Phosphoinositide Signalling in a Neuroblastoma Cell Line

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by

Stephen T. Safrany

Department of Pharmacology and Therapeutics
University of Leicester

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Stephen T. Safrany.

In the studies described in this thesis, the ability of muscarinic agonists to initiate phosphoinositide metabolism, subsequently leading to the mobilisation of Ca^{2+} from intracellular stores was examined in permeabilised human neuroblastoma SH-SY5Y cells. Muscarinic receptors, determined as being of the M3 subtype, were found to possess different levels of receptor reserve for the production of the second messenger, inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and the mobilisation of Ca^{2+} in this permeabilised cell preparation.

The effects of agonist pretreatment of intact cells with the muscarinic agonist carbachol has also been examined. Such pretreatment attenuated the ability of muscarinic agonists to elicit Ca^{2+} mobilisation in permeabilised cells. The rate of desensitisation was dependent on the dose of agonist used, the temperature at which pretreatment was performed and was affected by the extracellular Ca^{2+} concentration. The mechanism of such receptor-mediated desensitisation was studied.

The structure-activity relationships of a number of inositol phosphates and inositol phosphate analogues have also been studied with regards their interactions with the Ca^{2+}-mobilising Ins(1,4,5)P₃ receptor, Ins(1,4,5)P₃ 5-phosphatase and Ins(1,4,5)P₃ 3-kinase. This work has identified partial agonists at the Ins(1,4,5)P₃ receptor, an inhibitor of Ins(1,4,5)P₃ 3-kinase which interacts poorly with the Ins(1,4,5)P₃ receptor, and a highly potent and selective inhibitor of Ins(1,4,5)P₃ 3-phosphatase.

In conclusion, the results obtained characterise a permeabilised cell preparation in which receptor coupling to phospholipase C is maintained, leading to the formation of Ins(1,4,5)P₃ and mobilisation of Ca^{2+} from intracellular stores, and identifies this as a useful model in which this coupling can be modulated by cell membrane-impermeant agents.
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CHAPTER 1.
Introduction.

1.1 Synthesis and Metabolism of Phosphoinositides.

One of the major constituents of plasma membranes are the phospholipids. Of these, a minor component, phosphatidylinositol (PtdIns), has been the subject of much research in recent years. PtdIns is unique, in that its head group, inositol, can be further phosphorylated. Phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) are synthesised in series from PtdIns by the lipid kinases PtdIns 4-kinase and PtdIns(4)P 5-lipidase respectively (see Downes and Michell, 1985), the first purification and detailed characterisation of the former being reported by Porter et al. (1988).

Resting levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P2 are maintained in a futile cycle by the action of specific kinases and phosphatases. Cytidine monophosphorylphosphatidate (CMP-PA) is the key intermediate in phosphoinositide biosynthesis. It is formed from the phosphorylation product of diacylglycerol (DAG), phosphatidic acid (PA) and cytidine triphosphate (CTP) in the presence of CTP-PA cytidylyltransferase. PtdIns biosynthesis from CMP-PA and myo-inositol, via PtdIns synthase occurs at the E.R., from which it is transported to the plasma membrane (see Abdel-Latif, 1986). Phosphoinositides are rich in arachidonoyl residues. Indeed, about 75% of all phosphoinositides contain an arachidonoyl group at the sn-2 position of the glycerol backbone (Majerus et al., 1984).

The role of these minor phospholipids (PtdIns, PtdIns(4)P2 and PtdIns(4,5)P2), which, as a whole constitute <10% of total phospholipids, appears to be as a precursor for the second messengers D-myo inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and DAG, produced on activation of a number of Ca^2+ mobilising receptors, via phospholipase C (PLC) (Fig. 1.1).

The first evidence for a receptor-mediated effect on inositol phospholipid metabolism was provided by Hokin and Hokin (1953) in experiments which showed that acetylcholine stimulated the incorporation of 32P into the phospholipids of pigeon pancreas and guinea pig cerebral cortical slices. It was subsequently shown that this stimulated incorporation was mainly associated with PtdIns and phosphatidic acid (see Hokin, 1985). These
Identified metabolic enzymes:

1. PI-PLC  6. PtdIns(4)P 5-kinase
2. DAG-kinase  7. PtdIns 3-kinase
3. Phospholipase C  8. PtdIns(3)P 4-kinase
cytidylyltransferase  9. PtdIns(3,4)P_2 5-kinase
4. PtdIns synthase
5. PtdIns 4-kinase

Figure 1.1 Phosphoinositide Metabolism and Synthesis
phenomena have been referred to as 'the phospholipid effect' and have been observed in a large variety of tissues (see Michell, 1975; Downes, 1982, 1983; Hokin, 1985; Downes and Michell, 1985; Abdel-Latif, 1986; Fisher and Agranoff, 1987; Fisher et al., 1992). From these early studies it was proposed that metabolic turnover of PtdIns served some function in the membrane fluxes essential to exocytotic secretion (see Hokin, 1985).

However, the idea that inositol lipids may be involved in a novel transduction mechanism emerged throughout the late 1960's and into the late 1970's. The majority of evidence at this time favoured a PLC cleavage of PtdIns to yield DAG, Ins(1)P and Ins(1:2)P, indicating that the stimulated incorporation of $^{32}$P into the phospholipids is secondary to the initial hydrolysis (see Michell, 1975). Michell added further to this hypothesis in proposing that the hydrolysis of phosphoinositides might serve as a link coupling receptor activation to an elevation of intracellular Ca$^{2+}$.

The first suggestion that polyphosphoinositides and not just PtdIns itself, may be hydrolysed in response to agonists came from studies by Durell et al. (1969) in guinea pig brain, where acetylcholine was shown to stimulate the production of InsP$_2$ and later by Abdel-Latif and colleagues (reviewed by Abdel-Latif, 1986), who demonstrated agonist-stimulated decreases in $^{32}$P labelled PtdIns(4,5)P$_2$ in rabbit iris smooth muscle.

Following the identification of Ins(1,3,4)P$_3$ (Irvine et al., 1984) and Ins(1,3,4,5)P$_4$ (Batty et al., 1985) isomers in tissue extracts, the question of the presence of additional polyphosphoinositides was raised, and in particular, whether PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ were the precursors of Ins(1,3,4,5)P$_4$ and Ins(1,3,4)P$_3$. This, however, was not found to be the case (Downes et al., 1986). The presence of a PtdIns 3-kinase has been reported in a number of tissues, allowing for the formation of PtdIns(3)P, PtdIns(3,4)P$_2$, and PtdIns(3,4,5)P$_3$. These lipids however, appear to be resistant to activity of any PLC yet identified, and their precise function is unclear (see Downes and Carter, 1991). PtdIns 3-kinase is activated through mechanisms involving tyrosine phosphorylation or the stimulation of G-protein-linked receptors. The appearance of PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ occurs within seconds of agonist stimulation, but slower than the observed rises in Ins(1,4,5)P$_3$. This suggests that the 3-phosphorylated inositol phospholipids may be acting as second messengers, one possible role being in cell mitosis (see Downes and Carter, 1991).

In earlier studies, it was shown that the binding of agonist to receptors known to activate PtdIns(4,5)P$_2$ hydrolysis was modulated by GTP and its non-hydrolysable analogues (Evans et al., 1985). Later, Cockcroft and Gomperts (1985) and Litosch et al. (1985) demonstrated the ability of guanine nucleotides to directly stimulate PI hydrolysis in $^{32}$P
labelled membranes from neutrophils and \(^{3}H\) inositol-labelled blowfly salivary glands—providing direct evidence that the hydrolysis of endogenous membrane phosphoinositides by a membrane-bound PI-PLC was regulated by guanine nucleotides, hence indicating the probable involvement of a G protein.

1.2.1 G-Protein Involvement in Phospholipase C Activation.

The suggested involvement of one or more G-proteins in activation of PI-PLC resulted from a number of findings. Analogues of GTP, such as GTP\(_{y}\)S, have been reported to activate inositol phosphate formation (Cockcroft and Gomperts, 1985; Litosch et al., 1985), and decrease the affinity of agonists for receptors which are known to activate PI-PLC (Evans et al., 1985), in a fashion analogous to receptors linked to the adenylyl cyclase system (see Taylor and Merritt, 1986). The involvement of two different G-proteins was first suggested by studies using pertussis toxin. In most cells the G-protein linked to PI-PLC was pertussis toxin-insensitive, in others hormone-activated PI hydrolysis was blocked (reviewed by Meldrum et al., 1991; Cockcroft and Thomas, 1992). More than one pertussis toxin-insensitive G-protein has been identified from brain (Pang and Sternweis, 1989, 1990).

The G-proteins involved in signal transduction pathways are a large family of heterotrimeric receptors each comprising \(\alpha\), \(\beta\), and \(\gamma\) subunits. The \(\alpha\) subunits readily dissociate from the \(\beta\gamma\) dimer, which are tightly associated.

In a resting state, the heterotrimeric G-proteins are tightly associated, and the \(\alpha\) subunit is bound by GDP (Fig. 1.2(i)). Under non-stimulated conditions, GDP dissociates slowly (half time ~hours for transducin, see Taylor, 1990). Activation of an associated cell-surface receptor, in concert with the actions of intracellular magnesium (Mg\(^{2+}\), increases the rate of GDP dissociation from the \(\alpha\) subunit, and its replacement with GTP (Fig. 1.2(ii)). The activated receptor has high affinity for a conformation of the G-protein in which its \(\alpha\) and \(\beta\gamma\) subunits are associated, but no guanine nucleotide is bound (Fig. 1.2(iii)) (see Wessling-Resnick et al., 1987). This 'activated' form of the G-protein may have increased affinity for GTP relative to GDP (Florio and Sternweis, 1989). Its effects are, however, rapidly attenuated by a GTPase activity, hydrolysing the GTP in the GTP-\(\alpha\) complex, yielding inactive GDP-\(\alpha\), which has high affinity for the \(\beta\gamma\) subunit, leading to reassociation and continuation of the G-protein cycle. PLC\(_{B1}\), an isozyme of
Figure 1.2 Receptor interactions with G-proteins.
PI-PLC (see Meldrum et al., 1991) has recently been identified as a GTPase-activating protein (GAP) for $G_q/11$. A mixed population of $G_q$ and $G_{11}$ were found to have intrinsic GTPase activity, giving a half life for GTP-activated $G_q/11$ of about 1 minute (Bernstein et al., 1992b). This GTPase activity was further stimulated by the addition of recombinant PLC$_{B1}$ in a dose dependent manner. A role for receptor mediated GTPase activation was also identified (Bernstein et al., 1992a). Reconstitution of $G_q/11$ into lipid vesicles abolished all GTPase activity, either in the absence or presence of PLC$_{B1}$. Agonists enhanced GTPase activity, in a dose dependent manner, which was synergistic in the presence of PLC$_{B1}$. The GAP property of PLC$_{B1}$ was specific for $G_q/11$, having no effect on $G_s$ or $G_o$, consistent with the inability of $G_s$ or $G_o$ to activate PLC$_{B1}$. PLC$_{B1}$ did not affect GDP-GTP exchange rates.

Although only the $\alpha$ subunit undergoes exchange, the $\beta\gamma$ complex was believed to be essential in presenting the $\alpha$ subunit to the receptor, without it there is little effect of stimulation by a receptor on the $\alpha$ subunit (Florio and Sternweis, 1989). The ternary complex of agonist, receptor and G-protein is normally transient because GTP binds within milliseconds to the $\alpha$ subunit (May and Ross, 1988), the affinity of the receptor for the G-protein is decreased and the two dissociate. However, using cell membranes or permeabilised cells, it is possible to remove guanine nucleotides, and stabilise this high affinity state of the receptor. If guanine nucleotide is then added, it binds the vacant site on the $\alpha$ subunit and the transition state is lost, the G-protein dissociates and high affinity binding of agonist for its receptor is also lost. Antagonist-occupied receptors cannot bind more tightly than empty receptors to the guanine nucleotide-free form of the G-protein and, hence, do not activate GDP-GTP exchange. Equally, antagonists do not alter the equilibrium between receptor and G-protein, and hence antagonists binding to receptors is guanine nucleotide insensitive (but see Horn et al., 1990). Alternatively, antagonists have been shown to have negative intrinsic activity, inhibiting basal turnover (Costa and Herz, 1989). In this case, it is reasonable to assume that antagonist-receptor complexes bind more tightly to and stabilise inactive forms of the G-protein, or displace endogenously produced agonist.
1.2.2 G-Protein Activation.

Binding of GTP to the $\alpha$ subunit of a G-protein, leading to dissociation produces the active GTP-G$\alpha$ complex. It is unclear whether this GTP-G$\alpha$ complex dissociates from the inner leaflet of the plasma membrane, although work by Levitzki and co-workers (Tolkovsky and Levitzki, 1978; Arad et al., 1984; Levitzki, 1986) shows that G$\alpha$ remains associated with the plasma membrane throughout the G-protein cycle. The G-protein acts as a shuttle between the receptor and the effector protein. This effector protein can be an enzyme (e.g. adenylyl cyclase, PLC), or ion channel (G$\alpha$ gated K$^+$ channels, G$\alpha$ gated Ca$^{2+}$ channels). The GTP-bound form is the active conformer, it is in this form only that the G-protein can interact with its target molecule. A large number of distinct G-proteins have been identified the roles of which are under investigation.

1.2.2(i) $\alpha$ Subunits of G-proteins.

More than 30 different cDNA's have been cloned for this large family. When aligned, about 20% identity is observed throughout the whole family, all of which appear to be of molecular weight 39-50 kDa (Birnbaumer et al., 1990). Simon et al. (1991) have collated data on 17 of the subtypes, and shown how they are related to one another. They have divided this family into 4 classes, G$\beta$, G$i$, G$q$, and G$\gamma$, each composed of different isotypes.

Isotypes of the G$\alpha$ class of G-proteins, of which four have been identified (see Birnbaumer et al., 1990) couple receptors to the activation of adenylyl cyclase, leading to an increase in the formation of cyclic AMP, and are substrates for cholera toxin, ADP ribosylation occurring at an arginine residue.

Members of the G$i$ class of subunit couple receptors that lower cyclic AMP levels. They may play a role in the activation of PLC, increasing formation of inositol phosphates. Pertussis toxin is known to uncouple receptors from these specific G$\alpha$ subunits, hence blocking signal transduction occurring through G$i$ (see Birnbaumer et al., 1990).
GTP-dependent signals activating PLC which are resistant to pertussis toxin are mediated through the \(G_q\) and \(G_{12}\) \(\alpha\) subunits. All of these lack the cysteine residue 4 amino acids from the carboxy-terminal end that is the target for pertussis toxin-mediated ADP ribosylation in the \(G_i\) subclass of \(G\) \(\alpha\) subunits. Of the \(G_q\) family, \(G_{\alpha q}\) and \(G_{\alpha n}\) appear to be important in pertussis toxin-insensitive activation of PLC, however, little else is known about the \(G_{12}\) family.

The amino-terminal region of the \(G\) \(\alpha\) subunit is thought to interact with the \(\beta\gamma\) subunit as proteolysis of this terminus prevents \(\alpha\beta\gamma\) association. The amino terminus is also the site for myristoylation on some \(G_i\) \(\alpha\) subunits. Myristoylation increases the affinity of the \(\alpha\) subunit for the \(\beta\gamma\) subunit, facilitating heterotrimer formation (discussed by Simon et al., 1991).

It has been suggested that the carboxy-terminal region of the \(G\) \(\alpha\) subunit is involved in receptor interactions. This is supported by the observation that pertussis toxin modification blocks interactions with receptors. Antibodies or peptides that specifically interact with the carboxy terminus of some \(G\) \(\alpha\) proteins also block interactions with receptors (Simon et al., 1991).

1.2.2(ii) \(\beta\gamma\) Subunit Diversity and Function.

In mammals, four \(\beta\) subunit isoforms have been found, sharing over 80% identity. \(\beta_1\), \(\beta_2\) and \(\beta_3\) are ubiquitously expressed, while \(\beta_4\) is abundant in brain and lung tissue, but found in low levels elsewhere. All \(\beta\) subunits are made up of 8 segments, each consisting of a repetitive 40 amino acid sequence motif, the significance of which is still unknown (see Simon et al., 1991). That \(\beta\gamma\) dimers are actively involved in transmembrane signalling was first described in yeast cells (Dietzel and Kurjan, 1987), and it is only recently that the role of the subunits is being studied in mammalian cells (see above; Camps et al., 1992a,b; Katz et al., 1992)

Four distinct isotypes of \(\gamma\) subunits have been isolated as cDNA clones. Peptide sequences from purified proteins suggest the existence of at least two more. These proteins are most divergent at their amino-terminal region, and contain substantial homology at the carboxy-terminal region. \(\gamma\) subunits are all modified by removal of the three carboxy-terminal amino acid residues adjacent to a cysteine (fourth amino acid
residue from the carboxy terminus). This cysteine requires carboxymethylation and isoprenylation. Replacement of the cysteine residue with a leucine residue, which cannot undergo such modification, removes all activity of the βγ-stimulated PLCγ2 activation, associated with the loss of ability of this βγ dimer to bind to membranes (Katz et al., 1992).

1.3 Role of Ins(1,4,5)P3 as a Second Messenger.

The first direct report that Ins(1,4,5)P3 was a Ca^{2+}-mobilising agent came from Stieb et al. (1983). Upon addition of Ins(1,4,5)P3 to permeabilised pancreatic acinar cells, a release of Ca^{2+} from non-mitochondrial stores was observed. Subsequent studies confirmed that Ins(1,4,5)P3 had similar effects in many other cell types (see reviews, Berridge and Irvine 1984, 1989).

Evidence that Ins(1,4,5)P3 stimulated a Ca^{2+} channel rather than by affecting a carrier system came from demonstrations that Ca^{2+} mobilisation by Ins(1,4,5)P3 was relatively temperature insensitive (Smith et al., 1985; Meyer et al., 1988), very rapid (Champeil et al., 1989; Ogden et al., 1990) and finally, from electrophysiological recordings of Ins(1,4,5)P3-stimulated channel activity (Ehrlich and Watras, 1988).

As evidence accumulated against an important role for mitochondria as Ins(1,4,5)P3-sensitive stores of Ca^{2+}, the E.R., almost by default, was ascribed the role. There is evidence from both morphological (Wakasugi et al., 1982) and sub-cellular fractionation (Bayerdorffer et al., 1984) studies supporting an important role for the rough E.R. in some cell types, but not in others (Krause and Lew, 1987). While further evidence using electron probe microanalysis of liver showed Ca^{2+} content of rough E.R. decreased upon agonist stimulation (Bond et al., 1987), it has been suggested that the Ins(1,4,5)P3-sensitive stores may be present in discrete stores-Calciosomes (Krause et al., 1989). These structures account for some 1-2% of cytoplasmic volume. The status of these 'organelles' is still unclear- are they discrete organelles or specialised parts of other organelles? Immunocytochemical studies with antibodies to the Ins(1,4,5)P3 receptor specifically labelled structures which were clearly not these calciosomes (Satoh et al., 1990), and Volpe et al. (1991) have identified certain of these structures to be sensitive to ryanodine. Equally, Volpe et al. (1991) have shown that Ins(1,4,5)P3 receptors are localised to areas of the E.R. which are enriched with a Ca^{2+}-binding protein.
Ins(1,4,5)P₃ binding and Ca²⁺ mobilisation properties have also been identified in nuclear membranes prepared from liver (Malviya et al., 1990) and plasma membranes from lymphocytes (Kuno and Gardner, 1987). A number of other studies have shown the Ins(1,4,5)P₃ receptor to be localised in a large number of organelles (see Ferris and Snyder, 1992b). The ryanodine receptor was first identified in smooth muscle cells, located on the sarcoplasmic reticulum. This receptor is responsible for the release of Ca²⁺ from internal stores via a Ca²⁺-induced Ca²⁺ mobilisation mechanism following Ca²⁺ influx. The ryanodine receptor, like the Ins(1,4,5)P₃ receptor (see below) exists as a homotetramer producing an integral Ca²⁺ channel (see Berridge, 1993). Three ryanodine receptors have been cloned, two of which, isolated from skeletal (RYR1) and cardiac (RYR2) muscle, showed 66% homology (Otsu et al., 1990). A third ryanodine receptor (RYR3) appears to be insensitive to caffeine (Giannini et al., 1992), and appears to be much smaller than RYR1 or RYR2. The ryanodine receptor has now been identified in non-muscle cells, including neurons (Otsu et al., 1990; Walton et al., 1991).

1.4 Ins(1,4,5)P₃ Binding Sites.

Early suggestions that Ins(1,4,5)P₃ may be recognised by a specific intracellular receptor protein (Berridge and Irvine, 1984) have been supported by a number of studies indicating high affinity [³H] and [³²P] Ins(1,4,5)P₃ binding sites in a range of peripheral and central tissues which show strict stereo- and positional specificity for Ins(1,4,5)P₃ (reviewed by Nahorski and Potter, 1989; Taylor and Richardson, 1991; Ferris and Snyder, 1992a,b). Although the rank order of potency of inositol phosphates at these binding sites is very similar to that found for Ca²⁺ mobilisation, equilibrium binding occurs with some 50-fold higher affinity than Ca²⁺ release (see Nunn and Taylor, 1990). The discrepancies may, however, merely reflect the very different conditions under which radioligand binding and Ca²⁺ flux assays are performed (Mauger et al., 1989; Nunn and Taylor, 1990). However, Mauger et al. (1989) have identified two binding affinities for Ins(1,4,5)P₃ in membranes obtained from rat liver. Pretreatment of the liver with agonists which stimulated Ins(1,4,5)P₃ production caused an increase in the high affinity binding, which was stabilised at low temperature and not present at 37°C. Mauger et al. (1989) suggested that this high affinity site may be a desensitised form of the receptor. Equally, Rouxel et al. (1992), have shown that the Ins(1,4,5)P₃ receptor can exist in a low affinity and high affinity state. The inactive state required ~1 second before returning to the high
affinity form. This process was Ca\(^{2+}\) sensitive, high levels of Ca\(^{2+}\) inhibiting interconversion. Ca\(^{2+}\) regulation of the Ins\((1,4,5)\)P\(_3\) receptor had been previously shown by Worley et al. (1987), who showed that physiologically relevant levels of Ca\(^{2+}\) inhibited Ins\((1,4,5)\)P\(_3\) binding to membranes prepared from brain, giving rise to the possibility of a negative feedback mechanism. Ins\((1,4,5)\)P\(_3\) binding was also found to be highly pH sensitive, with affinity tripling between pH 7.5 and 8.5 (Worley et al., 1987). This may, however, merely reflect changes in the protonation of the phosphate groups of Ins\((1,4,5)\)P\(_3\), which fully ionise within this pH range (Joseph et al., 1989b). Some Ins\((1,4,5)\)P\(_3\) receptors appear to be targets for phosphorylation by cyclic AMP-dependent protein kinase (protein kinase A), protein kinase C and Ca\(^{2+}\)-calmodulin dependent protein kinase, each of these appear to phosphorylate at different sites (Ferris et al., 1991).

Evidence now shows that the Ins\((1,4,5)\)P\(_3\)-binding site is the ligand recognition site of the Ca\(^{2+}\) mobilising receptor as:-

1. Ins\((1,4,5)\)P\(_3\) binding sites and Ca\(^{2+}\) mobilisation have similar agonist and antagonist recognition profiles.

2. Ins\((1,4,5)\)P\(_3\) binding sites have been shown not to be metabolic enzymes (Nunn et al., 1990).

3. Purification and functional reconstitution of the Ins\((1,4,5)\)P\(_3\) receptor (Ferris et al., 1989; Maeda et al., 1991), together with expression of the cloned gene have provided conclusive evidence that the Ins\((1,4,5)\)P\(_3\) binding site is the ligand recognition domain of the Ca\(^{2+}\) mobilising receptor.

The identification of specific high affinity Ins\((1,4,5)\)P\(_3\) binding sites which are integral membrane proteins has allowed for their successful solubilisation and purification. The native molecular weight of the Ins\((1,4,5)\)P\(_3\) receptor is about 1,000 kDa (Supattapone et al., 1988b). The receptor protein has been purified to homogeneity from rat cerebellum (Supattapone et al., 1988b) and, subsequently many other sources (reviewed by Taylor and Richardson, 1991; Ferris and Snyder, 1992a,b). The purified Ins\((1,4,5)\)P\(_3\) receptors from these tissues appears to be very similar. They are homotetramers of noncovalently linked subunits (Mignery and Sudhof, 1990; Maeda et al., 1991). The size of the subunits determined using SDS-PAGE are similar in peripheral (Mr 224 kDa or 260 kDa) and central tissues (260 kDa). Recent, chemical cross-linking studies has confirmed the tetrameric nature of the Ins\((1,4,5)\)P\(_3\) receptor, subunits of which were found to be of 320 kDa in mouse cerebellum (Maeda et al., 1991).
Ins(1,4,5)P$_3$ binding to the rat cerebellar receptor was identified as being Ca$^{2+}$ sensitive in crude preparations, a property which was lost once the receptor was purified (Supattapone et al., 1988b), suggesting that a closely associated protein binds Ca$^{2+}$ and confers sensitivity of the Ins(1,4,5)P$_3$ receptor to Ca$^{2+}$. This Ca$^{2+}$ sensitivity was not observed with Ins(1,4,5)P$_3$ receptors previously studied from peripheral tissues (see Supattapone et al., 1988b). Maeda et al. (1991) have identified Ca$^{2+}$ sensitive calmodulin binding to the Ins(1,4,5)P$_3$ receptor purified from mouse cerebellum, which was lost during long term storage.

The Ins(1,4,5)P$_3$ receptor has been incorporated into phospholipid vesicles where it binds Ins(1,4,5)P$_3$ and has been shown to mediate Ca$^{2+}$ fluxes (Ferris et al., 1989, 1990), and into lipid bilayers where Ins(1,4,5)P$_3$ has been shown to open a large Ca$^{2+}$ channel, which is also permeable to Na$^+$ (Maeda et al., 1991). Thus, it is the Ins(1,4,5)P$_3$ receptor which constitutes both the Ins(1,4,5)P$_3$ binding site and the associated Ca$^{2+}$ channel. This receptor has been shown to bind inositol phosphates and permit Ca$^{2+}$ fluxes with the same rank order of potency and selectivity as those previously found in other systems (Ferris et al., 1989).

The Ins(1,4,5)P$_3$ receptor has been sequenced and cloned from rat brain (Mignery et al., 1990) and mouse cerebellum (Furuichi et al., 1989). Transfection of cell lines which normally express low levels of the Ins(1,4,5)P$_3$ receptor, show increased Ins(1,4,5)P$_3$ binding (Furuichi et al., 1989; Mignery and Sudhof, 1990) and increased sensitivity to Ins(1,4,5)P$_3$-stimulated Ca$^{2+}$ mobilisation (Miyawaki et al., 1990).

Marks et al., (1990) have shown that the purified bovine smooth muscle Ins(1,4,5)P$_3$ receptor has high homology with the mouse cerebellar receptor. Indeed, the Ins(1,4,5)P$_3$ receptor from many species shows great homology. Rat and mouse cerebellar receptors differ by only 21 residues from a total of 2749 residues (Mignery and Sudhof, 1990) and over 85% of the human brain Ins(1,4,5)P$_3$ receptor is identical to these (Danoff et al., 1988). Mignery et al. (1990), using an antibody targeted at the carboxy-terminal 19 amino acid residues, have identified proteins of similar weights which interact with this antibody in heart, liver, testis and spinal cord, suggesting that the Ins(1,4,5)P$_3$ receptors in these regions are closely related to that identified from cerebellum. Hydrophobicity studies of the cerebellar Ins(1,4,5)P$_3$ receptor identified 8 lipophilic regions. The amino-terminal region is undoubtedly cytoplasmic (Mignery et al., 1990), and it is this region which binds Ins(1,4,5)P$_3$. Indeed, deletion of the amino terminal 418 residuesof the rat brain Ins(1,4,5)P$_3$ receptor removes all Ins(1,4,5)P$_3$ binding (Mignery et al., 1990), and a protein comprising only the terminal 788 residues specifically binds Ins(1,4,5)P$_3$.
Deletion of 2225-2604, the putative transmembrane domain, causes loss of membrane association producing soluble monomers (Mignery and Sudhof, 1990). The 1400 amino acid tail between the Ins(1,4,5)P3-binding region and the putative transmembrane region contains a protein kinase consensus site described by Danoff et al (1991), and adenine nucleotide binding sites (see Ferris and Snyder, 1992a). Mignery and Sudhof (1990) have shown that the apparent molecular weight of the receptor is substantially reduced upon agonist stimulation. Accordingly, Ins(1,4,5)P3 binding must elicit a significant conformational change of the receptor over this 1400 amino acid tail.

Recent studies have identified multiple forms, derived from alternative splicing (Danoff et al., 1991; Nakagawa et al., 1991). The long form identified by Danoff et al. (1991) appears, so far, to be exclusively neuronal, and a shorter form (lacking 39-40 residues) near 2 consensus sites for phosphorylation by protein kinase A, is more widely expressed. The functional significance of this differential splicing and of a smaller splicing event (15 residues within the Ins(1,4,5)P3 binding region) is not yet clear. Nakagawa et al. (1991) also identified a number of rat brain Ins(1,4,5)P3 receptors derived from alternative splicing. They identify 2 segments of the Ins(1,4,5)P3 receptor at which splicing occurs, producing six heterogeneous Ins(1,4,5)P3 receptors. Ratios of these proteins changed during the animals development. Finally, they suggested that tetramers of the Ins(1,4,5)P3 receptor, making up the Ca^{2+} channel, may be combinations of different splice variants, generating a wide diversity of possible heterogeneous tetramers.

In total, the Ins(1,4,5)P3 receptor is now believed to be derived from at least four different gene products. Over 70% homology exists between the Ins(1,4,5)P3 receptor described by Furuichi et al. (1989) and Mignery et al. (1990) (InsP3R1) and that described by Sudhof et al. (1991) and Ross et al. (1992) (InsP3R2). Partial sequences exist for a further two gene products (Sudhof et al., 1991; Ross et al., 1992) (labelled InsP3R3 and InsP3R4 by Berridge, 1993). The function of these receptors is, as yet, unknown. They appear to be expressed at lower levels than InsP3R1 and InsP3R2 (Ross et al., 1992). Clearly, the opportunity of splice variants within these four gene products, as has been described for the InsP3R1 (see Berridge, 1993), allow a large family of Ins(1,4,5)P3 receptors to exist. Pharmacological characterisation of Ins(1,4,5)P3 receptors has also shown substantial differences in binding properties of receptors prepared from rat cerebellum and thymus and Jurkat human T-cells (Khan et al., 1992). Rat cerebellar membranes bound Ins(1,4,5)P3 with a single affinity (KD=40nM), whereas thymus membranes bound Ins(1,4,5)P3 with a high- (KD=45nM) and low-
(K_D=1.2μM) affinity. Jurkat human T-cell plasma membranes revealed a single, low affinity site (K_D=0.8-1μM), and microsomal membranes revealed a high affinity site (K_D=25nM). The low affinity site appears to correspond to Ins(1,4,5)P_3 receptors associated with the plasma membrane and the high affinity sites appear to correspond to Ins(1,4,5)P_3 receptors in the E.R. (Khan et al., 1992). Equally, those receptors with low affinity for Ins(1,4,5)P_3 had high affinity for Ins(1,3,4,5)P_4, with little selectivity, and receptors with high affinity for Ins(1,4,5)P_3 had low affinity for Ins(1,3,4,5)P_4 (Khan et al., 1992). This suggests that the plasma membrane binding site may be involved in the gating of extracellular Ca^{2+} in an Ins(1,4,5)P_3/Ins(1,3,4,5)P_4-sensitive manner, as previously suggested by Irvine and Moor (1987).

1.5 Ins(1,4,5)P_3-induced Ca^{2+} Mobilisation.

Work by Meyer et al. (1988) has shown that on average three Ins(1,4,5)P_3 molecules must bind the tetrameric receptor to cause opening of the Ca^{2+} channel. The rapid kinetics and limited temperature dependence of Ca^{2+} release show that Ins(1,4,5)P_3 mobilises Ca^{2+} directly via a channel opening mechanism, rather than a carrier mechanism (Smith et al., 1985). Smith et al. (1985) also claimed the requirement for adenine nucleotides for Ca^{2+} mobilisation, further work suggests that, while playing an important role in Ins(1,4,5)P_3-induced Ca^{2+} mobilisation, there is not an absolute requirement.

ATP appears to interact with at least two sites on the Ins(1,4,5)P_3 receptor, as well as being involved in the loading of the endoplasmic reticulum by a Ca^{2+}/Mg^{2+} ATPase. It binds with low affinity to the same site as Ins(1,4,5)P_3, behaving as a competitive antagonist (Willcocks and Nahorski, 1988; Nunn and Taylor, 1990) (K_D=50μM for free ATP, however Mg^{2+} chelated ATP was without effect). ATP also binds with high affinity (K_D=17μM) to a distinct site distal to the Ins(1,4,5)P_3 binding region. ATP binding to this site is not displaceable by Ins(1,4,5)P_3 (Ferris et al., 1990; Maeda et al., 1991). ADP and AMP also bind this site, however, GTP was without effect (Nunn and Taylor, 1990; Maeda et al., 1991). Low levels of ATP (EC_{50}<10μM) enhance Ins(1,4,5)P_3-stimulated Ca^{2+} fluxes through the purified receptor (Ferris et al., 1990). Maeda et al. (1991) suggest that this occurs by adenine nucleotides increasing the open probability of the Ca^{2+} channels in the presence of Ins(1,4,5)P_3, without affecting the open probability themselves.
Another factor involved with Ins(1,4,5)P3-induced Ca^2+ release is the levels of Ca^2+ present in the E.R. and cytosol. Intraluminal Ca^2+ appears to control, in some way, the sensitivity of the Ins(1,4,5)P3 receptor to Ins(1,4,5)P3. That luminal Ca^2+ is essential before Ins(1,4,5)P3 can open the channel has been confirmed by Nunn and Taylor (1991). Cytosolic Ca^2+ appears to inhibit Ins(1,4,5)P3 binding in some, but not all, systems (see Taylor and Richardson, 1990). The discrepancy may be due to the distribution of a membrane protein, calmodulin. Calmodulin, a monomer with molecular weight of 15 kDa (Fennis and Snyder, 1992b), confers Ca^2+ sensitivity upon receptors which are not sensitive in its absence (see Danoff et al., 1988; Mouray, 1990). Recent studies on the Ca^2+ sensitivity of binding show that its effects are biphasic. Indeed, Ca^2+ enhances binding affinity up to 300nM, whereas higher levels are inhibitory (Iino, 1987, 1990; Bezprozvanny et al., 1991). The site at which Ca^2+ binds, is, as yet, unidentified, although the brain Ins(1,4,5)P3 receptor characterised by Furuichi et al. (1989a) appears not to possess any sequences recognised as Ca^2+ binding sites of previously characterised Ca^2+ binding proteins.

This may explain why binding assays, performed in membrane preparations in the presence of high levels of Ca^2+ chelating agents give Hill slopes of unity, whereas Ins(1,4,5)P3-induced Ca^2+ mobilisation studies are performed in systems where Ca^2+ levels are increased. However, work by Nunn and Taylor (1990) has compared binding and Ca^2+ mobilisation under identical conditions, and still found a discrepancy between slopes of the two curves. Contrary results were obtained by Finch et al. (1991), who showed that in a flow system, where Ca^2+ levels at the Ins(1,4,5)P3 receptor are not permitted to rise, Ins(1,4,5)P3 induces Ca^2+ mobilisation with a Hill coefficient of unity.

1.6 Models of Regulation of Ins(1,4,5)P3-induced Ca^2+ Mobilisation.

A number of theories concerning Ins(1,4,5)P3-induced Ca^2+ mobilisation have attempted to explain the complexity of mechanisms involved, these are discussed below.

In 1989, Muallem et al. described the effects of plasma membrane-derived Ins(1,4,5)P3 as being quantal. That is, suboptimal doses of Ins(1,4,5)P3 mobilised a discrete 'quantum' of Ca^2+. A suboptimal dose of Ins(1,4,5)P3 may have been expected to cause a submaximal opening of Ca^2+ channels which would mobilise the same net amount of Ca^2+, but with a slower rate of efflux. Such behaviour is observed with the Ca^2+...
ionophore, ionomycin (Muallem et al., 1989; Taylor and Potter, 1990), whereas Ins(1,4,5)P$_3$ or a non-metabolisable analogue (inositol 1,4,5-trisphosphorothioate) rapidly mobilised a fraction of the Ca$^{2+}$ stores, this fraction depending on the dose of agonist used. In 1990, Irvine proposed a hypothesis to explain the data obtained by Muallem et al. (1989) and Taylor and Potter (1990). The central proposal of this hypothesis was that the Ins(1,4,5)P$_3$ receptor, or a closely associated protein, possessed a binding site for Ca$^{2+}$ on the intraluminal side, with low affinity for Ca$^{2+}$. He proposed that the binding of Ins(1,4,5)P$_3$ to its receptor increased the affinity of the intraluminal site of the receptor for Ca$^{2+}$ and, likewise, the binding of Ca$^{2+}$ to this intraluminal site increased the affinity of the receptor for Ins(1,4,5)P$_3$. Ins(1,4,5)P$_3$-induced Ca$^{2+}$ mobilisation lowered the concentration of intraluminal Ca$^{2+}$, such that the affinity of the receptor for Ins(1,4,5)P$_3$ was reduced, and no further Ca$^{2+}$ mobilised, until Ca$^{2+}$ was resequenced, allowing a steady state to be attained. The addition of further Ins(1,4,5)P$_3$ would, again, establish a new steady state. However, Ferris et al. (1992) have shown, using Ins(1,4,5)P$_3$ receptors reconstituted into lipid vesicles, that responses to Ins(1,4,5)P$_3$ are heterogeneous even when Ca$^{2+}$ levels are held constant on both sides of the receptor, although recent reports by Missiaen et al. (1992 a,b), in agreement with Irvine’s hypothesis (1990), claim that depleted stores are less sensitive to Ins(1,4,5)P$_3$ than fully loaded stores. Missiaen et al.(1992 a,b) showed a biphasic response, a rapid release, being complete within the first second of Ins(1,4,5)P$_3$ addition, followed by a much slower, long term release in permeabilised A7r5 smooth muscle cells, this response being sensitive to changes in intraluminal Ca$^{2+}$ levels.

Cytosolic Ca$^{2+}$ has also been shown to have biphasic effects on the binding of Ins(1,4,5)P$_3$. Bezprozvanny et al. (1991) have shown that cytosolic Ca$^{2+}$ both enhances (0.01-0.3μM) and inhibits (>0.3μM) Ins(1,4,5)P$_3$-induced Ca$^{2+}$ channel opening in vesicles prepared from canine cerebellum. Similarly, Finch et al. (1991) have shown that extraluminal Ca$^{2+}$ acts as a coagonist at the Ins(1,4,5)P$_3$ receptor. Superfusion techniques allowed Finch et al. (1991) to remove any Ca$^{2+}$ mobilised from the vicinity of the Ins(1,4,5)P$_3$ receptor. Dose response relationships prepared under conditions where superfusion rates were high showed a Hill coefficient of 1.0, due to the positive effects of Ca$^{2+}$ being absent (but see Nunn and Taylor, 1990). Meyer et al. (1988) had previously shown a strong positive cooperativity of Ins(1,4,5)P$_3$-induced Ca$^{2+}$ mobilisation.

More recently it has been suggested that ‘quantal’ Ca$^{2+}$ release occurs by discrete stores fully mobilising all their Ca$^{2+}$ following stimulation of an Ins(1,4,5)P$_3$ receptor (all-or-nothing release, Parker and Ivorra, 1990; Bootman et al., 1992). A large number of stores may exist associated with Ins(1,4,5)P$_3$ receptors which have different affinities for...
Ins(1,4,5)P$_3$. Thus, low levels of Ins(1,4,5)P$_3$ would fully mobilise Ca$^{2+}$ from stores associated with the most sensitive Ins(1,4,5)P$_3$ receptors, with higher concentrations required to mobilise Ca$^{2+}$ from stores associated with the less sensitive Ins(1,4,5)P$_3$ receptors. This pattern of response has been confirmed in Xenopus oocytes (Parker and Ivorra, 1990).

1.7 Metabolism of Ins(1,4,5)P$_3$.

The metabolism of Ins(1,4,5)P$_3$, although initially thought to involve a simple dephosphorylation pathway to Ins(1,4)P$_2$ and Ins(1)P/Ins(4)P to free inositol (Storey et al., 1984) is now understood to be considerably more complex (Fig. 1.3). In mammalian cells, Ins(1,4,5)P$_3$ is metabolised via two major routes, a 5-phosphatase pathway to Ins(1,4)P$_2$, and a 3-kinase pathway to Ins(1,3,4,5)P$_4$ (reviewed by Shears, 1989, 1991). The inositol polyphosphate 5-phosphatase (referred to as Ins(1,4,5)P$_3$ 5-phosphatase throughout this thesis) is a Mg$^{2+}$-dependent, 2,3 bisphosphoglycerate (2,3 BPG) -inhibitable enzyme able to hydrolyse Ins(1,3,4,5)P$_4$, Ins(1,2,4,5)P$_3$, and Ins(4,5)P$_2$ in addition to Ins(1,4,5)P$_3$ (reviewed by Shears, 1991; Taylor and Richardson, 1991). Since Ins(1,4)P$_2$, Ins(1,2,4)P$_2$, Ins(4)P and Ins(1,3,4)P$_3$ are unable to mobilise Ca$^{2+}$, this enzyme plays a crucial role in the termination of the Ca$^{2+}$ mobilising signal. A number of distinct Ins(1,4,5)P$_3$ 5-phosphatase enzymes have been isolated and purified which differ not only in their Mr but also in their affinity for Ins(1,3,4,5)P$_4$ and susceptibility to protein kinase C-mediated phosphorylation. The specificity of Ins(1,4,5)P$_3$ 5-phosphatase to cleave the 5-phosphate was first shown by Downes et al. (1982) in human erythrocytes and later in insect salivary glands (Berridge et al., 1983), followed by the identification of a Mg$^{2+}$-dependent Ins(1,4,5)P$_3$ 5-phosphatase in a number of tissues (reviewed by Shears, 1989). Following the identification of Ins(1,3,4,5)P$_4$ (Batty et al., 1985), it was noted that this too was a substrate for Ins(1,4,5)P$_3$ 5-phosphatase from human erythrocyte ghosts, although no characterisation of this was made. So far, there have only been a handful of reports of an Ins(1,4,5)P$_3$ 5-phosphatase which recognises Ins(1,3,4,5)P$_4$ poorly. One is a soluble Ins(1,4,5)P$_3$ 5-phosphatase, and is one of two purified by Hansen et al. (1987) from rat brain. The soluble type 1 of around 60 kDa has $K_m$ values for Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ of 3 and 0.8μM, the type 2, of around 160 kDa, has $K_m$ values of 18 and...
Identified metabolic enzymes:
1. PI-PLC
2. 5-phosphatase
3. 3-kinase
4. 3-phosphatase
5. 1-phosphatase
6. 4-phosphatase
7. 3-phosphatase
8. hydrolase
9. monophosphatase
10. 6-kinase
11. 5-kinase

Figure 1.3 Metabolism of PtdIns(4,5)P₂ and Inositol Phosphates (for further details see Shears, 1989, 1991).
PtdIns(4,5)P2 5-phosphatase is also reported to act upon Ins(1,4,5)P3 (reviewed by Shears, 1989).

Ins(1,4,5)P3 5-phosphatases have also been purified from bovine brain (Erneux et al., 1989). A soluble and a particulate Ins(1,4,5)P3 5-phosphatase (37 and 36 kDa) both have Km values of 11 and 1 μM for Ins(1,4,5)P3 and Ins(1,3,4,5)P4. A second soluble Ins(1,4,5)P3 5-phosphatase (115 kDa) appears not to recognise Ins(1,3,4,5)P4, and has an affinity of 72 μM for Ins(1,4,5)P3. Takimoto et al. (1989) have also purified a particulate Ins(1,4,5)P3 5-phosphatase, from rat liver. They, like Erneux et al. (1989), find this enzyme very similar to the soluble type 1 species, with respect to molecular weight (32 kDa), kinetic parameters (Km 5-6 and 0.8 μM for Ins(1,4,5)P3 and Ins(1,3,4,5)P4 respectively), and the isoelectric point, but quite different from the soluble type 2 species (69 kDa, Km 8 and 130 μM respectively). Takimoto et al. (1989) also identified that soluble type 2 enzyme activity was substantially increased in the presence of CoCl₂ (1 mM).

Similarly, Mitchell et al. (1989) identified two soluble Ins(1,4,5)P3 5-phosphatases from human platelets. A 75 kDa protein with Km values for Ins(1,4,5)P3 and Ins(1,3,4,5)P4 of 24 and 7.5 μM, and a 45 kDa protein with Km values of 7.5 and 0.5 μM respectively were identified. The 45 kDa protein had been previously identified as a substrate for protein kinase C, leading to activation, observed as an increase in Vmax with no change in Km (Connolly et al., 1986), this was not observed with the 75 kDa protein. Both the 45 and 75 kDa proteins hydrolysed Ins(4,5)P2, but at <1% of the rate of Ins(1,4,5)P3.

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The major route of metabolism of Ins(1,4)P2 is an inositol polyphosphate 1-phosphatase. This has also been purified (Inhorn and Majerus, 1987 (Mr 44 kDa), Gee et al., 1988 (Mr 40 kDa)) and found to have activity against Ins(1,3,4)P3 as well as Ins(1,4)P2, but not Ins(1,4,5)P3 or Ins(1,3,4,5)P4. Km values for Ins(1,4)P2 and Ins(1,3,4)P3 are approximately 1-5 and 5-20 μM respectively, with Vmax values the same for both phosphates (Shears, 1991). The inositol polyphosphate 1-phosphatase is mostly cytosolic, with less than 20% particle bound, and, like the monophosphatase (see below) is uncompetitively inhibited by Li⁺ (reviewed by Shears, 1989, 1991).

