in 2.3.A. They were included for comparative analysis and are represented by lanes 13-15. The membranes in lanes 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14 were [^{32}P]ADP-ribosylated by thiol-preactivated pertussis toxin using [^{32}P]NAD+ as outlined in 2.11. In lanes 1, 4, 7, 10 and 13 the reaction was performed in the absence of GTP (denoted as "-"), whereas in lanes 2, 5, 8, 11 and 14, GTP was present (denoted as "+") in the incubation. Lanes 3, 6, 9, 12 and 15 represent non-ribosylated, ice precipitated membrane samples.

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7. The gel was then immunoblotted as described in section 2.10., with a mixture of primary antipeptide antisera SG1(1: 2000 dilution) and I3B(1: 200 dilution) and is represented in panel A. The primary antisera were detected as described in section 2.10.2. After development the dried nitrocellulose was autoradiographed for 48 hours and the resulting autoradiograph is represented by panel B. This experiment was performed once.

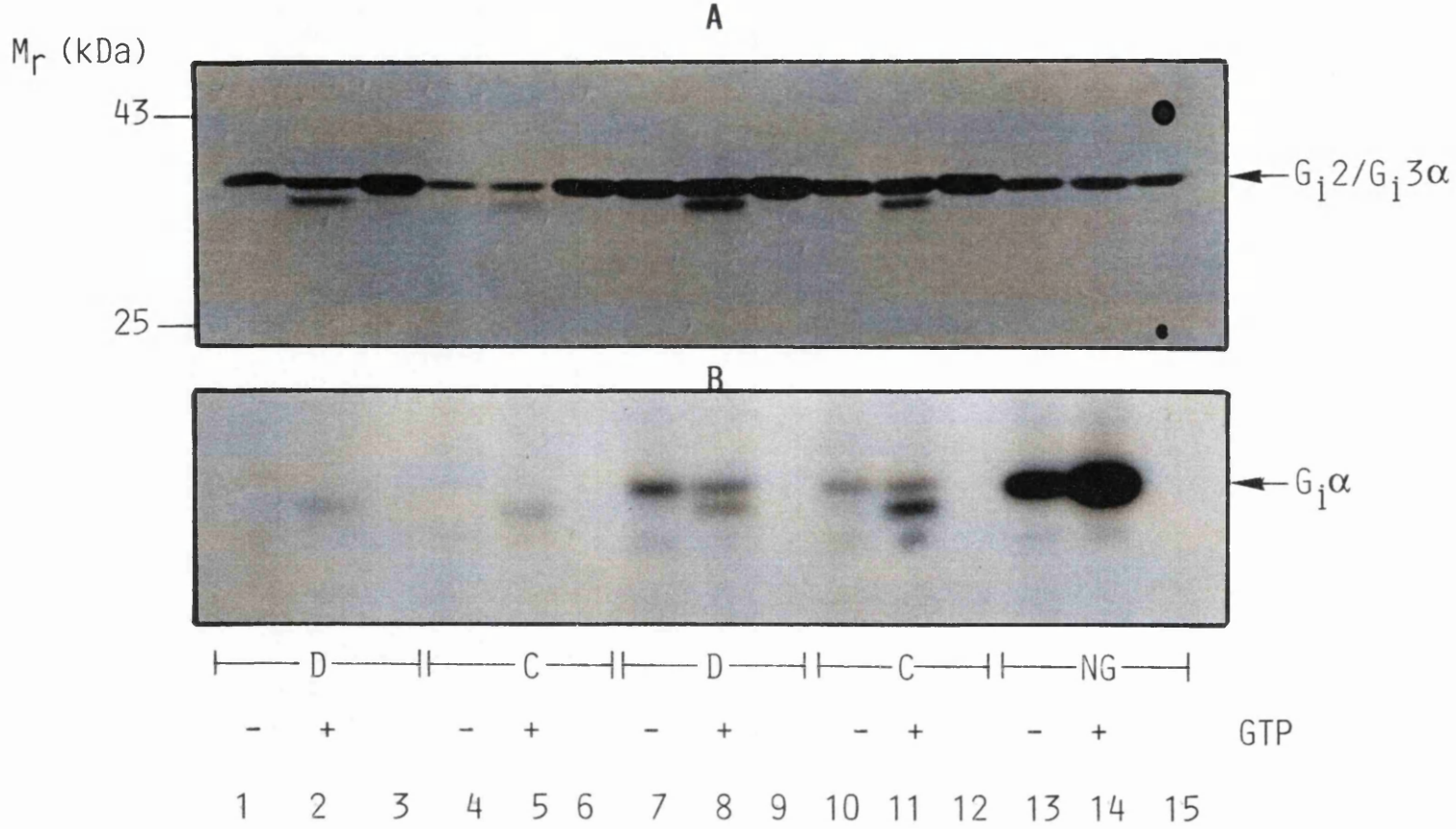

A
M_r (kDa)
43
25
$G_i2/G_i3\alpha$
B
$G_i\alpha$
D
C
D
C
NG
- + - + - + - + - +
GTP
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3.4.

IMMUNOLOGICAL INVESTIGATION OF THE Giα SUBUNITS RESISTANCE TO PROTEOLYTIC DEGRADATION IN CONTROL U937 CELL MEMBRANES PREPARED BY AN ALTERNATIVE METHOD.

Lanes 1-4 represent control U937 cell membranes prepared in the presence of protease inhibitors according to the method outlined in section 2.3.B. This method was also employed to prepare another set of control U937 cell membranes, represented in lanes 5-8. Lanes 9-12 represent 24 hour DMSO-differentiated membranes prepared in the absence of protease inhibitors, as described in section 2.3.A. Cells were treated with DMSO as outlined in 2.2.3. Lanes 13-15 represent control U937 cell membranes, prepared as in 2.3.A.

The membranes (30μg /lane) were TCA precipitated on ice after a 1 hour incubation at 37°C (lanes 3, 4, 7, 8, 11, 12, 14 and 15), or a 1 hour incubation on ice (lanes 1, 2, 5, 6, 9, 10 and 13). These incubations were conducted in the absence, denoted as "-", (lanes 2, 4, 6, 8, 10, 12 and 15) or presence, denoted as "+", (lanes 1, 3, 5, 7, 9, 11, 13 and 14) of the protease inhibitors detailed in 2.2.6.B. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and immunoblotted as described in sections 2.7 and 2.10. The primary antipeptide antiserum was a mixture of SG1(1: 2000 dilution) and I3B(1: 200 dilution) and the immunoreactive proteins detected as in 2.10.2.

This experiment is representative of two other sets of membranes which had been prepared from control U937 cells in the presence of protease inhibitors as in 2.3.B and similarly tested for Gi2/3α proteolysis.

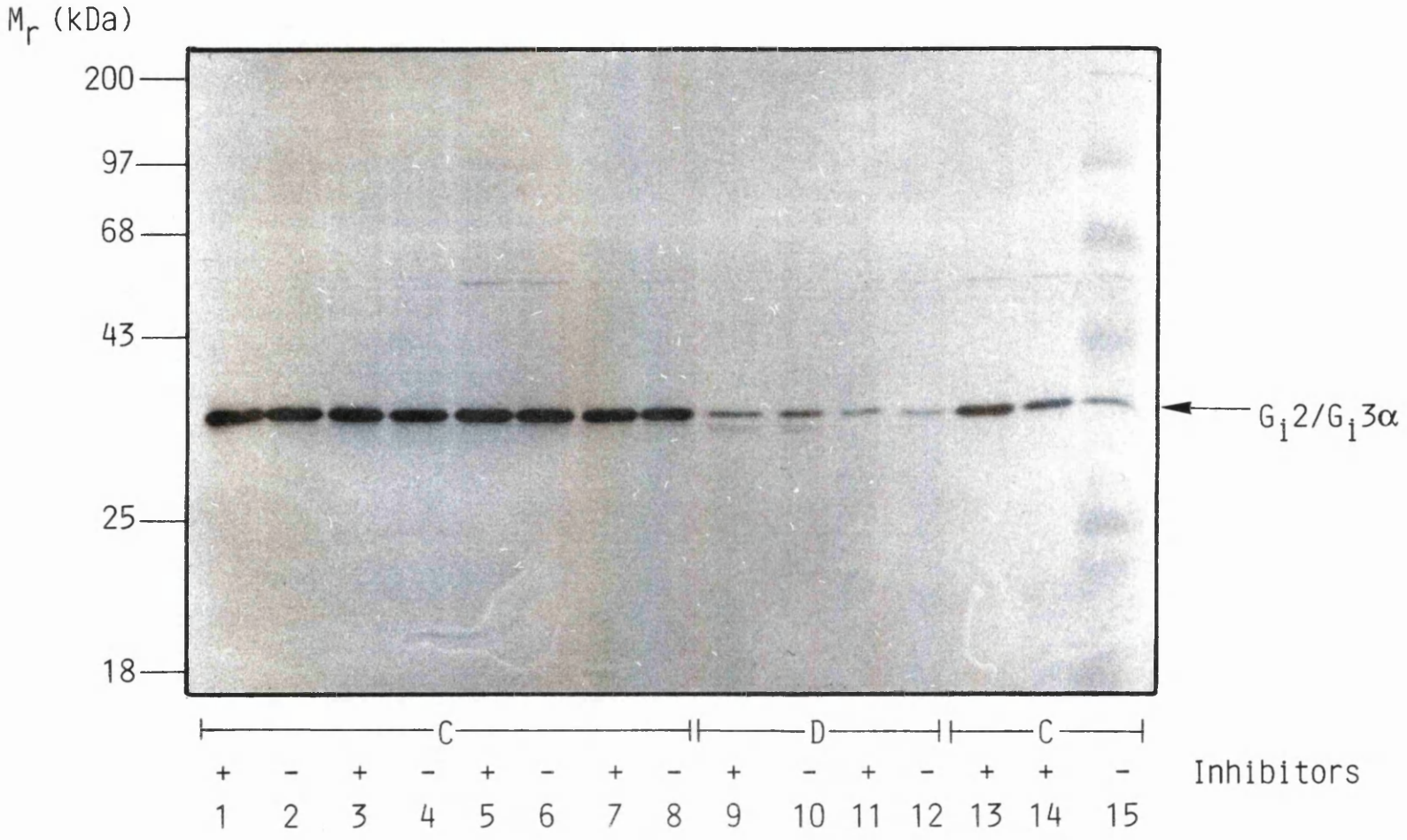

M_r (kDa)
200
97
68
43
25
18
$G_i2/G_i3\alpha$
C
D
C
+ − + − + − + − + − + − + + −
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Inhibitors

Figure 3.5.

IMMUNOLOGICAL IDENTIFICATION OF THE G-PROTEIN α SUBUNITS EXPRESSED IN BOTH CONTROL AND DIFFERENTIATED U937 CELL MEMBRANES.

Lane 1 represents rat cerebral cortex membranes, 200μg in panel A and 50μg in panel D. These membranes were prepared as described in 2.3.A The U937 cell membranes were prepared as outlined in 2.3.B. Lane 2 represents control U937 cell membranes, 200μg in panel A, 100μg in panel B, 50μg in panel C and 100μg in panel D. Lane 3 represents 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes, 200μg in A, 100μg in B, 20μg in C and 100μg in D. Cells were treated with DMSO as outlined in 2.2.3. The samples were resolved by SDS-PAGE using a 12.5% [w/v] acrylamide, 0.06% [w/v] bisacrylamide, 20cm x 20cm resolving gel in panels A and B and a 10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel in panels C and D, as in 2.7. In panels A and B, the membrane samples were subjected to alkylation prior to SDS-PAGE, as described in 2.7.3.B. The gels were then immunoblotted as described in 2.10.2 for A, B and D and as outlined in section 2.10.2.B for panel C. The primary antisera used were I1A (1: 60 dilution) in panel A, a mixture of LE2 (1: 200 dilution) and I3B (1: 200 dilution) in panel B, CS1 (1: 200 dilution) in panel C and IM1 (1: 200 dilution) in panel D. The primary antisera were detected as outlined in section 2.10.2. The molecular weight standards are relevant to panel A and are omitted from the other panels for clarity.

Each panel is representative of at least three experiments, each performed with a different membrane preparation.

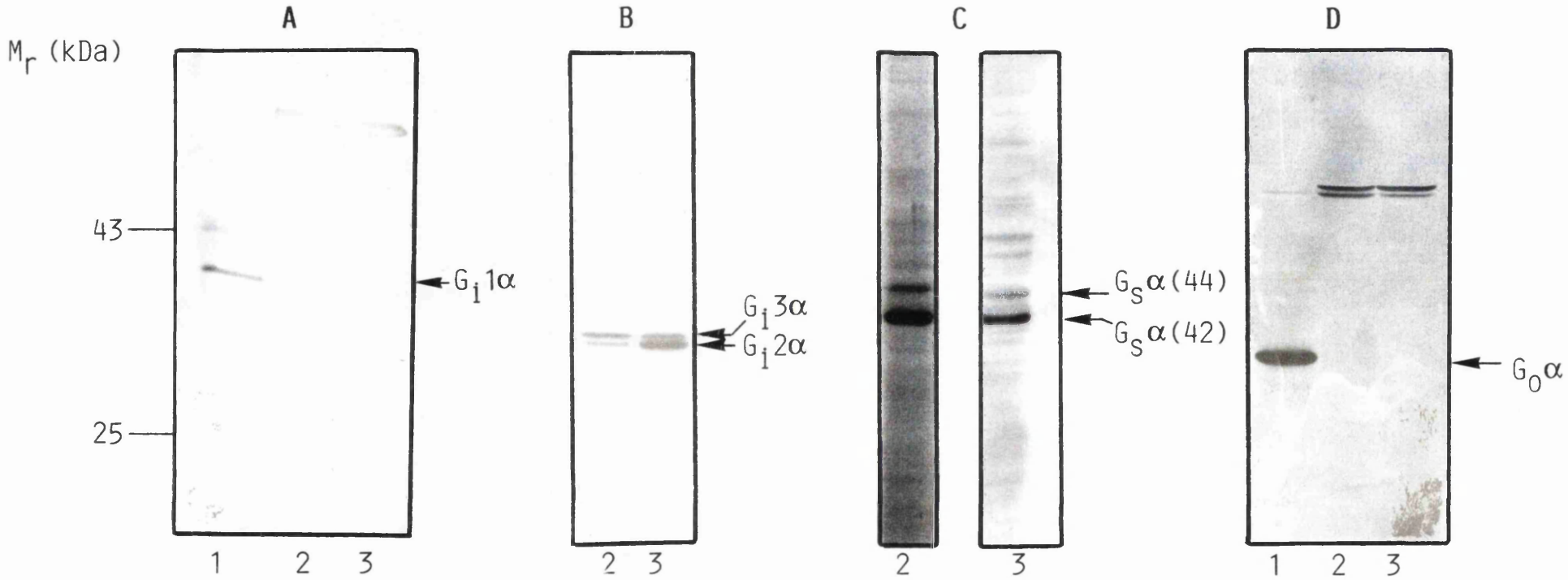

A
B
C
D
M_r (kDa)
43
25
$G_i1\alpha$
$G_i3\alpha$
$G_i2\alpha$
$G_s\alpha(44)$
$G_s\alpha(42)$
$G_o\alpha$
1
2
3
2
3
2
3
1
2
3

Figure 3.6.

IMMUNOLOGICAL INVESTIGATION OF Goα EXPRESSION IN CONTROL AND DIFFERENTIATED U937 CELL MEMBRANES.

Lanes 1, 7 and 13 represent rat cerebral cortex membranes, 10μg in panel A, 5μg in panel C and 30μg in panel E. U937 cell membranes were prepared as outlined in 2.3.B. Lanes 2, 5, 8, 11 and 14 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes, 100μg in panels A, C and E, 30μg in B and 10μg in panel D. Cells were treated with DMSO as outlined in 2.2.3. Lanes 3, 6, 9, 12 and 15 represent control U937 cell membranes, at equivalent concentrations to the differentiated membranes for each panel. Prestained molecular weight markers were also present in lanes 1, 4, 7, 10 and 13. The samples were resolved by SDS-PAGE (12.5% [w/v] acrylamide, 0.06% [w/v] bisacrylamide, 20cm x 20cm resolving gel) as described in 2.7. The gel was immunoblotted as in 2.10.2. The primary antipeptide antisera employed were OC1 (1: 200 dilution) in panel A, I3B (1: 200 dilution) in B, ON1 (1: 200 dilution) in C, SG1 (1: 200 dilution) in D and IM1 (1: 200 dilution) in panel E.

This experiment was repeated, using a different membrane preparation and essentially identical results observed.

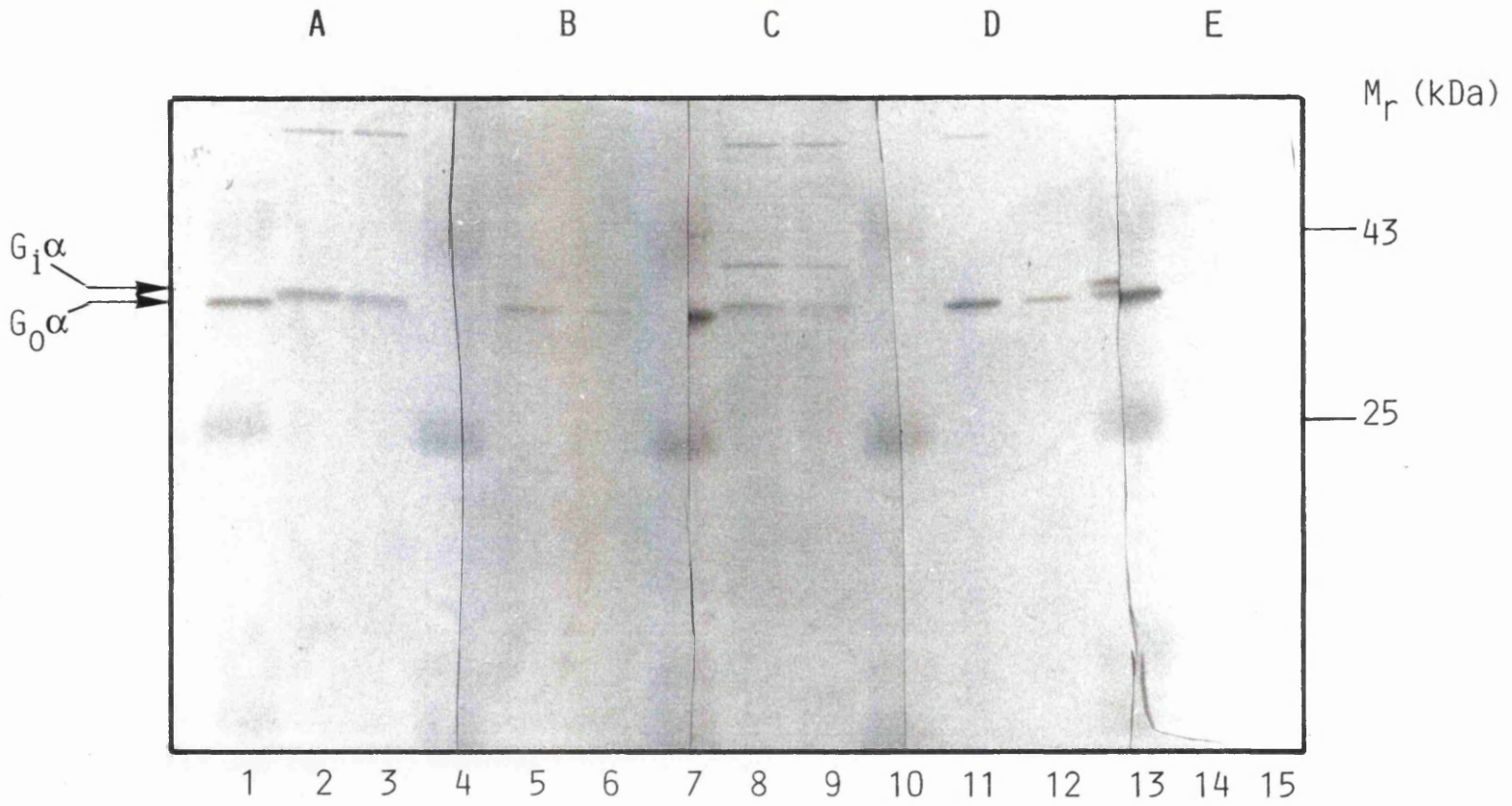

A
B
C
D
E
M_r (kDa)
43
25
$G_i\alpha$
$G_o\alpha$
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3.7.

IMMUNOLOGICAL IDENTIFICATION OF A β SUBUNIT IN CONTROL AND DIFFERENTIATED U937 CELL MEMBRANES.

U937 cell membranes (25μg/lane) were prepared as in 2.3.B. Lane 1 represents control U937 cell membranes, lanes 2-4 represent 24, 72 and 96 hour DMSO (1.25% v/v)-differentiated U937 cell membranes, respectively. Cells were treated with DMSO as outlined in 2.2.3. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and immunoblotted as described in section 2.10.2. B. The primary antipeptide antiserum used was BN2 (1: 200 dilution).

This immunoblot is representative of three others, using different membrane preparations, which gave essentially identical results.

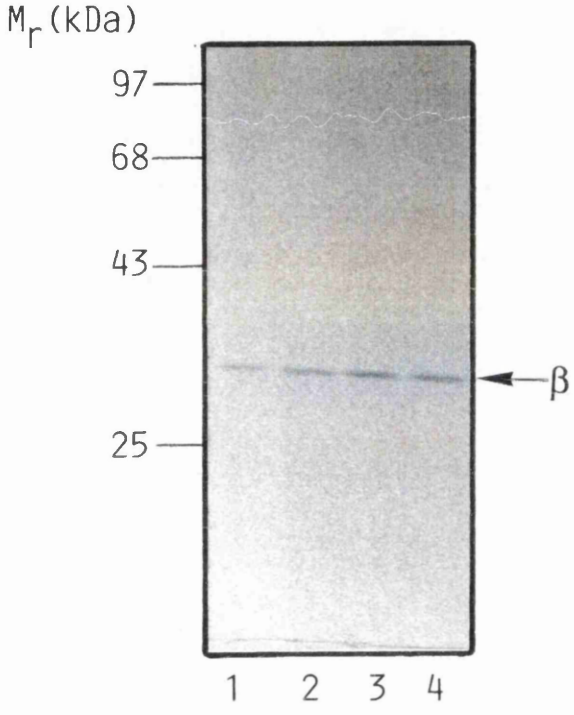

M_r (kDa)
97
68
43
25
β
1
2
3
4

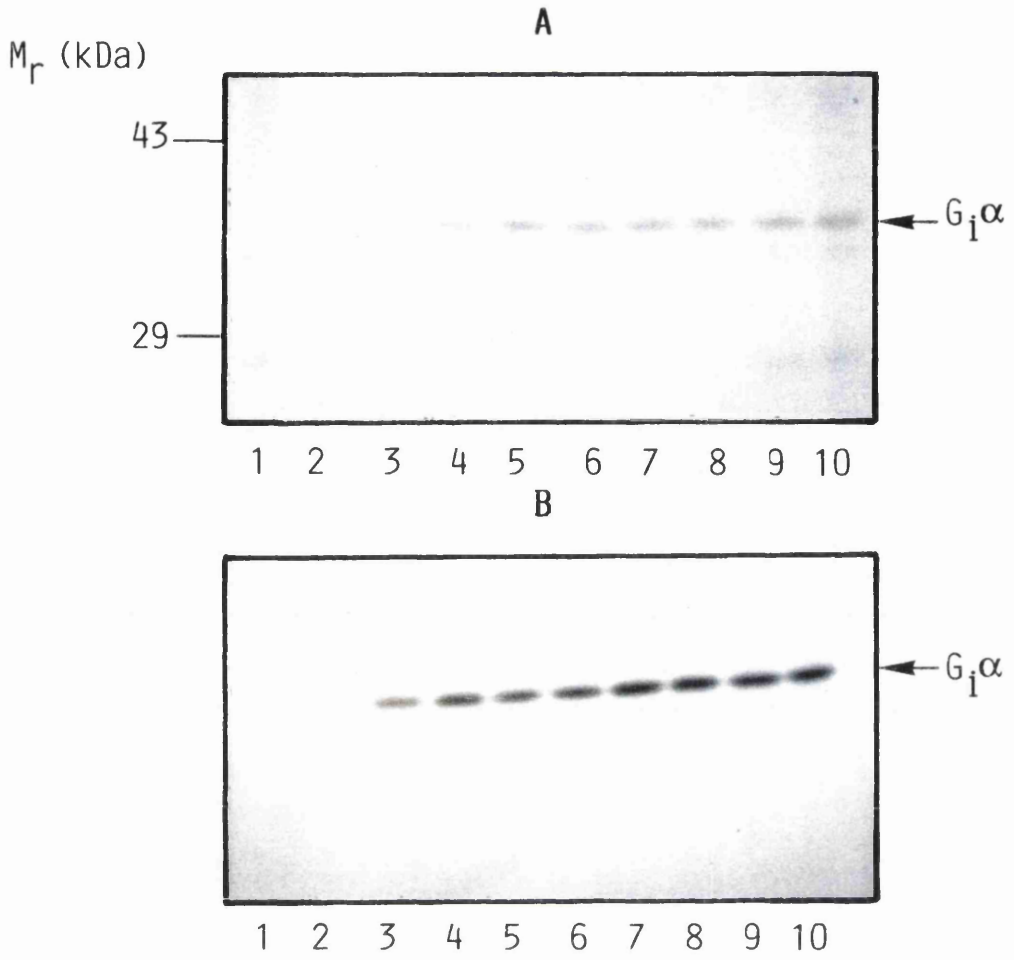
Figure 3.8.

PERTUSSIS TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF U937 CELL MEMBRANES - TITRATION OF MEMBRANE PROTEIN.

Increasing concentrations of membranes from either control (panel A) or 72 hour DMSO (1.25% v/v)-differentiated U937 cells (panel B) were [32P]ADP-ribosylated as described in section 2.11., although the final pertussis toxin concentration was reduced to 2μg/ml. Cells were treated with DMSO as outlined in 2.2.3 and membranes prepared as in 2.3.B.

Lane 1 represents [32P]ADP-ribosylation of membranes (50μg) performed in the absence of bacterial toxin. Lanes 2-10 represent 0, 5, 10, 15, 20, 25,30, 40 and 50μg of membrane protein, respectively. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18 x 16cm resolving gel) as described in 2.7. The dried gels were autoradiographed for 3 days as described in 2.9. The experiments shown in panels A and B were performed once.

Panel C represents the graphs of the densitometric scan analysis of the titrations in panel A (□) and panel B (■).


A
M_r (kDa)
43
29
$G_i\alpha$
1 2 3 4 5 6 7 8 9 10
B
$G_i\alpha$
1 2 3 4 5 6 7 8 9 10

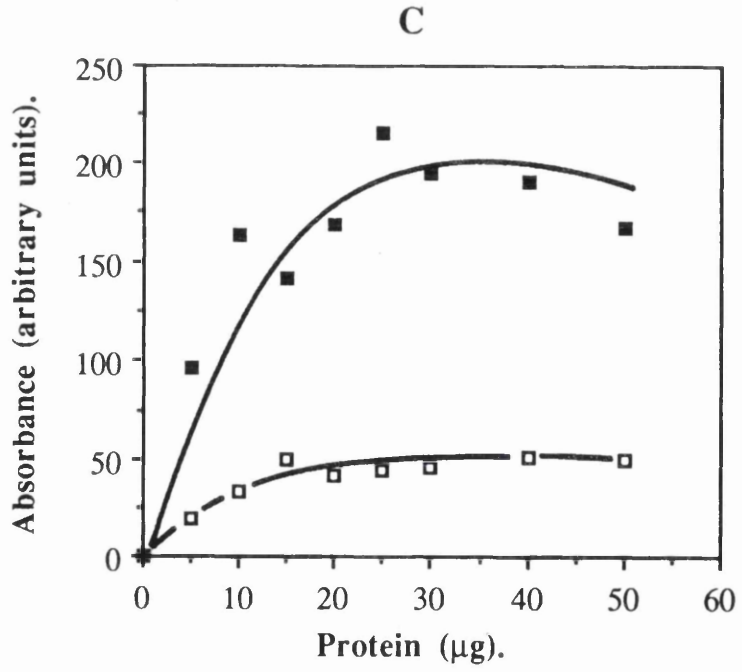

C
Absorbance (arbitrary units).
250
200
150
100
50
0
0 10 20 30 40 50 60
Protein (µg).

Figure 3.9.

IMMUNOLOGICAL DETECTION OF Gi2α IN CONTROL U937 CELL MEMBRANES WITH INCREASING CONCENTRATIONS OF MEMBRANE PROTEIN.

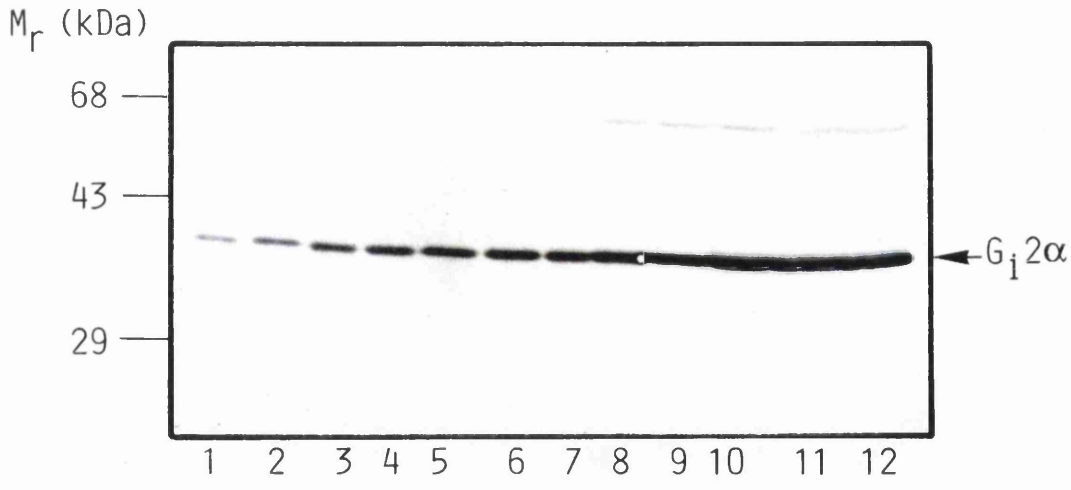
Lanes 1-12 represent 5, 7.5, 10, 12.5, 15, 20, 25, 50, 75, 100, 150 and 200μg of control U937 cell membranes, respectively. The membranes were prepared as in 2.3.B. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7.

Panel (A) represents the peroxidase stained immunoblot as performed by the method described in section 2.10.2.B. The primary antipeptide antiserum, SG1, was used at a 1: 2000 dilution.

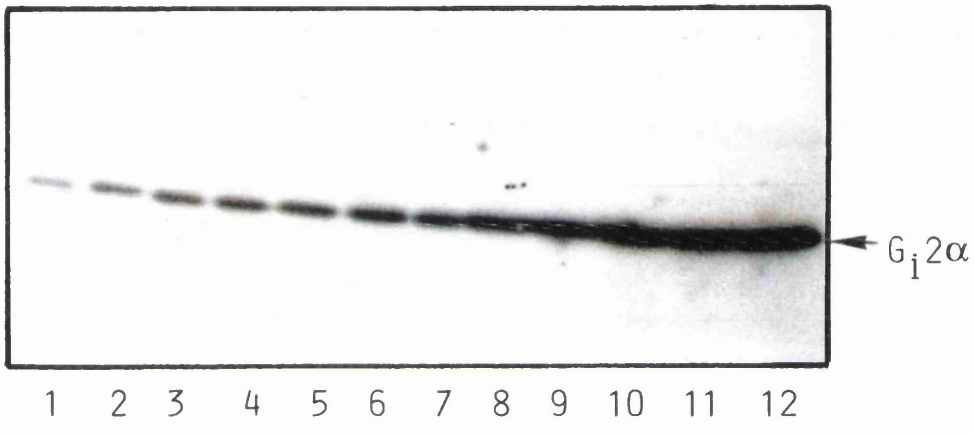
Panel (B) represents the autoradiograph of the immunoblot, exposed for 16 hours, after re-probing it with [^{125}I]-labelled anti-rabbit IgG as described in 2.10.3.

Panel (C) describes panel (B) graphically. It represents the [^{125}I] counts obtained from the excised Gi2α immunoreactive bands, corrected for background signal, as described in 2.10.3. The experiment was performed once.

A


M_r (kDa)
68
43
29
$G_i2\alpha$
1 2 3 4 5 6 7 8 9 10 11 12

B


$G_i2\alpha$
1 2 3 4 5 6 7 8 9 10 11 12

C

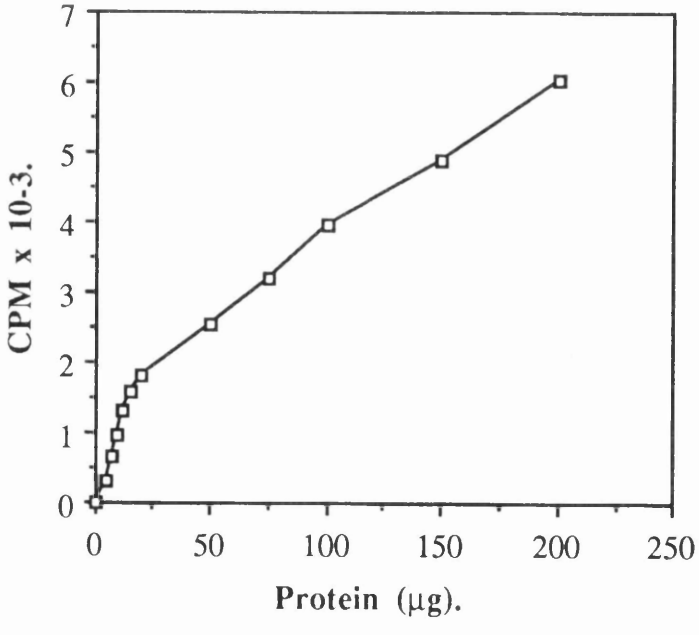

CPM x 10-3.
0
1
2
3
4
5
6
7
0
50
100
150
200
250
Protein (µg).

Figure 3.10.

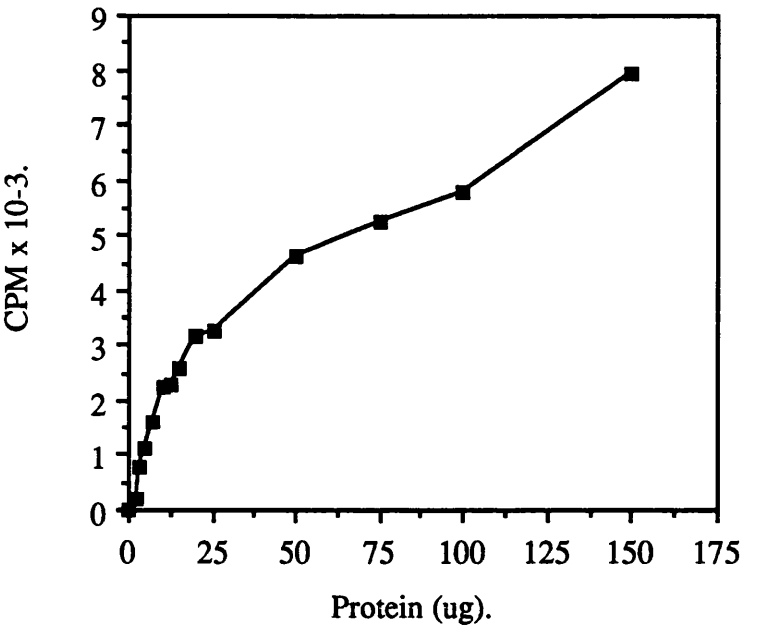
IMMUNOLOGICAL DETECTION OF Gi2α AND Gi3α IN U937 CELL MEMBRANES WITH INCREASING CONCENTRATIONS OF MEMBRANE PROTEIN - [125I] QUANTIFICATION OF THE IMMUNOREACTIVE SIGNAL.

Immunoblots of increasing concentrations of U937 cell membrane protein versus a constant antipeptide antiserum concentration were performed as described in sections 2.10.2.B. The membrane proteins were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7. The immunoblots were then probed with an [^{125}I]-labelled second antibody, the immunoreactive bands excised and counted in a gamma counter, as described in 2.10.3. This quantification procedure is represented graphically correcting for background signal as described in 2.10.3.

In panel A, membranes were prepared from 72 hour DMSO (1.25% v/v)-differentiated U937 cells as in 2.3.B. The cells were treated with DMSO as in 2.2.3. The primary antipeptide antiserum, SG1, specific for $G_i2\alpha$ in these cell membranes, was used at a 1: 2000 dilution.

Panel B represents membranes from control (□) and 72 hour DMSO (1.25% v/v)-differentiated U937 cells (■), prepared as in 2.3.B. The cells were treated with DMSO as in 2.2.3. The primary antipeptide antiserum, I3B, specific for $G_i3\alpha$, was used at a 1: 200 dilution.

A


CPM x 10-3.
9
8
7
6
5
4
3
2
1
0
0
25
50
75
100
125
150
175
Protein (ug).

B

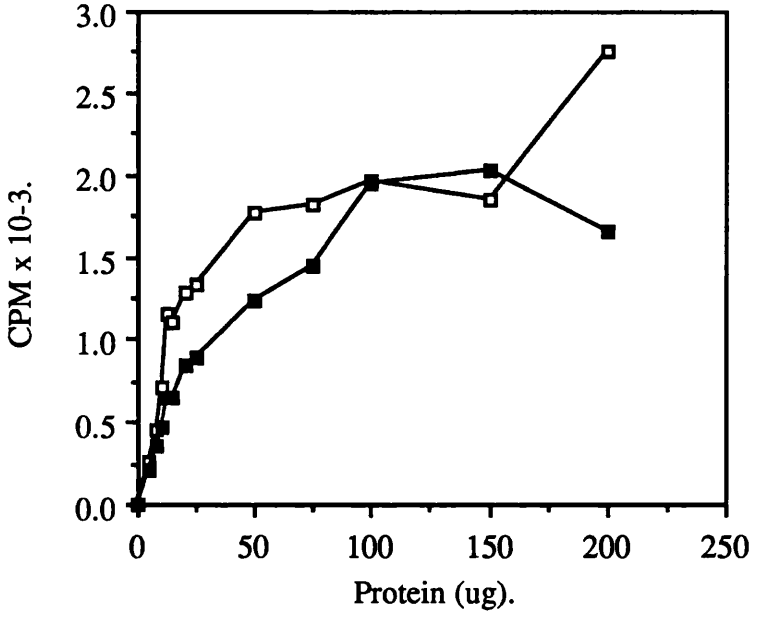

CPM x 10-3.
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
Protein (ug).

Figure 3.11.

ALTERATIONS IN THE EXPRESSION OF PERTUSSIS TOXIN SUBSTRATES UPON DMSO-INDUCED DIFFERENTIATION OF U937 CELLS.

Lanes 1-6 represent membranes from control, 24, 48, 72, 96 and 120 hour DMSO (1.25% v/v)-differentiated U937 cells, prepared in the presence of protease inhibitors as outlined in section 2.3.B. The cells were treated with DMSO as outlined in 2.2.3. The membrane proteins were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7.

Panel A represents pertussis toxin-catalysed [^{32}P]ADP-ribosylation of the membranes (5μg/lane), performed as in 2.11., except that the final pertussis toxin concentration was reduced to 2μg/ml. "-Tx" designates the ribosylation reaction performed with 120 hour DMSO (1.25% v/v)-differentiated membranes in the absence of pertussis toxin. After SDS-PAGE the dried gel was autoradiographed for 5 hours as in 2.9.

Panel B represents an immunoblot of the time course of differentiation (5μg/lane), performed as in 2.10.2.B. The primary antipeptide antiserum SG1, which recognises $G_i2\alpha$ in these cells, was used at a dilution of 1: 2000.

Panel C represents an immunoblot of the time course of differentiation (10μg/lane), performed as in 2.10.2.B. The primary antipeptide antiserum I3B, which recognises $G_i3\alpha$, was used at a dilution of 1: 200.

All panels are single experiments representative of experiments performed at two different membrane concentrations for each of three different membrane preparations of the DMSO-induced differentiation time course. The cell membranes were prepared and experiments performed on the same day as the cells were harvested. Quantification of these experiments is described overleaf.

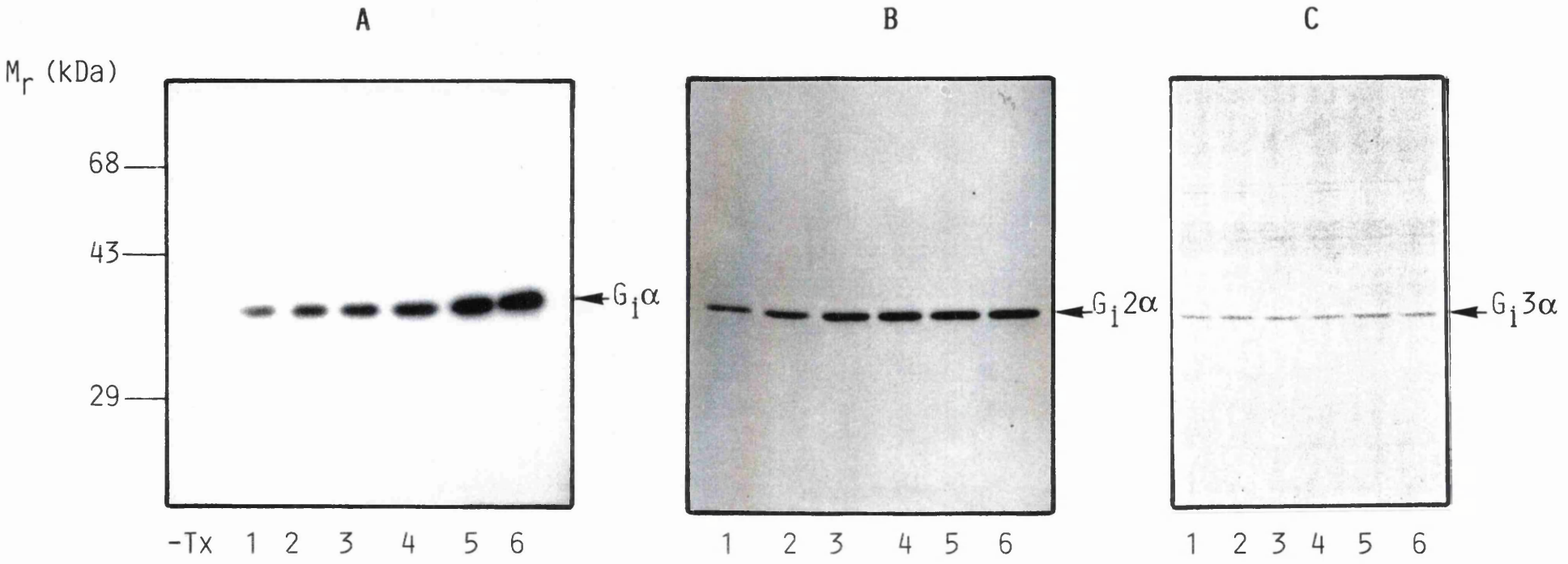
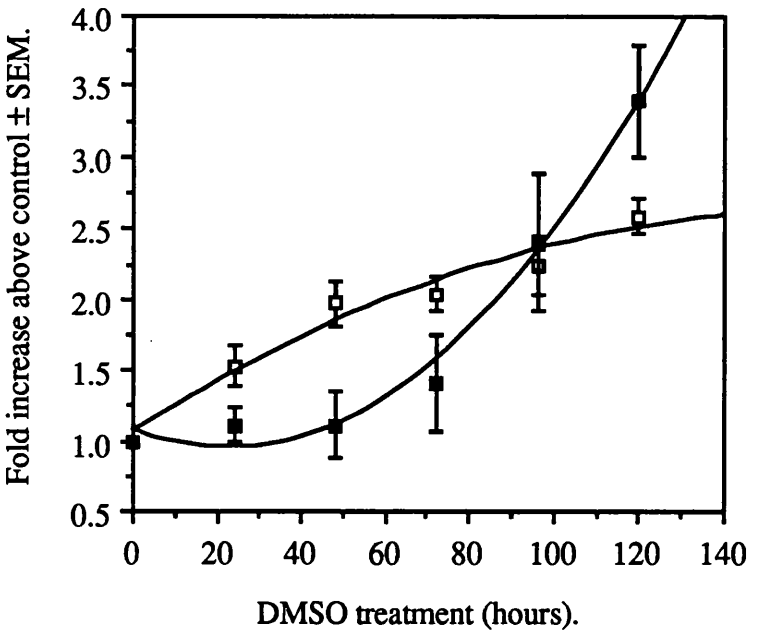

A
B
C
M_r (kDa)
68
43
29
$G_i\alpha$
$G_i2\alpha$
$G_i3\alpha$
-Tx 1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6

Figure 3.11 (continued).

Panel D represents quantification of four experiments represented by panel A (■) and three experiments represented by panel B (□). Densitometric scan analysis was used to quantify the pertussis toxin-catalysed [^{32}P]ADP-ribosylation experiments, as described in 2.9. An [^{125}I]-labelled goat anti-rabbit secondary antibody was employed to quantify immunoblots for $G_i2\alpha$. The [^{125}I] counts obtained from the excised $G_i2\alpha$ immunoreactive band were corrected for background signal and represented graphically, as described in 2.10.3. Data is expressed as fold increases above the expression of $G_i\alpha$ in control, non DMSO-differentiated U937 cell membranes. After 120 hours DMSO treatment the increased expression of the $G_i\alpha$ subunit was significant, as assessed by both methods of detection ($p < 0.01$).

Panel E represents quantification of four experiments represented by panel C. Alterations in the levels of expression of $G_i3\alpha$ were quantified using an [^{125}I]-labelled goat anti-rabbit secondary antibody. The [^{125}I] counts obtained from the excised $G_i3\alpha$ immunoreactive band were corrected for background signal and represented graphically, as described in 2.10.3. The results are expressed as fold increase above the level of $G_i3\alpha$ expressed in control, non-DMSO differentiated U937 cell membranes. $G_i3\alpha$ expression in 120 hours DMSO-differentiated cell membranes was not significantly lower than that of control cell membranes.

