MOLECULAR MECHANISMS OF LITHIUM ACTION ON PHOSPHOINOSITIDE SIGNALLING.

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by

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This thesis is dedicated to my
Mother and late Father to whom I owe so much.
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Molecular Mechanisms of Lithium Action on Phosphoinositide Signalling.

Stephen Jenkinson.

The work described in this thesis examines the phosphoinositide (PI) signalling system and its disruption by the anti-manic agent lithium. The effects of lithium upon the accumulation of labelled and unlabelled inositol (poly)phosphates in muscarinic cholinoreceptor-stimulated rat brain slices and Chinese hamster ovary (CHO) cells expressing the human M1-muscarinic receptor subtype (CHO-M1) were examined. Similarly, the effects of this ion on other intermediates of this second messenger signalling system were examined in order to give an overall picture of the action of lithium. These included the accumulation of CMP-phosphatidic acid (CMP-PA), a precursor of the (poly)phosphoinositide lipids, and the agonist-stimulated levels of the (poly)phosphoinositide lipids.

Initial experiments examined phosphoinositide metabolism in cortex, hippocampus and striatum to determine whether there were regional variations in both this signalling system and the effects which lithium had upon it. Both cortical and hippocampal PI metabolism were similar, however, striatum was significantly different such that in the continued presence of agonist this region was unable to maintain the initial elevated levels of Ins(1,3,4,5)P4, unlike the other regions examined. Lithium appeared to have a similar disruptive effect on PI metabolism in all regions, with statistically similar EC50 values for the accumulation of InsP1 in all regions.

The effects of lithium upon PI metabolism stimulated by a variety of different agonists was examined to determine whether the action of lithium was agonist dependent. Lithium appeared to have a similar disruptive effect upon PI metabolism stimulated by these various agonists.

Studies examining the effects of lithium upon the carbachol-stimulated accumulation of the inositol (poly)phosphate isomers revealed the presence of a lithium-sensitive accumulation of the inositol bisphosphate Ins(4,5)P2. This study was unable to determine the source of this isomer, however, the formation of this isomer in both cerebral cortex slices and CHO cells suggests the possibility that Ins(4,5)P2 may be a dephosphorylation product of Ins(1,4,5)P3. The lithium-sensitivity of the accumulation of this isomer also suggests that a novel lithium-sensitive 4- or 5-phosphatase activity may be present in these preparations.

The effects of lithium on phosphoinositide metabolism in CHO cells expressing the human M1-muscarinic receptor subtype was also examined to determine whether this cell line would represent a suitable model of cerebral PI metabolism. It was hoped that the use of this cell-line would result in clearer more interpretable data. Indeed, a definitive analysis of PI metabolism in this cell line clearly demonstrated that the addition of lithium to agonist-stimulated cells resulted in a decrease in the intracellular myo-inositol reserves within the cell which resulted in a decrease in the (poly)phosphoinositide precursor of Ins(1,4,5)P3, PtdIns(4,5)P2. In turn this resulted in a time-dependent decrease in the agonist-stimulated levels of Ins(1,4,5)P3 after a lag period of 5-10 min, similar to that observed in cerebral cortex slices. The data demonstrate that the CHO-M1 cell-line is a valuable tool in elucidating the actions of lithium upon PI signalling.

In conclusion, the results described in this thesis clearly demonstrate the profound effects that this monovalent ion has on phosphoinositide signalling in the preparations examined. The main action of lithium appears to be the inhibition of the inositol monophosphatase, however, this agent may also have other more subtle effects upon this complex system. These possibilities and their implications are also discussed.
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Chapter 1

Introduction
Introduction.

The evolution of multicellular organisms has relied upon the ability of the individual cells within that organism being able to communicate with each other. Communication between cells is essential for the regulation of such processes as development, cell differentiation, growth and the control of cellular activity which must be tightly controlled in order to maintain the viability of each individual cell within such an organism. One way in which cells have overcome this communication problem is by the use of chemical signals which relay the message from one cell to another. These external stimuli are detected by receptors situated in the plasma membrane of the cell and upon detection the signal is transduced to, and amplified within, the cell's cytosol by means of second messenger systems. Several such systems have been shown to exist within a single cell type and these include the inhibition or stimulation of adenylyl cyclase, the modulation of ion channel opening, and protein kinase stimulation. The work described in this thesis involves such a second messenger system, the phosphoinositide system, which in recent years has been shown to be involved in a large range of diverse cellular processes from controlling secretion and smooth muscle contraction to regulation of cellular growth itself.

1.1 History.

In 1930, Anderson and Roberts demonstrated the existence of inositol-containing lipids in mycobacterial membranes. Folch and Woolly later went on to isolate an inositol phospholipid fraction from bovine brain thus demonstrating that eukaryotic cell membranes also contain inositol phospholipids (Folch & Woolly, 1942). The actual components of this fraction were later shown to be phosphatidylinositol 4-phosphate (PtdIns(4)P), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) and the most abundant of the inositol containing lipids, phosphatidylinositol (PtdIns). By 1975 these three lipids had been isolated in a variety of cell types including both animal and plant cells (see Michell, 1975 and refs. therein) and it is now known, at least in eukaryotic membranes, that as a whole they constitute only a minor (< 10%)
component of the total membrane phospholipid of these cells.