The other route of Ins(1,4,5)P3 metabolism involves a 3-kinase, which appears highly specific for Ins(1,4,5)P3. Shortly after the discovery of Ins(1,3,4,5)P4, Ins(1,4,5)P3 3-kinase activity was found in the soluble fraction of a wide variety of cells and has been found to have a higher affinity for Ins(1,4,5)P3 (Km 0.2-1.5 μM) than any of the Ins(1,4,5)P3 5-phosphatases yet identified (Km 10-100 μM) (see Shears, 1989, 1991).
With such a high affinity for Ins(1,4,5)P₃, it is somewhat surprising that high levels of Ins(1,3,4,5)P₄ are not found in cells under resting conditions. Levels of Ins(1,4,5)P₃ have been found in the region of 1-4μM (Challiss et al., 1990; Zhao et al., 1990), rising to ~25μM upon agonist stimulation. Thus, even under resting conditions, Ins(1,4,5)P₃ 3-kinase should be operating at near its maximal rate, in contrast to Ins(1,4,5)P₃ 5-phosphatases which encounter Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ well below their Km values. This, in concert with evidence indicating that increases in Ca²⁺ across the physiological intracellular range markedly stimulates Ins(1,4,5)P₃ 3-kinase activity, largely by increasing its Vₘₐₓ, indicates the potential of this enzyme in regulating Ins(1,4,5)P₃ metabolism (Biden and Wollheim, 1986). However, following purification (Lee et al., 1990) and cloning (Choi et al., 1990), Ins(1,4,5)P₃ 3-kinase was also identified as a substrate for the Ca²⁺-dependent protease, calpain, containing six PEST regions common to all calpain substrates. This 53 kDa protein was subject to proteolysis by calpain, producing 51, 40, 33, and 32 kDa fragments, which still possessed Ins(1,4,5)P₃ 3-kinase activity. Indeed, following calpain cleavage of 90% of the 53 kDa protein, only 25% loss of activity was observed. The 53 kDa and 40 kDa proteins were also shown to be activated by calmodulin in the presence of Ca²⁺. This same group have also shown that Ins(1,4,5)P₃ 3-kinase is a substrate for protein kinase C, phosphorylation leading to a decrease in Vₘₐₓ by some 75%. Protein kinase A phosphorylation was also observed, at a different site, increasing Vₘₐₓ by 80% (Sim et al., 1990). Thus, it appears that receptor stimulation leading to rises in cytosolic Ca²⁺ concentration activates Ins(1,4,5)P₃ 3-kinase, followed by a protein kinase C-induced phosphorylation and deactivation.

The role of Ins(1,3,4,5)P₄ in Ca²⁺ homeostasis is under much debate. It may mobilise Ca²⁺ from internal stores via a distinct Ins(1,3,4,5)P₄ receptor (Donie and Reiser, 1989; Challiss et al., 1991), or it may act via the Ins(1,4,5)P₃ receptor (Wilcox et al., 1993). Ins(1,3,4,5)P₄ is reported to mobilise Ca²⁺ from internal stores (Joseph et al., 1989; Gawler et al., 1990), resequester mobilised Ca²⁺ to internal stores (Hill and Boynton, 1990), induce influx of Ca²⁺ into cells (Irvine and Moor, 1987) and produce synergistic effects with Ins(1,4,5)P₃ in some but not other systems (Joseph et al., 1987; Morris et al., 1987; Cullen et al., 1990).

Backconversion of Ins(1,3,4,5)P₄ may occur via a 3-phosphatase to Ins(1,4,5)P₃ with Kₘ≈2.4μM (Oberdisse et al., 1990; McIntosh and McIntosh, 1990), although the product, Ins(1,4,5)P₃, appears to inhibit its own formation (Oberdisse et al., 1990; Hoer et al., 1990). This 3-phosphatase is Ca²⁺-insensitive, but can be further stimulated by thrombin or the protein kinase C-activating phorbol esters. These effects were not
additive, and, therefore, may be due to the activation of protein kinase C, although this is in contradiction to the observation that GTPγS, which persistently activates G-proteins (and may, therefore, also cause Ins(1,4,5)P3 and DAG formation leading to protein kinase C activation), acts as an inhibitor, as did UTP (Oberdisse et al., 1990). Alternatively, Ins(1,3,4,5)P4 appears to be metabolised almost exclusively by the above Ins(1,4,5)P3 5-phosphatase to Ins(1,3,4)P3, an isomer which plays no role in Ca2+ homeostasis, which then can be either dephosphorylated to Ins(3,4)P2 and/or Ins(1,3)P2 or phosphorylated back to Ins(1,3,4,5)P4 (to generate an Ins(1,3,4,5)P4/Ins(1,3,4)P3 futile cycle), or to Ins(1,3,4,6)P4, which can be further phosphorylated to Ins(1,3,4,5,6)P5—this latter pathway may form an important pathway between Ins(1,4,5)P3 and the higher phosphates (reviewed by Shears, 1989, 1991).

The existence of an inositol monophosphatase has been known for many years (Eisenberg, 1967). Recent purification studies indicate that this soluble enzyme is dimeric in structure with a subunit Mr of 29-31 kDa, and is able to hydrolyse Ins(5)P as well as Ins(1)P and Ins(4)P, binding with a similar affinity to all (Km 0.1-0.2 mM). Ins(1:2)P and Ins(2)P appear to be the only two inositol monophosphates which are resistant to this enzyme (see Shears, 1989). The activity of this enzyme has additional significance in tissues that have limited access to extracellular inositol, where the major source of inositol is from recycling of the inositol phosphates or the cyclisation of glucose 6-phosphate, which is converted to L-Ins(1)P (D-Ins(3)P) by the enzyme L-Ins(1)P synthase (Eisenberg, 1967).

The discovery that a number of the above inositol polyphosphate phosphatases (notably the 1-phosphatase) and inositol monophosphatase are uncompetitively blocked by Li+ ions (at clinically relevant doses) was seen as a major advance in the understanding of the possible mechanisms underlying the therapeutic effects of LiCO3 (Shermen et al., 1981; Berridge et al., 1982; reviewed by Nahorski et al., 1991). Indeed, there is now compelling evidence that Li+, through its uncompetitive inhibition of the monophosphatase enzyme, deprives cells of myo-inositol. Due to the nature of its inhibition, depletion will occur in the cells where PI turnover is greatest. Correspondingly, this will reduce the pools of phosphoinositides available for second messenger production and subsequently reduce the intracellular signal. This inhibition can only be effective in cells where recycling of inositol is the major source and uptake from extracellular sources is absent or insufficient. This is the case in the CNS, as inositol traverses the blood-brain barrier poorly (see Nahorski et al., 1991), and it is in the treatment of manic-depressive psychosis that Li+ has considerable success. This phenomenon has also allowed the development of assays of PI breakdown where any
inositol phosphate produced is trapped at the monophosphate level (Berridge et al., 1982).

A minor route of Ins(1,4,5)P3 metabolism may be a 1-phosphatase, since relatively low levels of Ins(4,5)P2 have been identified in GH3 cells (Hughes and Drummond, 1987) and cerebral cortical slices (Batty et al., 1989). Recent evidence (Jenkinson et al., 1992) suggests that this phosphatase is Li+ sensitive. At lower levels of Li+, the unidentified enzyme involved in the metabolism of Ins(4,5)P2 is inhibited, enhancing accumulation of Ins(4,5)P2. At higher levels, the formation of Ins(4,5)P2 is also inhibited. Ins(4,5)P2 appears to be some 60-fold less potent at Ca2+ mobilisation (Irvine et al., 1986; Nunn and Taylor, 1990). It is unclear whether this 1-phosphatase can hydrolyse Ins(1,3,4,5)P4 to yield Ins(3,4,5)P3. Ins(3,4,5)P3 has been identified in avian erythrocytes, but the evidence provided suggests it is formed by a 6-phosphatase activity on Ins(3,4,5,6)P4 (Stephens et al., 1989).

1.8 Activation of Phospholipase C.

Hydrolysis of PtdIns(4,5)P2 is catalysed by the activation of specific phospholipases C. The phospholipases C are a superfamily of phosphodiesterases which hydrolyse the glycerophosphate bond of intact phospholipids to generate diacylglycerol and an aqueous group carrying the phosphate.

Initial studies concerning PLC s which acted specifically upon phosphoinositides (PI-PLC s) suggested that they were mostly cytosolic, had an acidic pH optimum and required a supraphysiological levels of Ca2+ to be activated (Kemp et al., 1961; Atherton and Hawthorne, 1968), although later, cytosolic PLC from lymphocytes was found to have pH optima of 7 and required Ca2+ in the micromolar range for activation (Allan and Michell, 1974). During the 1970's, several reports of membrane-associated PI-PLC appeared (reviewed by Meldrum et al., 1991). Like the cytosolic PLC described by Allan and Michell (1974), their pH optima were neutral, and they were sensitive to low levels of Ca2+. Furthermore, this Ca2+ dependence was not only influenced by pH, but also by substrate concentration (Allan and Michell, 1974; Irvine and Dawson, 1983). In the early 1980's, simple purifications of PI-PLC activity from animal cells showed multiple forms within one tissue. The first report of a purified PI-PLC was from Takenawa and Nagai (1981), with a 68-70 kDa protein from rat liver cytosol. Other reports of 65-70 kDa and
80 kDa PLC’s from sheep seminal vesicles (Hofman and Majerus, 1982) and a 143 kDa protein from bovine platelets (Hakata et al., 1982) suggested a large family may exist.

At this time PtdIns(4,5)P$_2$ was identified as the target substrate for receptor driven PI turnover. Most studies showed that PtdIns(4)P and PtdIns(4,5)P$_2$ were preferred substrates (see Meldrum et al., 1991), although Carter and Smith (1987) reported on a PLC from lymphocytes which preferentially acted upon PtdIns under all conditions, and Manne (1987) reported on a platelet PI-PLC which did not hydrolyse PtdIns. In the late 1980’s, a large number of PI-PLC’s were identified (see Meldrum et al., 1991).

From primary structure comparisons, it appears possible to divide the PI-PLC superfamily into three classes of isoforms, $\beta$(1-3), $\gamma$(1&2), and $\delta$(1-3). The existence of further isoforms has been suggested, based on enzyme purification and biochemical characterisation. These include a large number of $\alpha$ and $\epsilon$ isozymes, with the possibility of further isoforms in the $\gamma$ and $\delta$ families (reviewed by Cockcroft and Thomas, 1992). Comparing the predicted amino acid sequences of individual PLC’s, the overall level of identity of these proteins is low. However, $\beta$, $\gamma$ and $\delta$ isoforms have two regions of high homology, termed X (~170 amino acid residues) and Y (~260 amino acid residues) regions by Rhee et al. (1989). It was proposed that these two, highly conserved regions form the catalytic unit within the protein. Indeed, deletion of either or both of these regions from bovine brain PLC$_{\beta 1}$ removes all activity (see Meldrum et al., 1991). Bovine brain $\beta_1$, $\gamma_1$ and $\delta_1$ have overall identity in these regions of 43 and 33% respectively. However, PLC$\alpha$ has no identity in these regions, and one has recently been reported to have no PLC activity (see Rhee and Choi, 1992).

The role of each individual within this superfamily is currently under investigation, with clear patterns emerging.

PLC$_{\beta 1}$ has been shown to have the same $K_m$ for PtdIns(4)P and PtdIns(4,5)P$_2$, however, the $V_{max}$ for hydrolysis is some 30 fold higher for PtdIns(4,5)P$_2$ (Katan and Parker, 1987), suggesting considerable specificity towards polyphosphoinositides. PLC$_{\gamma 1}$ hydrolyses PtdIns(4,5)P$_2$ less efficiently than PLC$\beta$ or PLC$\delta$ family members, such that PtdIns and PtdIns(4,5)P$_2$ are hydrolysed at similar rates (Ryu et al., 1987). PLC$\delta$ s show a catalytic preference for polyphosphoinositides at low Ca$^{2+}$ levels.

However, at mM levels of Ca$^{2+}$, PLC$\delta$(1 & 2), unlike PLC$_{\beta 1}$, will hydrolyse PtdIns efficiently. PLC$\alpha$ also appears to preferentially hydrolyse polyphosphoinositides at low Ca$^{2+}$ levels. In vitro studies, using single lipid vesicles of PtdIns(4,5)P$_2$ have indicated that purified enzymes are capable of catalysing phospholipid hydrolysis at a high rate.
(Rhee et al., 1989), in good agreement with earlier data using semipurified proteins and PtdIns as substrate (Allan and Michell, 1974). Such was the rate of hydrolysis, that Rhee et al. (1989) calculated the lifetime of preformed PtdIns(4,5)P2 to be under 20 seconds under resting conditions. Clearly, this is energetically unfavourable, and suggests that some form of negative control is missing in these purified preparations. Two suggestions were made by Meldrum et al. (1991), firstly, an inhibitor of PLC, active and present in crude preparations, may be dissociated or become inoperative upon receptor stimulation, and secondly, PI-PLCs may be active but unable to gain access to their substrates. Such properties may be lost in a purified preparation.

The production of cyclic inositol phosphates (Ins(1,2)P, Ins(1,2,4)P2 and Ins(1,2,4,5)P3) has been observed in cell systems, accumulating to high levels in some cells (see Willcocks et al., 1989). It has been reported that PLCβ1, PLCγ1 and PLCδ1 form cyclic and non-cyclic inositol phosphates in different ratios (Kim et al., 1989), with PLCβ1 producing the greatest proportion of cyclic phosphates followed by PLCδ1 and PLCγ1. Ins(1,2)P was the predominant cyclic phosphate, with less Ins(1,2,4)P2 and Ins(1,2,4,5)P3 being produced.

As the new class of phosphoinositides emerge, phosphorylated in the 3-position (PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3), studies have, so far, been unable to identify any PLC which is capable of hydrolysing these lipids (see Meldrum et al., 1991, and Rhee and Choi, 1992 for reviews).

### 1.8.1 Activation of PLCβ

Recent work has identified the nature of the G-protein subtypes which couple to PI-PLCs. Simon et al. (1991) have sequenced a number of cDNA s corresponding to previously uncharacterised α subunits of the Gq subfamily, Gαq, Gα11, Gα14, and Gα16. None of these members contain a site for pertussis toxin modification. It appears that this subfamily of Gq proteins are involved in regulation of PI-PLC s (Taylor et al., 1990; Smrcka et al., 1991), activating PLCβ (Blank et al., 1991). The mechanism by which heterotrimeric G-proteins are associated with signal transduction has recently taken on new significance. Previously (see above), it was believed that these heterotrimers (αβγ) dissociate into a GTP-Gα subunit and a βγ dimer, and it was the GTP-Gα which
was solely associated with signal transduction (reviewed by Taylor, 1990; Birnbaumer, 1990; Birnbaumer et al., 1990).

PLCβ1 is activated by association with the GTP-bound forms of Gα11, Gα14 and Gαq, catalysing PtdIns(4,5)P2 hydrolysis. Blank et al. (1991) purified a mixture of Gαq and Gα11 and found specific activation of PLCβ1 with no activation of PLCγ1 or PLCζ1. Half maximal activation of PLCβ1 required 4μM GTPγS, a non-hydrolysable GTP analogue, suggesting that the affinity of these G-proteins for the GTP analogue is low. PLCζ2 is activated by association with Gα16. However, data are accumulating which identify PLCζ2 as the target for activation by the βγ dimer. Camps et al. (1992a) identified that one PI-PLC purified from HL60 cells was stimulated by this βγ dimer, whereas another form was unaffected. The βγ-dependent PI-PLC was later identified as PLCζ2 (see Sternweis and Smrcka, 1992). Later, Camps et al. (1992b) identified both a PLCβ1 (~165 kDa) and a PLCζ2 (~155 kDa), transfected into COS-1 cells, as targets for activation by βγ dimers obtained from retinal transducin. Activation of inositol phosphate formation was greatly stimulated by, and dependent on, free Ca2+ in broken cell preparations, with PLCζ2 causing greatest inositol phosphate formation (>20 fold, compared with ~3-fold for PLCβ1). Equally, less PLCζ2 was required to stimulate inositol phosphate formation. Introduction of the GDP-liganded α1 completely inhibited βγ-stimulated inositol phosphate formation with a 1:1 stoichiometry, showing the requirement of the free βγ dimer for PLCζ2 activation. PLCβ1 was unaffected by the inclusion of a βγ dimer under stimulation conditions. In the same edition of Nature, Katz et al. (1992) identified that βγ dimer activation of PLCζ2, transfected into COS-7 cells, was pertussis toxin sensitive. As previously described (Camps et al., 1992a), Katz et al. (1992) founds that βγ dimers were unable to activate PLCβ1. They also gave evidence that β subunits or γ subunits of the G-protein were unable to activate PLCζ2 alone, and, as found by Camps et al. (1992b), these effects were inhibited by the addition of a corresponding free α subunit, Gα2. The physiological role of βγ-dimer activation of PLCζ2 is, as yet, unclear, as the expression of PLCζ2 may be localised to cells which also contain high levels of Gα16 (Amatruda et al., 1992).

The receptors that activate PLCζ via Gαq and Gα11 include those for acetylcholine (muscarnic m1, m3, and m5), histamine (H1), thromboxane A2, bradykinin, angiotensin, and vasopressin (see Rhee and Choi, 1992).
1.8.2 Activation of PLCγ.

PLCγ activation appears to occur upon stimulation of receptors which act through tyrosine kinase, directly, without the involvement of a G-protein.

Activation of PLCγ by the growth factors PDGF, EGF and NGF, requires intrinsic tyrosine kinase activity of the receptor. Treatment of a number of cells with PDGF, EGF or NGF leads to increased phosphorylation of PLCγ (but not of PLCβ1, or PLCδ1). This correlates well with activation of PtdIns(4,5)P2 hydrolysis, EGF treatment also causes a tight association between its receptor and PLCγ. PLCγ activation appears to be essential for its activation (see Rhee and Choi, 1992; Cockcroft and Thomas, 1992).

1.8.3 Activation of PLCδ.

Neither the receptors nor the transducing proteins that are coupled to any of the PLCδ members are known (see Rhee and Choi, 1992).

1.9.1 Receptor Activation.

A great number of agents, acting through a wide variety of receptors, activate hydrolysis of the membrane phospholipid PtdIns(4,5)P2 (Table 1.1). An important, and well studied, example of agents which causes hydrolysis of PtdIns(4,5)P2 is acetylcholine, this will be discussed in greater detail in later chapters.

The majority of receptors which are ultimately involved in the mobilisation of Ca2+ from internal stores via the above G-proteins, contain seven membrane-spanning domains. These receptors are remarkable in their structural and functional homology, despite their variety of stimuli. It is believed that these receptors (some 350-500 amino acid residues long) contain seven membrane spanning regions linked by three cytoplasmic and three extracellular loops (Wang et al., 1989). The extracellular amino-terminal tail may contain...
one or more glycosylated residues and the third intracellular loop and the carboxy-terminal tail contain several sites suitable for phosphorylation (serine and threonine). The transmembrane regions are unusual, in that they have many proline and glycine residues. Whilst it is unclear how these receptors sit in the membrane, these residues may form kinks in helices that help form the ligand binding pocket (Hulme et al., 1990).

That receptors coupling to PI hydrolysis are coupled through a G-protein, in a manner analogous to the highly studied adenylyl cyclase-linked receptors (see Taylor and Merritt, 1986), was first suggested by the observation that guanine nucleotides affected the binding of agonists which did not affect the activity of adenylyl cyclase. Guanine nucleotide sensitivity of muscarinic agonist binding was observed in 1321N1 cells (Evans et al., 1985), which was insensitive to pertussis toxin, an agent which blocks the effects of GTP on receptors which are linked to adenylyl cyclase in an inhibitory manner.
Table 1.1 G-Protein-Coupled Receptors Activating PtdIns(4,5)P_2 Breakdown.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Receptor</th>
<th>Selective Agonist</th>
<th>Selective Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5-HT1A^*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5-HT2A^*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>M1^*</td>
<td>McN-A 343</td>
<td>pirenzepine</td>
</tr>
<tr>
<td></td>
<td>M3^*</td>
<td>-</td>
<td>HHSD</td>
</tr>
<tr>
<td></td>
<td>(M3^b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>AT1^*</td>
<td>Angiotensin II</td>
<td>Losartan</td>
</tr>
<tr>
<td>Bombesin</td>
<td>^*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>B2^*</td>
<td>[Phe^8,γ(CH2-NH)Arg^9]BK</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DArg[Hyp^3,Thi^5,8,D-Phe^7]BK</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>CCK_A</td>
<td>A71623</td>
<td>devazepide</td>
</tr>
<tr>
<td>Endothelin</td>
<td>ETA^*</td>
<td>-</td>
<td>BE18257B</td>
</tr>
<tr>
<td></td>
<td>ETB^*</td>
<td>[Ala^1,3,11,15]ET-1</td>
<td>-</td>
</tr>
<tr>
<td>Glutamate</td>
<td>mGluR1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leukotrienes</td>
<td>LTB4</td>
<td>LTB4</td>
<td>LY255283</td>
</tr>
<tr>
<td></td>
<td>LTD4</td>
<td>-</td>
<td>ICI198615</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>^*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>α1A^*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>α1B^*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>α1C^*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1.25
Table 1.1 (Cont.)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Receptor</th>
<th>Selective Agonist</th>
<th>Selective Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleotides</td>
<td>P_{2Y}</td>
<td>ADPβS</td>
<td>-</td>
</tr>
<tr>
<td>PAF</td>
<td>*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prostanoids</td>
<td>EP_1</td>
<td>17-phenyl-o-troinor-PGE_2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP_2</td>
<td>butaprost</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP_3</td>
<td>enprostil</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FP</td>
<td>fluprostanol</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>TP^*</td>
<td>U46619</td>
<td>GR32191</td>
</tr>
<tr>
<td>Tachykinins</td>
<td>NK_{1,4}^*</td>
<td>-</td>
<td>SP methyl ester</td>
</tr>
<tr>
<td></td>
<td>NK_{2,4}^*</td>
<td>-</td>
<td>[β-Ala^8]NKA_{4,10}</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>V_{1A}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V_{OT}</td>
<td>[Thy^4, Gly^7]OT</td>
<td>d(CH_2)_2[Tyr(Me)<em>2, Thr^4, Otm^8]OT</em>{1,8}</td>
</tr>
<tr>
<td></td>
<td>V_{IB}</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The receptors marked * have been identified as having seven transmembrane domains. No information is yet available for the others. Data are taken from Watson and Abbott, Trends. Pharmacol. Sci. Receptor Nomenclature Supplement, 1991, from which abbreviations can be obtained.
1.10 Aims of this Thesis.

The studies described in this thesis are concerned with the role of the polyphosphoinositide second messenger system in neuronal cells.

The role of the second messenger, Ins(1,4,5)P₃, in Ca²⁺ homeostasis is now well established (for reviews, see Berridge and Irvine, 1984, 1989; Abdel-Latif, 1986; Taylor and Richardson, 1991; Berridge, 1993), and I have compared the effects of a number of analogues with the effects of endogenously produced Ins(1,4,5)P₃.

These novel analogues (prepared by the groups of Profs. B.V.L. Potter and A.P. Kozikowski) have been synthesised to further the understanding of the structure-activity relationships of inositol phosphates with three recognition proteins, the Ca²⁺-mobilising Ins(1,4,5)P₃ receptor, Ins(1,4,5)P₃ 5-phosphatase and Ins(1,4,5)P₃ 3-kinase, and aid the development of agents which interact selectively with each protein. Inositol phosphates are plasma membrane-impermeant, and, therefore, Ca²⁺ mobilisation studies were performed using permeabilised cells.

A major aim of this thesis was to determine whether muscarinic receptor coupling to phospholipase C, leading to the production of Ins(1,4,5)P₃ and, subsequently, its effects on Ca²⁺ mobilisation, were maintained following a similar permeabilisation protocol, and how these responses were related to one another. Thus, this same preparation was used to study the effects of muscarinic agonists on these responses. The use of permeabilised cells has enabled the introduction of other plasma membrane-impermeant agents (for example, guanine nucleotides) which act inside the cells, and may modulate the effects of receptor activation. This permeabilised cell preparation has also been used to study the effects of agonist pretreatment on cells, in an attempt to further the understanding of mechanisms of receptor desensitisation.
CHAPTER 2.
2.1 Culture of SH-SY5Y Cells.

SH-SY5Y cells (Fig. 2.1) were obtained from Dr. J.L. Biedler (Sloan Kettering Cancer Center, New York, NY). They are a thrice subcloned derivative of the SK-N-SH cell line, originally established from bone marrow metastases from a four year old girl (Biedler et al., 1973). SK-N-SH cells comprise two morphologically and biochemically distinct cell types, a neuroblast-like cell with small-to-medium length neurites which extend radially from the cell body, and a large epithelioid cell. Population doubling time is approximately 44 hours (Biedler et al., 1973). Both SK-N-SH and SH-SY5Y cell lines have been characterised as human neuroblastoma cell lines with high levels of dopamine β-hydroxylase (Ross et al., 1983), an important enzyme involved in the conversion of dopamine to noradrenalin. Both lines also have low levels of tyrosine hydroxylase, an enzyme involved in the production of dopa (dihydroxyphenylalanine) (Ross et al., 1983).

As both cells express activities for tyrosine hydroxylase and dopamine β-hydroxylase, enzymes specific to noradrenergic neurons, and morphologically they resemble human foetal sympathetic ganglion cells in vitro (Ross et al., 1983 and refs. therein), it is believed that these cells can act as a good model for sympathetic ganglia. Further evidence for the suitability of these cells, is the pattern of GTP-binding protein (G-protein) α-subunits present. Klinz et al. (1987) have shown that SH-SY5Y cells contain high levels of G0a (39 kDa) and G0a (41 kDa) and low levels of G0a (40 kDa). This profile is very similar to that of cells obtained from human frontal lobe cortex (Klinz et al., 1987).

The advantage of using the SH-SY5Y subclone over the SK-N-SH parent cell is that the two cell types present in the SK-N-SH cell line interconvert readily (Ross et al., 1983). The SH-SY5Y subclone appeared relatively stable, with less than 5% of colonies grown containing epithelioid-like cells 44 weeks (19 passages) after cloning (Ross et al., 1983). The relevance of this small percentage of non-neuroblast-like cells in experiments described herein can be considered to be of little importance.

SH-SY5Y cell cultures were initiated from stocks of cryogenically preserved cells as follows. The contents of a frozen ampoule of cells were rapidly warmed to 37°C and
Figure 2.1. Electron micrograph of SH-SY5Y neuroblastoma cells in monolayer. Photograph courtesy of Dr. D.G. Lambert.
were aseptically transferred to a 50cm² tissue culture flask (Nunc) containing 20ml of fresh culture medium. Cells were maintained in culture medium comprising minimum essential medium supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100μg/ml streptomycin, 2.5mg/ml amphotericin B (fungizone), and 10% foetal calf serum. Cultures were maintained at 37°C in 5% CO₂, 95% humidified air. Once confluent, cells were harvested in sterile 10mM HEPES, 0.9% NaCl, pH 7.4 containing 0.02% EDTA (HBS/EDTA), and subcultured into 175cm² flasks containing 30ml of supplemented medium. Subsequently, foetal calf serum was gradually removed from the medium and replaced with newborn calf serum over a number of passages. For experimental work, SH-SY5Y cells were harvested as above, using 10ml of HBS/EDTA. 9ml of cell suspension was removed and 30ml of medium was added to the remaining 1ml of cell suspension, allowing further cultivation of cells. Flasks were reused a maximum of 10 times. No morphological or biochemical selection was observed following continued and repeated use of flasks or replacement of foetal calf serum with newborn calf serum. Indeed, assays described below, utilising SH-SY5Y cells (Ins(1,4,5)P₃ and carbachol-induced Ca²⁺ mobilisation) and determination of number and type of cell-surface muscarinic receptors present performed on cells grown in foetal calf serum and cells grown in newborn calf serum for 5 and 10 passages gave identical results (data not shown).

2.2 ⁴⁵Ca²⁺ Release Assay.

To assess the Ca²⁺ mobilising effects of Ins(1,4,5)P₃ and its analogues from intracellular stores, it was first necessary to gain access to the internal receptor. Cells were permeabilised using the detergent, saponin, as described by Strupish et al. (1988) or using high voltage electrical discharges, as described by Wojciliewicz et al. (1990b).

Cells were harvested from tissue culture flasks as described above, centrifuged at 600xg for 2 minutes, and washed in an intracellular buffer (ICB) comprising 120mM KCl, 20mM HEPES, 6mM MgCl₂, 5mM sodium succinate, 5mM Na₂ATP, 2mM KH₂PO₄, 10-30μM EGTA (to reduce free Ca²⁺ concentration to 70-300nM), pH 6.9, based upon a method described by Gershengorn et al. (1984) and modified as described by Strupish et al. (1988). Initially, oligomycin (2μg/ml of cell suspension) was included to prevent ⁴⁵Ca²⁺ uptake into mitochondria. Its omission was,
however, without effect on Ca\textsuperscript{2+} mobilisation and inositol phosphate metabolism, therefore, in the studies described herein, oligomycin was omitted.

Cells permeabilised with saponin, were treated with the detergent (100μg/ml) for 1 minute, after which the cells were harvested (600xg, 2 minutes) and the supernatant removed. Alternatively, aliquots (0.8ml) of cell suspensions were exposed to 3-12 discharges of a 3μF capacitor with a field strength of 3.75kV/cm and a time constant of 0.1ms across a 0.4cm cuvette to permeabilise cells electrically, as described and characterised previously by Wojcikiewicz et al. (1990b). Cells were then spun down (600xg, 2 minutes) and the supernatant removed. Following either of these procedures >90% of cells were unable to exclude trypan blue, whereas 70-80% of cells incubated in ICB were unable to exclude trypan blue. Equally, Ins(1,4,5)P\textsubscript{3} was unable to evoke a response in cells which had not been permeabilised by one of the above routes.

Detergents, like the plant glycoside, saponin, disrupt the plasma membrane by removing cholesterol (Gogelhein and Huby, 1984), causing the membrane to break down, producing pores of ~8nm (see Gogelhein and Huby, 1984). However, detergents are also capable of disrupting intracellular membranes once they have gained access by destroying the plasma membrane. This damage is less severe than that observed with the plasma membrane, as intracellular membranes contain lower levels of cholesterol (Menashi et al., 1981). Clearly, any damage to intracellular Ca\textsuperscript{2+} stores would greatly compromise experiments in which the emptying of these stores is being studied. As stated by Knight and Scrutton (1986), the protocol for cell permeabilisation by detergents requires empirical determination. Such problems are limited using electrical permeabilisation techniques, in which permeabilisation is dependent on the induction of potential across a membrane. The radius of a cell is, clearly, much greater than the radius of any intracellular organelle, and so a potential difference of 1.5V, that required for permeabilisation, across a cell would give considerably smaller potential difference across any intracellular organelle (see Knight and Scrutton, 1986). Electrical permeabilisation appears to produce smaller pores in the plasma membrane (4nm) than detergents, allowing some proteins to be maintained within the cell (see Knight and Scrutton, 1986).

The cells were then incubated in ICB, supplemented with \textsuperscript{45}Ca\textsuperscript{2+} (approx. 4,000,000 d.p.m./ml) at room temperature to allow uptake of \textsuperscript{45}Ca\textsuperscript{2+} into intracellular stores. The effects of Ins(1,4,5)P\textsubscript{3}, its analogues, or muscarinic agonists, were assessed by adding aliquots of cells to polypropylene tubes containing buffer and stimulus. Incubations, performed in duplicate, were terminated by the addition of a silicone oil mixture (Dow Corning 550:556, 9:11 v/v), which was less dense than the cells, but more dense than the aqueous solution (density at 20°C, 1.02g/ml). On centrifugation
at 16,000g for 2 minutes, the cells were separated from the aqueous buffer containing stimulus and any $^{45}\text{Ca}^{2+}$ released or not sequestered. The cells were then assayed for radioactivity following addition of 1ml scintillation cocktail (Optiphase X), as previously described by Strupish et al. (1988).

In experiments where the effects of Ins(1,4,5)P$_3$ or its analogues were examined, cells were loaded with $^{45}\text{Ca}^{2+}$ at a density of 0.5-1 mg of protein/ml. 50µl aliquots of cells were then dispensed using an Eppendorf Multipette into tubes containing 50µl of inositol phosphate.

In experiments where the effects of muscarinic agonists were being examined, cells were loaded with $^{45}\text{Ca}^{2+}$ at a density of 8-16mg of protein/ml. 30µl aliquots of cells were then dispensed into tubes containing 20µl of muscarinic agonist, antagonist or guanine nucleotide.

2.3 Ins(1,4,5)P$_3$ Mass Assay.

2.3.1 Preparation of Ins(1,4,5)P$_3$ Binding Protein.

Fresh bovine adrenal glands were demedullated and decapsulated. The cortex was homogenised in 8 volumes of ice-cold 20mM NaHCO$_3$, 1mM dithiothreitol (DTT), pH 8.0 in an Atomix blender. The homogenate was centrifuged at 5,000xg for 15 minutes at 4°C. The supernatant was removed and the pellet rehomogenised in 4 volumes of NaHCO$_3$/DTT as above. The pooled supernatant fraction was centrifuged at 38,000xg for 20 minutes at 4°C, and the P$_2$ pellet obtained was washed with NaHCO$_3$/DTT as above. The pellet was then resuspended in homogenisation buffer at a protein concentration of 20 mg/ml and frozen in 1ml batches at -20°C before use. This preparation has been previously described by Challiss et al. (1990).
2.3.2 Preparation of Samples.

SH-SY5Y cells were prepared as above for $^{45}$Ca$^{2+}$ release assay (2.2). However, $^{45}$Ca$^{2+}$ was omitted and reactions were terminated by the addition of an equal volume of ice-cold 1M trichloroacetic acid (TCA). The samples were maintained at 4°C for 10-15 minutes and centrifuged at 16,000xg for 5 minutes. 80μl were removed and added to tubes containing 25μl 10mM EDTA. TCA was removed from the supernatant solution by the addition of 100μl of 1,1,2-trichloro 1,2,2-trifluoroethane (Freon)/tri-n-octylamine (1:1 v/v), as previously described by Downes et al. (1986). After thorough mixing, the samples were centrifuged at 16,000xg for 2 minutes and 80μl of the upper, aqueous phase were removed and neutralised with NaHCO$_3$.

2.3.3 Ins(1,4,5)P$_3$ Binding Assay.

Assays were routinely performed, in duplicate, at 4°C in a total volume of 120μl as previously described and characterised by Challiss et al. (1990). A 30μl portion of sample of TCA-extracted buffer containing standard amounts of Ins(1,4,5)P$_3$ (0.12-36pmol or 1.2nmol to determine non-specific binding) was added to 30μl of 100mM Tris HCl/4mM EDTA, pH7.8 and 30μl of $^3$H-Ins(1,4,5)P$_3$ (44 Ci/mmol) (~7,000 d.p.m./assay) (Fig. 2.2). Then 30μl (0.2-0.4mg of protein) of the adrenal cortical binding protein preparation was added, and the samples were incubated for 30 minutes-3 hours at 1-2°C. Bound and free ligand were separated by rapid filtration through Whatman GF/B glass fibre filters with 3x3ml washes of ice-cold 25mM Tris HCl, 5mM NaHCO$_3$, 1mM EDTA, pH 7.8. Scintillant (Optiphase X) was added to the filters and radioactivity was determined after a 6-12 hour extraction period by liquid scintillation counting.

These methods for assaying levels of Ins(1,4,5)P$_3$ have been previously described and characterised by Challiss et al. (1990), who have shown that, of all the naturally occurring inositol phosphates, $^3$H Ins(1,4,5)P$_3$ binding was only displaced with high affinity by Ins(1,4,5)P$_3$. Of other inositol phosphates tested, Ins(1,3,4,5)P$_4$ displaced Ins(1,4,5)P$_3$ with the highest affinity, but was recognised with some 140-fold lower affinity (Challiss et al., 1990). Thus, other phosphates present will not significantly interfere with binding.
Figure 2.2 Displacement of $[^3H]\text{Ins}(1,4,5)P_3$ binding from adrenal cortical membranes by unlabelled $\text{Ins}(1,4,5)P_3$. Assays were performed as described (2.3.3). Values represent means of a single experiment, performed in duplicate, with similar results obtained in many further experiments.
2.4[^3H] Ins(1,4,5)P₃ Metabolism Studies.

SH-SY5Y cells were prepared as described above for ⁴⁵Ca²⁺ release assay. However ⁴⁵Ca²⁺ was omitted, and[^3H] Ins(1,4,5)P₃ (approximately 10,000 d.p.m.) was included. Reactions were terminated, and samples prepared as described above for the Ins(1,4,5)P₃ mass assay.[^3H] Inositol phosphates were then separated using gravity fed ion exchange chromatography (2.6.2) or HPLC (2.6.3).

2.5 Data Analysis.

The EC₅₀ values for ⁴⁵Ca²⁺ release and Ins(1,4,5)P₃ formation, maximal release (⁴⁵Ca²⁺), maximal production (Ins(1,4,5)P₃) and Hill coefficients were obtained by computer-assisted curve fitting using ALLFIT (DeLean et al., 1978) for each individual curve. Mean values of (log EC₅₀) were taken and back-transformed (antilog (log EC₅₀)) to give a geometric mean for the data.

95% Confidence limits were calculated as follows:-

lower limit = antilog[(log EC₅₀) - t₀.₀⁵(n-1)√(s²/n)]
upper limit = antilog[(log EC₅₀) + t₀.₀⁵(n-1)√(s²/n)]

where:-
t₀.₀⁵ = t value for n-1 at 5%
s² = variance of (log EC₅₀), i.e. (standard deviation)²

K₅₀ and EC₅₀ values are presented as mean followed by 95% confidence limits in parentheses.

Maximal Ins(1,4,5)P₃ formation and Ca²⁺ release values were calculated as arithmetic means and are presented as mean ± standard error of mean.
2.6 Ins(1,4,5)P3 5-Phosphatase Studies.

Human erythrocyte ghosts (HEGs), rich in Ins(1,4,5)P3 5-phosphatase, were used to determine whether analogues of Ins(1,4,5)P3 were recognised by Ins(1,4,5)P3 5-phosphatase and whether they were substrates or inhibitors.

2.6.1 Preparation of HEGs.

The method employed was essentially as described by Downes et al. (1982). 2x 43ml of fresh human blood was mixed with 10ml ice-cold 2% disodium citrate (prepared by adjusting 2% trisodium citrate to pH 5.0 with HCl) and 2ml 15% glucose. All subsequent procedures were performed at 4°C. Following centrifugation (2,500xg, 5 minutes), the plasma and white cells were aspirated and the red cells washed three times with 30ml 0.154M NaCl, 0.0015M HEPES, pH 7.2. The packed cells (8x 5.5ml) were then lysed over 15 minutes with 10mM Tris, 1mM EDTA, pH 7.4 (36ml/5.5ml packed red blood cells). Red cell membranes were centrifuged at 50,000xg for 20 minutes and washed four times with lysis buffer. The membranes (white pellet) were then pooled, discarding the lower red pellet, washed again with the lysis buffer and finally resuspended and washed twice with 0.154M NaCl, 0.0015M HEPES, pH 7.2. The membranes in the final pellet (approx. 5-7ml, 20mg of protein/ml) were adjusted to a suitable concentration, and aliquots stored at -70°C.

2.6.2 Ins(1,4,5)P3 5-Phosphatase Assay.

Inhibition of [32P] Ins(1,4,5)P3 hydrolysis by Ins(1,4,5)P3 analogues was performed, in quadruplicate, as described by Cooke et al. (1989). Assays (50µl) were performed in a buffer comprising 30mM HEPES, 2mM MgSO4, pH 7.2. [32P] Ins(1,4,5)P3 10-40µM was incubated with HEGs at 37°C under conditions where no more than 30% of Ins(1,4,5)P3 was hydrolysed, liberation of 32P-inorganic phosphate (32Pi) being linear (Fig. 2.3). For competition experiments, appropriate concentrations of inhibitors were included, in duplicate, in the reaction mixture. The release of 32P_i was monitored as follows: mixtures were diluted with 200µl ice cold buffer, and quenched
Figure 2.3 Ins(1,4,5)P₃ 5-phosphatase-catalysed dephosphorylation of Ins(1,4,5)P₃. [³²P] Ins(1,4,5)P₃ (40μM, filled squares; 16μM filled diamonds; 10μM open squares) was incubated with HEGs (0.4mg of protein/ml). Liberated ³²Pᵢ was complexed with ammonium molybdate and separated from [³²P] Ins(1,4,5)P₃ as described (2.6.2). Values represent a single experiment, performed in duplicate, with similar results obtained in one further experiment.
with 250μl HClO₄ (2M) and centrifuged at 16,000xg for 5 minutes. 450μl of the supernatant were taken and 50μl ammonium molybdate (100mg/ml) were added. The complexed inorganic phosphate (soluble in an organic solvent) was separated from remaining inositol phosphates (water soluble) by the addition of isobutanol (400μl) and toluene (400μl). After vigorous mixing and phase separation by centrifugation, 500μl of the upper phase were taken, added to 4.5ml of scintillation cocktail (Optiphase X) and assessed for radioactivity.

Inhibition of [³H] Ins(1,4,5)P₃ hydrolysis was performed in a similar fashion. However, reactions were terminated by boiling. 100μl of water were then added and inositol mono-, bis-, tris-, and tetrakis phosphates were separated using ion-exchange chromatography on Dowex AG1-X8 resin (see below, 2.6.2). Rates of Ins(1,4)P₂ formation were calculated, from which Lineweaver-Burk plots were constructed, and Kᵢ values were obtained, as described below (2.5.3).

Two procedures were used to determine whether analogues of Ins(1,4,5)P₃ were substrates for, or inhibitors of, Ins(1,4,5)P₃ 5-phosphatase. Firstly, Ins(1,4,5)P₃ or its analogues were incubated with active or heat inactivated HEGs. Their ability to mobilise ⁴⁵Ca²⁺ from permeabilised SH-SY5Y cells was then determined, ascribing loss of activity to hydrolysis by Ins(1,4,5)P₃ 5-phosphatase. Alternatively, assays were performed, in duplicate, as described by Cooke et al. (1989), using a colorimetric assay to follow the liberation of inorganic phosphate. 10ml Ammonium molybdate (4.2% w/v in 4.2M HCl) and 30ml malachite green hydrochloride (0.045% w/v) were stirred together for 20 minutes and filtered through a 0.45μm Acrodisc. 1.2ml Tween 20 (1.5% w/v) were then added. Inorganic phosphate release from Ins(1,4,5)P₃ or its analogues (40-80μM) was determined following incubations with HEGs (0.25mg/ml) for 20 minutes in an assay volume of 250μl. Reactions were terminated by boiling for 10 minutes. The samples were then centrifuged at 16,000xg for 5 minutes and 200μl of the supernatant were taken and 1.2ml of the colour reagent added. Samples were incubated at 37°C for 1 hour, and allowed to cool. The absorbance at 630nm was measured and compared to a standard curve using 0-50μM KH₂PO₄ which had been taken through the entire procedure (Fig. 2.4), as described by Cooke et al. (1989).
Figure 2.4 Typical standard curve from ammonium molybdate-inorganic phosphate assay. KH$_2$PO$_4$ was incubated with ammonium molybdate (4.2% in 4.2M HCl) at 37°C for 1 hour. After cooling, absorbance was assessed at 630nm. Values represent means from a single experiment, with similar results obtained in many further experiments.
2.7 Ins(1,4,5)P₃ 3-Kinase Studies.

2.7.1 Preparation of Brain Homogenate Supernatant.

The Ins(1,4,5)P₃ 3-kinase preparation was obtained as a supernatant from whole rat brain homogenate, using a modification of the method described by Irvine et al. (1986). Two whole brains from male Wistar rats were removed on ice, blotted dry and weighed. They were minced and suspended in 0.15M sucrose solution. They were then homogenised using a glass-Teflon homogeniser (10 strokes), and diluted in further 0.15M sucrose solution to give a final tissue concentration of 20% w/v. Supernatant fractions obtained by centrifugation at 100,000xg for 90 minutes, and aliquots stored at -70°C.

2.7.2 Ins(1,4,5)P₃ 3-Kinase Assay.

Assays (50µl volume) were performed, in quadruplicate, in a buffer comprising 50mM Tris maleate, 20mM MgCl₂, 10mM Na₂ATP, 5mM D-2,3 bisphosphoglycerate (2,3 BPG), 0.1% bovine serum albumin, pH 7.5. 2,3 BPG, a competitive inhibitor of Ins(1,4,5)P₃ 5-phosphatase (Kᵢ=350µM, Downes et al., 1982) was present to inhibit any Ins(1,4,5)P₃ 5-phosphatase activity present in the supernatant preparation. Guillemette et al. (1990), have shown that 2,3 BPG also acts as a competitive inhibitor of Ins(1,4,5)P₃ 3-kinase. The affinity of Ins(1,4,5)P₃ 3-kinase for 2,3 BPG was found, however, to be over 30 times lower than that of Ins(1,4,5)P₃ 5-phosphatase (Guillemette et al., 1990). It is believed, therefore, that the inclusion of 5mM 2,3 BPG would greatly inhibit any Ins(1,4,5)P₃ 5-phosphatase present without substantially inhibiting Ins(1,4,5)P₃ 3-kinase.

[³H] Ins(1,4,5)P₃ (1-10µM) was incubated with this supernatant preparation at 37°C, under conditions where <30% conversion to Ins(1,3,4,5)P₄ occurred, reactions were terminated by boiling. Under these conditions, Ins(1,3,4,5)P₄ formation was linear (Fig. 2.5). For competition experiments, appropriate concentrations of inhibitor were included in the mixtures. The accumulation of [³H] Ins(1,3,4,5)P₄ was monitored following separation of tris- and tetrakis phosphates using ion-exchange.
Figure 2.5 Ins(1,4,5)P₃ 3-kinase-catalysed phosphorylation of Ins(1,4,5)P₃. [³H] Ins(1,4,5)P₃ (3µM, open squares; 10µM, filled diamonds; 30µM, filled squares) was incubated with a supernatant preparation (0.1% w/v) from rat brain homogenates as described (2.7.2). Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were separated using ion-exchange chromatography as described (2.9.2). Values represent a single experiment, performed in duplicate, with similar results obtained in one further experiment.
chromatography on Dowex AG1-X8 resin (see 2.6.2). No mono- or bis-phosphates were detected. Rates of \( \text{Ins}(1,3,4,5)\text{P}_4 \) accumulation were calculated, from which Lineweaver-Burk plots were constructed and \( K_i \) values obtained, as described below (2.5.3).

Substrate properties of inositol phosphate analogues were determined by incubating \( \text{Ins}(1,4,5)\text{P}_3 \) or its analogues (1mM) in the presence of the supernatant preparation (5\% w/v solution) for prolonged periods (60 minutes) at 37°C in the above buffer. Their ability to mobilise \( ^{45}\text{Ca}^{2+} \) from permeabilised SH-SY5Y cells was then determined, ascribing loss of activity to phosphorylation to \( \text{Ins}(1,3,4,5)\text{P}_4 \). This was not possible with analogues which did not mobilise \( ^{45}\text{Ca}^{2+} \) potently, as constituents of the buffer were found to interfere with the \( \text{Ca}^{2+} \) mobilisation assay. Guillemette et al. (1990) have shown that 2,3 BPG not only interacts with \( \text{Ins}(1,4,5)\text{P}_3 \) 5-phosphatase, but inhibits \( \text{Ins}(1,4,5)\text{P}_3 \)-induced \( \text{Ca}^{2+} \) mobilisation at concentrations above 100\( \mu \)M. Likewise, a 1 in 10 dilution of the \( \text{Ins}(1,4,5)\text{P}_3 \) 3-kinase buffer was found to attenuate the ability of \( \text{Ins}(1,4,5)\text{P}_3 \) to mobilise \( \text{Ca}^{2+} \). Thus, in the experiments described herein, the maximal dose of inositol phosphate was no higher than 30\( \mu \)M.