D


Fold increase above control ± SEM.
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
20
40
60
80
100
120
140
DMSO treatment (hours).

E

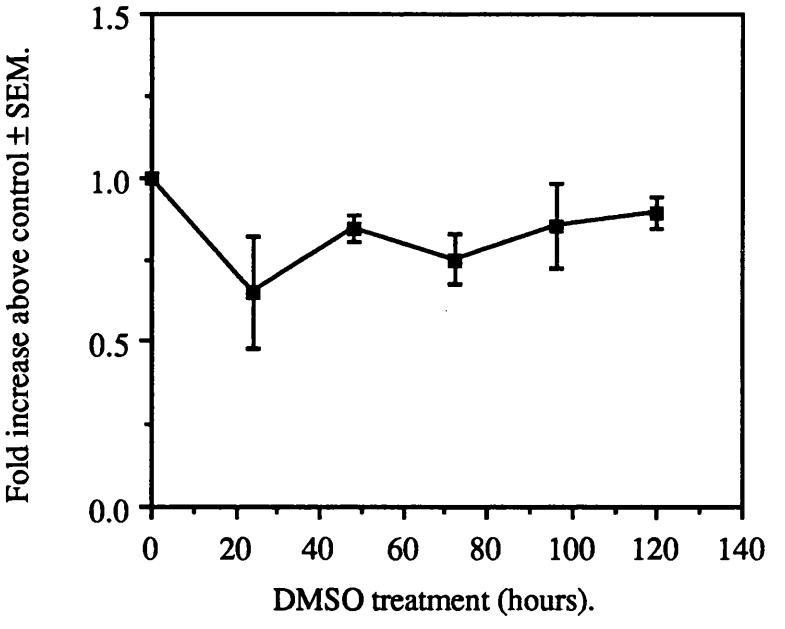

Fold increase above control ± SEM.
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
140
DMSO treatment (hours).

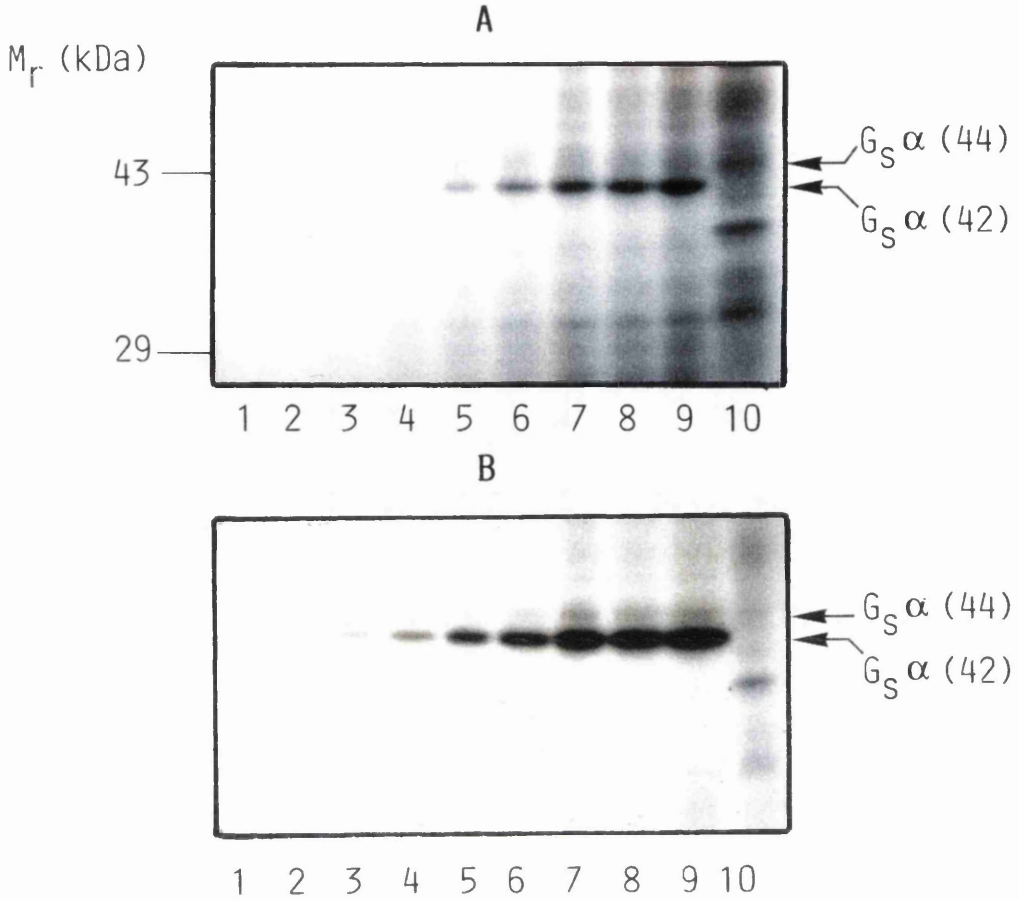
Figure 3.12.

CHOLERA TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF U937 CELL MEMBRANES - TITRATION OF MEMBRANE PROTEIN.

Increasing concentrations of membranes from either control (panel A) or 72 hour DMSO (1.25%)-differentiated U937 cells (panel B) were [^{32}P]ADP-ribosylated as described in section 2.11, with slight modifications. The final NAD+ concentration was 0.78μM and the assay was performed in the presence of 2.5mM $MgCl_2$. The U937 cells were DMSO-treated as described in 2.2.3.

Lane 10 represents ADP-ribosylation of membranes (150μg) performed in the absence of bacterial toxin. Lanes 1-9 represent 0, 5, 10, 15, 25, 50, 75, 100 and 150μg of membrane protein, respectively. The samples were resolved by SDS-PAGE (10% acrylamide [w/v], 0.25% bisacrylamide [w/v], 18 x 16cm resolving gels) as described in 2.7. The dried gels were autoradiographed for 9 days as described in 2.9. The experiments were performed once.

Panel C represents the graphs of the densitometric scan analysis of the [^{32}P]ADP-ribosylated $G_s\alpha$(42kDa) polypeptide in the titrations represented by panel A (□) and panel B (■), respectively. Densitometry was performed as in 2.9.


A
M_r (kDa)
43
29
$G_s\alpha$ (44)
$G_s\alpha$ (42)
1 2 3 4 5 6 7 8 9 10
B
$G_s\alpha$ (44)
$G_s\alpha$ (42)
1 2 3 4 5 6 7 8 9 10

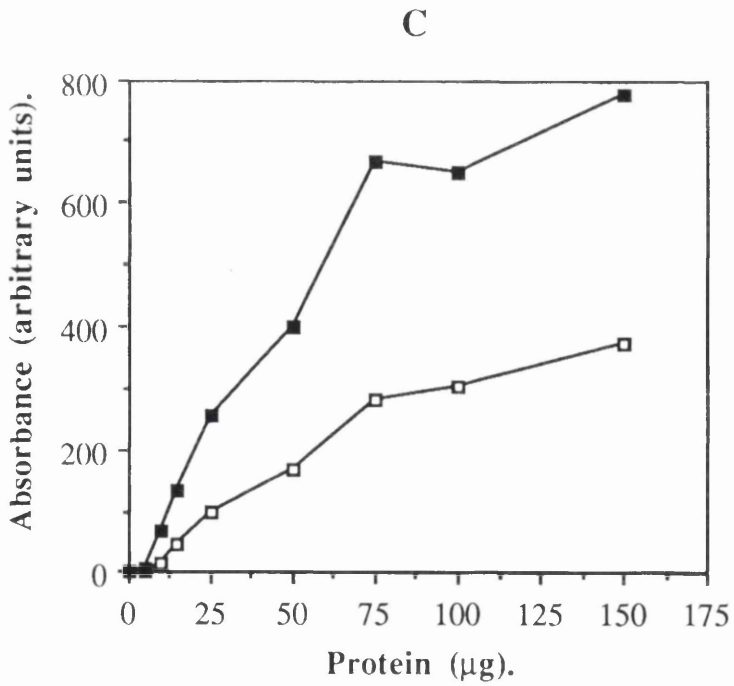

C
Absorbance (arbitrary units).
800
600
400
200
0
0 25 50 75 100 125 150 175
Protein (µg).

Figure 3.13.

IMMUNOLOGICAL DETECTION OF Gsα(42kDa) IN U937 CELL MEMBRANES WITH INCREASING CONCENTRATIONS OF MEMBRANE PROTEIN.

Increasing concentrations of U937 cell membranes were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and immunoblots were performed as described in sections 2.7 and 2.10.2. B. The primary antipeptide antiserum CS1, which recognises both the 42kDa and the 44kDa forms of Gsα, was present at a 1: 200 dilution. The immunoblots were then probed with [^{125}I]-labelled second antibody and the [^{125}I] counts obtained from the 42kDa form of Gsα were corrected for background signal and represented graphically, as described in 2.10.3.

The antiserum was titrated against control U937 cell membranes (□) and against membranes from 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (■), prepared as in 2.3.B. The cells were DMSO-treated as described in 2.2.3. These experiments were performed once.

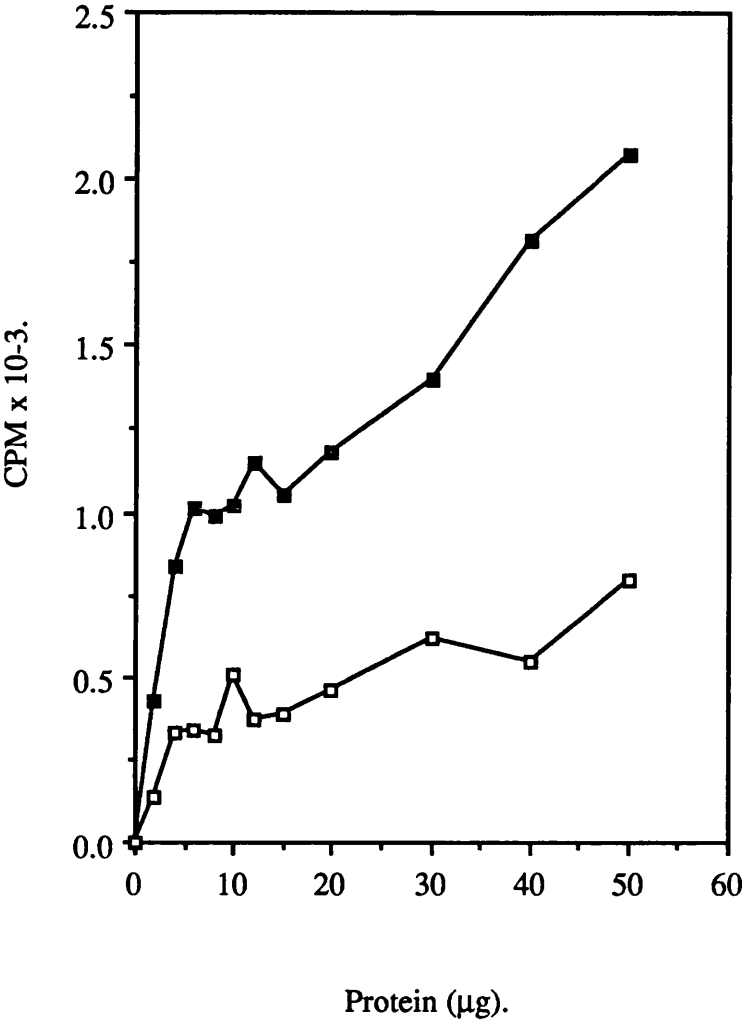

2.5
2.0
1.5
1.0
0.5
0.0
CPM x 10-3.
0
10
20
30
40
50
60
Protein (µg).

Figure 3.14.

ALTERATIONS IN THE EXPRESSION OF CHOLERA TOXIN SUBSTRATES UPON DMSO-INDUCED DIFFERENTIATION OF U937 CELLS.

Lanes 1-6 represent membranes from control, 24, 48, 72, 96 and 120 hour DMSO (1.25% v/v)-differentiated U937 cells, prepared in the presence of protease inhibitors as outlined in section 2.3.B and resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7.

Panel A represents an autoradiograph of the cholera toxin-catalysed [^{32}P]ADP-ribosylation of the membranes (15μg/lane), performed as in 2.11. "-Tx" designates the ribosylation reaction performed in the absence of cholera toxin using 120 hour DMSO-differentiated membranes. The dried gel was autoradiographed for 10 days as described in 2.9.

Panel B represents an immunoblot of the time course of differentiation (4μg/lane), performed as in 2.10.2.B. The primary antipeptide antiserum CS1, which recognises both the 42kDa and 44kDa forms of $G_{s}\alpha$, was used at a dilution of 1: 200.

Both panels are single experiments representative of experiments performed at two different membrane concentrations for each of three different membrane preparations of the DMSO-induced differentiation time course. The cell membranes were prepared and these experiments performed on the same day as the cells were harvested.

Densitometric analysis and an approach to ensure equivalent protein concentrations at each differentiation time point are described overleaf.

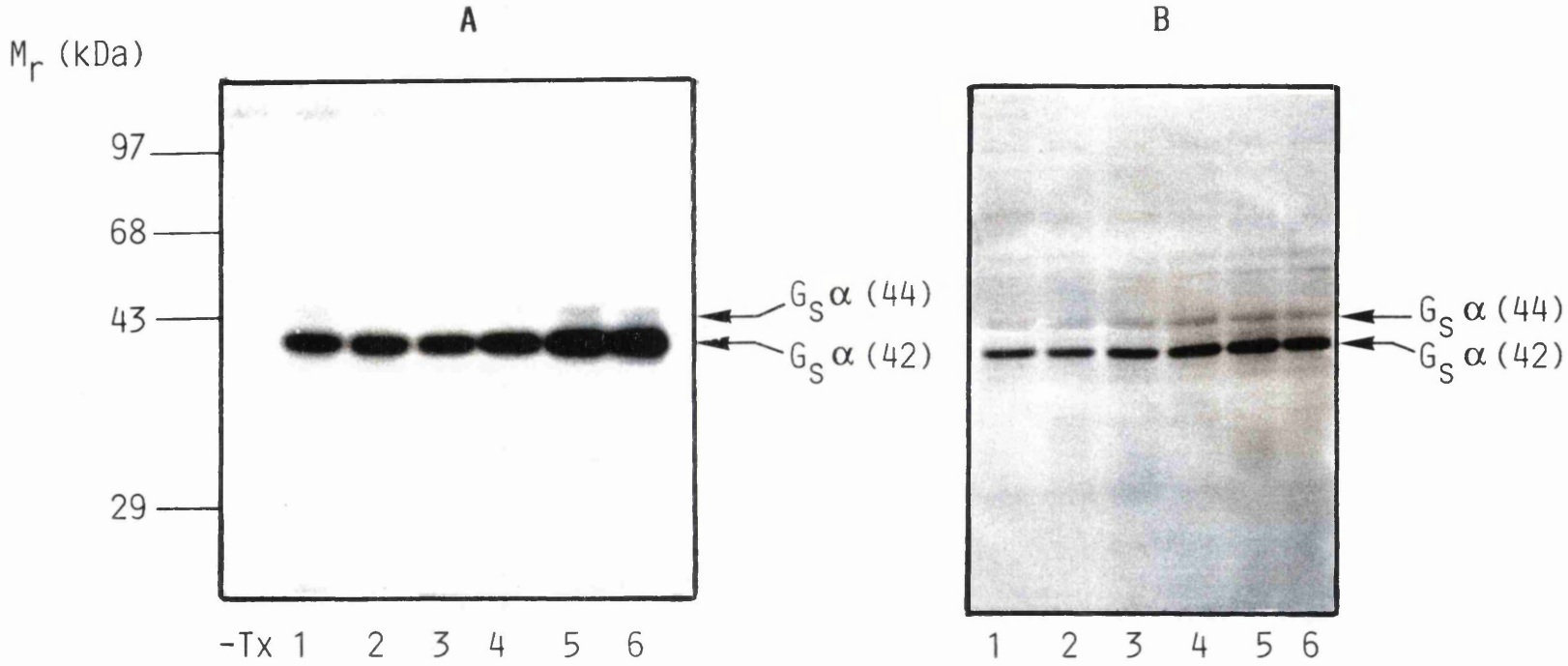
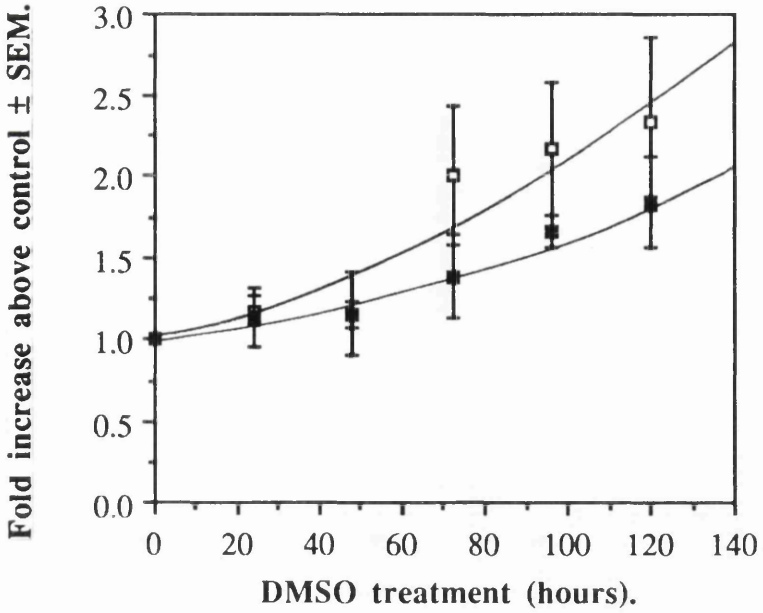

A
B
M_r (kDa)
97
68
43
29
$G_s\alpha$ (44)
$G_s\alpha$ (42)
-Tx 1 2 3 4 5 6
1 2 3 4 5 6

Figure 3.14. (continued).

Quantification of the alterations in the level of expression of $G_S\alpha$42kDa upon DMSO-induced differentiation is represented graphically in Panel C. Results are plotted as fold increase over the control value ± SEM, n = 4 experiments for both the cholera toxin-catalysed [^{32}P]ADP-ribosylation time course (□) and the analysis of the immunoblot data (■). The [^{32}P]ADP-ribosylation profile was densitometrically scanned, as described in 2.9. Alterations in the levels of expression of $G_S\alpha$42kDa were quantified using an [^{125}I]-labelled goat anti-rabbit secondary antibody. The [^{125}I] counts obtained from the excised $G_S\alpha$ immunoreactive band were corrected for background signal and represented graphically, as described in 2.10.3. The value for the increased expression of $G_S\alpha$ after 120 hours DMSO exposure was significant only to the level of $p < 0.1$ for the analysis of both the ribosylation and antipeptide antiserum data.

Panel D represents the Coomassie Brilliant Blue R stained gel of the ribosylation time course performed in panel A. The lanes are as described for panel A and the method used outlined in 2.8.1.

C


Fold increase above control ± SEM.
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
140
DMSO treatment (hours).

D

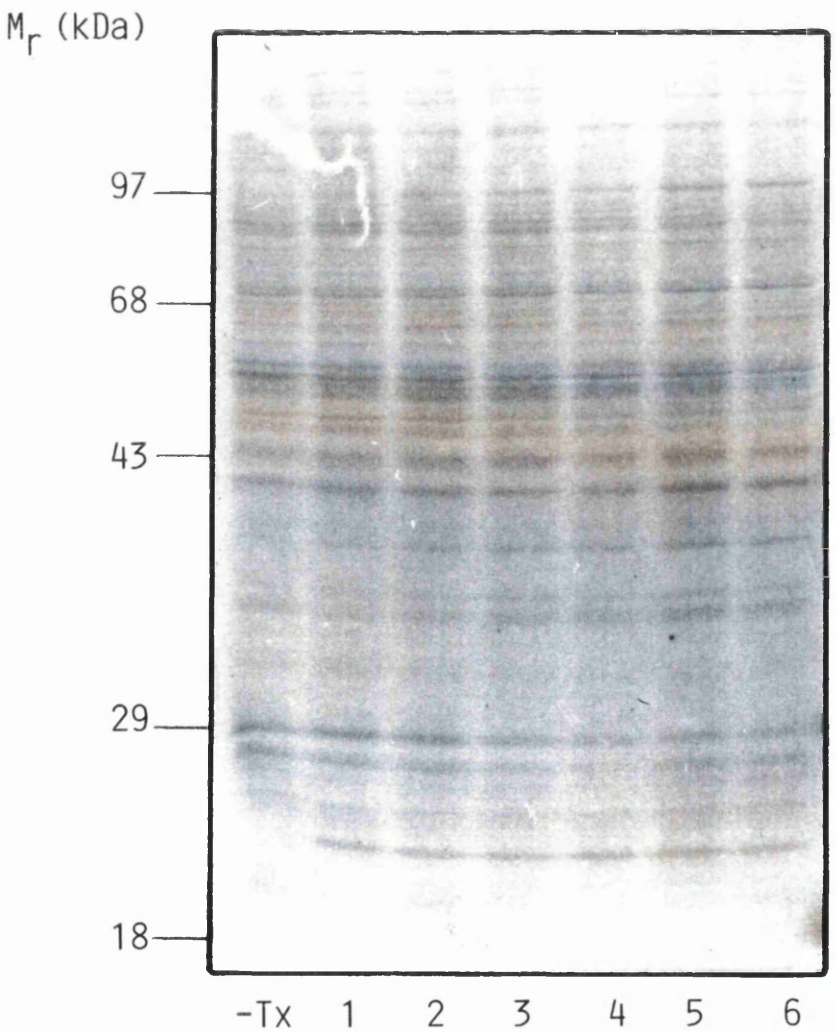

Mr (kDa)
97
68
43
29
18
-Tx
1
2
3
4
5
6

Figure 3.15.

IMMUNOLOGICAL DETECTION OF THE β SUBUNIT IN CONTROL AND DIFFERENTIATED U937 CELL MEMBRANES WITH INCREASING CONCENTRATIONS OF MEMBRANE PROTEIN.

Increasing concentrations of U937 cell membrane protein were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and immunoblotted as described in sections 2.7 and 2.10.2.B, respectively. The immunoblots were then probed with [^{125}I]-labelled second antibody. The [^{125}I] counts obtained from the excised β subunit immunoreactive band were corrected for background signal and represented graphically, as described in 2.10.3.

The antiserum was titrated against control U937 cell membranes (□) and against membranes from 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (■), prepared as in 2.3.B. The U937 cells were DMSO-treated as described in 2.2.3. The primary antipeptide antiserum BN1, which was raised against a peptide sequence common to both the 35kDa and 36kDa forms of the β subunit, was used at a 1: 200 dilution. These titration experiments were performed once.

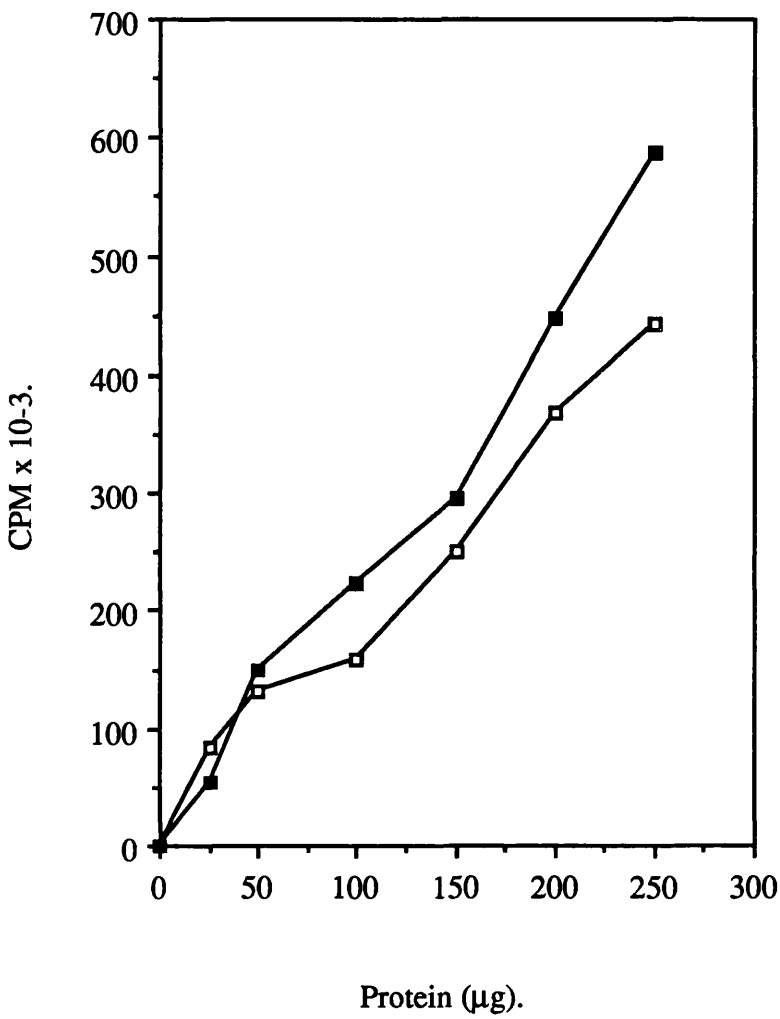

700
600
500
400
300
200
100
0
CPM x 10-3.
0
50
100
150
200
250
300
Protein (µg).

Figure 3.16.

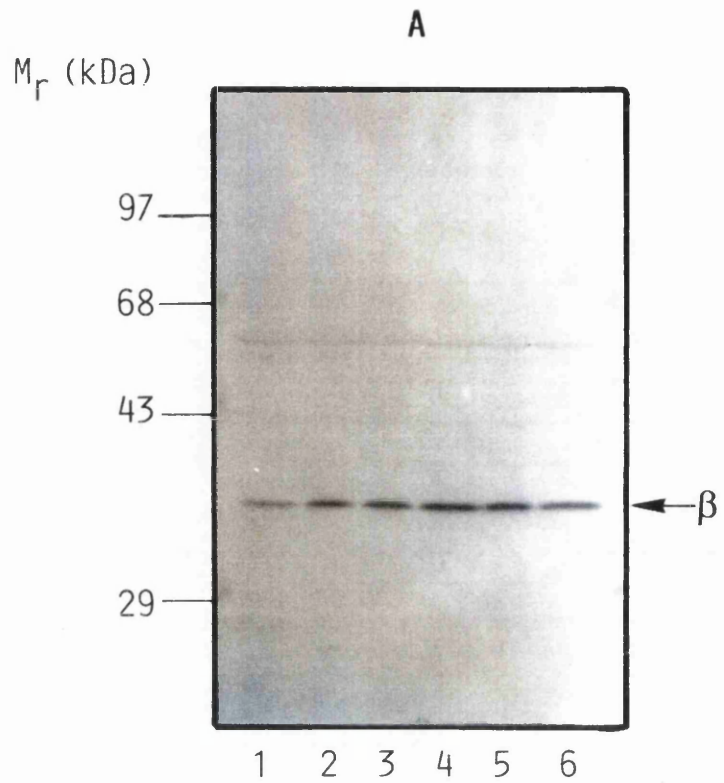
IMMUNOLOGICAL INVESTIGATION OF ALTERATIONS IN THE EXPRESSION OF THE β SUBUNIT UPON DMSO-INDUCED DIFFERENTIATION OF U937 CELLS.

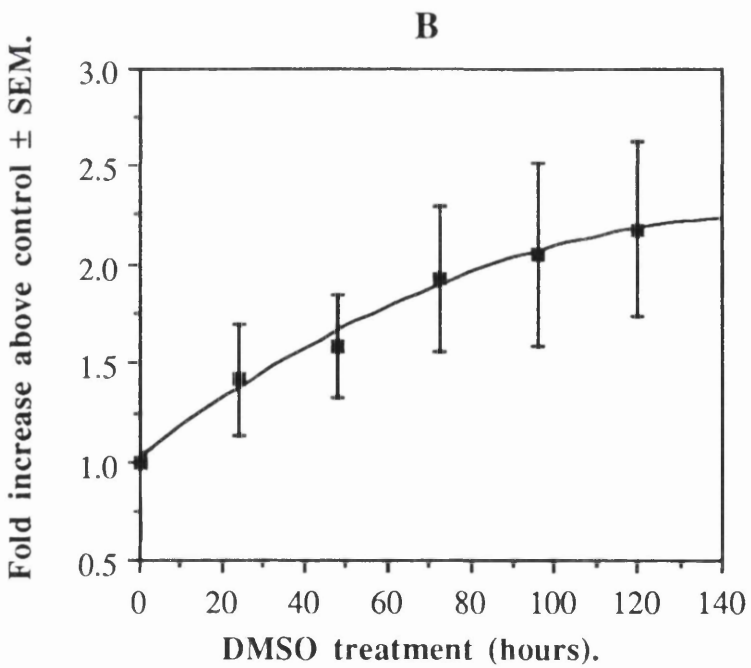
Lanes 1-6 represent membranes (50μg/lane) from control, 24, 48, 72, 96 and 120 hour DMSO (1.25% v/v)-differentiated U937 cells, prepared in the presence of protease inhibitors as outlined in section 2.3.B. The membrane samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as described in 2.7.

Panel A represents an immunoblot performed as in 2.10.2.B. The primary antiserum, BN1, was used at a 1: 200 dilution. This panel is representative of experiments performed at two different membrane concentrations for each of three different membrane preparations of the DMSO-induced differentiation time course. The cell membranes were prepared and experiments performed on the same day as the cells were harvested.

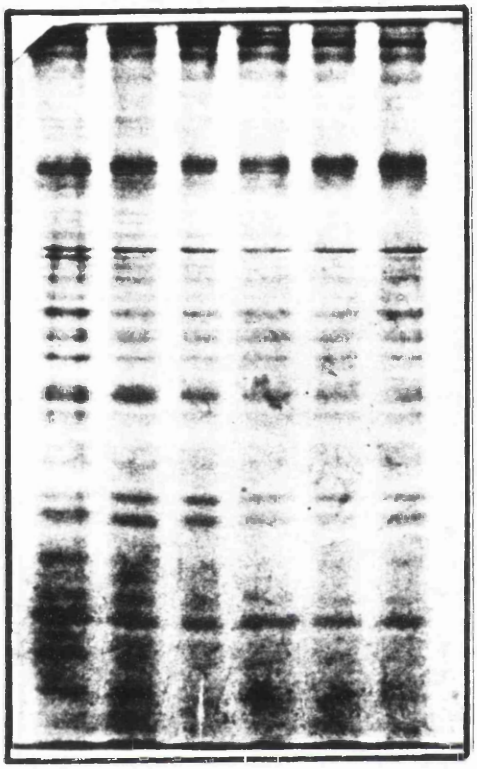
Panel B represents quantification of alterations in the levels of expression of the β subunit using an [^{125}I]-labelled goat anti-rabbit secondary antibody. The [^{125}I] counts obtained from the excised β subunit immunoreactive band were corrected for background signal and represented graphically, as described in 2.10.3. The results are plotted as fold increase over control values ± SEM (n = 4 experiments). The fold increase in β subunit expression at 120 hours, relative to control, is low and only significant to $p < 0.1$.

Panel C represents the silver stained gel of the immunoblot performed in panel A. The lanes are as described for panel A and the method of staining was as described in 2.8.2.


A
M_r (kDa)
97
68
43
29
β
1 2 3 4 5 6


B
Fold increase above control ± SEM.
3.0
2.5
2.0
1.5
1.0
0.5
0 20 40 60 80 100 120 140
DMSO treatment (hours).

C



1 2 3 4 5 6

3.3. DISCUSSION.

It is now apparent that a given cell type may express a considerable repertoire of highly homologous G-proteins, evident from cDNA cloning information and the use of discriminating antipeptide antisera (Jones & Reed, 1987; McKenzie & Milligan, 1990). Thus it was necessary to define the complement of G-proteins expressed in both control (non DMSO-treated) and differentiated (72 hour DMSO-treated) membranes prepared from U937 cells. The relevance of the 72 hour DMSO treatment was that this was the condition used by Pollock and co-workers when demonstrating a totally pertussis toxin-sensitive Ins(1,3,4)P_3, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 production in response to the chemotactic peptide, fMLP in U937 cells (Pollock *et al.*, 1990).

One could envisage that the phagocytic nature of these cells may predispose their own proteins to proteolytic degradation, especially upon cell disruption and plasma membrane preparation. Indeed the neutral protease, elastase, has been shown to be present within the U937 cell (Harris & Ralph, 1985). More recently, Kumatori and co-workers have demonstrated that human leukaemic cell lines exhibit an abnormally high expression of proteosomes (Kumatori *et al.*, 1990). However, initially a range of experimental approaches, including immunoblotting, toxin-catalysed [^{32}P]ADP-ribosylations and GTPase activity measurements, utilised crude plasma membranes prepared in the absence of any protease inhibitors. Indeed, it was in part the initial success of these experiments, outlined later in Chapter 4, that prevented more immediate recognition of the possibility of a degradative activity in the membrane preparations.

The initial observation of G-protein degradation was the GTP-dependent appearance of a pertussis toxin-catalysed, [^{32}P]ADP-ribosylated "doublet", under SDS-PAGE conditions that should not resolve the individual pertussis toxin substrates. This "doublet" need not necessarily have been due to proteolysis and the possibility that the more rapidly migrating [^{32}P]ADP-ribosylated protein

represented a "novel" pertussis toxin substrate was also considered. In experiments not represented, doublet formation occurred to varying degrees in several membrane preparations examined. Such variation may explain why some of the initial experiments proved successful and suggested that the "novel" substrate proposal was an unlikely explanation. From initial studies using both control and differentiated membranes, prepared in the absence of protease inhibitors, it was apparent that the only known pertussis toxin substrates expressed in these cells were $G_i2\alpha$ and $G_i3\alpha$. Using a combination of antipeptide antisera, which in these cells were selective for these $G_i\alpha$ subunits, the GTP-dependent pertussis toxin-catalysed [^{32}P]ADP-ribosylated "doublet" was found to be immunoreactive, again suggesting that this "additional" polypeptide did not represent a novel pertussis toxin substrate. Indeed the preservation of the C-terminal immunoreactive site, the C-terminal site for pertussis toxin-catalysed [^{32}P]ADP-ribosylation and the reduction in relative amounts of substrate after the 37^{o}C incubations implies an amino terminal-directed proteolytic action. Interestingly, this more rapidly migrating form of $G_i\alpha$ displayed equivalent and in some preparations greater [^{32}P]ADP-ribose incorporation than the $G_i2\alpha/G_i3\alpha$ polypeptides. In experiments not included this proteolysis was found to also occur in the absence of GTP and on ice. Further, using discriminatory antipeptide antisera the proteolysis of both $G_i2\alpha$ and $G_i3\alpha$ was individually demonstrated.

To overcome this proteolysis problem the membrane preparation was initially altered to more closely mimic that used in studies of the human leukaemic, HL-60 cell line (Gierschik *et al.*, 1989b). The "cocktail" of protease inhibitors included within their preparation procedure, did prevent degradation of G-proteins but the increased number and extended duration of the centrifugation spins was detrimental to functional coupling, as assessed by fMLP-stimulated GTPase activity (results not included). To combat these problems the original crude plasma membrane preparation procedure was employed with the inclusion of the "inhibitor

cocktail", used by Gierschik and co-workers, throughout the cell harvesting and membrane preparation procedure. This preparation did exhibit a low level of fMLP-stimulated GTPase activity (see later in Chapter 5) and did preserve the integrity of the $G_i\alpha$ subunits expressed by these cells as assessed by immunoblotting with specific antipeptide antisera.

Thus having established a membrane preparation which prevented proteolysis of the native $G_i\alpha$ subunits, the complement of G-proteins expressed by both control and 72 hour DMSO (1.25% v/v)-differentiated U937 cells was redefined using the antipeptide antisera outlined previously in Table 2.1.

The rationale behind the generation of antipeptide antisera which are selective for the G-protein subunits has been introduced previously in 1.13.1. Membranes of brain, neutrophil, U937 and HL-60 cell lines were instrumental in defining the specificity of the first antipeptide antiserum raised. This antipeptide antiserum was directed against a synthetic peptide equivalent to the extreme C-terminal decapeptide of the rod transducin α subunit and was shown to recognise what can now be assigned retrospectively as both $G_i1\alpha$ and $G_i2\alpha$ (Goldsmith *et al.*, 1987; Falloon *et al.*, 1986). Indeed these studies demonstrated the presence of $G_i2\alpha$ but not $G_i1\alpha$ in leukocyte cells. An equivalent antipeptide antiserum, SG1, was employed in the experiments presented in this Chapter. U937 cells were also one source of a $G_i2\alpha$ cDNA clone (Didsbury *et al.*, 1987). With the sequence information provided by these clones came the generation of antipeptide antisera discriminatory for $G_i1\alpha$ and $G_i2\alpha$ (Goldsmith *et al.*, 1987 & 1988). Again an equivalent antipeptide antiserum, LE2/3 specific for $G_i2\alpha$, was employed in the experiments presented in this Chapter. The antipeptide antiserum, I1A, which was raised against a synthetic peptide corresponding to the sequence of residues 159-168 of $G_i1\alpha$ was shown to be specific for the detection of $G_i1\alpha$ in the work of this Chapter and also in that of Chapter 6. After completion of these experiments, an antipeptide antiserum generated against the identical conjugate has been shown to specifically recognise recombinant $G_i1\alpha$, but not $G_i2\alpha$, $G_i3\alpha$ or $G_o\alpha$ subunits

(Milligan, 1992). Another pertussis toxin substrate, $G_i3\alpha$, differs in only two of the ten residues at its extreme C-terminus but certain antipeptide antisera that identify both $G_i1\alpha$ and $G_i2\alpha$, e.g., SG1, do not recognise $G_i3\alpha$. This is demonstrated in the experiments of this Chapter and that of Chapter 6. Antipeptide antisera raised against a synthetic peptide equivalent to the extreme C-terminal decapeptide of $G_i3\alpha$ are specific for this α subunit. This specificity is again demonstrated in the experiments of this Chapter and those of Chapter 6 and by more recent work with recombinant G-proteins (McClue *et al.*, 1992).

Prior to the work of this Chapter it had been shown that the major pertussis toxin substrate of the U937 cell was not $G_i1\alpha$ and in retrospect can now be identified as $G_i2\alpha$. This study also demonstrated the presence of a β subunit in U937 cells (Falloon *et al.*, 1986). The experiments presented in this Chapter demonstrated that the only known pertussis toxin-sensitive G-proteins expressed by both control and differentiated U937 cells were $G_i2\alpha$ and $G_i3\alpha$. The other known pertussis toxin substrates, $G_i1\alpha$ and $G_o\alpha$ were not detected with specific antipeptide antisera. The specificity of the antipeptide antiserum I1A was also demonstrated in that it did not cross react with the other $G_i\alpha$ forms expressed in the U937 cells. Further, under electrophoretic conditions designed to resolve the individual pertussis toxin substrates, a combination of the antipeptide antisera discriminatory for $G_i2\alpha$ and $G_i3\alpha$ did identify mutually exclusive polypeptides of the previously reported apparent molecular weight and relative electrophoretic mobility of these $G_i\alpha$ subunits.

The antipeptide antiserum raised against the peptide corresponding to residues 22-35 of $G_o\alpha$ did not identify this polypeptide in the U937 cells, nor did it cross react with any of the other pertussis toxin substrates expressed by these cells. Indeed, this antipeptide antiserum has recently been shown to recognise recombinant $G_o\alpha$ protein but not any of the recombinant G_i1,G_i2 or $G_i3\alpha$ subunits (Milligan, 1992). However, in an attempt to confirm that $G_o\alpha$ was not

expressed in U937 cell membranes, two antipeptide antisera which were raised against the N- and C-terminal amino acid sequences of this protein did detect immunoreactive proteins in both control and differentiated U937 cell membranes (Mullaney & Milligan, 1990). However, these polypeptides comigrated with the $G_i\alpha$ subunits expressed in these cells and not the $G_o\alpha$ protein expressed in rat cortical membranes. Such a lack of specificity of anti-$G_o\alpha$ antipeptide antisera has been demonstrated previously (Rouot *et al.*, 1989). These conclusions are supported by the detection of mRNA's corresponding to $G_i2\alpha$ and $G_i3\alpha$, but not $G_i1\alpha$ or $G_o\alpha$ in control and dibutryl cAMP-differentiated HL-60 cells (Murphy *et al.*, 1987). However, it should be noted that this treatment of HL-60 cells will differentiate them to neutrophil-like cells (Chaplinski & Niedel, 1982).

Antipeptide antiserum CS1 was generated against a synthetic decapeptide corresponding to the C-terminal sequence of $G_s\alpha$, which is common to all four of the splice variant forms of $G_s\alpha$ (Bray *et al.*, 1986). This antipeptide antiserum recognised the 42kDa and 44kDa forms of $G_s\alpha$ demonstrating the first immunological identification of these subunits in both control and DMSO-differentiated U937 cell membranes.

Antipeptide antiserum BN1 was raised against the N-terminal sequence of the β_1 subunit, which differs by one amino acid substitution from β_2 (Levine *et al*, 1990). This antipeptide antiserum can recognise both the β subunit forms which can be resolved under suitable conditions (Evans *et al.*, 1986). Under the conditions employed in this study only one immunoreactive polypeptide was detected in both control and DMSO-differentiated U937 cell membranes. Whether this represented β_1 and/or β_2 was not established.

Previous studies have demonstrated that in the dibutryl cAMP-differentiated HL-60 and U937 cells there was at least a two fold increase in the expression of a pertussis toxin substrate, when expressed on a per milligram of membrane protein basis. This was assessed both by pertussis toxin-catalysed [^{32}P]ADP-ribosylation

and immunoreactivity. In retrospect the antipeptide antiserum used in this study could not have distinguished between the pertussis toxin substrates, $G_i2\alpha$ and $G_i3\alpha$ (Falloon *et al.*, 1986). In a later study, Murphy and co-workers used an antipeptide antiserum specific for $G_i2\alpha$ and demonstrated this proteins increased expression upon differentiation of HL-60 cells to neutrophil-like cells. Northern blot analysis of dibutryl cAMP-differentiated HL-60 cells demonstrated that the abundance of the mRNA transcripts for $G_i2\alpha$, $G_i3\alpha$ and $G_s\alpha$ all increased with maturation. Using DMSO as the differentiating agent it was established that, at the protein level, $G_i2\alpha$ increased with HL-60 maturation over the 5 day time course. However at this time specific antipeptide antibodies recognising $G_i3\alpha$ were not available to examine the expression of this $G_i\alpha$ species (Murphy *et al.*, 1987). More recent analysis of 72 hour DMSO-induced differentiation of the U937 cell line demonstrated a five fold increase in $G_i2\alpha$ relative to uninduced cells, as assessed by use of a specific antipeptide antiserum. This study did not address whether the other G-protein subunits expressed by this cell line varied upon DMSO-induced differentiation (Pollock *et al.*, 1990).

Thus in an attempt to further these initial investigations U937 cells were induced to differentiate with dimethyl sulphoxide (1.25% v/v) over a five day time course. The choice of the concentration and the duration of the inducing stimulus was based upon the work of Collins and his co-workers. In a study of terminal differentiation of the HL-60 cell line, they demonstrated that a concentration of 1.3% (v/v) DMSO gave maximal induction of maturation without the inhibition of cell replication which was evident at a concentration of 1.5% (v/v) DMSO. Maximal differentiation occurred within five days of 1.25% (v/v) DMSO treatment, with cell death evident beyond this incubation period (Collins *et al.*, 1978).

To minimise protein degradation problems the immunoblot and toxin-catalysed [^{32}P]ADP-ribosylation experiments were conducted on the same day as the cells were harvested, with protease inhibitors included at every stage in the

procedure. With the exception of $G_{i}3\alpha$, whose expression remained unaltered, the expression of $G_{i}2\alpha$, $G_{s}\alpha$ 42kDa and 44kDa forms and β subunit increased upon cell maturation, when expressed per milligram of membrane protein. It was appreciated that for general scientific purposes a value of $p < 0.05$ is considered to indicate a significant effect. The *t* values calculated for the changes in β and $G_{s}\alpha$ subunit expression were nearer to the corresponding $p = 0.05$ level than $p = 0.1$ and were tentatively considered as significant. The *t* value for the $G_{i}3\alpha$ subunit expression was far less than that required for a $p = 0.1$ and hence the hypothesis that there were significant changes in expression of this subunit rejected.

The most reproducible increase in $G\alpha$ subunit expression was that of $G_{i}2\alpha$. The level of this subunit increased 2.6 fold in membranes of 120 hour DMSO-differentiated U937 cells relative to levels expressed by control (non DMSO-treated) cells on a per milligram of membrane protein basis. This was assessed by quantification of immunoblots probed with an antipeptide antiserum specific for $G_{i}2\alpha$ in these cells. Such an increase in $G_{i}2\alpha$ expression was observed in every preparation of DMSO-differentiated U937 cell membranes and as such was used as an indicator of cell maturation.

Quantification of the immunoreactive band detected using an antipeptide antiserum specific for $G_{i}3\alpha$ proved more difficult than for $G_{i}2\alpha$, due to a higher level of non specific [^{125}I] labelling of the immunoblot and a low incorporation of specific [^{125}I] counts. These problems were not encountered when examining the same membrane preparations for expression of $G_{i}2\alpha$ and using the equivalent [^{125}I]-labelled second antibody. However the antipeptide antiserum specific for $G_{i}2\alpha$ in these cells was used at a ten fold lower dilution than that specific for $G_{i}3\alpha$. As such, an explanation of the quantification problems may reside in a degree of non-specificity of the first antipeptide antiserum that is only evident when probed with the [^{125}I]-labelled second antibody. An alternative explanation could be that $G_{i}3\alpha$ may be less abundant than $G_{i}2\alpha$, thus decreasing the "signal to noise" ratio. This study has not attempted to quantify the absolute concentrations of G-protein

subunits expressed in the U937 cell membrane but this would now be possible with the availability of recombinant G-protein subunits over-expressed by *E.coli* strains (McClue *et al.*, 1992).