The first evidence for a receptor-mediated effect on inositol phospholipid metabolism was provided by Hokin and Hokin (1953) when they demonstrated that the agonist acetylcholine could stimulate the incorporation of $^{32}$P into the phospholipids of pigeon pancreas and guinea pig cerebral cortex slices. It was subsequently shown that this stimulated incorporation was mainly associated with PtdIns and phosphatidic acid (PA) (see Hokin, 1985). These phenomena were referred to as the "phospholipid effect". Closer investigation of this response revealed that whilst the incorporation of labelled phosphate was larger in the presence of agonist there was little, if any, concomitant increase in the incorporation of label into the glycerol backbone (see Hokin, 1968). Thus it was established that the reaction involved labelling of the inositol headgroup. This "phospholipid effect" has since been observed in a large variety of tissues (see Michell, 1975; Downes, 1982, 1983; Berridge & Irvine, 1984; Hokin, 1985; Downes & Michell, 1985; Abdel-Latif, 1986; Fisher & 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, during the late 1960's and 1970's the idea emerged that inositol lipids may be involved in a novel signal transduction mechanism. The overwhelming evidence at that time favoured the cleavage of PtdIns by a phospholipase C to yield sn-1,2-diacylglycerol, InsP$_1$ and cyclic Ins(1,2)P$_1$, indicating that incorporation of $^{32}$P into the phospholipids was secondary to the initial hydrolysis (see Michell, 1975). In addition, a connection was made between agonists which were known to elicit a rise in intracellular Ca$^{2+}$ and those generating a change in PtdIns metabolism (Michell, 1975) from which Michell proposed that PtdIns turnover actually induced the Ca$^{2+}$ rise.

The initial suggestion that the polyphosphoinositides (PtdIns(4)P and PtdIns(4,5)P$_2$) may be hydrolysed in response to receptor activation, not just PtdIns itself, was originally reported in crude mitochondrial fractions of guinea-pig brain (Durell et al., 1968). Durell et al.
demonstrated that acetylcholine could not only increase the accumulation of InsP₁ but also InsP₂, which may be formed from the metabolism of PtdIns(4)P by PLC. Moreover Schacht and Agranoff (1972) added weight to this hypothesis when they observed that addition of carbachol to guinea-pig cerebral cortex resulted in a decrease in the labelling of PtdIns(4)P with ³²P, suggesting receptor mediated metabolism of this lipid. Furthermore, it was shown in rabbit iris smooth muscle that muscarinic or alpha-adrenergic receptor activation could produce a loss of ³²P radioactivity from PtdIns(4,5)P₂ with a concomitant increased labelling of PtdIns and PA (Abdel-Latif & Akhtar, 1976; Abdel-Latif et al., 1977, 1978). The realization that the initial target for phospholipase C was in fact PtdIns(4,5)P₂ and not PtdIns(4)P or PtdIns did not, however, come until 1981 (Michell & Kirk, 1981; Kirk et al., 1981). The hydrolysis of PtdIns(4,5)P₂ leads to the production of not only DAG but also inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃). Studies by Streb et al. (1983) indicated that in rat pancreatic acinar cells it was Ins(1,4,5)P₃ that was responsible for intracellular Ca²⁺ mobilization from non-mitochondrial intracellular stores following receptor-activated inositol lipid hydrolysis, whilst the DAG metabolite was subsequently found to activate endogenous protein kinase C (PKC) (for reviews see Nishizuka, 1984, 1986).

More recently it has been shown that the binding of agonist to receptors known to activate PtdIns(4,5)P₂ hydrolysis can be modulated by GTP and its non-hydrolysable analogues (Evans et al., 1985). Cockcroft & Gomperts (1985) and Litosch et al. (1985) demonstrated the ability of guanine nucleotides to directly stimulate phosphoinositide (PI) hydrolysis in ³²P labelled membranes from neutrophils and [³H]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. This has since been confirmed in a number of studies (for review see Taylor, 1990).

Since these early studies, the progress in the field of inositol lipid signalling has been rapid.
Figure 1.1: The Phosphoinositide Cycle: Metabolism of PtdIns(4,5)P$_2$ and Inositol (poly)phosphates.

Identified metabolic enzymes are:

1. PI-PLC.
2. 5-phosphatase.
3. 3-kinase.
4. 3-phosphatase.
5. 1-phosphatase.
6. 4-phosphatase.
7. 3-phosphatase.
8. 5-kinase.
9. monophosphatase.
10. 6-kinase.
11. PtdIns synthase.
12. PtdIns 4-kinase.
13. PtdIns(4)P 5-kinase.
15. PtdIns(4,5)P$_2$ diesterase.
16. DAG kinase.
17. Phosphatidate cytidylyltransferase.

DAG: sn-1,2-diacylglycerol; PA: phosphatidic acid;
CMPPA: CMP-phosphatidate; PIP$_2$: PtdIns(4,5)P$_2$;
PIP: PtdIns(4)P; PI: PtdIns.
Figure 1.1. illustrates the possible enzymic steps which occur upon agonist stimulation of PLC as it is presently understood.

The aim of this introduction is to discuss the constituent elements of this metabolic pathway. The known therapeutic and pharmacological aspects of the disruption of this pathway caused by lithium will also be discussed.

1.2. Muscarinic Receptors.

In 1914 Dale discovered two types of response to acetylcholine, one mimicked by muscarine and the other by nicotine (Dale, 1914; Dale & Ewin, 1914). This led to the subsequent discovery of nicotinic and muscarinic acetylcholine receptors. In addition to their pharmacological differences, muscarinic and nicotinic receptors can be differentiated by the mechanism and speed by which their cellular signals are transduced. Nicotinic receptors have a central pore through which sodium and potassium ions pass, resulting in depolarisation of the cell membrane. Acetylcholine activates nicotinic receptors by opening the channel, and thus the response is as fast as the channel opening rate (ms). Muscarinic responses are more diverse, both hyperpolarising and depolarising cells by a variety of mechanisms. Muscarinic responses are slower (in the order of 100's of milliseconds to seconds), due to their interaction with GTP-binding proteins (G-proteins) through which the cellular response is transduced. The slowest signals involve second messengers activated via G-proteins. Examples include inhibition of adenylate cyclase (reducing cAMP levels) and stimulation of polyphosphoinositide hydrolysis. However there are also muscarinic responses that are independent of second messengers. The activation of an inwardly rectifying potassium channel in the heart provides an example of a channel coupling directly with a G-protein (see Caulfield, 1993).