HPLC analysis (using a gradient described by Batty et al., 1989, which isolates individual inositol tetrakisphosphates) of \( \text{Ins}(1,4,5)\text{P}_3 \) treated with this \( \text{Ins}(1,4,5)\text{P}_3 \) 3-kinase preparation showed only \( \text{Ins}(1,4,5)\text{P}_3 \) and \( \text{Ins}(1,3,4,5)\text{P}_4 \) present. The inclusion of heparin (10\( \mu \)g/ml), a non-competitive \( \text{Ins}(1,4,5)\text{P}_3 \) 3-kinase inhibitor (Guillemette et al., 1989) was found to totally inhibit any inositol tetrakisphosphate formation (data not shown).

2.8 Data Analysis.

\( K_{50} \) values obtained for agents which interacted potently with \( \text{Ins}(1,4,5)\text{P}_3 \) 5-phosphatase and 3-kinase were calculated by means of a Lineweaver-Burk transformation. Rates of \( \text{InsP}_2 \) (\( \text{Ins}(1,4,5)\text{P}_3 \) 5-phosphatase assay) and \( \text{InsP}_4 \) formation (\( \text{Ins}(1,4,5)\text{P}_3 \) 3-kinase assay) were calculated, and means of inverse rate values were plotted. \( K_{50} \) and \( V_{\text{max}} \) values were then obtained from Lineweaver-Burk transformations (see Fig. 2.6).
Figure 2.6 Ideal Lineweaver-Burk Plot for a competitive inhibitor, from which $K_m$ and $V_{max}$ of an enzyme-catalysed reaction, and $K_i$ of an inhibitor may be obtained.
K₉₀ values for agents which bound poorly to the enzymes were calculated using a single dose of substrate (Ins(1,4,5)P₃) and competitive inhibition was assumed. IC₉₀ values were obtained and K₉₀ values calculated using the equation

\[ K_{90} = \frac{IC_{90}}{1 + \left( \frac{S}{K_m} \right)} \]

for each individual curve, as described by Cheng and Prusoff (1978). Mean values of \( \log EC_{90} \) values were taken and back transformed (antilog(\( \log EC_{90} \))) to give a geometric mean for the data.

### 2.9 Separation of \([³H]\) Inositol mono-, bis-, tris-, and tetrakis phosphates by Ion-Exchange Chromatography.

#### 2.9.1 Preparation of Ion-Exchange Columns.

Inositol phosphates were separated using down flow, fixed bed, gravity fed open column anion exchange chromatography, as described by Richards et al. (1979), and modified by Batty et al. (1985). Dowex AG1-X8 (200-400 mesh, formate form, 0.8ml 50% slurry) was settled under water in glass columns with an internal diameter of 5mm, containing a glass wool plug. Before initial usage, the columns were washed with 3x 5ml of water, regenerated with 10ml 2M NH₄COOH/0.1M HCOOH, and washed again with 10ml water, as previously described by Batty et al. (1985).

#### 2.9.2 Elution of Inositol Phosphates.

Neutral samples containing \([³H]\) inositol phosphates were applied to the column and washed with 10ml 0.025M NH₄COOH, inositol phosphates were eluted as follows:

- monophosphates: 10ml 0.2M NH₄COOH,
- bisphosphates: 10ml 0.5M NH₄COOH/0.1M HCOOH,
- trisphosphates: 10ml 0.8M NH₄COOH/0.1M HCOOH and
- tetrakisphosphates: 10ml 1M NH₄COOH/0.1M HCOOH.
The columns were then regenerated with 10ml 2M NH₄COOH/0.1M HCOOH, and washed with 10ml water, as previously described by Batty et al. (1985). Following the preparation of each batch of new columns, [³H] standards were eluted from the columns to ensure these profiles had not changed substantially. Routinely, 10% of each phosphate would be collected in the fractions immediately before and after that ascribed to it.

The eluant at each stage was collected and 2ml fractions were assessed for radioactivity following the addition of 18ml scintillation cocktail (Optiphase X).

2.9.3 High Performance Liquid Chromatography of Inositol Phosphates.

HPLC analysis of [³H] inositol phosphates was performed as described by Batty et al. (1989). Separation was achieved with a Partisil SAX analytical column equipped with a pre-column packed with Whatman pellicular anion-exchange resin and eluted with gradients comprising water and (NH₄)H₂PO₄, adjusted to pH 3.7 with H₃PO₄.

Following sample injection, [³H] inositol phosphates were separated by applying three consecutive gradients at a flow rate of 1ml/min. Monophosphates were resolved by applying a linear gradient of 0-60mM (NH₄)H₂PO₄ over 30 minutes. Bisphosphates were then separated by isocratic elution at 190mM (NH₄)H₂PO₄ for 15 minutes, followed by a linear increase in eluant concentration to 300mM over a further 15 minutes. Trisphosphates were separated by isocratic elution for 35 minutes at 500mM (NH₄)H₂PO₄. Tetakisphosphates were removed from the column by a 15 minute wash with 1.4M (NH₄)H₂PO₄ and then the column was washed for a further 20 minutes with water before the injection of the following sample. Between 20 and 95 minutes of the separation, 30 second fractions were collected, followed by 1 minutes fractions between 95 and 125 minutes. This gradient was achieved using 0.5M (NH₄)H₂PO₄ during the first 95 minutes of the gradient, followed by the use of 1.4M (NH₄)H₂PO₄ between 95 and 110 minutes, exactly as described and characterised by Batty et al. (1989) (Fig. 2.7).

Nucleotides were routinely added to samples before injection, having similar elution profiles to inositol phosphates, and were used to estimate elution times of inositol phosphates. Times of elution were in close agreement with those found by Batty et al. (1989) (Table 2.1).
Figure 2.7 Profile of elution gradient used for HPLC. Mixtures of 0.5M \( \text{NH}_4\text{H}_2\text{PO}_4 \) and water were used to elute inositol mono-, bis- and tris phosphates and 1.4M \( \text{NH}_4\text{H}_2\text{PO}_4 \) was used to elute inositol tetrakisphosphates. For elution times see Table 2.1.
Table 2.1 Time of elution of nucleotides and inositol phosphates.

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>Elution time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batty et al. (1989)</td>
</tr>
<tr>
<td>AMP</td>
<td>20</td>
</tr>
<tr>
<td>GMP</td>
<td>26</td>
</tr>
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<tr>
<td>AP₄</td>
<td>106</td>
</tr>
<tr>
<td>GP₄</td>
<td>110</td>
</tr>
</tbody>
</table>

Inositol Phosphates.

| Ins(l)P     | 22                     | 23         |
| Ins(2)P     | 24                     | N.D.       |
| Ins(4)P     | 26                     | 26         |
| Ins(1,3)P₂  | 48                     | 49         |
| Ins(1,4)P₂  | 51                     | 51         |
| Ins(3,4)P₂  | 55                     | N.D.       |
| Ins(4,5)P₂  | 60                     | N.D.       |
| Ins(1,3,4)P₃| 80                     | 80         |
| Ins(1,4,5)P₃| 85                     | 85         |
| Ins(1,3,4,5)P₄| 110                  | 110        |

2.10 Quantitation and Analysis of Cell-Surface Receptors.

Cell-surface muscarinic receptor number was determined by the use of the tritiated antagonist, N-methyl-scopolamine ([³H] NMS). NMS is a non-selective muscarinic antagonist, binding to all subtypes with similar affinity (Buckley et al., 1989).

Ligand binding studies were carried out in polystyrene tubes at 1-2°C in 250μl of appropriate buffer. Incubations were terminated 1-24 hours after commencement, by rapid filtration on glass fibre (Whatman GF/B) filters, which were then washed with 2x1ml of appropriate buffer (1-2°C). Radioactivity bound to the filters was then
measured after 6-12 hours equilibration in 3.5ml of scintillation cocktail (Optiphase X), at approximately 30% counting efficiency.


The binding of $[^3]$H NMS to intact cells was studied as described by Lambert et al. (1989), except a 250|il assay volume was used, and assays were performed at 1-2°C in Krebs-HEPES solution (NaCl, 118.6mM; KCl, 4.7mM; MgSO₄·6H₂O, 1.2mM; KH₂PO₄, 1.2mM; NaHCO₃, 25mM; glucose, 11.7mM; CaCl₂, 1.3mM, plus HEPES free acid, 10mM, pH 7.4), stopping incubations 6 hours after commencement. Non-specific binding was defined in the presence of 1μM atropine sulphate. Saturation studies were performed using 0.02-6nM $[^3]$H NMS. Total binding was determined in duplicate, non-specific binding was determined in single tubes.

Displacement of $[^3]$H NMS with selective, unlabelled agents (pirenzepine, methoctramine and pF-HHSD) was performed in much the same way, except 0.5-2nM $[^3]$H NMS was used, with increasing amounts of unlabelled displacer. Non-specific binding was determined in the presence of 1μM atropine sulphate.


Cells were permeabilised in ICB (see 2.2), and were washed in MgSO₄, 1mM; HEPES, 10mM, pH 6.9. Binding assays were performed at 1-2°C, for 6 hours in this buffer, in the absence or presence of GTP (1mM).

Incubations were terminated by rapid filtration through glass fibre (Whatman GF/B) filters, and were then washed with 2x1ml of ice cold buffer. These filters had been previously soaked in 1% Sigmacote, 5% bovine serum albumin, for 2 hours, washed with water, and allowed to dry in air. This pretreatment was found to substantially decrease non-specific binding of label to the glass fibre filters, as previously characterised by Harden et al. (1983), studying $[^3]$H oxotremorine binding to rat heart membranes.
Displacement of $[^{3}H]$ NMS with muscarinic agonists was performed in much the same way, except 0.5-2nM $[^{3}H]$ NMS was used, with increasing amounts of agonist in the absence or presence of GTP (1mM). Non-specific binding was defined in the presence of 1µM atropine sulphate.

2.10.3 Data Analysis.

Saturation isotherms were analysed according to Scatchard (1949), to yield the equilibrium dissociation constant, $K_D$, and the maximum binding capacity, $B_{\text{max}}$. The IC$_{50}$, the concentration of displacer producing 50% inhibition of total specific binding and the slope factor, $n_H$, were obtained by computer-assisted curve fitting using ALLFIT (DeLean et al., 1978) for each individual curve. The K$_{50}$ values (IC$_{50}$ values corrected for the competing mass of radioligand) were calculated according to Cheng and Prusoff (1973), and are equivalent to the inhibitor constant, $K_i$, when slope factors are close to unity. K$_{50}$ values are presented as mean followed by 95% confidence limits in parentheses, as described in section 2.3.4.

Statistical differences between K$_{50}$ values were compared using Students t-test and considered significant when $p<0.05$.

2.11 $[^{35}S]$ GTP$\gamma$S Binding.

The binding of GTP$\gamma$S to permeabilised cells was determined as described and characterised by Hilf et al. (1989) and modified by Lazareno et al. (1993). Cells were permeabilised in ICB, as described (2.2). Cells were then washed in a buffer comprising HEPES, 10mM; NaCl, 0.1mM; MgCl$_2$, 10mM, pH 6.9.

Assays were performed at 20°C in 250µl volumes. Incubations were terminated 10-90 minutes after commencement by rapid filtration onto glass fibre (Whatman GF/B) filters, which were then washed with 2x1ml distilled water (1-2°C). Non-specific binding was defined in the presence of 10µM unlabelled GTP$\gamma$S. All tubes contained 1µM GDP to enhance specific binding (Hilf et al., 1989).
To determine the effects of agonists and antagonists on the binding of GTPγS, either CCh (1mM), atropine (1μM) or both were included in incubations, and time courses of GTPγS binding were constructed.

2.12 Desensitisation Protocols.

Intact cells were harvested and washed in Krebs buffer (NaCl, 118.6mM; KCl, 4.7mM; MgSO4·6H2O, 1.2mM; KH2PO4, 1.2mM; NaHCO3, 25mM; glucose, 11.7mM; CaCl2, 1.3mM, pH 7.4), or nominally Ca²⁺-free Krebs-like buffer (as above, omitting CaCl₂). Cells were then incubated at 37, 20 or 1-2°C in the presence of carbachol (1μM-1mM) for 5 minutes-5 hours. Pretreatment was terminated by washing the cells in ice-cold Krebs buffer to remove all carbachol present. Cells were then treated in one of four ways.

1. Cells were washed in ice-cold Krebs-HEPES buffer, and the binding of [³H] NMS was monitored as described in (2.7.1). Alternatively, cells were washed in ice-cold ICB, permeabilised electrically as described above (2.2) and treated as follows:

2. Cells were incubated in ⁴⁵Ca²⁺-containing ICB, pH 6.9 at 20°C for 12 minutes and ⁴⁵Ca²⁺ release experiments were performed as described above (2.2).

3. Cells were washed in Mg/HEPES buffer, pH 6.9 and binding of [³H] NMS to permeabilised cells at 1-2°C was examined as described above (2.10.2).

4. Cells were washed in NaCl/MgCl2/HEPES buffer, pH 6.9 and the binding of [³⁵S] GTPγS at 20°C was examined as described (2.11).

2.13 Protein Determinations.

Protein concentrations were determined using adaptations of methods previously described by Lowry et al. (1951) or Bradford (1976).

The density of cells used in ⁴⁵Ca²⁺-release assays (as described in (2.2)) were determined by the method described by Bradford (1976). Coomassie Brilliant Blue G-250 (100mg) was dissolved in 50ml of 95% ethanol. To this solution, 100ml 85% w/v orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. The solution was then filtered under gravity through a Whatman Grade 40
ashless filter. Assays were performed by adding 500μl of sample to 2.5ml of the above colour reagent and mixing thoroughly. The samples were left for 5 minutes at room temperature, and absorbance determined at 595nm. Comparison with standards (0-25μg bovine serum albumin/ml) treated in an identical manner enabled protein concentration in each sample to be determined.

All other determinations were performed using an adaptation of the method described by Lowry et al. (1951). Four solutions were required:

Solution A. 2% Na₂CO₃ in 0.1M NaOH.
Solution B. 1% CuSO₄.
Solution C. 2% NaK tartrate.
Solution D. Folin/Ciocalteu reagent (diluted 1:3 in water).

Immediately before the assay, A, B and C were mixed (100:1:1) to give Solution E. 500μl of sample were then added to 2.5ml of Solution E. After 10 minutes, 250μl of Solution D were added and each sample was mixed thoroughly. The samples were left for 30 minutes at room temperature, and absorbance determined at 750nm. Comparison with standards (0-250μg bovine serum albumin/ml) treated in an identical manner enabled protein concentration in each sample to be determined.
CHAPTER 3.
3.1 Introduction.

In 1983, Stieb et al. showed directly that the application of low micromolar levels of Ins(1,4,5)P$_3$ to 'leaky' pancreatic cells triggered a rapid release of Ca$^{2+}$ from stores with considerable selectivity. This was corroborated by a number of other studies in other cell types, whereby very few naturally occurring inositol phosphates mobilised Ca$^{2+}$ from permeabilised cell preparations (see Taylor and Richardson, 1991). However, reuptake of this Ca$^{2+}$ occurred. Ins(1,4,5)P$_3$ had previously been found to be a substrate for a phosphatase, attacking specifically the 5-position (Downes et al., 1982), thus producing the inactive bisphosphate, Ins(1,4)P$_2$. Later, Batty et al. (1985) discovered a higher phosphate, Ins(1,3,4,5)P$_4$, and this was soon followed by identification of a kinase which specifically phosphorylated Ins(1,4,5)P$_3$ at the 3-position (Irvine et al., 1986).

Little was known about the structure-activity relationships of the three proteins involved in Ca$^{2+}$ mobilisation, and metabolism by dephosphorylation and phosphorylation. Work described in the chapter has attempted to identify and separate specific recognition requirements of these three proteins and substrate/inhibitor profiles of the two metabolic enzymes. The initial work described involves the characterisation of a model which could be used to determine the Ca$^{2+}$ mobilisation properties of the inositol phosphate analogues. This is followed by studies comparing data obtained using novel inositol phosphate analogues, prepared in the laboratories of Profs. B.V.L. Potter (Leicester and Bath, U.K.) and A.P. Kozikowski (Jacksonville, U.S.A.), with those previously published by other groups.
3.2 Background.

The Ins(1,4,5)P$_3$ receptor shows great stereo- and positional specificity (For structures of inositol phosphates and their analogues, see Appendix A II). L-Ins(1,4,5)P$_3$ (also known as D-Ins(3,5,6)P$_3$), (Strupish et al., 1988; Polokoff et al., 1988), Ins(1,3,4)P$_3$ (Strupish et al., 1988; Polokoff et al., 1988), Ins(1,2,6)P$_3$ (Authi et al., 1989), Ins(1,3,5)P$_3$ (Polokoff et al., 1988) and 3-deoxy Ins(1,5,6)P$_3$ (Seewald et al., 1990) all being ineffective, as are inositol, Ins(1)P, Ins(2)P, Ins(1:2)P, Ins(1,4)P$_2$, Ins(2,4)P$_2$, Ins(1,3,4,5,6)P$_5$ and phytic acid (InsP$_6$) (see Berridge and Irvine, 1984, 1989). Ins(4,5)P$_2$ is a weak agonist, 650-fold (Nunn and Taylor, 1990) weaker than Ins(1,4,5)P$_3$. Ins(1:2,4,5)P$_3$ has also been shown to be 13- (Hirata et al., 1989) to 15-fold (Willcocks et al., 1989) less potent than Ins(1,4,5)P$_3$, but acid labile producing a mixture of Ins(1,4,5)P$_3$ and Ins(2,4,5)P$_3$ some 0.4-fold weaker than Ins(1,4,5)P$_3$ (Willcocks et al., 1989). Ins(2,4,5)P$_3$ has also been shown to be a full and relatively potent Ca$^{2+}$ mobilising agent, some 12-68 fold less potent than Ins(1,4,5)P$_3$ (Willcocks et al., 1989; Hirata et al., 1989; Polokoff et al., 1988; Nunn and Taylor, 1990; Denis and Ballou, 1991), suggesting that the phosphate grouping on the axial 2-position can interact with a site on the receptor normally approached by the equatorial 1 phosphate. Glycerophospho-myositol 4,5-bisphosphate (Gro-P-Ins(4,5)P$_2$) is also a potent agonist, 3- (Willcocks et al., 1989) to 10-fold (Henne et al., 1988) weaker than Ins(1,4,5)P$_3$. Work by Denis and Ballou (1991) has shown that D-chiro inositol 1,3,4-trisphosphate is also a potent Ca$^{2+}$ mobilising agent, with EC$_{50}$ equal to that of Ins(2,4,5)P$_3$ in the same study. It bears a close resemblance to Ins(2,4,5)P$_3$, except that, if likened to Ins(2,4,5)P$_3$, it possesses an axial 1 hydroxyl group. The L-enantiomers of Ins(2,4,5)P$_3$ and ch Ins(1,3,4)P$_3$ are only 25-30 fold weaker than their D-enantiomers, far more active than any L-Ins(1,4,5)P$_3$ analogue. The most significant difference between these compounds is that all of the phosphate groups in L-Ins(1,4,5)P$_3$ are equatorial, while one phosphate group in both Ins(2,4,5)P$_3$ and ch Ins(1,3,4)P$_3$ is axial. DL-Ins(1,3,4)P$_3$, Ins(1,4)P$_2$, and Ins(1,2,4,5)P$_3$ are not phosphorylated, and appear to be poorly recognised by Ins(1,4,5)P$_3$ 3-kinase (see Shears, 1989). Ins(2,4,5)P$_3$ appears to be a poor substrate for Ins(1,4,5)P$_3$ 5-phosphatase, with similar affinity to Ins(1,4,5)P$_3$ (Polokoff et al., 1988) and is very poorly recognised by Ins(1,4,5)P$_3$ 3-kinase, being a poor substrate (Ryu et al., 1987b; Morris et al., 1988). Removal of the 1-phosphate group, producing Ins(4,5)P$_2$, greatly decreases affinity of Ins(1,4,5)P$_3$ 3-kinase (see Shears, 1989).
The non-specific nature of Ins(1,4,5)P3 5-phosphatase is exemplified by its binding to L-Ins(1,4,5)P3 with similar affinity to Ins(1,4,5)P3 itself (Polokoff et al., 1988). L-Ins(1,4,5)P3, is, however, an inhibitor of Ins(1,4,5)P3 5-phosphatase. Polokoff et al. (1988) found DL-Ins(1,3,4)P3 to be as potent an inhibitor ($K_i=32\mu M$). This may be due to L-Ins(1,3,4)P3 (or D-Ins(1,3,6)P3), which is produced in avian cells, as a metabolite of Ins(1,3,4,6)P4. Ins(2,4,5)P3 is also well recognised by Ins(1,4,5)P3 5-phosphatase, inhibiting Ins(1,4,5)P3 dephosphorylation with $K_i=23-131\mu M$ (Hirata et al., 1989; Polokoff et al., 1988). It is, however, only slowly metabolised (Polokoff et al., 1988). Likewise, Ins(1;2,4,5)P3 is recognised with a slightly lower affinity and hydrolysed at a slower rate than Ins(1,4,5)P3 (Hirata et al., 1989). Ins(1,3,5)P3, a synthetic analogue of Ins(1,4,5)P3 was also recognised with low affinity by Ins(1,4,5)P3 3-kinase and the Ins(1,4,5)P3 receptor, but bound Ins(1,4,5)P3 5-phosphatase with similar affinity to Ins(1,4,5)P3 itself, inhibiting Ins(1,4,5)P3 dephosphorylation with $K_i=32\mu M$ (Polokoff et al., 1988).

The discovery of Ins(1,3,4,5)P4 (Batty et al., 1985) quickly led to the detection of Ins(1,4,5)P3 3-kinase activity in cells (Irvine et al., 1986; Biden and Wollheim, 1986; Hansen et al., 1986). In cells studied so far, it appears that the enzyme is predominantly soluble. Significant particulate activity has been found in turkey erythrocyte membranes (Morris et al., 1987), but even this only amounted to ~25% of total kinase activity. The 3-position of Ins(1,4,5)P3 is of major importance, being the site of phosphorylation by Ins(1,4,5)P3 3-kinase. The product, Ins(1,3,4,5)P4, has a controversial role in Ca2+ homeostasis and is thought to interact poorly with the Ins(1,4,5)P3 receptor. It appears that Ins(1,3,4,5)P4 is capable of mobilising Ca2+ from internal stores, albeit weakly ($EC_{50}=2.76\pm0.01\mu M$ in SH-SY5Y cells, Gawler et al., 1991; $EC_{50}=4.6\mu M$ in cerebellar microsomes, Joseph et al., 1989a). Some groups have claimed that Ins(1,3,4,5)P4 may not be an important second messenger per se, but have its effects in combination with Ins(1,4,5)P3. Thus, it is most important to produce analogues which, whilst mimicking Ins(1,4,5)P3, cannot become phosphorylated, or which are potent, selective Ins(1,4,5)P3 3-kinase inhibitors. Despite this, few studies have concentrated on chemical modification of this highly important position. Preliminary studies using 3-deoxy InsP3 have shown it to be a potent Ca2+ mobilising agent (Seewald et al., 1990), with, like 2-deoxy InsP3 (Hirata et al., 1989), only moderate loss of activity. Equally, 3-fluoro InsP3 has been claimed to be equipotent to Ins(1,4,5)P3 (Kozikowski et al., 1990).

Inositol 1,3,4,6-tetrakisphosphate, normally the product of 6-kinase activity on Ins(1,3,4)P3, appears to act as a partial agonist at the Ins(1,4,5)P3 receptor, maximally releasing some 80-90% of the Ins(1,4,5)P3-sensitive pool with $EC_{50}=5.9\pm0.5\mu M$. 

3.3
(Gawler et al., 1991). It is not obvious, at first sight, why \text{Ins}(1,3,4,6)\text{P}_4 should mobilise \text{Ca}^{2+}, as it does not possess the vicinal 4,5-bisphosphate pairing normally required for agonist activity. However, two alternative binding conformations of \text{Ins}(1,3,4,6)\text{P}_4 to the \text{Ins}(1,4,5)\text{P}_3 receptor have been suggested by Gawler et al. (1991), in which a number of important recognition features of \text{Ins}(1,4,5)\text{P}_3 can be mimicked, including, most importantly, a pseudo 4,5-bisphosphate pairing (Fig. 3.1).

(1) has an equatorial 6 hydroxyl group being replaced by an axial group. (2) possesses an axial 3 hydroxyl group.

From the above data, it appears that \text{Ca}^{2+} mobilising properties of naturally occurring inositol phosphates are dependent on a vicinal D 4,5 bisphosphate pairing. The presence of a monoester phosphate at position 1 greatly enhances receptor recognition, although this can be mimicked by the presence of a phosphate group at the 2-position. Replacement of the three phosphate groups with sulphate, sulphonamide, methylphosphonate or carboxymethyl groups (Westerduin et al., 1992) removes all affinity for the \text{Ins}(1,4,5)\text{P}_3 receptor.

3.2.1 \text{Ins}(1,4,5)\text{P}_3 Receptor Antagonists.

So far, no inositol phosphate analogue discovered or synthesised has been found to be an \text{Ins}(1,4,5)\text{P}_3 receptor antagonist. The polysulphated polysaccharide, heparin was the first antagonist of \text{Ins}(1,4,5)\text{P}_3 to be recognised (Worley et al., 1987), binding with reasonable potency and inhibiting \text{Ins}(1,4,5)\text{P}_3 induced mobilisation in a number of systems (K_{50}=86\text{mM} in beet microsomes (Brosnan and Sanders, 1990); IC_{50}=10\mu\text{g/ml} in bovine adrenal cortex (Guillemette et al., 1989); IC_{50}=4.5\mu\text{g/ml} in rat liver microsomes (Cullen et al., 1988); K_{IC}=100\text{nM} (0.6\mu\text{g/ml}) in cerebellar microsomes (Joseph et al., 1989a); and 2.7\text{nM} (approximately 14\text{ng/ml}) in microsomes from DDT_{1-MMF-2} cells (Ghosh et al., 1988)). This suggests that \text{Ins}(1,4,5)\text{P}_3-induced \text{Ca}^{2+} mobilisation occurs via similar mechanisms in animal and plant tissues. However, \text{Ins}(1,4,5)\text{P}_3-induced \text{Ca}^{2+} mobilisation in fungi is heparin insensitive (Cornelius et al., 1989). It is tempting to speculate that the negatively charged sulphate groups of heparin may interact with those parts of the \text{Ins}(1,4,5)\text{P}_3 receptor responsible for binding the phosphate groups of \text{Ins}(1,4,5)\text{P}_3. Indeed, O- and N-desulphated heparins are relatively ineffective (Challiss et al., 1991). Heparin has also been reported to be a potent inhibitor of \text{Ins}(1,4,5)\text{P}_3 3-kinase. Guillemette et al. (1989) have shown heparin to be a non-competitive inhibitor of \text{Ins}(1,4,5)\text{P}_3 3-kinase,
Figure 3.1  Possible binding conformations of Ins(1,3,4,6)P_4, courtesy of Professor B.V.L. Potter. The structure of Ins(1,4,5)P_3 is shown in the upper panel, and two possible binding conformations of Ins(1,3,4,6)P_4 (1) and (2) are given below.
lowering the apparent $V_{max}$ but not the $K_m$ of the enzyme for Ins(1,3,4,5)P$_4$
production. The effects of heparin were such that whilst it inhibits Ins(1,4,5)P$_3$
receptor binding and Ca$^{2+}$ mobilisation, it is some 10 times more potent on inhibition
of Ins(1,4,5)P$_3$ 3-kinase. It does not, however, interfere with Ins(1,4,5)P$_3$ 5-
phosphatase. Decavanadate has also been found to act as a competitive antagonist at
the Ins(1,4,5)P$_3$ receptor, with $K_i$=1.2µM (Strupish et al., 1991).

A number of intracellular components have been identified as weak antagonists, but
are of little pharmacological use. ATP competitively inhibits binding of Ins(1,4,5)P$_3$
($IC_{50}$=51µM on inhibition of Ins(1,4,5)P$_3$ binding in rat cerebellum, Willcocks et al
(1988), $K_i$=220-350µM on rat cerebellum and liver membranes, Nunn and Taylor
(1990)), although at 30mM, some Ca$^{2+}$ mobilisation was observed (Nunn and Taylor,
1990), and 2,3 bisphosphoglycerate (2,3 BPG) ($IC_{50}$=0.5 and 2mM on Ins(1,4,5)P$_3$
binding and Ca$^{2+}$ mobilisation from bovine adrenal cortex microsomes, Guillemette
et al., 1990, $K_i$=60-200µM on Ins(1,4,5)P$_3$ binding, Nunn and Taylor, 1990) may play
some role in setting the sensitivity of Ins(1,4,5)P$_3$ for its receptor. ATP inhibiting
Ins(1,4,5)P$_3$ 5-phosphatase has been reported by a number of groups. However, full
characterisation of its effects have not been published. It is unclear whether ATP acts
in a competitive or non-competitive fashion (see Shears, 1989). As Mg$^{2+}$ is required
for Ins(1,4,5)P$_3$ 5-phosphatase to function, does ATP merely chelate free Mg$^{2+}$, or is
ATP involved in phosphorylation of the protein? 2,3 BPG has also been identified as
a competitive inhibitor of Ins(1,4,5)P$_3$ 5-phosphatase with "$IC_{50}$"=0.3mM
(Guillemette et al., 1990) and $K_i$=978µM (Cooke et al., 1989). It does, however, also
inhibit Ins(1,4,5)P$_3$ receptor binding ("$IC_{50}$"=0.5mM), Ins(1,4,5)P$_3$-induced Ca$^{2+}$
release ("$IC_{50}$"=2mM) and Ins(1,4,5)P$_3$ 3-kinase ("$IC_{50}$"=10.5mM) (Guillemette et
al., 1990). Ironically, 2,3 BPG has been used in Ins(1,4,5)P$_3$ 3-kinase studies to
inhibit Ins(1,4,5)P$_3$ 5-phosphatase activity. The selectivity of 2,3 BPG to inhibit
Ins(1,4,5)P$_3$ 5-phosphatase over Ins(1,4,5)P$_3$ 3-kinase is some 30-40 fold, and the
dose used in this study (5mM) is believed to inhibit any Ins(1,4,5)P$_3$ 5-phosphatase
activity almost maximally, while not interfering with Ins(1,4,5)P$_3$ 3-kinase activity
(Guillemette et al., 1990).

Whether a single enzyme catalyses the dephosphorylation of Ins(1,4,5)P$_3$ and
Ins(1,3,4,5)P$_4$ is a problem best resolved in a purified enzyme preparation. Of two
such phosphatases purified, the 'type 1' soluble enzyme has been identified as having
high affinity for Ins(1,4,5)P$_3$, and higher affinity for Ins(1,3,4,5)P$_4$ (3 and 0.8µM
respectively, $V_{max}$=1.1 and 0.09 µmol/min/mg), and the 'type 2' lower affinity for
Ins(1,4,5)P$_3$ and very low affinity for Ins(1,3,4,5)P$_4$ (18 and >150µM respectively,
$V_{max}$=8 µmol/min/mg, Hansen et al., 1987) (see Introduction, Chapter 1).
Most reports put the $K_m$ of Ins(1,4,5)P$_3$ 3-kinase between 0.2 and 1.5$\mu$M, some 10-fold lower than the $K_m$ values for Ins(1,4,5)P$_3$ 5-phosphatase (see Shears, 1989). Early studies showed that, like the Ca$^{2+}$ mobilising Ins(1,4,5)P$_3$ receptor, Ins(1,4,5)P$_3$ 3-kinase exhibits high stereo- and positional selectivity (see Nahorski and Potter, 1989).

The purpose of the studies described in this chapter is to attempt to identify agents which selectively bind the Ins(1,4,5)P$_3$ receptor, selective inhibitors of Ins(1,4,5)P$_3$ 3-kinase and 5-phosphatase, or metabolism-resistant Ca$^{2+}$-mobilising agents. These agents may then be used in furthering the understanding of the roles of the Ins(1,4,5)P$_3$ receptor, Ins(1,4,5)P$_3$ 3-kinase and 5-phosphatase in the second messenger signalling systems studied in later sections.

### 3.3 Methods.

To assess the Ca$^{2+}$ mobilising effects of Ins(1,4,5)P$_3$ and its analogues from intracellular stores, it was first necessary to gain access to the internal receptor. Cells were permeabilised using the detergent, saponin, as described by Strupish et al. (1988) or using high voltage electrical discharges, as described by Wojcikiewicz et al. (1990b) (see Chapter 2(2.2)). The cells were then incubated in ICB, supplemented with $^{45}$Ca$^{2+}$ (approximately 4,000,000 d.p.m./ml) at room temperature to allow uptake of $^{45}$Ca$^{2+}$ into intracellular stores. Cells were loaded with $^{45}$Ca$^{2+}$ at a density of 0.5-1 mg of protein/ml. 50$\mu$l aliquots of cells were then dispensed using an Eppendorf Multipette into tubes containing 50$\mu$l of inositol phosphate. Incubations were terminated by the addition of a silicone oil mixture (Dow Corning 550:556, 9:11 v/v). On centrifugation at 16,000g for 2 minutes, the cells were separated from the aqueous buffer containing stimulus and any $^{45}$Ca$^{2+}$ released or not sequestered. The cells were then assayed for radioactivity following addition of 1ml scintillation cocktail (Optiphase X), as previously described by Strupish et al. (1988).

The rates and routes of Ins(1,4,5)P$_3$ metabolism were monitored using SH-SY5Y cells prepared as described above for the $^{45}$Ca$^{2+}$ release assay. However $^{45}$Ca$^{2+}$ was omitted, and [$^3$H]Ins(1,4,5)P$_3$ (approximately 10,000 d.p.m.) was included. Reactions were terminated, and samples prepared as described above for the Ins(1,4,5)P$_3$ mass assay. [$^3$H]Inositol phosphates were then separated using gravity fed ion exchange chromatography (2.6.2) or HPLC (2.6.3).
Inhibition of [32P] Ins(1,4,5)P3 hydrolysis by Ins(1,4,5)P3 analogues was performed as described by Cooke et al. (1989). Assays (50μl) were performed in a buffer comprising 30mM HEPES, 2mM MgSO4, pH 7.2. [32P] Ins(1,4,5)P3 10-40μM was incubated with HEGs at 37°C. For competition experiments, appropriate concentrations of inhibitors were included in the reaction mixture. The release of 32P1 was monitored as described in Chapter 2(2.6.2). Inhibition of [3H] Ins(1,4,5)P3 hydrolysis was performed in a similar fashion, as described in Chapter 2(2.6.2). Rates of Ins(1,4)P2 formation were calculated, from which Lineweaver-Burk plots were constructed, and K1 values were obtained. Two procedures were used to determine whether analogues of Ins(1,4,5)P3 were substrates for, or inhibitors of, Ins(1,4,5)P3 5-phosphatase. Firstly, Ins(1,4,5)P3 or its analogues were incubated with active or heat inactivated HEGs. Their ability to mobilise 45Ca2+ from permeabilised SH-SY5Y cells was then determined, ascribing loss of activity to hydrolysis by Ins(1,4,5)P3 5-phosphatase. Alternatively, assays were performed as described by Cooke et al. (1989), using a colorimetric assay to follow the liberation of inorganic phosphate as described in Chapter 2(2.6.2). Ins(1,4,5)P3 was bound by HEG Ins(1,4,5)P3 5-phosphatase with Km=15-40μM, and Vmax=1.1nmol/min/mg of protein, in good accordance with Cooke et al. (1989) (40μM and 1.1nmol/min/mg of protein) and Downes et al. (1982) (25μM and 2.8nmol/min/mg of protein).

The Ins(1,4,5)P3 3-kinase preparation was obtained as a supernatant from whole rat brain homogenate, using a modification of the method described by Irvine et al. (1986), as described in Chapter 2(2.7.1). Assays were performed in a buffer consisting 50mM Tris maleate, 20mM MgCl2, 10mM Na2ATP, 5mM 2,3 BPG, 0.1% bovine serum albumin, pH 7.5 as described in Chapter 2(2.7.2). Rates of Ins(1,3,4,5)P4 accumulation were calculated, from which Lineweaver-Burk plots were constructed and K1 values obtained. Substrate properties of inositol phosphate analogues were determined by incubating Ins(1,4,5)P3 or its analogues (1mM) in the presence of the supernatant preparation (5% w/v solution) for prolonged periods (60 minutes) at 37°C in the above buffer. Their ability to mobilise 45Ca2+ from permeabilised SH-SY5Y cells was then determined, ascribing loss of activity to phosphorylation to Ins(1,3,4,5)P4. This was not possible with analogues which did not mobilise 45Ca2+ potently, as constituent(s) of the buffer were found to interfere with the Ca2+ mobilisation assay. Ins(1,4,5)P3 was bound with high affinity by Ins(1,4,5)P3 3-kinase, with Km=0.85-3.2μM and Vmax=3.1nmol/min/mg of protein (570nmol/min/g fresh weight of tissue).
3.4 Results.

3.4.1 Validation of the $^{45}\text{Ca}^{2+}$ Mobilisation Assay

$^{45}\text{Ca}^{2+}$ uptake into permeabilised cells occurred in a time and ATP dependent manner. Removal of exogenous ATP markedly attenuated $^{45}\text{Ca}^{2+}$ uptake by over 50% and increased the time required for loading. It is unclear how much uptake was due to the presence of endogenous ATP. Unpermeabilised cells 'bound' about 10% and 25% of $^{45}\text{Ca}^{2+}$ sequestered by permeabilised cells in the absence and presence of exogenous ATP, respectively.

Both saponised (100μg/ml, 1 minute) and electroporated (3 or 12 pulses) cells sequestered $^{45}\text{Ca}^{2+}$ with a half time of less than 1 minute (Fig. 3.2). A slow, steady rate of $^{45}\text{Ca}^{2+}$ uptake was still apparent after 10 minutes. However, $\text{Ca}^{2+}$ mobilising agents were routinely added at 12-20 minutes. Ionomycin (10μM) released 88±1.2% (n=7) of this sequestered $^{45}\text{Ca}^{2+}$, suggesting it was intravesicular (Fig. 3.2). Data obtained with cells exposed to 3 or 12 electrical discharges gave highly complementary results in all cases, except in the absence of ATP, cells exposed to 3 pulses sequestered 33% more $^{45}\text{Ca}^{2+}$ (48% of those containing ATP) than those exposed to 12 pulses. This may be due to increased leakage of ATP from cells with a greater number of pores.

The addition of Ins(1,4,5)P$_3$ (1μM) caused rapid release of sequestered $^{45}\text{Ca}^{2+}$ (50-60%), which was maximal by 30 seconds, the earliest time point studied. Upon prolonged incubation in the presence of the cells, reuptake of $^{45}\text{Ca}^{2+}$ was observed (Fig. 3.3).

3.4.2 $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ Metabolism by Permeabilised SH-SY5Y Cells.

To establish whether $^{45}\text{Ca}^{2+}$ reuptake was due to the metabolism of Ins(1,4,5)P$_3$, SH-SY5Y cells were incubated with $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (1μM) and the rate and route of
Figure 3.2 Time course of $^{45}\text{Ca}^{2+}$ uptake by permeabilised cells. Cells were permeabilised electrically using 12 discharges of a 3µF capacitor across a 0.4cm cuvette in the ICB described in Chapter 2 (2.2). Cells were then incubated in the presence of $^{45}\text{Ca}^{2+}$ (2µCi/ml) in buffer containing 5mM ATP (open squares) or with ATP omitted (filled diamonds). Ionomycin (10µM) (filled squares) was added to the former at 20 minutes. Incubations were terminated by centrifugation of cells through a silicone oil mixture (see Chapter 2 (2.2)). Data represent mean ±S.E.M. for 6 experiments.
Figure 3.3 Time course of $^{45}$Ca$^{2+}$ release and reuptake following addition of Ins(1,4,5)P$_3$. Electrically permeabilised cells (12 discharges) were loaded with $^{45}$Ca$^{2+}$ for 20 minutes and treated with 1μM Ins(1,4,5)P$_3$ for 30 seconds-30 minutes (filled diamonds). Incubations were terminated as described (Chapter 2 (2.2)). Data represent mean±S.E.M. for 6 experiments.
Ins(1,4,5)P3 metabolism followed, by separating inositol mono-, bis-, tris- and tetrakisphosphates.

Permeabilised cells rapidly caused loss of radioactivity in the InsP3 fraction, a majority of metabolites eluting as bisphosphates. Electroporated cells appeared unable to metabolise the bisphosphates at room temperature. A small amount of tetrakisphosphates were accumulated (Fig. 3.4).

Ins(1,4,5)P3 metabolism was more rapid in saponised cells, greater levels of bisphosphates were observed than with electroporated cells, with slight increases in monophosphates also being observed. Similar levels of tetrakisphosphates to those in electroporated cells were observed (Fig. 3.5).

A more detailed examination, using HPLC, of the routes of Ins(1,4,5)P3 metabolism in electrically permeabilised cells at a higher cell density (3-4mg of protein/ml) showed that, while Ins(1,3,4,5)P4 was formed, Ins(1,4)P2 was the major metabolite. Thus, dephosphorylation by Ins(1,4,5)P3 5-phosphatase is the predominant route of Ins(1,4,5)P3 destruction in permeabilised cells (Fig. 3.6).

Bisphosphatase activity, while minimal at 20°C, was observed at 37°C. Ins(4)P was the major metabolite, with only trace levels of Ins(1)P being observed (Fig. 3.7). These monophosphates appeared not to be metabolised in permeabilised cells, as no loss in total radioactivity was apparent. As discussed in the introduction (Chapter 1), Ins(4)P is a direct product of dephosphorylation of Ins(1,4,5)P3, whereas Ins(1)P is formed via dephosphorylation of Ins(1,3,4,5)P4. Thus, it is not surprising that little Ins(1)P is formed, as relatively little Ins(1,3,4,5)P4 is also observed (but see Batty and Nahorski, 1992).

Comparing the rates of [3H] Ins(1,4,5)P3 metabolism and 45Ca2+ reuptake at the lower cell density, it appears that Ca2+ reuptake parallels Ins(1,4,5)P3 metabolism.

It is apparent that no substantial metabolism of Ins(1,4,5)P3 or reuptake of 45Ca2+ had occurred during the first 2 minutes of incubation. Therefore, dose response relationships were assessed, stopping reactions at 2 minutes.
Figure 3.4 Metabolism of $[^3]$H Ins(1,4,5)P$_3$ by electrically permeabilised cells. Cells were permeabilised, washed and incubated in ICB, after 20 minutes the cells were treated with $[^3]$H Ins(1,4,5)P$_3$ (1µM) at 20°C as described (Chapter 2 (2.2)). Reactions were terminated by the addition of an equal volume of TCA 1M. Inositol mono-(open squares), bis-(filled diamonds), tris-(filled squares) and tetrakis phosphate(open diamonds) fractions were separated using gravity fed ion-exchange chromatography as described (Chapter 2 (2.9.2)). Data represent mean ±S.E.M. for 6 experiments.
**Figure 3.5** Metabolism of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ by saponin permeabilised cells. Cells were permeabilised with the detergent, saponin (100µg/ml for 1 minute), washed and incubated in ICB, after 20 minutes the cells were treated with $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (1µM) at 20°C as described (Chapter 2 (2.2)). Reactions were terminated by the addition of an equal volume of TCA (1M). Inositol mono-(open squares), bis-(filled diamonds), tris-(filled squares) and tetrakis phosphate(open diamonds) fractions were separated using gravity fed ion-exchange chromatography as described (Chapter 2 (2.9.2)). Data represent mean ±S.E.M. for 6 experiments. Inositol bisphosphate levels rose rapidly, mirroring trisphosphate metabolism. Low, and equal, levels of inositol mono- and tetrakis-phosphates were also observed.
Figure 3.6 HPLC of metabolites of [3H]Ins(1,4,5)P₃ following incubation with electrically permeabilised cells at 20°C. Cells (4 mg of protein/ml) were treated as described (see Fig. 3.4), but incubated with 5 μM [3H]Ins(1,4,5)P₃ for 1 minute (middle panel) or 5 minutes (lower panel). The upper panel shows Ins(1,4,5)P₃ which had not been incubated with permeabilised cells. Inositol phosphates were separated using the elution protocol described in Chapter 2 (2.9.3). Values represent a single experiment, with similar results obtained in 2 further experiments. Elution times can be compared with those in Table 2.1.
d.p.m. per fraction.

Time (minutes).
Figure 3.7 HPLC of metabolites of [3H] Ins(1,4,5)P3 following incubation with electrically permeabilised cells at 37°C. Cells (4mg of protein/ml) were treated as described (see Fig. 3.4), but incubated with 5μM [3H] Ins(1,4,5)P3 for 1 minute (middle panel) or 5 minutes (lower panel). The upper panel shows Ins(1,4,5)P3 which had not been incubated with permeabilised cells. Inositol phosphates were separated using the elution protocol described in Chapter 2 (2.9.3). Values represent a single experiment, with similar results obtained in 2 further experiments. Elution times can be compared with those in Table 2.1.
3.4.3 Recognition of Inositol Phosphate Analogues by the Ca²⁺-Mobilising Ins(1,4,5)P₃ Receptor, Ins(1,4,5)P₃ 5-Phosphatase and Ins(1,4,5)P₃ 3-Kinase.

3.4.3(i) Characterisation of Ins(1,4,5)P₃-induced ⁴⁵Ca²⁺ Mobilisation.

Ins(1,4,5)P₃ was a potent Ca²⁺ mobilising agent with EC₅₀ values and Hill coefficients obtained from saponised and electroporated (3 and 12 pulses) of 0.08 (0.06-0.12) μM, 1.4 (n=7) (Fig 3.8), 0.09 (0.07-0.13) μM, 1.6 (n=7) (Fig 3.9) and 0.10 (0.08-0.14) μM, 1.2 (n=9) (Fig 3.10) respectively. Maximal mobilisation of loaded ⁴⁵Ca²⁺ was 78±1%, 64±3% and 59±3% for saponised and electroporated (3 and 12 pulses) cells, respectively.

After 15 minutes incubation of Ins(1,4,5)P₃ with the cells, the dose response relationships were shifted to the right, again indicating metabolism of Ins(1,4,5)P₃ and subsequent Ca²⁺ reuptake. EC₅₀ values for Ins(1,4,5)P₃ in saponised and electroporated (3 and 12 pulses) cells were shifted to the right 6-, 3-, and 3-fold respectively, again indicative that Ins(1,4,5)P₃ metabolism is more rapid in saponised cells (Figs 3.8-10).

Using this ⁴⁵Ca²⁺ mobilisation technique, a large number of Ins(1,4,5)P₃ analogues have been utilised to investigate further the structural requirements for Ins(1,4,5)P₃ receptor recognition.

3.4.3(ii) Effects of Phosphorothioate Substitution on Recognition Properties of Ins(1,4,5)P₃ Analogues.