As $G_i2\alpha$ and $G_i3\alpha$ represent the only known G-protein pertussis toxin substrates in the U937 cell it was anticipated that, over the differentiation time course, the pertussis toxin-catalysed [^{32}P]ADP-ribosylation signal would reflect the contribution each of these substrates made to any alterations in $G_i\alpha$ subunit expression. However, from a comparative plot of the increase in $G_i2\alpha$ relative to the increase in pertussis toxin substrate(s) it was apparent that this was an over simplification. As $G_i3\alpha$ expression did not alter significantly upon cell maturation the increase in toxin-catalysed [^{32}P]ADP-ribosylation was presumably due to $G_i2\alpha$. However the increase in pertussis toxin-catalysed [^{32}P]ADP-ribosylated substrate exhibited a significantly different time course from that of the increase in expression of $G_i2\alpha$, only reaching equivalence at the 96 hour time point.

There are a number of possible explanations for this observation. Ribeiro-Neto and his co-workers demonstrated that pertussis toxin-catalysed [^{32}P]ADP-ribosylation is facilitated most effectively by GTP, GDP and non-hydrolysable analogues of GDP (Ribeiro-Neto *et al.*, 1987). As toxin-catalysed [^{32}P]ADP-ribosylation is further augmented by β subunits it has been suggested that the preferred pertussis toxin substrate is the GDP-liganded $\alpha\beta\gamma$ complex (Neer *et al.*, 1984; Tsai *et al.*, 1984). One consideration is that alterations in α–$\beta\gamma$ ratio may alter the pertussis toxin-catalysed [^{32}P]ADP-ribosylation of the $G_i\alpha$ substrates. However, the general trend of the increased β subunit expression did mimic that of the $G_i2\alpha$ subunit in both time course and relative fold increased expression, despite considerable preparation to preparation variations. Such variations were possibly again attributable to the high background [^{125}I] levels observed in attempts to quantify the immunoblots. Whether this increased expression reflects a contribution from both β subunit forms that the antipeptide antisera can recognise

was not established. However assuming that both the Gsα subunits and the Giα subunits contact this β subunit population, which may be an incorrect assumption from emerging evidence regarding αβγ specificities, then it could be argued that an increase in Gsα "early" in the differentiation time course may cause a reduction in β subunit accessible to the pertussis toxin substrates. This hypothesis also relies on the fact that the Gsα subunits would have to be expressed at a significant concentration relative to the Giα species. However from the immunoblot and cholera toxin-catalysed [^{32}P]ADP-ribosylation data, Gsα42kDa expression does not appear to increase significantly until between 72-96 hours of DMSO treatment. Interestingly these two methods of detecting altered expression of Gsα42kDa did not produce significantly different profiles. The preferred cholera toxin substrate is free Gsα, perhaps implying that βγ effects are indeed some component of the discrepancies previously outlined for the pertussis toxin substrates (Kahn & Gilman, 1984b; Ribeiro-Neto *et al.*, 1987). Although assessed more qualitatively, the differentiated-associated increase in Gsα44kDa expression did appear to occur more rapidly than that of the Gsα42kDa form. In retrospect it may have been informative to have attempted to have quantified this Gsα form, perhaps possible by increasing the concentration of the membrane protein and using a lower dilution of the primary antipeptide antiserum.

A range of other factors influence the toxin-catalysed [^{32}P]ADP-ribosylation and one can speculate may contribute to the initial "low" ribosylation status of Giα in the differentiating U937 cells. Alterations, with ontogeny, in the activity of an endogenous NADase activity in rat brain membranes has been demonstrated (Milligan *et al.*, 1987a). The possibility of an increased endogenous membrane associated NADase activity in the initial stages of U937 cell differentiation was considered but is also difficult to assess without direct measurement and is perhaps discredited by the correlation between the Gsα42kDa expression data obtained from both *in vitro* [^{32}P]ADP-ribosylation and immunoblotting approaches. Ribeiro-

Neto and his co-workers have demonstrated that a non ionic detergent augments the pertussis toxin-catalysed [^{32}P]ADP-ribosylation of Giα in membranes but inhibits the action of cholera toxin (Ribeiro-Neto *et al.*, 1987). Thus the physiochemical nature of the membrane would appear to be important and maturation induced or perhaps DMSO-induced alterations in these properties may have initially obscured a pertussis toxin-catalysed [^{32}P]ADP-ribosylation. More recently an endogenous ADP-ribosyl transferase activity has been isolated from erythrocytes (Tanuma *et al.*, 1988). In support of the hypothesis that endogenous ribosylation may play a physiological role, is the isolation of activators and inhibitors of this enzyme and also an enzyme that cleaves mono-ADP-ribosyl linkages (Brune & Lapetina, 1990; Hara-Yokoyama & Furuyama, 1988 and Tanuma & Endo, 1990). It is conceivable that any of these enzymes and factors, if present in this cell line, could be modulated upon differentiation of the U937 cell line. This would render the pertussis toxin-catalysed [^{32}P]ADP-ribosylation reaction a reflection of ribosylation "site" availability rather than the concentration of Giα.

In attempting to investigate alterations in G-protein subunit expression it was discovered that absolute quantification of even relative changes in expression suffers from both technical and biological uncertainty. A larger sample size may have improved the significance of the effects observed and it was appreciated that in expressing fold increases over control values one assumes negligible preparation to preparation variation in expression of G-protein subunits in control membranes. In retrospect, it was also appreciated that in systems in which there are alterations in cell size how one expresses changes in the level of expression of membrane associated proteins is extremely important. Indeed, whether it is more valid to express such changes with respect to cell number equivalents rather than on a per milligram of membrane protein basis was another factor considered. In an early study, discussed previously, which examined dibutryl cAMP-induced differentiation of U937 cells, it was observed that there was a notable difference in

the alterations in pertussis toxin substrate expression depending on how such changes were expressed. Indeed similar levels of this substrate were observed when compared on a cell equivalent basis but there was an approximate doubling in this substrate when expressed as per milligram of membrane protein (Falloon *et al.*, 1986). The differentiation strategy described in 2.2.3 was employed to at least attempt to achieve a similar cell number at each point on the differentiation time course. Further, the finding that $G_i3\alpha$ expression did not alter significantly did suggest that the observed increases in expression of the other G-protein subunits was not merely a consequence of the increase in cell size that has been reported to accompany U937 cell differentiation. Interestingly, the quantification problems experienced in this work were highlighted by the more recent work of Bohm and co-workers. In their opinion quantification of immunoblots by densitometry is hampered by a lack of precision and they too discovered discrepancies between immunodetectable $G_i\alpha$ levels and the intensity of pertussis toxin labelling in membranes from various sources, in particular human lung and leukocyte membranes. To overcome these problems they have developed a radioimmunoassay for the quantification of $G_i\alpha$ in cell membranes (Böhm *et al.*, 1991).

Despite these caveats, what is suggested by the work of this Chapter is that DMSO-treatment of the U937 cell line does alter the relative expression of G-protein subunits. The mechanism of action of DMSO and other highly polar compounds which act to induce terminal differentiation is unknown (Collins *et al.*, 1978). Their cryoprotective properties has led to the suggestion that these compounds may act through perturbation of the cell membrane, whereas others suggest that they may diffuse into the nucleus and elicit effects upon gene transcription (Lyman *et al.*, 1976; Tanaka *et al.*, 1975). In Friend virus-infected murine erythroleukaemia cells, DMSO treatment induces a transient 5-6 fold increase in intracellular cAMP levels (Gazitt *et al.*, 1978). One could speculate that a similar effect of DMSO in the U937 cell line may alter gene transcription by

activation of some of the cAMP-responsive elements introduced previously. Interestingly the $G_{i}2\alpha$ promoter contains possible DNA binding domains for AP2 (Weinstein *et al.*, 1988). However, although one can speculate on possible mechanisms for DMSO-induced cell maturation and changes in gene expression these processes remain largely unresolved. Whether alterations in G-protein expression are consequences of the differentiation process or are in some way causal is not at present understood.

There is substantial precedent for alteration in G-protein expression upon differentiation of a variety of systems. During differentiation of the 3T3-L1 cell from fibroblast to adipocyte increased expression of β, $G_{s}\alpha$, $G_{i}\alpha$ and $G_{o}\alpha$ subunits has been demonstrated (Watkins *et al.*, 1987). In the murine neuroblastoma x rat glioma hybrid cell line NG108-15, dibutryl cAMP-induced differentiation, elevates $G_{o}\alpha$ expression but does not alter $G_{i}2\alpha$ expression (Mullaney *et al.*, 1988). This study demonstrates that an increase in intracellular cAMP does not necessarily lead to altered $G_{i}2\alpha$ expression and demonstrates a differential regulation of G-protein α subunit expression.

Indeed modulation of G-protein levels in general is a widespread phenomenon, evident under various conditions such as in pathological states, under the influence of the autonomic nervous system or upon prolonged agonist exposure (Ohisalo & Milligan, 1989; Reithmann *et al.*, 1990; Milligan & Green, 1991). It would appear that alteration in the expression of G-proteins is potentially a site at which cells may regulate the response to extracellular signals. It is tempting to speculate that the differentiation-associated, increased expression of the pertussis toxin substrate, $G_{i}2\alpha$, may contribute to the mediation of maturation-specific responses e.g., the response to the chemotactic peptide, fMLP. Chapters 4 and 5 will address this issue.

CHAPTER 4.

IDENTIFICATION OF THE G-PROTEIN COUPLING TO THE fMLP RECEPTOR IN U937 CELL MEMBRANES.

4.1. INTRODUCTION.

Phagocytic cells perform a diversity of complex functions, briefly introduced in the previous Chapter. They migrate from the circulation to the sites of inflammation in response to chemoattractants which are produced through humoral or cellular immunological processes.

Well defined chemoattractants, acting via distinct receptors, include the complement product, C5a, leukotriene B4 (LTB4), platelet-activating factor (PAF), and synthetic *N*-formylated-methionyl peptides (NFP). The chemoattractant properties of NFP's were predicted by the fact that much of the proteinaceous chemoattractant activity present in culture supernatants of *Escherichia coli* had blocked NH_2-terminal amino acids (Schiffmann *et al.*, 1975). Studies of structure and function demonstrated a strict relationship between the amino acid sequence of the NFP's and their biological activity, an extremely potent tripeptide being *N*-formyl-methionyl-leucyl-phenylalanine, fMLP (Showell *et al.*, 1976).

The observation that receptors for fMLP on phagocytic leukocytes existed in high and low affinity states which were interconvertible by guanine nucleotides was indicative of a G-protein coupling interaction with this receptor (Koo *et al.*, 1983; Synderman *et al.*, 1984). As previously stated, numerous studies in a variety of systems have demonstrated that the G-protein coupled to the fMLP receptor is a substrate for pertussis toxin and that *in vivo* treatment of leukocytic cells with this toxin can inhibit phospholipid metabolism and many of the functional responses of these cells (Lad *et al.*, 1985; Volpi *et al.*, 1985; Williamson *et al.*, 1988). Indeed pertussis toxin pretreatment of guinea pig polymorphonuclear leukocytes (PMN's) inhibited an fMLP-stimulated high affinity GTPase activity in membranes prepared from these cells (Okajima *et al.*, 1985). The half-maximal concentration of the toxin to inhibit this GTPase activity correlated with that of the toxin to inhibit the cellular arachidonate-releasing response and to catalyse ADP-ribosylation of an approximately 41kDa membrane protein (Okajima & Ui, 1984). The observation

that $G_i\alpha$, "purified" from rat brain, reconstituted an fMLP-dependent GTPase activity in membranes from pertussis toxin pretreated PMN membranes suggested that the G-protein coupling to the fMLP receptor may be equivalent to that which mediated the inhibitory input to adenylyl cyclase (Okajima *et al.*, 1985). However, this was discredited by the fact that the chemotactic peptide neither inhibited nor stimulated this enzyme in human PMN's (Verghese *et al.*, 1985b).

As discussed previously, pertussis toxin and cholera toxin were of key importance in the original identification of the G-proteins which couple stimulatory and inhibitory receptors to adenylyl cyclase, G_s and G_i, respectively (discussed in 1.1). These toxins have more recently proved useful in assigning coupling specificity of a G-protein to a particular receptor.

Pertussis toxin catalyses the mono-ADP-ribosylation of its substrates at a cysteine residue four amino acids removed from the C-terminus (Medynski *et al.*, 1985). With the knowledge that the C-terminus plays a major role in receptor coupling it can now be appreciated why such a covalent modification prevents productive coupling between receptor and G-protein. However, the realisation that $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_{o}\alpha$ and the transducins all have a conserved C-terminal cysteine residue suggested that it was an oversimplification to assume that only $G_i\alpha$ could be implicated in coupling to effector systems that were perturbed by this toxin (Kaziro, 1990).

Cholera toxin catalyses the mono ADP-ribosylation of transducin at Arg_{174} (Van Dop *et al.*, 1984b). The corresponding amino acid residue in $G_s\alpha$ is $Arg_{187/188}$ or $Arg_{201/202}$, depending on the splice variant form of $G_s\alpha$ (Bray *et al.*, 1986). These are the presumed sites of ADP-ribosylation by cholera toxin and mutation of these residues does reduce toxin-catalysed ADP-ribosylation, although additional arginine residues may also provide sites for modification (Landis *et al.*, 1989; Freissmuth & Gilman, 1989) Apart from the transducins, under "normal" conditions other pertussis toxin-sensitive G-protein α subunits are not substrates

for ADP-ribosylation catalysed by cholera toxin. It is not that these proteins lack the target for the addition of a mono-ADP-ribose group, as a conserved arginine residue is found at the position equivalent to that in which it is ribosylated in Gsα. This amino acid is believed to be proximal to a section of primary sequence that forms part of the guanine nucleotide binding site and as discussed previously the maintenance of this residue is crucial to GTPase activity (see 1.6.1). Considering these properties, it can be postulated that this arginine residue may become a site for cholera toxin-catalysed ADP-ribosylation if it could be rendered free of "blocking " guanine nucleotides.

This hypothesis would agree with the work of Graves and co-workers. They demonstrated that cholera toxin, in the absence of exogenously added GTP, catalysed an *in vitro* [32P]ADP-ribosylation of a protein of approximately 41kDa in adipocyte membranes (Graves *et al.*, 1983). This work will be discussed further in Chapter 6. Chemotaxis in the RAW264 mouse macrophage cell line was found to be inhibited by cholera toxin in a cAMP-independent manner. Further, in the absence of guanine nucleotides, this toxin catalysed the *in vitro* [32P]ADP-ribosylation of an approximately 40kDa substrate that co-migrated with a pertussis toxin substrate in RAW264 membranes (Aksamit *et al.*, 1985). Such an *in vitro* [32P]ADP-ribosylation reaction was also demonstrated in membranes of human PMNs, undifferentiated HL-60 and U937 cell lines and human monocytes, but interestingly not in erythrocyte or bovine brain membranes. Again this [32P]ADP-ribosylation was only observed when the reaction was performed in the absence of GTP (Verghese *et al.*, 1986).

Adapting this assay, Gierschik and Jakobs demonstrated that this effect of cholera toxin could be enhanced upon agonist stimulation. Using membranes from DMSO-differentiated HL-60 cells they also observed that, in the absence of guanine nucleotides, there was a significant cholera toxin-dependent [32P]ADP-ribosylation of a 40kDa substrate even in the absence of fMLP. However, fMLP markedly stimulated cholera toxin-dependent [32P]ADP-ribosylation of this protein and this

was functionally correlated with a marked inhibition of fMLP-stimulated GTPase activity. There was a significant decrease in basal and an almost complete loss of fMLP-stimulated ADP-ribosylation of the 40kDa protein in membranes from cells pre-treated with pertussis toxin. These observations implicated the 40kDa protein as a substrate for both toxins in HL-60 membranes (Gierschik & Jakobs, 1987). The authors suggested that the pertussis toxin sensitivity of the fMLP-stimulated phospholipase C activity in these cells implicated this 40kDa protein as a potential coupling candidate. This is perhaps too simplistic in view of the current knowledge of the variety of pertussis toxin-sensitive effector systems with which the activated fMLP receptor interacts. However, as the authors suggest, this reaction does appear to provide a means of determining which G-protein couples to a given receptor within the native plasma membrane.

The agonist-induced enhancement of cholera toxin-catalysed ADP-ribosylation of what are "classically" considered as pertussis toxin substrates may be explained by the cycle of G-protein activation and deactivation (for more detail refer to 1.5.2.). Performing the ribosylation reactions in the presence of GTP which undergoes hydrolysis to GDP, the Gα subunits are most likely to be in the Gα-GDP state. Agonist occupancy of a receptor promotes a conformational change in the G-protein α subunit which reduces its affinity for GDP. This serves to increase the rate of release of GDP and therefore enhance the rate of binding of the activating guanine nucleotide, GTP (Gilman, 1987). However, if the cholera toxin-catalysed [^{32}P]ADP-ribosylation reaction is performed upon well-washed membranes in the presence of agonist, but in the absence of exogenously added GTP, the agonist-stimulated release of GDP will not be replaced with GTP. This will result in an α subunit devoid of bound guanine nucleotide and in which an arginine residue may become an accessible site for cholera toxin-catalysed ADP-ribosylation.

Since these initial studies the ability of agonists, in the absence of

exogenous GTP, to stimulate the cholera toxin-catalysed [^{32}P]ADP-ribosylation of "classical" pertussis toxin substrates has been observed in membranes of the neuroblastoma x glioma hybrid cell line, NG108-15 cell line stimulated with opiate peptides and those of the C6BU1 glial cell line stimulated with calf serum (Milligan & McKenzie, 1988; Milligan, 1989).

As mentioned previously Verghese and co-workers did demonstrate that in non-differentiated U937 cell membranes, in the absence of exogenous GTP, cholera toxin-catalysed [^{32}P]ADP-ribosylation of 40kDa protein that co-migrated with a pertussis toxin substrate in these cells (Verghese *et al.*, 1986). The work presented in this chapter aimed to extend these observations by investigating whether fMLP stimulated this cholera toxin-catalysed ADP-ribosylation reaction. The fact that responsiveness to this chemotactic peptide is dependent on maturation of these cells made it pertinent to examine whether there was any difference in the ability of DMSO-differentiated cells to support such an fMLP-stimulated ribosylation reaction relative to the non-differentiated, immature U937 cells. With the knowledge that the known pertussis toxin substrates in this cell were $G_{i}2\alpha$ and $G_{i}3\alpha$, a further aim was to use electrophoretic conditions that resolve these substrates and discriminatory antipeptide antisera to identify which particular $G_{i}\alpha$ subtype was covalently modified by cholera toxin and hence identify the G-protein(s) interacting with the fMLP receptor.

4.2. RESULTS.

A. CHOLERA TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Giα.

As previously described in Chapter 3, the treatment of both control and differentiated U937 cell membranes with thiol-activated toxins, in the presence of [32P]NAD+ and added guanine nucleotide (GTP), led to the incorporation of radioactivity into predominantly two polypeptides. Incorporation of radioactivity into other proteins, in particular those of approximately 25kDa, occurred in a toxin-independent manner in both control and differentiated membranes (lanes 1 and 2, Fig. 4.1). However, toxin-dependent [32P]ADP-ribosylation of an approximately 18kDa protein may represent auto-ADP-ribosylation of the A1 subunit of cholera toxin itself (lanes 5, 6 and 9-12, Fig. 4.1).

The substrate for pertussis toxin displayed an apparent molecular weight of approximately 40kDa, whereas the cholera toxin substrate was approximately 42kDa in molecular weight. From the immunological approaches discussed in Chapter 3 these substrates represent the pertussis toxin substrates $G_i2\alpha$ and $G_i3\alpha$ and the cholera toxin substrate $G_s\alpha$42kDa. The increased expression of these substrates is again evident upon differentiation as was discussed in Chapter 3. Further, in the presence of GTP, both cholera and pertussis toxin are apparently specific in their ability to ADP-ribosylate $G_s\alpha$ and in this cell line $G_i2\alpha/G_i3\alpha$, respectively (lanes 3-6, Fig. 4.1).

However, when the ribosylation reactions were performed in well-washed membranes from both control and differentiated U937 cells, in the absence of any exogenously added guanine nucleotides, an alternative ribosylation pattern emerged. Cholera toxin catalysed the incorporation of [32P]ADP-ribose into two polypeptides with apparent molecular weights of 42kDa and 40kDa, again with slightly enhanced incorporation of radioactivity into the polypeptides in differentiated relative to control cell membranes (lanes 11 and 12, Fig. 4.1). The

40kDa polypeptide modified by cholera toxin comigrated with the only apparent polypeptide to be specifically [32P]ADP-ribosylated by pertussis toxin in these membranes (compare lanes 11 and 12 with lanes 7 and 8, Fig. 4.1). In both control and differentiated U937 cell membranes addition of the chemotactic peptide, fMLP (10^{-5}M final), enhanced the cholera toxin-catalysed [32P]ADP-ribosylation of the 40kDa polypeptide relative to that observed upon addition of the vehicle for the peptide, DMSO, but did not significantly affect the incorporation of radioactivity into the 42kDa substrate (compare lanes 9 and 10 to lanes 11 and 12, Fig.4.1). The fMLP-stimulated, cholera toxin-catalysed [32P]ADP-ribosylation of the 40kDa polypeptide was more apparent in membranes from differentiated U937 cells relative to those from control U937 cells (compare lanes 9 and 10, Fig. 4.1).

This effect of fMLP was specific to cholera toxin as the chemotactic peptide had no effect upon pertussis toxin-catalysed [32P]ADP-ribosylation of its substrates, neither in the absence nor presence of GTP (lanes 1-6, Fig. 4.2).

Mimicking the ribosylation conditions which supported fMLP-stimulated cholera toxin-catalysed [32P]ADP-ribosylation of the 40kDa polypeptide, but without the addition of [32P]NAD+, fMLP nor its vehicle (DMSO) had any significant effect upon the expression of $G_s\alpha$44kDa and $G_s\alpha$42kDa in membranes of differentiated U937 cells (lanes 1-8, panel A, Fig. 4.3). Further indication that the 40kDa polypeptide [32P]ADP-ribosylated by cholera toxin was not a form of $G_s\alpha$ was evident from the observation that *in vivo* pre-treatment of differentiated U937 cells with cholera toxin prevented this toxin, in the absence of GTP and presence of fMLP, from catalysing an *in vitro* [32P]ADP-ribosylation of the 42kDa $G_s\alpha$ substrate but not a 40kDa polypeptide (lane 3, panel B, Fig. 4.3). This 40kDa polypeptide comigrated with a protein in non toxin-pretreated differentiated U937 cell membranes whose incorporation of radiolabel increased, albeit only slightly in this experiment, upon fMLP stimulation (lanes 1-3, panel B, Fig 4.3).

Further indication that the 40kDa substrate [32P]ADP-ribosylated by cholera toxin may be a pertussis toxin substrate was demonstrated by the effect of *in vivo*

pertussis toxin pretreatments upon [32P]ADP-ribosylation of this protein. This *in vivo* pre-treatment of intact cells completely abolishes the ability of pertussis toxin to catalyse an *in vitro* incorporation of [32P]ADP-ribose into its Giα substrates in differentiated U937 cell membranes (lane 3 relative to lane 2, panel A, Fig. 4.4). Further, this pretreatment prevented the cholera toxin-catalysed [32P]ADP-ribosylation of the approximately 40kDa polypeptide, but not the Gsα42kDa substrate in an experiment performed in the absence of GTP and presence of various concentrations of fMLP (panel B, Fig. 4.4).

B. IDENTIFICATION OF THE Giα SUBSTRATE(S) FOR CHOLERA TOXIN.

As demonstrated in Chapter 3, the known pertussis toxin substrates expressed in the U937 cell line are Gi2α and Gi3α. Under the SDS-PAGE conditions used in Figure 4.1., the cholera toxin-catalysed [32P]ADP-ribosylation of the 40kDa polypeptide could reflect incorporation of radioactivity into either or both of these Giα subunits. It was demonstrated in Chapter 3 that these subunits can be resolved by alkylation of the membrane proteins and altering the electrophoretic conditions. These approaches successfully resolved Gi2α and Gi3α, evident from the immunoblot in panel A, Figure 4.5. It was anticipated that by immunoblotting the toxin-catalysed [32P]ADP-ribosylated proteins under these "resolving" conditions that the polypeptide(s) incorporating radioactivity could be identified by overlaying the autoradiograph upon the immunoblot. This was possible for the pertussis toxin-catalysed [32P]ADP-ribosylation of Gi2α and Gi3α, with Gi2α incorporating a greater amount of radioactivity and displaying more mobility in the gel matrix relative to Gi3α (lanes 1 and 3, panels A and B, Fig. 4.5). The chemotactic peptide, fMLP, stimulated cholera toxin-catalysed [32P]ADP-ribosylation of a polypeptide, tentatively identifiable as Gi2α by virtue of its co-migration with pertussis toxin-catalysed [32P]ADP-ribosylated Gi2α (lanes 5-11, panels A and B, Fig. 4.5). However as cholera toxin-catalysed

incorporation of radioactivity into Giα was much less than that catalysed by pertussis toxin it was necessary to increase the exposure of the immunoblot accordingly. This decreased the resolution of the cholera toxin-catalysed [32P]ADP-ribosylated proteins, rendering the unambiguous identification of the Giα labelled in response to fMLP difficult (lanes 5-11, panel B, Fig. 4.5).

Prior to using an alternative approach aimed at definition of the 40kDa polypeptide [32P]ADP-ribosylated in response to fMLP various conditions were employed to attempt to increase the level of incorporation of radioactivity into this protein. Indeed on examining the effect of incubation period upon the fMLP-stimulated,cholera toxin-catalysed [32P]ADP-ribosylation of the 40kDa protein, the radioactivity incorporated into this protein actually decreased with increasing incubation period at 37°C. Surprisingly, this was also evident for the cholera toxin-catalysed [32P]ADP-ribosylation of the Gsα42kDa substrate, despite the anticipated increase in the level of toxin-independent incorporation of radioactivity into lower molecular weight proteins with increasing incubation period (lanes 7-12, Fig. 4.6). Further, a doublet of [32P]-labelled proteins was observed upon pertussis toxin-catalysed [32P]ADP-ribosylation of membranes in the presence of GTP, under SDS-PAGE conditions which should not resolve the known substrates for this toxin (lane 2, Fig. 4.6). These were the initial indications of the proteolytic problems previously described in detail in Chapter 3. The membrane preparation was thus altered to combat this problem as previously described in Chapter 3. Indeed even using membranes prepared in this manner performing the subsequent ribosylation reactions in the presence of protease inhibitors prevented any further degradation that occurred over the incubation at 37°C (lanes 3, 5, 7 and 9 as compared to lanes 4, 6, 8 and 10 in Fig. 4.7).

C. IMMUNOPRECIPITATION OF THE Giα SUBSTRATE(S) FOR CHOLERA TOXIN.

An alternative approach aimed at identification of the 40kDa polypeptide

ribosylated by cholera toxin in response to fMLP was to attempt to immunoprecipitate this protein with antipeptide antisera specific for each of $G_{i}2\alpha$ and $G_{i}3\alpha$. The concentration of antipeptide antiserum required to maximally immunoprecipitate these substrates was assessed by titrating the antipeptide antisera against a constant amount of pertussis toxin-catalysed [^{32}P]ADP-ribosylated membranes from 72 hour DMSO (1.25% v/v)-differentiated U937 cells. Pre-immune whole rabbit serum did not immunoprecipitate any [^{32}P]ADP-ribosylated protein from the differentiated U937 cell membranes (lane 3, panel B, Fig. 4.8). Addition of 20μl of whole serum from antipeptide antiserum SG1, specific for $G_{i}2\alpha$ in these cells, removed the maximum amount of substrate from the membrane, as assessed from the least amount of non-immunoprecipitated pertussis toxin-catalysed [^{32}P]ADP-ribosylated substrate remaining after this treatment (lane 9, panel A, Fig. 4.8). However, greater recovery of $G_{i}2\alpha$ was observed after addition of 5μl of whole serum from SG1, despite relatively more [^{32}P]ADP-ribosylated substrate remaining as non-immunoprecipitated material (lane 7, panel B and compare lanes 7 and 9, panel A, Fig. 4.8). This was apparent despite efforts to recover more immunoprecipitated $G_{i}2\alpha$ by varying the final wash and extraction conditions (results not shown). $G_{i}3\alpha$ was immunoprecipitated in a specific manner from the differentiated U937 cell membranes. Using the antipeptide antiserum I3B, 10μl of whole serum immunoprecipitated the maximum amount of $G_{i}3\alpha$ (lane 5, panel B, Fig. 4.9). Again, from the relative levels of [^{32}P]ADP-ribosylated, non-immunoprecipitated substrate it was observed that maximal recovery of $G_{i}3\alpha$ did not correspond exactly to the lowest relative level of non-immunoprecipitated substrate remaining (compare lane 7, panel A to lane 8, panel B, Fig. 4.9). Altering the recovery procedures did not improve the yield of $G_{i}3\alpha$ from the sample immunoprecipitated with addition of 15μl of the antipeptide antiserum, I3B (results not shown). The choice of the optimal concentrations of antipeptide antisera used to immunoprecipitate the $G_{i}\alpha$ polypeptides was thus determined from both the

maximal recovery of the substrate and the minimisation of residual non-immunoprecipitated pertussis toxin-catalysed [^{32}P]ADP-ribosylated substrate. Using these estimations and after conducting an fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of the 40kDa polypeptide in differentiated U937 cell membranes, the [^{32}P]ADP-ribosylated proteins were immunoprecipitated with the antipeptide antiserum, SG1. In the autoradiograph of the non-immunoprecipitated [^{32}P]ADP-ribosylated proteins the anticipated increased incorporation of radioactivity into a polypeptide co-migrating with $G_i\alpha$, with increasing concentrations of fMLP, was evident, although not as apparent as had been previously observed (lanes 3-9, panel A, Fig. 4.10). Upon immunoprecipitation of these samples with an antipeptide antiserum which is specific for $G_i2\alpha$ in these cells, [^{32}P]-labelled $G_i2\alpha$, but not $G_s\alpha$42kDa, was recovered from both the pertussis toxin-catalysed [^{32}P]ADP-ribosylated sample and the samples [^{32}P]ADP-ribosylated by cholera toxin. However although cholera toxin-catalysed [^{32}P]ADP-ribosylated $G_i2\alpha$ was specifically immunoprecipitated the protein was not recovered in a dose-dependent manner. Indeed the sample stimulated with DMSO yielded a comparable amount of immunoprecipitated $G_i2\alpha$ relative to that recovered from the fMLP-stimulated samples (lanes 2-9, panel B, Fig. 4.10).

In a repeat of this experiment, but using the $G_i3\alpha$ specific antipeptide antiserum I3B, a pertussis toxin-catalysed [^{32}P]ADP-ribosylated protein was immunoprecipitated, however no fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylated substrate was recovered (results not shown). These observations may reflect a lower level of the $G_i3\alpha$ substrate expressed in the cell membranes and thus available for [^{32}P]ADP-ribosylation catalysed by cholera toxin. Thus prior to concluding that the fMLP receptor did not interact with this protein several approaches were employed to improve the level of [^{32}P] labelling of the 40kDa polypeptide in response to the chemotactic peptide and ensure that the lack of immunoprecipitation of $G_i3\alpha$ was not merely a reflection of assay

sensitivity.

D. APPROACHES TO IMPROVE THE LEVEL OF CHOLERA TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Giα.

Studies of the fMLP receptor described later in Chapter 5 demonstrated that addition of a further three protease inhibitors to the ligand binding assay buffer prevented loss of binding activity. These inhibitors were then included in the cell harvesting and membrane preparation procedures to ensure that lack of fMLP-stimulated cholera toxin-catalysed [32P]ADP-ribosylation of Giα was not a reflection of low peptide binding activity.

It was observed that when cholera toxin-catalysed [32P]ADP-ribosylation of Gsα was at a high level, then this toxins ability to catalyse the incorporation of [32P]ADP-ribose into Giα was improved. Thus a variety of approaches were employed to maximise the cholera toxin-catalysed ribosylation of Gsα. Perhaps the most obvious limitation upon the assay was that the concentration of cholera toxin may have been limiting the reaction. In experiments not included increasing the cholera toxin concentration four fold did not result in any significant increase in the incorporation of [32P]ADP-ribose into Gsα.

Inclusion of the magnesium chloride in the cholera toxin-catalysed [32P]ADP-ribosylation assay significantly increased the level of incorporation of radioactivity into the Gsα substrates, with the 44kDa form of Gsα incorporating [32P]ADP-ribose under these conditions. The level of incorporation of radioactivity into the 40kDa polypeptide did increase significantly, although these conditions removed the agonist-dependency of the [32P] labelling reaction which was evident in the absence of the salt (lanes 1-15, Fig. 4.11).

Similarly, a variety of different guanine nucleotides and their non-hydrolysable analogues increased the level of incorporation of radioactivity into the Gsα proteins but abolished the ability of fMLP to stimulate cholera toxin-catalysed [32P]ADP-ribosylation of a 40kDa polypeptide (lanes 7-12, Fig. 4.12). However

in two different membrane preparations examined, fMLP did not stimulate a cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{i}\alpha$, even in the absence of GTP (lanes 3-6, Fig. 4.12).

As anticipated, increasing the unlabelled NAD^{+} concentration in the ribosylation assay decreased the ability of cholera toxin to catalyse a [^{32}P]ADP-ribosylation of both $G_{s}\alpha$42kDa and the 40kDa polypeptide [^{32}P]ADP-ribosylated in response to fMLP. Interestingly, inhibition of $G_{s}\alpha$ [^{32}P]ADP-ribosylation occurred at lower NAD^{+} concentrations than inhibition of $G_{i}\alpha$ [^{32}P]ADP-ribosylation by cholera toxin (lanes 1-15, Fig. 4.13).

Testing the same stock of fMLP, it was found to significantly stimulate the cholera toxin-catalysed [^{32}P]ADP-ribosylation of a polypeptide that comigrated with the pertussis toxin substrate(s) in membranes prepared from dibutryl cAMP-differentiated HL-60 cells (lanes 3, 5 and 7 relative to lanes 2, 4 and 6,respectively, panel A, Fig. 4.14). Upon immunoprecipitation of these samples with antipeptide antisera that specifically recognise $G_{s}\alpha$, $G_{i}2\alpha$ and $G_{i}3\alpha$ in these cells, a number of cholera toxin-catalysed [^{32}P]ADP-ribosylated proteins were recovered. As anticipated the 42kDa form of $G_{s}\alpha$ was immunoprecipitated (lanes 2 and 3, panel B, Fig. 4.14). Further cholera toxin-catalysed [^{32}P]ADP-ribosylated $G_{i}2\alpha$ was specifically immunoprecipitated from the sample stimulated with fMLP (lane 5, panel B, Fig. 4.14). An extremely low level of $G_{i}3\alpha$, which upon fMLP stimulation had been [^{32}P]ADP-ribosylated by cholera toxin, was also immunoprecipitated (lane 7, panel B, Fig. 4.14). These immunoprecipitated, cholera toxin-catalysed [^{32}P]ADP-ribosylated $G_{i}\alpha$ substrates did comigrate with the $G_{i}\alpha$ species [^{32}P]ADP-ribosylated by pertussis toxin in these cell membranes (lanes 5 and 7 as compared with lanes 1 and 8, respectively, panel B, Fig. 4.14).

Figure 4.1.

fMLP-STIMULATED CHOLERA TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF A 40kDa SUBSTRATE IN U937 CELL MEMBRANES.

Lanes 1, 3, 5, 7, 9 and 11 represent control U937 cell membranes (50μg/lane), denoted as "C". Lanes 2, 4, 6, 8, 10 and 12 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane), denoted as "D". These membranes were prepared as in 2.3.A. The cells were treated with DMSO as described in 2.2.3.

Toxin-catalysed [^{32}P]ADP-ribosylations were performed as described in 2.11, with a 2 hour incubation period. Ribosylation reactions in lanes 1-6 were performed in the presence of GTP, denoted as "+GTP", whereas those in lanes 7-12 were performed in the absence of GTP, denoted as "-GTP". In lanes 1 and 2, the [^{32}P]ADP-ribosylations were performed in the absence of bacterial toxin. Pertussis toxin-catalysed the [^{32}P]ADP-ribosylation reactions in lanes 3, 4, 7 and 8, denoted as "PTx", whereas cholera toxin catalysed the [^{32}P]ADP-ribosylation reactions performed in lanes 5, 6 and 9-12, denoted as "CTx". In lanes 9 and 10, fMLP (10^{-5}M final) was added at the initiation of the incubation. fMLP was dissolved in DMSO and this vehicle (0.1% v/v final) was added at the initiation of the incubation in lanes 11 and 12.

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7. The dried gel was autoradiographed for 13 days as described in 2.9. This experiment is representative of three others, performed with different membrane preparations, which gave essentially identical results.

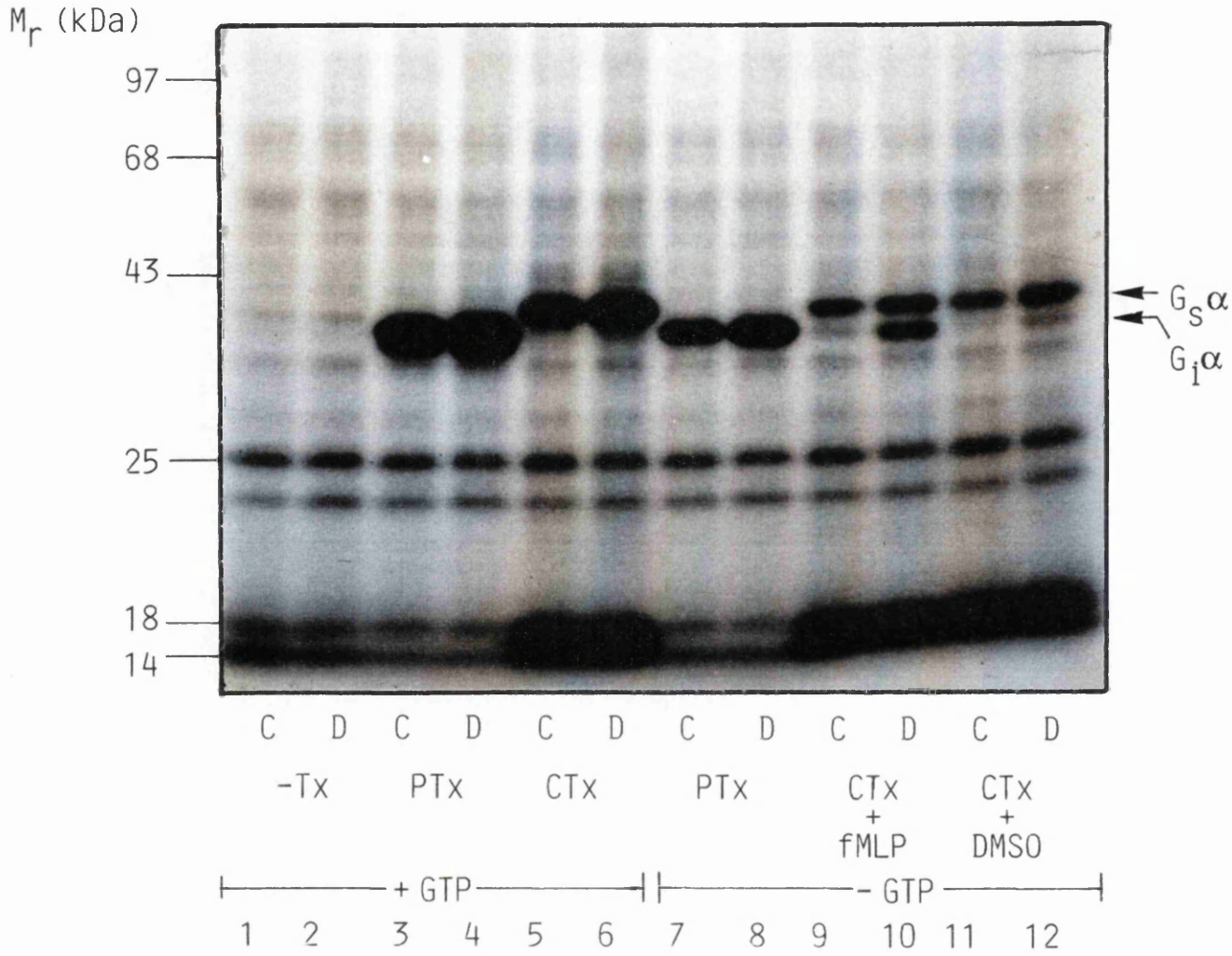

M_r (kDa)
97
68
43
25
18
14
$G_s\alpha$
$G_i\alpha$
C D C D C D C D C D C D C D
-Tx
PTx
CTx
PTx
CTx + fMLP
CTx + DMSO
+ GTP
- GTP
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4.2.

THE EFFECT OF fMLP STIMULATION ON THE PERTUSSIS TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Giα IN DIFFERENTIATED U937 CELL MEMBRANES.

Lanes 1-6 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane). These membranes were prepared as in 2.3.A. The cells were treated with DMSO as described in 2.2.3.

Pertussis toxin-catalysed [^{32}P]ADP-ribosylations were performed as described in 2.11., with a 2 hour incubation period. Ribosylation reactions in lanes 1-3 were performed in the presence of GTP, whereas those in lanes 4-6 were performed in the absence of GTP. Lanes 1 and 4 were incubated in the absence of either agonist or vehicle. Lanes 2 and 5 were incubated in the presence of the vehicle, DMSO (0.1% v/v final), as a control for fMLP (10^{-5}M final) which was included in lanes 3 and 6.

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7. The dried gel was autoradiographed for 6 hours, as described in 2.9. This experiment is representative of two experiments performed with different membrane preparations.

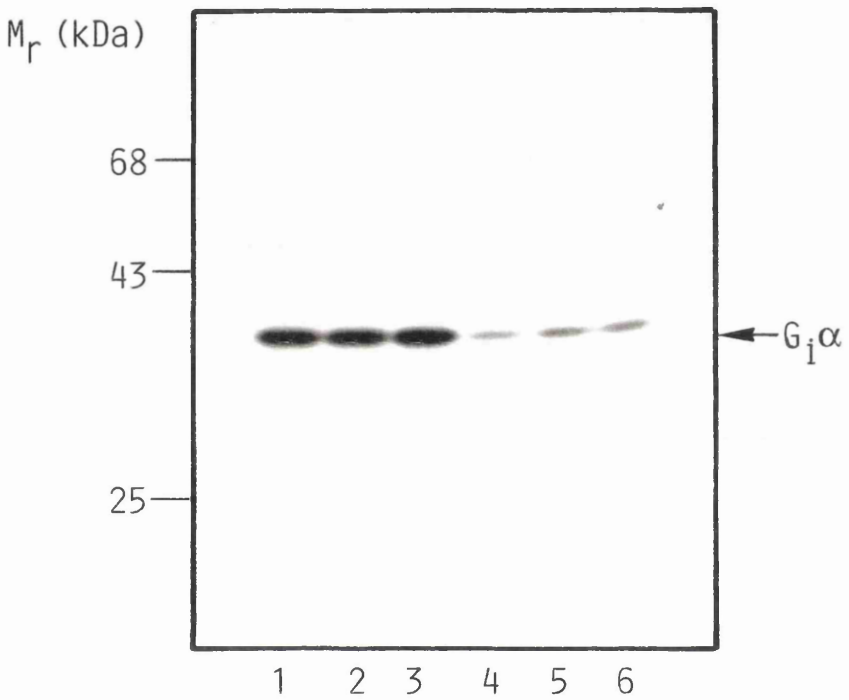

M_r (kDa)
68
43
25
$G_i\alpha$
1 2 3 4 5 6

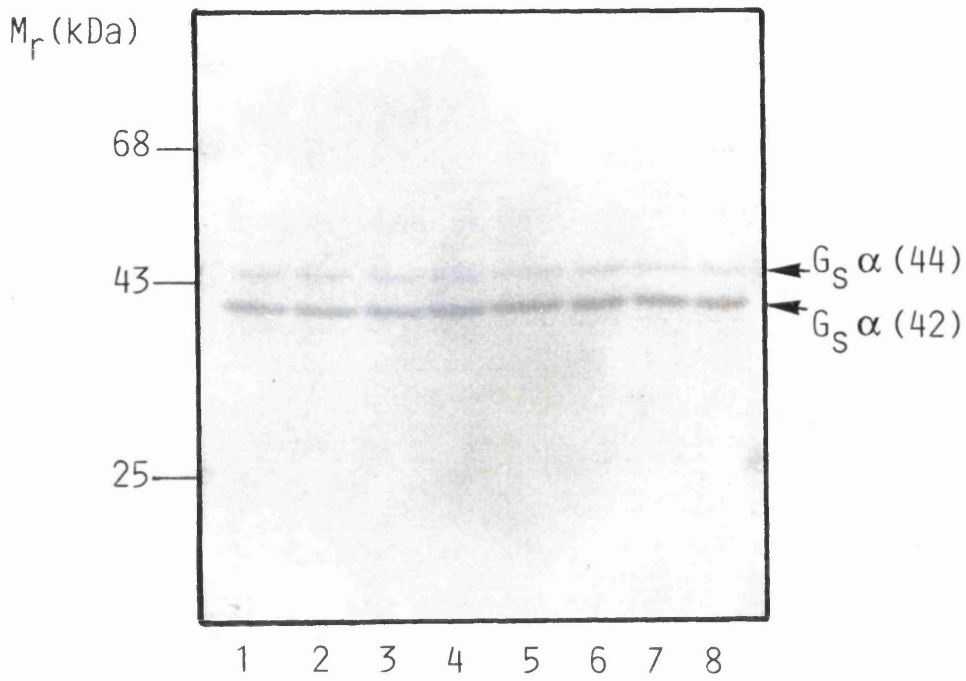
Figure 4.3.

INVESTIGATION AS TO WHETHER THE 40kDa POLYPEPTIDE RIBOSYLATED BY CHOLERA TOXIN UPON fMLP STIMULATION WAS A FORM OF Gsα.