In addition to this functional diversity, muscarinic receptors can be pharmacologically differentiated. For example, muscarinic receptors in cerebral cortex, salivary glands, and heart have high, moderate and low affinities for the antagonist pirenzepine (PZP), respectively (Hammer & Giachetti, 1982). On the other hand, the antagonist AF-DX 116 has higher affinity for muscarinic receptors expressed in the heart compared with those expressed in the other
tissues. These and other studies have led to the division of muscarinic receptors into three pharmacological subtypes (M₁, e.g., cerebral cortex; M₂, e.g., heart; M₃, e.g., glands) (see Caulfield, 1993).

Because of the pharmacological differences between muscarinic receptors located in heart and brain (Hammer et al., 1980), these tissues were initially targeted for purification efforts. Muscarinic receptors were purified to homogeneity from porcine brain (Haga & Haga, 1983) and atria (Peterson et al., 1984). Amino acid sequences were obtained from fragments of these pure proteins, and the corresponding cDNA's were cloned (Kubo et al., 1986a,b; Peralta et al., 1987b). Comparison of the two sequences indicates that atrial and brain muscarinic receptors are highly related but distinct proteins and that they are homologous with all members of the G-protein-coupled receptor superfamily. By screening various libraries at moderate stringency, species homologues of these two receptors, as well as three closely related receptor sequences, were identified in rat and human (Bonner, et al., 1987, 1988). Based on the pharmacology of the receptors encoded by these five related genes, they have all been shown to encode muscarinic acetylcholine receptors (Bonner, et al., 1987, 1988). The cloning of all five of the receptor subtypes has been confirmed (Peralta et al., 1987a; Liao et al., 1989). The assignment of receptor number has been made on the basis of their affinity for a variety of different muscarinic receptor antagonists. Broadly speaking, the receptors expressed by m₁, m₃ and m₅ genes preferentially couple to the stimulation of (poly)phosphoinositide metabolism via a pertussis (PTX)-insensitive G-protein (Brann et al., 1988b; Conklin et al., 1988; Peralta et al., 1988; Novotny & Brann, 1989; Jones et al., 1991); whilst m₂ and m₄ receptors both appear to inhibit the adenylate cyclase system via a PTX-sensitive G-protein (Brann et al., 1988b; Peralta et al., 1988; Wess et al., 1990). Several exceptions to this generalization do exist, however. m₂ and m₄ receptors can weakly stimulate (poly)phosphoinositide hydrolysis, via a PTX-sensitive G-protein, although this is noticeably less efficient than their coupling to adenylate cyclase inhibition and is only observed when receptors are expressed at high levels (Ashkenazi et al., 1987; Peralta et al., 1988; Ashkenazi et al., 1989). Moreover, stimulation of
RAT-1 cells (Pinkras-Kramarski et al., 1988; Stein et al., 1988) transfected with M1 receptors or A9L cells (Branne et al., 1988b; Novotny & Brann, 1989) transfected with M1 or M3 receptors results in the stimulation of both (poly)phosphoinositide hydrolysis and adenylate cyclase, although at least in A9L cells this is believed to be mediated indirectly by the rise in intracellular calcium concentration (Felder et al., 1989).

Cerebral cortex, which is the tissue used in the majority of the experiments described here, has been shown by in situ hybridization to express mainly M1 receptors with lower levels of M2 through to M5 also being expressed (Levey et al., 1991). The other cell type which is predominantly used in the following chapters is the Chinese hamster ovary cell line which has been stably transfected with human m1 receptor cDNA. For the pharmacological data obtained with cloned receptors to be physiologically relevant, the receptors must have similar properties when expressed endogenously in tissues and in transfected cell lines. Fortunately, at least with respect to antagonist binding, the available data indicate a virtual independence on cell type for the pharmacological properties of muscarinic receptors. For example, it has been shown that no differences exist in the affinities of several muscarinic antagonists for muscarinic receptors expressed by CHO-K1, COS-7, A9L, RBL 2H3 and NIH 3T3 cells (Jones et al., 1992).

1.3 G-Proteins.

The first reports that the cAMP signal transduction system was not only regulated by hormones but by GTP were published in 1971 (Rodbell et al., 1971a,b). Rodbell provided evidence that at least in the cAMP system the receptor and effector were separate proteins that communicated through a guanine nucleotide-dependent regulatory protein or G-protein. The developments that followed Rodbell's pioneering studies have established that many different receptors regulate many intracellular effectors through a family of closely related G-proteins (see Simon et al., 1991). The suggested involvement of one or more G-proteins in the activation of PI-PLC resulted from a number of findings, including the observation that analogues of the guanine nucleotide GTP, such as GTP-gamma-S, can activate inositol
phosphate formation (Cockcroft & Gomperts, 1985; Litosch et al., 1985).

The G-proteins that transmit information from receptors to their intracellular effector systems have now been shown to belong to a large homologous family of heterotrimeric proteins each with an alpha-subunit that binds guanine nucleotides, and beta- and gamma-subunits that are always tightly associated (Casperson & Bourne, 1987; Gilman, 1987; Holbrook & Kim, 1989). Different G-proteins are most readily distinguished by their alpha-subunits, though there are also more subtle structural and functional differences in some beta- and gamma-subunits (Cerione et al., 1987). Activation of G-proteins requires their association with the membrane. Attachment of the alpha-subunit to the cytoplasmic face of the membrane may be mediated by the beta/gamma-complex (Sternweis, 1986) or by fatty acids or a poly isoprene group covalently linked to the N-termini of some alpha-subunits (Buss et al., 1987; Lochrie & Simon, 1988; Mumby et al., 1990).