The 5-position of Ins(1,4,5)P₃ is clearly of major importance, as it is the site of attack by Ins(1,4,5)P₃ 5-phosphatase, which appears to play the major role in Ins(1,4,5)P₃ metabolism in permeabilised cells (see above). The introduction of phosphorothioate groups to replace phosphate groups has been used previously to produce phosphatase-resistant analogues of many other short-lived agents, ATP₇S, ADP₇S, GTP₇S and GDP₇S being prime examples. ADP₇S and ATP₇S have been used extensively to study purinergic receptors (Welford et al., 1987; Boyer et al., 1989; Wilkinson et al., 1993), while GDP₇S and GTP₇S have been used to stabilise G-protein states, in either
Figures 3.8-10 Dose-response relationships for Ins(1,4,5)P3 in electrically- and saponin permeabilised cells. Cells were permeabilised using (Fig.3.8) 3 pulses of a 3μF capacitor (Fig.3.9) 12 pulses of a 3μF capacitor or (Fig.3.10) saponin (100μg/ml, 1 minute) and loaded with 45Ca2+ as described (Chapter 2 (2.2)). Incubations were terminated after 2 minutes (open symbols) or 15 minutes (closed symbols), and 45Ca2+ release determined. Data represent mean±S.E.M. of 6 experiments.
Fig. 3.8

Fig. 3.9

Fig. 3.10
inactivated or activated states (Cockcroft and Taylor, 1987; Taylor et al., 1988). However, the most analogous example is that of the phosphorothioate derivative of the second messenger, cyclic AMP. cAMP\textsubscript{S} has been used extensively to probe the role and effects of cyclic AMP. cAMPS does have advantages as a tool when compared to cyclic AMP, in that these phosphorothioate groups are more hydrophobic than phosphate groups. In the case of cAMPS, this produces an analogue which is membrane permeant. \textsubscript{S}p-cAMPS activates cyclic AMP-dependent kinases, whereas \textsubscript{R}p-cAMPS is an antagonist (reviewed by Potter, 1990). A number of analogues of Ins(1,4,5)P\textsubscript{3} were, therefore, synthesised, in an attempt to make phosphatase-resistant analogues. These included inositol 1,4-bisphosphate 5-phosphorothioate (InsP\textsubscript{3}-5\textsubscript{S}), inositol 1-phosphate 4,5-bisphosphorothioate (InsP\textsubscript{3}-(4,5)\textsubscript{S}2) and inositol 1,4,5-trisphosphorothioate (Ins(1,4,5)PS\textsubscript{3}). Inositol 1-phosphorothioate 4,5-bisphosphate (InsP\textsubscript{3}-1\textsubscript{S}) was also produced which allowed the introduction of a fluorescent moiety, nitrobenzoxadiazole, at this 1-position (NBD InsP\textsubscript{3}-1\textsubscript{S}). However, Ins(1,4,5)P\textsubscript{3} is much more polar than cyclic AMP, and the addition of one, or indeed three, phosphorothioate groups, was not expected to produce a membrane-permeable analogue.

Introduction of a phosphorothioate group into position 5 (InsP\textsubscript{3}-5\textsubscript{S}) produced an analogue which was resistant to dephosphorylation. This introduction causes a decrease in the ability to mobilise \textsuperscript{45}Ca\textsuperscript{2+}, giving an EC\textsubscript{50}=0.9 \mu M. The introduction of a second phosphorothioate group into the 4-position causes further attenuation in \textsuperscript{45}Ca\textsuperscript{2+} mobilising potency, InsP\textsubscript{3}-(4,5)\textsubscript{S}2 having an EC\textsubscript{50}=1.3 \mu M. Total phosphorothioate replacement in all 3 phosphate groups caused a further decrease in potency, Ins(1,4,5)PS\textsubscript{3} having an EC\textsubscript{50}=2.4 \mu M. Substitution of this 1 phosphate with a phosphorothioate group essentially caused no loss of activity, EC\textsubscript{50} for DL-InsP\textsubscript{3}-1\textsubscript{S} being 0.21 \mu M. Introduction of the fluorescent moiety, NBD, at this 1 phosphorothioate caused only a slight loss of activity (EC\textsubscript{50}= 0.6 \mu M).

InsP\textsubscript{3}-5\textsubscript{S} was found to be a potent inhibitor of Ins(1,4,5)P\textsubscript{3} 5-phosphatase, with K\textsubscript{m}=6.8\mu M. The introduction of a second phosphorothioate group at the 4-position caused further increase in affinity, K\textsubscript{m}(InsP\textsubscript{3}-(4,5)\textsubscript{S}2)=1.4\mu M. Total phosphorothioate replacement in all three phosphate groups did not appear to further enhance the affinity of Ins(1,4,5)P\textsubscript{3} 5-phosphatase for the analogue (K\textsubscript{m}(Ins(1,4,5)PS\textsubscript{3})=1.7\mu M). The introduction of a phosphorothioate group to replace the 5-phosphate slightly attenuated recognition by Ins(1,4,5)P\textsubscript{3} 3-kinase for the analogue (K\textsubscript{m}=3.2\mu M). However, treatment of InsP\textsubscript{3}-5\textsubscript{S} with Ins(1,4,5)P\textsubscript{3} 3-kinase caused loss of Ca\textsuperscript{2+} mobilising ability, suggesting it is a substrate for Ins(1,4,5)P\textsubscript{3} 3-kinase. Work performed by J. McBain (Safrany et al., 1991) identified that InsP\textsubscript{3}-5\textsubscript{S} was clearly
phosphorylated more slowly, some 15% the rate of Ins(1,4,5)P₃, as determined by the
time required to produce a similar shift in Ca²⁺ mobilising ability. A 5 minute
incubation of Ins(1,4,5)P₃ produced a 6-fold shift in EC₅₀, while a 30 minute
incubation of InsP₃-5S caused a 4.3-fold shift. The introduction of a second
phosphorothioate group into the 4-position attenuated recognition by Ins(1,4,5)P₃ 3-
kinase (Kᵢ=46μM). InsP₃-(4,5)S₂ was also a poor substrate for Ins(1,4,5)P₃ 3-kinase,
appearing to be phosphorylated some 10% of the rate of Ins(1,4,5)P₃. Total
phosphorothioate replacement markedly diminished Ins(1,4,5)P₃ 3-kinase recognition
(Kᵢ=230μM), and produced an analogue which was resistant to phosphorylation (as
well as dephosphorylation by Ins(1,4,5)P₃ 5-phosphatase). Equally, dose response
relationships prepared following incubation of electrically permeabilised cells with
InsP₃-5S and Ins(1,4,5)P₅S₃ for 2 and 15 minutes, as performed for Ins(1,4,5)P₃ (Fig
3.10), were affected to different extents. The dose-response relationship for InsP₃-5S,
which is resistant to Ins(1,4,5)P₃ 5-phosphatase, but a substrate for Ins(1,4,5)P₃ 3-
kinase, was shifted from 0.87(0.62-1.22)μM to 2.12(1.42-3.17)μM (p=0.02, paired t
test) (Fig 3.11). The dose-response relationship for Ins(1,4,5)P₅S₃ was unaffected by
such incubations with EC₅₀ values of 2.38(1.72-3.30)μM and 3.09(2.35-4.07)μM
following incubations of 2 and 15 minutes (Fig 3.12) (see Summary Table 3.1).

In the search for a selective Ins(1,4,5)P₃ 5-phosphatase inhibitor it is, therefore, worth
bearing in mind that replacement of phosphate groups with phosphorothioate groups
substantially reduces the ability of an analogue to mobilise Ca²⁺ or interact with
Ins(1,4,5)P₃ 3-kinase, whilst imbuing resistance to Ins(1,4,5)P₃ 5-phosphatase and
substantially enhancing the affinity of Ins(1,4,5)P₃ 5-phosphatase for the analogue.
Figures 3.11, 12  Dose-response relationships for InsP3-5S (Fig. 3.11) and Ins(1,4,5)PS3 (Fig. 3.12) in electrically permeabilised cells. Cells were permeabilised using 12 pulses of a 3μF capacitor and loaded with 45Ca2+ as described (Chapter 2 (2.2)). Incubations were terminated after 2 minutes (open symbols) or 15 minutes (closed symbols), and 45Ca2+ release determined. Data represent mean±S.E.M. of 6 experiments.
Fig. 3.11

Initial $[\text{InsP}_3 - 5S]$ (μM) vs. % $^{45}\text{Ca}^{2+}$ Released

Fig. 3.12

Initial $[\text{Ins}(1,4,5)\text{PS}]_3$ (μM) vs. % $^{45}\text{Ca}^{2+}$ Released
Table 3.1 Phosphorothioate Substitution Summary Table.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>EC50(μM)(^a)</th>
<th>Kᵢ(μM)</th>
<th>S/R(^b)</th>
<th>Kᵢ(μM)</th>
<th>S/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ins(1,4,5)P₃</td>
<td>0.09</td>
<td></td>
<td>S</td>
<td></td>
<td>s</td>
</tr>
<tr>
<td>InsP₃-1S</td>
<td>0.21(0.13-0.34)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>NBD InsP₃-1S</td>
<td>0.6(0.2-1.8)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>InsP₃-5S</td>
<td>0.9(0.6-1.2)</td>
<td>6.8(^*)</td>
<td>R</td>
<td>3.2</td>
<td>S</td>
</tr>
<tr>
<td>InsP₃-(4,5)S₂</td>
<td>1.4(0.21-6.8)</td>
<td>1.4(^*)</td>
<td>R</td>
<td>46</td>
<td>S</td>
</tr>
<tr>
<td>Ins(1,4,5)P₅S₄</td>
<td>2.5(1.7-3.3)</td>
<td>1.7(^*)</td>
<td>R</td>
<td>230</td>
<td>R</td>
</tr>
</tbody>
</table>

\(\text{a} \) EC5₀ for \(^{45}\text{Ca}^{2+}\) mobilisation from permeabilised SH-SY5Y cells, expressed as mean, followed by 95% confidence limits in parentheses.
\(\text{b} \) S, substrate; R, resistant.
\(\text{*} \) Assuming competitive inhibition.

3.4.3(ii) Effects of 2-Position Modification on Recognition Properties of Ins(1,4,5)P₃ Analogues.

A substantial number of studies have concentrated upon introduction of groups at the unique 2-position of the myo-inositol ring. This is the only position which possesses an axial group in the native molecule. A number of such analogues have been produced by B.V.L. Potter and colleagues. Hirata et al. (1989) have previously shown that DL-2-deoxy InsP₃ is a potent Ca\(^{2+}\) mobilising agent (EC₅₀=0.5μM, compared with EC₅₀(Ins(1,4,5)P₃)=0.2μM), which is bound by Ins(1,4,5)P₃ 3-kinase with only minimal loss of affinity whilst being recognised by Ins(1,4,5)P₃ 5-phosphatase with a similar profile to Ins(1,4,5)P₃ itself.

The introduction of a fluorine into this myo 2-position (DL-2-deoxy-2-fluoro myo inositol 1,4,5-trisphosphate, myo 2F InsP₃) moderately attenuated its ability to
mobilise Ca^{2+} (EC_{50}=0.24\mu M). myo 2F InsP_3 was a substrate for Ins(1,4,5)P_3 3-kinase but was bound with lower affinity. Treatment of myo 2F InsP_3 with the Ins(1,4,5)P_3 3-kinase preparation which caused a 29-fold shift in EC_{50} in the ability of Ins(1,4,5)P_3 to mobilise ^{45}Ca^{2+} in a subsequent assay caused a 40-fold shift in the ability of myo 2F InsP_3. myo 2F InsP_3 inhibited the phosphorylation of Ins(1,4,5)P_3 with an apparent K_i=3.0\mu M. Interestingly, however, myo 2F InsP_3 was a potent inhibitor of Ins(1,4,5)P_3 5-phosphatase, with K_i=14.4\mu M. This observation implicates the 2-position of Ins(1,4,5)P_3-like molecules as being important with regards Ins(1,4,5)P_3 5-phosphatase substrate properties. Introduction of a second fluorine at the equatorial 2-position (DL-2-deoxy-2,2-difluoro scyllo inositol 1,4,5-trisphosphate, DL-2,2F^2 InsP_3) again had little effect on Ca^{2+} mobilisation properties (EC_{50}=0.36\mu M). DL-2,2F^2 InsP_3 was resistant to Ins(1,4,5)P_3 5-phosphatase, with a K_i value of 26\mu M. DL-2,2F^2 InsP_3 was, however, a substrate for Ins(1,4,5)P_3 3-kinase, with an affinity of 11\mu M. Separation of the enantiomers of DL-2,2F^2 InsP_3 identified D-2,2F^2 InsP_3 as a potent Ca^{2+} mobilising agent, with EC_{50}=0.27\mu M and L-2,2F^2 InsP_3 as a weak agonist with EC_{50}=52\mu M. D-2,2F^2 InsP_3 was a substrate for both Ins(1,4,5)P_3 3-kinase and 5-phosphatase, having affinities of 10.2 and 60\mu M respectively. D-2,2F^2 InsP_3 appeared to be hydrolysed slightly more slowly than Ins(1,4,5)P_3, as a 1 hour treatment of 100\mu M Ins(1,4,5)P_3 and D-2,2F^2 InsP_3 caused a 168- and 71-fold shift respectively in their abilities to mobilise ^{45}Ca^{2+} from permeabilised SH-SY5Y cells. D-2,2F^2 InsP_3 was a substrate for Ins(1,4,5)P_3 3-kinase with an affinity of 10.2\mu M. L-2,2F^2 InsP_3 was a potent inhibitor of Ins(1,4,5)P_3 5-phosphatase, with K_i=19\mu M. L-2,2F^2 InsP_3 also inhibited Ins(1,4,5)P_3 3-kinase-induced phosphorylation of Ins(1,4,5)P_3, with K_i=11.9\mu M. It is, however, most difficult to ascertain whether such an analogue is a substrate for, or an inhibitor of Ins(1,4,5)P_3 3-kinase. The only technique used in these studies to determine whether analogues were susceptible to phosphorylation was, as described in Chapter 2, whether treatment with the Ins(1,4,5)P_3 3-kinase preparation caused an attenuation in Ca^{2+} mobilising properties. This assumes that the tetrakisphosphate product is a considerably less potent Ca^{2+}-mobilising agent than the initial analogue. However, such treatment can only be performed on analogues which mobilise Ca^{2+} potently, and, therefore, such analysis is not possible for agents such as L-2,2F^2 InsP_3.

The introduction of a single fluorine into an equatorial 2-position, replacing the axial group by a hydrogen, produced DL-2-deoxy-2-fluoro scyllo inositol 1,4,5-trisphosphate (sc 2F InsP_3). Such modification further reduced the ability of this analogue to mobilise Ca^{2+} (EC_{50}=0.7\mu M). sc2F InsP_3 was also found to be a substrate, although poor, for both Ins(1,4,5)P_3 3-kinase and 5-phosphatase, having affinities of 8.8 and 0.71\mu M respectively. Replacement of this fluorine with an
equatorial hydroxyl group (scylo inositol 1,2,4-trisphosphate, sc Ins(1,2,4)P₃) substantially enhanced Ca²⁺ mobilisation properties, sc Ins(1,2,4)P₃ releasing Ca²⁺ with EC₅₀=0.34(0.24-0.47)µM. Ins(1,4,5)P₃ 3-kinase recognition was also enhanced, sc Ins(1,2,4)P₃ being a substrate, with an affinity of 4.0µM. The rate of phosphorylation of sc Ins(1,2,4)P₃ appeared to be slightly slower than that of Ins(1,4,5)P₃. A treatment of sc Ins(1,2,4)P₃, which caused a 29-fold shift in EC₅₀ for Ins(1,4,5)P₃, caused a 7-fold shift in EC₅₀ for sc Ins(1,2,4)P₃. However, Ins(1,4,5)P₃ 5-phosphatase recognition was substantially reduced, sc Ins(1,2,4)P₃ being an inhibitor with Kᵢ=24µM.

The introduction of an axial phosphate group at this position (DL-myo inositol 1,2,4,5-tetrakisphosphate, Ins(1,2,4,5)P₄) produced another agent which potently mobilised Ca²⁺ (EC₅₀=0.36(0.11-1.1)µM). Ins(1,2,4,5)P₄ was recognised very poorly by Ins(1,4,5)P₃ 3-kinase (Kᵢ>200µM), and was resistant to phosphorylation. Ins(1,2,4,5)P₄ was also resistant to Ins(1,4,5)P₃ 5-phosphatase, but was a potent inhibitor (Kᵢ=2.9µM) (see Summary Table 3.2). Assuming that the L-enantiomers of myo 2F InsP₃, sc Ins(1,2,4)P₃ and Ins(1,2,4,5)P₄ are, like L-Ins(1,4,5)P₃, only weakly active at the Ins(1,4,5)P₃ receptor, this suggests that such modifications cause no loss of activity.
Table 3.2 2-Position Modification Summary Table.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>$^{45}$Ca$^{2+}$ Release</th>
<th>5-Phosphatase</th>
<th>3-Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$(μM)$^a$</td>
<td>K$_i$(μM) S/R$^b$</td>
<td>K$_i$(μM) S/R</td>
</tr>
<tr>
<td>Ins(1,4,5)P$_3$</td>
<td>0.09</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>myo 2F InsP$_3$</td>
<td>0.24(0.11-0.48)</td>
<td>14.4 R</td>
<td>3.0 S</td>
</tr>
<tr>
<td>DL 2,2F$_2$ InsP$_3$</td>
<td>0.36(0.27-0.49)</td>
<td>26 R</td>
<td>11 S</td>
</tr>
<tr>
<td>D 2,2F$_2$ InsP$_3$</td>
<td>0.27(0.17-0.44)</td>
<td>60 S</td>
<td>10.2 S</td>
</tr>
<tr>
<td>L 2,2F$_2$ InsP$_3$</td>
<td>52(37-76)</td>
<td>19 R</td>
<td>11.9 N.D.</td>
</tr>
<tr>
<td>sc 2F InsP$_3$</td>
<td>0.71(0.53-0.92)</td>
<td>0.7 S</td>
<td>8.8 S</td>
</tr>
<tr>
<td>sc Ins(1,2,4)P$_3$</td>
<td>0.34(0.24-0.47)</td>
<td>24 R</td>
<td>4.0 S</td>
</tr>
<tr>
<td>Ins(1,2,4,5)P$_4$</td>
<td>0.36(0.11-1.1)</td>
<td>2.9 R</td>
<td>&gt;&gt;200 R</td>
</tr>
</tbody>
</table>

$^a$ EC$_{50}$ for $^{45}$Ca$^{2+}$ mobilisation from permeabilised SH-SY5Y cells, expressed as mean, followed by 95% confidence limits in parentheses.

$^b$ S, substrate; R, resistant; N.D. not determined.
3.4.3(iv) Effects of 3-Position Modification on Recognition Properties of
Ins(1,4,5)P$_3$ Analogues.

The 3-position of Ins(1,4,5)P$_3$ is of great interest, being the site of phosphorylation by
Ins(1,4,5)P$_3$ 3-kinase. Despite this, there are few reports of inositol phosphate
analogues in which this 3-position has been targeted for chemical modification. The
product of phosphorylation, Ins(1,3,4,5)P$_4$, is a poor inhibitor of Ins(1,4,5)P$_3$ 3-
kinase, suggesting it plays no role in any 'feedback' mechanism. D-Ins(1,3,4,5)P$_4$
competitively inhibited Ins(1,4,5)P$_3$ phosphorylation with a $K_i$ of 26.5µM, in close
agreement with that which can be derived from work by Polokoff et al. (1988) with
DL-Ins(1,3,4,5)P$_4$ ($IC_{50}=90$µM). Whilst earlier data suggested that Ins(1,3,4,5)P$_4$
was a substrate for HEG Ins(1,4,5)P$_3$ 5-phosphatase (Batty et al., 1985),
Ins(1,3,4,5)P$_4$ appeared to be a very poor substrate for Ins(1,4,5)P$_3$ 5-phosphatase
when incubated with HEGs. Indeed, under conditions where 10µM (2.5nmol)
Ins(1,4,5)P$_3$ liberated 1.4±0.5nmol P$_i$ when incubated with HEGs, 10µM (2.5nmol)
Ins(1,3,4,5)P$_4$ liberated only 0.11nmol P$_i$. It did, however, potently inhibit
Ins(1,4,5)P$_3$ dephosphorylation, with a $K_i$ value of 1.9µM. The introduction of a
fluorine atom at the 3-position (3F InsP$_3$), produced, as expected, an analogue
resistant to Ins(1,4,5)P$_3$ 3-kinase. The affinity of Ins(1,4,5)P$_3$ 3-kinase for 3F InsP$_3$
($K_i=8.6$µM) was slightly lower than that for Ins(1,4,5)P$_3$. 3F-InsP$_3$ appears to be less
potent than Ins(1,4,5)P$_3$ in 45Ca$^{2+}$ mobilisation studies using permeabilised SH-
SY5Y cells ($EC_{50}=0.36$ µM). 3F InsP$_3$ was, however, a good substrate for
Ins(1,4,5)P$_3$ 5-phosphatase, binding with substantially higher affinity than
Ins(1,4,5)P$_3$ ($K_m=3.9$µM), but was hydrolysed at some 60% of the rate of the rate
Ins(1,4,5)P$_3$.

Inversion of the stereochemistry at this 3-position (producing L-chiro inositol 2,3,5-
trisphosphate, L-ch Ins(2,3,5)P$_3$) also produced an Ins(1,4,5)P$_3$ 3-kinase-resistant
analogue, with a slight decrease in affinity ($K_i=7.1$µM). This also caused substantial
loss of Ca$^{2+}$ mobilising activity ($EC_{50}=1.3$ µM, n=6). More surprisingly, L-ch
Ins(2,3,5)P$_3$ was found to be an inhibitor of Ins(1,4,5)P$_3$ 5-phosphatase, with
$K_i=7.7$µM. Replacement of the three phosphate groups with phosphorothioate groups
causcd a slight decrease in potency, L-ch Ins(2,3,5)P$_3$S$_3$ having $EC_{50}=4.3$ µM, (n=8).
More surprisingly, however, L-ch Ins(2,3,5)P$_3$S$_3$ was clearly a partial agonist,
releasing only some 25% of loaded 45Ca$^{2+}$ (see below, Fig. 3.13). Such replacement
also enhanced the affinity of Ins(1,4,5)P$_3$ 5-phosphatase, L-ch Ins(2,3,5)P$_3$S$_3$
inhibiting Ins(1,4,5)P$_3$ dephosphorylation with a $K_i=0.21$µM, making it the most

3.17
Figure 3.13 Dose-response relationships for Ins(1,4,5)P₃ (open squares), L-ch Ins(2,3,5)PS₃ (open circles) and inhibition of Ins(1,4,5)P₃ (0.7µM) effects by L-ch Ins(2,3,5)PS₃ (filled circles). Electrically permeabilised cells were loaded with $^{45}$Ca$^{2+}$ and then challenged with Ins(1,4,5)P₃, L-ch Ins(2,3,5)PS₃ or both. Incubations were terminated after 2 minutes, at which time the amount of $^{45}$Ca$^{2+}$ released was assessed. Data shown are mean±S.E.M. from at least 3 experiments.
potent Ins(1,4,5)P$_3$ 5-phosphatase inhibitor yet described, whilst not affecting the affinity of Ins(1,4,5)P$_3$ 3-kinase ($K_i=0.82\mu$M) (see Summary Table 3.3).

Table 3.3 3'Position Modification Summary Table.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>$^{45}$Ca$^{2+}$ Release</th>
<th>5-Phosphatase</th>
<th>3-Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$(μM)$^a$</td>
<td>$K_i$(μM)</td>
<td>S/R$^b$</td>
</tr>
<tr>
<td>Ins(1,4,5)P$_3$</td>
<td>0.09</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Ins(1,3,4,5)P$_4$</td>
<td>N.D.</td>
<td>1.9</td>
<td>S</td>
</tr>
<tr>
<td>3F InsP$_3$</td>
<td>0.36(0.26-0.50)</td>
<td>3.9</td>
<td>S</td>
</tr>
<tr>
<td>L-ch Ins(2,3,5)P$_3$</td>
<td>1.3(0.66-2.5)</td>
<td>3.9</td>
<td>R</td>
</tr>
<tr>
<td>L-ch Ins(2,3,5)PS$_3$</td>
<td>4.3(2.5-7.5)</td>
<td>0.21</td>
<td>R</td>
</tr>
</tbody>
</table>

$^a$ EC$_{50}$ for $^{45}$Ca$^{2+}$ mobilisation from permeabilised SH-SY5Y cells, expressed as mean, followed by 95% confidence limits in parentheses.

$^b$ S, substrate; R, resistant; N.D. not determined.

3.4.3(v) Effects of 6-Position Modification on Recognition Properties of Ins(1,4,5)P$_3$ Analogues.

The 6 hydroxyl group present within Ins(1,4,5)P$_3$ appears to play a crucial role in Ins(1,4,5)P$_3$ receptor recognition. Removal of this group (6-deoxy InsP$_3$) produces a weak full agonist (EC$_{50}=6.0\mu$M, n=3), some 70-fold less potent than Ins(1,4,5)P$_3$. The removal of this 6-hydroxyl group, however, did not greatly affect Ins(1,4,5)P$_3$ 3-kinase or 5-phosphatase recognition, inhibiting with $K_i$ values of 5.7 and 88.6μM respectively. 6-deoxy InsP$_3$ was resistant to Ins(1,4,5)P$_3$ 5-phosphatase, liberating no inorganic phosphate when incubated with Ins(1,4,5)P$_3$ 5-phosphatase. I was unable to determine whether 6-deoxy InsP$_3$ was a substrate for Ins(1,4,5)P$_3$ 3-kinase, due to its lack of potency at the Ins(1,4,5)P$_3$ receptor.
The introduction of a phosphate group at this important 6-position, forming Ins(1,4,5,6)P₄ produced an analogue which was without effect at the Ins(1,4,5)P₃ receptor. Clearly, the presence of such a large, negatively charged group in this position will greatly affect intramolecular bonding, thus changing the solution conformation quite markedly. The trisphosphorothioate analogue of 6-deoxy InsP₃, 6-deoxy InsPS₃ was, as expected from the discussion earlier, less potent than 6-deoxy InsP₃ at mobilising ⁴⁵Ca²⁺, with EC₅₀=16.0 µM (n=7). It was, however, clearly a partial agonist, releasing some 30% of loaded ⁴⁵Ca²⁺ loaded, further evidence that not all small molecules which bind the Ins(1,4,5)P₃ receptor are full agonists (see below; Fig. 3.14).

The observation that phosphorothioate group introduction enhances the affinity of Ins(1,4,5)P₃ 5-phosphatase for Ins(1,4,5)P₃ analogues has been tested and used in the search for selective, potent Ins(1,4,5)P₃ 5-phosphatase inhibitors. 6-deoxy InsPS₃, while being a relatively impotent partial agonist at the Ins(1,4,5)P₃ receptor, is an inhibitor of Ins(1,4,5)P₃ 5-phosphatase, being some 84-fold more potent than 6-deoxy InsP₃ (Kᵢ=1.4µM). Phosphorothioate introduction was without effect on Ins(1,4,5)P₃ 3-kinase affinity, 6-deoxy InsPS₃ giving a Kᵢ of 7.9µM (see Summary Table 3.4).

Table 3.4 6-Position Modification Summary Table.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>⁴⁵Ca²⁺ Release</th>
<th>5-Phosphatase</th>
<th>3-Kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀(µM)</td>
<td>Kᵢ(µM)</td>
<td>S/R</td>
</tr>
<tr>
<td>Ins(1,4,5)P₃</td>
<td>0.09</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>6-deoxy InsP₃</td>
<td>6.0(2.2-16.4)</td>
<td>88.6</td>
<td>R</td>
</tr>
<tr>
<td>6-deoxy InsPS₃</td>
<td>16(10-25)</td>
<td>1.4</td>
<td>R</td>
</tr>
<tr>
<td>Ins(1,4,5,6)P₄</td>
<td>&gt;&gt;100</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

³ EC₅₀ for ⁴⁵Ca²⁺ mobilisation from permeabilised SH-SY5Y cells, expressed as mean, followed by 95% confidence limits in parentheses.

S, substrate; R, resistant; N.D., not determined.
Figure 3.14 Dose-response relationships for Ins(1,4,5)P₃ (open squares), 6-deoxy InsPS₃ (open circles) and inhibition of Ins(1,4,5)P₃ (0.7 µM) effects by 6-deoxy InsPS₃ (filled circles). Electrically permeabilised cells were loaded with ⁴⁵Ca²⁺ and then challenged with Ins(1,4,5)P₃, 6-deoxy InsPS₃ or both. Incubations were terminated after 2 minutes, at which time the amount of ⁴⁵Ca²⁺ released was assessed. Data shown are mean±S.E.M. from at least 3 experiments.
3.4.3(vi) Other Modifications Affecting Recognition Properties.

Following the observation by Polokoff et al. (1988), that Ins(1,3,5)P₃ was a moderate Ins(1,4,5)P₃ 5-phosphatase inhibitor, which interacted poorly with the Ins(1,4,5)P₃ receptor and Ins(1,4,5)P₃ 3-kinase, its trisphosphorothioate derivative was synthesised. Ins(1,3,5)PS₃ was found to be a potent inhibitor of Ins(1,4,5)P₃ 5-phosphatase (Kᵢ=0.5μM), with little interaction with the Ins(1,4,5)P₃ receptor (<100pM) and Ins(1,4,5)P₃ 3-kinase (Kᵢ=250μM). The production of DL-myo inositol 1-phosphate 4,5-diphosphate (InsP₃(PP)) removed all Ca²⁺-mobilising ability (<300μM) and Ins(1,4,5)P₃ 3-kinase recognition (<300μM). InsP₃(PP), however, also bound Ins(1,4,5)P₃ 5-phosphatase very poorly (Kᵢ~200μM) and appeared resistant to dephosphorylation.

A number of analogues based on a cyclohexane nucleus have also been produced. Cyclohexane 1,2,4-trisphosphate (CH(1,2,4)P₃) has been previously shown to be almost devoid of Ca²⁺ mobilising ability, with only slight attenuation of affinity for Ins(1,4,5)P₃ 5-phosphatase (Polokoff et al., 1988). Polokoff et al. (1988) showed that CH(1,2,4)P₃ inhibited Ins(1,4,5)P₃ 5-phosphatase with an IC₅₀=137μM, affinity of the Ins(1,4,5)P₃ receptor and Ins(1,4,5)P₃ 3-kinase were substantially reduced. The insertion of methylene groups between the cyclohexane ring and phosphate groups of CH(1,2,4)P₃, producing cyclohexane 1,2,4-trismethylene phosphate (CH(1,2,4){CH²PO₄}₃) which had similar properties to those reported for CH(1,2,4)P₃ (Polokoff et al., 1988), being resistant to Ins(1,4,5)P₃ 5-phosphatase, with Kᵢ=144μM. Replacement of the bridging oxygen by a methylene group (cyclohexane 1,2,4-trismethylenephosphonate, CH(1,2,4){CH²PO₃}₃) produced a resistant analogue which was also bound very poorly (Kᵢ=535μM). If the phosphate groups are replaced with sulphonate groups (giving cyclohexane 1,2,4-trismethylenesulphonate, CH(1,2,4){CH²SO₃}₃) a highly potent ‘inhibitor’ is produced (Kᵢ=3.9μM) (N.B. I was unable to determine whether CH(1,2,4){CH²SO₃}₃ was a substrate for, or inhibitor of, Ins(1,4,5)P₃ 5-phosphatase, as the assay is performed in a buffer already containing 2mM MgSO₄). No other reports of inositol sulphate/sulphonate analogues interacting with Ins(1,4,5)P₃ 5-phosphatase exist, and, therefore this appears to be a potential lead in the production of high affinity agents interacting with Ins(1,4,5)P₃ 5-phosphatase. All of the above agents did not inhibit Ins(1,4,5)P₃ 3-kinase (<1μM). CH(1,2,4){CH²PO₃}₃, CH(1,2,4){CH²PO₄}₃, and CH(1,2,4){CH²SO₃}₃ were all very poorly recognised by
the enzyme, once again suggesting that Ins(1,4,5)P₃ 3-kinase cannot tolerate steric bulk at these positions.

Decavanadate also inhibits Ins(1,4,5)P₃ 5-phosphatase, and inhibited Ins(1,4,5)P₃ dephosphorylation with $K_i=1.5\mu M$, while orthovanadate was without effect ($\leq 30\mu M$). This suggests that decavanadate offers a cage of negatively charged oxygen atoms and the relative separation of charges can correspond approximately to the presumed separation in conformational space between the three phosphate groups of Ins(1,4,5)P₃ (Fig. 3.15). Decavanadate was also found to inhibit Ins(1,4,5)P₃ 3-kinase with $K_i=5\mu M$. However, as described above, decavanadate interacts with all Ins(1,4,5)P₃ recognition sites with $K_i=1.2(Ca^{2+} \text{ mobilisation})-5\mu M$ (Ins(1,4,5)P₃ 5-phosphatase). Thus, while interacting potently with all aspects of Ins(1,4,5)P₃-mediated signalling, decavanadate cannot be considered to be a selective at any one locus.

However, by far the most selective phosphate-containing analogue which acts as an Ins(1,4,5)P₃ 5-phosphatase inhibitor is L-ch-Ins(1,4,6)P₃. L-ch-Ins(1,4,6)P₃ which possesses a diaxial phosphate pairing, is unable to mobilise Ca²⁺ (<100μM) or interact with Ins(1,4,5)P₃ 3-kinase (<300μM). It does however, inhibit Ins(1,4,5)P₃ 5-phosphatase competitively with $K_i=44\mu M$. Replacement of the three phosphate groups with phosphorothioate groups produces an analogue which inhibits Ins(1,4,5)P₃ 5-phosphatase with $K_i=0.3\mu M$ (this figure can be obtained from Fig. 3.16) whilst not interacting with the Ins(1,4,5)P₃ receptor or Ins(1,4,5)P₃ 3-kinase at concentrations $\leq 30\mu M$ (see Summary Table 3.5).
Figure 3.15 A comparison of the molecular structures of (a) decavanadate and (b) Ins(1,4,5)P₃. Molecular parameters are comparable. Ionic charges are not shown for simplicity. Figure courtesy of Professor B.V.L. Potter.
Figure 3.16 Lineweaver-Burk Plot of Ins(1,4,5)P$_3$ 5-phosphatase inhibition by L-ch Ins(1,4,6)PS$_3$. [${}^3$H] Ins(1,4,5)P$_3$ was incubated with HEGs in the presence of L-ch Ins(1,4,6)PS$_3$ as described (Chapter 2 (2.6.2)). Rates of Ins(1,4)P$_2$ formation were calculated. Data shown are reciprocals of means of rates from 4 experiments. Calculations, as described in Chapter 2 (2.8), yielded a $K_i$ of 0.3µM.
### Table 3.5 ‘Other Modifications’ Summary Table.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>$^{45}$Ca$^{2+}$ Release EC$_{50}$(μM)$^a$</th>
<th>5-Phosphatase $K_{i}(μM)$</th>
<th>S/R$^b$</th>
<th>3-Kinase $K_{i}(μM)$</th>
<th>S/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ins(1,4,5)P$_3$</td>
<td>0.09</td>
<td>S</td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ins(1,3,5)PS$_3$</td>
<td>&gt;&gt;100</td>
<td>0.5</td>
<td>R</td>
<td>250</td>
<td>N.D.</td>
</tr>
<tr>
<td>InsP$_3$(PP)</td>
<td>&gt;&gt;300</td>
<td>~200</td>
<td>R</td>
<td>&gt;&gt;300</td>
<td>N.D.</td>
</tr>
<tr>
<td>(CH(1,2,4)P$_3$</td>
<td>40</td>
<td>IC$_{50}$=137</td>
<td>R</td>
<td>IC$_{50}$=327</td>
<td>N.D.$^a$</td>
</tr>
<tr>
<td>CH(1,2,4)(CH$_2$PO$_4$)$_3$</td>
<td>N.D.</td>
<td>144</td>
<td>R</td>
<td>&gt;&gt;1000</td>
<td>N.D.</td>
</tr>
<tr>
<td>CH(1,2,4)(CH$_2$PO$_3$)$_3$</td>
<td>N.D.</td>
<td>535</td>
<td>R</td>
<td>&gt;&gt;1000</td>
<td>N.D.</td>
</tr>
<tr>
<td>CH(1,2,4)(CH$_2$SO$_3$)$_3$</td>
<td>N.D.</td>
<td>3.9</td>
<td>R</td>
<td>&gt;&gt;1000</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-ch Ins(1,4,6)P$_3$</td>
<td>&gt;&gt;100</td>
<td>44</td>
<td>R</td>
<td>&gt;&gt;300</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-ch Ins(1,4,6)PS$_3$</td>
<td>&gt;&gt;30</td>
<td>0.3</td>
<td>R</td>
<td>&gt;&gt;30</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

$^a$ Data from Polokoff et al., 1988.

$^b$ EC$_{50}$ for $^{45}$Ca$^{2+}$ mobilisation from permeabilised SH-SY5Y cells.

S, substrate; R, resistant; N.D. not determined.
3.5 Discussion.

SH-SY5Y cells, like most cells (see Berridge and Irvine, 1989), contain Ins(1,4,5)P₃ receptors located intracellularly, possibly on the E.R. These are acted upon by the water soluble second messenger, Ins(1,4,5)P₂. The addition of Ins(1,4,5)P₃ to unpermeabilised cells was without effect (data not shown), indicating that cells did not become permeable to such molecules when incubated in ICB. Permeabilisation of the cell membrane, using either saponin or electrical discharges, greatly enhanced ⁴⁵Ca²⁺ loading, and rendered the cells sensitive to Ins(1,4,5)P₃. Ins(1,4,5)P₃ mobilises some 75% of the ⁴⁵Ca²⁺ loaded into vesicular stores. However, reuptake occurs and this correlates well with the ability of these cells to metabolise Ins(1,4,5)P₃. Comparing the rates of Ins(1,4,5)P₃ metabolism, it appears that cells permeabilised with the detergent, saponin are able to metabolise Ins(1,4,5)P₃ more rapidly than cells permeabilised electrically. That ⁴⁵Ca²⁺ reuptake is dependent on Ins(1,4,5)P₃ metabolism is confirmed by the observation that ⁴⁵Ca²⁺ reuptake in saponin-permeabilised cells is also more rapid. Further confirmation is obtained by the observations that the dose-response relationships for Ins(1,4,5)P₃ (an analogue resistant to metabolism) and, to a lesser extent, InsP₃-5S (an analogue resistant to dephosphorylation), were affected less by a prolonged incubation in the presence of the cells. This suggests that under these conditions the Ins(1,4,5)P₃ receptor does not undergo rapid functional desensitisation as has been observed for cell-surface receptors (see Oldershaw et al., 1992; Chapter 5).

The introduction of phosphorothioate groups into the inositol nucleus decreased their ability to mobilise Ca²⁺. As phosphorothioate groups are more hydrophobic than phosphate groups, this suggests that the Ins(1,4,5)P₃ binding site is in a relatively hydrophilic environment of the Ins(1,4,5)P₃ receptor. This idea complements data obtained with NBD InsP₃-1S, which does not appear to fluoresce when bound to the Ins(1,4,5)P₃ receptor (S.M. Smith, J. Strupish, unpublished observations.). Alternatively, it may fluoresce, but the NBD chromophore may be too weak to detect. NBD is not naturally fluorescent, becoming so only in a lipophilic environment. Studies using this analogue showed no increase in fluorescence when it bound to the Ins(1,4,5)P₃ receptor from rat cerebellar preparations, despite displacing [³H]Ins(1,4,5)P₃ from these sites (S.M. Smith, J. Strupish, unpublished observations.). This would suggest that Ins(1,4,5)P₃ is not held proximal to a lipophilic environment.
when bound to a receptor. These data complement those by Mignery et al. (1990), who produced a hydrophobicity plot of the Ins(1,4,5)P₃ receptor, and have shown that the Ins(1,4,5)P₃ binding region is hydrophilic. Judging by the increased affinity of Ins(1,4,5)P₃ 5-phosphatase for phosphorothioate-containing analogues of Ins(1,4,5)P₃, this appears not to be the case with Ins(1,4,5)P₃ 5-phosphatase, and this increase in affinity may reflect enhanced hydrophobic interactions between Ins(1,4,5)P₃ 5-phosphatase and the phosphorothioate groups.

The 2-position possesses the only axial grouping of myo-inositol. It, like the 1-position, can tolerate the introduction of large groups with only a slight attenuation of potency in Ins(1,4,5)P₃ analogues. Hirata et al. (1989) discussed the profiles of a number of analogues containing aliphatic or aromatic groups attached to the 2-position by an ester bond, as preliminary work in order to produce an affinity column for solubilised Ins(1,4,5)P₃ receptors, and compared their profiles to 2-deoxy InsP₃, Ins(1,2,4,5)P₃, Ins(2,4,5)P₃ and Ins(1,4,5)P₃ itself. From these data, it is believed that the 2-hydroxyl group is relatively unimportant with respect to recognition by the Ca²⁺ mobilising receptor. Work by Denis and Ballou (1991) has shown that D-chiro inositol 1,4,3-trisphosphate is also a potent Ca²⁺ mobilising agent, with EC₅₀ equal to that of Ins(2,4,5)P₃ in the same study. It bears a close resemblance to Ins(2,4,5)P₃, except that, if drawn like Ins(2,4,5)P₃, it possesses an axial 1-hydroxyl group. The L-enantiomers of Ins(2,4,5)P₃ and ch Ins(1,3,4)P₃ are only 25-30 fold weaker than their D-enantiomers, far more active than any L-Ins(1,4,5)P₃ analogue. The most significant difference between these compounds is that all of the phosphate groups in L-Ins(1,4,5)P₃ are equatorial, while one phosphate group in both Ins(2,4,5)P₃ and ch Ins(1,3,4)P₃ is axial. Likewise, removal of the hydroxyl group (Hirata et al., 1989) or replacement with a fluorine (myo 2F InsP₃ and 2,2F₂ InsP₃) also only causes a slight loss of potency at the Ca²⁺-mobilising receptor. Introduction of an equatorial fluorine into the 2-position of sc Ins(1,2,4)P₃ caused a marginally greater loss of potency (EC₅₀ DL-sc 2F InsP₃=0.70 μM), suggesting that, unlike sc 2F InsP₃, the axial fluorine of myo 2F InsP₃ and 2,2F₂ InsP₃ can mimic the 2-OH group of Ins(1,4,5)P₃, allowing H-bonds to be formed. The introduction of aromatic and aliphatic groups into the 2-position of the myo-inositol ring of Ins(1,4,5)P₃ decreased the rate of hydrolysis by both particulate (HEG) and soluble (rat brain cytosol) Ins(1,4,5)P₃ 5-phosphatase, however, affinity for the analogues was increased (Hirata et al., 1989). Fluoro substitution at this 2-position identified L-enantiomers of Ins(1,4,5)P₃ analogues (L-2,2F₂ InsP₃) having higher affinities for Ins(1,4,5)P₃ 5-phosphatase than the corresponding D-enantiomer, inhibiting dephosphorylation in a racemic mixture. Indeed, similar results had been found earlier by Hirata et al. (1990), with L-209 (L-2(4-aminobenzoyl) InsP₃) partially inhibiting the hydrolysis of D-209 in a
racemic mixture. However, D- and L-209 were bound with similar affinities (K$_{i}$(DL-209)=3µM, L-209=3.8µM, therefore D-209~2.5µM). D-209 was hydrolysed at a similar rate to Ins(1,4,5)P$_3$. This is clearly not the case with all L-analogues of Ins(1,4,5)P$_3$, as L-2-deoxy InsP$_3$ does not inhibit dephosphorylation of D-2-deoxy InsP$_3$ in a racemic mixture (Hirata et al., 1989). L-2(4-aminocyclohexanecarbonyl) InsP$_3$ (L-206) is also a weak inhibitor of Ins(1,4,5)P$_3$ 5-phosphatase (K$_{i}$=102µM), which only partially inhibits the hydrolysis of D-206 in a racemic mixture. DL-206 inhibited Ins(1,4,5)P$_3$ dephosphorylation with K$_{i}$=16µM, from this one can assume D-206 to have a K$_{m}$=8.3µM.

The phosphatase-resistant properties of Ins(1,2,4,5)P$_4$ and Ins(2,4,5)P$_3$ suggest that the resistant properties of Ins(2,4,5)P$_3$ are due to the presence of a 2-phosphate group, and not the absence of the 1-phosphate group. The introduction of this phosphate group, however, dramatically increased the affinity for Ins(1,4,5)P$_3$ 5-phosphatase (K$_{i}$= 2.9µM), unlike Ins(2,4,5)P$_3$ which bound Ins(1,4,5)P$_3$ 5-phosphatase with lower affinity than Ins(1,4,5)P$_3$ (Polokoff et al., 1988; Hirata et al., 1989). This, in concert with the observation that sc Ins(1,2,4)P$_3$, sc 2F InsP$_3$, and myo 2F InsP$_3$ are also resistant to Ins(1,4,5)P$_3$ 5-phosphatase identify the 2-position to be highly important with respect to Ins(1,4,5)P$_3$ 5-phosphatase substrate properties. However, larger substitutions at this axial 2-position by Hirata et al. (1989), while decreasing the rate of hydrolysis identified a large number of such analogues which remained substrates for Ins(1,4,5)P$_3$ 5-phosphatase. The identification in this and previous studies (Hirata et al., 1990) of L-enantiomers of Ins(1,4,5)P$_3$ analogues being potent inhibitors of Ins(1,4,5)P$_3$ 5-phosphatase may explain why so many Ins(1,4,5)P$_3$ analogues appear to be resistant. DL-2,2F$_2$ InsP$_3$, which appeared to be resistant to Ins(1,4,5)P$_3$ 5-phosphatase, identified a relatively good substrate, D-2,2F$_2$ InsP$_3$, and a potent inhibitor, L-2,2F$_2$ InsP$_3$.

Thus, it is possible that while D-enantiomers may be substrates for Ins(1,4,5)P$_3$ 5-phosphatase, their dephosphorylation is inhibited by the presence of an equal amount of the L-enantiomer. Indeed, comparing structures of the L-enantiomers of such analogues, it is possible to suggest that they bind in an orientation whereby the molecule approaches the enzyme, offering this 2-position towards the active site (Fig. 3.17). Equally, L-Ins(1,4,5)P$_3$ was bound by Ins(1,4,5)P$_3$ 5-phosphatase with relatively high affinity (K$_{i}$=15µM, this study), and was resistant to dephosphorylation. In such an orientation many features would be maintained, these include:- 1. The ring pucker would be correct, 2. The 1- and 4-phosphate groups would mimic the 4- and 1-phosphate groups, 3. The 6- and 3-hydroxyl groups would mimic the 3- and 6-hydroxyl groups, 4. The 5-equatorial phosphate group could, to
Figure 3.17 Possible inhibition of Ins(1,4,5)P₃ 5-phosphatase by analogues of L-Ins(1,4,5)P₃. The structure of Ins(1,4,5)P₃ is given in the upper panel, with the conformation of analogues of L-Ins(1,4,5)P₃ which may inhibit Ins(1,4,5)P₃ 5-phosphatase given below.
Analogue

L-Ins(1,4,5)P₃
L-myo 2F InsP₃
L-2,2F₂ InsP₃
L-sc 2F InsP₃
L-sc Ins(1,2,4)P₃
L-Ins(1,2,4,5)P₄

X  Y
H   OH
H   F
F   F
F   H
OH  H
H   PO₄
some extent, mimic an axial 2-phosphate group and 5. The 2-substituted electronegative group could mimic a 5-phosphate group and inhibit dephosphorylation. Indeed, previous reports (see Shears, 1989) have shown Ins(1,4)P₂ to be an inhibitor of Ins(1,4,5)P₃ 5-phosphatase, with only a marginally lower affinity than Ins(1,4,5)P₃ itself. The analogues described by Hirata et al. (1989) contain far larger groups in the 2-position which may be too big to fit the active site, and, therefore, would not be expected to inhibit dephosphorylation of the D-enantiomer in a similar manner.