In panel A, membranes (50μg/lane) from 72 hour DMSO (1.25% v/v)-differentiated cells were prepared as in 2.3.A. The cells were treated with DMSO as described in 2.2.3. These membranes were from the preparation used previously in the experiment represented by Figure 4.1. Cholera toxin-catalysed [^{32}P]ADP-ribosylation reactions were incubated for 2 hours and performed as described in 2.11., but with the exclusion of [^{32}P]NAD^{+}. The chemotactic peptide, fMLP, was included at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11}M final in lanes 2-8, respectively. The vehicle, DMSO (0.1% v/v final), was included in lane 1 at an equivalent concentration to the maximal fMLP concentration tested. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and then immunoblotted as described in 2.10.2.B. The primary antiserum (CS1) was used at a 1: 200 dilution.

In panel B, lanes 1 and 2 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes and lane 3 represents membranes from 72 hour DMSO (1.25% v/v)-differentiated U937 cells pretreated *in vivo* for 16 hours with cholera toxin (100ng/ml final) as outlined in 2.2.5. The membranes (50μg/lane) were prepared as in 2.3.A. Cholera toxin-catalysed [^{32}P]ADP-ribosylation was performed in the absence of GTP as in 2.11., with a 2 hour incubation. In lanes 2 and 3, fMLP(10^{-5}M final) was included during the incubation, whereas the vehicle, DMSO (0.1% v/v), was included in the incubation represented by lane 1. The samples were resolved by SDS-PAGE as described for panel A and the dried gel autoradiographed for 72 hours as in 2.9. Both panels are representative of two experiments which gave essentially identical results.

A


M_r (kDa)
68
43
25
$G_s\alpha$ (44)
$G_s\alpha$ (42)
1 2 3 4 5 6 7 8

B

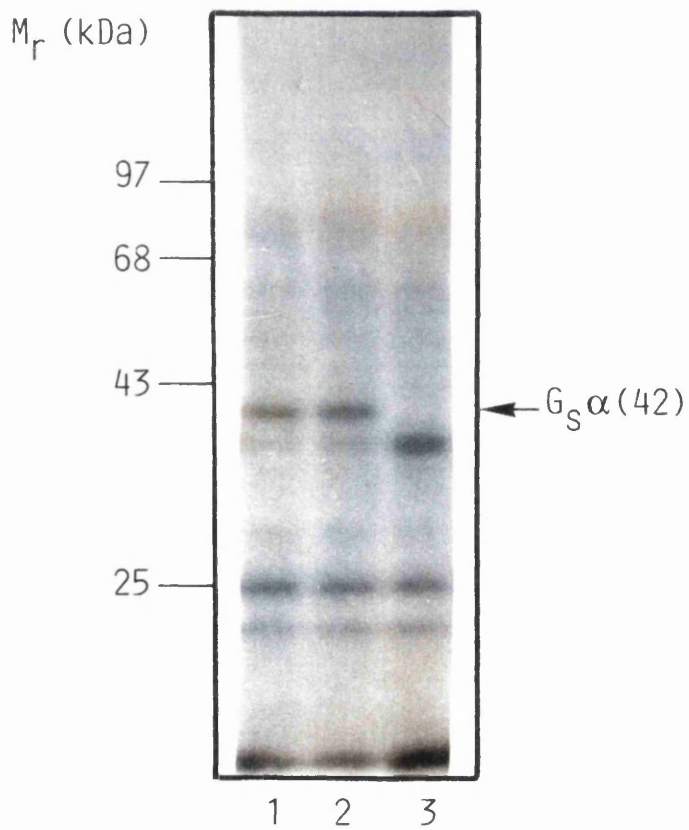

M_r (kDa)
97
68
43
25
$G_s\alpha$ (42)
1 2 3

Figure 4.4.

THE EFFECT OF *IN VIVO* PERTUSSIS TOXIN PRETREATMENT UPON THE *IN VITRO* TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Giα IN U937 CELL MEMBRANES.

Panel A represents a pertussis toxin-catalysed [32P]ADP-ribosylation reaction performed as in 2.11, in the presence of GTP and with a 2 hour incubation period. Toxin was omitted from the reaction in lane 1. Lanes 1 and 2 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane). Lane 3 represents membranes (50μg), from 72 hour DMSO (1.25% v/v)-differentiated U937 cells pre-treated *in vivo* with pertussis toxin (50ng/ml, 16 hours), prepared as in 2.3.A. The cells were treated with DMSO as described in 2.2.3. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and the dried gel autoradiographed for 4 days as in 2.9. This experiment is representative of three others which gave essentially identical results.

Panel B represents toxin-catalysed [32P]ADP-ribosylation reactions as performed in 2.11., with a 2 hour incubation. Lanes 1-13 represent membranes (50μg/lane) from the same preparation as in lane 3, panel A. The reaction was performed in the presence of GTP in lanes 1-3 and absence of GTP in lanes 4-13. Lane 1 represents the [32P]ADP-ribosylation reaction performed in the absence of bacterial toxin. Pertussis toxin catalysed the [32P]ADP-ribosylation reaction in lanes 2 and 4, whereas cholera toxin was included in lanes 3 and 5-13. fMLP was added at the initiation of the incubation at concentrations of 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M final, in lanes 6-13, respectively. DMSO (0.1% v/v), was included in lanes 4 and 5. The samples were resolved as in panel A and the gel autoradiographed for 8 days as in 2.9. Molecular weight standards were included in the experiment but were excluded for clarity. This experiment was performed twice with essentially identical results.

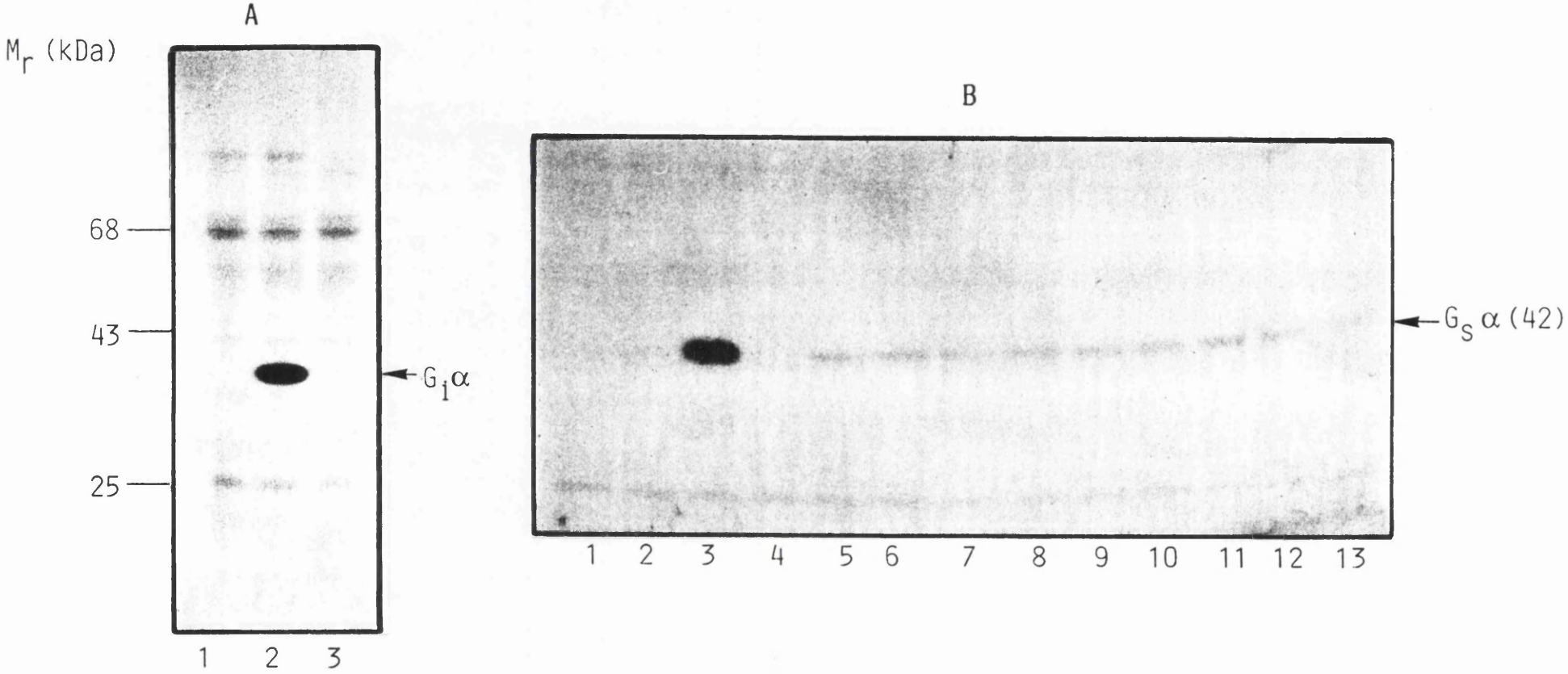

A
M_r (kDa)
68
43
25
$G_i\alpha$
1 2 3
B
$G_s\alpha$ (42)
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.5.

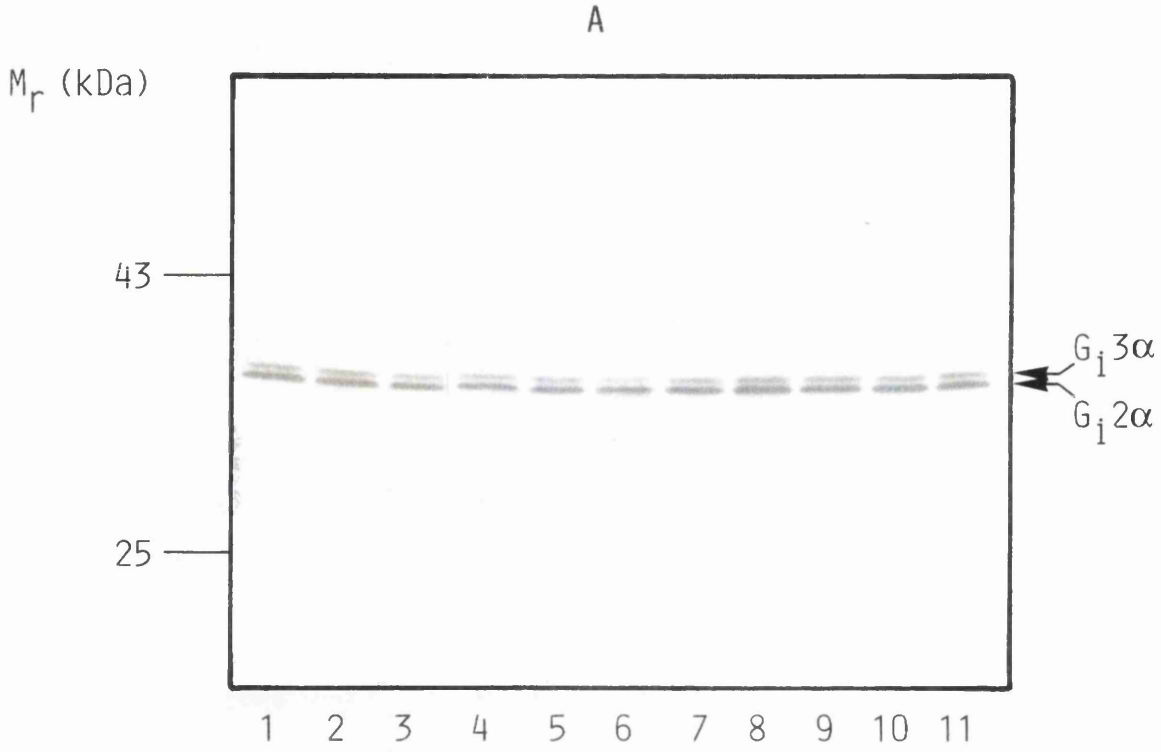
fMLP-STIMULATED CHOLERA TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Giα IN DIFFERENTIATED U937 CELL MEMBRANES - SEPARATION OF Gi2α AND Gi3α.

In both panels A and B, lanes 1-11 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (100μg/lane) prepared as in 2.3.A. The cells were treated with DMSO as described in 2.2.3. Toxin-catalysed [^{32}P]ADP-ribosylation reactions were performed as in 2.11, with a 2 hour incubation period. GTP was included in the assay mix for lanes 1 and 2, denoted as "+GTP" in panel B, but was excluded from the assays performed in lanes 3-11, denoted as "-GTP" in panel B. Lanes 1 and 3 represent the [^{32}P]ADP-ribosylation reactions performed in the presence of pertussis toxin, denoted as "PTx" in panel B. In lanes 2 and 4-11 the [^{32}P]ADP-ribosylation reactions were performed in the presence of cholera toxin, denoted as "CTx" in panel B. fMLP at 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M final was included at the initiation of the incubation in lanes 5-11, respectively and DMSO (0.1% v/v) was included in lane 4, as denoted in panel B.

After completion of the ribosylation reactions the samples were alkylated and resolved by SDS-PAGE separation (12.5% acrylamide, 0.06% bisacrylamide, 20cm x 20cm resolving gel) as outlined in 2.7.3.B and 2.7.1, respectively.

Panel A represents an immunoblot of this gel performed as in 2.10.2. The primary antiserum (1: 200 dilution) was a mixture of antipeptide antisera specific for Gi2α and Gi3α, namely LE2 and I3B, respectively.

Panel B represents an autoradiograph of this immunoblot. Lanes 1-3 represent a 5 day exposure, whereas lanes 4-11 represent a 14 day exposure both performed as described in 2.9. This experiment is representative of two others which gave essentially identical results.


A
M_r (kDa)
43
25
$G_i3\alpha$
$G_i2\alpha$
1 2 3 4 5 6 7 8 9 10 11

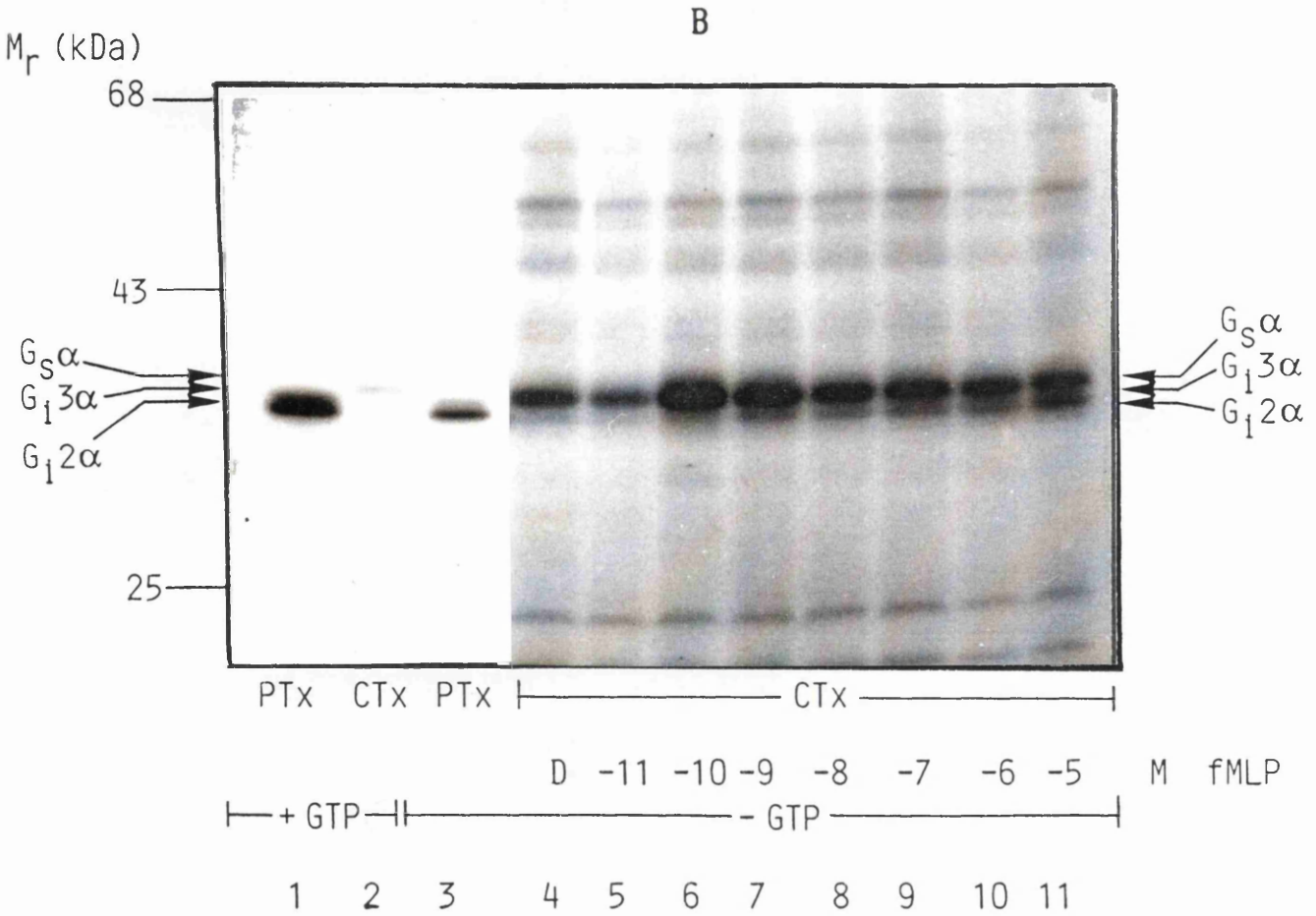

B
M_r (kDa)
68
43
25
$G_s\alpha$
$G_i3\alpha$
$G_i2\alpha$
PTx CTx PTx
CTx
D -11 -10 -9 -8 -7 -6 -5 M fMLP
+ GTP
- GTP
1 2 3 4 5 6 7 8 9 10 11

Figure 4.6.

fMLP-STIMULATED LABELLING OF Giα BY CHOLERA TOXIN - TIME COURSE OF THE REACTION INCUBATION PERIOD.

Lanes 1-12 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) prepared as in 2.3.A. The cells were treated with DMSO as described in 2.2.3.

Toxin-catalysed [^{32}P]ADP-ribosylation reactions were performed as outlined in 2.11., in the presence of GTP in lanes 1-3, denoted as "+GTP" and in the absence of GTP in lanes 4-12, denoted as "-GTP". In lane 1, the [^{32}P]ADP-ribosylation reaction was performed in the absence of bacterial toxin, denoted as "-Tx". Pertussis toxin catalysed the [^{32}P]ADP-ribosylation reactions in lanes 2 and 4, denoted as "PTx", whereas cholera toxin catalysed the [^{32}P]ADP-ribosylation reactions in lanes 3 and 5-12, denoted as "CTx". The peptide, fMLP (10^{-5}M final), was included at the initiation of the reaction in lanes 6-12 and the samples incubated for 0, 20, 30, 45, 60, 90 and 120 minutes, respectively. In lane 5, the sample was incubated with the vehicle, DMSO (0.1% v/v), for 120 minutes. The samples in lanes 1-4 were incubated for 120 minutes.

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and the dried gel autoradiographed for 8 days, as described in 2.7 and 2.9, respectively. This experiment was performed twice with different membrane preparations.

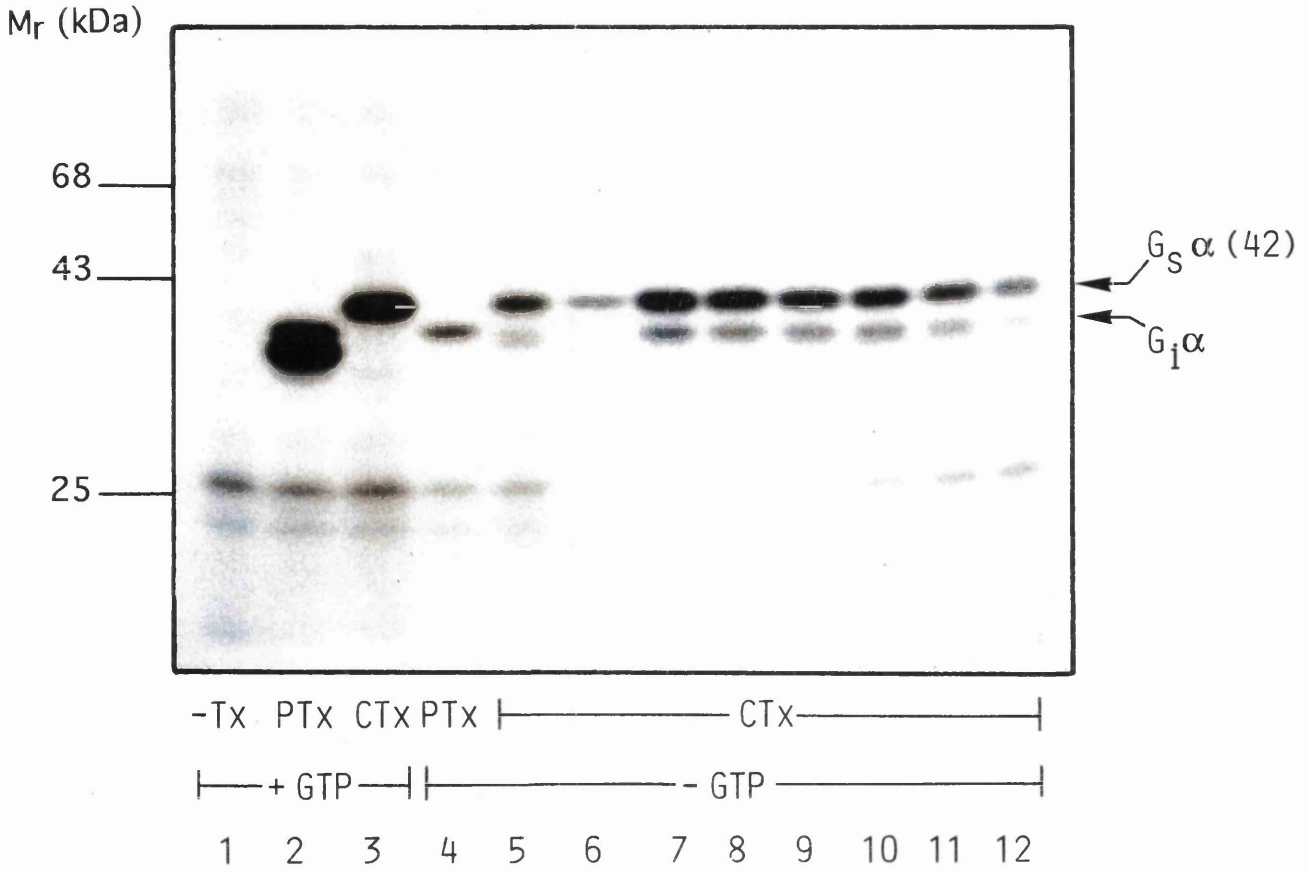

Mr (kDa)
68
43
25
$G_s\alpha$ (42)
$G_i\alpha$
-Tx PTx CTx PTx CTx
+ GTP
- GTP
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4.7.

THE EFFECT OF PROTEASE INHIBITORS ON THE TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Gα SUBUNITS.

Lanes 1-10 represent membranes (50μg/lane) from 72 hour DMSO (1.25% v/v)-differentiated cells prepared as in 2.3.B. The cells were treated with DMSO as described in 2.2.3.

Toxin-catalysed [^{32}P]ADP-ribosylation reactions were performed as in 2.11., for a 2 hour incubation period, with lanes 1-6 performed in the presence of GTP, denoted as "+GTP" and lanes 7-10 performed in the absence of GTP, denoted as "-GTP". The reactions represented by lanes 1 and 2 were performed in the absence of bacterial toxin. Pertussis toxin-catalysed the ribosylation reaction performed in lanes 3, 4, 9 and 10, whereas cholera toxin was included in lanes 5-8. A "cocktail" of serine and cysteine protease inhibitors, equivalent to those used in the membrane preparation outlined in 2.3. B and at equivalent final concentrations, was included in the reaction buffer in samples represented by lanes 1, 3, 5, 7 and 9, denoted as "+". The reactions in lanes 2, 4, 6, 8 and 10 were performed in the absence of protease inhibitors during the incubation, denoted as "-".

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and the dried gel autoradiographed for 4 days, as described in 2.7 and 2.9, respectively. This experiment was performed once.

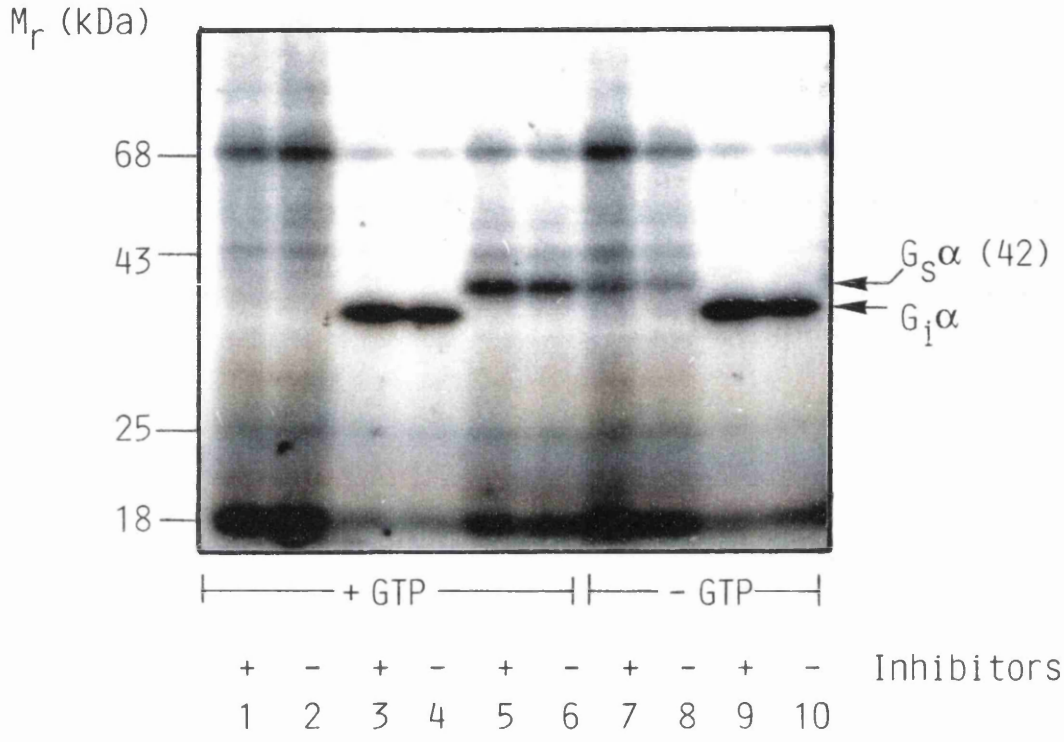

M_r (kDa)
68
43
25
18
$G_s\alpha$ (42)
$G_i\alpha$
+ GTP
- GTP
+ - + - + - + - + -
Inhibitors
1 2 3 4 5 6 7 8 9 10

Figure 4.8.

IMMUNOPRECIPITATION OF PERTUSSIS TOXIN-CATALYSED [32P]ADP-RIBOSYLATED Gi2α FROM DIFFERENTIATED U937 CELL MEMBRANES.

In panel A, lanes 1-10 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) prepared as outlined in 2.3.B. A pertussis toxin-catalysed [32P]ADP-ribosylation reaction was performed as in 2.11, in the presence of GTP and with a 2 hour incubation period. Bacterial toxin was omitted from the sample in lane 1 and pertussis toxin included in lanes 2-10. The protease inhibitors detailed in 2.2.6.B were included throughout the experiment. The [32P]ADP-ribosylated samples were immunoprecipitated as described in 2.12. The samples were incubated with 25μl of pre-immune rabbit whole serum in lane 2 and 0.1, 0.5, 1, 5, 10, 15, 20 and 25μl of the whole serum from the antipeptide antiserum SG1 in lanes 3-10, respectively. SG1 (25μl of whole serum) was included in lane 1. This panel represents the [32P]ADP-ribosylated substrate not immunoprecipitated by the antipeptide antiserum.

Panel B represents Gi2α specifically immunoprecipitated from the samples represented in panel A, with the exception of lane 2 which represents the pertussis toxin-catalysed [32P]ADP-ribosylation of 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg) which had not been immunoprecipitated. Lane 1 represents the immunoprecipitate from the sample ribosylated in the absence of toxin. Immunoprecipitated Gi2α from samples incubated with preimmune serum and 0.1, 0.5, 1, 5, 10, 15, 20 and 25μl of the whole serum from antipeptide antiserum SG1 are represented by lanes 3-11, respectively. Both panels represent autoradiographs of gels (10% acrylamide/0.25% bisacrylamide, as in 2.7) exposed for 4 days and are representative of one other experiment performed which gave identical results.

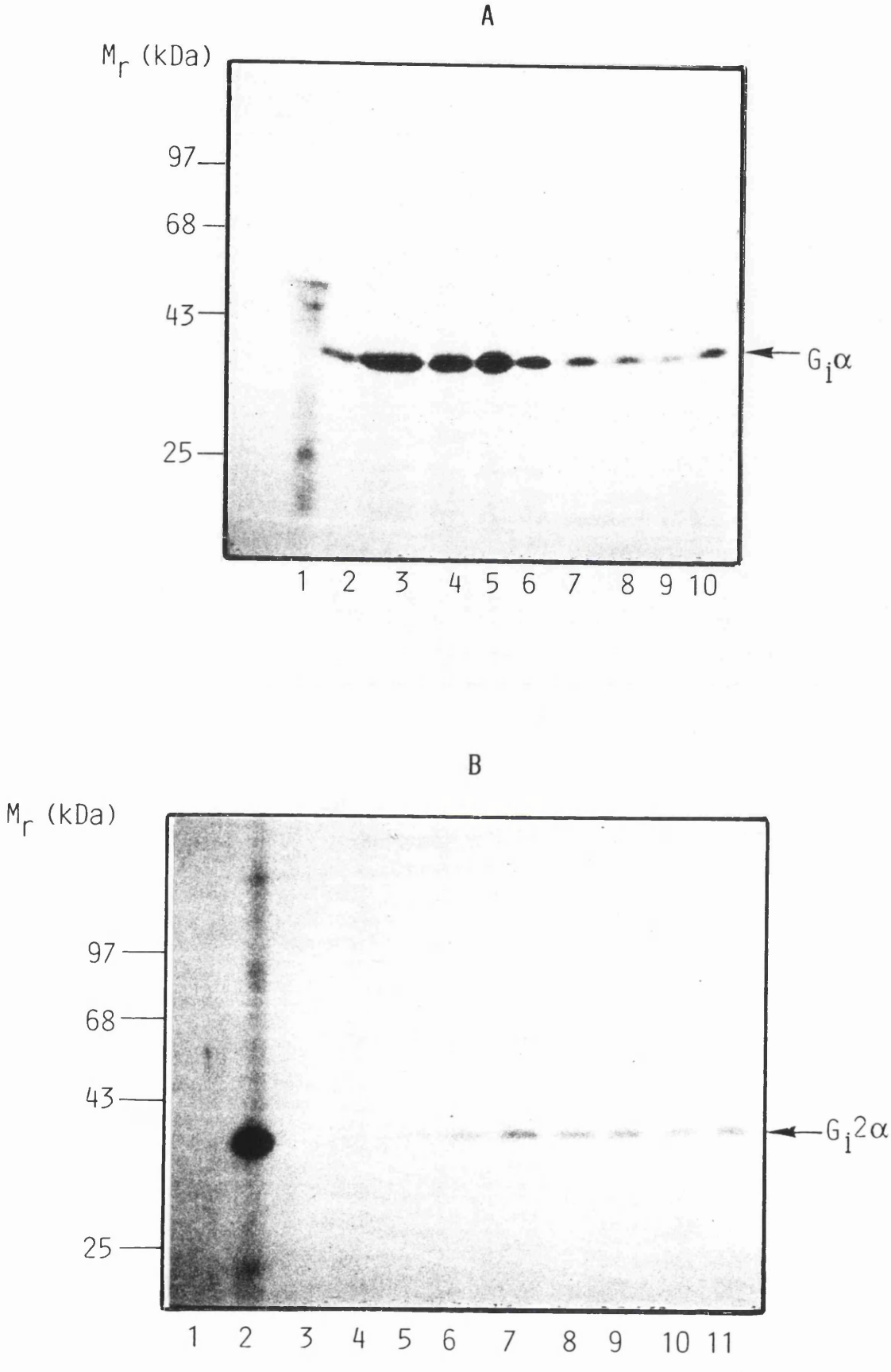

A
M_r (kDa)
97
68
43
25
$G_i\alpha$
1 2 3 4 5 6 7 8 9 10
B
M_r (kDa)
97
68
43
25
$G_i2\alpha$
1 2 3 4 5 6 7 8 9 10 11

Figure 4.9.

IMMUNOPRECIPITATION OF PERTUSSIS TOXIN-CATALYSED [32P]ADP-RIBOSYLATED Gi3α FROM DIFFERENTIATED U937 CELL MEMBRANES.

In panel A, lanes 1-9 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) prepared as outlined in 2.3.B. Upon completion of a 2 hour toxin-catalysed [32P]ADP-ribosylation reaction, performed as in 2.11 in the presence of GTP, an immunoprecipitation reaction was performed as in 2.12. Bacterial toxin was omitted from the sample in lane 1 and pertussis toxin included in lanes 2-9. The protease inhibitors detailed in 2.2.6.B were included throughout the experiment. After completion of the toxin-catalysed [32P]ADP-ribosylation reaction the samples were incubated with 25μl of pre-immune rabbit whole serum in lane 2 and 0.5, 1, 5, 10, 15, 20 and 25μl of whole serum from the antipeptide antiserum (I3B) in lanes 3-9, respectively. I3B (25μl of whole serum) was included in lane 1. This panel represents the [32P]ADP-ribosylated substrate not immunoprecipitated by the antipeptide antiserum.

Panel B represents Gi3α specifically immunoprecipitated from the samples represented in panel A, with the exception of lane 2 which represents the pertussis toxin-catalysed [32P]ADP-ribosylation of 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg), not immunoprecipitated. Lane 1 represents the immunoprecipitate from the sample ribosylated in the absence of toxin. Immunoprecipitated Gi3α from samples incubated with preimmune serum and 0.5, 10, 5, 1, 15, 20 and 25μl of the antipeptide antiserum I3B are represented by lanes 3-10, respectively. Lanes 5 and 7 were as indicated. Both panels represent autoradiographs of dried gels (10% acrylamide, 0.25% bisacrylamide SDS-PAGE performed as in 2.7) exposed for 10 days and are representative of one other experiment performed.

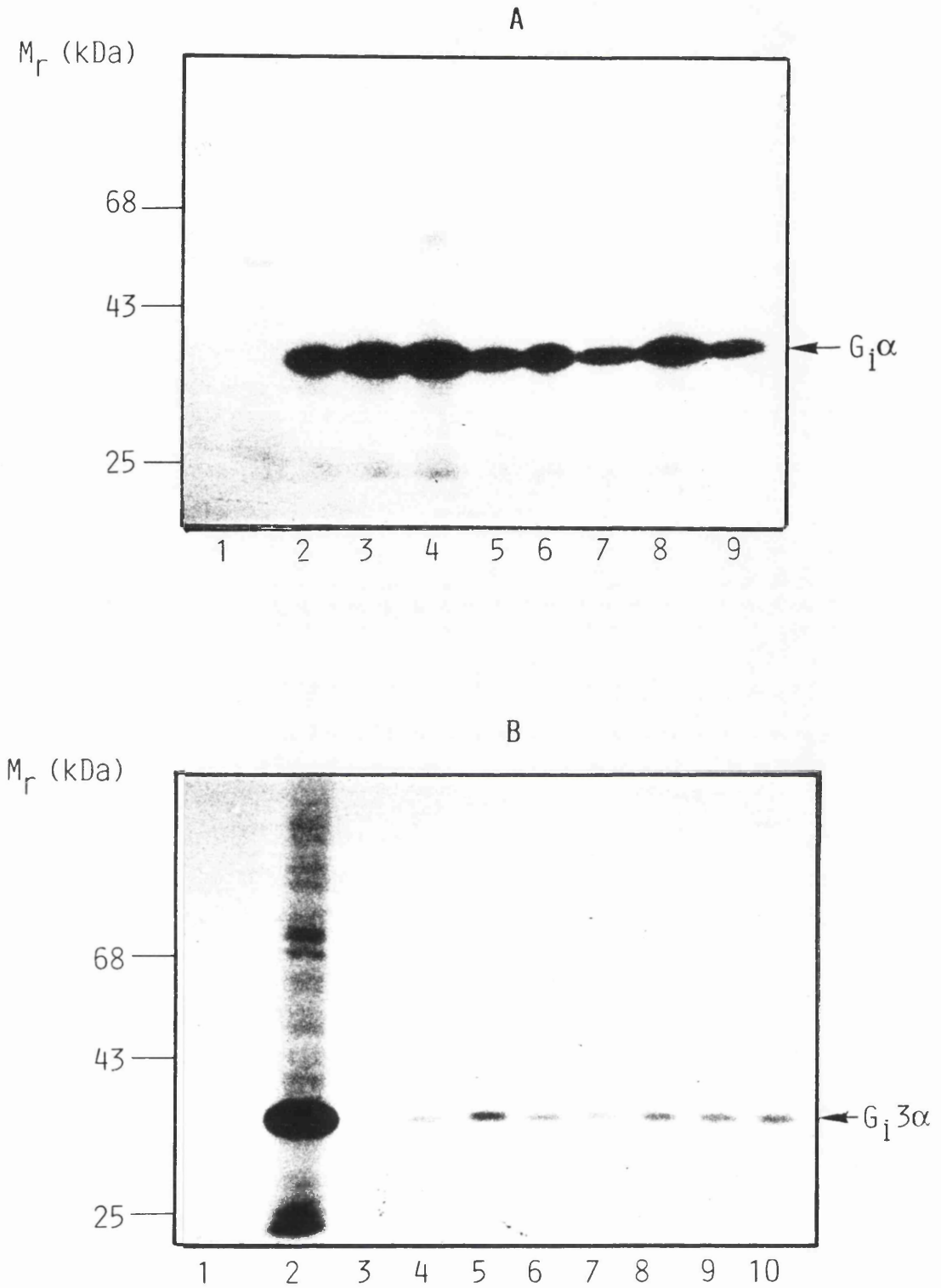

A
M_r (kDa)
68
43
25
$G_i\alpha$
1 2 3 4 5 6 7 8 9
B
M_r (kDa)
68
43
25
$G_i3\alpha$
1 2 3 4 5 6 7 8 9 10

Figure 4.10.

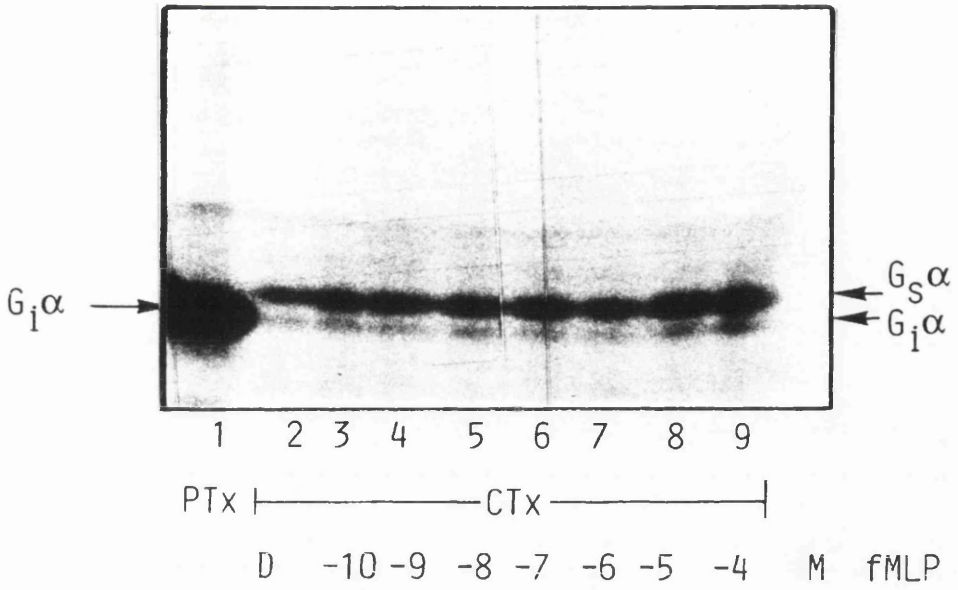
fMLP-STIMULATED [32P]ADP-RIBOSYLATION OF Giα BY CHOLERA TOXIN IN DIFFERENTIATED U937 CELL MEMBRANES - IMMUNOPRECIPITATION OF Gi2α.

Lanes 1-9 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) prepared as in 2.3.B. The cells were treated with DMSO as described in 2.2.3. Toxin-catalysed [^{32}P]ADP-ribosylation reactions were performed as detailed in 2.11., in the absence of GTP and the presence of the protease inhibitors described in 2.2.6.B, for a 2 hour incubation period. Lane 1 represents the [^{32}P]ADP-ribosylation reaction performed in the presence of pertussis toxin, denoted as "PTx", whereas lanes 2-9 represent the [^{32}P]ADP-ribosylation reactions performed in the presence of cholera toxin, denoted as "CTx". The chemotactic peptide, fMLP was added at the initiation of the incubation to final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M in lanes 3-9, respectively as indicated. The vehicle, DMSO (0.1% v/v) was added at the initiation of the incubation in lane 2, denoted as "D". Upon completion of the ribosylation reaction 15μl of whole serum, from the antipeptide antiserum SG1, was added to the samples and immunoprecipitation reactions performed as outlined in 2.12.

Panel A represents the toxin-catalysed [^{32}P]ADP-ribosylated substrates, Gsα and Giα, not immunoprecipitated with the antipeptide antiserum.

Panel B represents the toxin-catalysed [^{32}P]ADP-ribosylated Gi2α immunoprecipitates. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7. The dried gels were autoradiographed for 5 days in panel A and 9 days in panel B, as in 2.9. This experiment was performed once.

A


$G_i\alpha$
$G_s\alpha$
$G_i\alpha$
1 2 3 4 5 6 7 8 9
PTx
CTx
D -10 -9 -8 -7 -6 -5 -4 M fMLP

B

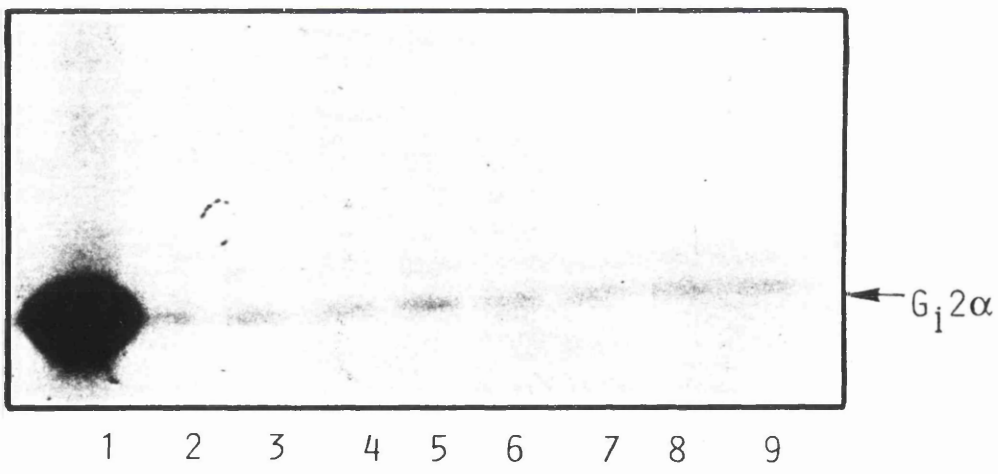

$G_i2\alpha$
1 2 3 4 5 6 7 8 9

Figure 4.11.

THE EFFECT OF $MgCl_2$ ON THE fMLP-STIMULATED CHOLERA TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Giα IN DIFFERENTIATED U937 CELL MEMBRANES.

Lanes 1-15 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) prepared as in 2.3.C. The cells were treated with DMSO as described in 2.2.3.

Cholera toxin-catalysed [^{32}P]ADP-ribosylation reactions were performed in the presence of the protease inhibitors described in 2.2.6.C, for a 2 hour incubation period, with slight modifications of the protocol in 2.11. GTP was included in the incubations for lanes 1, 4, 7, 10 and 13, but was omitted from the other samples. Magnesium chloride ($MgCl_2$) was included to a final concentration of 1mM, 2.5mM, 5.0mM and 10.0mM in lanes 4-6, 7-9, 10-12 and 13-15, respectively as indicated. Samples in lanes 1-3 were incubated in the absence of $MgCl_2$. A maximal fMLP concentration (10^{-5}M final) was added at the initiation of the incubation to samples in lanes 3, 6, 9, 12 and 15. The vehicle, DMSO (0.1% v/v final) was added at the initiation of the incubation to samples in lanes 2, 5, 8, 11 and 14.

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) and the dried gel autoradiographed for 6 days, as described in 2.7 and 2.9, respectively. This experiment is representative of two others which gave essentially identical results.