GDP bound to the G protein alpha-subunit normally dissociates only very slowly (half-time of 1-5 min for Gs and hours for transducin) (Brandt & Ross, 1985; Gilman, 1987; Stryer, 1988; Chabre & Detterre, 1989), however the concerted actions of intracellular Mg2+ and activated receptors catalyse G-protein activation by increasing the rate of GDP dissociation from the alpha-subunit and its replacement by GTP. When an agonist interacts with its receptor part of the free energy of binding is used to deform the receptor protein (Jencks, 1975; Burgen, 1981), switching it to an "active" form. The activated receptor has a high affinity for a conformation of the G-protein in which its alpha and beta/gamma subunits are associated and the single guanine nucleotide-binding site of the alpha-subunit is empty (Wessling-Resnick et al., 1987; Chabre et al., 1988). Activated receptors therefore do more than promote release of bound GDP, they also hold open the guanine nucleotide binding site (Bimbaumer et al., 1980; May & Ross, 1988) and may even increase the affinity for GTP relative to GDP (Florio & Sternweis, 1989). Association of GTP with the alpha-subunit results in the dissociation of the alpha- and beta/gamma-subunits of the G-protein and activation of the alpha-subunit. The ternary complex of agonist, receptor and G-protein (DeLean et al., 1980) is transitory since GTP binds within milliseconds to the alpha-subunit (May & Ross, 1988) which results in a decrease in the
affinity of the receptor for the G-protein, and the two dissociate. The hydrolysis of GTP bound to the activated alpha-subunit, by the intrinsic GTPase activity present in the alpha-subunit (Godchaux & Zimmerman, 1979; Cassel & Selzinger, 1976), yields the inactive form of the alpha-subunit (i.e. G-alpha bound to GDP) which has a high affinity for the beta/gamma subunit, leading to reassociation and continuation of the cycle (see Fig. 1.2.; for review see Birnbaumer, 1990). It has been calculated that one "activated" receptor can interact with several G-proteins over a period of a few seconds (Pederson & Ross, 1982; Hekman et al., 1984), possibly leading to the amplification of the signal.

The original proposal that G-proteins couple to PI-PLC was made in 1983 when Gomperts described their involvement in histamine release from rat mast cells (Gomperts, 1983). Since then several lines of evidence have implicated a mandatory role for a G-protein in transduction of the signal from certain receptor types to PLC. However, the nature of these PLC-activating G-proteins remained elusive until only recently, when the G-protein subfamily Go was characterized independently by several laboratories.

Simon et al. (1991) obtained and sequenced a number of cDNAs corresponding to previously uncharacterised alpha-subunits of the Go subfamily, increasing the total number of identified mammalian G-alpha-subunits to 15 (see Simon et al., 1991 for review). These 15 G-alpha-subunits have been classified on the basis of amino acid sequence homology into four G-protein subfamilies, Go, Go/G, Gq and G12. It has been shown that there are four distinct members in the Gq family (G-alpha-q, G-alpha-n, G-alpha-u and G-alpha-ig). The amino acid sequences of G-alpha-q and G-alpha-u are 88% identical, whereas the other two are more distantly related (amino acid identity of only 55-60%). None of the four members of the Gq family contains a site for pertussis toxin modification.

Smrcka et al. (1991) purified a mixture of G-alpha-q and G-alpha-u from rat brain with the use of an affinity matrix containing immobilized G-protein beta/gamma-subunits, and they subsequently demonstrated that these brain G-proteins activated partially purified PLC. At the same time, Taylor et al. (1991) purified a mixture of G-alpha-q and G-alpha-u, on the basis of
Receptor Interactions With G-Proteins.

The interactions between agonist (A), receptor (R), and G-protein are described in the text.
its ability to activate partially purified PLC from bovine liver membranes. When reconstituted in the presence of GTP-gamma-S with isozymes of PLC, the mixture of G-alpha-q and G-alpha-11 specifically activated PLC-81, but not PLC-gamma-1 or PLC-91 (Taylor et al., 1991). More recently, it has been shown in COS-7 cells, that PI-PLC can be activated by the alpha-subunits of G_q and G_11 when they are transiently transfected into the cell line (Wu et al., 1992).

The domains within the M1-subtype of the muscarinic acetylcholine receptor (which is the receptor subtype most frequently utilized in the studies described in this thesis) that are responsible for interacting with the PLC-activating G-protein have been proposed to be the second and third intracellular loops of the receptor (Wong et al., 1990). Interaction with the ligand-occupied receptor causes dissociation of the heterotrimeric GDP-bound G_q to yield GTP-bound G-alpha_q. PLC-81 then binds the GTP-bound G-alpha_q, probably via the carboxyl-terminal regions of both proteins, which results in the activation of PLC-81 (see Rhee and Choi, 1992b). The carboxyl-terminal region of PLC-81 is thought to be important in the activation of PLC-81 by G-alpha_q since the removal of this region by calpain abolishes activation of this PLC-81 by G-alpha_q (Park et al., 1993a).

Members of the G_i class of G-protein couple to receptors that lower cAMP levels. However, it has been suggested that they may also play a role in the activation of PLC, increasing the formation of the inositol phosphates. Pertussis toxin is known to uncouple receptors from these specific G-alpha subunits, hence blocking signal transduction occurring through G_i (see Bimbamer et al., 1990).