The axial 2-position of Ins(1,4,5)P₃ appears to be important with respect to Ins(1,4,5)P₃ 3-ldnase recognition. While Hirata et al. (1989) showed that DL-2-deoxy InsP₃ was recognised with much the same affinity by Ins(1,4,5)P₃ 3-kinase, no data showing it to be a substrate or inhibitor were given. Ins(2,4,5)P₃ is, however, poorly recognised (Polokoff et al., 1988; Hirata et al., 1989) and has been shown to be a relatively weak substrate for Ins(1,4,5)P₃ 3-kinase (Ryu et al., 1987b). Likewise, Ins(1,2,4,5)P₄ is poorly recognised by Ins(1,4,5)P₃ 3-kinase, indicating steric interference by the phosphate group in the 2-position. Hirata et al. (1989), have shown differing effects of substituting aliphatic and aromatic groups at the 2-position. Reducing free Ca²⁺ levels in the assay buffer from 17 to <0.01μM appeared to enhance inhibitory effects of these 2-position modified analogues, without affecting the affinity of Ins(1,4,5)P₃ 3-kinase for Ins(1,4,5)P₃ or 2-deoxy InsP₃. Two closely related analogues, D-206 (D-2(4-aminocyclohexanecarbonyl) InsP₃) and D-209 (D-2(4-aminobenzoyl) InsP₃) were found to typify the stringent substrate specificity of Ins(1,4,5)P₃ 3-kinase. The cyclohexane derivative (D-206) was a substrate for Ins(1,4,5)P₃ 3-kinase, while the benzoyl derivative (D-209) was a potent inhibitor (Hirata et al., 1990).

That L-ch Ins(2,3,5)P₃ is resistant to Ins(1,4,5)P₃ 5-phosphatase is surprising, since the change in orientation of the 3-hydroxyl group is remote from the site of attack by Ins(1,4,5)P₃ 5-phosphatase. Two possibilities have been suggested by Potter (Safrany et al., 1992) to explain this phenomenon: The configuration of L-ch Ins(2,3,5)P₃ in solution and/or bound to Ins(1,4,5)P₃ 5-phosphatase is sufficiently different from Ins(1,4,5)P₃ to interfere with the catalytic mechanism of the enzyme, but nevertheless, the analogue binds to the enzyme in a similar mode to Ins(1,4,5)P₃ (Fig. 3.18a,b). Alternatively, the inhibition may be the result of ‘non-productive’ binding of L-ch Ins(2,3,5)P₃ in an inverted, rotated mode (Fig. 3.18c). This arrangement of L-ch Ins(2,3,5)P₃ would mimic four elements of Ins(1,4,5)P₃ correctly, namely: 1 the ring pucker, 2 the vicinal 4,5 bisphosphate pairing (as the 2,3 bisphosphate pair) and 3 the 3-hydroxyl group (as the 4-hydroxyl group). The 5-phosphate group of L-ch
Figure 3.18 Possible binding of L-ch Ins(2,3,5)P₃ to Ins(1,4,5)P₃ 5-phosphatase. Schematic illustration of the binding of (i) Ins(1,4,5)P₃ to Ins(1,4,5)P₃ 5-phosphatase, (ii) L-ch Ins(2,3,5)P₃ in a similar mode and (iii) L-ch Ins(2,3,5)P₃ in an inverted and rotated 'non-productive' mode. Figure courtesy of Professor B.V.L Potter.
Ins(2,3,5)P₃ mimics an equatorial 2-phosphate group of an Ins(1,4,5)P₃ analogue. Such a phosphate could, presumably, still make interactions with an acceptor group normally disposed to binding the equatorial 1-phosphate of Ins(1,4,5)P₃. As discussed above, Ins(2,4,5)P₃, whilst binding with similar affinity to Ins(1,4,5)P₃ itself, is only slowly hydrolysed by Ins(1,4,5)P₃ 5-phosphatase. In this inverted binding mode, the axial 1-hydroxyl group of L-ch Ins(2,3,5)P₃ now mimics an axial 6-hydroxyl group of an Ins(1,4,5)P₃ analogue, which, again would be expected to imbue inhibitory properties towards Ins(1,4,5)P₃ 5-phosphatase, as described for 6-deoxy InsP₃ below.

The 6-hydroxyl group present within Ins(1,4,5)P₃ appears to play a crucial role in Ins(1,4,5)P₃ receptor recognition. Removal of this group (6-deoxy InsP₃) produces a weak full agonist, some 70-fold less potent than Ins(1,4,5)P₃. DL-6-methoxy InsP₃ is even less active, some 215-fold weaker than Ins(1,4,5)P₃ (Polokoff et al., 1988). This contrasts markedly with data obtained with 2- (Hirata et al., 1989) and 3-deoxy InsP₃ (Seewald et al., 1990). Indeed, multiple deletion of hydroxyl groups was shown to produce an analogue with similar loss of activity to removal of the 6 hydroxyl alone, CH(1,2,4)P₃ being some 130-fold weaker than Ins(1,4,5)P₃ (Polokoff et al., 1988). D-6-deoxy InsP₃ and DL-6-methoxy InsP₃, being of some 2- and 4-fold lower affinity for Ins(1,4,5)P₃ 5-phosphatase underline the relative non-selectivity of this enzyme. Both are, however, resistant to Ins(1,4,5)P₃ 5-phosphatase, suggesting that a free equatorial 6-hydroxyl group of Ins(1,4,5)P₃ plays an important role in the catalytic mechanism of hydrolysis, and that a change in configuration at this centre would be expected to imbue the resulting analogue with inhibitory properties.

Like the 2 and 3 hydroxyl groups, the 6 hydroxyl group can potentially act as a hydrogen bond donor or acceptor. In addition, these hydroxyl groups may be involved in fixing the solution conformation of Ins(1,4,5)P₃ by intramolecular hydrogen bonding to neighbouring phosphate groups. Hydroxyl groups at positions 2 and 3 appear to play minor roles in Ins(1,4,5)P₃ receptor recognition, as their removal only marginally decreased Ca²⁺-mobilising potency at the Ins(1,4,5)P₃ receptor (Hirata et al., 1989; Seewald et al., 1990). However, removal of the 6 hydroxyl group, neighbouring the highly crucial 5 phosphate and the important 1 phosphate, may affect conformation of these phosphates and population of appropriate conformer for receptor binding. No data are, however, yet available concerning the conformation of Ins(1,4,5)P₃ bound to its receptor. 6-deoxy InsP₃ was found to inhibit phosphorylation of Ins(1,4,5)P₃ by Ins(1,4,5)P₃ 3-kinase potently, with apparent Kᵢ=5.7μM. Thus, as seen with CH(1,2,4)P₃, removal of the 6-hydroxyl group causes only slight attenuation of Ins(1,4,5)P₃ 3-kinase affinity. It was, however, not possible to determine whether it was a cosubstrate or an inhibitor. In contrast, DL-6-methoxy
InsP₃ inhibited phosphorylation of Ins(1,4,5)P₃ only weakly (Polokoff et al., 1988). Since hydrogen bonding potential is removed by hydroxyl deletion or methylation, it seems likely that the reason for the low Ins(1,4,5)P₃ 3-kinase affinity for 6-methoxy InsP₃ lies with a low tolerance of the enzyme for increased steric bulk at this position.

In contrast to the marked loss of affinity of Ins(1,4,5)P₃ 3-kinase for InsPS₃ compared to Ins(1,4,5)P₃, 6-deoxy InsPS₃ was recognised with only marginally lower affinity than 6-deoxy InsP₃.

Following the bridging of the 4- and 5-phosphate groups, producing InsP₃(PP), it is noteworthy that the affinity of all three recognition sites was markedly decreased. On electrostatic grounds, the 4- and 5-phosphate groups of Ins(1,4,5)P₃ are expected to be diametrically opposed (see Nahorski and Potter, 1989). Further evidence that this is so comes from the ability of decavanadate to act as a potent antagonist at the Ins(1,4,5)P₃ receptor. As discussed by Strupish et al. (1991), the decavanadate molecule provides a cage of negatively charged oxygen atoms, and the relative separation of these charges can correspond approximately to this presumed separation in conformational space between the three phosphate groups of Ins(1,4,5)P₃.

Likewise, it can be anticipated that the 5-phosphate group is required to be free for Ins(1,4,5)P₃ 5-phosphatase activity. Ins(1,4,5)P₃ 5-phosphatase recognition may also require diametrically opposed 4- and 5-phosphate groups. Similarly, that Ins(1,4,5)P₃ 3-kinase no longer recognises InsP₃(PP) suggests that the 4-phosphate group makes a hydrophilic, ionic interaction with the enzyme. Limiting the rotational freedom of this 4-phosphate, holding it close to the 5-phosphate group, prevents this likely interaction. Likewise, the introduction of a phosphorothioate group into this 4-position also decreases Ins(1,4,5)P₃ 3-kinase recognition. As described above, phosphorothioate groups are less hydrophilic than their corresponding phosphates, and so a decrease in hydrophilic interactions may also explain the decrease in affinity for Ins(1,4,5)P₃ 3-kinase. Alternatively, the conformation in solution and bound to the three recognition proteins are unknown, it is possible that conformational changes may be required for receptor and enzyme binding. As InsP₃(PP) contains one 6-membered ring and one 7-membered ring, producing a more rigid structure, it may be unlikely that the conformation required for binding can be induced.

The broad specificity of Ins(1,4,5)P₃ 5-phosphatase is further recognised by its relatively high affinity for cyclohexane-based molecules, which are virtually devoid of affinity for the Ins(1,4,5)P₃ receptor and Ins(1,4,5)P₃ 3-kinase. Of greatest interest is the observation that CH(1,2,4){CH₂SO₃}₃ is recognised with 20-fold higher affinity than its corresponding phosphorus-containing analogue, CH(1,2,4){CH₂PO₃}₃. If all sulphonate analogues of inositol phosphates are
recognised with some 20-fold higher affinity than the original phosphates, highly potent and selective inhibitors can now be synthesised. Ins(1,3,4,5)P₄ is currently the inositol phosphate which has the highest known affinity for Ins(1,4,5)P₃ 5-phosphatase. Initial studies should be involved with synthesising inositol 1,3,4,5-tetralds methylene sulphonate and sulphate, the former may be expected to inhibit Ins(1,4,5)P₃ 5-phosphatase with Kᵢ~100nM.

3.5.1 Partial Agonists at the Ins(1,4,5)P₃ Receptor.

The existence of partial agonists at ligand-gated channels is extremely rare. Ins(1,3,4,6)P₄ has been previously reported to be some 80% as efficacious as Ins(1,4,5)P₃ at the Ins(1,4,5)P₃ receptor (Gawler et al., 1991), and a number of partial agonists at the nicotinic acetylcholine receptor, including suxamethonium and decamethonium (Marshall et al., 1991) have been reported. Indeed, a number of similarities between the Ins(1,4,5)P₃ and nicotinic receptors are observed. Firstly, the Hill slope for binding of acetylcholine to the nicotinic receptor is unity, despite the belief that the nicotinic receptor has two binding sites for acetylcholine in its pentameric structure. Equally, the binding of Ins(1,4,5)P₃ to its tetrameric receptor, which is believed to have four binding sites also shows a Hill slope of one. Secondly, the binding of agonists to the nicotinic receptor opens an integral ion channel, allowing the flux of Na⁺. Experiments concerning the dose relationship of a number of agonists consistently find a Hill coefficient of 2 (Neubig and Cohen, 1980). This would suggest that two molecules of agonist are required to bind a single receptor to enhance the probability of the channel to open. In the case of the Ins(1,4,5)P₃ receptor, which, when bound to Ins(1,4,5)P₃, increases the probability of the opening of an integral ion channel permeable to Ca²⁺, Hill coefficients of 1.5-2.0 have been observed throughout this study. Thus, like the nicotinic receptor, on average, 2 molecules need to bind a single receptor to open the channel. To explain the above observations for the nicotinic receptor, Neubig et al. (1982) suggested that the receptor exists in two different, interconvertible conformations. Rₑ, a state which is functional in opening the Na⁺ channel but had low affinity for the agonist, and Rᵈ, a desensitised state with higher affinity for the agonist. Thus, equilibrium binding studies only display the high affinity, functionally desensitised state. When, however, kinetics of binding are studied, three distinct rates are observed. A rapid rate, presumably to receptors pre-existing in Rᵈ, followed by binding to receptors in Rₑ, which are then desensitised.
Similarly, the K\text{D} observed for binding of Ins(1,4,5)P\text{3} to its receptor is routinely substantially lower than the EC\text{50} forIns(1,4,5)P\text{3}-induced Ca\text{2+} mobilisation (see Chapter 1). Indeed, Mauger and co-workers (Mauger et al., 1989; Pietri et al., 1990; Rouxel et al., 1992) have identified two states of the Ins(1,4,5)P\text{3} receptor prepared from rat hepatocytes. These two states were interconvertible, this interconversion being mediated by temperature and Ca\text{2+} levels. The high affinity state (K\text{D}=3\text{nM}) was stabilised in conditions of raised Ca\text{2+} (0.7\text{μM}). This high affinity state was associated with a desensitised form of the receptor. The low affinity state of the receptor returned on incubation at 37°C. This low affinity state appears to be responsible for Ca\text{2+} mobilisation, and, with K\text{D}=490\text{nM} (Rouxel et al., 1992), appears to have little receptor reserve. Thus, it appears that, like the nicotinic receptor, the Ins(1,4,5)P\text{3} receptor is capable of undergoing conformational change leading to the formation of a high affinity, desensitised state.

L-ch Ins(2,3,5)PS\text{3} and 6-deoxy InsPS\text{3} have been identified as partial agonists at the Ins(1,4,5)P\text{3} receptor. The rate of 45Ca\text{2+} release appeared similar to that of Ins(1,4,5)P\text{3}, and dose-response relationships were compared at 2 minutes, at which time point all responses were maximal (data not shown). L-ch Ins(2,3,5)PS\text{3} and 6-deoxy InsPS\text{3} mobilised only 34 and 42% respectively of that maximally mobilised by Ins(1,4,5)P\text{3} (Figs. 3.13, 3.14). The ability of these agents to inhibit 45Ca\text{2+} mobilising properties of Ins(1,4,5)P\text{3} was also investigated. L-ch Ins(2,3,5)PS\text{3} and 6-deoxy InsPS\text{3} were found to inhibit Ca\text{2+} mobilisation induced by Ins(1,4,5)P\text{3} (0.7μM) in a dose-dependent manner, with IC\text{50} values of 26±6 and 171±25μM (n=3) (Figs. 3.13, 14). Assuming this to be due to competition between Ins(1,4,5)P\text{3} and L-ch Ins(2,3,5)PS\text{3}/6-deoxy InsPS\text{3} at the same site, an estimate of the K\text{D} for L-ch Ins(2,3,5)PS\text{3} and 6-deoxy InsPS\text{3} of 5.7 (1.7-18.4) μM and 32.6 (17.5-60.6) μM can be obtained from the equation derived by Cheng and Prusoff (1973), in relatively close agreement with the EC\text{50} values obtained for 45Ca\text{2+} release (4.3 and 16 μM respectively). L-ch Ins(2,3,5)PS\text{3} and 6-deoxy InsPS\text{3} were also found to move the Ins(1,4,5)P\text{3} dose response curve to the right (data not shown).

Watras et al. (1991) have shown there to be four conductance states of the Ins(1,4,5)P\text{3} receptor. These four states may reflect the interactions among the four receptors thought to comprise the integral ion channel, and not merely due to independent channel opening events. That L-ch Ins(2,3,5)PS\text{3} and 6-deoxy-Ins(1,4,5)PS\text{3} show Hill coefficients equal to Ins(1,4,5)P\text{3} suggests that, as partial agonists, they do not alter the relative proportions of the four open states. Although the importance of both intraluminal and extraluminal Ca\text{2+} levels cannot be discounted for the high Hill coefficient observed (see Introduction, Chapter 1), a role
for Ca^{2+} is difficult to envisage in light of the above data. While less Ca^{2+} is mobilised by the partial agonists, the relative profiles of Ca^{2+} mobilisation appear identical to Ins(1,4,5)P₃, with no difference in Hill coefficients observed. Thus, less Ca^{2+} would be required to increase release to the same extent in the case of the partial agonists as that observed in the case of a full agonist. Equally, the all-or-nothing release theory described by Parker and Ivorra (1990), by which the binding of Ins(1,4,5)P₃ to its receptor mobilises fully that Ca^{2+} which is associated with it, cannot account for the existence of partial agonists. L-ch Ins(2,3,5)PS₃ and 6-deoxy InsPS₃ fully displace [³H] Ins(1,4,5)P₃ (Kᵢ=6.5nM) from bovine adrenal cortical membranes with Kᵢ=0.5nM and 5.3μM (Safrany et al., 1993), and thus, would be expected to displace Ins(1,4,5)P₃ fully from the Ins(1,4,5)P₃ receptor in SH-SY5Y cells. This is confirmed by the ability of both agents to fully inhibit the Ca^{2+} mobilisation elicited by Ins(1,4,5)P₃, decreasing maximal release to that observed solely in the presence of the partial agonist. That [³H] Ins(1,4,5)P₃ binds the receptor with some 27-fold higher affinity than its EC₅₀ for Ca^{2+} mobilisation described in parallel studies, whereas L-ch Ins(2,3,5)PS₃ and 6-deoxy Ins(1,4,5)PS₃ displace [³H] Ins(1,4,5)P₃ with only 9.6 and 2-fold higher affinity than their EC₅₀ values for Ca^{2+} release, is indicative of these partial agonists acting in a similar way to partial agonists acting at cell-surface receptors (see Chapter 4). Partial agonists are less able to induce a conformational change of the target receptor than corresponding full agonists, and this may explain why the affinity of these partial agonists for the receptor is only slightly lower than the EC₅₀ for Ca^{2+} release. Indeed, these Kᵢ values may be the first true affinity values of inositol phosphates for the active form of the Ins(1,4,5)P₃ receptor.

It is also difficult to explain the effects of caffeine using the quantal release theory. While Parker and Ivorra (1991) identified caffeine as inhibiting Ins(1,4,5)P₃-induced Ca^{2+} fluxes in Xenopus oocytes, and identified that in the presence of caffeine the dose-response relationship for Ins(1,4,5)P₃ was more shallow, they did not find a suitable explanation for these observations. Likewise, Brown et al. (1992) found that caffeine increased the EC₅₀ and decreased the Hill coefficient for Ins(1,4,5)P₃-induced Ca^{2+} mobilisation, without affecting Ins(1,4,5)P₃ binding characteristics. However, this may merely suggest the presence of an allosteric binding site for caffeine, associated with the Ca^{2+} channel, which regulates the sensitivity of the receptor to Ca^{2+}.

No inositol phosphate analogue yet described acts as an Ins(1,4,5)P₃ receptor antagonist. A number of other agents (ATP, 2,3 BPG, heparin and decavanadate) have been identified as receptor antagonists, however, none of the above agents are in
any way selective, inhibiting the binding of Ins(1,3,4,5)P₄, metabolism of
Ins(1,4,5)P₃ by 3-kinase or 5-phosphatase, indeed decavanadate inhibits Ins(1,4,5)P₃
and Ins(1,3,4,5)P₄ binding, Ins(1,4,5)P₃ 3-kinase and Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄
5-phosphatase with Ki between 0.6 and 5μM.

Ins(1,4,5)P₃ binding inhibition curves obtained with decavanadate and heparin
showed high Hill slopes (1.35-1.40 and 1.26 respectively, Strupish et al., 1991; but
see Oldershaw et al., 1992). This may reflect that individual molecules of
decavanadate and heparin interact with more than one Ins(1,4,5)P₃ binding site, as
described by Strupish et al. (1991). This may equally explain why orthovanadate,
oligovanadate and smaller heparin fragments do not inhibit Ins(1,4,5)P₃ receptor
binding (Fohr et al., 1989; Challiss et al., 1991; Strupish et al., 1991), as they are
unable to bridge Ins(1,4,5)P₃ binding sites. From work performed by Pettersson et al.
(1983), it is expected that decavanadate will exist as VIO₂⁺ at pH 7. As heparin is
a polysulphated polysaccharide, it is tempting to suppose its effects result from
binding of its sulphate groups, which are less well charged than phosphate groups, at
sites on the Ins(1,4,5)P₃ receptor which normally bind the phosphates of Ins(1,4,5)P₃
Indeed, removal of these sulphate groups (Challiss et al., 1991) destroys Ins(1,4,5)P₃
receptor antagonist properties. The structure of VIO₂⁺ would provide a cage of
negatively charged oxygen atoms, and the relative separation of these charges can
correspond approximately to the presumed separation in conformational space
between the three phosphate groups of Ins(1,4,5)P₃.
3.6 Summary of Results.

3.6.1 Ins(1,4,5)P3 Receptor Recognition Requirements.

From the above data, it is clear that the Ca$^{2+}$ mobilising Ins(1,4,5)P3 receptor shows a high degree of stereospecificity. D-Ins(1,4,5)P3 analogues, of which a summary of recognition requirements are given in Fig 3.19, mobilise Ca$^{2+}$ potently, L-Ins(1,4,5)P3 analogues being poor agonists.

The vicinal, 4,5-bisphosphate pairing is essential for activity, although this may be mimicked by the 6- and 1- (or 1- and 6-) phosphate groups in Ins(1,3,4,6)P4. The 2- and 3-hydroxy groups are of little importance, as their removal only slightly attenuates Ca$^{2+}$ mobilising properties, while the 6-hydroxy group contributes greatly to Ca$^{2+}$ mobilising properties. However, work performed with 2-position modified Ins(1,4,5)P3 analogues suggests that the 2-hydroxyl group acts as a hydrogen bond acceptor from the Ins(1,4,5)P3 receptor, as sc Ins(1,2,4)P3, 2,2F2 InsP3, Ins(1,2,4,5)P4 and sc 2F InsP3, which are unable to act as hydrogen bond donors, are potent Ca$^{2+}$ mobilising agents.

The 4- and 5-phosphate groups may be diametrically opposed on binding, as InsP3(PP), in which the two phosphate groups are linked and held close to one another, is totally inactive.

The studies described herein identify two novel partial agonists at the ion-gated Ins(1,4,5)P3 receptor, L-ch Ins(2,3,5)P3 and 6-deoxy InsP3, designed after phosphorothioate introduction was seen to decrease intrinsic efficacy (decrease the ability of agonists to cause an effect without as great an effect on binding) of Ins(1,4,5)P3 analogues.

Further studies are required to identify a small molecule antagonist at the Ins(1,4,5)P3 receptor, and this must be considered as a major objective.
Ins(1,4,5)P₃ Receptor Recognition Requirements

Equatorial 4-phosphate
Little information available.
Required to be present, free and equatorial.

Equatorial 3-hydroxyl
Relatively unimportant.
Cannot tolerate replacement with large groups.

Axial 2-hydroxyl
Relatively unimportant.
Removal or replacement with PO₄ or large uncharged groups changes affinity marginally.

Equatorial 5-phosphate
Most important.
Removal or bridging with R₄ removes all activity.
Replacement with PO₃S tolerated, but attenuates activity.

Equatorial 6-hydroxyl
Important.
Removal or replacement with OMe severely attenuates activity.

Equatorial 1-phosphate
Removal dramatically reduces affinity.
Replacement with:-
PO₄ in R₂, GlnP or PO₃S returns activity.

Figure 3.19 Summary of Ins(1,4,5)P₃ receptor recognition requirements.
3.6.2 Ins(1,4,5)P3 5-Phosphatase Studies.

From the above data, knowledge of the structural requirements for Ins(1,4,5)P3 5-phosphatase recognition and substrate properties are emerging (Fig 3.20).

3.6.2(i) Recognition Requirements.

All the inositol phosphates and analogues yet described recognised by Ins(1,4,5)P3 5-phosphatase appear to be substrates or competitive inhibitors of the enzyme. Some recognition requirements are now becoming apparent.

The role of the three hydroxyl groups seem relatively unimportant, as their removal (2-deoxy InsP3, 3-deoxy InsP3, 6-deoxy InsP3, CH(1,2,4)P3) does not markedly affect Ins(1,4,5)P3 5-phosphatase affinity. Stereospecificity of Ins(1,4,5)P3 5-phosphatase is relatively poor, as L-Ins(1,4,5)P3 binds with much the same affinity as does Ins(1,4,5)P3, indeed, this study has identified an L-enantiomer with higher affinity for Ins(1,4,5)P3 5-phosphatase than its D-enantiomer (L- and D- 2,2F2 InsP3).

The 1-phosphate group is highly important, but not essential, as removal or replacement in the 2-position, attenuates binding affinity.

The 3-hydroxyl group is of interest, inverting the stereochemistry at this point increases Ins(1,4,5)P3 5-phosphatase affinity, as does replacement of the hydroxyl group with a fluorine or hydrogen.

The introduction of phosphorothioate groups markedly enhanced Ins(1,4,5)P3 5-phosphatase recognition, suggesting hydrophobic interactions between Ins(1,4,5)P3 5-phosphatase and its target are important.

Finally, while the mechanism of L-ch Ins(1,4,6)P3 and L-ch Ins(1,4,6)PS3 inhibition of Ins(1,4,5)P3 5-phosphatase remains without explanation, L-ch Ins(1,4,6)PS3 is of great interest, in that it is the first totally selective inhibitor of Ins(1,4,5)P3 5-phosphatase, with an increase in affinity some 3000-fold over the currently used Ins(1,4,5)P3 5-phosphatase inhibitor, 2,3 BPG. It also has the advantage over 2,3 BPG, in that 2,3 BPG is also an antagonist at the Ins(1,4,5)P3 receptor.
3.6.2(ii) Substrate Requirements.

Few analogues of Ins(1,4,5)P$_3$ appear to be substrates for Ins(1,4,5)P$_3$ 5-phosphatase. Removal of the 1 phosphate, or replacement in the 2-position produces poor substrates for Ins(1,4,5)P$_3$ 5-phosphatase. However, the presence of phosphates in the 1- and 2-positions (Ins(1,2,4,5)P$_4$) produces a resistant analogue (similar to Ins(2,4,5)P$_3$). Equally, amending the stereochemistry at the 2-position (sc Ins(1,2,4)P$_3$) produces an Ins(1,4,5)P$_3$ 5-phosphatase-resistant analogue. However, such observations may suggest the identification of L-enantiomers as potent inhibitors of Ins(1,4,5)P$_3$ 5-phosphatase. The 3-hydroxyl group is of interest, as inverting the stereochemistry here produces a potent inhibitor. Possible reasons for this are discussed above.

Removal of the 6-hydroxyl group again confers resistance, suggesting intramolecular bonding with this group is important in holding the molecule in the correct orientation for it to be a substrate.

Ins(1,4,5)P$_3$ 5-phosphatase has become an enzyme of great interest in recent years. In 1990, Nye and Pinching identified that HIV infection caused a reduction in the number and function of CD4+ lymphocytes and functional abnormalities in other cells. Chronic infection led to decreases in InsP and increases in InsP$_2$, InsP$_3$ and, most substantially, InsP$_4$. These data suggested abnormalities in InsP$_4$ metabolism. Indeed, incubation of $[^{3}H]$ Ins(1,3,4,5)P$_4$ with cell lysates of HIV infected cells showed an inability of these cells to cause dephosphorylation by Ins(1,4,5)P$_3$ 5-phosphatase, although some 3-phosphatase activity was observed. Treatment of HIV infected patients with zidovudine (AZT) was seen to progressively increase both 3- and 5-phosphatase activity (Nye et al., 1991), with a time course which paralleled 'clinical benefit' and changes in lymphocyte function. Further work in identifying the mechanisms of this dysfunction are progressing (Nye et al., 1992).

Lowes oculocerebrorenal syndrome, a human X-linked developmental disorder affecting the lens, brain and kidney, has also been identified as a condition in which inositol phosphate metabolism may be affected. A novel protein produced from the region proximal to X-q25-q26, the OCRL locus, is highly homologous to a 75 kDa
Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase, and is thought to correspond to a 100 kDa 5-phosphatase found in brain (Attree et al., 1992).
Ins(1,4,5)P$_3$ 5-Phosphatase Recognition Requirements

- **Equatorial 4-phosphate**: Little information available. Bridging with $R_3$ removes all affinity. Diagonal 4,5-like bisphosphate pair produces analogue with high affinity.

- **Equatorial 3-hydroxyl**: Replacement with PO$_4$ or axial -OH greatly increases affinity, but reduces rate of hydrolysis.

- **Axial 2-hydroxyl**: Removal or replacement with equatorial -OH of little effect. Replacement with PO$_4$ or axial-F greatly enhances recognition.

- **Equatorial 5-phosphate**: Removal slightly attenuates affinity. Introduction of PO$_3$S increases affinity, but renders analogue resistant.

- **Equatorial 6-hydroxyl**: Removal or replacement with OMe attenuates affinity, renders analogue resistant.

- **Equatorial 1-phosphate**: Important for recognition, but not for substrate properties.

Figure 3.20 Summary of Ins(1,4,5)P$_3$ 5-phosphatase recognition requirements.
3.6.3 Ins(1,4,5)P₃ 3-Kinase Studies.

Ins(1,4,5)P₃ 3-kinase is, at present, the least well understood Ins(1,4,5)P₃ recognition protein, requirements for binding and substrate properties are summarised in Fig 3.21.

3.6.3(i) Recognition Requirements.

All the inositol phosphates and analogues yet described recognised by Ins(1,4,5)P₃ 3-kinase appear to be substrates or competitive inhibitors of the enzyme. Some recognition requirements are now becoming apparent.

The stereospecificity of Ins(1,4,5)P₃ 3-kinase is marked, with some 30-100 fold selectivity for the D-isomer. However, relatively minor changes to the inositol nucleus can destroy this selectivity, as seen with D- and L-2,2F₂ InsP₃, which are bound with equal affinity.

Positional specificity appears higher than with the Ins(1,4,5)P₃ receptor or Ins(1,4,5)P₃ 5-phosphatase, Ins(1,4)P₂, Ins(2,4,5)P₃, Ins(1,3,4,6)P₄ and Ins(1,3,4,5)P₄ being bound with low affinity.

The role of the three hydroxyl groups of Ins(1,4,5)P₃ in Ins(1,4,5)P₃ 3-kinase recognition is relatively unimportant. Removal of the 2-, 3-, or 6-hydroxyl groups, or replacement of the 3-hydroxyl group with a fluorine atom (3F InsP₃) causes only minor changes in affinity of Ins(1,4,5)P₃ 3-kinase.

The introduction of phosphorothioate groups gives a most confusing picture. While removing all recognition from InsP₅₃, only marginal loss of affinity is observed between 6-deoxy InsP₃ and 6-deoxy InsP₅₃, and a slight enhancement of affinity is observed between L-ch Ins(2,3,5)P₅ and L-ch Ins(2,3,5)P₅₃.

As with Ins(1,4,5)P₃ receptor recognition, the introduction of diaxial phosphate groups at positions 4- and 5- (L-ch Ins(1,4,6)P₃) removes all affinity.

However, L-2,2F₂ InsP₃ is the first agent which interacts potently with Ins(1,4,5)P₃ 3-kinase and not with the Ins(1,4,5)P₃ receptor. L-2,2F₂ InsP₃, therefore represents a
real lead compound in the search for a potent small molecule Ins(1,4,5)P₃ 3-kinase inhibitor.

3.6.3(ii) Substrate Requirements.

Ins(1,4,5)P₃ 3-kinase has the strictest requirements concerning binding and substrate properties. A vast majority of analogues made so far appear to be resistant to Ins(1,4,5)P₃ 3-kinase, and all are recognised with lower affinity than Ins(1,4,5)P₃ itself. From this work, little can be drawn to identify which regions are involved in substrate/inhibitor profiles.
**Ins(1,4,5)P₃ 3-Kinase Recognition Requirements**

**Equatorial 4-phosphate**
- Little information available.
- Bridging with R₅ or dixial 4,5-like bisphosphate pair removes all affinity.

**Equatorial 3-hydroxyl**
- Replacement with axial -OH has little effect. All other modifications reduce affinity.

**Axial 2-hydroxyl**
- Removal has little effect. All other modifications reduce affinity.

**Equatorial 5-phosphate**
- Introduction of PO₃⁻ reduces affinity and rate of phosphorylation, further loss following similar replacement at R₄ and R₁.

**Equatorial 6-hydroxyl**
- Removal has little effect on affinity. Replacement with OMe reduces affinity.

**Equatorial 1-phosphate**
- Removal or replacement with axial PO₄ at R₅ dramatically reduces affinity.

Figure 3.21 Summary of Ins(1,4,5)P₃ 3-kinase recognition requirements.
CHAPTER 4.
Muscarinic Receptor Responses in Permeabilised SH-SY5Y Cells.

4.1 Introduction and Background.

In 1914, Dale had distinguished between two types of cholinergic receptors, those acted upon by nicotine and blocked by curare, and those acted upon by muscarine and blocked by atropine. These were later defined as the nicotinic and muscarinic receptors, respectively. These receptors are now known to exist as multiple variants of two distinct gene superfamilies, with activation by acetylcholine being the only shared property (see Hulme et al., 1990; Richards 1991). This chapter describes attempts to study the role of one of the variants of the muscarinic receptor in second messenger production and subsequent mobilisation of Ca^{2+} from internal stores.

The first indication that muscarinic receptors did not form a homogeneous class came from the observations that gallamine blocked the effects of acetylcholine in the heart, but not in other peripheral tissues (Riker et al., 1951). These effects were later described as allosteric, gallamine binding to a site on the muscarinic receptor other than the ligand-binding domain (Stockton et al., 1983). Subsequently, a number of antagonists to which different tissues showed differing sensitivities identified a number of different receptor subtypes. Initially, the 'M1' receptor subtype was defined as having high affinity for the antagonist, pirenzepine, and 'M2' receptor subtypes as being of low affinity. 'M1' receptor subtypes were also found to be activated by McN-A 343, a selective agonist identified by Roszkowski (1961). Indeed, this is still one of the few agonists which shows selectivity between subtypes (see Ringdahl, 1989). An analogue of pirenzepine, AF-DX 116, which was considered to be 'M2' selective, was also found to discriminate between 'M2' receptor subtypes in the heart, for which it had higher affinity than 'M2' receptors in the exocrine glands, whereas receptors in the cerebral cortex were of intermediate affinity (Hammer et al., 1986). This led to the classification of 'M1', 'heart M2' and 'gland M2'. The 'gland M2' subtype is now termed the 'M3', and has been found to have high affinity for 4-DAMP and HHSD (and its p-fluorinated analogue). A fourth potential subtype of the muscarinic receptor has been pharmacologically identified. While no selective agents have yet been described, this 'M4' subtype, found in rabbit
lung (Lazareno et al., 1990) does not fit antagonist affinity data obtained for the M1, M2 and M3 subtypes (Buckley et al., 1989; Hulme et al., 1990; Dorje et al., 1991).

A far more powerful tool, receptor cloning, has more recently been employed to distinguish between the muscarinic receptor subtypes (as well as many other systems). In the past six years, genes for five different receptor subtypes have been identified, and designated m1-m5. The proteins encoded by m1, m2, m3 and m4 genes correspond to the previously described M1, M2, M3, and M4 receptors respectively. Although mRNA for m5 has been identified in rat CNS, no pharmacological studies of this receptor occurring naturally in tissues have been reported, so that the cloned m5 receptor is, so far, the only way in which to study this receptor (see Hulme et al., 1990; Richards, 1991; Jones et al., 1992). Bonner et al. (1987) suggested that there may be more than these five distinct subtypes, as they have identified a site for potential splice variants in the structures of m1 to m4.

The primary structures of m1 to m5 receptor subtypes are known, and a high degree of homology is observed, not only among them, but also with α2 and β2 adrenoceptors and the rhodopsin receptor (see Hulme et al., 1990; Jones et al., 1992). Each has seven regions of 20-25 amino acid residues of high hydrophobicity and homology (p>0.75). It was suggested that these are membrane spanning domains, linking three intracellular and three extracellular loops. The amino-terminal region projects into the extracellular space and the carboxy-terminal region projecting into the cytosol (Kubo et al., 1986). The second intracellular loop and all three extracellular loops also show high conservation in the muscarinic receptor subfamily (p>0.85), with greatest variation in the third intracellular loop and the amino- and carboxy-terminals. m1, m3 and, to a lesser extent, m5 show high homology in the third intracellular loop, as do m2 and m4 with one another. It is here that the sequences for m1, m3 and m5 show the greatest divergence from those of m2 and m4, providing the mechanism of selectivity between m1, m3 and m5, and m2 and m4. m1, m3 and m5 are believed to couple the G-protein, Gq, to activate PI-PLC of the β subtype (see Chapter 1), leading to the formation of the second messengers Ins(1,4,5)P3 and DAG (see Richards, 1991). Activation also leads to increases in adenylyl cyclase activity (see Baumgold, 1992), through an uncharacterised pathway. Stimulation of m2 and m4 receptors is seen to lower cyclic AMP levels, acting through a pertussis toxin-sensitive family of G-proteins, Gi (see Richards, 1991). However, CHO cells transfected with cDNA for the m4 receptor pretreated with pertussis toxin were also observed to increase cyclic AMP levels following receptor stimulation, suggesting that the m4 receptor can couple via other G-proteins as well as Gi (Jones et al., 1991). Unlike m1-, m3- and m5 receptor-activated increases in

4.2
cyclic AMP, this effect of m4 receptor stimulation cannot be accounted for by increases in cytosolic Ca^{2+}, activating a Ca^{2+} calmodulin-sensitive adenylyl cyclase (see Baumgold, 1992). The coupling of m1, m3 and m5 receptors to G_q has been identified and determined as involving the amino-terminal 17 amino acid residues of the third intracellular loop for m3 receptors (Wess et al., 1989).

The initial observations that activation of muscarinic receptors in brain slices resulted in an increase in PI turnover (Hokin and Hokin, 1955) has since been amply confirmed in a number of neural preparations (see Fisher and Agranoff, 1987; Fisher et al., 1992). Within the CNS there exists a strong correlation between the density of muscarinic receptors, as determined by radioligand binding studies, and the magnitude of stimulated inositol lipid turnover (Brown and McDonough, 1989). These effects are totally sensitive to the muscarinic agonist atropine (see Hokin and Hokin, 1955). A number of cell lines of neural origin have also been identified as expressing muscarinic receptors. These include N1E 115 (Cohen et al., 1983), SK-N-SH (Fisher and Snider, 1987) and SH-SY5Y neuroblastoma cells (Lambert et al., 1989), and NCB-20 neurohybridoma cells (Chuang, 1986).

A wide variety of agonists, both full and partial, have been used to characterise the PI response in brain tissue and cell lines. The ability of these agents to enhance PI turnover correlates well with their binding characteristics. This is in great contrast to the effects of muscarinic agonists to inhibit adenylyl cyclase activation, where only a few receptors are required to bind for a large inhibition (for example, Brown and Goldstein, 1986), but more closely related to muscarinic receptor stimulation of guanylyl cyclase (McKinney et al., 1985). It has been routinely noted that high concentrations of agonists are required to activate PI turnover, carbachol-induced turnover ranging from 3μM in rat parotid (Gil and Wolfe, 1985) to over 300μM in the cerebral cortex (Fisher and Snider, 1987).

Agonists which greatly stimulate inositol phosphate production have been found to bind the muscarinic receptor with two affinity states, while less efficacious agonists appear less able to promote the stabilisation of a higher affinity state (Fisher et al., 1983, 1984). This clearly shows that muscarinic agonists differ in their abilities to promote a conformational change in the receptor, hence stimulating GTP/GDP exchange on the relevant G-protein and activating PI-PLC to differing degrees. Many other receptors are linked to PI turnover. α1 Adrenergic, 5-HT2-serotonergic and H1 histaminergic receptors also potently stimulate PI turnover in the CNS. Like the muscarinic receptors, there is little, if any, receptor reserve, as agonists show EC50 values for stimulation of PI turnover similar to K50 values for binding their respective receptors, and irreversible inactivation of a proportion of these receptors leads to a
comparable decrease of receptor-mediated PI turnover (see Fisher and Agranoff, 1987; Fisher et al., 1992).

Upon stimulation of muscarinic receptors and production of Ins(1,4,5)P_3, the second messenger involved in mobilisation of Ca^{2+} from internal stores, a ‘peak’ of Ca^{2+} is observed in the cytosol. This has been identified as originating from intracellular stores, presumably via an Ins(1,4,5)P_3-sensitive mechanism, as removal of extracellular Ca^{2+} causes only slight attenuation. The peak in [Ca^{2+}]_i occurs rapidly (~10 seconds) and then declines to a ‘plateau’ (maintained phase) above basal, which continues as long as the receptor is occupied. This plateau phase is dependent on extracellular Ca^{2+} being present (Fisher et al., 1989; Lambert et al., 1991).

The mechanism by which Ca^{2+} influx occurs is unclear. Initially, Ins(1,3,4,5)P_4 was believed to play some role in Ca^{2+} influx (Irvine and Moor, 1987), but more recently it has been claimed that other factors may gate extracellular Ca^{2+} (see Taylor, 1990). A number of routes have been proposed for Ca^{2+} influx, these include second messenger operated channels (as above, Irvine, 1990), receptor operated Ca^{2+} channels (Rink, 1990), G-protein coupled channels (Dolphin and Scott, 1989) and capacitive refilling of intracellular stores (Putney, 1986). It is clear that Ca^{2+} uptake occurs immediately upon receptor activation, and synchronous to release from intracellular stores. Lambert and Nahorski (1990) have shown that partial agonists appear not to initiate the peak response of Ca^{2+} in SH-SY5Y cells, however, the plateau phase is still observed. This is despite Ins(1,4,5)P_3 production occurring, albeit at lower levels.

These spike and plateau Ca^{2+} phases appear to be closely correlated to the transient rise in Ins(1,4,5)P_3 levels in SH-SY5Y cells, which also peaks at ~10 seconds, and reaches a lower but maintained plateau for many hours. Levels of Ins(1,3,4,5)P_4 are also raised, peaking at ~30 seconds and remaining relatively high for at least 5 minutes (Lambert et al., 1991). It is unclear whether this rapid decline from peak levels of Ins(1,4,5)P_3 are due to enhanced metabolism to other phosphates, or if the rate of production of Ins(1,4,5)P_3 is higher over the first few seconds of receptor stimulation. As inositol phosphate accumulation (in the presence of Li^+) appears to be linear for far longer (90 minutes in 1321N1 cells, Masters et al., 1985; 120 minutes in SK-N-SH cells, Thompson and Fisher, 1990), this second suggestion would imply that phospholipids other than PtdIns(4,5)P_2 are being metabolised at later time points. Imai and Gershengorn (1986) have previously shown that, while PtdIns(4,5)P_2 was the major substrate for PI-PLC during the first 2 minutes of agonist stimulation in TRH-stimulated GH_3 cells, PtdIns and PtdIns(4)P were the major substrates after this period. Thus, while the rate of inositol phosphate formation appeared linear, rates of
formation of the second messenger, Ins(1,4,5)P3, were severely attenuated within 2 minutes. Similarly, Menniti et al. (1991) have shown that inositol phosphate accumulation is not linear in bombesin-stimulated AR4 2J cells, in which a slower phase followed a rapid, but transient (20 seconds) burst. The profile of Ins(1,4,5)P3 accumulation was greatly different, depending on the receptor stimulated, such that in the case of cholecystokinin and bombesin receptors, Ins(1,4,5)P3 levels were rapidly raised and returned to an elevated plateau, substance P elicited only a peak response, and methacholine produced a slowly developing response which then waned slightly after 5 minutes. Nakahata and Harden (1987) had previously shown that, while carbachol induced inositol phosphate formation with a long time course (>60 minutes), histamine-induced inositol phosphate formation was complete within 5 minutes. However, Fisher et al. (1990) have shown that, in SK-N-SH cells, polyphosphoinositides are the major substrates for PI-PLC throughout agonist stimulation, due to the accumulation of Ins(4)P, which, they claim, can only be formed as a dephosphorylation product of higher inositol phosphates. The addition of a large excess of Ins(1,4,5)P3 greatly enhanced levels of [3H] inositol trisphosphates accumulating in permeabilised cells over a 5 minute period, however, the time course of such accumulation was not shown. Thus, it is clear, that different receptors in different tissues elicit a wide variety of responses. There is evidence that Ca2+ activates Ins(1,4,5)P3 3-kinase and that both Ins(1,4,5)P3 5-phosphatase and 3-kinase are substrates for, and activated by, protein kinase C (see Shears, 1989, 1991), thus the production of Ins(1,4,5)P3 and subsequent Ca2+ mobilisation and influx and DAG-induced protein kinase C activation may also lead to a termination of the Ca2+ signal.

This Chapter is concerned with the ability of muscarinic agonists to increase inositol phosphate accumulation and to mobilise Ca2+ from internal stores from SH-SY5Y cells. However, contrary to much of the previous work performed, these studies were performed in permeabilised cells, so that modulatory agents which cannot traverse the cell membrane can gain access to intracellular sites. While the permeabilisation techniques used appear to maintain coupling of receptors to PI-PLC (Wojciechiewicz et al., 1990b), it is unknown how such treatment affects other cellular parameters. Indeed, as described in the previous chapter, little Ins(1,4,5)P3 is phosphorylated to Ins(1,3,4,5)P4 by permeabilised cells as prepared here. This can be explained by the dilution of the soluble Ins(1,4,5)P3 3-kinase. Other effects of permeabilisation are unknown. Very few reports of the effects of cell-surface receptor stimulation in permeabilised cells had appeared prior to the work described herein.
4.2 Methods.