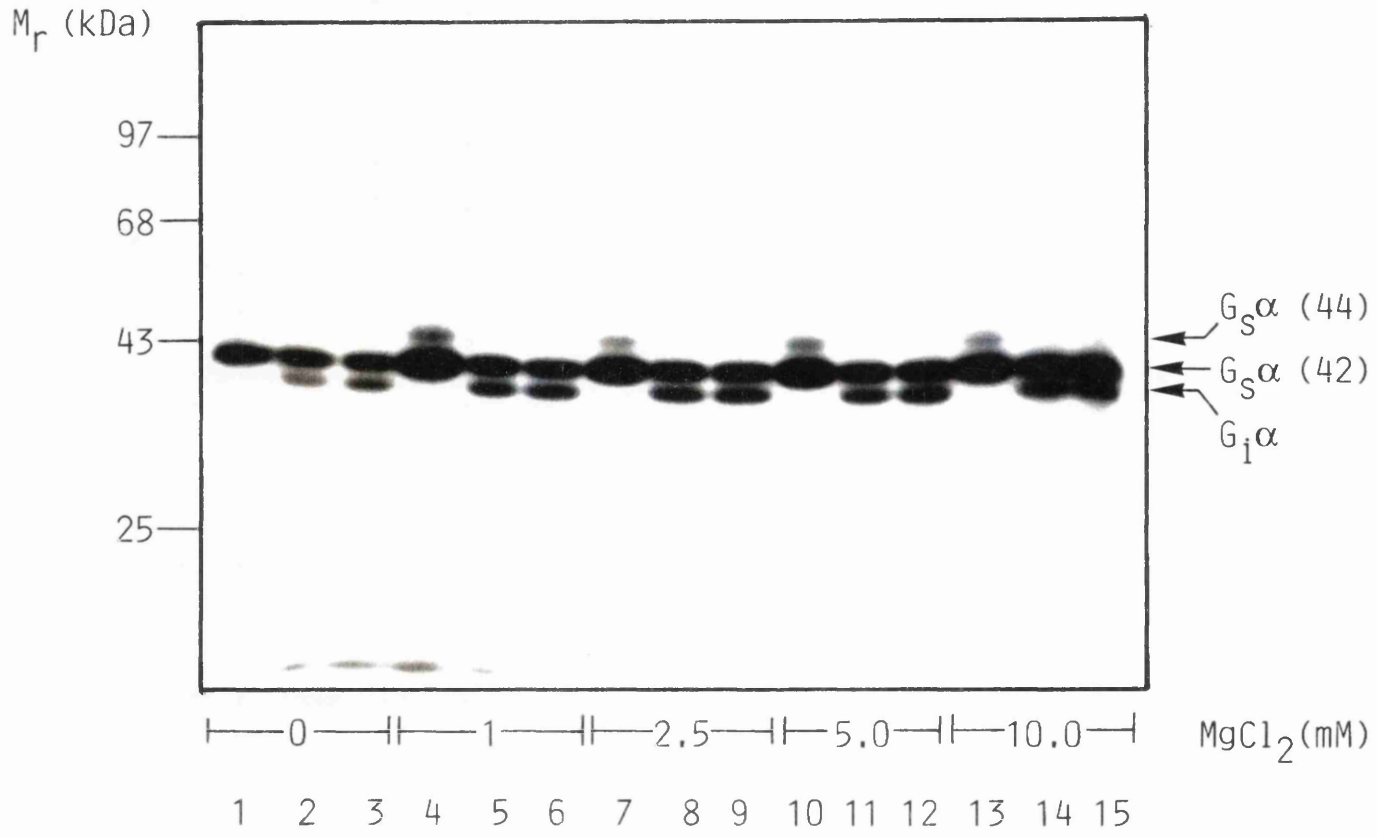

M_r (kDa)
97
68
43
25
$G_s\alpha$ (44)
$G_s\alpha$ (42)
$G_i\alpha$
0
1
2.5
5.0
10.0
$MgCl_2$(mM)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4.12.

THE EFFECT OF GUANINE NUCLEOTIDES ON THE fMLP-STIMULATED [32P]ADP-RIBOSYLATION OF Giα IN DIFFERENTIATED U937 CELL MEMBRANES.

Lanes 1-4 and 7-12 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) prepared as described in 2.3.C. The cells were treated with DMSO as described in 2.2.3. Lanes 5 and 6 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) from a different membrane preparation also prepared as in 2.3.C. Toxin-catalysed [^{32}P]ADP-ribosylation reactions were performed as in 2.11, with modifications. The protease inhibitors described in 2.2.6.C were included in the incubations. The assays were conducted in the absence of GTP in lanes 1-6; in the presence of GTP (10^{-4}M final) in lanes 7 and 8; in the presence of Gpp[NH]p (10^{-4}M final) in lanes 9 and 10 and in the presence of GTPγS (10^{-4}M final) in lanes 11 and 12. The ribosylation reaction was performed in the absence of bacterial toxin in lane 1. Pertussis toxin catalysed the [^{32}P]ADP-ribosylation reaction in lane 2, whereas cholera toxin catalysed the [^{32}P]ADP-ribosylation reaction in lanes 3-12. The chemotactic peptide, fMLP (10^{-5}M final), was included at the initiation of the incubation in the samples in lanes 4, 6, 8, 10 and 12. The vehicle, DMSO (0.1% v/v final), was added at the initiation of the incubation in lanes 3, 5, 7, 9 and 11.

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7 and the dried gel autoradiographed for 4 days, as described in 2.9. This experiment is representative of two experiments which gave essentially identical results.

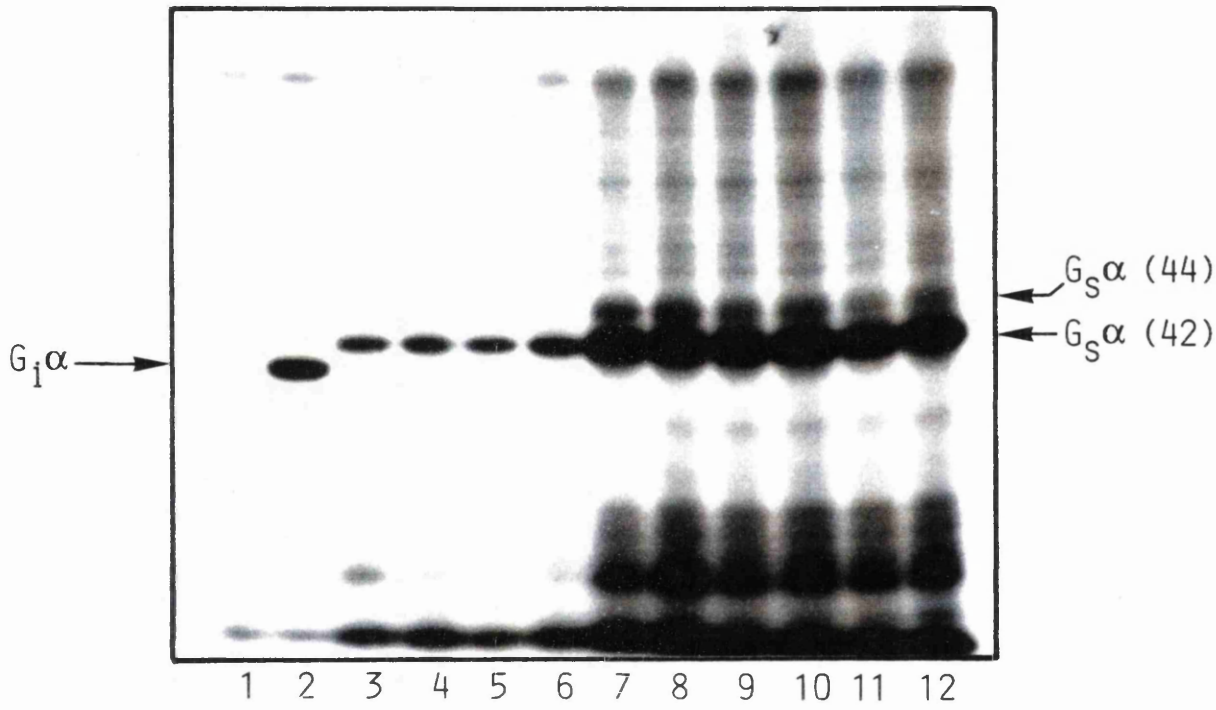

$G_i\alpha$
$G_s\alpha$ (44)
$G_s\alpha$ (42)
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4.13.

THE EFFECT OF NAD+ CONCENTRATION ON THE fMLP-STIMULATED [32P]ADP-RIBOSYLATION OF Giα IN DIFFERENTIATED U937 CELL MEMBRANES.

Lanes 1-15 represent 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (50μg/lane) prepared as in 2.3.C. The cells were treated with DMSO as described in 2.2.3.

Cholera toxin-catalysed [32P]ADP-ribosylation reactions were performed as in 2.11, with modifications. The assay was conducted in the absence of GTP and presence of the protease inhibitors described in 2.2.6.C, for a 2 hour incubation period. Unlabelled NAD+ was included in the assay at increasing concentrations i.e; 0.67μM final in lanes 1-3, 1μM in lanes 4 and 5, 5μM in lanes 6 and 7, 10μM in lanes 8 and 9, 50μM in lanes 10 and 11, 100μM in lanes 12 and 13 and 500μM in lanes 14 and 15. The chemotactic peptide, fMLP (10-5M final), was included upon initiation of the reaction to samples in lanes 3, 5, 7, 9, 11, 13 and 15. The vehicle, DMSO (0.1% v/v final), was added at the initiation of the incubation to lanes 2, 4, 6, 8, 10, 12 and 14.

The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7 and the dried gels autoradiographed for 10 days, as described in 2.9. This experiment was performed once.

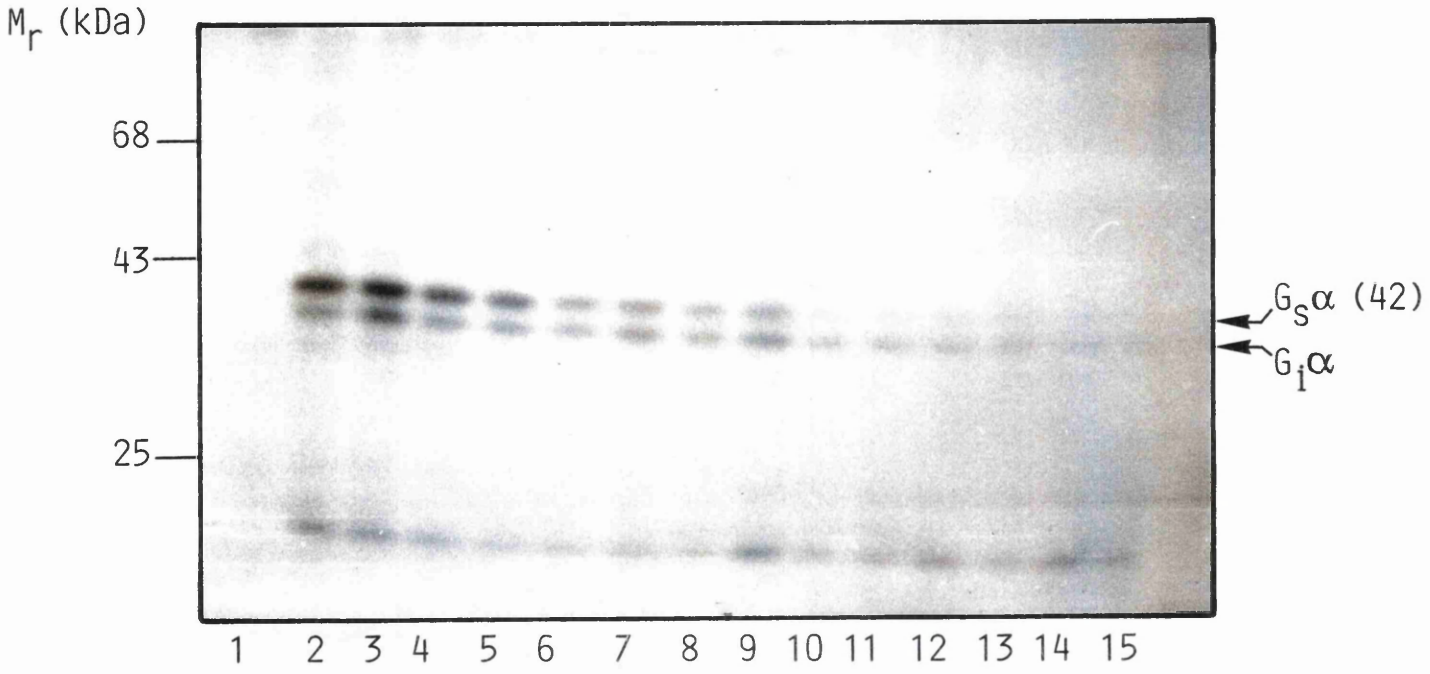

M_r (kDa)
68
43
25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
$G_s\alpha$ (42)
$G_i\alpha$

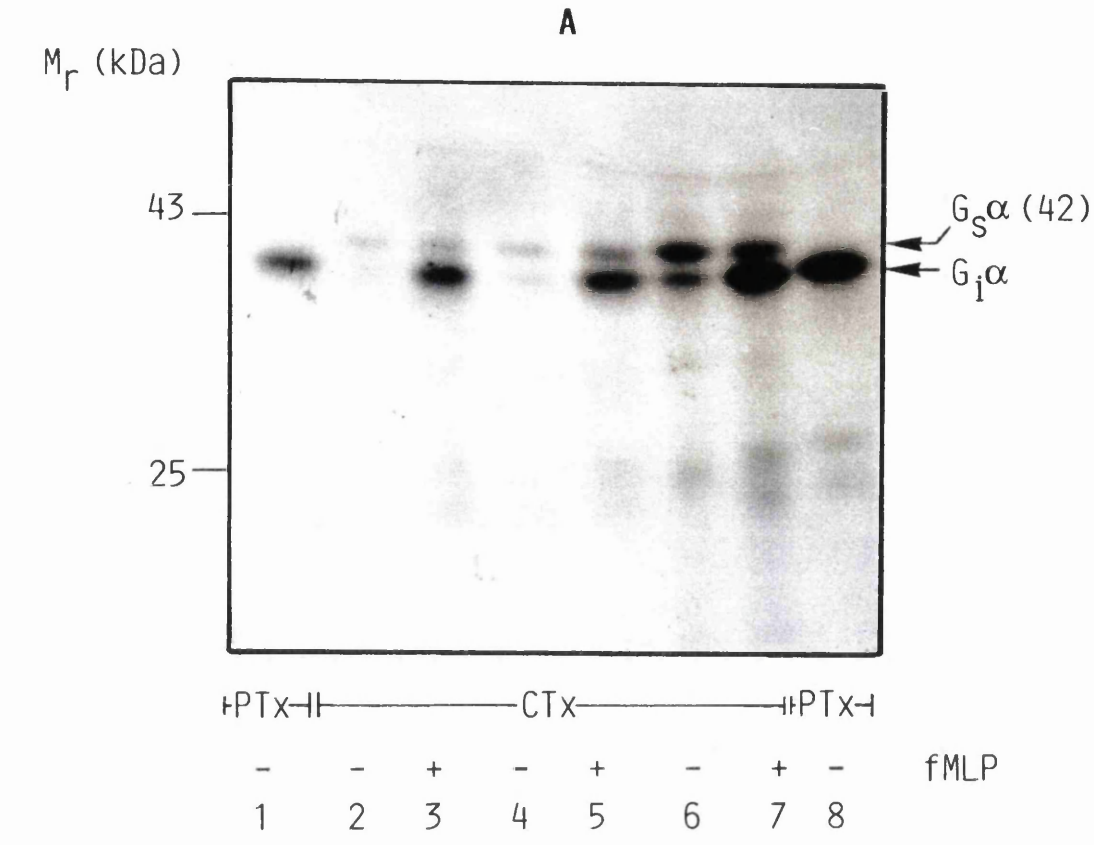
Figure 4.14.

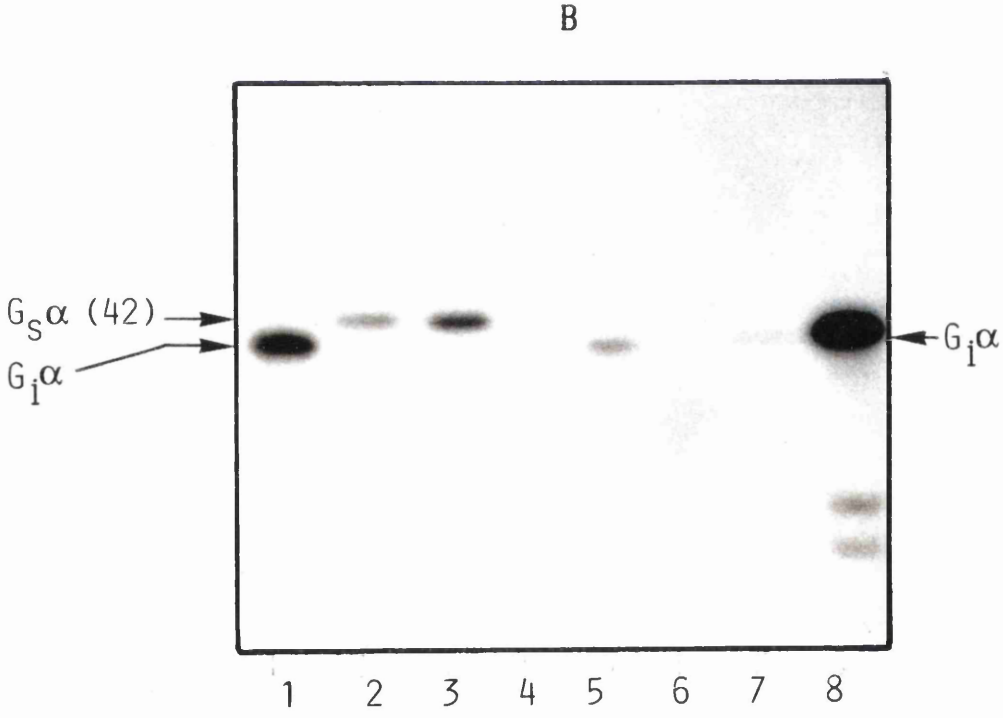
fMLP-STIMULATED [32P]ADP-RIBOSYLATION OF Giα BY CHOLERA TOXIN IN DIFFERENTIATED HL-60 CELL MEMBRANES - IMMUNOPRECIPITATION OF Gsα, Gi2α AND Gi3α.

In panel A, lanes 1-8 represent dibutryl cAMP-differentiated HL-60 cell membranes (50μg/lane). These membranes were a kind gift from Dr. S. Cockcroft, as detailed in 2.3.D. Toxin-catalysed [32P]ADP-ribosylation reactions were performed as detailed in 2.11., in the absence of GTP and presence of the protease inhibitors described in 2.2.6.C, for a 2 hour incubation period.

Lanes 1 and 8 represent the [32P]ADP-ribosylation reaction performed in the presence of pertussis toxin, denoted as "PTx", whereas lanes 2-7 were performed in the presence of cholera toxin, denoted as "CTx". fMLP (10^{-5}M final) was added at the initiation of the incubation to lanes 3, 5 and 7, as indicated. DMSO (0.1% v/v final), was added at the initiation of the incubation in lanes 2, 4 and 6. Upon completion of the ribosylation immunoprecipitation reactions were performed as outlined in 2.12. 10μl of whole serum, from the antipeptide antiserum CS1, specific for Gsα, was added to the samples in lanes 2 and 3. Similarly 10μl of whole serum from the antipeptide antiserum SG1, specific for Gi2α, was added to lanes 1, 4 and 5 and 15μl of whole serum from the antipeptide antiserum I3B, specific for Gi3α, was added to lanes 6 and 7.

Panel A represents the toxin-catalysed [32P]ADP-ribosylated substrates, not immunoprecipitated with the antipeptide antisera. Panel B represents the immunoprecipitated proteins, with the exception of lane 8 which represents pertussis toxin-catalysed [32P]ADP-ribosylated substrates not subjected to immunoprecipitation. The samples were resolved by SDS-PAGE (10% acrylamide, 0.25% bisacrylamide) as in 2.7 and the dried gels autoradiographed for 14 days in panel A and 7 days in panel B, as in 2.9. This experiment was performed once.


A
M_r (kDa)
43
25
$G_s\alpha$ (42)
$G_i\alpha$
PTx
CTx
PTx
- - + - + - + - fMLP
1 2 3 4 5 6 7 8


B
$G_s\alpha$ (42)
$G_i\alpha$
$G_i\alpha$
1 2 3 4 5 6 7 8

4.3. DISCUSSION.

It is now appreciated that the initial assumption that pertussis and cholera toxin were highly specific in their ability to catalyse a NAD^+-dependent mono-ADP-ribosylation of $G_{i}\alpha$ and $G_{s}\alpha$, respectively, is an oversimplification. The possibility that cholera toxin was not absolutely specific for $G_{s}\alpha$, but was also able to ADP-ribosylate the "classical" pertussis toxin substrates was initially raised by the work of Graves and co-workers, as introduced previously (Graves *et al.*, 1983).

The results presented in this chapter demonstrate that in control, non-differentiated U937 cell membranes, cholera toxin, in the absence but not in the presence of GTP, catalysed the [^{32}P]ADP-ribosylation of a polypeptide that co-migrates with the pertussis toxin substrates expressed in these cells. This was only apparent upon stimulation with the chemotactic peptide, fMLP and represented an extremely low level of [^{32}P] incorporation relative to that observed in the 72 hour DMSO-differentiated membranes. However, the fact that cholera toxin-catalysed [^{32}P]ADP-ribosylation of this substrate occurred at all seems to contradict the view that in the control, non-differentiated cell the fMLP receptor is not expressed (Fischer *et al.*, 1980; Pike *et al.*, 1980). Further evidence in support of the non-differentiated U937 cell expressing a fMLP receptor - G-protein interaction is presented in the next Chapter.

In membranes prepared from the 72 hour DMSO-differentiated U937 cells, cholera toxin, in the absence but not the presence of GTP, catalysed the [^{32}P]ADP-ribosylation of a protein that not only co-migrated with the pertussis toxin substrates of these cells, but was also a substrate for pertussis toxin. This was demonstrated by the observation that in membranes prepared from differentiated U937 cells that had been pretreated with pertussis toxin *in vivo*, cholera toxin was unable to catalyse an *in vitro* [^{32}P]ADP-ribosylation of a 40kDa protein, even in the presence of fMLP. Under these conditions cholera toxin still modified the

42kDa form of $G_s\alpha$. This inhibitory effect of *in vivo* pertussis toxin pretreatment is not explicable by the loss of a common target residue for each toxin. As previously discussed in Chapter 3, the ability of these toxins to catalyse mono-ADP-ribosylation of their α subunit substrates is modified by their state of association with $\beta\gamma$ subunits. From studies of both transducin and $G_s\alpha$ the preferred substrate for cholera toxin is the monomeric α subunit whereas pertussis toxin displays a preference for the holomeric, heterotrimeric form of $G_i\alpha$ (Abood *et al.*, 1982; Owens *et al.*, 1985; Ribeiro-Neto *et al.*, 1987). Pertussis toxin pretreatment may stabilise the holomeric form of G_i and reduce the ability of cholera toxin to catalyse its ADP-ribosylation. An alternative explanation, more plausible with respect to the hypothesised mode of action of cholera toxin on $G_i\alpha$, is that pertussis toxin pretreatment is preventing receptor-driven stimulation of guanine nucleotide exchange by virtue of its ability to attenuate coupling between the receptor and $G_i\alpha$. The presence of GDP in the active site of the $G_i\alpha$ may be sufficient to prevent the cholera toxin-catalysed [^{32}P]ADP-ribosylation of the protein.

Two observations suggested that the 40kda polypeptide [^{32}P]-labelled by cholera toxin was not a form of $G_s\alpha$. Mimicking the conditions which supported the cholera toxin-catalysed [^{32}P]ADP-ribosylation of the 40kDa polypeptide, but excluding [^{32}P]NAD^+ from the incubation, $G_s\alpha$ expression was shown not to alter significantly, suggesting that this polypeptide was not simply a proteolytic product of $G_s\alpha$. Further *in vivo* pretreatment of differentiated U937 cells with cholera toxin prevented *in vitro* fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_s\alpha$, but not the 40kDa polypeptide, again suggesting these peptides were mutually exclusive.

This agonist-driven effect was specific for cholera toxin in that the chemotactic peptide, fMLP, had no effect upon pertussis toxin-catalysed [^{32}P]ADP-ribosylation of $G_i\alpha$, neither in the presence nor absence of GTP.

Interestingly, although stimulation of the membranes with fMLP did significantly increase the level of [32P] labelling of the pertussis toxin substrate by cholera toxin relative to that with the vehicle for the peptide, DMSO, there was a significant level of [32P] incorporation upon challenge with the vehicle in the differentiated U937 cell membranes. It seems unlikely that DMSO interacts at a specific receptor suggesting that even in the absence of agonist a proportion of the $G_i\alpha$ population is accessible to cholera toxin in membranes of the differentiated U937 cells. This further implies that GDP is being released from the $G_i\alpha$ subunits and that perhaps an unoccupied receptor can couple to these proteins to promote such GDP release. Further observations suggesting this possibility are presented in Chapter 5. Indeed this concept that G-proteins interact with and are activated by receptors, even in the absence of agonists, is suggested by various observations in a number of systems. In the HL-60 cell membranes, both sodium ions and pertussis toxin treatment markedly reduce basal GTPase activity. Sodium appeared to be acting at the level of the receptor and pertussis toxin acts to uncouple receptors from their G-proteins contradicting the belief that basal activity of G-proteins is receptor-independent (Gierschik *et al.*, 1989c). Indeed reconstitution of the purified β-adrenoreceptor into lipid vesicles containing purified G_s, significantly enhanced GTPase activity of G_s, even in the absence of a receptor agonist (Cerione *et al.*, 1984). Thus there is a precedent for an unoccupied receptor coupling to a G-protein in the differentiated U937 cell membranes.

Initial attempts to identify which pertussis toxin substrate was interacting with the fMLP receptor utilised the ability to electrophoretically resolve pertussis toxin-catalysed [32P]ADP-ribosylated $G_i2\alpha$ and $G_i3\alpha$. The radioactive proteins were then immunoblotted and identified using specific antipeptide antisera. The chemotactic peptide, fMLP, stimulated cholera toxin-catalysed incorporation of [32P]ADP-ribose into a polypeptide that comigrated with pertussis toxin-catalysed [32P]ADP-ribosylated $G_i2\alpha$. However, cholera toxin-catalysed a lower level of

[^{32}P]ADP-ribosylation of $G_i\alpha$ relative to that of pertussis toxin-catalysed [^{32}P]ADP-ribosylation, presumably reflecting the fact that only that proportion of the $G_i\alpha$ pool interacting with the fMLP receptor would act as a substrate for [^{32}P]ADP-ribosylation by cholera toxin. This necessitated increased exposure of the immunoblot, decreasing the resolution of the cholera toxin-catalysed [^{32}P]ADP-ribosylated proteins and rendering it difficult to assess whether $G_i3\alpha$ was interacting with the fMLP receptor.

Interestingly, subsequent to these attempts to resolve which $G_i\alpha$ was [^{32}P]ADP-ribosylated in response to fMLP stimulation, a similar approach was used in the HL-60 cell line. These cells were induced to differentiate to neutrophil-like cells by DMSO treatment. Gierschik and co-workers resolved the [^{32}P]ADP-ribosylated proteins on a long, 8% (w/v) acrylamide SDS-PAGE separation system supplemented with 4M deionised urea. Using this approach they achieved resolution of the fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylated $G_i\alpha$ polypeptides from each other and from $G_s\alpha$. They identified the $G_i\alpha$ polypeptides as two different proteins by performing peptide mapping studies and suggested that they represented $G_i2\alpha$ and $G_i3\alpha$ by their equivalent mobility in the gel matrix to the pertussis toxin substrates of these cells. The authors further suggested that the similarity in the half maximal and maximal concentration of fMLP required to stimulate labelling of both $G_i2\alpha$ and $G_i3\alpha$, implies activation of two G-proteins by a single fMLP receptor population. Indeed, as these authors state this concept of a single receptor interacting with more than one G-protein is not without precedence in the literature, but they further suggest that the subsequent interaction of these different G-proteins with different effector systems may represent the point at which the complex range of phagocytic functions can be regulated (Gierschik *et al.*, 1989a).

Although the U937 cell line exhibits many differences from the HL-60 cell line, not least in differentiating along an alternative lineage upon treatment with DMSO, the work of Gierschik and co-workers did suggest the possibility that $G_i3\alpha$

could also be interacting with the fMLP receptor in the U937 cell (Gierschik *et al.*, 1989a). An alternative and potentially more definitive approach to address the question of which Giα substrate(s) were interacting with the fMLP receptor was to immunoprecipitate the Giα polypeptides from differentiated U937 cell membranes after fMLP-induced cholera toxin-catalysed [^{32}P]ADP-ribosylation. The antipeptide antisera specific for Gi2α and Gi3α were tested initially for their ability to immunoprecipitate these substrates from DMSO-differentiated U937 cell membranes after a prior *in vitro* pertussis toxin-catalysed [^{32}P]ADP-ribosylation reaction. Initial attempts using at least three different membrane preparations for each antipeptide antiserum were unsuccessful, despite successful immunoprecipitation of these substrates from other cell lines tested in conjunction. In retrospect, it can now be appreciated that these were the initial indications of the G-protein proteolysis that was occurring in these membranes, possibly most prolific in the immunoprecipitation procedure which does include an overnight incubation period at 4°C. Although the protease inhibitors PMSF and aprotinin were routinely included in the procedure for any cell type, they did not prevent degradation in this cell type. This proteolytic problem was further highlighted by the decreasing cholera toxin-catalysed [^{32}P]ADP-ribosylation of both Gsα42kDa and Giα, with increasing incubation period at 37°C. Many other experiments illustrated the proteolytic degradation of particularly the Giα proteins in the U937 cell line. Some of these and the solution to the problem have already been discussed in detail in Chapter 3.

However it should be noted that the experiments of this chapter discussed to date were performed upon membranes that were prepared in the absence of protease inhibitors. It was perhaps only fortuitous that these membrane preparations did not exhibit a high degree of proteolytic activity, enabling the effects of fMLP upon cholera toxin-catalysed [^{32}P]ADP-ribosylation of Giα to be observed. However it was obviously necessary to repeat this work on membranes prepared in the

presence of protease inhibitors. It was also observed that the inclusion of these inhibitors within the incubations further prevented degradation occurring within the toxin-catalysed [32P]ADP-ribosylation assays.

Using these modifications it was then possible to specifically immunoprecipitate both Gi2α and Gi3α from differentiated U937 cell membranes. The yield of these proteins was relatively low despite investigating a variety of approaches designed to improve recovery of these substrates.

However, when the ability of fMLP to stimulate the cholera toxin-catalysed [32P]ADP-ribosylation of Giα in the absence of GTP was re-examined in the presence of protease inhibitors this effect was found to vary from preparation to preparation. Repeating the fMLP-stimulated cholera toxin-catalysed [32P]ADP-ribosylation of Giα, [32P]-labelled Gi2α was immunoprecipitated from the U937 cell membranes. Although cholera toxin catalysed the incorporation of radioactivity into Gi2α, it was not apparent that fMLP had stimulated the toxins ability to catalyse this reaction. This perhaps implies that Gi2α was interacting with an unoccupied receptor, but provides no information as to the nature of the Giα species interacting with the agonist occupied receptor. Similar attempts to examine whether Gi3α fulfilled this coupling role were hampered by a low initial incorporation of radioactivity into both the Gsα and the Giα polypeptides catalysed by cholera toxin.

Surprisingly, the loss of cholera toxins ability to ribosylate both Gsα and Giα was apparent in membranes prepared in the presence of additional protease inhibitors and assayed on the same day to further minimise protein degradation, in particular that of the fMLP receptor (see Chapter 5). Indeed, even in membranes in which a receptor - G-protein coupling event was implicated by a Gpp[NH]p-mediated displacement of fMLP binding (see Chapter 5), cholera toxins ability to catalyse [32P]ADP-ribosylation was reduced. To combat these problems conditions were altered towards maximising cholera toxins ability to ribosylate its *in vivo*

substrate $G_s\alpha$, as a high level of ribosylation of this substrate seemed to be a prerequisite for incorporation of radioactivity into $G_i\alpha$.

Magnesium ions, over a range of concentrations, have been shown to exert multiple effects upon both the receptor and the G-protein in a number of systems. Indeed magnesium ions and other divalent cations at millimolar concentrations, convert many G-protein coupled receptors into high affinity states for agonists, although the mechanism for this is currently unknown. It has been speculated that this effect of Mg^{2+} may be a consequence of Mg^{2+} interaction with the lipid components of the membrane, altering the local lipid environment surrounding the receptor and G-protein and facilitating coupling interactions (Herrmann *et al.*, 1989). There are multiple effects of Mg^{2+} upon G-proteins, with low (nM) concentrations required for nucleotide hydrolysis *per se* and higher (μM) concentrations required to maximise hormone - receptor (HR) catalysed nucleotide exchange (Brandt & Ross, 1986). Some of these multiple effects have been summarised by Gilman who speculates that Mg^{2+} has a low affinity binding site on the G-protein which in the absence of HR is of the order of 1-100mM. At this concentration it is proposed that Mg^{2+} facilitates dissociation of GDP, with HR lowering the concentration of Mg^{2+} required for this effect (Gilman, 1987).

Thus the effect of Mg^{2+} was tested primarily with a view to inducing a high affinity state of the fMLP receptor and possibly increasing the fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of the 40kDa polypeptide(s). Magnesium in the low mM concentration range did increase incorporation of radioactivity into all the polypeptides [^{32}P]ADP-ribosylated by cholera toxin, even enabling the visualisation of the $G_s\alpha$44kDa polypeptide. These effects have been observed previously (Ribeiro-Neto *et al.*, 1987). However, possibly due to its ability to increase the rate of GDP dissociation, Mg^{2+} appeared to remove the agonist-dependency of the cholera toxin-catalysed [^{32}P]ADP-ribosylation of the 40kDa polypeptide(s). The removal of fMLP-dependency rendered this approach

less useful than initially anticipated. Other workers have found that the effect of Mg^{2+} upon fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{i}\alpha$ and $G_{s}\alpha$ was biphasic, with increased ribosylation up to 1mM and decreased ribosylation beyond this concentration (Iiri *et al.*, 1989).

An alternative approach was to examine the effect of GTP and non-hydrolysable GTP analogues upon fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation in the differentiated U937 cell membranes. These nucleotides promote the formation of the monomeric α subunit which as discussed previously is the preferred substrate for cholera toxin. The anticipated increase in incorporation of radioactivity into both forms of $G_{s}\alpha$, relative to that obtained in the absence of GTP, was observed. However, as might be expected there was no apparent labelling of a 40kDa polypeptide, probably due to the "blocking" of the site of ADP-ribosylation by the GTP analogues. These observations were in agreement with several groups studying the ability of cholera toxin to catalyse [^{32}P]ADP-ribosylation of both $G_{s}\alpha$ and $G_{i}\alpha$ (Ribeiro-Neto *et al.*, 1987; Owens *et al.*, 1985). However, in HL-60 membranes GTPγS and GTP, but not Gpp[NH]p, all present at 100μM, inhibited the effect of fMLP on labelling of the $G_{i}\alpha$ protein (Gierschik & Jakobs, 1987). These effects of the GTP analogues seem contradictory but fundamental differences between the behaviour of these analogues has been observed in other systems. Indeed, cholera toxin-catalysed ADP-ribosylation of transducin is stimulated by light-activated rhodopsin. This reaction is further stimulated by Gpp[NH]p, but not by GTPγS (Abood *et al.*, 1982). These observations suggest that the Gpp[NH]p bound form of these proteins is accessible to cholera toxin. Again using HL-60 membranes, Iiri and co-workers found that although the fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{i}\alpha$ was stimulated upon addition of GTP or its analogues, these effects displayed quite varied potencies, with inhibition evident at concentrations of GTPγS above 0.1μM, Gpp[NH]p above 3μM, GTP above 10μM and Gpp[CH_2]p above 100μM (Iiri *et al.*, 1989). Although not tested, with

respect to these observations, perhaps lower concentrations of the analogues may have improved cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{i}\alpha$ in response to fMLP.

On comparison of several methods for the toxin-catalysed [^{32}P]ADP-ribosylation of membrane G-proteins there appeared to be variation in the concentration of NAD^+ used in the ribosylation assay i.e., ranging between 1-25μM (Gierschik & Jacobs, 1987; Hudson & Johnson, 1980). In the initial experiments of this chapter although a constant amount of radioactivity was used the actual NAD^+ concentration was dependent on the decay of the isotope. However, as it appeared that in experiments in which fMLP did stimulate cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{i}\alpha$ the NAD^+ concentration was in the range 1-10μM, the effect of NAD^+ concentration was investigated. As anticipated increasing the NAD^+ concentration decreased both the cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{s}\alpha$ and $G_{i}\alpha$, interestingly more effectively inhibiting the ribosylation of $G_{s}\alpha$.

Further unsuccessful attempts at eliminating this problem of loss of toxin-catalysed [^{32}P] incorporation into $G_{s}\alpha$ and $G_{i}\alpha$ included increasing the membrane concentrations tested, increasing the incubation period, ensuring the viability of the fMLP and the ribosylation reagents and even use of the irreversible serine protease inhibitor, di-isopropyl fluorophosphate in the initial cell harvesting steps. Dibutryl cAMP-differentiation of the U937 cells has been demonstrated to induce higher amounts of fMLP receptor relative to DMSO-differentiated cells (Kay *et al.*, 1983). However using this alternative differentiating agent did not improve the cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{i}\alpha$ in membranes prepared from these cells (results not included).

Another consideration was that ADP-ribosylation factor (ARF), an intrinsic membrane protein required for the cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_{s}\alpha$, was being adversely affected by the alternative membrane preparation or

conditions. However, ARF has been found in membranes prepared in a similar way to those examined in this work. It is sensitive to tryptic digestion and is thermolabile, with a half time for the loss of activity of 3.7 hours at 37°C (Kahn & Gilman, 1984a). From these few characteristics it would appear that, if anything, the conditions of the latter experiments should have been more conducive to this factors function.

Paradoxically, protecting the signal transducing proteins under investigation from proteolytic degradation appeared to inhibit the fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of Giα in differentiated U937 cell membranes. Loss of this effect is not without precedence and Iiri and co-workers observed that this ability of fMLP to stimulate ribosylation of purified Giα could not be observed in phospholipid vesicles, containing ARF and functional fMLP receptors, although the bacterial toxins did catalyse ADP-ribosylation of their "classical" Gα substrates under these conditions. The authors propose that a labile membrane "factor(s)" is lost upon the process of membrane extraction and that this is essentially required for cholera toxin to ADP-ribosylate the Giα coupled to stimulated fMLP receptors (Iiri *et al.*, 1989). Whether deprivation of such a "factor" was responsible for the problems encountered in the work of this Chapter is purely speculative.

Interestingly, preliminary experiments aimed primarily at establishing whether such problems were specific to the U937 cell line, demonstrated that in membranes prepared from the dibutryl cAMP-differentiated HL-60 cell line, differentiated to neutrophil-like cells, the fMLP receptor interacted with Gi2α and tentatively, Gi3α. This would agree with previous observations using this cell line (Gierschik *et al.*, 1989a). Successful immunoprecipitation of fMLP-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylated substrates demonstrated that this experimental approach can be informative with respect to definition of coupling specificities and that the problems encountered with the U937 cell line may have been cell line or lineage specific. However, it is notable that using the HL-60 cell

line fMLP stimulates a considerably greater incorporation of [^{32}P]ADP ribose into Giα relative to both the vehicle control and that observed using the U937 cell line. Whether such discrepancies represent differences between the signal transducing components of these cell lines, i.e., variation in their levels of expression, affinities and viability, was not investigated.

In conclusion, the experiments presented in this Chapter demonstrate a novel approach to elucidating receptor - G-protein coupling specificity that may be of general usefulness in systems that display pertussis toxin-sensitive receptor-mediated signalling pathways. In control U937 cell membranes the activated fMLP receptor stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of a protein that co-migrated with the pertussis toxin substrates of these cells indicating a coupling interaction. Such an interaction was more apparent in membranes from differentiated U937 cells and may reflect an increased expression of the coupling components (see previous Chapter and Chapter 5). In the differentiated U937 cell membranes the fMLP receptor interacted with a pertussis toxin substrate, tentatively identifiable as Gi2α, although possible interaction with Gi3α was not successfully addressed.

CHAPTER 5.

ALTERNATIVE APPROACHES TOWARDS IDENTIFICATION OF THE G-PROTEIN COUPLED TO fMLP RECEPTOR.

5.1. INTRODUCTION.

From the literature discussed in the previous chapters it can be appreciated that there is considerable evidence to implicate a coupling interaction between the fMLP receptor, a pertussis toxin-sensitive G-protein(s) and phospholipase effector enzymes. The experiments presented in this Chapter investigated alternative approaches towards definition of the specificity of such coupling interactions.

An average of 50,000 chemotactic peptide receptors are expressed in the human polymorphonuclear leukocyte (PMN) whole cell, which bind the chemoattractant with a single dissociation constant (K_d) of approximately 20nM. However, when membranes of these cells were studied the binding data was more consistent with the presence of two classes of binding sites with average K_d values of 0.5nM and 20nM. The higher affinity (lower K_d) receptors accounted for approximately 25% of the binding sites. Interestingly, in contrast to intact, viable PMN cells, formalin permeabilised whole PMN's also demonstrated heterogeneity of binding sites with K_d values in good agreement with those found in isolated membranes. Several interpretations would account for the heterogeneous steady state binding data obtained in this study but using a kinetic approach developed by DeMeyts, it was established that heterogeneity of binding was not a reflection of negative cooperativity (DeMeyts *et al.*, 1976). However this study did not address whether the binding heterogeneity represented two distinct receptor classes or two affinity states of a single receptor population (Koo *et al.*, 1982).

A later study by the same group discovered that guanine nucleotides modulated the binding affinity of the oligopeptide chemoattractant receptor on human PMN's and established that the two classes of binding sites were at least in part composed of interconvertible states of one population of receptors (Koo *et al.*, 1983). Equilibrium binding of fMLP to membranes from guinea pig macrophages is also best fit by a model defining two affinity states. In this study preincubation of membranes with Gpp[NH]p resulted in a 90% reduction in the percentage of

high affinity sites without a change in total receptor number. The intact cells displayed a single affinity of receptor statistically indistinguishable from the lower affinity site in membranes. The levels of GTP and GDP in guinea pig macrophages were estimated as 114μM and 67μM, respectively. The authors propose that these nucleotide levels are sufficient to allow receptor interconvertability *in vivo* and thus expression of one affinity state of the receptor in the intact macrophage. In membrane preparations the removal of guanine nucleotides by washing allows an additional population of receptors to be detected, since interconvertability is blocked (Synderman *et al.*, 1984). An early study using the U937 cell line compared whole cell receptor number and affinity between undifferentiated and differentiated cells. The undifferentiated cells expressed no detectable receptors whereas 72 hour DMSO-induced cells expressed approximately 4000 receptors per cell, with a Kd of approximately 20nM. A variety of differentiating agents displayed differential abilities to increase receptor number but the receptor Kd value remained constant (Kay *et al.*, 1983).

An initial attempt to biochemically characterise the N-formylated peptide receptor was made by Niedel and co-workers. Using a variety of techniques to covalently label the receptor they identified a polypeptide with an apparent molecular weight between 55 and 70kDa (Niedel *et al.*, 1980). Other groups have also succeeded in covalently photolabelling and further characterising the N-formylated peptide receptor in a number of systems. Indeed in the study of the U937 cell line previously discussed, Kay and co-workers demonstrated no detectable binding of the covalent affinity label, fNle-Leu-Phe-Nle-[^{125}I]-Tyr-Lys, to undifferentiated cells whereas it coupled to proteins with molecular weights ranging from 76kDa to 91kDa in DMSO-differentiated cells. These covalently labelled proteins were detected in normal human monocytes and neutrophils (Kay *et al.*, 1983).

More recently the receptor has been solubilised from rabbit neutrophils and

fMLP high affinity binding reconstituted in detergent solubilised membranes and phospholipid vesicles, with exogenous G-proteins purified from bovine brain. The ability of brain $G_{i}\alpha/G_{o}\alpha$ to reconstitute high affinity binding is consistent with the findings of Kikuchi and co-workers who had demonstrated that these proteins were capable of reconstituting fMLP induced PtdIns(4,5)P_2 hydrolysis in pertussis toxin-treated neutrophil membranes (Williamson *et al.*, 1988; Kikuchi *et al.*, 1986). Although the reconstituted system may indicate coupling interactions that do not occur in the *in vivo* situation, as $G_{o}\alpha$ expression has not been demonstrated in cells that express fMLP receptors, this work importantly demonstrated that the receptor could be resolved in a functionally operable condition.

Recently, a novel approach has been employed to clone genes of proteins which have not been isolated in large quantities. This involves the expression of the functional protein from the mRNA in *Xenopus laevis* oocytes (Masu *et al.*, 1987). Several calcium mobilising receptors have been cloned by such functional expression approaches e.g., the platelet-activating factor and V1a arginine vasopressin receptors (Honda *et al.*, 1991; Morel *et al.*, 1992). Two separate groups have functionally reconstituted fMLP receptors into *Xenopus laevis* oocytes by microinjecting them with RNA isolated from dibutryl cAMP-differentiated HL-60 cells. Coats and co-workers monitored the fMLP-induced calcium mobilisation by measurement of the photon emission elicited by aequorin. Size fractionation of the RNA and microinjection of the individual fractions indicated that messenger RNA for the fMLP receptor is between 1.5 and 2.0 kilobases (Coats & Navarro, 1990). Murphy and co-workers isolated a 2 kilobase messenger RNA that when injected into *Xenopus* oocytes resulted in their acquisition of a formyl peptide-activatable inward transmembrane current consistent with chloride conductance. The opening of chloride channels is dependent upon intracellular calcium released by Ins(1,4,5)P_3-mediated mechanisms. Interestingly the calcium mobilising activity was completely abolished by pertussis toxin (Murphy *et al.*, 1990).

This reconstitution strategy has been used as one of the criteria to characterise a cDNA encoding the fMLP receptor from neutrophils. As discussed previously (see 1.8.2), the high degree of amino acid homology between G-protein coupled receptors in their putative transmembrane domains has provided the basis for the design of oligonucleotide probes used to isolate cDNA's encoding other G-protein coupled receptors. A cDNA encoding the fMLP receptor was isolated by screening a rabbit neutrophil cDNA library with an oligonucleotide probe deduced from the second transmembrane domain of G-protein coupled receptors (Thomas *et al.*, 1990). Interestingly, Boulay and co-workers have recently characterised two cDNA isolates which code for two variants of the human N-formyl peptide receptor. The analysis of genomic DNA with a cDNA probe revealed a complex pattern consistent with the presence of either two genes encoding the fMLP receptor or a single gene with introns in the coding sequence. As the authors suggest, alternate splicing could direct the expression of two isoforms of the fMLP receptor lending credence to the observations of possible receptor heterogeneity at the biochemical level (Boulay *et al.*, 1990).