Recently, attention has been turned to the role of the beta/gamma complex in signal transduction. Although not as diverse in nature as the alpha-subunits there are a number of isoforms of both beta- and gamma-subunits. In mammals, four beta subunit isoforms have been found, sharing over 80% sequence identity. beta_1, beta_2 and beta_3 are ubiquitously expressed, while beta_4 is abundant in brain and lung tissue, but is only found at low levels elsewhere (see Simon et al., 1991). Similarly there are four isoforms of the gamma-subunits, isolated as cDNA clones (see Simon...
et al., 1991). Originally it was thought that the signal was transduced from receptor to effector solely by the alpha-subunit of the respective G-protein which interacted with a specific effector molecule (e.g. adenylate cyclase or PLC). However recent reports have demonstrated that the alpha/gamma-subunits of certain G-proteins can activate PLC isozymes (Camps et al., 1992; Blank et al., 1992) with PLC-63 being activated to a greater extent than PLC-B2 or PLC-B1 (Park et al., 1993b). This adds yet further complexity to the role of G-proteins in signal transduction.

1.4 Phospholipase C Isoenzymes.

The phospholipases C are phosphodiesterases which hydrolyse the glycerophosphate bond of intact phospholipids to generate DAG and the aqueous soluble head group carrying the phosphate(s) (for review see Rhee & Choi, 1992b).

With the discovery of the "phospholipid effect", attention was focused on isolating a phospholipase C activity against PtdIns. Initial studies indicated that the PtdIns-PLC activity in animal tissues was predominantly cytosolic, required Ca^{2+} (in the supraphysiological range) and had an acidic pH optimum (Kemp et al., 1961; Atherton & Hawthorne, 1968; Friedel et al., 1969; Thompson, 1967). Furthermore, it was shown that this activity also behaved as a cyclizing phosphotransferase, resulting in the production of cyclic Ins(1:2)P_1 as well as Ins(1)P_1 from the cleavage of PtdIns (Dawson et al., 1971; Jungalwala et al., 1971; Lapetina & Michell, 1973).

During the 1970's, several reports of membrane-associated PtdIns-PLCs appeared in the literature (Achtar & Abdel latif, 1978; Canessa de Scamati & Rodriguez de Lores Arnaiz, 1972; Keough & Thompson, 1972; Lapetina & Michell, 1973). One indicated that the activity in the particulate fraction of rat brain, unlike cytosolic activities reported previously, had a neutral pH optimum (Oran et al., 1975). Furthermore, a cytosolic activity from lymphocytes was also found to have a pH optimum of approximately 7 (Allan & Michell, 1974). At neutral pH, instead of requiring millimolar levels of Ca^{2+}, this activity was maximal in the micromolar range for Ca^{2+}, from which it was argued that the rise in intracellular Ca^{2+} induced by agonist
stimulation was sufficient to activate the PtdIns-PLC (Allan & Michell, 1974).

Preliminary purifications of PtdIns-PLC activity from animal cells revealed that instead of a single activity, multiple chromatographically distinct activities were found in one tissue, stressing the need to perform characterisations with purified enzymes rather than cellular extracts (Hofmann & Majerus, 1982; Low et al., 1984; Ryu et al., 1986). The first report of a purified PI-PLC activity was from Takenawa & Nagai (1981), who isolated a 68-70 KDa protein from rat cytosol. Other reports of 65-70 KDa and 80 KDa PLC's from sheep seminal vesicles (Hofmann & Majerus, 1982) and a 143 KDa protein from bovine platelets (Hakata et al., 1982) suggested that a large family of PLC isozymes may exist.

It was at this time that PtdIns(4,5)P₂ was identified as the target substrate for receptor-activated phospholipid hydrolysis. Although many purification procedures used PtdIns to screen column fractions, purified activities were also assessed for their ability to act against PtdIns(4,5)P₂. The majority of studies indicated that the activities which acted against PtdIns, also acted against PtdIns(4)P and PtdIns(4,5)P₂, and that at physiological Ca²⁺ concentrations the polyphosphoinositides were, in fact, preferred substrates (Katan & Parker, 1987; Nakanishi et al., 1985; Wilson, et al., 1984).

Several distinct PLC enzymes have since been purified from a variety of mammalian tissues (Rhee et al., 1989; and refs. therein), and a total of 16 amino acid sequences, of which 14 are mammalian enzymes and 2 are Drosophila enzymes, have been deduced from the nucleotide sequences of their corresponding cDNA's (Rhee & Choi, 1992a). Comparison of the deduced amino acid sequences has indicated that the enzymes can be divided into three types, namely PLC-β, PLC-gamma and PLC-δ (see Fig. 1.3.), which in turn contain more than one subtype (designated 1, 2 etc.). Other isoforms may exist and on the basis of enzyme purification and biochemical characterisation two other PLC have been proposed, PLC-alpha and PLC-epsilon (see Cockcroft & Thomas, 1992). However, it is now thought that PLC-alpha carries no PLC activity and indeed recent studies have suggested that cDNA for PLC-alpha actually encodes a thiol-protein disulfide oxidoreductase (Srivastava et al., 1991).
Figure 1.3 Linear display of three types of PLC's represented by PLC-81, PLC-gamma and PLC-31.
Although the overall amino acid sequence similarity between the different PLC types is low, significant similarity is observed in two regions which are designated the X and Y regions by Rhee (see Rhee & Choi, 1992). Whereas PLC-β and PLC-δ contain short sequences of 50-70 amino acids separating the X and Y regions, PLC-gamma has a long sequence of ~400 amino acids, which contains the src homology (SH2 and SH3) domains (Koch et al., 1991). Also, whereas the carboxyl-terminus sequence following the Y region is ~450 amino acids long in PLC-β, this region is very short in PLC-δ. Between the PLC subtypes (i.e. PLC-B1 and PLC-B2), amino acid sequence identity is much higher than that between the three PLC types, not only in the conserved X and Y regions but also in the other regions.