These studies were performed in SH-SY5Y cells cultured, as described in Chapter 2 (2.1), in culture medium comprising minimum essential medium supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100μg/ml streptomycin, 2.5mg/ml amphotericin B (fungizone), and 10% foetal calf serum. Cultures were maintained at 37°C in 5% CO₂, 95% humidified air. Prior to use, cells were harvested and washed in Krebs-HEPES solution, in which binding assays to intact cells were performed (see Chapter 2(2.7.1)). Alternatively, cells were washed in intracellular buffer (ICB), comprising 120mM KCl, 20mM HEPES, 6mM MgCl₂, 5mM sodium succinate, 5mM Na₂ATP, 2mM KH₂PO₄, 10-30μM EGTA (to reduce free Ca²⁺ concentration to 70-300nM), pH 6.9. Cells were permeabilised using 3 discharges of 1.50kV through a 3μF capacitor across a 0.4cm cuvette using a Bio Rad gene pulsor, or using the detergent saponin (100μg/ml, 1 minute). The effects of muscarinic agonists and antagonists, and guanine nucleotides were studied in these permeabilised cell preparations. Binding properties, and the abilities of these agents to produce Ins(1,4,5)P₃ and mobilise Ca²⁺ were then examined.

Ca²⁺ release assays were performed with saponised and electroporated cells to determine whether one technique would maintain more efficiently the coupling between receptors and PI-PLC. Ca²⁺ release profiles were determined in permeabilised cells washed in ICB and loaded with ⁴⁵Ca²⁺ for 12 minutes, after which stimuli were added. Incubations were terminated by spinning the cells through a silicone oil mixture, separating cells from the stimulus-containing supernatant. Cells were then assessed for radioactivity (Chapter 2 (2.2)). Ins(1,4,5)P₃ production profiles were determined in permeabilised cells washed, and incubated in ICB for 12 minutes, after which stimuli were added. Incubations were terminated by the addition of 1M TCA. Samples were prepared as described in Chapter 2 (2.3.3). Binding characteristics of agents to permeabilised cells was determined following washing the cells in a Mg²⁺ and HEPES-containing buffer, in which receptor binding assays were performed, as has been previously described for membrane preparations (Lambert et al., 1989).
4.3 Results.

4.3.1 $[^3H]$ NMS Binding.

Specific binding of $[^3H]$ NMS to intact cells at 1-2°C was time and concentration dependent and saturable. Equilibrium binding was found to be obtained within approximately 6 hours. Subsequently, all assays were terminated 6 hours after commencement. Analysis of binding revealed a relatively high density of specific binding sites, with $B_{\text{max}}$ 384±89 fmol/mg of protein, and a Hill slope of 1.06±0.03. The affinity for $[^3H]$ NMS at this reduced temperature was 0.65 (0.41-1.02) nM (Fig. 4.1).

The type(s) of muscarinic receptor represented by this binding was determined by studying the displacement of a fixed concentration of $[^3H]$ NMS by various muscarinic antagonists. $[^3H]$ NMS binding was inhibited in a concentration dependent manner by the selective competitive antagonists, pirenzepine, methoctramine and parafluoro-hexahydrodilufenidol (pF-HHSD). These agents were chosen to identify the nature of the expressed muscarinic receptors.

Pirenzepine has been widely used as an antagonist to distinguish the $M_1$ receptor subtype, since its affinity is 4- and over 10-fold higher for the $M_1$ than the $M_4$ and other muscarinic receptor subtypes respectively. Methoctramine has over 10-fold higher affinity for $M_2$ receptor subtypes over other receptor subtypes. AF-DX 116 also shows selectivity towards the $M_2$ receptor, but binds with only 4-fold higher affinity than with the $M_3$ receptor subtype. AF-DX 116 also does not distinguish between $M_2$ and $M_4$ receptor subtypes, having equal affinity for both, however, binding to $M_4$ receptor subtypes shows a low Hill slope. HHSD shows at least a 3-fold selectivity towards $M_3$ receptor subtypes over all other muscarinic receptor subtypes. Its pF analogue, as used in this study, is believed to have a slightly higher selectivity. No selective agent has yet been found to distinguish pharmacologically the $M_4$ and ‘$M_3$’ receptor subtypes (Buckley et al., 1989; Dorje et al., 1991; Jones et al., 1992). The $M_4$ subtype binds most antagonists in a complex manner (Buckley et al., 1989; Dorje et al., 1991). Data thus far have mostly been obtained using $M_4$ cDNA. More than one receptor protein may be manufactured from this single cDNA (‘alternative splicing’) and a mixture of these receptor subtypes may be expressed, as suggested by Bonner et al. (1987). Indeed, such splicing has been observed for the
Figure 4.1 Binding of $[^{3}\text{H}]$ NMS to intact SH-SY5Y cells at 1-2°C. $[^{3}\text{H}]$ NMS was incubated with SH-SY5Y cells at 1-2°C for 6 hours in an assay volume of 250µl (filled squares). Non specific binding (open squares) was defined in the presence of 1µM atropine sulphate, from which specific binding (filled diamonds) could be calculated. Data represent means from a single experiment, with similar results being obtained in many further experiments.
dopamine D\textsubscript{2} receptor, another seven transmembrane domain receptor protein (Dellavedova et al., 1992).

Pirenzepine and pF-HHSD both generated simple displacement curves with Hill slopes of unity. Methoctramine also displaced \[^{3}H\] NMS with a single affinity state. The Hill slope for methoctramine was, however, 1.3, as has been found by a colleague (N.T. Burford, personal communication) in other cells expressing a single recombinant muscarinic receptor subtype. No satisfactory explanation for this has been found. \[^{3}H\] NMS was displaced with low affinity by pirenzepine and methoctramine, and with high affinity by pF-HHSD, suggesting that the receptors expressed on these cells are of the M\textsubscript{3} subtype (Fig 4.2, Table 4.1).

Table 4.1. Antagonist affinity for M\textsubscript{3} Receptors on SH-SY5Y cells.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>K\textsubscript{D}(nM)\textsuperscript{a}</th>
<th>nH\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS (n=15)</td>
<td>0.65(0.41-1.02)</td>
<td>1.03±0.02</td>
</tr>
<tr>
<td>Antagonist</td>
<td>K\textsubscript{50}(nM)\textsuperscript{c}</td>
<td>nH</td>
</tr>
<tr>
<td>PZ (n=3)</td>
<td>510(330-780)</td>
<td>0.92±0.07</td>
</tr>
<tr>
<td>Methoc. (n=3)</td>
<td>7100(3500-14600)</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>pF-HHSD (n=6)</td>
<td>290(170-490)</td>
<td>0.97±0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Dissociation constant, expressed as mean, followed by 95% confidence limits in parentheses.

\textsuperscript{b} Hill slope of binding, expressed as mean±S.E.M.\n
\textsuperscript{c} IC\textsubscript{50} corrected for mass of radioligand, expressed as mean, followed by 95% confidence limits in parentheses.
Figure 4.2 Inhibition of $[{}^3H]$ NMS (0.7nM) binding to intact SH-SY5Y cells by pF HHSD (open squares), methoctramine (filled diamonds) and pirenzepine (filled squares). Incubations were performed at 1-2°C for 6 hours in an assay volume of 250μl. Non-specific binding, defined in the presence of 1μM atropine sulphate, was 7% of total $[{}^3H]$ NMS binding. Data represent means from a single experiment, performed in duplicate, with similar results obtained in a further 2 experiments.
Specific binding of the tritiated muscarinic antagonist, $[^3H]$NMS to electroporated cells was saturable, with a $K_D$ of 0.35 (0.20-0.60) nM, a $B_{max}$ of 299±40 fmol/mg of protein, and a Hill slope of 1.08±0.03 (mean ± S.E.M., n=15). The ability of $[^3H]$NMS to bind to these receptors was not affected by the addition of GTP (1mM) ($K_D=0.41$ (0.27-0.61) nM, $B_{max}=283±43$ fmol/mg of protein, Hill slope=1.08±0.05, n=6). The binding of $[^3H]$NMS was inhibited by carbachol, arecoline and oxotremorine (Table 4.2). The displacement by carbachol appeared to be complex, exhibiting a low Hill slope. Two site analysis revealed the existence of high- ($K_H=3.5$ (1.2-10.2) µM, $P_H=32±8\%$) and low- ($K_L=102$ (64-102) µM, $P_L=67±8\%$) affinity binding sites. Competition curves generated in the presence of GTP (1mM) caused a decrease in affinity and increase in the Hill slope for carbachol, but not arecoline or oxotremorine, caused by a reduction in the proportion of high-affinity binding sites (Table 4.2).
Table 4.2 Binding Profiles of Muscarinic Agonists to Permeabilised SH-SY5Y Cells.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$K_{50}(\mu M)^a$</th>
<th>$n_H^b$</th>
<th>$K_{H}(\mu M)^c$</th>
<th>$P_H(%)^d$</th>
<th>$K_{L}(\mu M)^e$</th>
<th>$P_L(%)^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCh</td>
<td>65</td>
<td>0.81±0.03</td>
<td>3.5</td>
<td>32±8</td>
<td>102</td>
<td>67±8</td>
</tr>
<tr>
<td></td>
<td>(43-99)</td>
<td></td>
<td>(1.2-10.2)</td>
<td></td>
<td>(64-162)</td>
<td></td>
</tr>
<tr>
<td>+GTP$^g$</td>
<td>85</td>
<td>0.94±0.05$^*$</td>
<td>7.1</td>
<td>11±5</td>
<td>104</td>
<td>89±4</td>
</tr>
<tr>
<td></td>
<td>(56-126)</td>
<td></td>
<td>(3.8-13.1)</td>
<td></td>
<td>(74-145)</td>
<td></td>
</tr>
<tr>
<td>Areca</td>
<td>26.9</td>
<td>1.04±0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(15.5-46.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+GTP</td>
<td>26.7</td>
<td>1.08±0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(15.8-45.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxo</td>
<td>1.1</td>
<td>0.96±0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.62-1.99)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+GTP</td>
<td>1.6</td>
<td>0.91±0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.93-2.68)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- $^a$ IC$_{50}$ corrected for mass of radioligand, expressed as mean, followed by 95% confidence limits in parentheses.
- $^b$ Hill slope of binding, expressed as mean±S.E.M.
- $^c$ IC$_{50}$ of carbachol for high affinity binding sites, corrected for mass of radioligand.
- $^d$ Proportion of high affinity binding sites.
- $^e$ IC$_{50}$ of carbachol for low affinity binding sites, corrected for mass of radioligand.
- $^f$ Proportion of low affinity binding sites.
- $^g$ Assays performed in the presence of GTP (1mM).
- $^*$ Significantly different from $n_H$ for carbachol alone (p<0.05).
4.3.2 Ins(1,4,5)P3 Formation and Ca2+ Mobilisation.

Treatment of electrically permeabilised cells with maximal doses of carbachol (1mM), arecoline (10mM) or oxotremorine (10μM) induced Ins(1,4,5)P3 accumulation which peaked at 80-150 seconds (Fig 4.3 a, b, c, n=4-5), after which the response waned slightly. Levels of Ins(1,4,5)P3 (basal, 10±2 pmol/mg of protein, n=20) were increased maximally to 115±28, 27±4, and 17±3 pmol/mg of protein respectively. The inclusion of a maximal dose of the metabolism-resistant GTP analogue, GTPγS (100μM) increased maximal Ins(1,4,5)P3 accumulation to 169±61, 86±13 and 86±16 pmol/mg of protein respectively (n=4-5), but did not affect the time required for the response to peak. GTPγS alone raised Ins(1,4,5)P3 levels modestly to 68±12 pmol/mg of protein at 4 minutes (n=4), after a lag period of about 40 seconds (Fig 4.3.).

Using the same protocol as that used for Ins(1,4,5)P3 and its analogues (cell density 0.25-0.5mg of protein/ml) (Chapter 3), the muscarinic agonist carbachol failed to elicit 45Ca2+ mobilisation, whether cells were permeabilised using electrical discharges or the detergent saponin. However, at higher cell densities (2-10mg of protein/ml) carbachol was able to mobilise 45Ca2+, both from electrically permeabilised and saponised cells with similar EC50 values. Using cells permeabilised with 3 discharges, dose-response data obtained using cells at a density of 4 mg and 8mg of protein/ml (final concentration) gave EC50 values of 35 (23-54) μM and 51 (20-134) μM respectively (n=6). In the presence of GTPγS (100μM), the dose-response curves were shifted to the left to 0.24 (0.06-0.89) μM and 0.15 (0.02-1.02) μM respectively (n=5). From these data, it is apparent that, whilst a high cell density is required for 45Ca2+ mobilisation to occur, minor variations in cell density do not affect the EC50 values obtained. All subsequent work was performed at a cell density of 4-6 mg of protein/ml.

Agonist-induced mobilisation of Ca2+, followed using trace levels of 45Ca2+, showed similar time courses to that observed for Ins(1,4,5)P3 mass, peaking between 1 and 2 minutes (Fig 4.4 a, b, c). Maximal release was enhanced by the inclusion of GTPγS (100μM), which, again, had no effect on the time course for the response to peak. GTPγS-induced 45Ca2+ mobilisation peaked at 8 minutes (19±3 % release), after a lag time of about 1 minute. A close correlation between Ins(1,4,5)P3 accumulation and 45Ca2+ mobilisation was observed, carbachol being the most efficacious agonist, releasing 29±3 % (n=7) of loaded 45Ca2+ at 2 minutes. Arecoline was slightly more
Figure 4.3 Time course of Ins(1,4,5)P$_3$ production following stimulation of electrically permeabilised SH-SY5Y cells with muscarinic agonists. Permeabilised cells were treated with carbachol (1mM) (upper panel), arecoline (10mM) (middle panel) or oxotremorine (10µM) (lower panel) in the absence (open squares) or presence (filled diamonds) of GTPγS (100µM), or GTPγS (100µM) alone (crosses). Incubations were terminated by the addition of TCA (1M), as described (Chapter 2 (2.3.2)). Levels of Ins(1,4,5)P$_3$ were then determined as described (Chapter 2 (2.3.3)). Data represent means from 6 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 15%).
Figure 4.4 Time course of $^{45}$Ca$^{2+}$ following stimulation of electrically permeabilised SH-SY5Y cells with muscarinic agonists. Permeabilised cells were treated with carbachol (1mM) (upper panel), arecoline (10mM) (middle panel) or oxotremorine (10μM) (lower panel) in the absence (open squares) or presence (filled diamonds) of GTPγS (100μM), or GTPγS (100μM) alone (crosses). Incubations were terminated as described (Chapter 2 (2.2)) and $^{45}$Ca$^{2+}$ release was calculated. Data represent means from 6 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 3%).
efficacious than oxotremorine, mobilising 13±2 % (n=7), compared with 6±2 %
(n=7). In the presence of GTPγS, carbachol maximally released 34±2 %, arecoline
29±2 % and oxotremorine 31±3 % of loaded 45Ca2+.

In order to further investigate the effects of the three agonists on Ins(1,4,5)P3
accumulation and Ca2+ release, dose-response curves for carbachol, arecoline and
oxotremorine were obtained, stopping incubations at 2 minutes.

Carbachol alone stimulated release of 32±2% of 45Ca2+ with an EC50 of 49 (36-67)
μM (Fig 4.5a, n=20). GTPγS alone released maximally 13±2% of 45Ca2+ in a dose
dependent manner with an EC50 value of 4.0 (2.6-6.1) μM (Table 4.3). Co-incubation
of carbachol and GTPγS (100μM) produced a synergistic response, maximal release
was 33±2% above GTPγS alone (41±2 % in total), however, the EC50 for carbachol
was moved 195-fold to the left (EC50=0.25 (0.18-0.34) μM, n=20) (Fig 4.5a).

Measurement of Ins(1,4,5)P3 mass levels in parallel incubations with cells showed
there to be a close correlation between Ins(1,4,5)P3 accumulation and 45Ca2+
mobilisation (Fig 4.5 a, b). In the absence of GTPγS, carbachol induced Ins(1,4,5)P3
accumulation with an EC50 of 47 (26-85) μM (n=8). Co-incubation with GTPγS
increased maximal Ins(1,4,5)P3 accumulation by over 40%, to 111±20 pmol/mg of
protein over basal, and moved the dose response curve 10-fold to the left (EC50=4.1
(2.1-7.9) μM, n=8) (Fig 4.5b). At this time point (2 minutes), GTPγS alone raised
Ins(1,4,5)P3 levels by 6±2 pmol/mg of protein.

The partial agonist arecoline was found to mobilise 45Ca2+ in a dose dependent
manner with an EC50=46 (32-66) μM (Fig 4.6a), in reasonable agreement with the
K50 values obtained for the displacement of [3H] NMS (27 (15-47) μM, n=6),
suggesting that SH-SY5Y cells do not possess a receptor reserve for arecoline.
Maximal release was 13±2% (n=5) of sequestered 45Ca2+. In the presence of GTPγS,
maximal release again approached the upper limit (29±4% over GTPγS alone). The
EC50 was shifted 52-fold to the left (EC50=0.86 (0.60-1.22) μM, n=7) (Fig 4.6a), a
substantially smaller shift than that seen with carbachol (195-fold). Again,
measurement of Ins(1,4,5)P3 in cells showed a close correlation with 45Ca2+
mobilisation. In the absence of GTPγS, arecoline stimulated Ins(1,4,5)P3
accumulation with an EC50=51 (13-201) μM (n=7). Co-incubation with GTPγS
increased maximal Ins(1,4,5)P3 accumulation by over 200%. The dose-response
curve was shifted 13-fold to the left (EC50=3.6 (1.9-6.8) μM, n=7) (Fig 4.6b).

Oxotremorine was the most potent agonist at this receptor, giving an EC50 for 45Ca2+
mobilisation of 0.36 (0.11-1.24) μM, in close agreement with binding data, from
which oxotremorine gave a K50 of 1.1-1.6 μM. It appeared, however, to be the least

4.12
Figure 4.5 Carbachol-induced $^{45}\text{Ca}^{2+}$ release (upper panel) and Ins(1,4,5)P$_3$ accumulation (lower panel) in permeabilised SH-SY5Y cells. Cells were prepared as described in the legends to Figs. 4.3 and 4.4, and $^{45}\text{Ca}^{2+}$ and Ins(1,4,5)P$_3$ accumulation were followed in the absence (open squares) and presence (filled diamonds) of GTPyS (100µM), from which the effects of GTPyS alone have been subtracted. Data represent mean ±S.E.M. for 6-20 experiments.
Figure 4.6 Arecoline-induced $^{45}\text{Ca}^{2+}$ release (upper panel) and 
Ins(1,4,5)P$_3$ accumulation in permeabilised SH-SY5Y cells. Cells were 
prepared as described in the legends to Figs. 4.3 and 4.4, and $^{45}\text{Ca}^{2+}$ and Ins(1,4,5)P$_3$
accumulation were followed in the absence (open squares) and presence (filled 
diamonds) of GTPyS (100µM), from which the effects of GTPyS alone have been 
subtracted. Data represent mean ±S.E.M. for 6-8 experiments.
EC₅₀ = 0.86 μM

EC₅₀ = 46 μM

EC₅₀ = 3.6 μM

EC₅₀ = 51 μM

[Arecoline] (μM)
efficacious, releasing only 11±4% of sequestered $^{45}$Ca$^{2+}$ (n=8) (Fig 4.7a). In the presence of GTP$\gamma$S, there was no significant shift in EC$_{50}$ (0.10 (0.02-0.36) μM), however, maximal release had increased to 26±4% (n=8) above GTP$\gamma$S alone (Fig 4.7a). Monitoring Ins(1,4,5)P$_3$ mass accumulation in parallel studies, it was found that including GTP$\gamma$S again did not significantly affect the EC$_{50}$ values (0.52 (0.18-1.46) μM in the absence (n=5) and 0.28 (0.13-0.64) μM in the presence (n=4) of GTP$\gamma$S) (Fig 4.7b).

Table 4.3 Effects of Agonists on GTP$\gamma$S-Induced $^{45}$Ca$^{2+}$ Release From Permeabilised SH-SY5Y Cells.

<table>
<thead>
<tr>
<th>EC$_{50}$ (μM)</th>
<th>Max. Rel. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>4.0</td>
</tr>
<tr>
<td>+ CCh$^b$</td>
<td>0.76</td>
</tr>
<tr>
<td>100μM</td>
<td>0.08</td>
</tr>
<tr>
<td>+ Arex</td>
<td>0.27</td>
</tr>
<tr>
<td>1mM</td>
<td>0.12</td>
</tr>
<tr>
<td>+ Otox</td>
<td>0.1μM</td>
</tr>
<tr>
<td>10μM</td>
<td>0.31</td>
</tr>
</tbody>
</table>

$^a$ EC$_{50}$ of GTP$\gamma$S-induced $^{45}$Ca$^{2+}$ release from permeabilised cells, expressed as means, followed by 95% confidence limits in parentheses.

$^b$ $^{45}$Ca$^{2+}$ release assay performed in the presence of muscarinic agonists at stated concentration.

The addition of threshold or near maximal doses of the three agonists used co-incubated with GTP$\gamma$S in increasing concentrations was found to affect both the EC$_{50}$
**Figure 4.7** Oxotremorine-induced $^{45}$Ca$^{2+}$ release (upper panel) and Ins(1,4,5)P$_3$ accumulation in permeabilised SH-SY5Y cells. Cells were prepared as described in the legends to Figs. 4.3 and 4.4, and $^{45}$Ca$^{2+}$ and Ins(1,4,5)P$_3$ accumulation were followed in the absence (open squares) and presence (filled diamonds) of GTPyS (100μM), from which the effects of GTPyS alone have been subtracted. Data represent mean ±S.E.M. for 4-6 experiments.
45Ca²⁺ Released (%)

[Oxotremorine] (µM)

EC50 = 0.10 µM
EC50 = 0.36 µM

InsP3 / mg of protein

[Oxotremorine] (µM)

EC50 = 0.28 µM
EC50 = 0.52 µM
and maximal GTPyS-induced $^{45}$Ca$^{2+}$ release. A threshold dose of agonist (1μM carbachol, 10μM arecoline or 0.1μM oxotremorine) greatly enhanced $^{45}$Ca$^{2+}$ release, and lowered the EC$_{50}$ for GTPyS. Greater occupancy of the muscarinic receptors with maximal or near maximal doses of agonists (100μM carbachol, 1mM arecoline, 10μM oxotremorine) caused a further leftward shift in EC$_{50}$ (Table 4.3). In contrast, GDPβS (1mM), which stabilises the inactive form of G-proteins (see Fig 1.2, Chapter 1), blocked the effects of carbachol, arecoline and oxotremorine (Table 4.4).

The effects of carbachol (1mM), arecoline (1mM) and oxotremorine (10μM) were totally blocked by the inclusion of the non-selective muscarinic antagonist, atropine (10μM) (Table 4.4.). Atropine also inhibited carbachol- (1mM), arecoline- (1mM), and oxotremorine- (10μM) stimulated enhancement of responses to GTPyS (100μM) (Table 4.4). However, GTPyS-induced $^{45}$Ca$^{2+}$ release was unaffected.

Under identical conditions, exogenous Ins(1,4,5)P$_3$ induced Ca$^{2+}$ mobilisation with an EC$_{50}$=0.21 (0.13-0.35) μM, maximal release being 54±4% (n=10). GTP (1mM), GTPyS (10μM) GDPβS (1mM) and atropine (10μM) had no significant effect on Ins(1,4,5)P$_3$-induced Ca$^{2+}$ mobilisation, EC$_{50}$ values and maximal release of $^{45}$Ca$^{2+}$ obtained being 0.21 (0.13-0.35) μM, 48±3% (n=3), 0.23 (0.10-0.55) μM, 46±4% (n=4), 0.17 (0.06-0.44) μM, 47±7% (n=3), 0.14 (0.03-0.72) μM, 52±8% (n=5) respectively (Data not shown).
Table 4.4 Effects of Atropine and GDPβS on Muscarinic Agonist- and GTPγS-induced $^{45}$Ca$^{2+}$ Mobilisation.

<table>
<thead>
<tr>
<th>Buffer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>100μM</th>
<th>1mM CCh</th>
<th>1mM AreC</th>
<th>10μM Oxo</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTPγS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-GTPγS</td>
<td>+15±4</td>
<td>-2±1</td>
<td>26±3</td>
<td>1±2</td>
</tr>
<tr>
<td>+GTPγS</td>
<td>26±3</td>
<td>1±2</td>
<td>27±5</td>
<td>2±2</td>
</tr>
<tr>
<td>+GTPγS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23±2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Control          0   13±3   38±5   41±4   18±4   43±4   11±3   35±3
+10μM Atr.       -1±1  15±3   -2±1   26±3   1±2   27±5   2±2   23±2
+1mM GDPβS       4±1   14±4   8±5   23±2   10±4   24±5   4±2   22±1

<sup>a</sup> Release of $^{45}$Ca$^{2+}$ from permeabilised SH-SY5Y cells, values represent mean±S.E.M. for 4-6 experiments.

<sup>b</sup> In the presence of GTPγS (100μM).
4.4 Discussion.

The pharmacological characterisation of receptors present on SH-SY5Y cells has identified only a handful of receptor types. α2-Adrenergic receptors have been identified in SK-N-SH cells (Baron and Siegel, 1989) and SH-SY5Y cells (Kazmi and Mishra, 1989). α2 receptor activation decreases levels of cyclic AMP elevated by the non specific adenylyl cyclase activator forskolin. Pertussis toxin pretreatment attenuated this inhibition, suggesting that α2 receptors are coupled through Gi, which has been previously identified as a substrate for pertussis toxin (see Chapter 1). Radioligand binding studies of SH-SY5Y cells has shown that two subtypes of the α2 receptor are present, α2A comprising 38%, α2B, 60% of total α2 receptors (Kazmi and Mishra, 1989). Opioid receptors are also present on SH-SY5Y cells, μ and δ subtypes have been reported in a 9:2 ratio (Yu et al., 1990). These are also negatively coupled to adenylyl cyclase.

Two receptor systems present in SH-SY5Y cells which couple positively to adenylyl cyclase, hence raising cyclic AMP levels, (presumably through Gs) are VIP receptors (Müller et al., 1989) and prostaglandin E1 receptors (Yu et al., 1988, 1990). Agonist binding to the prostaglandin E1 receptor, activating adenylyl cyclase and causing a rise in the levels of cyclic AMP can be attenuated by μ and δ opioid receptor agonists (Yu et al., 1990).

Most recently, after the work for this thesis was started, nicotinic receptors were identified in the parent SK-N-SH and SH-SY5Y cell lines. Activation of these nicotinic receptors was found to transiently increase intracellular Ca2+ levels, via a Ca2+-influx mechanism in some (Noronha-Blob et al., 1989), but not other (Forsythe et al., 1992) cells.

Adem et al. (1987) found there to be two muscarinic receptor subtypes present on SH-SY5Y cells, 34% M1 and 64% non M1. This was however disputed by Serra et al. (1988) and Mei et al. (1989) who suggested that the M1 subtype was the major subtype. The source of SH-SY5Y cells was not divulged in either of these papers. Further problems arose when Lambert et al. (1989) published data stating only M3 receptors were present. It is these cells (obtained directly from Dr. J.L. Biedler) which have been used in the studies described herein. The differences observed could be related to different sources of cells, or the different culture conditions used. This latter postulation suggests that the culture conditions may induce differential gene
expression. Further credence for this has come to light using subtype selective antisera. Wall et al. (1991), have found m1, m2 and m3 receptor subtypes (5:8:74) in SH-SY5Y cells, thus, SH-SY5Y cells are clearly capable of expressing m1, m2 and m3 receptors.

The SH-SY5Y human neuroblastoma cell line used in this study appears to express muscarinic receptors of the M3 subtype, exemplified by pirenzepine, methoctramine and pF-HHSD all displacing the non-selective muscarinic antagonist, [3H] NMS, with a single affinity, as has been previously reported by Lambert et al. (1989). The affinities for pirenzepine (520nM), methoctramine (7400nM) and pF-HHSD (330nM) obtained in this study, are lower than those obtained for the cloned m3 subtype expressed in CHO-K1 cells, where pirenzepine was found to have an affinity of 180nM (Buckley et al., 1988), methoctramine, 118 and 214nM (Buckley et al., 1988; Dorje et al., 1991) and pF-HHSD, 15nM (Dorje et al., 1991). However, the affinity of agents reported in these two papers (Buckley et al., 1988; Dorje et al., 1991) appear higher than those found with endogenous receptors, where pirenzepine was found to bind with an affinity of 254-1570nM (Doods et al., 1987; Giraldo et al., 1988; Lambert et al., 1989) and HHS with an affinity of 63nM (Doods et al., 1987) to M3 receptors. Likewise, in this study, the affinity of agents appears lower in general, however, the higher affinity for pF-HHSD is indicative of receptors of the M3 subtype. This does not, however, eliminate the possibility of a small population of muscarinic receptors of another subtype. Indeed, Wall et al. (1991), using selective antisera, have also identified SH-SY5Y cells as expressing predominantly m3 receptors (74%). However, with this more sensitive method, they also identify the presence of m1 and m2 subtypes (5 and 8% of total muscarinic receptor number). In total, 13% of the receptor number was not identified by the use of the three antisera, it was suggested that some posttranslational modification may occur, such that the remaining receptors are no longer recognised by the antisera used. The relative roles of the m1, m2 and m3 receptors in the functional studies described is unknown.

The binding of [3H] NMS to receptors on permeabilised cells was found to be similar to that observed when binding to intact cells, and is unaffected by guanine nucleotides. This is not unexpected, as most antagonists are believed not to cause conformational changes of the receptor which enable them to activate G-proteins (see Chapter 1). Displacement of [3H] NMS by the agonist carbachol, did, however, show sensitivity to guanine nucleotides. Under the conditions used, 32% of receptors expressed high affinity for carbachol, which was reduced to 11% in the presence of GTP(1mM). The affinity of [3H] NMS for these receptors does appear lower than that described previously, with KD routinely reported to be in the region of 0.2nM

4.17
It is tempting to explain the GTP-sensitive high-affinity sites for carbachol in terms of the ‘Ternary complex model’ of agonist/receptor/G-protein interaction, initially proposed for receptors linked to adenylyl cyclase, in which agonists induce a high-affinity but nucleotide-sensitive state of the receptor (Kent et al., 1980, Limbird, 1981). More recently, data from guinea pig cerebral cortex and human astrocytes indicate that this model may also be appropriate for receptor systems coupled to PI hydrolysis (Fisher et al., 1984, Evans et al., 1985). This is, however, not universally observed for receptors coupled to PI hydrolysis. In SH-SYSY cells (Wojcikiewicz et al., 1990b), and guinea pig cerebral cortex and ileal smooth muscle (Ek and Nahorski, 1988), guanine nucleotides cause a parallel shift in the displacement profile of $[^{3}H]$NMS by carbachol. In the case of Wojcikiewicz et al. (1990b), this was caused by a decrease in the affinity of the low affinity state and increase in the affinity of the high affinity state. A similar loss of high affinity sites was observed as in this study (from 31 to 13%) by the inclusion of the hydrolysis-resistant GTP analogue, GppNHp. As the role of $\beta\gamma$ dimers of G-proteins in enzyme activation becomes more clear, the ternary model complex model discussed here must be reevaluated with its significance regarding PI-PLC linked receptors and this may explain why earlier studies (for example, Ek and Nahorski, 1988) showed nucleotide-insensitive agonist binding.

Arecoline and oxotremorine, whilst unable to produce a significant shift to a GTP-sensitive high affinity state, did cause activation of the G-protein, and production of Ins(1,4,5)P$_3$, as observed in rat and guinea pig brain preparations (Fisher et al., 1983). In contrast, Lambert et al. (1989) found that arecoline bound muscarinic receptors on membrane preparations of SH-SYSY cells with a low Hill slope (0.66). The levels of arecoline-induced Ins(1,4,5)P$_3$ accumulation were, however, much smaller than that seen with carbachol. Fisher et al. (1984) have demonstrated, using analogues of oxotremorine, that the ability of agonists to cause breakdown of phospholipids correlates well with their ability to promote high affinity binding to the receptor in guinea pig cerebral cortex. Thus, the weak partial agonist oxo-1 bound muscarinic receptors with a single affinity and raised inositol phosphate levels by only 11% over basal, whereas oxo-2, -3, -4 and -M, which raised inositol phosphate levels by 25, 26, 143 and 191% induced 13, 18, 23 and 25% high affinity binding respectively. Oxo-5, a receptor antagonist, bound with single affinity and did not raise inositol phosphate levels. However, the role of different muscarinic receptor subtypes in the above is
uncertain, as guinea pig brain has at least two subtypes present. Pirenzepine
displacement of the non-selective antagonist, [3H] QNB, identified a large proportion
(>85%) of M1 receptors, and a smaller proportion of receptors of an undefined
subtype (Fisher and Bartus, 1985).

Receptor activation leads to the formation of the second messenger, Ins(1,4,5)P3. The
time required for the response to peak in permeabilised cells is much longer than that
observed for responses in intact cells. In intact cells, Ins(1,4,5)P3 levels are seen to
peak at 10 seconds in SH-SY5Y cells stimulated with carbachol (Lambert and
Nahorski, 1990; Lambert et al, 1991), UMR-106-01 cells stimulated with
prostaglandin F2α and endothelin in combination (Zhao et al., 1990) and in guinea pig
ileal slices stimulated with histamine (Bielkiewicz-Vollrath et al., 1987). Similarly,
Ca2+ levels are seen to peak at 10 seconds in intact SH-SY5Y cells (Lambert and
Nahorski, 1990) or even more rapidly (5 seconds) in SK-N-SH cells (Fisher et al.,
1989). Contraction of guinea pig ileum, an event downstream of Ins(1,4,5)P3
formation and Ca2+ release, peaked at 2.5 seconds (carbachol stimulation) and 5
seconds (Histamine stimulation) (Bielkiewicz-Vollrath et al., 1987). However, the
efflux of 45Ca2+ from intact 1321N1 cells was maximal at 2 minutes (Masters et al.,
1984) and in permeabilised UMR-106-01 cells appeared to be maximal at 1 minute
(Zhao et al., 1990). The differences observed may be methodological: Lambert and
coworkers (Lambert and Nahorski, 1990; Lambert et al., 1991) and Fisher et al.
(1989) monitored Ca2+ release using Ca2+-sensitive fluorophores (fura-2 and Quin-2),
Zhao et al. (1990) and I followed release of a radioactive tracer (45Ca2+) from internal
stores in permeabilised cells, and Masters et al. (1984) followed 45Ca2+ efflux from
intact cells. The influence of temperature does not account for the differences
observed, as Lambert et al. (1991), Fisher et al. (1989), Masters et al. (1984) and
Zhao et al. (1990) performed their experiments at 37°C. This work was performed at
20°C. At this temperature, carbachol induced Ca2+ mobilisation in intact SH-SY5Y
cells, monitored using fura-2, peaked at 25 seconds (D.G. Lambert, personal
communication). This would suggest that in a permeabilised cell system, second
messenger generation is less efficient, as Ins(1,4,5)P3 levels peak later. Metabolism is
also attenuated, as mentioned above, potentially due to the removal of Ins(1,4,5)P3 3-
kinease, and dilution of inositol phosphates, lowering concentrations at sites proximal
to membrane-bound phosphatases.

The lag in onset of the effects of GTPγS alone is common to other systems (for
example, Harden et al. 1988) and can be explained by the time required for
endogenous nucleotides bound to the G-protein to exchange with GTPγS (Gilman,
1987; Boyer et al., 1989; Plevin et al., 1990). This process is catalysed by the
presence of an agonist on the receptor. For this reason, the lag time observed in response to agonist alone, or with GTPyS, is greatly reduced. The EC50 for GTPyS is comparable with the EC50 values obtained for inositol phosphate production in permeabilised chick heart cells (1μM) (Jones et al., 1988), or 1321N1 human astrocytoma membranes (3-10μM) (Hepler and Harden, 1986), Ins(1,4,5)P3 production in permeabilised Swiss 3T3 cells (2μM) (Cattaneo and Vicentini, 1989) and inositol trisphosphate production in turkey erythrocyte membranes (0.5μM) (Rooney et al., 1989). Boyer et al. (1989) have shown that agonists induce enhancement of response and decrease in EC50 for GTPyS in a dose related manner, such that in the presence of a maximally effective concentration of 2-methylthioadenosine 5'-triphosphate, a potent agonist at the P2Y purinoceptor, the EC50 of GTPyS was shifted 50-fold to the left. The EC50 for GTPyS-induced inositol phosphate formation was lowered from 20μM to 3μM in permeabilised SH-SY5Y cells by the inclusion of 1mM carbachol, which also increased formation by ~10-fold (Wojcikiewicz et al., 1990b), whereas in permeabilised Swiss 3T3 cells, a shift in EC50 from 2μM to 0.2μM in the presence of maximal concentration of bombesin was not accompanied by increased levels of Ins(1,4,5)P3 (Cattaneo and Vicentini, 1989). That carbachol shifts the GTPyS dose relationship far more than arecoline which, again causes a greater shift than exotremorine, suggests that these effects are due to the abilities of these agonists to activate GDP-GTP (or GTPyS) -exchange on the relevant G-protein.

Rooney et al. (1989) have shown that alcohols, which act at the level of the G-protein, lower the EC50 for GTPyS to induce formation of InsP3 without increasing maximal stimulation. Inclusion of an agonist (as in this study and Boyer et al., 1989), however, caused a much greater shift in EC50 and an increase in maximal formation. That atropine, the muscarinic antagonist, blocks agonist-induced enhancement of the GTPyS response, but not the effects of GTPyS alone provides evidence that GTPyS is acting at a level beyond the muscarinic receptor.

The causal relationship between Ins(1,4,5)P3 accumulation and Ca2+ mobilisation has been previously shown and discussed (see Chapters 1 and 3). As described, Streb et al. (1983) showed that the addition of Ins(1,4,5)P3 to leaky pancreatic acinar cells caused a mobilisation of Ca2+ from stores. Equally, Wojcikiewicz et al., (1990c) have shown that the addition of Ins(1,4,5)P3 to permeabilised SH-SY5Y cells causes the mobilisation of Ca2+ in a dose dependent manner, whereas the addition of Ca2+, producing similar levels to that observed upon Ins(1,4,5)P3-stimulated release is unable to liberate Ins(1,4,5)P3 (Wojcikiewicz et al., 1990c). Similarly, in intact 1321N1 cells, Masters et al. (1984) have shown that Ca2+ mobilisation is secondary
to inositol phospholipid breakdown, as the Ca^{2+} ionophore A23187, which raises Ca^{2+} within cells to levels higher than would be obtained with agonists, does not cause the production of inositol phosphates.

The muscarinic agonist carbachol was unable to mobilise 45Ca^{2+} under the conditions initially used (cell density 0.25-0.5 mg of protein/ml). This may be due to the mechanism of Ca^{2+} mobilisation. Agonists produce Ins(1,4,5)P_{3} proximal to the plasma membrane. In a dilute suspension of permeabilised cells, this Ins(1,4,5)P_{3} may be diluted throughout the assay volume. Low cell densities may not generate sufficient Ins(1,4,5)P_{3} throughout to mobilise Ca^{2+}. That Ins(1,4,5)P_{3} provides a link between receptor-activation and Ca^{2+} mobilisation is supported by the close correlation, in the absence of GTPyS, between carbachol-induced Ins(1,4,5)P_{3} formation and Ca^{2+} mobilisation. Also, the concentration of Ins(1,4,5)P_{3} generated upon maximal stimulation with carbachol (at 6mg of protein/ml, 115 pmol/mg of protein would give approximately 0.7μM Ins(1,4,5)P_{3}) would be sufficient to mobilise almost fully Ca^{2+} from the Ins(1,4,5)P_{3}-sensitive stores. Indeed, half-maximal stimulation of 45Ca^{2+} release was apparent with a concentration of cell-membrane derived Ins(1,4,5)P_{3} of 60-80 pmol/mg of protein (which, at 6mg of protein/ml would give approximately 0.4μM Ins(1,4,5)P_{3}, similar to that of exogenous Ins(1,4,5)P_{3} (0.21μM) under identical condition. The dose-response curve for 45Ca^{2+} release was to the left of that for Ins(1,4,5)P_{3} accumulation in the presence of GTPyS. This may be explained, in part, by the fact that while Ins(1,4,5)P_{3} accumulation has increased by 43%, maximal 45Ca^{2+} mobilisation only increased by 28%, approaching the limit of 45Ca^{2+} mobilisation possible, after which the dose-response curve moves to the left. It does appear that endogenously produced Ins(1,4,5)P_{3} cannot access all the Ins(1,4,5)P_{3}-sensitive Ca^{2+} stores, as previously observed by Zhao et al. (1990). This GTPyS-induced decrease in EC_{50} and increase in Ins(1,4,5)P_{3} production appears to reflect increased efficiency in coupling between the muscarinic receptor and PLC. If it is assumed that the limit on the degree of Ins(1,4,5)P_{3} formation is governed by the activity of PLC, and that the limit is approached by carbachol alone, then increasing this coupling, by the addition of GTPyS, will only slightly increase the formation of Ins(1,4,5)P_{3} to this upper limit. This is followed by a decrease in the number of receptors required to be occupied to maximally stimulate Ins(1,4,5)P_{3} production. However, as carbachol-induced Ca^{2+} release appears to be already maximal, any enhancement of coupling will cause a leftward shift in EC_{50}. This is in contrast to permeabilised GH3 cells and Swiss 3T3 fibroblasts, where the EC_{50} for TRH- and bombesin-induced accumulation of inositol phosphates was unaltered by inclusion of a maximal dose of GTPyS, however,
maximal stimulation was substantially increased (Martin et al., 1986; Plevin et al., 1990).

Fisher and Snider (1987) have previously shown that the SK-N-SH cell line (of which the SH-SY5Y cell line is a subclone) have a small muscarinic receptor reserve for activation of phosphoinositide hydrolysis. The EC50 value for inositol phosphate formation was 10µM, marginally lower than the K50 value of 35µM. This appears also to be the case for SH-SY5Y cells, where the EC50 values for Ins(1,4,5)P3 formation (47µM) and Ca2+ release (49µM) are slightly lower than the K50 value for carbachol binding to the low affinity state of the receptor (102-104µM). This receptor reserve is much smaller than that observed for the consequences of activation of other biochemical systems, such as muscarinic receptor-induced inhibition of adenylyl cyclase. In chick heart membranes, low affinity carbachol binding (Kd=54µM) correlated well with the EC50 for inositol phosphate formation (20µM). However, a large receptor reserve for carbachol was observed for the inhibition of adenylyl cyclase (IC50=0.2µM) (Brown and Brown, 1984; Brown and Goldstein, 1986) and in 1321N1 cells, where oxotremorine acted as a full agonist for the inhibition of adenylyl cyclase, with little receptor reserve (Harden et al., 1986). Removal of receptors, by irreversible alkylation using propylbenzilylcholine mustard, confirmed there were large differences in receptor reserve for these two systems (some 100-fold), and no difference at the level of the muscarinic receptor (Brown and Goldstein, 1986).

Occupancy of 10-33% of available muscarinic receptors by carbachol in SK-N-SH cells was required for a half maximal stimulation of phosphoinositide turnover (Fisher and Snider, 1987). GTPγS, which, whilst decreasing the affinity of a receptor for an agonist (Evans et al., 1985; Birnbaumer et al., 1990; Wojciechowska et al., 1990b), vastly decreases the number of receptors required to be occupied to produce the same response. A second level at which the response to carbachol is amplified is the production of Ins(1,4,5)P3. It appears that while only 10-33% of muscarinic receptors need to be occupied to stimulate half-maximal production of inositol phosphates, ~30% of maximal formation is required to mobilise 50% of the available loaded Ca2+ from stores (Fisher and Snider, 1987). Similar results were obtained by Bielkiewicz-Vollrath et al. (1987) when looking at the ability of histamine to liberate inositol trisphosphates and cause contraction of guinea-pig ileum longitudinal muscle, where a dose of histamine liberating ~20% of maximal inositol trisphosphates caused half maximal contraction, and by Meurs et al. (1986) in bovine airway smooth muscle, where a maximal contractile response to methacholine was obtained when 3.5% of the maximal production of inositol phosphates was achieved. The partial agonists,
oxotremorine and McN A-343 liberated only 46.3 and 6.4% of the inositol phosphates liberated by methacholine, but caused 100 and 89% of the maximal contraction, identifying a large receptor reserve between Ins(1,4,5)P3 formation and muscle contraction (Meurs et al., 1988). In a similar system, Thomas et al. (1984) observed the EC50 for vasopressin-induced Ca2+ mobilisation was 25-fold less than that for Ins(1,4,5)P3 production in isolated rat hepatocytes, such that only 15% of the maximal Ins(1,4,5)P3 that could be generated was required to mobilise Ca2+ fully, and more recently by Thompson et al. (1991b), who found the EC50 of fMLP for Ca2+ mobilisation was 30-fold lower than that for Ins(1,4,5)P3 formation in human neutrophils. Less than 40% of receptors needed to be occupied to fully mobilise Ca2+, indicating the presence of a small receptor reserve for Ca2+ mobilisation. However, in intact 1321N1 cells, the correlation between inositol phosphate formation and Ca2+ mobilisation was very tight (Evans et al., 1985).

The possible cause of the discrepancy between EC50 values for carbachol-induced Ins(1,4,5)P3 accumulation and Ca2+ mobilisation being due to the overproduction of Ins(1,4,5)P3 in this system (more Ins(1,4,5)P3 being formed than was required to fully deplete the available Ca2+ stores) was then studied using the two partial agonists, arecoline and oxotremorine, which have been reported as being 64 and 45% of the efficacy of the full agonist carbachol for stimulation of 45Ca2+ efflux from 1321N1 human astrocytoma cells (Evans et al., 1985), which also contain muscarinic M3 receptors (Kunysz et al., 1989). Partial agonists are less able to transduce receptor occupancy into a response and must occupy more receptors than full agonists to achieve an optimum effect. This is consistent with partial agonists being less able to cause a conformational change of the receptor which imbues it with its catalytic properties regarding activation of a related G-protein. Ek and Nahorski (1988) have shown a correlation between the EC50 for carbachol-induced inositol phosphate formation and the efficacy of oxotremorine. Looking at guinea pig parotid gland, ileum and cerebral cortex, they observed an inverse log-linear relationship. In cortex, the EC50 for carbachol was 40μM and oxotremorine produced 9% of the maximal carbachol response with an EC50 of 0.8μM. In ileum and parotid gland, the EC50 for carbachol was 16μM and 4μM while oxotremorine produced 18 and 32% of the maximal carbachol response, with EC50 values of 0.6μM in both. This can be explained by increasing receptor reserves for carbachol from cortex to ileum to parotid gland. The EC50 for carbachol in SH-SY5Y cells in the present study was similar to that in guinea pig cortex, where only a small receptor reserve was observed.

Arecoline acted as a partial agonist in this system. Arecoline was bound by the muscarinic receptor with K50=27μM, identical to that observed in the presence of
It is of concern that both Ins(1,4,5)P₃ production and ⁴⁵Ca²⁺ mobilisation dose-response curves (EC₅₀ values 51 and 46µM respectively) lie to the right of the agonist binding curve. It is difficult to explain, once all the receptors are bound by agonist, how any further increase in agonist concentration can enhance the response. It is, therefore, proposed that the EC₅₀ values are subject to some error, as shown by the large range encompassed by 95% confidence limits. Equally, the inclusion of GTPγS is seen to shift the Ins(1,4,5)P₃ production curve greater for arecoline than for carbachol, whereas the shifts in the Ca²⁺ mobilisation curves for carbachol are almost 4-fold greater than for arecoline.