One early and fundamental observation was that G-protein linked receptors exhibited a reduced affinity for binding agonists, but not antagonists, in the presence of non hydrolysable analogues of GTP (see sections 1.1 and 1.8.1 for more detail). From the studies discussed already it can be appreciated that this applies to the fMLP receptor. In the case of pertussis toxin-sensitive G-proteins, a similar reduction in receptor affinity for agonist and a concomitant loss in guanine nucleotide sensitivity of agonist binding has been demonstrated following pretreatment of cells with this toxin (Kurose *et al.*, 1982; McKenzie & Milligan, 1990). Thus if the G-protein is prevented from interacting with its receptor, either by promoting the dissociation of the α subunit from the $\beta\gamma$ subunits with non hydrolysable analogues of GTP or by toxin-catalysed modification of the C-terminal region of the α subunit, the resultant receptor exhibits a lower affinity for

agonist. With the recent production of polyclonal, C-terminally directed antipeptide antisera it has been hypothesised that preincubation of the coupling components with these antisera may not only prevent productive interactions, but importantly perturb coupling in a highly specific manner.

Indeed at the receptor level, preincubation of cell membranes with C-terminally directed antipeptide antisera would be anticipated to convert the receptor to a form with reduced affinity, detectable as a reduction in agonist binding. This has been confirmed by a study of the δ opioid receptor of the neuroblastoma x glioma NG108-15 cell line. Preincubation of membranes of these cells with the IgG fractions from a C-terminally directed antipeptide antiserum capable of recognising only $G_i2\alpha$ in these cells, resulted in a reduction in the specific binding of the synthetic opioid peptide, D-ala^2-D-leu^5 enkephalin, [^{3}H] DADLE. This reduction in binding was due to an increase in the apparent K_d for the ligand and no alteration in maximal binding was observed. The increase in apparent K_d was equivalent to that produced by GTP analogues or pertussis toxin pretreatment of the cells and was specific for this antipeptide antiserum (McKenzie & Milligan, 1990). Using a similar approach the α_{2B} receptor was also specifically uncoupled from $G_i2\alpha$ in membranes from the NG108-15 cell line (McClue & Milligan, 1990).

An alternative approach towards defining the coupling specificity between a receptor and a G-protein has been to use antipeptide antisera to "uncouple" agonist-stimulated GTPase activity. In the study of the δ opioid receptor discussed previously preincubation of NG108-15 cell membranes with an antipeptide antiserum that recognised only $G_i2\alpha$ in these cells, completely abolished DADLE-stimulated GTPase activity (McKenzie & Milligan, 1990). More recently both $G_i2\alpha$ and $G_i3\alpha$ have been demonstrated to couple to the α_{2A}-C10 receptor by virtue of the ability of specific antipeptide antisera to inhibit agonist-stimulated high affinity GTPase activity (McClue *et al.*, 1992).

Antipeptide antisera have in certain systems been useful in defining G-

protein - effector coupling specificity (see 1.13.3). This is true of the adenylyl cyclase enzyme whose activity can be measured in a membrane system. Studies with C-terminally directed antipeptide antisera have demonstrated that inhibition of adenylyl cyclase is transduced by $G_{i}2\alpha$ in NG108-15 cells and human platelets (McKenzie & Milligan, 1990; Simonds *et al.*, 1989). Several membrane systems have been used to study the regulation of phospholipase C by guanine nucleotides and agonists as discussed briefly in 1.12.1. These systems are notoriously difficult to establish and in some cases interpret and in an effort to extend investigation of G-protein coupling to phospholipase C in the U937 cell line an alternative approach was employed. This was based on the observations of Iiri and co-workers who observed that cholera toxin-induced [^{32}P]ADP-ribosylation of $G_{i}\alpha$ in intact HL-60 cells stimulated with fMLP (Iiri *et al.*, 1989). The rationale was to establish if fMLP-stimulated a cholera toxin-induced [^{32}P]ADP-ribosylation of $G_{i}\alpha$ in intact U937 cells and what effect this had upon fMLP-stimulated Ins(1,4,5)P_3 generation.

The experiments performed in this chapter were thus aimed at establishing the potential for use of discriminatory antipeptide antisera in specifically "perturbing" [^{3}H]fMLP binding and fMLP-stimulated GTPase activities in membranes from U937 cells. Mass Ins(1,4,5)P_3 measurements were performed in whole U937 cells to confirm the pertussis toxin sensitivity of the fMLP-stimulated generation of this second messenger and to extend the possibility of defining coupling specificity in the presence of "effector" as opposed to just at the level of receptor-G-protein.

5.2. RESULTS.

A. EVIDENCE FOR AN fMLP RECEPTOR - G-PROTEIN INTERACTION.

Ligand binding experiments were performed in the presence of high concentrations of $MgCl_2$ (20mM) to convert the fMLP receptor into a high affinity agonist binding state. This concentration of $MgCl_2$ was found to be more effective than others tested (results not shown). As there are multiple effects of Mg^{2+} upon both receptors and G-proteins it was appreciated that the characteristics of these proteins interaction applied under these ionic conditions. In saturation binding experiments, specific binding of [^{3}H]fMLP to membranes of 72 hour DMSO (1.25% v/v)-differentiated U937 cells could be tentatively interpreted as being to two sites with quite different ligand binding affinities. Indeed one site ("high" affinity) appeared to approach saturation at approximately 5nM [^{3}H]fMLP, whereas the other site ("low" affinity) was not saturated at concentrations of 20nM [^{3}H]fMLP (panel A, Fig. 5.1). Analysis of the data, using a variation of the plot that Scatchard derived, suggested apparent dissociation constants (Kd) for the radioligand of 2.4 ± 1.1nM and 40.1 ± 3.3nM (mean ± range of two experiments),with B_{max} values of 23.7 ± 9.6 fmoles/mg and 205.9 ± 55.9 fmoles/mg (mean ± range of two experiments) for the "high" and "low" affinity sites, respectively (panel B, Fig. 5.1). This terminology of "high" and "low" affinity sites will be used in further discussion to implicate the use of lower nanomolar concentrations of [^{3}H]fMLP relative to use of higher nanomolar concentrations of the [^{3}H] ligand, respectively.

When statistical analysis was applied two mean values were compared using the unpaired Student's *t*-distribution test. The variances were unknown but assumed equal and a pooled estimation of variance calculated. The "n" value represents the "degrees of freedom", unless otherwise stated.

Binding experiments were performed in the presence of agents which act to uncouple receptor - G-protein interaction. Pertussis toxin pretreatment of DMSO-

differentiated U937 cells had a significant inhibitory effect on ligand binding performed using membranes prepared from these cells, reducing it by 87.4 ± 8.2% using 0.6nM [3H]fMLP ($p < 0.001$, $n = 5$) and by 39.5 ± 12.7% ($p < 0.01$, $n = 4$) when 17.9nM [3H]fMLP was used (panel A, Fig. 5.2). In the previous Chapter, this *in vivo* pertussis toxin pretreatment of intact cells has been demonstrated to completely ADP-ribosylate all the pertussis toxin G-protein substrate in the cell membrane. However in these and later experiments in this Chapter the *in vivo* pertussis toxin-treated membranes were always checked to ensure that complete ADP-ribosylation of the G-proteins had occurred (results not included). A five minute preincubation of differentiated U937 cell membranes with Gpp[NH]p (100μM final) significantly reduced the specific binding of [3H]fMLP at both affinity sites i.e., 82.6 ± 3.4% reduction ($p < 0.001$, $n = 4$) when using 0.4nM [3H]fMLP and 53.3 ± 12.2% reduction ($p < 0.001$, $n = 6$) when using 23.1nM [3H]fMLP (panel B, Fig. 5.2). This reduction in specific [3H]fMLP binding to differentiated U937 cell membranes after pertussis toxin pretreatment and Gpp[NH]p preincubation was non additive at the low affinity sites (panel C, Fig. 5.2).

On comparison of the results of several [3H]fMLP binding experiments at the low affinity site, performed on different days but using the same differentiated U937 cell membrane preparation, reduced radioligand binding was observed with increasing time after the initial experiment i.e, increasing membrane storage at -80°C. In each binding experiment membranes were used after only one freeze/thaw cycle. Examining the lower affinity site, even after 4 days storage there was a significant reduction in [3H]fMLP binding, (25.7 ± 7.4% reduction, $p < 0.002$, $n = 5$) relative to the initial binding experiment that had been performed with that preparation of DMSO-differentiated U937 cell membranes. After 21 days storage there was a highly significant decrease in the specific binding of [3H]fMLP, (61.3 ± 8.8% reduction, $p < 0.001$, $n = 5$), relative to the initial experiment (panel A,

Fig. 5.3). This decrease in ligand binding with increasing membrane storage was also apparent at the high affinity site, with significant effects observed after 11 days storage (16.3 ± 5.1% reduction, $p < 0.01$, $n = 5$), (panel B, Fig. 5.3). Upon prolonged storage i.e., 38 days, a highly significant 74.5 ± 4.9% reduction ($p < 0.001$, $n = 4$) in [^{3}H]fMLP binding was observed. Such a reduction in specific binding of [^{3}H]fMLP to differentiated U937 cell membranes was not explicable by a reduction in the affinity of the binding sites due to a loss of G-protein coupling, as preparations which exhibited reduced specific binding, relative to preparations which had been stored for less time, still exhibited a significant reduction in [^{3}H]fMLP binding upon pertussis toxin pretreatment as demonstrated previously in Figure 5.2, A. The membranes actually employed to perform this experiment were equivalent to those used after 11 and 4 days of storage, at the higher and lower affinity binding sites, respectively. This apparent loss of [^{3}H]fMLP binding was also observed in other membrane preparations examined (results not shown).

Testing the same [^{3}H]fMLP stock, at an average concentration of 21.8 ± 2.1nM over the three experiments represented, specific binding of 162.0 ±15.8 fmoles/mg was observed in freshly prepared membranes. However, again a highly significant decrease in specific binding of [^{3}H]fMLP was observed after only 1 day of membrane storage at -80°C, (50.6 ± 13.4% reduction, $p < 0.001$, $n = 6$). After a further two days membrane storage this apparent degradation of binding sites was prevented by altering the initial membrane thawing procedure and adding three further protease inhibitors to the binding reaction incubation buffer (Fig. 5.4). Despite this apparent preservation of [^{3}H]fMLP binding sites further studies employed membranes freshly prepared on the day that the binding assay was performed with these additional protease inhibitors included at all stages in the preparation and assay procedures.

B. SPECIFIC UNCOUPLING OF THE G-PROTEIN FROM THE fMLP RECEPTOR.

From previous studies using antipeptide antisera uncoupling as a method of defining the specificity of receptor - G-protein interaction it was appreciated that a prerequisite is a period of preincubation of the membranes with the IgG fractions of the antipeptide antisera, typically 1 hour at 37°C (McKenzie *et al.*, 1988; McClue *et al.*, 1992). In view of this different preincubation conditions were tested for their suitability in preserving [3H]fMLP binding activity and Gpp[NH]p sensitivity in freshly prepared 72 hour DMSO-differentiated U937 cell membranes. After a 1 hour preincubation at 25°C, Gpp[NH]p was unable to reduce the specific [3H]fMLP binding and although a suitable control was not included this specific binding was considerably lower than the average amount observed with freshly prepared 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes (panel A, Fig. 5.5). However, preincubation of freshly prepared membranes on ice did maintain [3H]fMLP binding, at the low affinity site, that was reduced upon preincubation with Gpp[NH]p. These small yet significant reductions of 25.1 ± 15.8% ($p < 0.05$, $n = 4$) and 26.5 ± 9.7% ($p < 0.01$, $n = 4$) after 30 and 5 hours preincubation, respectively, suggest that G-protein coupling interactions were still viable and could be perturbed by Gpp[NH]p. After 5 hours ice preincubation the fMLP antagonist, N-t-bocMLP (100μM final), almost totally inhibited [3H]fMLP binding (panel B, Fig. 5.5).

Membranes of 72 hour DMSO (1.25% v/v)-differentiated U937 cells were incubated either with or without Gpp[NH]p (100μM) or IgG fractions from each of preimmune rabbit serum, antipeptide antiserum AS7 (anti-Gi2α specific in these cells) and antipeptide antiserum I3B (anti-Gi3α) for 5 hours on ice. After this preincubation period the ability of a single concentration of [3H]fMLP (15.7nM), which was less than the apparent Kd for the radioligand, to bind specifically to the cell membranes was assessed. As anticipated, Gpp[NH]p significantly reduced the

specific binding of [3H]fMLP (54.6 ± 23.3% reduction, $p < 0.002$, $n = 6$), although none of the various IgG fractions tested were able to produce a similar inhibition (Fig. 5.6). In experiments not included immunoblotting approaches were used to ensure that the specificity of the antipeptide antisera remained unaltered after the affinity purification procedure.

Interestingly when freshly prepared membranes from control, non DMSO-treated U937 cells were examined for their ability to bind single, "less than Kd" concentrations of [3H]fMLP, specific binding of the radioligand was observed at the "lower" affinity site only. Upon a five minute preincubation of these control U937 cell membranes with Gpp[NH]p (100μM final) this binding was significantly reduced i.e., 31.4 ± 18.1% reduction ($p < 0.02$, $n = 6$), (panel A, Fig. 5.7). Interestingly, this [3H]fMLP binding was totally inhibited by preincubation with the fMLP antagonist, t-bocMLP (100μM final), (panel B, Fig. 5.7).

The ability of fMLP to stimulate GTPase activity in both non pertussis toxin-pretreated and pertussis toxin-pretreated (50ng/ml, 16 hours) control and 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes was examined. A maximal concentration of the chemotactic peptide (10^{-5}M final) induced an extremely low, yet significant level of stimulated GTPase activity in differentiated U937 cell membranes (25.1 ± 7.7% increase above the vehicle, $p < 0.01$, $n = 5$), but not in those from control cells (panels A and C, respectively, Fig. 5.8). The agonist vehicle, DMSO (0.1% v/v final), did not significantly alter the basal GTPase activity in either control or differentiated membranes. Pertussis toxin pretreatment of the membranes resulted in the abolition of the fMLP-stimulated GTPase activity in differentiated U937 cell membranes (panels D relative to C in Fig. 5.8). The basal GTPase activity was also sensitive to pertussis toxin pretreatment with a reduction of 68.8 ± 7.4% in control membranes and a 68.3 ± 12.4% reduction in differentiated membranes (panels B and D, Fig. 5.8). Prior to attempting to use antipeptide antisera to inhibit the fMLP-stimulated GTPase response various unsuccessful attempts were made to increase the level of the

response, primarily by decreasing the basal GTPase activity. Sodium ions and the anti-helaminthic drug, suramin, have been shown previously to decrease basal membrane GTPase activity (Gierschik *et al.*, 1989c; Butler *et al.*, 1988). However despite exerting this effect in U937 cell membranes these agents did not increase the fMLP-stimulated GTPase response (results not shown).

Preliminary experiments aimed at establishing preincubation conditions which would be suitable to test the effect of specific antipeptide antisera upon fMLP-stimulated GTPase activity, established that preincubation on ice for 75 minutes maintained significant fMLP-stimulated GTPase activity (40.7 ± 18.2% increase above the vehicle, $p < 0.02$, $n = 5$), although did reduce the overall GTPase activity of the membranes (panel A, Fig. 5.9). Preincubation for 1 hour at 37°C reduced the overall GTPase activity of the membranes and inhibited fMLP-stimulated GTPase activity above that of the vehicle-stimulated activity (panel B, Fig. 5.9).

C. MASS MEASUREMENT OF INS(1,4,5)P3.

Performing mass Ins(1,4,5)P_3 measurements on 72 hour DMSO (1.25% v/v)-differentiated U937 cells a 6.8 ± 3.6 fold increase in mass Ins(1,4,5)P_3 above basal levels was observed upon 15 seconds fMLP stimulation. A 5.8 ± 1.5 fold (mean ± SEM) stimulation in mass Ins(1,4,5)P_3 was typical of four experiments performed. It should be noted that basal levels of Ins(1,4,5)P_3 were measured throughout the experiment with, in this representative experiment, 15 observations of basal (i.e., 0 seconds fMLP stimulation) performed (see section 2.15.1. for further discussion). Interestingly, in this experiment, the response peaked at between 15-20 seconds and remained slightly elevated above basal up to 60 seconds after the addition of the chemotactic peptide (Fig. 5.10). Again this trend was typical of four experiments with, in one experiment, a more significant elevation of Ins(1,4,5)P_3 above basal levels observed even after 5 minutes of fMLP stimulation (data not included).

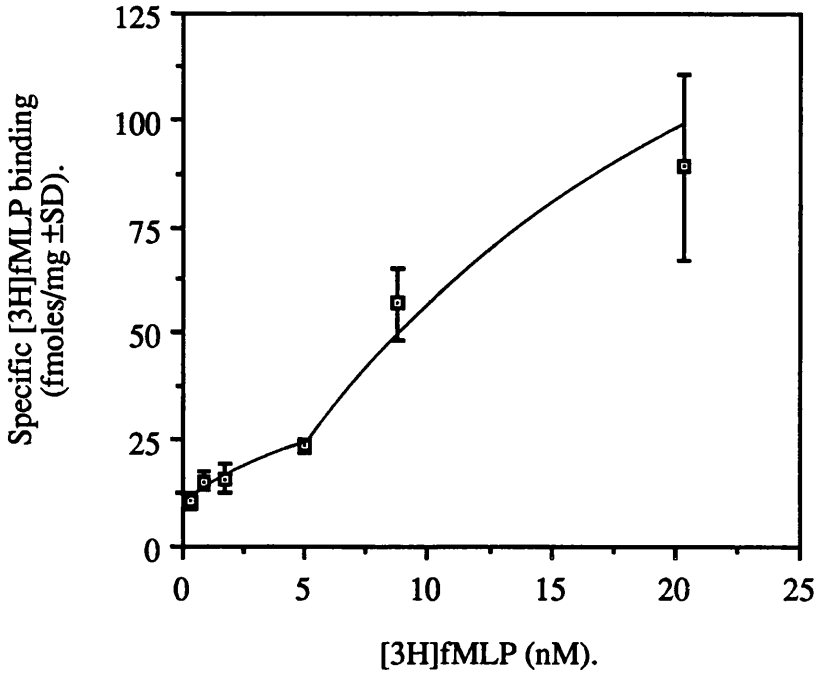
Figure 5.1.

SATURATION BINDING ANALYSIS OF [3H]fMLP TO DIFFERENTIATED U937 CELL MEMBRANES.

In panel A membranes of 72 hour DMSO (1.25% v/v)-differentiated U937 cells (75μg) were incubated with varying concentrations of [3H]fMLP (0.5nM-20nM) and specific [3H]fMLP binding assayed as described in 2.14 and in the presence of the protease inhibitors described in 2.2.6.B. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.B.

Panel B represents the data of panel A replotted after analysis as described by Scatchard (1949). Data are means ±SD taken from quadruplicate determinations in a single experiment which was performed twice.

A


125
100
75
50
25
0
0
5
10
15
20
25
Specific [3H]fMLP binding
(fmoles/mg ±SD).
[3H]fMLP (nM).

B

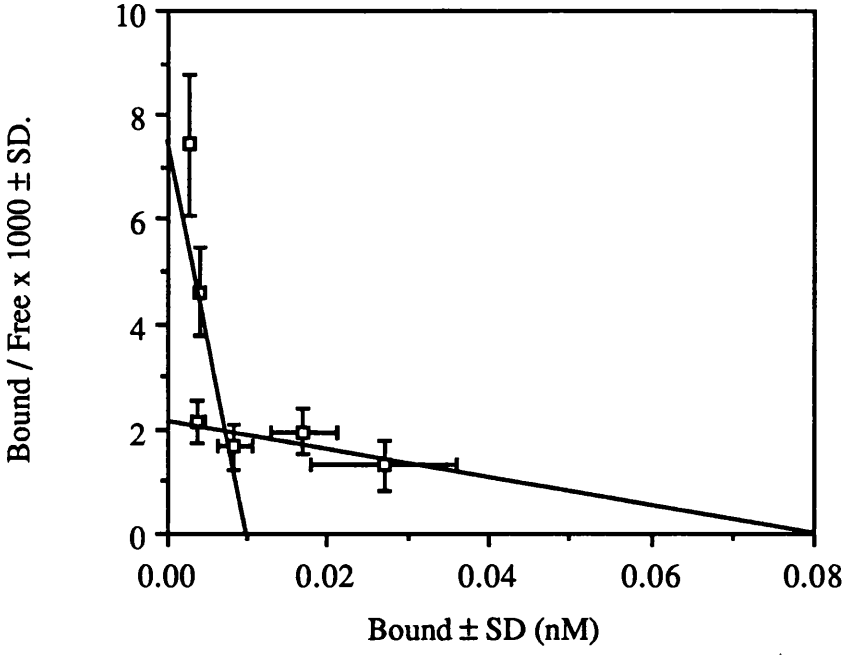

10
8
6
4
2
0
0.00
0.02
0.04
0.06
0.08
Bound / Free x 1000 ± SD.
Bound ± SD (nM)

Figure 5.2.

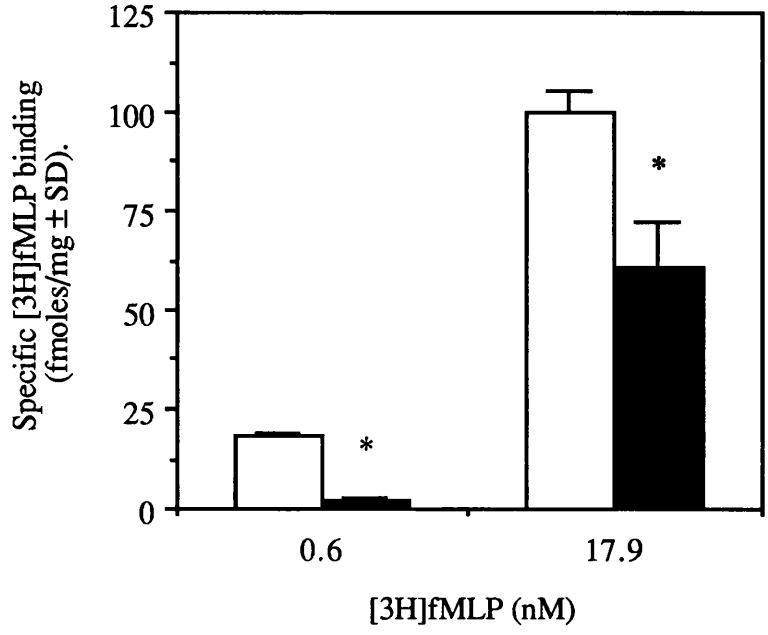
THE EFFECTS OF PERTUSSIS TOXIN AND Gpp[NH]p UPON SPECIFIC [3H]fMLP BINDING TO DIFFERENTIATED U937 CELL MEMBRANES.

Panel A represents membranes (200μg for the "high" affinity site and 100μg for the "low" affinity site) from either untreated 72 hour DMSO (1.25% v/v)-differentiated cells (□) or from cells pretreated *in vivo* with pertussis toxin (50ng/ml, 16 hours), (■), incubated with 0.6nM and 17.9nM [3H]fMLP as indicated. The data are means ±SD taken from quadruplicate determinations for the untreated cell membranes tested at 0.6nM and triplicate determinations for the other conditions represented. The pertussis toxin-induced reduction in specific [3H]fMLP binding observed at 0.6nM was statistically significant (* $p < 0.001$, $n = 5$), as was that at 17.9nM (*$p < 0.01$, $n = 4$).

Panel B represents 72 hour DMSO (1.25% v/v)-differentiated cell membranes (300μg for the "high" affinity site and 100μg for the "low" affinity site) incubated with 0.44nM and 23.1nM [3H]fMLP as indicated. Incubations were conducted in the absence (□) or presence (■) of Gpp[NH]p (100μM final) and binding experiments performed. The data are means ±SD taken from triplicate determinations for the cell membranes tested at 0.4nM [3H]fMLP and quadruplicate determinations for those tested at 23.1nM [3H]fMLP. The Gpp[NH]p-induced reduction in specific [3H]fMLP binding observed at 0.4nM was statistically significant (*$p < 0.001$, $n = 4$), as was that at 23.1nM (* $p < 0.001$, $n = 6$).

In both A and B specific [3H]fMLP binding was determined as described in 2.14, in the presence of the protease inhibitors described in 2.2.6.B. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.B. Both A and B are single experiments representative of two others which gave essentially identical results. Panel C is described overleaf.

A


125
100
75
50
25
0
Specific [3H]fMLP binding
(fmoles/mg ± SD).
*
*
0.6
17.9
[3H]fMLP (nM)

B

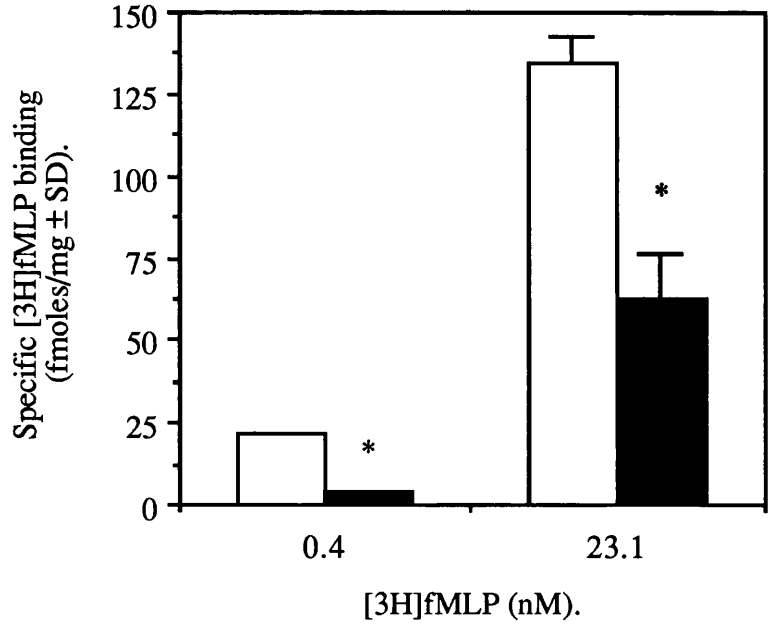

150
125
100
75
50
25
0
Specific [3H]fMLP binding
(fmoles/mg ± SD).
*
*
0.4
23.1
[3H]fMLP (nM).

Figure 5.2. (continued).

Panel C represents 72 hour DMSO (1.25% v/v)-differentiated cell membranes (50μg) either from untreated (□) or cells pretreated with pertussis toxin *in vivo* (50ng/ml, 16 hours), (■), incubated with a single concentration of [3H]fMLP (20.0nM). The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.B. Incubations were conducted in the absence or presence of Gpp[NH]p (100μM final) as indicated and specific [3H]fMLP binding assays performed as described in 2.14, in the presence of the protease inhibitors described in 2.2.6.B. This experiment is representative of two others which gave essentially identical results.

C

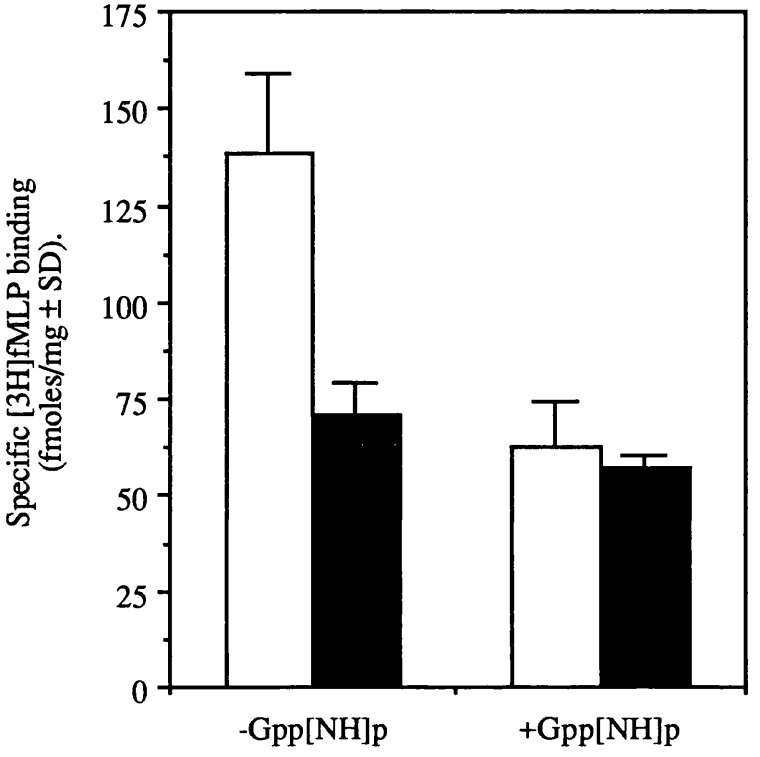

175
150
125
100
75
50
25
0
Specific [3H]fMLP binding
(fmoles/mg ± SD).
-Gpp[NH]p
+Gpp[NH]p

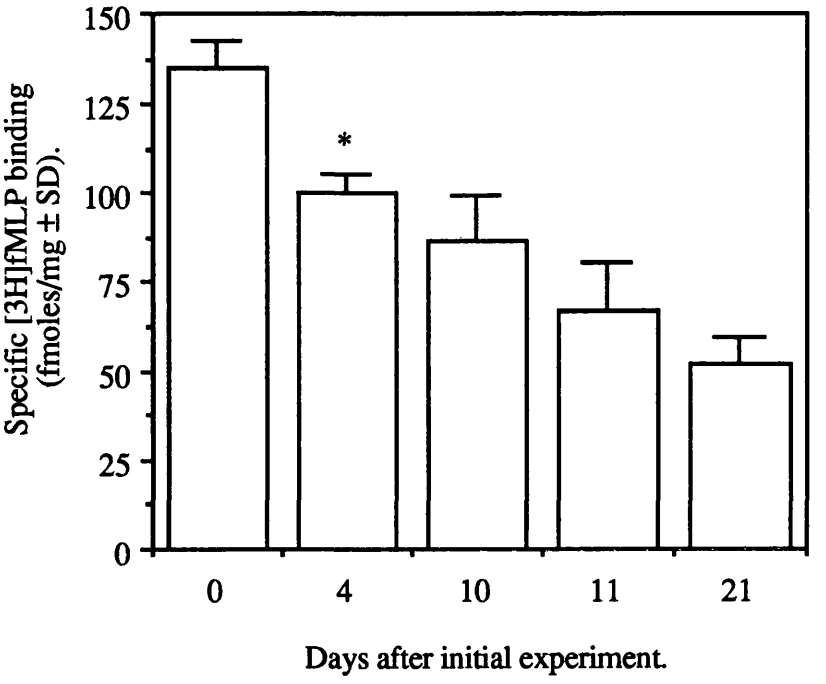
Figure 5.3.

COMPARISON OF THE ALTERATION IN SPECIFIC [3H]fMLP BINDING TO DIFFERENTIATED U937 CELL MEMBRANES WITH INCREASING PERIODS OF MEMBRANE STORAGE.

Panel A represents membranes (100μg) of 72 hour DMSO (1.25% v/v)-differentiated U937 cells incubated with [3H]fMLP (20.0 ± 2.0nM). Specific binding assays were performed as described in 2.14, in the presence of the protease inhibitors described in 2.2.6.B. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.B. The number of days after the initial experiment was performed was equivalent to the period of membrane storage at -80°C. The data represents mean ±SD from five separate experiments performed on different days but using the same preparation of membranes. The mean of quadruplicate determinations is represented for the experiments performed after 0, 10 and 11 days of membrane storage and triplicate determinations for the experiments performed after 4 and 21 days storage. The reduction in specific [3H]fMLP binding observed at 4 days was statistically significant ($*p < 0.002$, $n = 5$). The reduction in [3H]fMLP binding was significant to $p<0.001$ after all the other periods of membrane storage represented.

Panel B represents membranes (300μg) from the same preparation of 72 hour DMSO (1.25% v/v)-differentiated U937 cells incubated with [3H]fMLP (0.7 ± 0.3nM). Ligand binding assays were performed as for panel A. The mean of quadruplicate determinations is represented for the experiment performed after 11 days of membrane storage and triplicate determinations for the experiments performed after 0 and 38 days storage. The reduction in specific [3H]fMLP binding observed after 11 days was statistically significant ($*p < 0.01$, $n = 5$), as was that after 38 days membrane storage ($*p < 0.001$, $n = 4$).

A


150
125
100
75
50
25
0
*
0
4
10
11
21
Specific [3H]fMLP binding
(fmoles/mg ± SD).
Days after initial experiment.

B

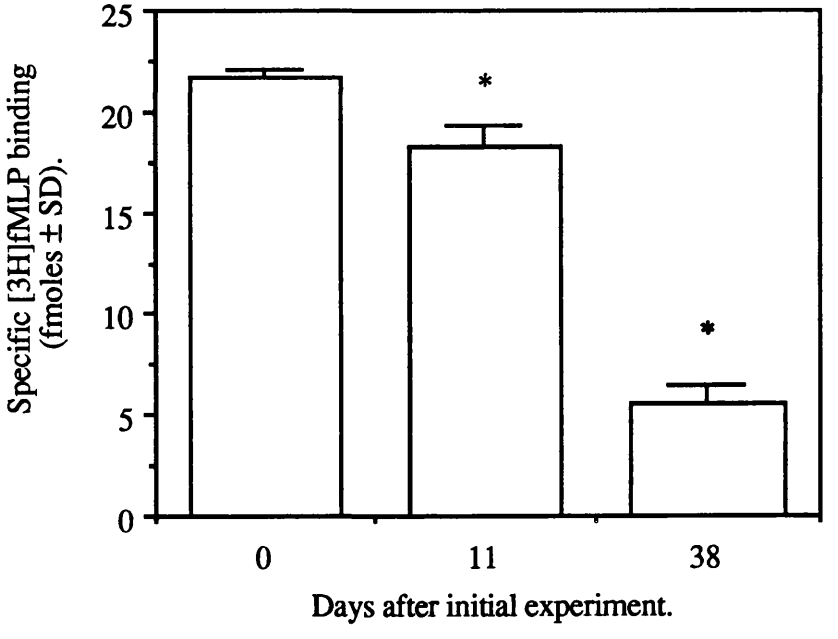

25
20
15
10
5
0
*
*
0
11
38
Specific [3H]fMLP binding
(fmoles ± SD).
Days after initial experiment.

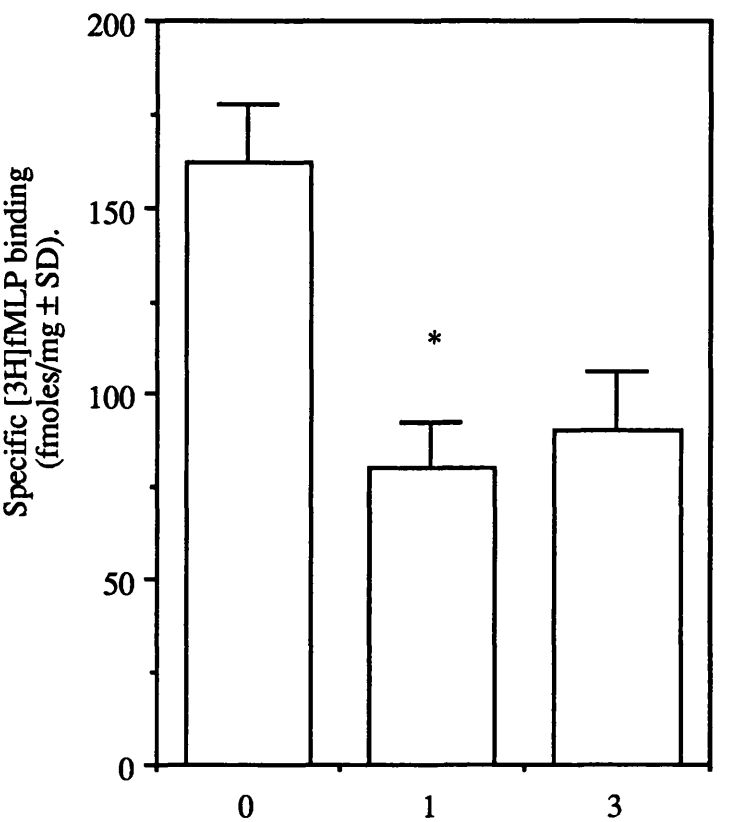
Figure 5.4.

MAINTENANCE OF BINDING OF [3H]fMLP TO MEMBRANES OF DIFFERENTIATED U937 CELL MEMBRANES.

Membranes (100μg) from 72 hour DMSO (1.25% v/v)-differentiated U937 cells were incubated with a single concentration of [3H]fMLP (21.7 ± 2.1nM) and ligand binding assayed as described in 2.14. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.B. The days after the initial experiment is equivalent to the period of membrane storage at -80°C.

The binding assays for the "0" and "1" day experiments were performed in the presence of the protease inhibitors described in 2.2.6.B. Whereas, in the experiment performed 3 days after the initial experiment the additional protease inhibitors described in 2.2.6.C were present in the assay and the membranes thawed in cold water.

The data represent means ±SD from quadruplicate determinations in single experiments performed with the same membrane preparation. The reduction in specific [3H]fMLP binding after 1 day of membrane storage at -80°C was statistically significant ($*p < 0.001$, $n = 6$).



Days after initial experiment.

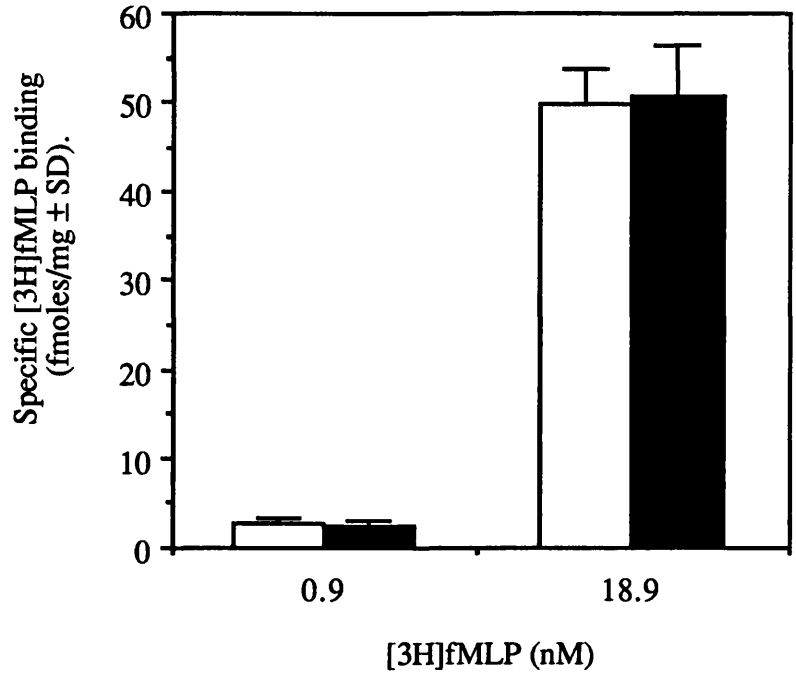
Figure 5.5.

THE EFFECT OF VARIOUS PREINCUBATION CONDITIONS UPON SPECIFIC [3H]fMLP BINDING IN DIFFERENTIATED U937 CELL MEMBRANES.

Panel A represents membranes (200μg) from 72 hour DMSO (1.25% v/v)-differentiated U937 cells preincubated at 25°C for 1 hour in the presence of binding assay buffer plus the protease inhibitors described in 2.2.6.C, but in the absence of ligand as described in 2.14. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.C. After preincubation, specific binding assays were performed using two concentrations of [3H]fMLP (as indicated) in the absence (□) and presence (■) of Gpp[NH]p (100μM final) as described in 2.14. The data are means ±SD taken from quadruplicate determinations in a single experiment representative of two experiments which gave essentially identical results.

Panel B represents membranes (200μg) of 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes which were incubated on ice for 30 hours or 5 hours, as indicated, in the presence of binding assay buffer plus protease inhibitors, as described for panel A. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.C. After preincubation, binding assays were performed using a single concentration of [3H]fMLP (16nM) in the absence (□) and presence (■) of Gpp[NH]p (100μM final) and the presence (▨) of the fMLP antagonist, t-BocMLP (100μM final). The data are means ±SD taken from triplicate determinations in a single experiment which was performed once. The Gpp[NH]p-induced reduction in the specific [3H]fMLP binding observed after 30 hours preincubation was statistically significant ($*p < 0.05$, $n = 4$), as was that observed after 5 hours preincubation ($*p < 0.01$, $n = 4$).

A


60
50
40
30
20
10
0
Specific [3H]fMLP binding
(fmoles/mg ± SD).
0.9
18.9
[3H]fMLP (nM)

B

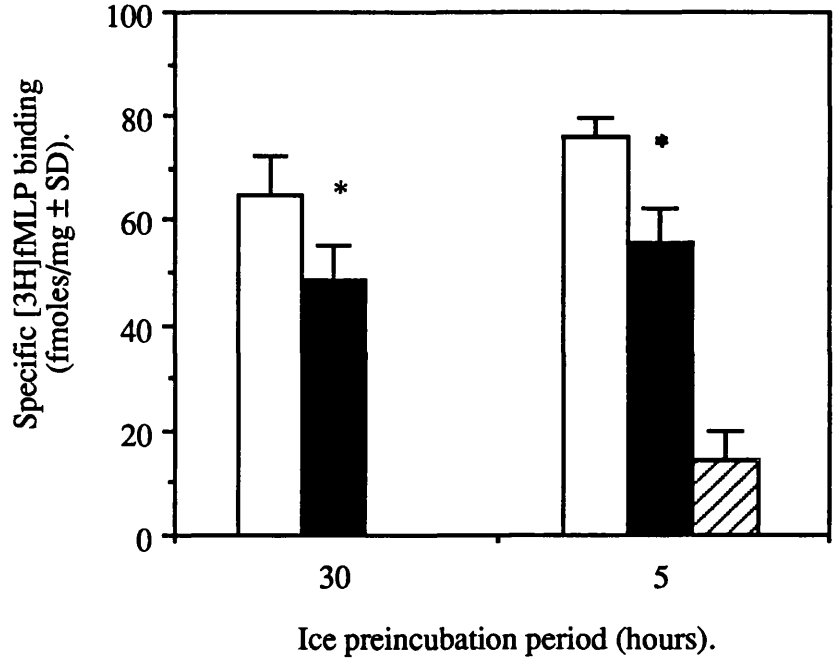

100
80
60
40
20
0
Specific [3H]fMLP binding
(fmoles/mg ± SD).
*
*
30
5
Ice preincubation period (hours).

Figure 5.6.

THE EFFECT OF PREINCUBATION OF MEMBRANES WITH ANTIPEPTIDE ANTISERA UPON THE SPECIFIC [3H]fMLP BINDING IN DIFFERENTIATED U937 CELL MEMBRANES .

Membranes (150μg) of 72 hour DMSO (1.25% v/v)-differentiated U937 cell membranes were preincubated on ice for 5 hours in the presence of binding assay buffer plus the protease inhibitors described in 2.2.6.C, but in the absence of ligand as described in 2.14. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.C.

After preincubation, specific binding assays were performed using a single concentration of [3H]fMLP (16nM) as described in 2.14. The binding assays were performed in the absence (1) or presence (2) of Gpp[NH]p (100μM final) or in presence of IgG fractions isolated from normal preimmune rabbit serum (3-5), antiserum AS7 (anti-$G_i2\alpha$) in (6) or antiserum I3B (anti-$G_i3\alpha$) in (7). The IgG fractions were prepared as in 2.6 and added at 15μg/150μg membrane in (3), (6) and (7), 20μg/150μg membrane in (4) and 40μg/150μg membrane in (5). Data are presented as means ±SD for quadruplicate determinations in a single experiment which was performed once. The Gpp[NH]p-induced reduction in [3H]fMLP binding was statistically significant ($*p < 0.002$, $n = 6$).

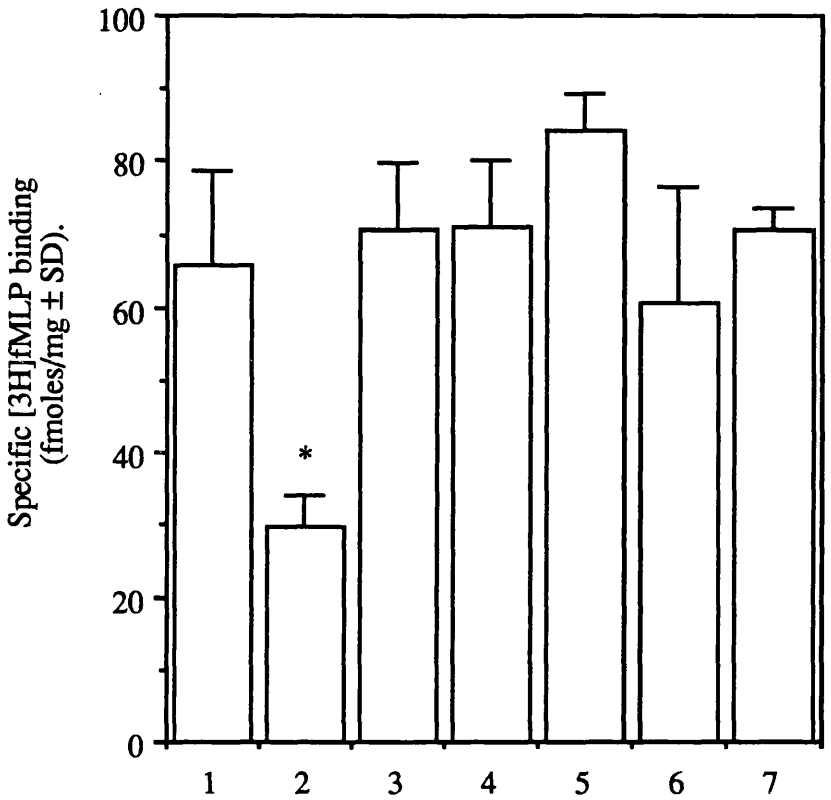

Specific [3H]fMLP binding
(fmoles/mg ± SD).
100
80
60
40
20
0
*
1
2
3
4
5
6
7

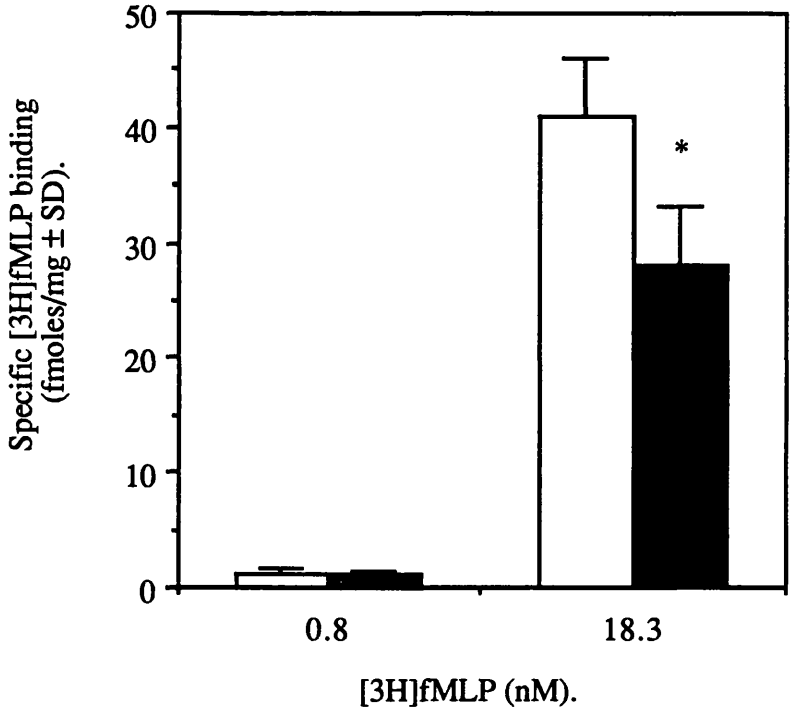
Figure 5.7.