The catalytic activities of all three types of PLC are dependent on Ca2+ and catalyze the hydrolysis of the three inositol phospholipids, PtdIns, PtdIns(4)P and PtdIns(4,5)P2. As mentioned previously, PtdIns(4,5)P2 is the preferred substrate, however the selectivity for PtdIns(4,5)P2 over PtdIns decreases in the order PLC-B1 > PLC-δ1 > PLC-gamma1. Interestingly, PLC-B1 has been shown to have the same K_m for PtdIns(4)P and PtdIns(4,5)P2 however, the V_max for hydrolysis is some 30-fold higher for PtdIns(4,5)P2 (Katan & Parker, 1987). PLC-gamma also hydrolyses PtdIns(4,5)P2 but at a similar rate as that for PtdIns(4)P (Ryu et al., 1987). Also mentioned previously, hydrolysis of PtdIns(4,5)P2 by all three types of PLC yields cyclic and noncyclic inositol phosphates (Kim et al., 1989). The ratio of cyclic to noncyclic inositol phosphates has been shown to differ between the individual subtypes of PLC, with the ratio of cyclic to noncyclic products decreasing in the order PLC-B1 > PLC-δ1 > PLC-gamma1 (Kim et al., 1989).

A number of receptors have been shown to activate PLC via activation of a G-protein (G_q), including those for acetylcholine, the muscarinic receptors (M1, M3 & M5). Interaction of the ligand-receptor complex with G_q causes dissociation of the heterotrimeric G_q to yield GTP-bound G-alpha_q which remains in the plane of the membrane. PLC-B1 can then bind the GTP-bound G-alpha_q, probably via the carboxyl-terminal regions of both proteins, which result in the activation of PLC-B1. At present, therefore, it is thought that when activation of a PLC
isozyme is via a G-protein then the PLC is probably of the PLC-8 type (see Rhee & Choi, 1992b).

PLC-gamma is activated by polypeptide growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and nerve growth factor (NGF) which mediate their action by binding to and activating cell surface receptors which have a cytoplasmic domain that contains a tyrosine kinase domain. Growth factor-induced stimulation of PLC appears to be independent of G-proteins and requires the intrinsic tyrosine kinase activity of the receptors. Treatment of a number of cell types with PDGF, EGF and NGF has been shown to lead to an increase in the phosphorylation of PLC-gamma1 (but not PLC-81 or PLC-91) with the increased phosphorylation occurring on both serine and tyrosine residues (Rhee & Choi, 1992a; and refs. therein). EGF-induced tyrosine phosphorylation of PLC-gamma1, which is mediated directly by the EGF receptor tyrosine kinase, occurs rapidly and correlates well with stimulation of PtdIns(4,5)P2 hydrolysis. The receptor-PLC-gamma1 association is mediated by a high affinity interaction between the SH2 domains of PLC-gamma1 and a specific tyrosine-auto-phosphorylation site on the receptor (Koch et al., 1991). Association of growth factors with the receptor has been shown to precede tyrosine phosphorylation of PLC-gamma1 by the receptor tyrosine kinase. It has now been demonstrated that the phosphorylation of PLC-gamma1 results in its activation (see Rhee & Choi, 1992b; Meldrum et al., 1991).

Although a great deal is known about the amino acid sequence of PLC-8 neither the receptors nor the transducer that this PLC couples to are known, however this enzyme has also been shown to be Ca2+ -dependent.

1.5 Ins(1,4,5)P3 and Ca2+ Release.

The first direct evidence that Ins(1,4,5)P3, the aqueous-soluble headgroup released following PLC-catalyzed hydrolysis of PtdIns(4,5)P2 was responsible for Ca2+ mobilization was made by Streb et al., (1983). They found that addition of Ins(1,4,5)P3 to permeabilised pancreatic acinar cells induced the release of Ca2+ from non-mitochondrial stores. Subsequent studies
confirmed that Ins(1,4,5)P3 had similar effects in many other cell types (for review see Berridge & Irvine, 1984, 1989). Early suggestions that Ins(1,4,5)P3 may be recognized by a specific intracellular receptor protein (Berridge & Irvine, 1984) have been supported by a number of studies indicating high affinity [3H] and [32P] Ins(1,4,5)P3 binding in a number of peripheral and central tissues which show strict stereo- and positional specificity for Ins(1,4,5)P3 (for review see Nahorski & Potter, 1989; Taylor & Richardson, 1991). The rapid kinetics (Champeil et al., 1989; Ogden et al., 1990) and limited temperature dependency (Smith et al., 1985; Meyer et al., 1991) of Ins(1,4,5)P3-induced Ca2+ release was thought to suggest that Ins(1,4,5)P3 stimulated a Ca2+ channel rather than affecting a carrier system. This was confirmed by electrophysiological studies (Ehrlich & Watras, 1988). More recent studies involving the purification and reconstitution of the Ins(1,4,5)P3 receptor (Ferris et al., 1989; Maeda, 1991), together with the expression of the cloned gene, have provided conclusive evidence that the Ins(1,4,5)P3 binding site is the ligand recognition domain of the Ca2+ mobilizing receptor.

The actual location of the Ins(1,4,5)P3 receptor has been debated over recent years. However, it is now generally believed that it is situated on the endoplasmic reticulum (E.R.). Evidence for this comes from a number of studies. Morphological (Wakasugi, 1982) and subcellular fractionation (Beyer dorffer et al., 1984) studies have supported an important role for the rough E.R. in some cells types. Electron probe micro-analysis of liver has also shown that upon agonist stimulation the Ca2+ content of the E.R. is significantly reduced. Studies by Volpe et al. (1991) have since confirmed that Ins(1,4,5)P3 receptors are localised to areas of the E.R.