Again, in the case of arecoline, the dose-response curve for ⁴⁵Ca²⁺ mobilisation was to the left of that for Ins(1,4,5)P₃ accumulation in the presence of GTPγS. An overproduction of Ins(1,4,5)P₃, as described above, may, once again, explain this discrepancy. It appears that in the presence of GTPγS, efficiency of receptor-PLC coupling is such that a large receptor reserve is produced. In the case of a full agonist, enhanced coupling and increased Ins(1,4,5)P₃ accumulation, cannot induce greater release of Ca²⁺ from internal stores, however, a large shift in EC₅₀ is observed. In the case of the partial agonist arecoline, which alone is unable to maximally mobilise Ca²⁺, the addition of GTPγS enhances coupling such that, firstly, maximal Ca²⁺ release is obtained and, secondly, a receptor reserve is produced. Clearly, the apparent receptor reserve is much smaller than for a full agonist, where only a shift in the EC₅₀ for Ca²⁺ release is observed. The effects of GTPγS on an even smaller partial agonist, such as oxotremorine, should, therefore, either only increase maximal Ca²⁺ mobilisation with no shift in EC₅₀ or a shift smaller than that observed with arecoline.

Oxotremorine alone stimulated the production of Ins(1,4,5)P₃ with EC₅₀ of 0.52µM and mobilised ⁴⁵Ca²⁺ with EC₅₀ of 0.36µM. This is in good agreement with the EC₅₀ values obtained for oxotremorine-induced increases in total inositol phosphate formation (EC₅₀=0.5µM) in guinea pig neostriatal slices (Fisher and Bartus, 1985) and in guinea pig cerebral cortex (0.8µM), ileum and parotid gland (0.6µM) (Ek and Nahorski, 1988). Maximal accumulation of Ins(1,4,5)P₃ was enhanced almost 5-fold by the inclusion of GTPγS with no significant shift in EC₅₀, to a level similar to that observed with carbachol alone. The absence of a shift in the EC₅₀ values for Ins(1,4,5)P₃ accumulation and Ca²⁺ mobilisation gives credence to the hypothesis that the discrepancies in EC₅₀ values for carbachol and arecoline in the presence of GTPγS are due to the limiting amount of Ca²⁺ being mobilised under these conditions.

The actions of GDPβS in inhibiting agonist-induced ⁴⁵Ca²⁺ mobilisation, and the ability of muscarinic agonists to mobilise ⁴⁵Ca²⁺ imply there is sufficient endogenous
GTP to enable Gq to activate PI-PLC as is observed in other permeabilised cell preparations (Martin et al., 1986; Jones et al., 1988; Fisher et al., 1989; Cattaneo and Vicentini, 1989) but not in membrane preparations (Hepler and Harden, 1986; Boyer et al., 1989; Claro et al., 1989), where PI-PLC activation is observed only in the presence of GTP or similar analogue. HPLC analysis revealed there to be ~50μM GTP in cell suspensions at the cell densities used throughout these studies (data not shown). Additional GTP (1mM) did not mobilise 45Ca2+ alone and did not significantly affect carbachol-induced Ca2+ mobilisation (n=4, data not shown).

In all the studies presented here, it is noteworthy that agonists were unable to mobilise all of the Ins(1,4,5)P3-sensitive pool. Similarly, Streb et al. (1983) found that maximal doses of the muscarinic agonist carbachol, were unable to mobilise fully the Ins(1,4,5)P3-sensitive pool. Equally, work by Zhao et al. (1990) found that, in permeabilised UMR-106-01 cells, a single agonist, even in the presence of GTPyS, was unable to mobilise fully the Ins(1,4,5)P3-sensitive pools. However, the combination of two agonists, working at different receptors, in the presence of GTPyS were able to mobilise all the Ins(1,4,5)P3-sensitive stores. Furthermore, Zhao et al. (1990) showed that, in UMR-106-01 cells, agonist-induced Ca2+ mobilisation was less sensitive to the Ins(1,4,5)P3 receptor antagonist, heparin, than Ca2+ mobilisation elicited by the addition of exogenous Ins(1,4,5)P3. Using heparin to block the effects of agonist and Ins(1,4,5)P3, they showed that, when produced in response to an agonist, Ins(1,4,5)P3 was not evenly distributed throughout the suspension of permeabilised cells. They interpreted such results as identification of compartmentalised generation and action of Ins(1,4,5)P3 during agonist stimulation. It may be possible that different receptors target overlapping, but different Ins(1,4,5)P3-sensitive pools, such that a single agonist cannot mobilise all the Ins(1,4,5)P3-sensitive pools. That is, Ins(1,4,5)P3 is produced in specific, localised regions within the cell, probably proximal to regions rich in Ins(1,4,5)P3 receptors, and is not produced diffusely throughout the cell. Similarly, quantification of resting Ins(1,4,5)P3 in intact cells, routinely in the μM range (Zhao et al., 1990; Challiss et al., 1990), which would be expected to fully mobilise Ca2+, appears unable to do so, and, therefore, may be compartmentalised and unable to access the Ins(1,4,5)P3 receptor. Earlier evidence, that inositol phosphates are not homogeneously distributed throughout the assay volume with permeabilised cells came from Martin et al. (1986), who showed that [3H] InsP, InsP2 and InsP3 levels were raised initially to a higher level within the disrupted cell, when compared with levels in the extracellular buffer. At later time points, however, levels of InsP2 and InsP3 were higher in the extracellular buffer than within the cells, presumably due to higher concentrations of metabolic enzymes retained within the cells. InsP levels remained higher within the
cells throughout (up to 20 minutes), presumably due to enhanced local production and inhibition of metabolism by the inclusion of the monophosphatase inhibitor, Li+, the effects of which did not appear to be affected by cell permeabilisation.

The observation that GTP was present in suspensions of permeabilised cells, at a level not less than 10% of that found in intact cells, despite extensive washing protocols (Martin et al., 1986), suggests that this GTP is tightly bound to proteins. Such explanations do not appear likely for Ins(1,4,5)P3, which, at levels of 1-2μM in intact cells is lowered to ~0.05μM in permeabilised cells (Zhao et al., 1990; this study). Alternatively, the protein(s) which bind(s) Ins(1,4,5)P3 may be soluble, and readily washed from the cells.

GTP and its analogues were unable to affect the dose response relationship for Ins(1,4,5)P3. This is further confirmatory evidence that the Ins(1,4,5)P3 receptor is not coupled to Ca2+ gating through a G-protein (Mignery et al., 1990 and refs. therein). It is of interest that while GTPγS alone mobilised 13% of 45Ca2+, these effects were neither synergistic with, nor additive to Ins(1,4,5)P3, thus suggesting they mobilise Ca2+ via the same mechanism, GTPγS activating PI-PLC to produce low levels of cell membrane-derived Ins(1,4,5)P3.

4.5 Summary.

In summary, these data provide evidence that not only is the linkage between receptors and PI-PLC maintained, but that Ca2+ stores remain sensitive to endogenously produced messenger, Ins(1,4,5)P3. Whilst the time course of Ins(1,4,5)P3 accumulation is different in permeabilised cells compared with intact cells, its role as a second messenger is still maintained, and it mobilises Ca2+ potently. Permeabilised cell preparations have the advantage over intact cells, in that agents can be introduced which interact with intracellular components of the signalling pathway. Thus, agents such as GTP, GTPγS, GDP, GDPβS, heparin and L-ch Ins(1,4,6)PS3 (see Chapter 3) can be used to further dissect aspects of receptor activated second messenger signalling. Similarly, when compared with membrane preparations, in which membrane-impermeant agents can also be used to affect receptor/G-protein/PI-PLC functions, permeabilised cell preparations are advantageous in that responses downstream of Ins(1,4,5)P3 and DAG production can be monitored and influenced, and this study identifies the potential of this useful model in which such experiments can be performed. This study, while characterising
muscarinic receptor responses in permeabilised cells also identifies different levels of receptor reserve for distinct levels within this second messenger pathway.
CHAPTER 5.
Desensitisation of Muscarinic Receptor Responses Following Agonist Stimulation.

5.1 Introduction.

In many systems, exposure of cells, tissues or whole animals to the continued presence of neurotransmitter or other receptor agonists can have both short and long term effects on both the number and function of receptors present. The loss of physiological responsiveness of receptors that occurs seconds or minutes after agonist exposure is termed desensitisation.

Receptors linked to PI-PLC show remarkable variability in their sensitivity to homologous desensitisation. This has been demonstrated most clearly in studies in which the stimulatory effects of more than one receptor have been examined in a particular cell type. For example, measurement of total inositol phosphate accumulation in the presence of Li⁺ in 1321N1 cells showed that H₁-histamine receptors were desensitised rapidly (within 5 minutes), whereas muscarinic receptors activated phosphoinositide hydrolysis for up to 1 hour (Nakahata and Harden, 1987). Similarly, in rat parotid acinar cells (Sugiya et al., 1987) and pancreatoma AR4-2J cells (Menniti et al., 1991) substance P receptor-mediated stimulation of inositol phosphate formation was rapidly and virtually completely desensitised within 1 minute, whereas stimulation via other receptors (for example, muscarinic and bombesin) was sustained. PI-PLC-linked receptor desensitisation has also been demonstrated in many other systems; for example, the platelet activating factor receptor in rabbit platelets (Morrison and Shulda, 1988), the P₂Y-purinergic receptor in turkey erythrocytes (Martin and Harden, 1989), the bradykinin receptor in NG108-15 cells (Wolsing and Rosenbaum, 1991), the serotonergic receptor in P11 and cerebellar granule cells (Ivins and Molinoff, 1991; Dillon-Carter and Chuang, 1989) and the α₁-adrenoceptor in cerebellar granule and smooth muscle cells (Dillon-Carter and Chuang, 1989; Leeb-Lundberg et al., 1987).

These differences in the susceptibility of PI-PLC-linked receptors to desensitisation are likely to be a reflection of distinct regulatory mechanisms. Details of PI-PLC-linked receptor modification (for example, by phosphorylation) are now emerging,
but the nature of these distinct mechanisms is still being established (Klueppelberg et al., 1991; Tobin and Nahorski, 1993).

In 1985, Masters et al. demonstrated that pre-exposure of 1321N1 astrocytoma cells to carbachol, whilst not resulting in a decrease in the rate of inositol phosphate production, resulted in a loss of stimulated Ca\(^{2+}\) efflux. They concluded that desensitisation occurred distal to PI turnover. Histamine-stimulated Ca\(^{2+}\) efflux was also lost in cells treated with carbachol, indicating that the desensitisation observed was heterologous. However, these results may merely be due to depletion of Ca\(^{2+}\) pools.

A change in the number of receptors at the cell surface is an obvious way in which the strength of a signal generated by a given agonist can be modulated. Initially, this is achieved by internalisation, a process which entails the rapid transfer of receptors from the cell surface to vesicular sites in the cell interior. Although receptors are recycled to the cell surface, it appears that long-term treatment with agonist can divert a proportion of these receptors to degradation sites. This leads to down regulation, a decrease in the total receptor number.

### 5.1.1 Loss of Cell Surface Receptors

Evidence that receptors involved in the activation of PI metabolism can be internalised after agonist stimulation comes from many studies (Maloteaux et al., 1983; Harden et al., 1985; Feigenbaum and El Fakahany, 1985; Sugiya et al., 1987; Cantau et al., 1988; Thompson and Fisher, 1990) and is consistent with other transmembrane signalling systems (see Benovic et al., 1988; Kobilka, 1992).

Internalisation of PI-PLC-linked receptors is known to occur rapidly, with \(t/2 \leq 10\) minutes for \(\alpha_1\) (Leeb-Lundberg et al., 1987), substance P (Sugiya et al., 1987), bombesin (Zhu et al., 1991), neurotensin (Turner et al., 1990) and muscarinic (Harden et al., 1985; Thompson and Fisher, 1990; Turner et al., 1990) receptors. The extent to which the number of cell-surface receptors can be reduced by internalisation is variable, but generally 40-75%. Internalisation and recycling are both temperature dependent and at reduced temperatures (below \(-20^\circ C\)) both processes are slowed markedly (El Fakahany and Richelson, 1980; Maloteaux et al., 1983; Feigenbaum and El Fakahany, 1985; Thompson and Fisher, 1991).
Although it appears that receptors are internalised following plasma membrane invagination and endocytosis and that they become localised in vesicles, it has not been established whether a universal mechanism of internalisation is applicable to all PI-PLC-linked receptors. In some cases, for example the V1 vasopressin receptor in WRK1 cells (Cantau et al., 1988), internalised receptors are localised in vesicles that are coated with clathrin. Such internalisation via 'coated pits' has been observed for other classes of receptor and is part of a fundamental endocytic process (Goldstein et al., 1985). In contrast, other receptors, for example muscarinic receptors in fibroblasts (Raposo et al., 1987), appear to be internalised by a similar mechanism but via 'non-coated pits'.

There is a good correlation between the efficacy of an agonist as a stimulus of PI-PLC and internalisation. Thus, full agonists more readily cause internalisation than do partial agonists (Thompson and Fisher, 1990; Stoll and Muller, 1991), and antagonists are ineffective (Feigenbaum and El Fakahany, 1985; Lutz et al., 1992). Interestingly, this may be unrelated to differing extents of PI-PLC activation, as recent evidence showing that removal of extracellular Ca\(^{2+}\) from SK-N-SH neuroblastoma cells attenuates inositol phosphate formation, but not receptor internalisation, indicates that internalisation is not dependent on PI hydrolysis (Thompson et al., 1991a). Rather, mere occupation with agonist, perhaps with the involvement of a G-protein, may provide the signal that targets the receptor for internalisation. This presents an intriguing parallel with the \(\beta\)-adrenoceptor system in which a \(\beta\)-adrenoceptor kinase phosphorylates preferentially the agonist occupied receptor (see Benovic et al., 1988; Kobilka, 1992). Whether a similar kinase phosphorylates some or all PI-PLC-linked receptors (Klueppelberg et al., 1991; Tobin and Nahorski, 1993) remains to be seen.

5.1.2 Down Regulation of Internalised Receptors.

Down regulation occurs much more slowly than internalisation and at markedly different rates in different tissues. For example, for PI-PLC-linked muscarinic receptors in 1321N1 cells (Harden et al., 1985; Hoover and Toews, 1989), SK-N-SH cells (Thompson and Fisher, 1990), N1E-115 cells (Taylor et al., 1979) and CHO cells transfected with m1 and m3 muscarinic receptors (Hu et al., 1991), the \(t_1/2\) of down regulation is approximately 4, 5, 4, and 13 hours, respectively.
In this chapter I have presented data in which I have used the permeabilised SH-SY5Y cell preparation described in Chapter 3 to study the mechanisms of desensitisation of muscarinic responses following agonist stimulation of intact cells.

5.2 Methods.

These studies were performed in SH-SY5Y cells cultured, as described in Chapter 2 (2.1), in culture medium comprising minimum essential medium supplemented with 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin, 2.5mg/ml amphotericin B (fungizone), and 10% foetal calf serum. Cultures were maintained at 37°C in 5% CO₂, 95% humidified air. Prior to use, cells were harvested and washed in Krebs-HEPES solution, in which binding assays to intact cells were performed (see Chapter 2(2.7.1)). Alternatively, cells were washed in intracellular buffer (ICB), comprising of 120mM KCl, 20mM HEPES, 6mM MgCl₂, 5mM sodium succinate, 5mM Na₂ATP, 2mM KH₂PO₄, 10-30µM EGTA (to reduce free Ca²⁺ concentration to 70-300nM), pH 6.9. Cells were permeabilised using 3 discharges of 1.50kV through a 3µF capacitor across a 0.4cm cuvette. The effects of muscarinic agonists and antagonists, and guanine nucleotides were studied in these permeabilised cell preparations. Binding properties, and the abilities of these agents to mobilise Ca²⁺ were then examined.

Ca²⁺ release assays were performed with electroporated cells. Ca²⁺ release profiles were determined in permeabilised cells washed in ICB and loaded with ⁴⁵Ca²⁺ for 12 minutes, after which stimuli were added. Incubations were terminated by spinning the cells through a silicone oil mixture, separating cells from the stimulus-containing supernatant. Cells were then assessed for radioactivity (Chapter 2 (section 2.2)). Binding characteristics of agents to permeabilised cells was determined following washing the cells in a Mg²⁺ and HEPES-containing buffer, in which receptor binding assays were performed, as has been previously described for membrane preparations (Lambert et al., 1989).

Pretreatment of intact cells was performed in Krebs buffer (NaCl, 118.6mM; KCl, 4.7mM; MgSO₄,6H₂O, 1.2mM; KH₂PO₄, 1.2mM; NaHCO₃, 25mM; glucose, 11.7mM; CaCl₂, 1.3mM, pH 7.4), or nominally Ca²⁺-free Krebs-like buffer (as above, omitting CaCl₂). Cells were incubated at 37, 20 or 1-2°C in the presence of carbobachol (1µM-1mM) for 5 minutes-5 hours. Pretreatment was terminated by
washing the cells in ice-cold Krebs buffer to remove all carbachol present. Cells were then treated in one of four ways.

1. Cells were washed in ice-cold Krebs-HEPES buffer, and the binding of $[^3H]NMS$ was monitored as described in Chapter 2 (section 2.7.1). Alternatively, cells were washed in ice-cold ICB, permeabilised electrically as described (Chapter 2, section 2.2) and treated as follows:-

2. Cells were incubated in $^{45}\text{Ca}^{2+}$-containing ICB, pH 6.9 at 20°C for 12 minutes and $^{45}\text{Ca}^{2+}$ release experiments were performed as described above.

3. Cells were washed in Mg$^{2+}$/HEPES buffer, pH 6.9 and binding of $[^3H]NMS$ to permeabilised cells at 1-2°C was examined as described in Chapter 2 (section 2.10.2).

4. Cells were washed in NaCl/MgCl$_2$/HEPES buffer, pH 6.9 and the binding of $[^35S]GTPyS$ at 20°C was examined as described in Chapter 2 (section 2.11).

5.3 Results.

5.3.1 Effects on Cell-Surface Receptors.

The effects of carbachol on both the number and affinity of muscarinic receptors, either specifically located at the cell surface or distributed throughout the cell was monitored by means of comparing the binding of $[^3H]NMS$ to intact cells at 1-2°C and 37°C.

Carbachol (1mM) caused a rapid loss of cell-surface receptors in a time- and temperature dependent manner. Cells exposed at 37°C to carbachol lost maximally 51±5% (at 40 minutes) of their cell-surface receptors with a half time of approximately 6 minutes. Cell-surface receptors of cells exposed at 20°C were lost more slowly, with a half time of 29 minutes, but maximal loss (attained within 3 hours) was no different (53±5%). Receptor internalisation followed similar kinetics if carbachol pretreatment at 20°C was performed in nominally Ca$^{2+}$-free Krebs-like buffer, 53±3% of cell-surface receptors were lost, with a $t_{1/2}$ of 26 minutes. (Fig. 5.1).

Cell surface receptor number was assessed by following the binding of $[^3H]NMS$ at 1-2°C, terminating incubations after a 6 hour incubation. In assays allowed to progress for 20 hours, cells pretreated with carbachol had less of an apparent loss of cell-surface receptors (23%). It appears that even at such temperatures internalised
Figure 5.1 Time course of loss of cell-surface receptors following treatment with carbachol. Cells were treated with carbachol (1mM) in Ca^{2+}-containing Krebs buffer at 37°C (filled squares) or 20°C (open squares), or in Ca^{2+}-free Krebs-like buffer at 20°C (filled diamonds) for 5-180 minutes. Subsequently, cell surface receptor number was determined by following the binding of [3H] NMS to intact cells at 1-2°C. Data represent means from at least 6 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 6%). All values were significantly different from control, except 20 minute point at 20°C in Krebs buffer. The rates of receptor sequestration at 20°C, whether pretreatment was performed in the presence or absence of extracellular Ca^{2+}, appear the same, with many coincident data points.
receptors are able to return to the cell-surface. Indeed, if, after carbachol pretreatment, cells were incubated alone at 1-2°C for 14 hours and then with [3H] NMS for 6 hours similar results were obtained as if cells were incubated with [3H] NMS for the whole 20 hours (data not shown). Thus, it appears, this increase of maximal binding is due to receptors returning to the cell-surface, and not [3H] NMS entering the cell. Binding assays performed at 37°C for 60 minutes showed no loss of [3H] NMS binding. This suggests that receptor 'internalised' were capable of rapidly returning to the cell-surface, as has been observed with the $\alpha_1$ adrenoceptor (Leeb-Lundberg et al., 1987).

To determine the nature of the remaining cell-surface receptors, the ability of the selective antagonists, pirenzepine, methoctramine and pF-HHSD to compete with [3H] NMS for binding sites was studied. Cells were pretreated with carbachol (1mM) for 60 minutes at 37°C, washed extensively, and competition binding assays were performed at 1-2°C for 6 hours. This pretreatment had no significant effect on the binding affinity or Hill slope for any of the four antagonists, suggesting that the predominantly expressed M3 receptor subtypes remain so after pretreatment (Table 5.1).

The effects of carbachol pretreatment on the binding characteristics of cells subsequently permeabilised was also studied. Permeabilised cells bound [3H] NMS with higher affinity than intact cells, this was not affected by the inclusion of GTP (1mM) (Table 5.2). After carbachol pretreatment a similar loss of binding sites was observed with intact cells, suggesting these receptors were taken into a lipophilic environment. The ability of carbachol to compete with [3H] NMS in the absence and presence of GTP was also studied. In cells not pretreated with agonist, carbachol displaced [3H] NMS in a complex manner. Two sites were observed, with some 10-fold separation in affinity. Inclusion of GTP did not affect the affinity of the two sites, but decreased the proportion of the high affinity sites. Carbachol pretreatment did not affect the high affinity state or the proportion of low to high affinity states. However, the lower affinity state bound carbachol with even lower affinity. Carbachol pretreatment also abolished the effects of including GTP (Table 5.2).
Table 5.1. Effects of carbachol (1mM) pretreatment on antagonist affinity in SH-SYSY Cells.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>B&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (Control)</th>
<th>Desensitised&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS (n=15)</td>
<td>365±35</td>
<td>155±36</td>
</tr>
<tr>
<td>KD(nM)</td>
<td>0.65</td>
<td>0.54</td>
</tr>
<tr>
<td>nH</td>
<td>1.03±0.02</td>
<td>1.03±0.03</td>
</tr>
<tr>
<td></td>
<td>(0.41-1.02)</td>
<td>(0.29-1.03)</td>
</tr>
<tr>
<td>PZ (n=3)</td>
<td>510</td>
<td>360</td>
</tr>
<tr>
<td>K&lt;sub&gt;S0&lt;/sub&gt;(nM)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.92±0.07</td>
<td>0.86±0.03</td>
</tr>
<tr>
<td>nH</td>
<td>1.3±0.1</td>
<td>1.12±0.04</td>
</tr>
<tr>
<td></td>
<td>(330-780)</td>
<td>(3900-8700)</td>
</tr>
<tr>
<td>Methoc. (n=3)</td>
<td>7100</td>
<td>5800</td>
</tr>
<tr>
<td>K&lt;sub&gt;S0&lt;/sub&gt;(nM)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.3±0.1</td>
<td>1.12±0.04</td>
</tr>
<tr>
<td>nH</td>
<td>1.3±0.1</td>
<td>1.12±0.04</td>
</tr>
<tr>
<td></td>
<td>(3500-14600)</td>
<td>(3900-8700)</td>
</tr>
<tr>
<td>pF-HHSD (n=6)</td>
<td>290</td>
<td>190</td>
</tr>
<tr>
<td>K&lt;sub&gt;S0&lt;/sub&gt;(nM)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.97±0.05</td>
<td>0.86±0.12</td>
</tr>
<tr>
<td>nH</td>
<td>1.97±0.05</td>
<td>0.86±0.12</td>
</tr>
<tr>
<td></td>
<td>(170-490)</td>
<td>(100-370)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells pretreated for 60 minutes with carbachol (1mM) at 37°C in Krebs buffer.

<sup>b</sup> Maximal specific binding of [3H] NMS (fmol/mg of protein) at 1-2°C, expressed as mean±S.E.M. (n=15).

<sup>c</sup> Dissociation constant, expressed as mean, followed by 95% confidence limits in parentheses.

<sup>d</sup> Hill slope of binding, expressed as mean±S.E.M.

<sup>e</sup> IC<sub>S0</sub> corrected for mass of radioligand, expressed as mean, followed by 95% confidence limits in parentheses.
Table 5.2. Effects of carbachol (1mM) pretreatment on agonist binding affinity in Permeabilised SH-SY5Y Cells.

For legends to table see overleaf.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Desensitised&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td>299±40</td>
<td>169±32</td>
</tr>
<tr>
<td>+GTP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>283±43</td>
<td>167±21</td>
</tr>
<tr>
<td>K&lt;sub&gt;p&lt;/sub&gt;(nM)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.35</td>
<td>0.35 nM</td>
</tr>
<tr>
<td>nH&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.08±0.03</td>
<td>1.07±0.03</td>
</tr>
<tr>
<td>NMS</td>
<td>0.35</td>
<td>0.38</td>
</tr>
<tr>
<td>(0.20-0.60)</td>
<td>(0.27-0.61)</td>
<td>(0.27-0.53)</td>
</tr>
<tr>
<td>+GTP</td>
<td>0.41</td>
<td>0.38</td>
</tr>
<tr>
<td>(0.27-0.61)</td>
<td>(0.27-0.53)</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;50&lt;/sub&gt;(μM)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.08±0.03</td>
<td>1.05±0.03</td>
</tr>
<tr>
<td>CCh</td>
<td>65</td>
<td>94</td>
</tr>
<tr>
<td>(42-101)</td>
<td>(52-170)</td>
<td></td>
</tr>
<tr>
<td>+GTP</td>
<td>86</td>
<td>94</td>
</tr>
<tr>
<td>(56-128)</td>
<td>(65-135)</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;H&lt;/sub&gt;(μM)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.81±0.03</td>
<td>0.70±0.04</td>
</tr>
<tr>
<td>P&lt;sub&gt;H&lt;/sub&gt;&lt;sup&gt;h&lt;/sup&gt;</td>
<td>67±8</td>
<td>67±8</td>
</tr>
<tr>
<td>(1.2-10.2)</td>
<td>(1.2-10.2)</td>
<td></td>
</tr>
<tr>
<td>+GTP</td>
<td>3.5</td>
<td>100</td>
</tr>
<tr>
<td>(1.2-10.2)</td>
<td>(60-160)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.5</td>
<td>100</td>
</tr>
<tr>
<td>(1.2-10.2)</td>
<td>(60-160)</td>
<td></td>
</tr>
<tr>
<td>+GTP</td>
<td>7.1</td>
<td>100</td>
</tr>
<tr>
<td>(3.8-13.1)</td>
<td>(70-140)</td>
<td></td>
</tr>
<tr>
<td>Desensitised</td>
<td>4.0</td>
<td>210</td>
</tr>
<tr>
<td>(1.5-10.6)</td>
<td>(130-350)</td>
<td></td>
</tr>
<tr>
<td>+GTP</td>
<td>4.0</td>
<td>190</td>
</tr>
<tr>
<td>(2.4-40.9)</td>
<td>(120-290)</td>
<td></td>
</tr>
</tbody>
</table>
Cells pretreated for 60 minutes with carbachol (1mM) at 37°C in Krebs buffer.

Maximal specific binding of [3H] NMS (fmol/mg of protein) at 1-2°C, expressed as mean±S.E.M.

Assay performed in the presence of GTP (1mM).

Dissociation constant, expressed as mean, followed by 95% confidence limits in parentheses.

Hill slope of binding, expressed as mean±S.E.M.

IC50 corrected for mass of radioligand, expressed as mean, followed by 95% confidence limits in parentheses.

IC50 of carbachol for high affinity binding sites, corrected for mass of radioligand.

Proportion of high affinity binding sites.

IC50 of carbachol for low affinity binding sites, corrected for mass of radioligand.

Proportion of low affinity binding sites.

p<0.05 compared with control cells in the absence of GTP.

not significantly different to desensitised cells in the absence of GTP.

5.3.2 Effects on Ca2+ Mobilisation.

Cells pretreated with carbachol at 20°C or 37°C for different times were then cooled to 4°C and washed extensively with KHS followed by ICB. After permeabilisation, cells were loaded with 45Ca2+ at 20°C for 12 minutes and 45Ca2+ mobilisation assays were performed. Carbachol pretreatment at 37°C caused a rapid loss of subsequent response which was dependent on the time of preexposure. A small rightward shift in EC50 was accompanied by a decrease in maximal release (Fig 5.2). However, this cannot be solely attributed to the effects of carbachol on the muscarinic receptor, because, as described by Wojcikiewicz and Nahorski (1991), such pretreatment also attenuates the ability of Ins(1,4,5)P3 to release Ca2+. Muscarinic agonists mobilise Ca2+ via the formation of Ins(1,4,5)P3 and so it appears that desensitisation observed is occurring at many levels. If, however, carbachol pretreatment was performed at 20°C, no desensitisation of the Ins(1,4,5)P3 response was observed (data not shown). Such pretreatment did not affect the loading of 45Ca2+ into Ins(1,4,5)P3-sensitive stores. Desensitisation of the Ca2+ mobilisation response was, as seen with the loss of cell-surface receptors, markedly slower than that seen at 37°C, such that after 20 minutes the dose response relationship was shifted to the right, with no significant
Figure 5.2 Carbachol-induced $^{45}$Ca$^{2+}$ release following pretreatment with carbachol at 37°C. Intact cells were incubated in Krebs buffer in the absence (open squares) or presence of carbachol (1mM) for 10 (filled triangles), 20 (open circles), 40 (filled circles) or 60 (open triangles) minutes, after which the cells were cooled, washed and permeabilised. $^{45}$Ca$^{2+}$ release assays were performed as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (Mean±S.E.M.) were as follows:- Control: 5.5(2.3-13)$\mu$M, 51±3%; 10 minutes: 40(19-87)$\mu$M, 35±3%; 20 minutes: 44(22-88)$\mu$M, 29±3%; 40 minutes: 92(12-707)$\mu$M, 18±3%; 60 minutes: 61$\mu$M(approximately), 11±4%. Ins(1,4,5)P$_3$ (10$\mu$M)-induced $^{45}$Ca$^{2+}$ release was as follows:- Control: 64±1%; 10 minutes: 58±3%; 20 minutes: 62±2%; 40 minutes 60±2% (all not significantly different from control); 60 minutes: 55±2% (p<0.05). Data represent means from at least 6 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 5%).
loss of maximal release (Fig 5.3). In contrast to the effects on \[^{3}H\] NMS binding, removal of Ca\(^{2+}\) from the pretreatment buffer caused a dramatic enhancement in the rate of desensitisation of this response (Fig 5.4). A 5 minute treatment caused a doubling in the EC\(_{50}\) and a decrease in maximal response (Fig. 5.4). A 20 minute treatment with carbachol all but obliterated the response to a second dose of carbachol. Once again, the loading of \(^{45}\)Ca\(^{2+}\) into Ins(1,4,5)P\(_{3}\)-sensitive stores and the response to exogenous Ins(1,4,5)P\(_{3}\) was unaffected. The effects of carbachol were dose-dependent. Indeed, pretreatment for 2 hours at 20\(^\circ\)C with 1\(\mu\)M-1mM carbachol caused a graded loss, such that 1\(\mu\)M carbachol caused a small rightward shift in EC\(_{50}\), with 10, 100\(\mu\)M and 1mM lowering the maximal release by increasing amounts (Fig 5.5).

Dose-response relationships for carbachol were also performed in the presence of GTP\(_{\gamma}\)S. As described previously, GTP\(_{\gamma}\)S shifts the carbachol dose response relationship greatly to the left (see Chapter 4), by virtue of its ability to stabilise the active form of the G-protein (Taylor, 1989), thus producing a large receptor reserve for carbachol-stimulated Ca\(^{2+}\) release. The inclusion of GTP\(_{\gamma}\)S made the response less sensitive to desensitisation, such that a 10 minute pretreatment with carbachol at 37\(^\circ\)C, which caused a decrease in maximal carbachol-stimulated Ca\(^{2+}\) mobilisation from 57 to 35% did not affect maximal release in its presence. However, the EC\(_{50}\) was shifted greatly, from 0.27\(\mu\)M to 9.0 \(\mu\)M. Further carbachol pretreatment caused no further shift in EC\(_{50}\), but a loss of maximal release was observed (Fig 5.6). Similar pretreatments at 20\(^\circ\)C showed a very slow rate of desensitisation, causing only a 9-fold shift in EC\(_{50}\) after 60 minutes, and only after 3 hours was a decrease in the maximal release observed (Fig 5.7). The omission of Ca\(^{2+}\) from the preincubation buffer, which was observed to have a dramatic effect on the response to carbachol alone did not greatly enhance the rate of desensitisation in the presence of GTP\(_{\gamma}\)S. A 60 minutes pretreatment with carbachol which caused a 9-fold shift in EC\(_{50}\) in the presence of Ca\(^{2+}\), caused a 20-fold shift in its absence. A 300 minute pretreatment was required to lower maximal carbachol-induced \(^{45}\)Ca\(^{2+}\) release in the presence of GTP\(_{\gamma}\)S (Fig 5.8).

It is clear from the above data that carbachol interacts with the muscarinic receptor such that a small receptor reserve exists. That is, not all receptors need to be occupied to maximally mobilise Ca\(^{2+}\), as removal of some of these receptors by carbachol pretreatment does not immediately lead to a loss in maximal release, but rather, a rightward shift in EC\(_{50}\) which is then followed by this loss. This receptor reserve is much enhanced in the presence of GTP\(_{\gamma}\)S. However, as previously discussed, the partial agonist, oxotremorine, which alone has been shown to mobilise Ca\(^{2+}\) with
Figure 5.3 Carbachol-induced $^{45}$Ca$^{2+}$ release following pretreatment with carbachol at 20°C. Intact cells were incubated in Krebs buffer in the absence (open squares) or presence of carbachol (1mM) for 20 (filled diamonds), 60 (filled squares) or 90 (open diamonds) minutes, after which the cells were cooled, washed and permeabilised. $^{45}$Ca$^{2+}$ release assays were performed as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (Mean±S.E.M.) were as follows:- Control: 6(3-13)$\mu$M, 49±3%; 20 minutes: 20(10-47)$\mu$M, 35±3%; 60 minutes: 158(31-802)$\mu$M, 23±4%; 90 minutes: 110$\mu$M (approximately), 12±4%. Ins(1,4,5)$P_3$ (10$\mu$M)-induced $^{45}$Ca$^{2+}$ release was as follows:- Control: 62±3%; 90 minutes 58±4% (not significantly different from control). Data represent means from at least 6 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 5%).
Figure 5.4 Carbachol-induced $^{45}\text{Ca}^{2+}$ release following pretreatment with carbachol at 20°C in the absence of extracellular $\text{Ca}^{2+}$. Intact cells were incubated in $\text{Ca}^{2+}$-free Krebs-like buffer in the absence (open squares) or presence of carbachol (1 mM) for 5 (filled diamonds), 10 (filled squares) or 20 (open diamonds) minutes, after which the cells were cooled, washed and permeabilised. $^{45}\text{Ca}^{2+}$ release assays were performed as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (Mean±S.E.M.) were as follows:- Control: 6(2-15) µM, 47±3%; 5 minutes: 15(11-21) µM, 28±4%; 10 minutes: 71(16-302) µM, 16±9%; 20 minutes: 110µM(approximately), 8±2%. Ins(1,4,5)P$_3$ (10 µM)-induced $^{45}\text{Ca}^{2+}$ release was as follows:- Control: 60±4%; 20 minutes: 56±2%. Data represent means from at least 5 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 5%).
Figure 5.5 Carbachol-induced $^{45}\text{Ca}^{2+}$ release following pretreatment with carbachol at 20°C for 2 hours. Intact cells were incubated in Krebs buffer in the absence (open squares) or presence of 1μM (filled triangles), 10μM (open circles), 100μM (filled circles) or 1mM (open triangles) carbachol for 2 hours, after which the cells were cooled, washed and permeabilised. $^{45}\text{Ca}^{2+}$ release assays were performed as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (Mean±S.E.M.) were as follows:- Control: 3.3(1.5-7.3)μM, 50±5%; 1μM carbachol: 14(6-35)μM, 47±6%; 10μM carbachol: 39(19-80)μM, 37±6%; 100μM carbachol: 79(11-548)μM, 22±6%; 1mM carbachol: 121(51-285)μM, 12±4%. Ins(1,4,5)P$_3$ (10μM)-induced $^{45}\text{Ca}^{2+}$ release was as follows:- Control: 62±3%; 1mM carbachol: 58±2% (not significantly different from control). Data represent means from at least 3 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 7%).
**Figure 5.6** Carbachol-induced $^{45}\text{Ca}^{2+}$ release in the presence of GTPγS (100μM) following pretreatment with carbachol at 37°C. Intact cells were incubated in Krebs buffer in the absence (open squares) or presence of carbachol (1mM) for 10 (filled triangles), 20 (open circles), 40 (filled circles) or 60 (open diamonds) minutes, after which the cells were cooled, washed and permeabilised. $^{45}\text{Ca}^{2+}$ release assays were performed in the presence of GTPγS (100μM) as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (over GTPγS alone) (Mean±S.E.M.) were as follows:- Control: 0.25(0.16-0.38)μM, 36±2%; 10 minutes: 9(8.5-9.5)μM, 35±6%; 20 minutes: 6(3-11)μM, 27±3%; 40 minutes: 4(1.5-11)μM, 15±5%; 60 minutes: 12(8-18)μM, 15±3%. Ins(1,4,5)P$_3$ (10μM)-induced $^{45}\text{Ca}^{2+}$ release was as follows:- Control: 62±4%; 60 minutes: 52±4% (not significantly different from control). Data represent means from at least 4 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 5%).
Figure 5.7 Carbachol-induced $^{45}$Ca$^{2+}$ release in the presence of GTPγS (100μM) following pretreatment with carbachol at 20°C. Intact cells were incubated in Krebs buffer in the absence (open squares) or presence of carbachol (1mM) for 60 (filled squares), 120 (open circles) or 180 (open diamonds) minutes, after which the cells were cooled, washed and permeabilised. $^{45}$Ca$^{2+}$ release assays were performed in the presence of GTPγS (100μM) as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (over GTPγS alone) (Mean±S.E.M.) were as follows: Control: 0.08(0.04-0.14)μM, 34±3%; 60 minutes: 0.41(0.04-3.8)μM, 31±10%; 120 minutes: 5.2(1.1-25.2)μM, 38±2%; 180 minutes: 3.4(1.0-12)μM, 28±3%. Ins(1,4,5)P$_3$ (10μM)-induced $^{45}$Ca$^{2+}$ release as follows: Control: 57±3%; 180 minutes: 55±4% (not significantly different from control). Data represent means from at least 4 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 5%).
Figure 5.8 Carbachol-induced $^{45}\text{Ca}^{2+}$ release in the presence of GTPγS (100μM) following pretreatment with carbachol at 20°C in Ca$^{2+}$-free Krebs-like buffer. Intact cells were incubated in Ca$^{2+}$-free Krebs-like buffer in the absence (open squares) or presence of carbachol (1mM) for 20 (filled diamonds), 60 (filled squares) or 300 (open diamonds) minutes, after which the cells were cooled, washed and permeabilised. $^{45}\text{Ca}^{2+}$ release assays were performed in the presence of GTPγS (100μM) as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (over GTPγS alone) (Mean±S.E.M.) were as follows:- Control: 0.11(.05-.24)μM, 35±3%; 20 minutes: 0.71(.60-.84)μM, 29±7%; 60 minutes: 2.3(0.73-7.3)μM, 31±4%; 300 minutes: 8.7(2.8-27.1)μM, 27±4%. Ins(1,4,5)P$_3$ (10μM)-induced $^{45}\text{Ca}^{2+}$ release as follows:- Control: 56±2%; 300 minutes: 48±4% (not significantly different from control). Data represent means from at least 4 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 4%).
34% of the efficacy of carbachol, mobilises 79% in the presence of GTP\(_{\gamma}S\), without appearing to possess a receptor reserve (Chapter 4). Carbachol pretreatment at 37°C caused a rapid loss of response induced by oxotremorine in the presence of GTP\(_{\gamma}S\). No change in EC\(_{50}\) was observed, as would be expected in a system where no receptor reserve is present. However, maximal release declined rapidly. A 60 minute pretreatment totally abolished Oxo-enhancement of the GTP\(_{\gamma}S\) response (Fig 5.9).

A 60 minutes pretreatment with carbachol also inhibited the ability of carbachol to enhance the binding of \([^{35}S]\text{GTP}\_\gamma\text{S}\) to permeabilised cells. In control conditions, the rate of GTP\(_{\gamma}S\) binding was enhanced by a maximal dose of carbachol (1mM). Following carbachol pretreatment, \([^{35}S]\text{GTP}\_\gamma\text{S}\) binding was decreased slightly, but carbachol-enhancement was totally abolished (Fig. 5.10).

5.4 Discussion.

A vast number of reports previously stated that receptors linked to PI hydrolysis such as muscarinic receptors, are only slowly desensitised. These conclusions have been drawn from the apparent linear accumulation of inositol phosphates after agonist stimulation, which has been reported to continue for up to 2 hours (Masters et al., 1985; Thompson and Fisher, 1990). This is despite the loss of some 50% of cell surface receptors, which may recycle at raised temperatures (37°C). However, at reduced temperatures (<20°C) this recycling is greatly attenuated (Maloteaux et al., 1983). At these lower temperatures the accumulation of \([^3\text{H}]\text{inositol phosphates}\) is reduced after carbachol pretreatment (Thompson and Fisher, 1991). However, many other PI-linked receptors show rapid desensitisation of their responses. Histamine-induced inositol phosphate formation is transient, with production halting some 5 minutes subsequent to agonist stimulation (Nakahata and Harden, 1987). The effects of carbachol itself appear to be complex in 1321N1 cells. Nakahata and Harden (1987) have shown that while total inositol phosphates appear to accumulate linearly for at least 1 hour, \(\text{Ins}(1,4,5)\text{P}_3\) accumulation was greatly attenuated within 5 minutes. This can be accounted for in 2 ways: firstly, while \(\text{Ins}(1,4,5)\text{P}_3\) production remains linear, its metabolism is enhanced, or secondly, phosphoinositides other than \(\text{PtdIns}(4,5)\text{P}_2\) are being metabolised, producing \(\text{Ins}(1,4)\text{P}_2\) and \(\text{Ins}(1)\text{P}\) directly (see Imai and Gershengorn, 1986). Equally, histamine-induced incorporation of \(^{32}\text{P}\) into PIs shows a rapid and total desensitisation which is dose-dependent (Cowlen et al.,
Figure 5.9 Oxotremorine-induced $^{45}$Ca$^{2+}$ release in the presence of GTP$\gamma$S (100µM) following pretreatment with carbachol at 37°C. Intact cells were incubated in Krebs buffer in the absence (open squares) or presence of carbachol (1mM) for 10 (filled squares), 20 (open circles) or 40 (filled circles) minutes, after which the cells were cooled, washed and permeabilised. $^{45}$Ca$^{2+}$ release assays were performed in the presence of GTP$\gamma$S (100µM) as described (Chapter 2 (2.2)). EC$_{50}$ values (mean followed by 95% confidence limits) and maximal release (over GTP$\gamma$S alone) (Mean±S.E.M.) were as follows:- Control: 0.25(0.04-1.5)µM, 25±3%; 10 minutes: 0.36(0.19-0.68)µM, 22±4%; 20 minutes: 0.39(0.1-1.5)µM, 15±5%; 40 minutes: 1.2µM(approx.), 7±4%. Ins(1,4,5)P$_3$ (10µM)-induced $^{45}$Ca$^{2+}$ release was as follows:- Control: 64±1%; 40 minutes 60±2% (not significantly different from control). Data represent means from at least 4 experiments, error bars are omitted for clarity (S.E.M. values were routinely within 4%).
Fig. 5.10 Binding of $^{35}$S GTPγS to permeabilised SH-SY5Y cells. Cells were incubated in the absence (upper panel) or presence (lower panel) of carbachol (1mM) for 60 minutes at 37°C, cooled, washed, permeabilised and $^{35}$S GTPγS binding followed as described (Chapter 2 (2.11)) in the absence (open squares) or presence (filled diamonds) of carbachol (1mM). Non-specific binding (filled squares) was defined in the presence of 10μM unlabelled GTPγS. Data represent means from a single experiment, with similar results obtained in a further 2 experiments.
1990), and noradrenaline-induced $^{32}$P incorporation has been shown to rapidly decay, with a half time of $<$5 minutes (Leeb-Lundberg et al., 1987).

More recently, Menniti et al. (1991) have shown that accumulation of total inositol phosphates after bombesin and cholecystokinin stimulation is not linear. After an initial ‘burst’ lasting some 20 seconds, a prolonged, but lower rate of inositol phosphate formation was observed, persisting for at least 20 minutes. The mechanisms of this desensitisation are unclear. Likewise, Tobin et al. (1992) showed that a 5 minute pretreatment of CHO cells transfected with the human m3 muscarinic receptor cDNA with carbachol (1mM) significantly reduced the Ca$^{2+}$ mobilisation response to a subsequent dose of carbachol. Following removal of this desensitising application, the subsequent response to carbachol recovered in a time- and Ca$^{2+}$ dependent fashion. This desensitisation may be due to depletion of intracellular stores, as no recovery was observed in the absence of extracellular Ca$^{2+}$. However, this short term treatment with carbachol also significantly affected peak levels of Ins(1,4,5)P$_3$, which recovered with a similar time course to that of the Ca$^{2+}$ response, thus suggesting that a true desensitisation process had occurred, and that the loss of Ca$^{2+}$ response was due to the decline in the ability of the cells to produce Ins(1,4,5)P$_3$.

Tobin et al. (1992) also identified a second rise in Ins(1,4,5)P$_3$ levels in cells stimulated with carbachol. Following a rapid peak (at 10 seconds) a lower plateau was established (by 1 minute), lasting some 12 minutes, this was followed by a further increase in Ins(1,4,5)P$_3$ to levels equivalent to those observed during the initial peak, lasting at least 2 hours. This second phase may be due to Ca$^{2+}$-activation of PI-PLC. This provides a further explanation as to why many reports have previously suggested that inositol phosphate accumulation, in the presence of Li$^+$, is linear for up to 2 hours (Masters et al., 1985; Thompson and Fisher, 1990).

Pearce et al. (1988) have shown that pretreatment of a neonatal rat cerebellar cortex preparation enriched in astrocytes with carbachol, whilst decreasing subsequent inositol phosphate formation, totally abolished a functional Ca$^{2+}$ mobilisation response. This pretreatment did not affect the labelling of phosphoinositide pool. However, this functional desensitisation appeared to be heterologous, as this treatment also inhibited noradrenaline-induced Ca$^{2+}$ mobilisation, suggesting a depletion of Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ stores.