THE EFFECT OF Gpp[NH]p AND AN fMLP ANTAGONIST UPON [3H]fMLP BINDING IN CONTROL U937 CELL MEMBRANES.

In panel A membranes (100μg) from control, undifferentiated U937 cells were incubated with two (less than apparent Kd) concentrations of [3H]fMLP, 0.8nM and 18.3nM in the absence (□) and presence (■) of Gpp[NH]p (100μM final). A specific [3H]fMLP binding reaction was performed as in 2.14, in the presence of the protease inhibitors described in 2.2.6.C. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3. C. The data are means ±SD taken from quadruplicate determinations in a single experiment representative of two others which gave essentially identical results. The reduction in [3H]fMLP binding observed at 18.3nM was statistically significant ($*p < 0.02$, $n = 6$).

In panel B membranes (100μg) from control, undifferentiated U937 cell membranes were incubated with a single, less than apparent Kd concentration of [3H]fMLP (17.1nM) in the absence (□) and presence (■) of the fMLP antagonist t-BocMLP (100μM final). A specific [3H]fMLP binding assay was performed as described for panel A. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3. C. The data are means ±SD taken from quadruplicate determinations in a single experiment which was representative of two other experiments performed with different membrane preparations.

A


Specific [3H]fMLP binding
(fmoles/mg ± SD).
50
40
30
20
10
0
*
0.8
18.3
[3H]fMLP (nM).

B

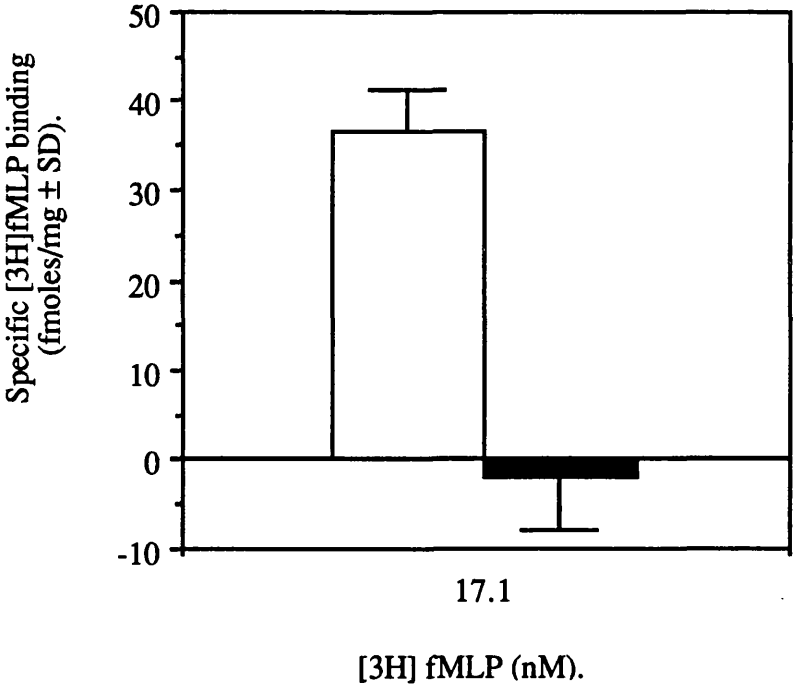

Specific [3H]fMLP binding
(fmoles/mg ± SD).
50
40
30
20
10
0
-10
17.1
[3H] fMLP (nM).

Figure 5.8.

THE EFFECT OF PERTUSSIS TOXIN UPON THE fMLP-STIMULATED GTPase ACTIVITY IN CONTROL AND DIFFERENTIATED U937 CELL MEMBRANES.

Membranes (10μg) from control (panels A and B) and 72 hour DMSO (1.25% v/v)-differentiated U937 cells (panels C and D) were stimulated with fMLP (10^{-5}M) and high affinity GTPase activity measured as described in 2.13. Panels B and D represent membranes from cells pretreated with pertussis toxin (16 hours, 50ng/ml). The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.B. (□) = basal GTPase activity, (■) = fMLP (10^{-5}M final)-stimulated GTPase activity and (▨) = DMSO (0.1% v/v final)-stimulated GTPase activity.

Results are the means ± SD of a triplicate determination for the fMLP-stimulated condition in DMSO-differentiated membranes but quadruplicate determinations for all the other conditions represented. The data are from a single experiment, representative of three others which gave essentially identical results. The stimulation of GTPase activity by fMLP in the DMSO-differentiated membranes, relative to that stimulated by the vehicle (DMSO), was statistically significant ($*p < 0.01$, $n = 5$) and is represented in "C".

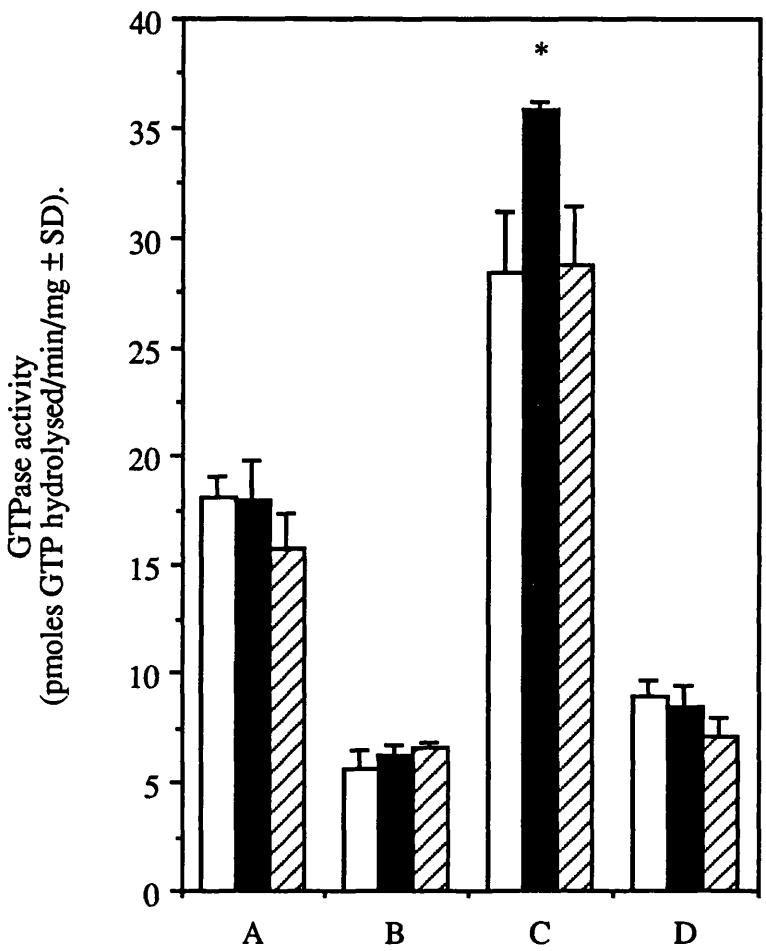

GTPase activity
(pmoles GTP hydrolysed/min/mg ± SD).
40
35
30
25
20
15
10
5
0
*
A
B
C
D

Figure 5.9.

THE EFFECT OF ICE PREINCUBATION UPON fMLP-STIMULATED GTPase ACTIVITY IN DIFFERENTIATED U937 CELL MEMBRANES.

Membranes (10μg) from 72 hour DMSO (1.25% v/v)-differentiated U937 cells were assayed for fMLP (10^{-5}M)-stimulated high affinity GTPase activity as described in 2.13. The cells were treated with DMSO as described in 2.2.3 and membranes were prepared as in 2.3.B. Panels A and B represent membranes preincubated for 75 minutes on ice and at 37°C, respectively as described in 2.13. (□) = basal GTPase activity, (■) = fMLP(10^{-5}M final)-stimulated GTPase activity and (▨) = DMSO (0.1% v/v final)-stimulated GTPase activity.

Results are the means ± SD of quadruplicate determinations, with the exception of the DMSO-stimulated GTPase activity in "A" which represents mean ±SD of a triplicate determination. The stimulation of GTPase activity by fMLP after ice preincubation, relative to DMSO-stimulated GTPase activity, was statistically significant ($*p < 0.02$, $n = 5$). This experiment was performed once.

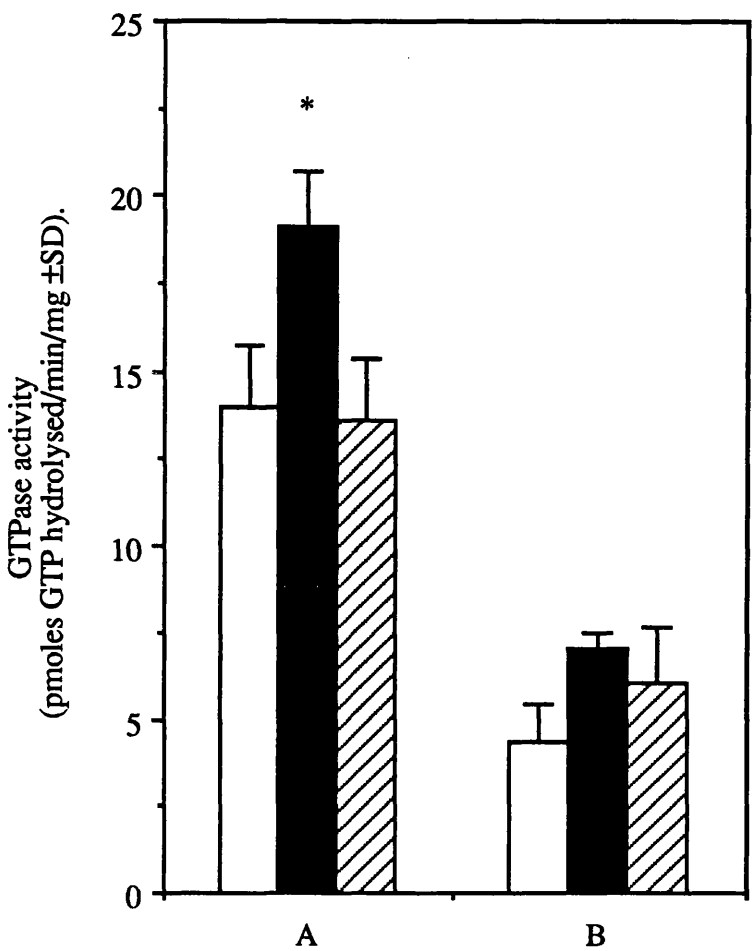
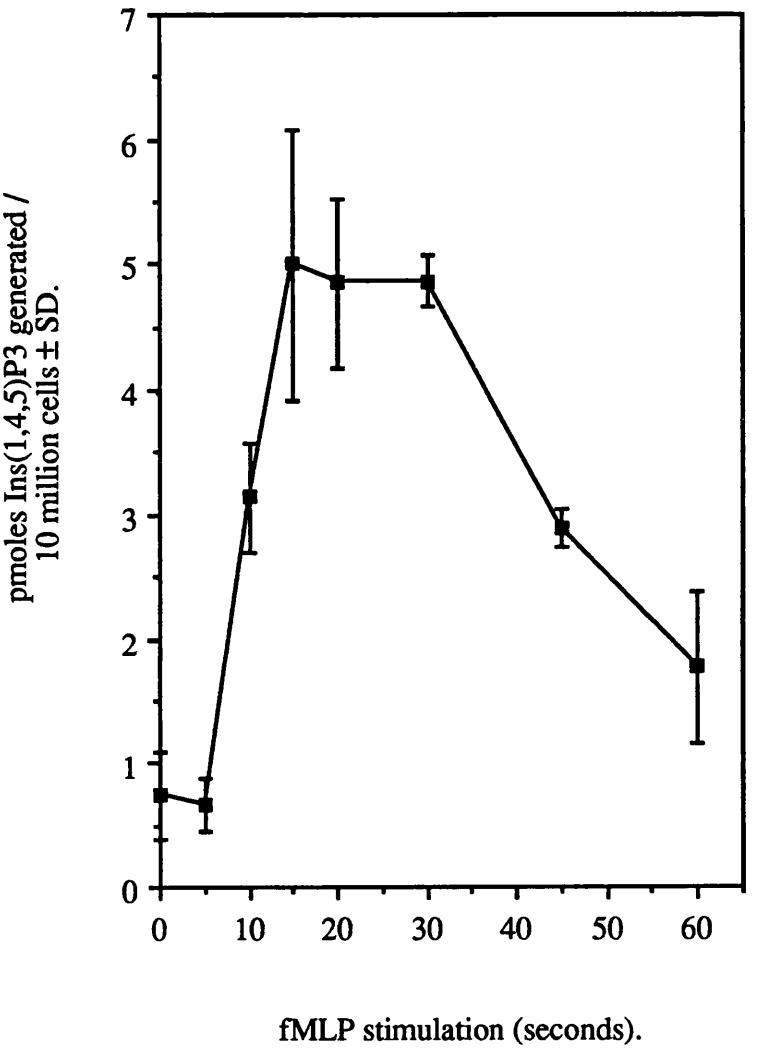

25
20
15
10
5
0
*
GTPase activity
(pmoles GTP hydrolysed/min/mg ±SD).
A
B

Figure 5.10.

MASS MEASUREMENT OF fMLP-STIMULATED Ins(1,4,5)P3 GENERATION IN DIFFERENTIATED U937 CELLS - TIME COURSE OF STIMULATION.

72 hour DMSO (1.25% v/v)-differentiated U937 cells (3.55 x 10^7 cells/sample) were stimulated with fMLP(10^{-5}M final) for the times indicated and mass Ins(1,4,5)P_3 measurements performed as described in 2.15. The cells were treated with DMSO as described in 2.2.3. The data are means ±SD taken from triplicate determinations in a single experiment representative of three others which gave essentially identical results.


pmoles Ins(1,4,5)P3 generated /
10 million cells ± SD.
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
fMLP stimulation (seconds).

5.3. DISCUSSION.

A study of the coupling specificity of the δ opioid receptor in neuroblastoma x glioma NG108-15 cell membranes using C-terminally directed antipeptide antisera was introduced previously. This work demonstrated that a convenient screen for the "uncoupling" effects of various antipeptide antisera was to measure the specific binding of a single concentration of the [3H] ligand, close to the Kd for that ligand. This concentration was chosen as representing that most sensitive to alterations in ligand binding as a reflection of receptor status (McKenzie & Milligan, 1990).

Saturation binding analysis of [3H]fMLP binding in 72 hour DMSO-differentiated U937 cell membranes was performed to provide an initial indication of binding parameters, in particular to estimate the Kd value(s). The assay incubation conditions were similar to those used by other investigators when investigating equilibrium binding parameters (Koo *et al.*, 1982; Herrmann *et al.*, 1989). Initial experiments indicated complex saturation binding phenomena, as was suspected from the extensive literature implicating a two affinity state for the fMLP receptor. Transformation of the binding data using a variation of the plot which Scatchard derived, resulted in a concave curve (Scatchard, 1949). The biological interpretations of such an observation include several possibilities namely; that negative cooperativity exists such that the affinity of the overall receptor population decreases with increasing occupancy of the receptors, that there are multiple, independent binding sites with unchanging and dissimilar affinities or that multiple interconvertible affinity states of the receptor exist. It was further appreciated that there are technical problems that can cause the artifactual appearance of such plots and that more experimentation was required before apparent Kd values and receptor densities from these arbitrarily defined "two populations" of binding sites could be justifiably calculated.

However, a considerable limiting factor for these binding studies was that the low receptor number necessitated the use of a large amount of membrane

protein. Thus preliminary studies were aimed towards establishing the feasibility of specifically uncoupling the fMLP receptor with antipeptide antisera prior to a more rigorous investigation of [3H] fMLP binding parameters. As such, the initial estimations of apparent Kd values of 2.4 ± 1.1nM (mean ± range of 2 experiments) for the "higher" affinity site and 40.1 ± 3.3nM (mean ± range of 2 experiments) for the "lower" affinity site were used as a basis to select single concentrations of [3H]fMLP that might be most sensitive to any potential alteration in receptor binding affinity i.e., at or lower than these apparent Kd values. These values do agree with the previously reported apparent Kd values for the fMLP receptor when investigating cell membranes, which range from 0.5-2nM and 20-40nM for the "high" and "low" affinity sites respectively (Koo *et al.*, 1983; Synderman *et al.*, 1984; Gierschik *et al.*, 1989b). Indeed, as previously discussed, in 72 hour DMSO-differentiated whole cells a fMLP receptor with a Kd of 20nM was observed (Kay *et al.*, 1983).

Mimicking the now classical approaches to implicate a receptor - G-protein coupling event, specific [3H]fMLP binding at both "high" and "low" affinity sites was reduced by preincubation of differentiated U937 cell membranes with the non hydrolysable analogue of GTP, Gpp[NH]p or upon pertussis toxin pretreatment of the membranes. These perturbing agents were non additive in their action, suggesting that they shared a common target coupling protein(s). Interestingly a lower affinity state of the so-called "low" affinity fMLP receptor could be expressed although it is possible that this reflects affinity "shifts" at the higher affinity binding site. There is some precedence for this in that studies of membranes of the HL-60 cells, differentiated to neutrophil-like cells, demonstrated that expression of the "high" affinity fMLP receptor was prevented by using micromolar concentrations of magnesium and that low affinity fMLP receptors were fully capable of functionally interacting with and activating the G-protein (Gierschik *et al.*, 1989b).

Obviously without performing full saturation binding experiments it can

only be suggested that one feasible explanation for the effect of these agents in causing reductions in radioligand binding may reflect their ability to uncouple the fMLP receptor from a pertussis toxin-sensitive G-protein, inducing an "isolated" receptor state which displays a lower agonist affinity than the G-protein coupled state of the receptor. In most studies, a decrease in receptor affinity reflects an increase in ligand dissociation rate (Delean & Robard, 1979). This has been demonstrated for the fMLP on human PMN membranes, where Gpp[NH]p was found to accelerate the dissociation rate of [3H]fMLP (Koo *et al.*, 1983).

These preliminary observations not only suggested that there was at least the potential to utilise the IgG fractions from C-terminally directed anti-Gα, antipeptide antisera to more specifically perturb receptor - G-protein coupling but also indicated that receptor degradation was occurring upon prolonged storage of the membrane preparations. This problem was overcome by use of freshly prepared membranes and the inclusion of additional protease inhibitors in the incubation buffer and subsequently throughout the cell harvesting and membrane preparation procedures.

Previous studies using these antipeptide antisera to interfere with the coupling of receptor - G-protein have established that preincubation of the membranes with the antisera is necessary. Obviously for these studies to be meaningful it is essential that the preincubation conditions *per se* do not merely perturb G-protein coupling interactions. This can be assessed by examining the ability of Gpp[NH]p to decrease the agonist affinity for the receptor after particular preincubation conditions. To limit membrane protein expenditure a single, "less than Kd" concentration of radioligand was tested and only the "low" affinity site examined, which required less membrane protein/assay. It was observed that G-protein coupling to the fMLP receptor in freshly prepared U937 cell membranes was destroyed by a 25°C incubation, but maintained by ice preincubations of 5 and 30 hours. As demonstrated in Chapter 3, U937 cells express the pertussis toxin substrates, $G_i2\alpha$ and $G_i3\alpha$. Thus IgG fractions of antipeptide antisera raised

against the C-terminal decapeptides of these proteins were employed. Previous studies using identical antipeptide antisera have established that 10μg of an IgG fraction of an antipeptide antiserum per 100μg of membrane protein is sufficient to uncouple both the δ-opioid receptor and the α_{2B} receptor from $G_i2\alpha$ in membranes from NG108-15 glioma cells (McKenzie & Milligan, 1990; McClue & Milligan, 1990). However, in a preliminary investigation equivalent concentrations of IgG fractions from antipeptide antisera capable of recognising both of the known pertussis toxin substrates expressed in U937 cells in a mutually exclusive manner and preimmune serum did not alter the specific binding of [^{3}H]fMLP to membranes preincubated with these antisera. It was appreciated that the high level of expression of the $G_i\alpha$ proteins, particularly $G_i2\alpha$, relative to other systems, the low level of expression of fMLP receptors and in general the high degradative activity that is inherent in U937 cell maturation are properties that may render the antipeptide antisera uncoupling approaches problematic. Indeed, the relatively high level of expression of the $G_i\alpha$ proteins may necessitate preincubation of membranes with higher concentrations of antipeptide antiserum than that found to be successful in other systems. In preparation for this possibility increased concentrations of preimmune serum did not alter fMLP binding. It was further appreciated that the kinetics of antigen-antiserum interaction are also likely to be altered by preincubation of membranes and antisera at 4^{o}C rather than at 37^{o}C. As such the 5 hour preincubation period used in these preliminary investigations may simply have been insufficient to enable any potential "uncoupling" interaction to occur.

Interestingly, contradictory to previous reports freshly prepared membranes from control, non-DMSO treated U937 cell membranes did exhibit [^{3}H]fMLP binding at the so-called "low" affinity site, albeit on average at approximately 30% of the level of binding in differentiated cell membranes. Even more surprisingly this binding was decreased by preincubation of the membranes with Gpp[NH]p and totally inhibitable by preincubation with the fMLP antagonist, N-t-butoxycarbonyl-Met-Leu-Phe (t-bocMLP). Although somewhat preliminary there are some

interesting points raised by these observations. Undifferentiated U937 cells have previously been shown not to exhibit the range of biological responses elicited by chemoattractants. They do not respond to fMLP chemotactically nor does this peptide enhance superoxide anion formation or stimulate enzyme secretion (Kay *et al.*, 1983). More specifically even if the U937 cells used in these experiments differed from those of other groups, this "clone" of undifferentiated U937 cells did not produce an fMLP-stimulated increase in inositol phosphate generation (Pollock *et al.*, 1990). The "lesion" was suggested to not be due to the phospholipase itself, as both control and differentiated cells displayed a similar increase in inositol phosphate generation in response to the calcium ionophore ionomycin, which acts to directly activate the phospholipase and "bypass" the receptor - G-protein input. Viewed simplistically, this "lesion" could be a reflection of the low level of fMLP binding site expressed or indeed the lack of the "correct" receptor affinity state. Indeed the transductional mechanisms of the chemoattractant receptor can be divided into motility related functions that require low doses of chemoattractant and secretory functions that require of the order of 20 fold higher concentrations of chemoattractant. Interestingly the EC_{50} for fMLP-stimulated inositol phosphate generation is similar to the equilibrium dissociation constant of the lower affinity state of the receptor. From these preliminary experiments it is apparent that this "low" affinity receptor is coupling to a G-protein as a Gpp[NH]p-mediated reduction in [^{3}H]fMLP binding occurred in the absence of expression of any high affinity binding sites. These observations do suggest that there is fMLP receptor - G-protein interaction in undifferentiated cell membranes. Further study of these undifferentiated U937 cell membranes may have indicated why fMLP-stimulated phosphoinositidase C activity was not observed and as such may have proved informative with respect to definition of the components involved in fMLP-stimulated phosphoinositidase C activation and indeed the stoichiometry of their interaction.

An alternative approach towards defining the coupling specificity between a receptor and a G-protein has been to use antipeptide antisera to "uncouple" agonist-stimulated GTPase activity. Using the U937 cell system, a fMLP-stimulated GTPase activity was only observed in differentiated cell membranes and was totally inhibited by pertussis toxin pretreatment of these membranes. Control membranes did not exhibit a fMLP-stimulated GTPase activity, which is interesting with respect to the previously discussed binding data. The basal GTPase activity was on average approximately two fold greater in the differentiated U937 cell membranes relative to the control membranes, which may reflect the previously presented increased expression of Gα subunits that may be contributing to this basal GTP hydrolytic activity. Indeed a pertussis toxin substrate(s) presumably is responsible for a large proportion of this basal GTPase activity, as pretreatment of membranes with this toxin results in, on average, a 70% decrease in this activity. As discussed briefly in Chapter 4 and again demonstrated by this pertussis toxin-sensitive basal GTPase activity, it would appear that G-proteins interact with and are activated by receptors even in the absence of agonists.

Although, as in the binding studies, ice preincubation did preserve the fMLP-stimulated GTPase response initial attempts using antipeptide antisera to specifically uncouple this response produced highly variable results (data not included). Again as for the binding studies, it was appreciated that there were a number of factors that would require further detailed study before this approach could be potentially useful in addressing the specificity of G-protein interaction with the fMLP receptor. Interestingly, the membranes used in these GTPase experiments were prepared in the presence of protease inhibitors, according to the method outlined in 2.3.B. When attempts were made to repeat this work in membranes prepared in the presence of additional inhibitors (as in 2.3.C), extremely low levels or in some cases no fMLP-stimulated GTPase activity was observed. This was despite the knowledge that in such preparations the G-proteins

expressed remained intact and the membranes exhibited a Gpp[NH]p-sensitive [3H]fMLP binding. Such reduction in agonist binding is classically believed to be a consequence of G-protein activation. However studies of the fMLP receptor in HL-60 membranes have demonstrated that guanine nucleotide occupancy and not G-protein activation is the critical determinant for a guanine nucleotide induced alteration of receptor binding properties (Gierschik *et al.*, 1989b). Thus it perhaps cannot be assumed that the G-proteins in this preparation were in a functionally active state and despite intensive effort the reason for loss of GTPase activity was not resolved. Indeed these observations may even provide an explanation for the lack of success of the latter experiments performed with cholera toxin in Chapter 4, as this reaction is proposed to be dependent upon agonist-driven release of bound guanine nucleotide, an initial step in the GTPase response.

In an attempt to address coupling specificity in the presence of the effector system i.e., functional phosphoinositidase C, mass measurements of Ins(1,4,5)P_3 were determined in whole cells using a specific binding assay. This assay was pioneered by Bradford and Rubin, who competed [^{32}P]Ins(1,4,5)P_3 with Ins(1,4,5)P_3 of extracts prepared from unstimulated and fMLP-stimulated neutrophils for the intracellular Ins(1,4,5)P_3 receptor of saponin-permeabilised neutrophils. Intracellular Ins(1,4,5)P_3 concentrations were found to be 0.05 pmoles/10^6 cells in control cells and rose to 0.55 pmoles/10^6 cells in fMLP-stimulated cells (Bradford & Rubin, 1986). The assay used in this study involves the competition of high specific activity [3H]Ins(1,4,5)P_3 with "cold" Ins(1,4,5)P_3, derived from the cells or tissue under investigation, for Ins(1,4,5)P_3-specific binding sites in adrenocortical membranes (Palmer *et al.*, 1989). Indeed a number of studies have made use of the Ins(1,4,5)P_3-specific binding assay (reviewed by Palmer & Wakelam, 1989).

Initial objectives in using the Ins(1,4,5)P_3-specific binding assay in this study were to confirm the pertussis toxin sensitivity of the fMLP-stimulated

generation of this second messenger and to expand upon an observation made by Iiri and co-workers. The cholera toxin-catalysed ribosylation of Giα has been described in detail in Chapter 4, however it is of note that Iiri and co-workers observed this effect in whole HL-60 cells stimulated with fMLP. They observed that *in vitro* fMLP-stimulated cholera toxin-induced [^{32}P]ADP-ribosylation of Giα was inhibited markedly by prior exposure of the membrane-donor HL-60 cells to the same toxin and chemotactic peptide (Iiri *et al.*, 1989). That this reaction can occur in a whole cell is perhaps somewhat surprising if, as has been well established, a "nucleotide free" Giα substrate is a prerequisite for cholera toxin as clearly free guanine nucleotide cannot be simply "washed" out of the cell in these circumstances. However it has been reported that induced maturation of the HL-60 cell line is associated with a depletion of intracellular GTP and GDP, whereas adenylate pools remain relatively unchanged (Lucas *et al.*, 1983). Indeed some investigators argue that such depletion of guanine nucleotide pools is involved in the regulation of induced myeloid cell maturation (Wright, 1987). Thus it may be somewhat fortuitous that this cholera toxin-catalysed ADP-ribosylation of the G-proteins coupling to the fMLP receptor is observed in this whole cell system.

Thus mass measurements of Ins(1,4,5)P_3 were made in the DMSO-differentiated U937 cell system with a view to testing the effect of prior exposure of whole cells to cholera toxin in the presence of fMLP, upon the fMLP-stimulated Ins(1,4,5)P_3 levels. The ribosylated G-proteins could then be identified, after membrane preparation from these cells, as described in Chapter 4. Obviously there were a number of assumptions inherent in this approach not least that Giα would be ADP-ribosylated by cholera toxin in the intact U937 cell and indeed that such a modification would perturb coupling with its potential phosphoinositidase C effector system. Another consideration was that the initial challenge with fMLP may desensitise the receptor. Interestingly after completion of these experiments more recent work by Iiri and co-workers demonstrated that in cell membranes cholera toxin-catalysed ribosylation of Gi2α actually enhanced the ability of this

protein to reconstitute high affinity fMLP binding, although did reduce its ability to hydrolyse GTP (Iiri *et al.*, 1992). In retrospect the proposed approach may have proved difficult to interpret with respect to G-protein - effector coupling specificity.

Unfortunately Ins(1,4,5)P_3 mass measurements were limited only to determining a time course of fMLP stimulation. The basal and fMLP-stimulated Ins(1,4,5)P_3 levels established were in close agreement with other studies of leukocytic cells (Bradford & Rubin, 1986; Michell *et al.*, 1989). It was of interest that although Ins(1,4,5)P_3 was clearly elevated after 10 seconds the response peaked at between 15-20 seconds of fMLP stimulation. This agreed with previous observations (Pollock *et al.*, 1990). The response also appeared not to desensitise rapidly. It would have been interesting to establish whether these were fMLP-specific properties or general to agonists that stimulate generation of Ins(1,4,5)P_3 in these cells. However the relatively large cell number required per sample again limited the practicality of this approach.

In conclusion, the work of this chapter represents initial exploratory experiments aimed at developing alternative strategies towards defining the coupling specificity between the fMLP receptor, the G-protein(s) and the effector(s) in the U937 cell line. The effect of discriminatory antipeptide antisera upon the [^{3}H]fMLP binding to U937 cell membranes was discovered to be potentially the most feasible approach towards achieving this goal with alternative approaches found to be less practical using the U937 cell line.

CHAPTER 6.

IDENTIFICATION OF A G-PROTEIN COUPLING RECEPTORS TO PHOSPHOINOSITIDASE C IN RAT ADIPOCYTE MEMBRANES.

6.1. INTRODUCTION.

As introduced previously there are a number of systems other than leukocytic cells in which agonist-stimulated inositol (1,4,5) trisphosphate generation is sensitive to pertussis toxin (see 1.12.1). Indeed early studies demonstrated a number of effects of pertussis toxin on the metabolism of rat adipocytes. Moreno and co-workers found that the toxin interfered with adenosine mediated inhibition of cAMP accumulation, but potentiated the catecholamine stimulated increases in cAMP. Pertussis toxin further increased the stimulatory effects of insulin upon adipocyte metabolism and inhibited α_1-catecholamine stimulation of phosphatidylinositol turnover. Alterations in the specific activity of a number of membrane phospholipids was examined, with pertussis toxin treatment resulting in a complete inhibition of adrenaline-stimulated increase in the specific activity of phosphatidic acid and a 70% reduction in the effect of adrenaline upon the specific activity of phosphatidylinositol plus phosphatidylserine (Moreno *et al.*, 1983).

These effects were observed in the presence of propanolol, a β-adrenergic antagonist, included to inhibit the potential activation of adenylyl cyclase that results from adrenaline interaction with β-adrenergic receptors. Alpha$_2$-adrenergic receptors are known to be coupled to the inhibition of adenylyl cyclase in a variety of systems (Sabol & Nirenberg, 1979; Garcia-Sainz *et al.*, 1980). Interestingly, it has been observed that there are no α_2-adrenergic mediated responses in rat white adipocytes suggesting that these adrenaline-stimulated effects on phospholipid turnover are α_1-receptor mediated (Fain, 1980). In several systems, including the rat renal cortex, guanine nucleotides have been shown to alter the affinity of the α_1-adrenergic receptor for adrenaline, providing further evidence for a G-protein interaction with this receptor (Snavely & Insel, 1982).

In a more recent analysis fat cells were isolated from control and pertussis toxin-treated rats, metabolically labelled with [3H] inositol and then challenged with

adrenaline. Analysis of the [^{3}H] inositol phosphates by h.p.l.c. separation identified a totally pertussis toxin-sensitive generation of Ins(1,4,5)P_3 (Rapiejko *et* al., 1986).

However, although there are several lines of evidence to suggest that a pertussis toxin-sensitive G-protein mediates α_1-adrenergic stimulated generation of inositol phosphates in rat white adipocytes, the nature of the pertussis toxin substrates expressed by this tissue has been an area of considerable debate. A number of studies have confirmed the expression of "Gi-like" G-proteins in adipocytes (Rapiejko *et* al., 1986; Milligan *et al.*, 1987b; Ros *et al.*, 1988). However, conflicting results have been obtained when assessing whether Goα is expressed in adipocytes (Hinsch *et al.*, 1988; Ros *et al.*, 1988).

Interestingly, as discussed previously in Chapter 4, cholera toxin-catalysed ADP-ribosylation of "classical" pertussis toxin substrates was initially demonstrated in adipocyte membranes (Graves *et al.*, 1983). A study by Owens and co-workers extended these initial observations to demonstrate that a doublet of 39kDa and 41kDa polypeptides act as substrates for both bacterial toxins. Further, cholera toxin-catalysed [^{32}P]ADP-ribosylation did have functional consequences in that hormonal and GTP-mediated inhibition of adenylyl cyclase was attenuated (Owens *et al.*, 1985). However, the ability of agonists to stimulate the cholera toxin-catalysed [^{32}P]ADP-ribosylation of pertussis toxin substrates has not been investigated.

Thus it appeared that the rat adipocyte represented another system where approaches similar to those described in the previous Chapters could be employed towards definition of the nature of the pertussis toxin-sensitive G-protein coupling to agonist-stimulated phospholipid turnover. An initial aim was to define the nature of the pertussis toxin-sensitive G-proteins expressed in rat white adipocyte membranes, using selective antipeptide antisera and membranes from other rat systems in which the G-proteins have been well characterised. Secondly, the

ability of cholera toxin to catalyse an NAD^+-dependent [^{32}P]ADP-ribosylation of a substrate(s) other than $G_S\alpha$ was examined with a view to assessing whether α_1-adrenergic receptor stimulation could enhance this effect and hence implicate a particular G-protein in coupling to phosphoinositidase C in rat white adipocyte membranes.

6.2. RESULTS.

A. IMMUNOLOGICAL DETECTION OF Gi1, Gi2 AND Gi3α SUBUNITS.

Pertussis toxin catalysed the [^{32}P]ADP-ribosylation of two distinct polypeptides of approximately 40kDa in rat white adipocyte membranes (lane 2, Fig. 6.1). The more mobile polypeptide comigrated with the major pertussis toxin-sensitive substrate of rat glioma C6BU1 cell membranes which has been shown previously to correspond to Gi2α. The polypeptide with the lower mobility co-migrated with the major form of Giα from rat cerebral cortex, namely Gi1α (lanes 1-3, Fig. 6.1).

Antipeptide antiserum, SG1, which recognises both Gi1α and Gi2α, but not Gi3α, recognised two polypeptides in rat adipocyte membranes of approximately 40kDa (lane 2, Fig. 6.2). This antipeptide antiserum cannot discriminate between Gi1α and Gi2α, as the sequence used to generate the antipeptide antiserum is conserved between the two polypeptides. Within the same experiment only a single co-migrating 40kDa polypeptide was identified in membranes from C6BU1 glial cell membranes, although two co-migrating polypeptides were identified in rat cerebral cortex (lanes 1-3, Fig. 6.2). The relative staining of the 40kDa to 41kDa polypeptide (40 < 41) was in agreement with previous studies of Gi2α relative to Gi1α in brain membranes. Employing a discriminatory antipeptide antiserum specific for Gi2α, a single polypeptide was identified in rat adipocytes, glioma C6BU1 cells and cerebral cortex membranes (lanes 1-3, panel A, Fig. 6.3). The relative intensity of staining of this polypeptide was similar to that observed for the 40kDa polypeptide identified by the antipeptide antiserum SG1 (compare Fig. 6.2 and panel A, Fig. 6.3). Antipeptide antiserum I1A, which specifically recognises Gi1α, identified a single polypeptide of approximately 41kDa in membranes of rat cerebral cortex and adipocytes, which was absent from C6BU1 cell membranes (lanes 1-3, panel B, Fig. 6.3). Some

cross reacting, lower molecular weight proteins were evident, particularly in the rat cortical and glioma cell membrane preparations. The identity of these proteins was not investigated further (lanes 1 and 3, panel B, Fig. 6.3). Two mutually exclusive polypeptides were identified in rat adipocytes using a mixture of these selective antipeptide antisera. Rat cerebral cortex membranes expressed predominately $G_{i}1\alpha$ and C6BU1 cell membranes expressed only $G_{i}2\alpha$. Again cross reacting, lower molecular weight polypeptides were observed, presumably reflecting a property of the I1A antipeptide antiserum (lanes 1-3, panel C, Fig. 6.3). The identity of these proteins was not investigated further.

The pertussis toxin substrate, $G_{i}3\alpha$, has a slightly higher apparent molecular weight than $G_{i}1\alpha$ and was identified in both rat adipocytes and C6BU1 cell membranes (lanes 1 and 2, panel A, Fig. 6.4). A mixture of antisera LE2 and I3B, specific for $G_{i}2\alpha$ and $G_{i}3\alpha$ respectively, identified a doublet of polypeptides in both adipocytes and C6BU1 cells, demonstrating that these antisera identified mutually exclusive polypeptides (lanes 1 and 2, panel B, Fig. 6.4). The less mobile protein co-migrated with the single polypeptide identified as $G_{i}3\alpha$ by the antiserum I3B, whereas the more mobile protein co-migrated with the single polypeptide identified by LE2 (compare panels A-C, Fig. 6.4). Cross reacting proteins of lower molecular weight than the $G_{i}\alpha$ subunits were identified by LE2, although their identity was not investigated further (panels B and C, Fig. 6.4) The antipeptide antiserum, I3B, was raised against the C-terminal decapeptide of $G_{i}3\alpha$ as outlined in Table 2.1. As I3B does not recognise $G_{i}2\alpha$, then it is unlikely to recognise $G_{i}1\alpha$ which has an identical C-terminal decapeptide sequence to $G_{i}2\alpha$.

Antipeptide antiserum IM1, which specifically recognises $G_{o}\alpha$, detected an extremely weakly immunoreactive polypeptide in adipocyte membranes which co-migrated with a strongly immunoreactive polypeptide in rat brain membranes (compare lanes 1 and 3, Fig. 6.5). This protein was absent in membranes prepared from C6BU1 glioma cells (lane 2, Fig. 6.5). An approximately 25kDa cross

reacting polypeptide was evident in rat brain membranes and proteins of greater molecular weight than $G_o\alpha$ were observed in the glioma and adipocyte membrane preparations. The identity of these proteins was not investigated further (lanes 1-3, Fig. 6.5).

B. CHOLERA TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF Giα.

In the presence and absence of the guanine nucleotide, GTP, pertussis toxin catalysed the [^{32}P]ADP-ribosylation of a broad band of proteins of approximate 40kDa in molecular weight, presumably representing the immunologically identifiable G_i1, G_i2 and $G_i3\alpha$ subunits (lanes 2 and 5, respectively, Fig. 6.6). Cholera toxin, in the presence of GTP, catalysed the incorporation of [^{32}P]ADP-ribose into $G_s\alpha$44kDa and $G_s\alpha$42kDa. Under these conditions this toxin also catalysed incorporation of radioactivity into a polypeptide that co-migrated with the pertussis toxin substrates and a more mobile polypeptide, although to a much lesser extent relative to $G_s\alpha$ (lane 3, Fig. 6.6). In the absence of GTP, cholera toxin catalysed [^{32}P]ADP-ribosylation of the $G_s\alpha$ polypeptides although to a lesser relative extent than in the presence of GTP. However, there was a slight enhancement of the incorporation of [^{32}P]ADP-ribose into the 40kDa polypeptide and an apparent loss of [^{32}P]-labelling of the most rapidly migrating protein (lane 4, Fig. 6.6). However, this ribosylation pattern was not affected by the presence of propanolol (10^{-5}M final), or adrenaline plus propanolol (both at 10^{-5}M final), (lanes 6 and 7, Fig. 6.6).

Figure 6.1.

PERTUSSIS TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF MEMBRANE PROTEINS IN RAT TISSUES.

Membranes from rat glioma C6BU1 cells (50μg), rat white adipocytes (20μg) and rat cerebral cortex (20μg), in lanes 1-3 respectively were [^{32}P]ADP-ribosylated with thiol-activated pertussis toxin as described in section 2.11. The adipocyte membranes were a kind gift from Dr. E. D. Saggerson as described in 2.3.D and the other membranes were prepared as in 2.3.A. The samples were alkylated and resolved by SDS-PAGE (12.5% [w/v] acrylamide, 0.06% [w/v] bisacrylamide, 20cm x 20cm resolving gel) as described in 2.7.3. B and 2.7. The dried gel was autoradiographed for 48 hours, as described in 2.9.

Tentative identities of the pertussis toxin-sensitive polypeptides are based on their relative electrophoretic mobility and previous immunological studies (see results and discussion sections for details). This experiment is representative of two others performed with different membrane preparations which gave essentially identical results.

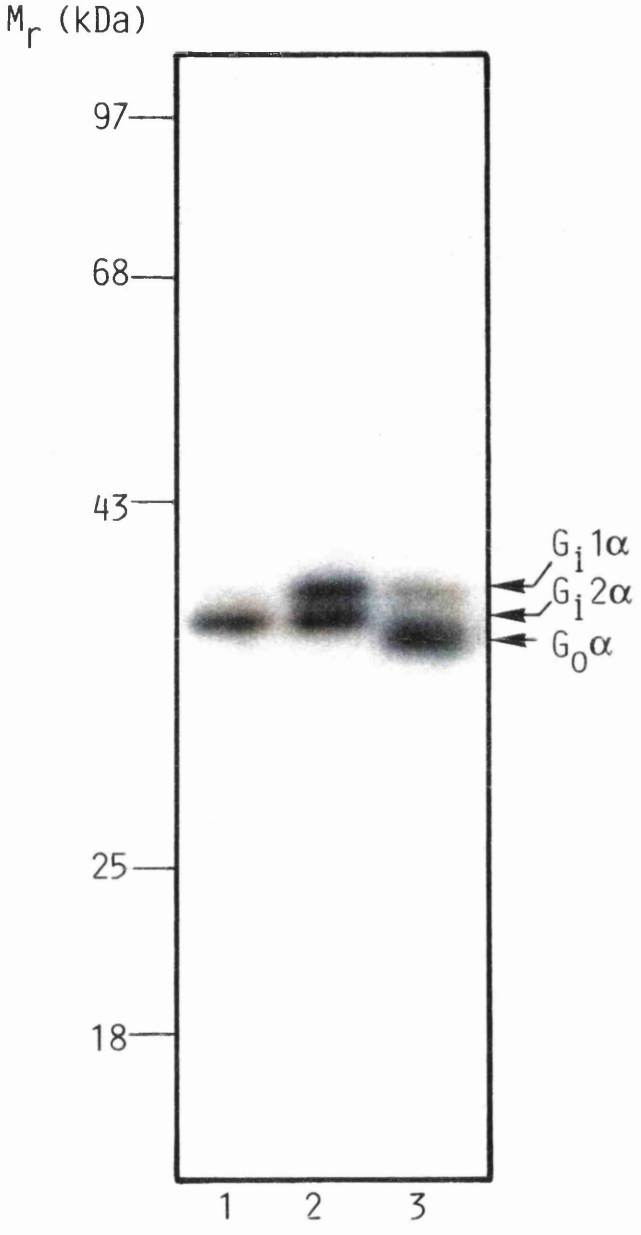

M_r (kDa)
97
68
43
25
18
$G_i1\alpha$
$G_i2\alpha$
$G_o\alpha$
1
2
3

Figure 6.2.

IMMUNOLOGICAL IDENTIFICATION OF Gi1α AND Gi2α IN RAT ADIPOCYTE MEMBRANES WITH AN ANTIPEPTIDE ANTISERUM THAT RECOGNISES BOTH Giα FORMS.