The Ins(1,4,5)P3 receptor has now been purified to homogeneity from rat cerebellum (Supattapone, 1988) and, subsequently many other sources (for review see Taylor & Richardson, 1991; Mikoshiba, 1993). The receptors from the various tissues appearing to be very similar. The Ins(1,4,5)P3 receptor has been incorporated into phospholipids vesicles where it binds Ins(1,4,5)P3 and has been shown to mediate Ca2+ fluxes (Ferris et al., 1989,
1990) and bilayers where Ins(1,4,5)P₃ has been shown to open a Ca²⁺ channel (Maeda et al., 1991). Thus, it is the receptor which constitutes both the Ins(1,4,5)P₃ binding site and the Ca²⁺ channel (i.e. an integral receptor/channel complex). The receptor/channel complex has been shown to be a homotetramer consisting of noncovalently linked subunits (Mignery & Sudhof, 1990; Maeda et al., 1991) with an aggregate molecular weight of about 1,000 kDa (Supattapone et al., 1988). Work by Meyer et al. (1988) has shown that on average three molecules of Ins(1,4,5)P₃ must bind to this tetrameric receptor in order to open the integral Ca²⁺ channel.

The Ins(1,4,5)P₃ receptor from rat brain has been sequenced and cloned (Mignery et al., 1990). It has been shown that transfection with the cDNA obtained into cell lines which normally express low levels of the Ins(1,4,5)P₃ receptor, show increased Ins(1,4,5)P₃ binding (Mignery & Sudhof, 1990) and increased sensitivity to Ins(1,4,5)P₃-induced Ca²⁺ mobilization (Miyawaki, et al., 1990).

A number of different forms of the Ins(1,4,5)P₃ receptor have been shown to exist, the different forms arising from alternative splicing of two segments (termed SI and SII) of the mRNA (see Mikoshiba, 1993). However, new types of Ins(1,4,5)P₃ receptors from separate genes have also been reported (Ross et al., 1992; Sudhof et al., 1991). The original Ins(1,4,5)P₃ receptor has been called the cerebellar type, or type I. The type II receptor has significant homology with the type I receptor, especially in the binding site and transmembrane domains (Sudhof et al., 1991). More recently, the genes for a further two Ins(1,4,5)P₃ receptors have been sequenced, giving rise to a type III and a type IV Ins(1,4,5)P₃ receptor (Ross et al., 1992). The Ins(1,4,5)P₃ receptors share significant partial homology with the ryanodine receptor (Mignery et al., 1989) of the sarcoplasmic reticulum in skeletal and cardiac muscle (Takeshima et al., 1989). This tetrameric receptor is also involved in the release of Ca²⁺ from Ins(1,4,5)P₃-insensitive intracellular stores and has a role in Ca²⁺-induced Ca²⁺ release (see Berridge, 1993). Thus the interplay between both these receptor subtypes is probably
involved in the complex Ca\textsuperscript{2+} oscillations observed in several cell types (Berridge, 1993).

Low affinity Ins(1,4,5)P\textsubscript{3} binding sites (K\textsubscript{D}= 0.8-1 \mu M) have been shown to correspond to Ins(1,4,5)P\textsubscript{3} receptors associated with the plasma membrane (Khan et al., 1992). These low affinity receptors for Ins(1,4,5)P\textsubscript{3} have been shown to have a relatively high affinity for Ins(1,3,4,5)P\textsubscript{4}, with little selectivity between these two inositol polyphosphate isomers (Khan et al., 1992). This suggests that the plasma membrane binding site may be involved in the gating of extracellular Ca\textsuperscript{2+} in an Ins(1,4,5)P\textsubscript{3}/Ins(1,3,4,5)P\textsubscript{4}-sensitive manner, as previously suggested by Irvine and Moor (1987).

1.6 Ins(1,4,5)P\textsubscript{3} Metabolism.

Originally the rapid metabolism of Ins(1,4,5)P\textsubscript{3} observed in cells was thought to proceed via a simple sequence of dephosphorylation reactions, whereby Ins(1,4,5)P\textsubscript{3} was dephosphorylated to Ins(1,4)P\textsubscript{2} and subsequently Ins(1)P\textsubscript{1} and Ins(4)P\textsubscript{1} (Storey et al., 1984). It is now known however that the metabolism of this Ca\textsuperscript{2+} releasing moiety is more complex and can proceed via at least two routes (see Fig 1.1; for review see Shears, 1991).

In mammalian cells, the major route for the dephosphorylation of Ins(1,4,5)P\textsubscript{3} is by a 5-phosphatase, although Ins(1,4,5)P\textsubscript{3} may also be phosphorylated by a 3-kinase to give rise to the putative second messenger Ins(1,3,4,5)P\textsubscript{4}. Thus increases in the cellular concentration of Ins(1,4,5)P\textsubscript{3} are usually closely followed by increases in the levels of Ins(1,3,4,5)P\textsubscript{4}. However, some cells such as Dictyostelium discoideum (van Haastert et al., 1989) and ram sperm (Harrison et al., 1990) appear to be devoid of 3-kinase activity. The Mg\textsuperscript{2+}-dependent 5-phosphatase activity which dephosphorylates Ins(1,4,5)P\textsubscript{3} to Ins(1,4)P\textsubscript{2} also dephosphorylates Ins(1,3,4,5)P\textsubscript{4}, producing Ins(1,3,4)P\textsubscript{2}. It has also been suggested that a minor route of metabolism for Ins(1,4,5)P\textsubscript{3} may be via a 1-phosphatase, since studies in GH3 cells and rat cerebral cortical slices have demonstrated the accumulation of significant levels of Ins(4,5)P\textsubscript{2} (Hughes & Drummond, 1987; Batty et al., 1989). More recent work (Jenkinson et
has demonstrated that agonist-stimulated accumulation of Ins(4,5)P$_2$ in rat cortical slices appears to be lithium-sensitive suggesting the possibility of a lithium-sensitive 1-phosphatase enzyme in this system.