Likewise, this study describes the desensitisation of a muscarinic receptor driven response. The rate of loss of response is far greater than the loss of cell-surface receptors. Indeed, only some 50% of cell-surface receptors are lost under the
conditions employed. This phenomenon has been observed in many other systems
(for example, bombesin, Zhu et al., 1991; muscarinic m1, Lameh et al., 1992;
muscarinic, Harden et al., 1985, subsequently found to be m3, Kunysz et al., 1989)
and has been linked to the desensitisation of cyclic GMP responses. The rate of
internalisation (t1/2 ~6 minutes) is in close agreement with that observed with
muscarinic receptors in SK-N-SH cells (7.5 minutes, Thompson and Fisher, 1990),
cerebellar neurones and 108CC18 cells (4.8 and 9.5 minutes respectively, Maloteaux
et al., 1983).

The shift in EC50 of the carbachol dose response curve after desensitisation shows
that there may be a receptor reserve. The affinity of carbachol for the active G-protein
linked form of the receptor appears to be approximately 100μM, as all desensitisation
protocols move the EC50 value of carbachol to ~10μM, after which maximal release
is diminished. This value is in close agreement with the affinity of carbachol for the
low affinity state of the receptor, as observed by its ability to compete with [3H] NMS
in binding studies (Kd=100μM). However, this was not observed in the presence of
GTPγS (100μM), where desensitisation protocols moved the EC50 values of
carbachol to ~10μM. A 100-fold shift, however, would require the effective removal
of 99% of functional receptors (see Kenakin, 1987). As discussed in Chapter 4, under
ideal conditions, cell-surface receptors appear unable to fully mobilise all the
Ins(1,4,5)P3-sensitive Ca2+ pools. Equally, Zhao et al. (1990) found that, in the
presence of GTPγS, two separate cell-surface receptor agonists (prostaglandin P2α
and endothelin) mobilised more Ca2+ than any single agonist alone. This may be due
to cell membrane-derived Ins(1,4,5)P3 being unable to access all the Ins(1,4,5)P3-
sensitive Ca2+ pools available to exogenous Ins(1,4,5)P3.

A similar phenomenon may also be occurring in these desensitised cells. Following
removal of 99% of functional receptors, the production of Ins(1,4,5)P3 would be far
more localised than under control conditions, and this Ins(1,4,5)P3 may only be able
to access a fraction of the Ins(1,4,5)P3-sensitive store.

It is possible that further prolonged pretreatment of cells with carbachol may have
caused a further shift in EC50, despite maximal release already being attenuated, as
was observed most clearly when the loss of the carbachol response following
carbachol pretreatment at 20°C was studied. A 20 minutes pretreatment caused a
small decrease in maximal release, however a 90 minutes treatment caused a further 4
cold shift in EC50 as well as lowering further maximal release.

Prolonged incubations of agonists with cells causes a decrease in total receptor
number (Taylor et al., 1979) by means of transport of internalised receptor to

5.13
degradation sites followed by receptor degradation (Ray et al., 1988). Agonist binding to these remaining receptors in permeabilised cells is insensitive to guanine nucleotides and so it appears that the remaining receptors are uncoupled from the G-proteins, despite some 11% expressing high affinity for carbachol. Other groups have shown that the loss of receptors from the cell-surface appear to be those which readily couple G-proteins in a guanine nucleotide-sensitive manner (Vinayek et al., 1990; Hootman et al., 1991). Indeed, Galper and Smith (1980) have previously observed that carbachol pretreatment causes an equal loss of affinity of agonist binding to muscarinic receptors in cultured chick heart cells as does the inclusion of GppNHp, a hydrolysis resistant GTP analogue, in the binding assay. This has also been observed in this study (see Table 5.2). Equally, carbachol-enhancement of [35S] GTPγS binding to permeabilised cells appeared to be totally abolished by a 60 minutes treatment of SH-SY5Y cells with 1mM carbachol at 37°C (Fig 5.10), suggesting that the remaining receptors were unable to couple to G-proteins and catalyse guanine nucleotide exchange. The remaining high affinity sites are mostly GTP-insensitive, and may be due to the binding assay being performed at low temperatures (1-2°C), which, as described by Jagadeesh and Deth (1988) for α1 adrenoceptors, may be stabilised in a GTP-insensitive manner at such temperatures.

The rate of loss of functional response, as monitored using loss of 45Ca2+ releasing activity, is far more rapid than the loss of cell-surface receptors. This is most clearly shown when pretreatment is performed in the absence of Ca2+, where removal of 9% of cell-surface receptors is accompanied by the doubling in EC50 and reduction of maximal release by 50%. For a system in which a receptor reserve is present, a 10-fold shift in EC50 for an agonist requires the effective removal of 90% of functional receptors. Equally, in the absence of a receptor reserve, removal of 50% of functional receptors in required to reduce the maximal response by 50% (see Kenakin, 1987). Thus, agents which act as partial agonists or full agonists without a receptor reserve, will be more sensitive to desensitisation than full agonists with a receptor reserve, and this has been observed with studies using oxotremorine in the presence of GTPγS (Fig. 5.9). This would indicate that internalisation is not the mechanism by which the muscarinic receptor response in these cells is desensitised. However, it is clear that these are true desensitisation effects, and not merely due to a depletion of intracellular Ca2+ stores, as the effects of exogenous Ins(1,4,5)P3 are, at 20°C, totally unaffected and even at 37°C, are affected to a far lesser degree. Phosphoinositides also appear not to be limiting, as, in the presence of GTPγS, a functional response is maintained for long periods (over 3 hours).
These data suggest that receptors are modified prior to internalisation. A number of receptor systems have shown similarly that they desensitise more rapidly than can be accounted for by receptor internalisation. Indeed, studying the β2 receptor, Perkins and coworkers (Toews et al., 1984; Hertel et al., 1985) showed that inhibiting receptor sequestration did not affect the rate of desensitisation, suggesting that internalisation was not a primary mechanism of desensitisation. This, the most studied receptor which is modified, rendering it desensitised, is phosphorylated by at least three kinases. The β2-adrenoceptor has been identified as a substrate for β-adrenoceptor kinase (βARK), cyclic AMP-dependent kinase (protein kinase A) and protein kinase C. Mutagenesis studies have identified that these three proteins preferentially phosphorylate the β2 receptor at different, specific sites. In the β2 receptor, two consensus sites for protein kinase A phosphorylation have been identified, one in the third intracellular loop and one in the carboxy-terminal tail (see Lefkowitz et al., 1990). Clark et al. (1989) have identified that 2 serines present in the third intracellular loop are the sites for phosphorylation by protein kinase A. Protein kinase A-induced phosphorylation is catalysed by the presence of agonist on the receptor (Bouvier et al., 1987). Such phosphorylation interferes with the ability of β2 receptors to couple with the G-protein, Gs (Benovic et al., 1985). Protein kinase A-induced phosphorylation appears to be preferentially activated in the presence of low levels of agonist. βARK, which causes receptor phosphorylation at sites on the carboxy-terminal tail appears to require higher doses of agonist for its activation. Receptor phosphorylation by βARK does not directly interfere with coupling to Gs (Benovic et al., 1987). β-Arrestin, a cytoplasmic protein, interacts with the phosphorylated receptor and disrupts the activation of Gs (Lohse et al., 1990). Protein kinase C has also shown to phosphorylate the β2 receptor. Such phosphorylation was not, however, sensitive to agonists (Bouvier et al., 1987), suggesting it plays no role in homologous desensitisation.

βARK has also been identified as an enzyme involved in the phosphorylation of muscarinic receptors present in chick heart preparations (Kwatra et al., 1989). These receptors (M2) are not, however, linked to PI-PLC. This receptor was also identified as a substrate for protein kinase C, greater phosphorylation occurring in a shorter time than that observed with βARK (Richardson and Hosey, 1990). The effects of protein kinase C and βARK were partially additive, indicative of protein kinase C and βARK preferentially phosphorylating the receptor at different sites. Phosphorylation of the PLC-linked α1 receptor has also identified different sites of phosphorylation, dependent on the stimulus used. The time course of agonist-induced receptor phosphorylation closely preceded the reduction in cell-surface receptor number and decrease in agonist-stimulated labelling of PI (Leeb-Lundberg et al., 1987). Leeb-
Lundberg et al (1987) also observed that receptor phosphorylation peaked (at ~15 minutes), and declined to basal (by ~60 minutes). They suggested that phosphorylated receptors were internalised and subject to dephosphorylation by various intracellular phosphatases. Agonist-induced receptor phosphorylation permitted up to 3 mol of phosphate/mol of receptor to be incorporated (Bouvier et al., 1987). Protein kinase C and protein kinase A activation also induced α₁ receptor phosphorylation. Inclusion of agonists enhanced the rate of phosphorylation induced by protein kinase C, which caused phosphorylation at multiple sites but not by protein kinase A, which specifically phosphorylated the receptor at 2 sites (Bouvier et al., 1987). Might this suggest that protein kinase A plays a role in agonist-induced receptor phosphorylation, leading to homologous desensitisation of the receptor stimulated, whereas protein kinase C phosphorylates multiple sites of receptors unspecifically, leading to heterologous desensitisation? Similarly, work performed on PLC-linked muscarinic receptors has identified regions involved in receptor activation and internalisation. Unlike the β₂ receptor, in which the carboxy-terminal region is involved in phosphorylation by βARK, removal of this region in human m1 receptor gene products appeared not to affect receptor internalisation. Using a number of receptor mutants, Lameh et al. (1992) identified a region within the third intracellular loop (284-292) consistently associated with a decrease in the ability to internalise. This (SMESLTSSE) is a region rich in potential phosphorylation sites and some homology between m1-m5 is observed within this region (see Lameh et al., 1992), suggesting phosphorylation in the region may play an important part in receptor internalisation. That activation of protein kinase C causes desensitisation of responses to agonists has been extensively studied. Protein kinase C activation with phorbol esters or synthetic diacylglycerols has also been shown to cause phosphorylation of transfected 5-HT₁A receptors in CHO cells (Raymond, 1991), leading to desensitisation of 5-HT-mediated inhibition of adenylyl cyclase. These effects were time and dose dependent and blocked by preincubation with the protein kinase C inhibitors H-7 and sphingosine. The data clearly showed that protein kinase C activation can lead to receptor phosphorylation and subsequent desensitisation. However, earlier work had already identified that receptor phosphorylation following agonist and phorbol ester pretreatment was different in the sites and extent of receptor phosphorylation (Leeb-Lundberg et al., 1987; Bouvier et al., 1987; Richardson and Hosey, 1990). While short-term pretreatments with phorbol esters which directly activate protein kinase C and muscarinic agonists result in similar losses of response, the mechanisms are greatly different (Lai et al., 1990). Functional studies also showed major differences between agonist- and phorbol ester-induced desensitisation in a number of different receptor systems. Long-term pretreatment with phorbol esters,
causing down regulation of protein kinase C itself, did not affect carbachol-induced
desensitisation in N1E 115 cells (Lai et al., 1990). The effects of the phorbol ester
occurred at a level distal to the receptor, as it induced heterologous desensitisation,
wheras the effects of carbachol were homologous, not affecting the responses to
histamine. Cowlen et al. (1990) showed similar effects in DDT1 MF-2 cells in which
histamine pretreatment caused homologous desensitisation of histamine-induced PI
labelling, whereas a protein kinase C-activating phorbol ester caused desensitisation
of both histamine and α1 receptor driven PI labelling.

Thus, it appears likely that protein kinase C does not play a major role in homologous
desensitisation of receptor responses. A role for protein kinase A has been
established, being predominant at low levels of agonist, whereas βARK or a closely
related kinase may be involved in receptor phosphorylation in the presence of higher
levels of agonist (see Lefkowitz et al., 1990).

Klueppelberg et al. (1991) have identified that the PI-PLC-linked cholecystokinin
(CCK) receptor is rapidly phosphorylated following agonist binding in a dose-
dependent manner, phosphorylation occurring at a serine residue. Phosphorylation of
the CCK receptor was, however, heterologous, enhancement also following
stimulation of muscarinic and bombesin receptors, both of which are coupled to PI-
PLC. A protein kinase C-activating phorbol ester was also seen to cause receptor
phosphorylation. The effects of the muscarinic agonist, carbachol and the phorbol
ester were inhibited by staurosporine, a potent protein kinase C inhibitor (Davis et al.,
1989). Recently, Tobin and Nahorski (1993) have identified that m3 muscarinic
receptors transfected into CHO cells are also substrates for phosphorylation. Protein
kinase C-activating phorbol esters increased receptor phosphorylation in a RO-
318220 (a selective protein kinase C inhibitor)-sensitive fashion, whereas agonist-
mediated phosphorylation was RO-318220-insensitive, suggesting that protein kinase
C does not play a major role in agonist-induced desensitisation. Roles for protein
kinase A and Ca2+/calmodulin-dependent protein kinase were also eliminated.
Receptor phosphorylation was extremely rapid following receptor stimulation, and
was maintained for at least 30 minutes, with a serine residue being the major substrate
for phosphorylation. The time course for receptor-mediated phosphorylation and
subsequent dephosphorylation following removal of agonist correlated well with
previously reported agonist-mediated desensitisation and resensitisation (Tobin et al.,
1992). This suggests that receptor phosphorylation plays a major role in m3 receptor
desensitisation. Thus, while protein kinase A does not play a role in agonist-mediated
muscarinic receptor phosphorylation, it appears that an unidentified kinase similar to
βARK or, indeed, βARK itself is involved in receptor phosphorylation.

5.17
That only 50% of receptors express high affinity for agonists has led to Potter et al. (1989) to suggest that muscarinic receptors (M₂ and M₁) may be paired, and only one receptor per pair can couple to G-proteins and exhibit high affinity for agonists. This would explain why, while only 50% of receptors are lost from the cell surface, the Ca²⁺ mobilisation response in these studies is totally obliterated, and why the ability of carbachol to enhance [³⁵S]GTPγS binding, after a pretreatment which only causes 50% decrease in cell-surface receptor number, is totally lost. This would also suggest that G-protein involvement is required for receptor internalisation. As 50% of the receptors appear incapable of G-protein involvement, they will not readily be internalised. However, Potter et al. (1989) thought this was unlikely, due to the simple characteristics of antagonist binding, and, therefore, these two sites had to be on separate receptor monomers. Indeed, alternative splicing of the mRNA for D₂ dopaminergic receptors has been noted in the region which generates the third intracellular loop of this protein (Dellavedova et al., 1992). It is this region which has been identified as that which couples to G-proteins (see Chapter 1). Hence, alterations in this region could produce receptors incapable of coupling to G-proteins. Potter et al. (1989) further suggested that, as there is no functional purpose for an inactive receptor, muscarinic receptors may, as may many other receptors, exist as dimers at some point in their posttranslational lifetimes.

Recent work by Thompson et al. (1991a), also suggests that internalisation of receptors requires the involvement of a G-protein. Whilst the removal of extracellular Ca²⁺ inhibited muscarinic receptor-stimulated phosphoinositide metabolism in SK-N-SH cells, this had no effect on the rate of receptor internalisation, as was also observed in this study, the aminosteroid U73122, which also prevented PI hydrolysis, but, it was claimed, by interacting with the G-protein, inhibited receptor sequestration. Thus, it appears that G-protein involvement plays an important role in receptor sequestration. However, in adenylyl cyclase-linked receptors this appears unlikely. Green and Clark (1981) found that homologous desensitisation of β receptors in lymphoma cells still occurs when G₅ in mutated and incapable of activating adenylyl cyclase.

Unlike the adenylyl cyclase-linked β-adrenoceptor, internalised muscarinic receptors in 1321N1 cells appear to bind agonists in the same GTP-sensitive manner as that seen for receptors present at the plasma membrane (Harden et al., 1985). Furthermore, binding of GTPγS, a hydrolysis-resistant GTP analogue, to a light vesicular fraction containing internalised receptors was enhanced when 1321N1 cells were pretreated with a muscarinic agonist (Nakahata et al., 1987). Thus, receptors may remain coupled to G-proteins after internalisation and if it is assumed that PI-PLC and
phosphoinositides are also localised in internalised vesicles, then it would be possible for these internalised receptors to stimulate phosphoinositide hydrolysis. Indeed, as PI-PLC-linked receptor agonists, for example bombesin, neurotensin and TRH are also internalised along with their receptors (Zhu et al., 1991; Turner et al., 1990), these receptors may even be persistently activated and still capable of producing second messengers providing that 1. they could still couple PI-PLC, 2. PtdIns(4,5)P₂ was still available and 3. agonists could access the receptor. This proposal could explain why inositol phosphate production can be maintained apparently linearly for hour-long periods when a large proportion the receptors (>50%) have been internalised (Masters et al., 1985; Harden et al., 1985; Nakahata et al., 1987). Although this phenomenon could be explained by other factors, for example, the presence of a "receptor reserve" at the cell surface or increased efficiency of coupling of the remaining receptors to phosphoinositide hydrolysis, perhaps involving feedback activation of PI-PLC by Ca²⁺, it is an attractive hypothesis that remains to be tested. However, no evidence for this has been reported in the case of the muscarinic receptor in SK-N-SH cells. Indeed, Thompson and Fisher (1991) have shown that these internalised receptors, whilst accessed by the lipophilic agonist L-670,548, are not involved in the production of inositol phosphates.

The dramatic effects of removal of extracellular Ca²⁺ on the rate of agonist-induced desensitisation of the Ca²⁺ mobilisation response, without any effect on the rate of receptor internalisation is of interest. This shows that an event prior to internalisation is involved with desensitisation, and this has been identified as a phosphorylation event in other receptor systems and may be the phosphorylation event observed by Tobin and Nahorski (1993) in the muscarinic m3 receptor system. The enhanced rate of desensitisation is unlikely to be due to enhanced rates of phosphorylation, as most kinases are either insensitive to or activated by increased levels of Ca²⁺ (Leeh-Lundberg et al., 1987; Bouvier et al., 1987; Kobilka, 1992). This would suggest that rather than the rate of phosphorylation increasing, the rate of dephosphorylation of the receptor is decreased. One family of protein phosphatases has been identified as Ca²⁺/calmodulin-sensitive (see Cohen and Cohen, 1989). Protein phosphatases may be capable of dephosphorylating phosphorylated receptors, hence causing their reactivation. Therefore, in the absence of Ca²⁺ the rate of deactivation may be maintained, but reactivation is greatly retarded. A large number of protein phosphatases which cause dephosphorylation at serine and threonine residues, sites at which receptors are phosphorylated, with broad substrate specificities are known (see Cohen and Cohen, 1989). Of these, PP2B is Ca²⁺/calmodulin dependent, whereas PP2C is Mg²⁺-dependent, and PP2A and PP1 have no dependence for divalent cations (see Cohen and Cohen, 1989). This suggests that PP2B may be involved in
the dephosphorylation of phosphorylated muscarinic receptors, resensitising them to agonist stimulation.

The level at which agonists cause a lesion in the signal transduction pathway has also been studied by Paris et al. (1988). Thrombin pretreatment of CCL39 Chinese hamster lung fibroblasts caused a rapid homologous desensitisation of thrombin-induced inositol phosphate formation, however serum and AIF₄⁻ induced inositol phosphate formation were unaffected, indicating that desensitisation was occurring at the level of the thrombin receptor itself. Desensitisation, whilst attenuated, was observed at 4°C, at such a temperature internalisation and phospholipid hydrolysis were absent. Therefore, removal of receptors and the depletion of the (agonist sensitive) phospholipid pool can be discounted as mechanisms of desensitisation, and, again, suggests that activation of the G-protein alone may be the sole requirement for agonist-induced desensitisation.

Pearce et al. (1988), showed that this was also true with muscarinic receptors in a neonatal rat cerebellar cortical preparation, where carbachol pretreatment did not affect the ability of GTPγS to stimulate inositol phosphate formation in membrane preparations, but inhibited the ability of carbachol to augment this response. Similarly, the maximal response to GTPγS was also unaffected by carbachol pretreatment in this study. I have not attempted to study the effects of carbachol-pretreatment on the small GTPγS dose response curve, which may have been affected in some way. However, Johnson et al. (1991) have shown that prolonged pretreatment of rat aorta with the adrenergic agonist, noradrenaline, decreased subsequent stimulation of G₉ and G₁₀. Agonist-stimulated GTPγS binding was, however, only subject to homologous desensitisation (Johnson et al., 1991). This suggests that under these conditions the G-protein involved in receptor coupling to PLC is not affected by agonist pretreatment. In contrast, stimulation of receptors coupled to the G₉ family of G-proteins have been seen to actually decrease levels of G₉ in some systems (McKenzie and Milligan, 1990). While data provided in this and other (Pearce et al., 1988) studies do not prove that G₉ function remains unaltered, they provide evidence that such alteration is minimal. More recently, such down-regulation of G-proteins coupled to PI-PLC has also been shown (Mullaney et al., 1993). However, such down-regulation was slow, G₉ down-regulation following a similar time course to that of down-regulation of the cell-surface receptor stimulated (human m1). The decrease in G₉ levels observed was seen to be due to an increase in the rate of breakdown of G₉, and not a decrease in the rate of formation. As with loss of cell-surface receptors described above, down-regulation maximally only removed some 50% of G₉ present.
In conclusion, desensitisation of the Ca\(^{2+}\) mobilising muscarinic receptor response is far more rapid than previously suggested, indeed, rather similar to that observed for the rate of desensitisation of the muscarinic receptor-induced accumulation of cyclic GMP (Cioffi and El Fakahany, 1989). Indeed, loss of cyclic GMP response, which appeared not to possess a receptor reserve, followed the same time course as loss of cell surface receptors. There is increasing evidence that the cyclic GMP response observed in cells is downstream from increases in levels of intracellular Ca\(^{2+}\) (see Surichamorn et al., 1990). Thus, desensitisation of the cyclic GMP response may be an accurate method in following the desensitisation of Ca\(^{2+}\) mobilisation in cells. The rate of desensitisation is greatly increased by the levels of Ca\(^{2+}\) and this may be due to the inactivation of Ca\(^{2+}\)-dependent protein phosphatase involved in dephosphorylating receptors phosphorylated in a manner similar to that described for the adenylyl cyclase-linked M\(_2\) (Kwatra et al., 1987) and \(\beta_2\) (Lefkowitz et al., 1990) receptors.

### 5.5 Summary

In summary, this permeabilised cell preparation is an ideal system in which to study the mechanisms of desensitisation. The effects of desensitisation on antagonist and agonist binding to cell-surface receptors can be monitored. The effects of guanine nucleotides on binding characteristics of agonists and antagonists can be assessed, as well as the effects of agonists and antagonists on guanine nucleotide binding. These can be studied in the same system in which functional responses (such as Ins(1,4,5)P\(_3\) accumulation and Ca\(^{2+}\) mobilisation) are also followed. Thus, this permeabilised cell preparation is, at present, the only simple system in which all the above functions can be assessed in a single system, providing advantages over two more established systems currently in use:-- intact cell preparations, from which guanine nucleotides are excluded (for example, Tobin et al., 1992), and membrane preparations, in which functional responses are absent (for example, Boyer et al., 1989).

The locus of desensitisation of this muscarinic receptor response is at the level of receptor-G-protein coupling. This uncoupling phenomenon appears to precede internalisation of receptors. A likely mechanism of this desensitisation appears to be receptor phosphorylation (see Tobin and Nahorski, 1993), identifying strong similarities between the PI-PLC-linked muscarinic receptor and the adenylyl cyclase-linked \(\beta_2\) receptor (see Kobilka, 1992).
CHAPTER 6.
Concluding Comment.

The work presented in this thesis can be adjudged to study two distinct aspects of phosphoinositide signalling in the neuronal cell line used.

In Chapter 3, studies using inositol phosphates and inositol phosphate analogues have attempted to identify recognition requirements for three key proteins: the Ca\(^{2+}\)-mobilising Ins(1,4,5)P\(_3\) receptor, Ins(1,4,5)P\(_3\) 5-phosphatase and Ins(1,4,5)P\(_3\) 3-kinase. These studies have identified potent Ca\(^{2+}\) mobilising agents which are resistant to dephosphorylation (for example, InsP\(_3\)-5S, InsP\(_3\)-4,5S\(_2\), se Ins(1,2,4)P\(_3\)), phosphorylation (for example, 3F InsP\(_3\)), or both (for example, L-ch Ins(2,3,5)P\(_3\), Ins(1,2,4,5)P\(_4\)). These studies have also identified the first Ins(1,4,5)P\(_3\) 3-kinase inhibitor which interacts poorly with the Ins(1,4,5)P\(_3\) receptor (L-2,2F\(_2\) InsP\(_3\)). L-2,2F\(_2\) InsP\(_3\) does, however, also inhibit Ins(1,4,5)P\(_3\) 5-phosphatase. Two highly potent and selective inhibitors of Ins(1,4,5)P\(_3\) 5-phosphatase have also been identified (L-ch Ins(1,4,6)PS\(_3\) and cyclohexane (1,2,4)(CH\(_2\)SO\(_3\))\(_3\)), L-ch Ins(1,4,6)PS\(_3\) being the most potent, selective inhibitor of Ins(1,4,5)P\(_3\) 5-phosphatase yet described. Two partial agonists at the Ins(1,4,5)P\(_3\) receptor have also been identified (L-ch Ins(2,3,5)PS\(_3\) and 6-deoxy InsPS\(_3\)), and these agents must now be used to further our understanding of the mechanisms involved in Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) mobilisation. No selective Ins(1,4,5)P\(_3\) 3-kinase inhibitor has, yet, been identified, and this must be considered a priority if the role of Ins(1,3,4,5)P\(_4\) in signal transduction is to be defined. These agents can then be used to study the specific roles of the Ins(1,4,5)P\(_3\) receptor, Ins(1,4,5)P\(_3\) 5-phosphatase and Ins(1,4,5)P\(_3\) 3-kinase in phosphoinositide signal transduction.

The preparation of antibodies against the Ins(1,4,5)P\(_3\) receptor, Ins(1,4,5)P\(_3\) 5-phosphatase and Ins(1,4,5)P\(_3\) 3-kinase, which would render them effectively removed from signal transduction, would also produce an attractive way in which phosphoinositide signal transduction could be manipulated. Antibodies have been prepared against the Ins(1,4,5)P\(_3\) receptor by Mikoshi and co-workers (Nakade et al., 1991; Miyazaki et al., 1992). Surprisingly, two antibodies which target the amino-terminal domain, proximal to the Ins(1,4,5)P\(_3\) binding site were without effect on Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) mobilisation or Ins(1,4,5)P\(_3\) binding, whereas an antibody which targets the carboxy-terminal domain slightly enhanced Ins(1,4,5)P\(_3\) binding. A further antibody targeting the carboxy-terminal domain actually increases Ins(1,4,5)P\(_3\)-induced effects (Fadool and Ache, 1992). However, the above methods
produce agents which are cell membrane-impermeant. This is a major drawback with all the analogues tested thus far. Microinjection or transient permeabilisation of cells, allowing incorporation of such agents have not been performed in these studies, and all the work described has used permeabilised cells which have not been allowed to reseal. The preparation of cell membrane-permeant analogues or cell membrane-permeant precursors of useful analogues, as has been described for the Ca\(^{2+}\) indicator, fura-2 (Tsien et al., 1982), must be considered a priority, if such agents are to become useful pharmacological tools.

Alternatively, the incorporation of modified inositols may be used to answer questions regarding the roles of specific inositol phosphates, and such an approach has already been tested, with varying degrees of incorporation into lipids, and effects on cell growth (Moyer et al., 1988; Kozikowski et al., 1990; McPhee et al., 1991). However, none of the above approaches will totally inhibit \(\text{Ins}(1,4,5)P_3\) and \(\text{Ins}(1,3,4,5)P_4\) formation, as all the analogues described in this thesis act as competitive inhibitors, and incorporation of inositol analogues into phospholipids would not exclude breakdown of pre-existing inositol phospholipids.

In Chapter 4, the muscarinic M_3 receptor mediated phosphoinositide response is studied in a continuous cell line, the SH-SY5Y human neuroblastoma. ‘Continuous’ cell lines should be an attractive way in which to study specific receptor effects on a second messenger system, with none of the complications inherent when using animal tissue, with mixed cell types within a preparation, and multiple subtypes of the receptors to be studied. However, despite the pharmacological characterisation of these cells as possessing muscarinic receptors solely of the M_3 subtype, these cells possess the ability to express a heterogeneous population of muscarinic receptors (see Wall et al., 1991). The effects of a small population of receptors of other subtypes, whilst not discussed here, may influence the responses observed in the work. This problem is compounded by the choice of muscarinic agonist used in this study. While being a widely used muscarinic agonist, carbachol is also a nicotinic receptor agonist. SH-SY5Y cells have also been identified as possessing nicotinic receptors (Noronha-Blob et al., 1989), once again, the role of this receptor in the carbachol responses described is unknown. Thus, the effects of carbachol may be explained by the co-stimulation of muscarinic and nicotinic receptors. However, the effects of carbachol were completely sensitive to atropine, a muscarinic receptor antagonist which does not interact with nicotinic receptors.

The work performed in this chapter characterised a muscarinic receptor-driven response in cells which had been permeabilised. Permeabilisation was routinely achieved by the discharged of a high voltage across a cell suspension. This
permeabilised cell preparation does possess properties which make it an attractive
tool in which to study receptor mediated effects on second messenger systems.
While the time course of Ins(1,4,5)P3 production and Ca2+ mobilisation appear much
slower than that observed in intact cells, agents which can interact with intracellular
components of the signalling pathway could be introduced. Indeed, this permeabilised
cell preparation enables the study of the phosphoinositide signal transduction pathway
at a number of sites. Cell-surface receptor binding of agonists and antagonists, as well
as the effects of guanine nucleotides on the binding of these agents, and vice versa,
has been studied in the same system as studies concerning the production of
membrane-derived Ins(1,4,5)P3 and the mobilisation of Ca2+ from internal stores.
This has previously not been possible with intact preparations, which exclude
exogenous guanine nucleotides from the cell interior, and membrane preparations
which no longer possess the equipment for Ca2+ mobilisation.

In this study, the effects of carbachol, arecoline and oxotremorine have been
examined. Carbachol is a full agonist for Ca2+ mobilisation, and induced Ins(1,4,5)P3
accumulation with similar EC50, but increasing coupling efficiency by the addition of
GTPyS (100μM) increased maximal levels of Ins(1,4,5)P3 produced and substantially
separate the EC50 values for Ins(1,4,5)P3 accumulation and Ca2+ mobilisation. This
strategy identifies different receptor reserves when studying the effects of agonists to
produce Ins(1,4,5)P3 and mobilise Ca2+. Similar, but less marked effects were
observed with the partial agonist, arecoline. Oxotremorine was the least efficacious
agonist, and GTPyS, whilst greatly enhancing maximal Ins(1,4,5)P3 accumulation and
Ca2+ mobilisation, was unable to affect the EC50 for either parameter. These agonists
also affected the dose-response relationship for GTPyS to mobilise Ca2+. Carbachol
raised maximal release and lowered the EC50 for GTPyS, as did arecoline and
oxotremorine, but less markedly, suggesting agonist efficacy is dependent on the
ability of agents to catalyse guanine nucleotide exchange on the G-protein influenced.
Parker and Ivorra (1990), and more recently Bootman et al. (1992), have suggested
that Ins(1,4,5)P3 mobilises Ca2+ from intracellular stores in an all-or-nothing fashion,
and that stores possessing different sensitivities to Ins(1,4,5)P3 enable the production
of dose-response relationships for agonist-mediated Ca2+ mobilisation. Studying
single cells, Bootman et al. (1992) identified that agonist-induced Ca2+ mobilisation
was an all-or-nothing response, with different cells having different sensitivities.
Thus, in a large population, these heterogeneous responses would summate, giving an
observed sigmoidal dose-response relationship to agonists. However, the
identification of partial agonists at the Ins(1,4,5)P3 receptor (Gawler et al., 1991; this
study) must encourage reevaluation of this all-or-nothing theory of release. The two
partial agonists described in this study have been shown to compete fully with
Ins(1,4,5)P$_3$ for all the Ins(1,4,5)P$_3$ receptors in bovine adrenal cortical membranes (Safrany et al., 1993), yet only release a fraction of the $^{45}\text{Ca}^{2+}$ loaded into Ins(1,4,5)P$_3$-sensitive stores. To satisfy the all-or-nothing mechanism of Ca$^{2+}$ mobilisation from discrete stores, these partial agonists must act as agonists at some receptors and as antagonists at other receptors, yet bind to both with equal affinity (as determined by the Hill slope of binding of these agents being similar to that of Ins(1,4,5)P$_3$ itself).

Other agents which can be introduced include inhibitors of Ins(1,4,5)P$_3$ and Ins(1,4,5)P$_3$ 3-lidnase or Ins(1,4,5)P$_3$ receptor antagonists, to further the understanding of the effects of endogenously produced Ins(1,4,5)P$_3$ (as described by Zhao et al., 1990) or assist in identifying which phosphoinositides are hydrolysed following agonist stimulation (see Wojcikiewicz et al., 1990a; Fisher et al., 1990), and it was to be with such studies that the inositol phosphate analogues described in Chapter 3 were to be used. The lack of identification of Ins(1,4,5)P$_3$ receptor antagonists or selective Ins(1,4,5)P$_3$ 3-kinase inhibitors, with only recent identification of a potent Ins(1,4,5)P$_3$ 5-phosphatase inhibitor has prevented such work being undertaken.

In Chapter 5, the effects of agonist pretreatment on a subsequent response have been studied. These studies identify that agonist-induced desensitisation is dependent on the dose of agonist used, the temperature at which pretreatment was performed, the time of pretreatment and was affected by the levels of extracellular Ca$^{2+}$ present. The loci of this desensitisation process appear to be firstly the cell-surface receptor, of which only 50% appear to be capable of coupling to the G-protein involved in PI-PLC activation, and secondly, the Ins(1,4,5)P$_3$ receptor, which, while not studied here, desensitises more slowly and requires physiological temperatures to do so (Wojcikiewicz and Nahorski, 1991). The cell-surface receptors are rapidly uncoupled from G-proteins, suggesting an event which precedes internalisation is involved in desensitisation. This appears to be rapid phosphorylation in CHO cells transfected with m3 muscarinic receptors (Tobin and Nahorski, 1993), and the nature of such phosphorylation in CHO-m3, SH-SY5Y and other cells is of great interest. The phosphorylating agent is yet to be identified. βARK has been found to phosphorylate not only β2 receptors, but also M2 receptors (Kwatra et al., 1989) and α1 receptors (Leeb-Lundberg et al., 1987). Determination of the kinase involved, be it βARK or another receptor kinase, must be undertaken to further the understanding of agonist-mediated desensitisation in this PI-PLC-linked system. The nature of desensitisation observed in these studies has been assumed to be receptor specific. However, prior to the conclusion of this work, no other receptors which couple to PI-PLC had been
identified in this cell type. It would, therefore be premature to describe these events as
being homologous (affecting solely the receptor stimulated) or heterologous (also
affecting other receptor systems, which would normally act through the same signal
transduction pathway).

In conclusion, this permeabilised cell preparation is an ideal system in which to study
the phosphoinositide signalling pathway. These studies represent the first detailed
analysis of the effects of muscarinic agonists on Ins(1,4,5)P₃ formation and
subsequent Ca²⁺ mobilisation in a permeabilised cell preparation, how these
responses are related to cell-surface receptor binding and how these responses are
affected by agonist pretreatment of intact cells. It is expected that the permeabilisation
techniques used in the thesis can be used for many other cell types, and it is,
therefore, likely that a similar approach could be used to study signal transduction
pathways in many other cell types.
APPENDICES.
**Abbreviations.**

The following abbreviations are used in this thesis.

**Phosphoinositides and inositol phosphates.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns(3)P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PtdIns(4)P</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PtdIns(3,4)P_2</td>
<td>Phosphatidylinositol 3,4-bisphosphate</td>
</tr>
<tr>
<td>PtdIns(4,5)P_2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P_3</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
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</tbody>
</table>

Inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphates are based on D-myo-inositol unless otherwise stated. InsP, InsP_2, InsP_3, InsP_4, InsP_5 and InsP_6 refer to myo-inositol mono, bis, tris, tetrakis, pentakis and hexakis phosphates, where appropriate the position of the phosphate locants around the inositol ring are given in parentheses to denote a specific isomer. The use of : indicates a cyclic phosphate, associated with the two positions. Abbreviations used for Ins(1,4,5)P_3 analogues can be found in A II, along with their structures.
Other abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>2,3 BPG</td>
<td>2,3 bisphosphoglyceric acid</td>
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<tr>
<td>4-DAMP</td>
<td>4-diphenylacetoxy-N-methylpiperazine methiodide</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ADPβS</td>
<td>adenosine 5'-O-2 thio-diphosphate</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>11-2[[2-(diethylamino)-methyl]-1-piperidinylacetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one</td>
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<tr>
<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>AP₄</td>
<td>adenosine 5'-tetraphosphate</td>
</tr>
<tr>
<td>Arec</td>
<td>arecoline</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPγS</td>
<td>adenosine 5'-O-3 thio-triphosphate</td>
</tr>
<tr>
<td>Atr.</td>
<td>atropine</td>
</tr>
<tr>
<td>βARK</td>
<td>β-adrenergic receptor kinase</td>
</tr>
<tr>
<td>Bₘₐₓ</td>
<td>maximal density of binding sites</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium, free ion</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>intracellular Ca²⁺ concentration</td>
</tr>
<tr>
<td>CCh</td>
<td>carbachol</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cAMPγS</td>
<td>adenosine 3':5'-cyclic monophosphorothioate</td>
</tr>
<tr>
<td>CMP</td>
<td>cytidine monophosphate</td>
</tr>
<tr>
<td>CMP-PA</td>
<td>cytidine monophosphorylphosphatidate</td>
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</table>
CNS  
central nervous system

cyclic AMP  
adenosine 3':5'-cyclic monophosphate

cyclic GMP  
guanosine 3':5'-cyclic monophosphate

DAG  
-sn-1,2-diacylglycerol

DTT  
dithiothreitol

E.R.  
endoplasmic reticulum

EC50  
concentration producing 50% of maximal response

EDTA  
ethylene diaminetetraacetic acid

EGF  
epidermal growth factor

EGTA  
ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N' tetraacetic acid

G-protein  
guanine nucleotide-binding protein

G\textsubscript{i}  
G-protein associated with inhibition of adenylyl cyclase

G\textsubscript{q/11}  
mixture of G\textsubscript{q} and G\textsubscript{11}

G\textsubscript{s}  
G-protein associated with activation of adenylyl cyclase

GAP  
GTPase activating protein

GDP  
guanosine 5'-diphosphate

GDP\textsubscript{\beta}\textsubscript{S}  
guanosine 5'-O-2 thio-diphosphate

GMP  
guanosine 5'-monophosphate

GP\textsubscript{4}  
guanosine 5'-tetraphosphate

GppNH\textsubscript{p}  
guanosine 5'-(\beta,\gamma-imido)triphosphate

GTP  
guanosine 5'-triphosphate

GTP\gamma\textsubscript{S}  
guanosine 5'-O-3 thio-triphosphate

HBS  
HEPES buffered saline
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HBS/EDTA</td>
<td>HEPES buffered saline containing 0.02% EDTA</td>
</tr>
<tr>
<td>HEG</td>
<td>human erythrocyte ghost</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)1-piperazineethanesulphonic acid</td>
</tr>
<tr>
<td>HHSD</td>
<td>hexahydrosiladifenidol</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration of agent inhibiting a response by 50%</td>
</tr>
<tr>
<td>ICB</td>
<td>intracellular buffer</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>dissociation constant for an inhibitor-enzyme complex</td>
</tr>
<tr>
<td>K&lt;sub&gt;S0&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; of agent not obeying Law of Mass Action, corrected for mass of competing radioligand</td>
</tr>
<tr>
<td>K&lt;sub&gt;H&lt;/sub&gt;</td>
<td>dissociation constant of high affinity binding site</td>
</tr>
<tr>
<td>K&lt;sub&gt;L&lt;/sub&gt;</td>
<td>dissociation constant of low affinity binding site</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis-Menton constant</td>
</tr>
<tr>
<td>M&lt;sub&gt;x&lt;/sub&gt;</td>
<td>muscarinic receptor of the x subtype, determined by antagonist affinities</td>
</tr>
<tr>
<td>mX</td>
<td>muscarinic receptor of the X subtype, determined by cDNA analysis</td>
</tr>
<tr>
<td>McN-A 343</td>
<td>4-m-chlorophenylcarbamoyloxy-2-butynyl trimethyl ammonium chloride</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>magnesium, free ion</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMS</td>
<td>N-methyl scopolamine</td>
</tr>
<tr>
<td>Oxo</td>
<td>oxotremorine</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>phosphate, inorganic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>$P_{\text{high}}$</td>
<td>proportion of high affinity sites</td>
</tr>
<tr>
<td>$P_{\text{low}}$</td>
<td>proportion of low affinity sites</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>pF-HHSD</td>
<td>parafluoro-hexahydrosiladifenidol</td>
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<tr>
<td>PI-PLC</td>
<td>phosphoinositide-specific phospholipase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>protein kinase A</td>
<td>cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
<td>QNB</td>
<td>quinuclidinyl benzilate</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin releasing hormone</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximum velocity of an enzyme-catalysed reaction</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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</table>
### Structures of Inositol Phosphates and Analogues

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<tr>
<th>Analogue</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
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<td>Ins(1)P</td>
<td>PO₄</td>
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<tr>
<td>Ins(2)P</td>
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<td>OH</td>
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<td>PO₄</td>
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<td>OH</td>
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<tr>
<td>Ins(1,2,6)P₃</td>
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<td>PO₄</td>
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<td>PO₄</td>
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<tr>
<td>Ins(1:2,4,5)P₃</td>
<td>-OP(O₂)O-</td>
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<td>PO₄</td>
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<tr>
<td>Ins(2,4,5)P₃</td>
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<td>PO₄</td>
<td>OH</td>
<td>PO₄</td>
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A II.1
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<th>Analogue</th>
<th>R₁</th>
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<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
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<tr>
<td>L-Ins(1,3,4)P₃</td>
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<td>PO₄</td>
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<td>HOCH₂C(OH)HCH₂OPO₄</td>
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<tr>
<td>Ins(1,3,4,5)P₄</td>
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<td>OH</td>
<td>PO₄</td>
<td>PO₄</td>
<td>PO₄</td>
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<td>Ins(1,3,4,6)P₄</td>
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<td>OH</td>
<td>PO₄</td>
<td>PO₄</td>
<td>OH</td>
<td>PO₄</td>
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<td>Ins(1,4,5,6)P₄</td>
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<td>OH</td>
<td>PO₄</td>
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<td>Ins(1,3,4,5,6)P₅</td>
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<td>PO₄</td>
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<td>OH</td>
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<td>PO₃S</td>
<td>PO₃S</td>
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<td>-OPO(O)OP(O)O-</td>
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<td>Analogue</td>
<td>R₁</td>
<td>R₂</td>
<td>R₃</td>
<td>R₄</td>
<td>R₅</td>
<td>R₆</td>
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<td>----</td>
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<tr>
<td>CH(1,2,4)P₃</td>
<td></td>
<td>PO₄</td>
<td>H</td>
<td>H</td>
<td>PO₄</td>
<td>PO₄</td>
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<tr>
<td>CH(1,2,4)(CH₂PO₄)₃</td>
<td>CH₂PO₄</td>
<td>H</td>
<td>H</td>
<td>CH₂PO₄CH₂PO₄</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>CH(1,2,4)(CH₂PO₃)₃</td>
<td>CH₂PO₃</td>
<td>H</td>
<td>H</td>
<td>CH₂PO₃CH₂PO₃</td>
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<tr>
<td>CH(1,2,4)(CH₂SO₃)₃</td>
<td>CH₂SO₃</td>
<td>H</td>
<td>H</td>
<td>CH₂SO₃CH₂SO₃</td>
<td>H</td>
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</tr>
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</table>
Analogue  \( R_1A \) \( R_1E \) \( R_2A \) \( R_2E \) \( R_3A \) \( R_3E \) \( R_4A \) \( R_4E \) \( R_5A \) \( R_5E \) \( R_6 \)

D-ch Ins(1,3,4)P\(_3\)
\[
\begin{align*}
&\text{OH} & \text{H} & \text{PO}_4 & \text{H} & \text{H} & \text{OH} & \text{H} & \text{PO}_4 & \text{H} & \text{PO}_4 & \text{OH} \\
\end{align*}
\]

D-2,2F\(_2\) InsP\(_3\)
\[
\begin{align*}
&\text{H} & \text{PO}_4 & \text{F} & \text{F} & \text{H} & \text{OH} & \text{H} & \text{PO}_4 & \text{H} & \text{PO}_4 & \text{OH} \\
\end{align*}
\]

L-2,2F\(_2\) InsP\(_3\)
\[
\begin{align*}
&\text{H} & \text{OH} & \text{F} & \text{F} & \text{H} & \text{PO}_4 & \text{H} & \text{OH} & \text{H} & \text{PO}_4 & \text{PO}_4 \\
\end{align*}
\]

sc 2F InsP\(_3\)
\[
\begin{align*}
&\text{H} & \text{PO}_4 & \text{H} & \text{F} & \text{H} & \text{OH} & \text{H} & \text{PO}_4 & \text{H} & \text{PO}_4 & \text{OH} \\
\end{align*}
\]

sc Ins(1,2,4)P\(_3\)
\[
\begin{align*}
&\text{H} & \text{PO}_4 & \text{H} & \text{OH} & \text{H} & \text{OH} & \text{H} & \text{PO}_4 & \text{H} & \text{PO}_4 & \text{OH} \\
\end{align*}
\]

L-ch Ins(2,3,5)P\(_3\)
\[
\begin{align*}
&\text{H} & \text{PO}_4 & \text{H} & \text{OH} & \text{OH} & \text{H} & \text{H} & \text{PO}_4 & \text{H} & \text{PO}_4 & \text{OH} \\
\end{align*}
\]

L-ch Ins(2,3,5)PS\(_3\)
\[
\begin{align*}
&\text{H} & \text{PO}_3\text{S} & \text{H} & \text{OH} & \text{OH} & \text{H} & \text{H} & \text{PO}_3\text{S} & \text{H} & \text{PO}_3\text{S} & \text{OH} \\
\end{align*}
\]

L-ch Ins(1,4,6)P\(_3\)
\[
\begin{align*}
&\text{H} & \text{PO}_4 & \text{H} & \text{OH} & \text{H} & \text{OH} & \text{PO}_4 & \text{H} & \text{PO}_4 & \text{H} & \text{OH} \\
\end{align*}
\]

L-ch Ins(1,4,6)PS\(_3\)
\[
\begin{align*}
&\text{H} & \text{PO}_3\text{S} & \text{H} & \text{OH} & \text{H} & \text{OH} & \text{PO}_3\text{S} & \text{H} & \text{PO}_3\text{S} & \text{H} & \text{OH} \\
\end{align*}
\]

A II.4
PUBLICATIONS ARISING FROM THIS THESIS.
Publications Arising from this Thesis.

Full Papers


Review Articles


Abstracts.

*Presented by S.T. Safrany


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