Membranes from rat glioma C6BU1 cells (30μg), rat white adipocytes (30μg) and rat cerebral cortex (100μg), in lanes 1-3 respectively, were alkylated and resolved by SDS-PAGE (12.5% [w/v] acrylamide, 0.06% [w/v] bisacrylamide, 20cm x 20cm resolving gel) as in 2.7.3. B and 2.7 and then immunoblotted as described in 2.10.2. A. The membranes were prepared as outlined previously in Fig. 6.1. The primary antiserum employed was SG1 (1: 200 dilution). This experiment is representative of two others performed with different membrane preparations which gave essentially identical results.

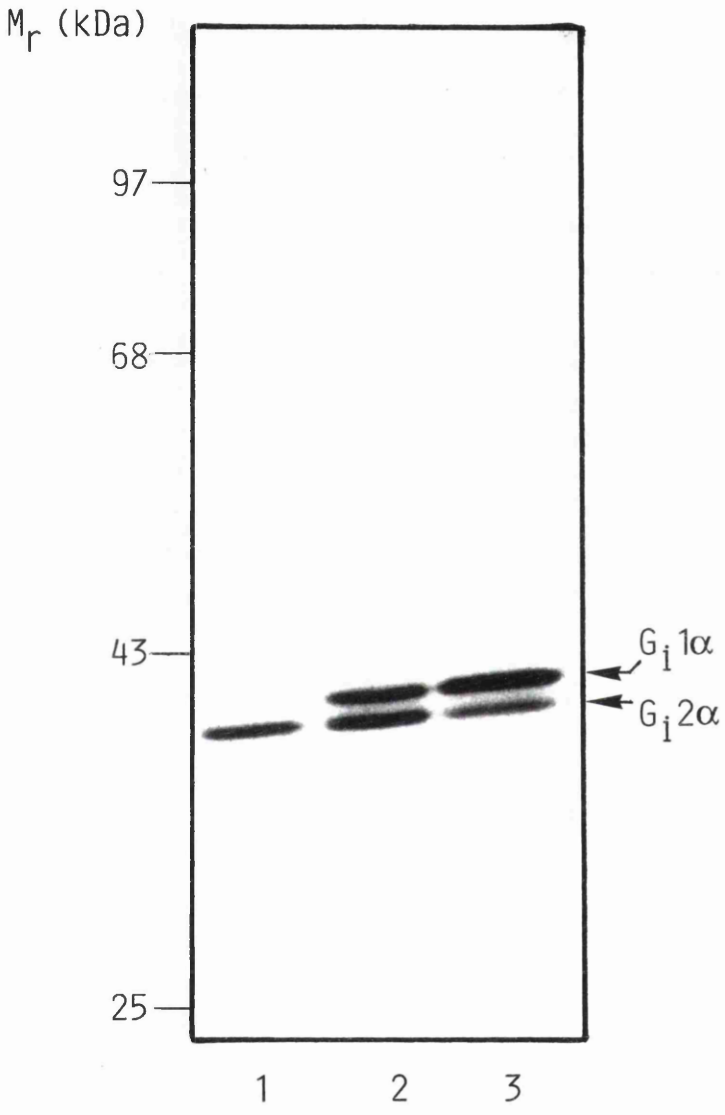

M_r (kDa)
97
68
43
25
$G_i1\alpha$
$G_i2\alpha$
1
2
3

Figure 6.3.

IMMUNOLOGICAL IDENTIFICATION OF BOTH Gi1α AND Gi2α IN RAT ADIPOCYTE MEMBRANES USING DISCRIMINATORY ANTIPEPTIDE ANTISERA.

Membranes from rat glioma C6BU1 cells, rat white adipocytes and rat cerebral cortex were alkylated and resolved by SDS-PAGE (12.5% [w/v] acrylamide, 0.06% [w/v] bisacrylamide, 20cm x 20cm resolving gel) as in 2.7.3. B and 2.7 and then immunoblotted as described in 2.10.2. A. The membranes were prepared as indicated in Fig. 6.1.

Panel A represents membranes from rat glioma C6BU1 cells (60μg), rat white adipocytes (60μg) and rat cerebral cortex (100μg) in lanes 1-3, respectively. The immunoblot was probed with the primary antiserum LE3 (1: 200 dilution). This antipeptide antiserum was raised against a peptide discriminatory for Gi2α.

Panel B represents membranes from rat glioma C6BU1 cells (200μg), rat white adipocytes (200μg) and rat cerebral cortex (200μg) in lanes 1-3, respectively. The primary antiserum employed was I1A (1: 60 dilution). This antipeptide antiserum was raised against a peptide discriminatory for Gi1α.

In panel C, membranes from rat cerebral cortex (200μg), rat white adipocytes (100μg) and rat glioma C6BU1 cells (100μg) in lanes 1-3, respectively, were immunoblotted and probed with a mixture of I1A and LE3 (1: 60 and 1: 200 dilution, respectively).

Molecular weight standards were included in the experiments performed for each panel, but for clarity are only represented for panel A. Each panel is representative of two other experiments performed with different membrane preparations which gave essentially identical results.

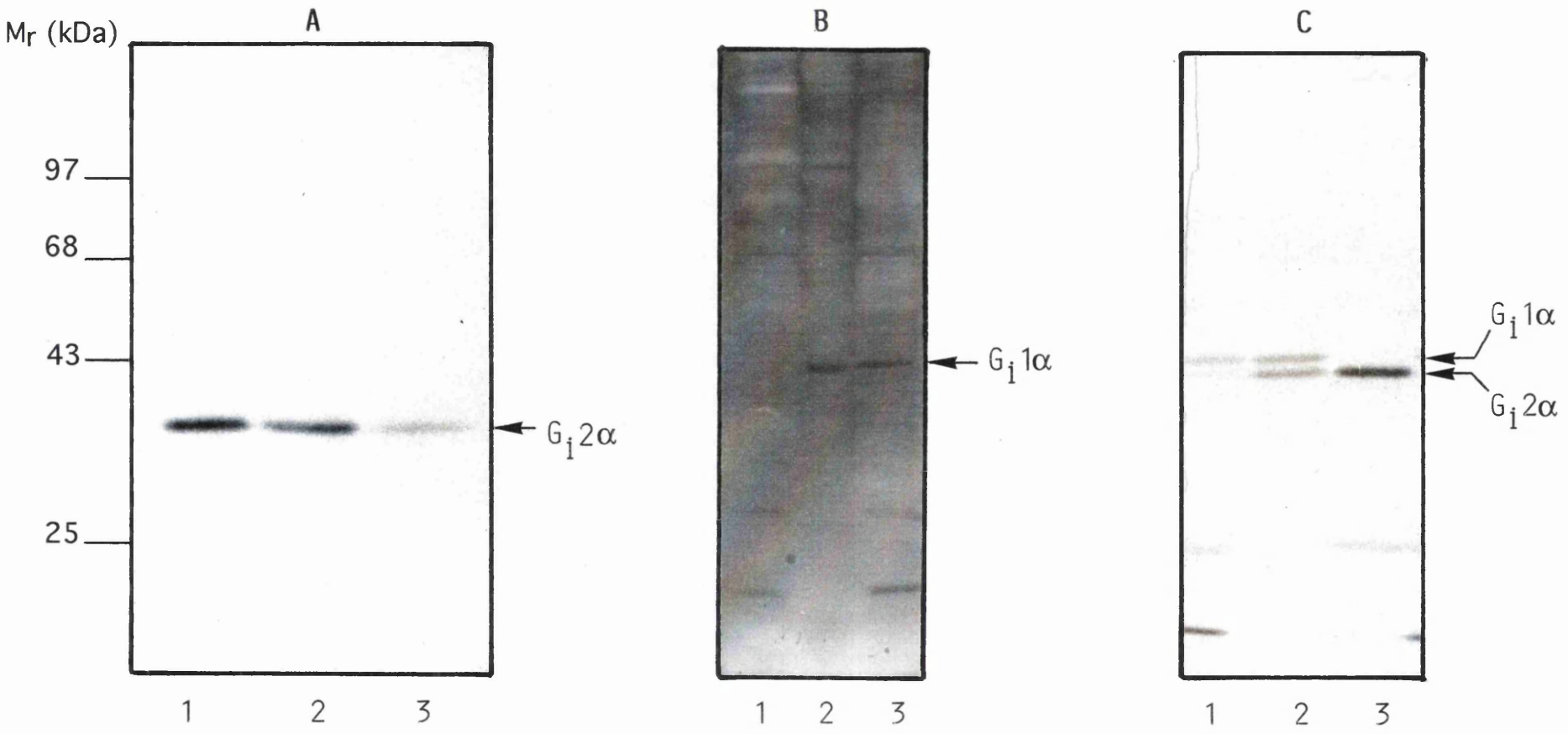

Mr (kDa)
A
97
68
43
25
Gi2α
1
2
3
B
Gi1α
1
2
3
C
Gi1α
Gi2α
1
2
3

Figure 6.4.

IMMUNOLOGICAL IDENTIFICATION OF Gi3α IN RAT ADIPOCYTE MEMBRANES.

Membranes of rat C6BU1 cells (100μg) and rat adipocytes (100μg) in lanes 1 and 2, respectively, were alkylated and resolved by SDS-PAGE (12.5% [w/v] acrylamide, 0.06% [w/v] bisacrylamide, 20cm x 20cm resolving gel) as in 2.7.3. B and 2.7 and then immunoblotted as described in 2.10.2. A. The membranes were prepared as outlined in Fig. 6.1.

In panel A the primary antiserum employed was LE2 (1: 200 dilution), whereas in panel C the primary antiserum employed was I3B (1: 200 dilution). These antipeptide antisera were raised against peptides discriminatory for Gi2α and Gi3α, respectively. In panel B a mixture of these antipeptide antisera, at the dilutions previously stated, was employed.

Molecular weight standards were included in the experiments performed for each panel, but for clarity are only represented for panel A. The experiments in panels A-C are each representative of two others performed with different membrane preparations which gave essentially identical results.

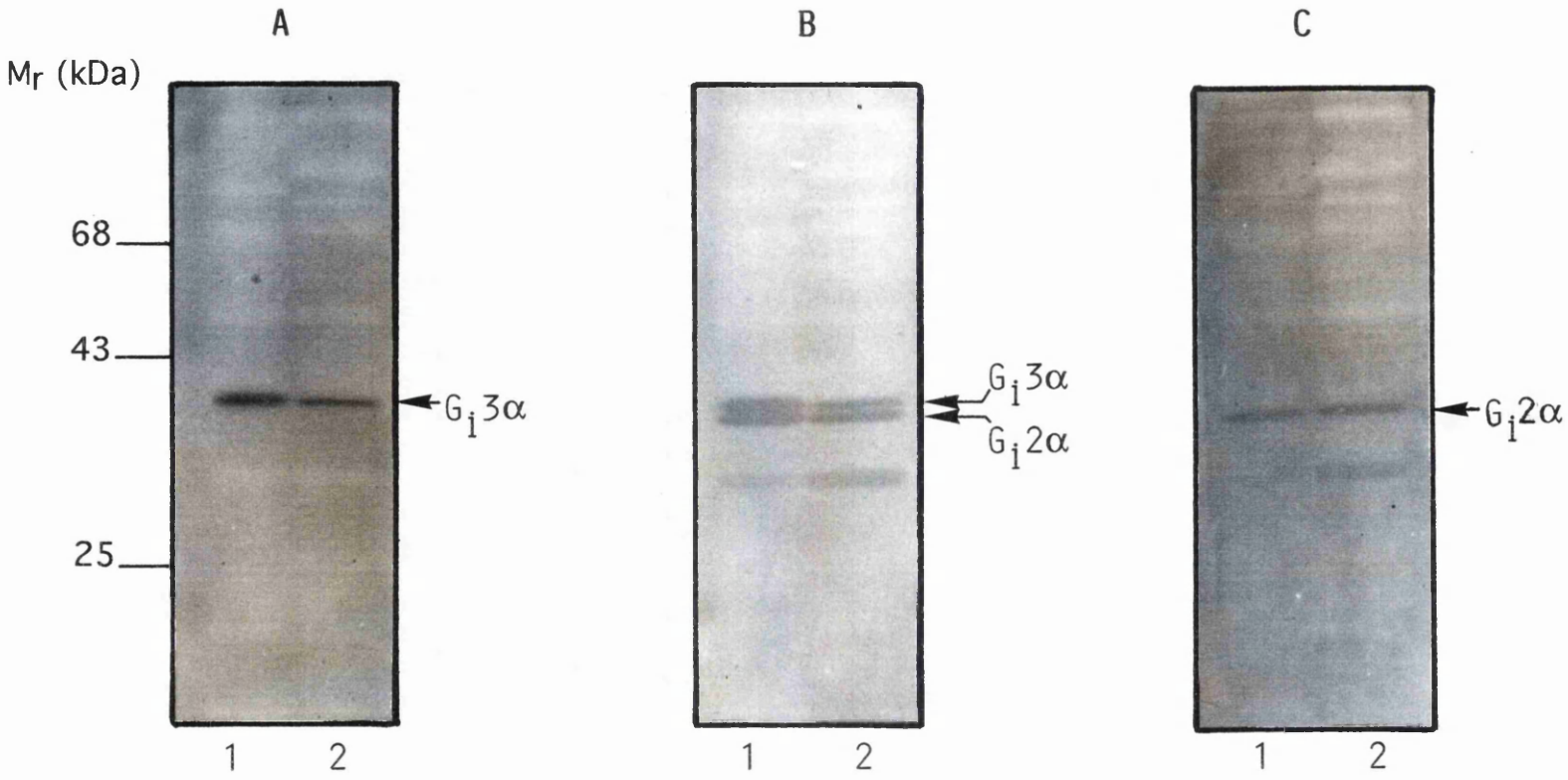

A
B
C
Mr (kDa)
68
43
25
Gi3α
Gi3α
Gi2α
Gi2α
1
2
1
2
1
2

Figure 6.5.

IMMUNOLOGICAL IDENTIFICATION OF Goα IN RAT ADIPOCYTE MEMBRANES.

Membranes from rat white adipocytes (100μg), rat glioma C6BU1 cells (80μg) and rat cerebral cortex (10μg) in lanes 1-3, respectively, were alkylated and resolved by SDS-PAGE (12.5% [w/v] acrylamide, 0.06% [w/v] bisacrylamide, 20cm x 20cm resolving gel) as in 2.7.3. B and 2.7 and then immunoblotted as described in 2.10.2.A. The membranes were prepared as outlined in Fig.6.1. The primary antipeptide antiserum was IM1 (1: 120 dilution). This experiment is representative of two others performed with different membrane preparations which gave essentially identical results.

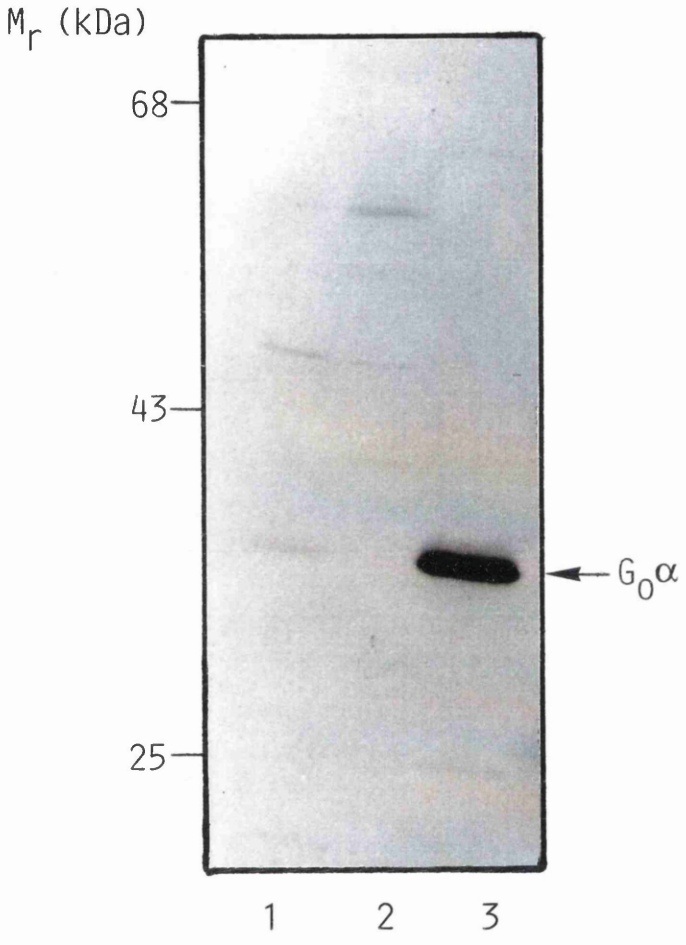
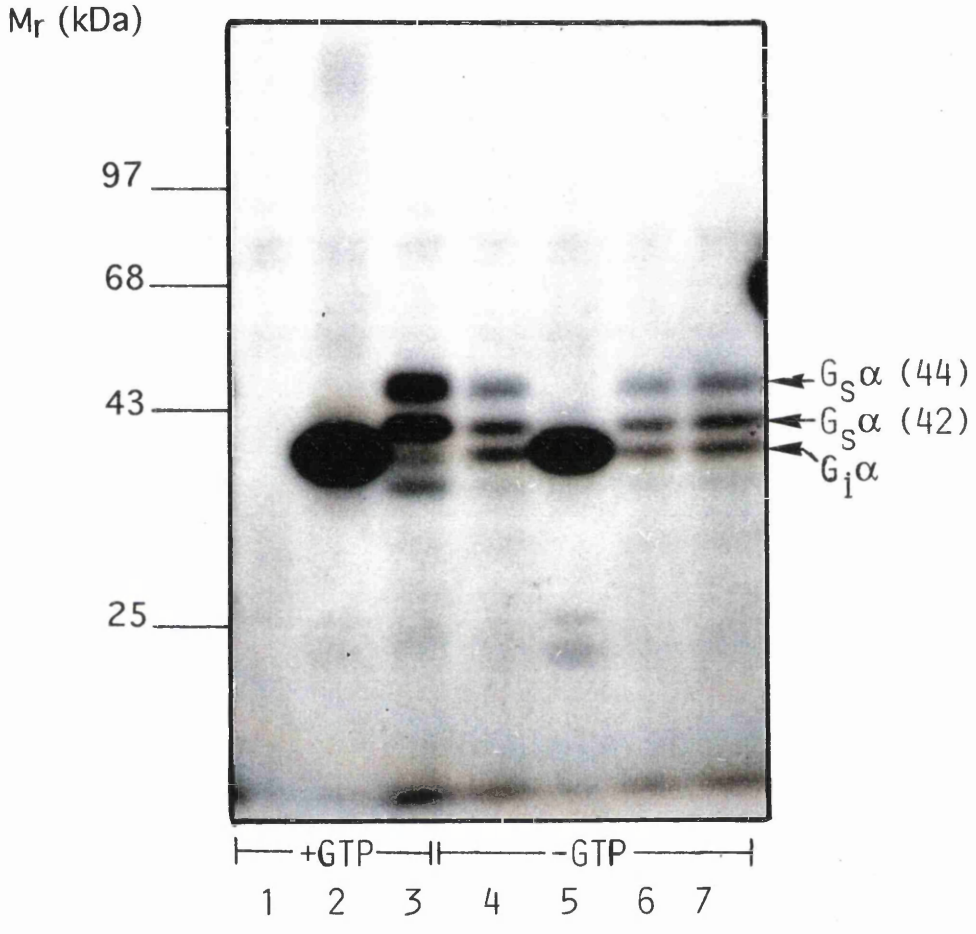

M_r (kDa)
68
43
25
$G_o\alpha$
1
2
3

Figure 6.6.

TOXIN-CATALYSED [32P]ADP-RIBOSYLATION OF RAT ADIPOCYTE MEMBRANES IN THE PRESENCE AND ABSENCE OF GTP AND ADRENALINE.

Membranes from rat white adipocytes (30μg) were [^{32}P]ADP-ribosylated with thiol-activated toxins for 2 hours, as described in section 2.10. The adipocyte membranes were a gift as described in 2.3.D. The samples were resolved by SDS-PAGE (10% [w/v] acrylamide, 0.25% [w/v] bisacrylamide, 18cm x 16cm resolving gel) as in 2.7 and the dried gel autoradiographed for 2 days, as described in 2.9.

Lanes 1-3 represent [^{32}P]ADP-ribosylation reactions performed in the presence of GTP, denoted as "+ GTP", whereas those in lanes 4-7 were performed in the absence of GTP, denoted as "-GTP". In lane 1, the [^{32}P]ADP-ribosylation reaction was performed in the absence of bacterial toxin. Pertussis toxin catalysed the [^{32}P]ADP-ribosylation reactions in lanes 2 and 5, whereas cholera toxin catalysed the [^{32}P]ADP-ribosylation reactions in lanes 3, 4, 6 and 7. Propanolol (10^{-5}M final) was included at the initiation of the incubation in lane 6. In lane 7, adrenaline (10^{-5}M final) and propanolol (10^{-5}M final) were added at the initiation of the reaction.


Mr (kDa)
97
68
43
25
Gsα (44)
Gsα (42)
Giα
+GTP
-GTP
1 2 3 4 5 6 7

6.3. DISCUSSION.

Theoretically any of the now known multiplicity of pertussis toxin-sensitive G-proteins could mediate receptor-generated signals which are attenuated by pretreatment of rat adipocytes with this toxin (Moreno *et al.*, 1983). These toxin-sensitive signals include receptor mediated inhibition of adenylyl cyclase and stimulation of inositol phosphate generation (Moreno *et al.*, 1983; Rapiejko *et al.*, 1986).

With the discovery of the expression of both $G_{i}1\alpha$ and $G_{o}\alpha$ in brain membranes it was initially presumed that the two distinct polypeptides in adipose tissue would be these forms (Sternweis & Robishaw, 1984; Neer *et al.*, 1984). However, with the recognition that the polypeptide identified as "G_i" in brain was immunologically distinct from the major pertussis toxin substrate in neutrophil and rat glioma C6BU1 cell membranes came the realisation that greater heterogeneity existed in the "G_i-like" family of G-proteins (Milligan *et al.*, 1986; Goldsmith *et al.*, 1987). These observations necessitated immunological approaches towards defining the G-proteins expressed in adipose tissue. As mentioned previously, several studies employing such approaches have confirmed the expression of "G_i-like" proteins in adipocytes although the question of whether $G_{o}\alpha$ is expressed in adipocytes has been a source of considerable debate. Malbon and co-workers argue strongly for its presence, whereas Hinsch and co-workers fail to detect expression of this G-protein (Ros *et al.*, 1988; Hinsch *et al.*, 1988).

In the experiments presented in this Chapter a panel of discriminatory antipeptide antisera and membranes prepared from two other relatively well characterised rat systems were used to attempt initially to clearly define the nature of the pertussis toxin-sensitive G-proteins expressed in rat adipocytes. Rat brain membranes express high levels of $G_{i}1\alpha$ and $G_{o}\alpha$ and lower levels of $G_{i}2\alpha$, whereas the rat glioma cell line, C6BU1, expresses high levels of $G_{i}2\alpha$ (Goldsmith *et al.*, 1988; Itoh *et al.*, 1986; Backlund *et al.*, 1988). The use of these

alternative rat systems clarified the identification of the adipocyte G-proteins by virtue of the co-migration of immunoreactive and toxin-sensitive polypeptides under SDS-PAGE conditions designed to resolve the G-protein α subunits.

Pertussis toxin-catalysed [^{32}P]ADP-ribosylation of adipocyte membranes identified a doublet of proteins of approximately 40kDa in molecular weight. These polypeptides could be tentatively assigned as $G_{i}1\alpha$ and the more rapidly migrating $G_{i}2\alpha$ from their electrophoretic mobility relative to the G-proteins of rat brain and C6BU1 cell membranes. This proved to be correct, assessed both by immunoreactivity with specific antipeptide antisera and by identification with an antipeptide antiserum incapable of distinguishing between the two gene products. In addition, $G_{i}3\alpha$ was also detectable in adipocyte membranes and in rat glioma cells. $G_{i}3\alpha$ was only barely detectable in rat cortical membranes (results not shown), an observation consistent with the inability to detect a pertussis toxin-sensitive G-protein in rat brain which co-migrates in a 2-dimensional gel system in a position equivalent to a protein expressed from a $G_{i}3\alpha$ cDNA (Goldsmith *et al.*, 1988). The fact that only two pertussis toxin [^{32}P]ADP-ribosylated polypeptides were observed in rat adipocyte membranes, despite immunological evidence for $G_{i}1$, $G_{i}2$ and $G_{i}3\alpha$, may be explained by the very similar relative mobilities of $G_{i}1\alpha$ and $G_{i}3\alpha$ under the SDS-PAGE conditions employed. $G_{i}1\alpha$ and $G_{i}3\alpha$ do possess two amino acid differences in their C-terminal decapeptide sequences. However the similar electrophoretic mobility of these α subunits necessitated evidence for the specificity of the discriminatory antipeptide antisera raised against these proteins. Such selectivity was indicated by the lack of detection of $G_{i}1\alpha$ in C6BU1 cell membranes, using a $G_{i}1\alpha$ specific antipeptide antiserum, despite the presence of $G_{i}3\alpha$ as detected by an antipeptide antiserum specific for $G_{i}3\alpha$. Indeed, after completion of these experiments, the specificity of these antipeptide antisera was confirmed by virtue of their specific recognition of recombinant G-protein α subunits (Milligan, 1992; McClue *et al.*, 1992). Thus the use of

discriminatory antipeptide antisera in conjunction with alternative, more well defined rat systems, has confirmed the presence of $G_{i}1\alpha$ and $G_{i}2\alpha$ subunits and further identified the expression of $G_{i}3\alpha$ in rat adipocyte membranes.

Using an antipeptide antiserum, IM1, which was raised against an N-terminal sequence of $G_{o}\alpha$, only an extremely weak immunoreactive signal was evident in adipocyte membranes relative to that from rat cortical membranes. These observations agree with the failure to detect a [^{32}P]ADP-ribosylated polypeptide in rat adipocyte membranes that co-migrated with the predominant pertussis toxin substrate of rat brain membranes. Interestingly, Hinsch and co-workers using an antipeptide antiserum raised against an identical peptide sequence, failed to detect an immunoreactive protein in rat adipocyte plasma membranes (Hinsch *et al.*, 1988). Quantification of the $G\alpha$ subunits has not been attempted in this study but assuming that the adipocyte G-protein is equally immunoreactive, it is apparent that $G_{o}\alpha$ is expressed in adipocyte membranes at an extremely low level relative to rat cerebral cortex membranes. In retrospect, it would perhaps have been informative to examine $G_{o}\alpha$ expression in adipocyte membranes using antipeptide antisera directed against other regions of $G_{o}\alpha$ amino acid sequence as described previously in Chapter 3.

As outlined previously, the adipocyte does express a totally pertussis toxin-sensitive α_1-adrenergic-mediated generation of Ins(1,4,5)P_3 (Rapiejko *et al.*, 1986). In an attempt to obtain an initial indication of which $G\alpha$ subunit interacts with this receptor and potentially serves to couple to phosphoinositidase C, the agonist-dependent cholera toxin-catalysed [^{32}P]ADP-ribosylation of substrates other than $G_{s}\alpha$ was examined. The rationale behind this assay has already been described in detail in Chapter 4, but basically depends on agonist "driving" guanine nucleotide from its binding site and, under the conditions used in the assay, allowing cholera toxin access to its substrate arginine residue. However, in several different membranes preparations tested, α_1-adrenergic stimulation failed to alter the cholera toxin-catalysed [^{32}P]ADP-ribosylation of proteins in adipocyte

membranes. This was also observed with oxytocin and vasopressin, other agonists known to stimulate phosphoinositide breakdown in adipocytes (results not shown). Interestingly cholera toxin catalysed the incorporation of [^{32}P]ADP-ribose into four polypeptides in the presence of GTP. Only two of these proteins are identifiable as forms of $G_s\alpha$ by virtue of their apparent molecular weight. Indeed, one, "non-$G_s\alpha$" polypeptide co-migrated with the pertussis toxin substrates, with incorporation of [^{32}P]ADP-ribose into this protein increasing in the absence of GTP in an agonist-independent manner. This may represent a $G_i\alpha$ protein(s) that cholera toxin can access preferentially in a guanine nucleotide free state and may implicate a GTP hydrolytic activity independent of receptor occupancy unless some endogenous ligand is present in the membrane preparation. The more mobile, "non-$G_s\alpha$" polypeptide was preferentially [^{32}P]-labelled in the presence of GTP and under these resolving conditions is unlikely to represent a known pertussis toxin substrate. The identity of this protein was not investigated further.

In conclusion, although preliminary attempts to define the $G\alpha$ coupling to agonist-stimulated, pertussis toxin-sensitive inositol phosphate generation in rat adipocytes were unsuccessful, this work has clearly defined the potential candidates for this role as $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$ and $G_o\alpha$.

CHAPTER 7.

CONCLUSIONS.

CONCLUSIONS.

This chapter serves to summarise the major observations of this thesis in a manner that places them in a wider context.

The research presented in this thesis was aimed towards definition of which pertussis toxin-sensitive G-protein couples to phosphoinositidase-specific phospholipase C (PIC). A number of different systems were initially examined but two were chosen for further investigation based on the criteria outlined previously in the research aims.

It was evident that both these systems expressed multiple pertussis toxin substrates. Indeed in the work of Chapter 6 it was demonstrated that rat white adipocyte membranes express all the currently known pertussis toxin substrates i.e., $G_{i}1\alpha$, $G_{i}2\alpha$, $G_{i}3\alpha$ and $G_{o}\alpha$. The transducins, Td_1 and Td_2, are also substrates for this toxin but it is generally accepted that their expression is limited to photoreceptor containing cells. This expression of the entire repertoire of potential coupling candidates was considered somewhat of a limitation. Cells of the human monocytic cell line, the U937 cell line were found not to express detectable levels of $G_{i}1\alpha$ or $G_{o}\alpha$, but did express detectable levels of the other known pertussis toxin substrates, $G_{i}2\alpha$ and $G_{i}3\alpha$ (Chapter 3). It was anticipated that the expression of only two potential candidates for mediators of PIC coupling would be useful towards defining whether one or indeed both of these proteins served such a function. The use of two-dimensional gel electrophoresis (2-D SDS-PAGE) has been fundamental to the discovery of charge variants of both $G_{s}\alpha$ and $G_{o}\alpha$ (Schleifer *et al.*, 1980; Mullaney & Milligan, 1990). The possibility of the expression of more than one isoform of $G_{i}2\alpha$ and $G_{i}3\alpha$ in U937 cell membranes was unsuccessfully addressed using 2D-SDS-PAGE (results not shown). There is however a precedence for only one form of these proteins being expressed in U937 cell membranes in that two-dimensional analysis of $G_{i}2\alpha$ and $G_{i}3\alpha$ purified from bovine brain has revealed the presence of a single polypeptide corresponding to

each of these proteins and at present only one cDNA sequence corresponding to each of these proteins and one gene has been identified (Goldsmith *et al.*, 1988; Strathmann *et al.*, 1989; Kaziro, 1990).

Dimethyl sulphoxide-induced differentiation of the U937 cells resulted in an increased expression of $G_{i}2\alpha$ but no significant alteration in the expression of $G_{i}3\alpha$. Increased expression of $G_{s}\alpha$ and β subunit forms was also observed, although these alterations were less significant than those observed for $G_{i}2\alpha$ (Chapter 3). Preliminary observations in Chapter 5 suggested that the fMLP receptor was expressed in two affinity states in differentiated cell membranes as opposed to one "lower" affinity state in control cell membranes. Under the binding conditions employed, this "lower" affinity state of the receptor represented the major form of the receptor in differentiated cell membranes and was present at a higher level in differentiated membranes relative to control cell membranes.

Previous work using the U937 cell line has demonstrated that a totally pertussis toxin-sensitive fMLP-stimulated generation of inositol phosphates is apparent in the DMSO-differentiated cells. This was not evident in the control cells, despite a functional PIC activity in these cells (Pollock *et al.*, 1990). The preliminary observations of Chapter 5 did suggest that the fMLP receptor in the control cells was interacting with a G-protein, although it should be noted that this was not reflected in an fMLP-stimulated high affinity GTPase activity. In combination with the observations of Chapter 4 on G-protein expression and the findings of Pollock and co-workers, it is interesting to speculate that the lack of functional response in the control cells may reflect the level of the coupling G-protein(s) and /or the affinity of the fMLP receptor expressed. It is further tempting to speculate that the differentiation-associated alterations in the expression of these signal transducing components may be of relevance to the onset of fMLP-stimulated PIC activity. It is however worth noting that PAF and LTB_4 receptors have been demonstrated to activate PIC in control, undifferentiated U937 cells (Creba *et al.*,

1989; Pollock *et al.*, 1989). Interestingly, pertussis toxin inhibited PAF stimulation of inositol phosphate generation equally well in both control and differentiated U937 cells, although only by some 60% in both cases (Pollock *et al.*, 1990). It would appear that these receptors do have some dependence, although not absolute, upon the pertussis toxin substrates of the control U937 cells which for some reason the fMLP receptor does not utilise.

As described previously the C-terminal region of the G-protein α subunit has been identified as a site of functional interaction with the receptor. It was anticipated that antipeptide antisera raised against synthetic peptides which were either homologous to, or corresponded to the C-terminal decapeptide of $G_i2\alpha$ and $G_i3\alpha$ would serve as selective tools to specifically prevent interaction of the corresponding G-protein with the fMLP receptor. However, although only preliminary attempts were made to use this approach, it was appreciated that the low receptor number and high expression of the pertussis toxin substrates in the U937 cell membranes were not ideal properties if the success of other systems was used as an indicator of "suitability". Indeed in one such successful system, one clone of Rat 1 fibroblast cells transfected with the α_{2A}-adrenergic receptor exhibited a B_{max} of some 3000 fmoles/mg of membrane protein as compared to an average of 100 fmoles/mg of membrane protein for the "lower" affinity form of fMLP receptor expressed in the differentiated U937 cell (McClue & Milligan, 1991). However, further investigation may have proved successful in utilising the large and reproducible Gpp[NH]p and pertussis toxin-mediated reductions in specific [^{3}H]fMLP binding, particularly at the "higher" affinity site, to address coupling specificity.

Antipeptide antisera were useful in adapting an assay which it is proposed relies upon agonist-driven removal of bound GDP to "free" a previously inaccessible residue which then becomes the target for a cholera toxin-catalysed [^{32}P]ADP-ribosylation reaction. Using this reaction G-protein α subunits that were

classically believed to be only pertussis toxin substrates can become cholera toxin substrates, but importantly do so in an agonist-dependent manner. Unfortunately attempts to use this assay to address the α_1-adrenergic receptor - G-protein interaction in rat adipocyte membranes were not successful. However, the experiments of Chapter 4 clearly defined that in membranes from U937 cells, differentiated along the macrophage lineage, the protein(s) [^{32}P]ADP-ribosylated by cholera toxin in the presence of fMLP was indeed a substrate for pertussis toxin. Altering the electrophoretic conditions to enable resolution of the $G_i\alpha$ subunits in conjunction with their selective identification and immunoprecipitation with specific antipeptide antisera, did indicate that $G_i2\alpha$ does interact with the fMLP receptor but did not successfully address the question of whether $G_i3\alpha$ can mediate fMLP responses in these cells. Interestingly in an experiment using membranes from the HL-60 cells, differentiated along the neutrophil lineage, fMLP-stimulated a cholera toxin-catalysed [^{32}P]ADP-ribosylation of $G_i2\alpha$ and $G_i3\alpha$. These substrates were identified by their specific immunoprecipitation, suggesting an interaction of the fMLP receptor with both of these G-proteins (Chapter 4). These conclusions would agree with the work of Gierschik and co-workers and further suggest that this technique of defining coupling specificity may be widely applicable to a variety of other G-protein mediated signalling systems (Gierschik *et al.*, 1989a). Indeed subsequent to the work of this thesis this approach did prove useful in defining an interaction of the α_{2A}-adrenergic receptor, transfected into Rat 1 fibroblast cells, with both $G_i2\alpha$ and $G_i3\alpha$ (Milligan *et al.*, 1991). The proposed arginine residue that acts as a substrate for cholera toxin in the "classical" pertussis toxin substrates is also conserved among the Gq class of G-proteins. As such it may be that this technique could prove useful in defining the coupling interaction between receptors and a pertussis toxin-insensitive Gp protein(s).

The tentative evidence to suggest that $G_i2\alpha$ played some role in coupling to the fMLP receptor was interesting with respect to the fact that this protein has been shown in a number of systems to couple receptors to inhibition of adenylyl cyclase,

as described previously (Simonds *et al.*, 1989b; McKenzie & Milligan, 1990). The chemotactic peptide, fMLP, neither stimulated basal nor inhibited forskolin-stimulated adenylyl cyclase activity in several different membrane preparations tested from both control and differentiated U937 cell membranes (F.R.McKenzie, personal communication). These observations then raised the question of whether the same $G_i\alpha$ subunit can serve to couple to different effector systems in different cells or whether these different cell types expressed different $G_i2\alpha$ isoforms. Unfortunately use of 2D-SDS-PAGE analysis did not successfully address whether the $G_i2\alpha$ subunit expressed in the U937 cell line was different from that expressed in the NG108-15 cell line, a system in which $G_i2\alpha$ acts to mediate inhibitory coupling to adenylyl cyclase (McKenzie & Milligan, 1990). However current evidence suggests that it is unlikely that cells express different $G_i2\alpha$ isoforms.

Interestingly, $G_i2\alpha$ is thought to be universally expressed and if it can interact with more than one effector system present in a single cell then it does question how specificity of signalling can occur in the *in vivo* situation. As introduced previously specificity of coupling interactions is further called into question by a number of reconstitution approaches where it is often observed that a range of G-proteins can couple receptors to a particular effector, although more recent studies do indicate a higher degree of specificity of interaction of G-proteins with receptors (Cerione *et al.*, 1986; Senogles *et al.*, 1990). In these *in vitro* assays then the high degree of sequence homology between the G-protein α subunits and the difficulty in exactly mimicking their native environment can perhaps explain some of these observations, but presumably in a cell there is some method of preventing such promiscuity.

Interestingly, membranes from both control and DMSO-differentiated U937 cells express a protein recognised by an antipeptide antisera raised against a sequence common to the $G_q\alpha$ and $G_{11}\alpha$ proteins (Mitchell *et al.*, 1991). A selection of the highly intensive series of investigations which have assigned a role

for these proteins in coupling to PICβ_1 has been discussed previously. It would appear that if these proteins have the same role in the U937 cell then clearly the fMLP receptor exerts some specificity in that it couples to PIC via a pertussis toxin-sensitive rather than an insensitive protein(s).

In more general terms it is interesting to consider whether receptors make a "choice" in the toxin sensitivity of the Gp protein they interact with or indeed whether they simply cannot interact with one or other of these proteins. In a relatively early study using *Xenopus* oocytes, where the receptor-evoked chloride current is a sensitive electrophysiological measure of receptor-stimulated PIC activity, "cross-talk" between the pertussis toxin-sensitive and insensitive pathways was demonstrated. The liver vasopressin (V_1)-receptor, which utilises a pertussis toxin-insensitive G-protein in its native environment utilised a pertussis toxin-sensitive pathway in the *Xenopus* oocyte. This was observed despite an operational pertussis toxin-insensitive pathway, demonstrated by toxin-insensitive cholecystokinin receptor-stimulated PIC activity (Moriarty *et al.*, 1989). Such "cross-talk" is perhaps even more surprising with the more recent appreciation that the "Gq" class of G-proteins are only some 50-60% homologous to the "Gi class" at the amino acid level (Figure 1.1 and Simon *et al.*, 1991). However these findings do imply that receptors that use the toxin-sensitive route may have the ability to use a toxin-insensitive coupling protein and therefore do exhibit some degree of coupling specificity.

One could further speculate that the particular effector enzymes that a cell expresses may also influence the coupling specificities. Is it possible that the effector phospholipase isoenzymes influence whether a pertussis toxin-sensitive or insensitive G-protein acts as the coupling protein ? Interestingly there is increasing evidence to suggest that the main PIC isoenzymes present in the HL-60 cell line differ structurally from the major forms identified in other tissues (Rhee *et al.*, 1991; Kriz *et al.*, 1990). Interestingly, using Chinese hamster ovary (CHO) cells,

it has been demonstrated that activation of phosphatidylinositol 4, 5-bisphosphate hydrolysis by receptors coupled to a pertussis toxin-insensitive G-protein results in substantially greater accumulation of inositol phosphates than the response mediated by the pertussis toxin-sensitive G-protein coupled receptors in these cells. Whether this reflected interaction with different PIC isoenzymes was not determined in this study. However, the authors suggest that the pertussis toxin-sensitive and insensitive pathways may have evolved to specify the nature of the cellular response mediated by different receptors that use a common second messenger system (Ashkenazi *et al.*, 1989). Interestingly there is more recent evidence to suggest that use of differentially pertussis toxin-sensitive coupling routes can mediate coupling of the same receptor to different phospholipase activities. Chemotactic peptide-mediated activation of phospholipase D (PLD) in rabbit neutrophils was only partially inhibited by pertussis toxin, whereas fMLP activated PIC via a totally pertussis toxin-sensitive protein (Kanaho *et al.*, 1991).

It would appear that although tissue or cell type-specific expression of signal transduction components may limit potential interactions and convey some degree of specificity, the co-expression of multiple isoforms of these components in a single cell requires more complex regulation of interaction specificity. Compartmentalisation may occur, where specificity of interaction would be limited to those components in a certain locale. However, as yet, there is limited evidence to support this attractive hypothesis. It has recently been demonstrated that in polarised epithelial cells $G_i2\alpha$ localised to the basolateral membrane and $G_i3\alpha$ to the apical membrane and Golgi. These cells have previously been shown to exhibit $G_i\alpha$ modulation of adenylyl cyclase activity at the basolateral membrane and regulation of an amiloride-sensitive sodium channel at the apical membrane. Transfection and overexpression of the $G_i2\alpha$ subunit in cells resulted in its "correct" targeting to the basolateral membrane (Ercolani *et al.*, 1990). More recent work by this group is beginning to address definition of the gene promoter

sequences which should aid elucidation of how such regulation can occur (Holzmann *et al.*, 1991). These observations do support the concept of at least G-protein compartmentalisation, although it is not known whether this occurs in an non polarised cell. Interestingly, polarisation is one of the primary responses of leukocytes to chemoattractants, with receptor redistribution to one end of the cell. Whether redistribution is evident for the G-proteins and effector enzymes has not been investigated, but it is tempting to speculate that this may be a method to achieve specificity of coupling and the activation of highly regulated physiological responses.

The approaches used in the work of this thesis concentrated on defining fMLP receptor - G-protein interaction in cell membrane systems. It was appreciated that even if coupling of this receptor had been conclusively demonstrated this would not necessarily define interaction with PIC, as this peptide interacts with other phospholipase enzymes in other cell models. Indeed as more is understood about phospholipid metabolism it is becoming apparent that selecting a PIC-specific agonist may actually not be possible. Obviously to define coupling specificity for an individual cell type all the signal transduction components have to be identified and their interactions dissected in that particular whole cell system. Antipeptide antisera have not, as yet, been routinely applicable to microinjection into a whole cell although development of such techniques would be extremely valuable in determining coupling specificity in the *in vivo* situation, in the presence of the effector. The development of agents that can "isolate" different components of phospholipid metabolism e.g., specific inhibitors of particular phospholipase isoenzymes is currently being investigated, although the highly integrated nature of this signalling pathway may restrict the value of the use of these agents.

Several recent studies have achieved definition of distinct modes of interaction of the chemotactic peptide receptor with different phospholipase activities in the whole cell. Recent work using permeabilised HL-60 cells differentiated along the neutrophil lineage has demonstrated that fMLP-stimulated

phospholipase C and A_2 activities can be distinguished and the degree of interaction of these signalling routes established. This was achieved using a range of approaches, some based on selective inhibition or activation of the PIC activity. The conclusions from these experiments were that although fMLP is linked to PLA_2 independent of PIC activation, second messengers associated with PIC activation are of major importance in regulating arachidonic acid release (Nielson *et al.*, 1991). More recently this group have also partially dissociated GTPγs-mediated activation of PLD from GTPγs-stimulated PIC activity in permeabilised, undifferentiated HL-60 cells. However, full activation of PLD did require protein kinase C, calcium and a G-protein (Geny & Cockcroft, 1992). Although these studies are beginning to "dissect" out the mechanisms of activation of the different phospholipases the interdependence of the phospholipid signalling pathways makes definition of the G-protein(s) involved extremely complex.

Such complexity has been highlighted further by an investigation which suggests that in the neutrophil the coupling of the fMLP receptor to PLD is distinct from its coupling to PIC and involves tyrosine phosphorylation. The fMLP receptor is not a member of the class of receptors with intrinsic tyrosine kinase activity, suggestive of a role for kinase or phosphatase links to the receptor. PLD activity is sensitive to pertussis toxin which suggests either that the products of the PIC reaction regulate this PLD activity or that a pertussis toxin-sensitive G-protein may be directly involved in coupling to the kinases or phosphatases (Uings *et al.*, 1992).

Recent studies have used antipeptide antisera directed against the different subunits of the G-protein to specifically immunoprecipitate the somatostatin (SRIF) receptor - G-protein complex. These investigations not only demonstrated specificity of coupling to $G_{i}\alpha$ and $G_{o}\alpha$ but further demonstrated that selectivity also exists in the association of β and γ subunits with the receptor (Law *et al.*, 1991). Indeed it would appear that the role of β and γ subunits in defining coupling

specificity has been somewhat neglected. As previously discussed in 1.7.3, it has recently been demonstrated that βγ subunits can differentially modulate the activity of different forms of adenylyl cyclase (Tang & Gilman, 1991; Federman *et al.*, 1992). The authors suggest that this could potentially make cAMP generation susceptible to stimulation by receptors not directly coupled to adenylyl cyclase but coupled, through G-proteins, to other second messenger generating pathways. If different receptors exhibit specificity in βγ interaction then the situation could be even more complex. Indeed, as βγ subunits are known to interact with phospholipase A_2, it would seem plausible to postulate a possible interaction of these subunits with other phospholipase enzymes, including phosphoinositidase C. If this proposal is correct then clearly the definition of the specificity of G-protein interaction with phospholipases may have acquired a new level of complexity.

CHAPTER 8.

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