Since Ins(1,4,5)P$_3$ is involved in the release of Ca$^{2+}$ a great deal of effort has been devoted to understanding the mechanisms by which the cell controls the levels of this inositol polyphosphate. In general, those cell types with a typical concentration range of Ins(1,4,5)P$_3$ (0.1-1 μM) the 3-kinase will operate not far from its $V_{\text{max}}$, since the $K_m$ of this enzyme for Ins(1,4,5)P$_3$ is 0.2-1.5 μM (see Biden & Wolheim, 1986; Ryu et al., 1987; Sim et al., 1990). In contrast, although there are several isoenzymes of the 5-phosphatase (see Erneux & Takazawa, 1991) kinetic data indicate that all of these isoenzymes operate in the cell well below their $V_{\text{max}}$. Furthermore, in intact cells the accumulation of Ins(1,3,4,5)P$_4$ reduces the dephosphorylation of Ins(1,4,5)P$_3$ in a competitive manner due to the high affinity of the particulate and type 1 soluble 5-phosphatases for Ins(1,3,4,5)P$_4$. In contrast, physiological levels of Ins(1,3,4,5)P$_4$ have little impact on 3-kinase activity (Polokoff et al., 1988).

A number of groups have demonstrated that the 3-kinase can be activated, in vitro, by increasing Ca$^{2+}$ concentrations and this is thought to be due to an interaction of the 3-kinase with Ca$^{2+}$/calmodulin (Biden et al., 1987; Ryu et al., 1987) resulting in an increase in the $V_{\text{max}}$ of the kinase (Biden & Wolheim, 1986; Johanson et al., 1988).

Protein kinase C has also been shown to regulate Ins(1,4,5)P$_3$ 3-kinase activity. The first evidence for this was obtained from studies where a human T-cell line was stimulated with either antibodies or phorbol esters (Imboden & Pattison, 1987); the partially purified 3-kinase from these cells exhibited a persistent activation. Similar studies in other cell lines have confirmed this effect (Biden, et al., 1988; King & Rittenhouse, 1989). However, these results have been questioned with the recent discovery that the 3-kinase is a calpain-sensitive enzyme. This Ca$^{2+}$-dependent protease (for review see Wang et al., 1989) may alter the regulatory or catalytic units of the kinase thereby altering the characteristics of the enzyme in vitro from what
they are in vivo. One subsequent study has demonstrated that the reconstitution of the purified and intact 3-kinase with PKC resulted in the phosphorylation of the enzyme and a subsequent decrease in the $V_{\text{max}}$ of the enzyme by 75% (Sim et al., 1990). The role of PKC in the control of this enzyme is therefore still uncertain.

Unlike the 3-kinase, there is little evidence for the regulation of the 5-phosphatase enzyme by changes in the intracellular Ca$^{2+}$ concentration (Biden et al., 1988). The type 1 soluble 5-phosphatase has been shown to be stimulated, at least in platelet extracts, by PKC activators such as phorbol esters (King & Rittenhouse, 1989). Indeed, it has been suggested that the inhibitory effect of lithium on agonist induced accumulations of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ (Kennedy et al., 1988) may be indirect and due to lithium producing an increase in DAG levels, which in turn activate PKC resulting in an enhancement of 3-kinase and 5-phosphatase activity.

Current evidence suggests that the products of 5-phosphatase activity against Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$, Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$, respectively, do not at physiological concentrations inhibit the 5-phosphatase enzyme (Downes et al., 1982; Shears et al., 1987). Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ produced from the dephosphorylation of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ can be further dephosphorylated. The major route of Ins(1,4)P$_2$ dephosphorylation is by a 1-phosphatase (Inhorn & Majerus, 1987, 1988; Gee et al., 1988b). This 1-phosphatase also converts Ins(1,3,4)P$_3$ to Ins(3,4)P$_2$ (Inhorn & Majerus, 1987, 1988) and this enzyme has since been renamed as the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase since it does not attack the 1-phosphate groups of other inositol phosphates (e.g. Ins(1,4,5)P$_3$). The $K_m$ for Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ of this enzyme is approximately 1-5 $\mu$M and 5-20 $\mu$M respectively, although some differences are detectable between different tissues. The value for Ins(1,3,4)P$_3$ is close to the concentration of this compound in maximally stimulated cells (Hughes et al., 1989), therefore the enzyme is not normally saturated. The enzyme has been shown to be inhibited by lithium at millimolar concentrations and is dependent upon Mg$^{2+}$ (Gee
A recent study by Batty & Nahorski (1992) has provided evidence that this may not be the only route of metabolism for Ins(1,4)P$_2$. They have provided indirect evidence that in cerebral cortex slices Ins(1,4)P$_2$ may also be metabolized by a 4-phosphatase to give Ins(1)P$_1$.

Ins(1,3,4)P$_3$ can also be dephosphorylated by a 4-phosphatase to give Ins(1,3)P$_2$ (Bansal et al., 1987; Dean & Moyer, 1988). Thus levels of both Ins(3,4)P$_2$ and Ins(1,3)P$_2$ increase upon receptor activation in a number of tissues. This 4-phosphatase also attacks Ins(3,4)P$_2$ giving Ins(3)P$_1$ (Bansal et al., 1990). Ins(1,3)P$_2$ is further metabolized by a 3-phosphatase (Bansal et al., 1987) of which two forms of different size have been found in brain, one Mg$^{2+}$-dependent and the other Mg$^{2+}$-independent (Howell et al., 1989).

It has also been shown that Ins(1,3,4)P$_3$ can be sequentially phosphorylated to produce Ins(1,3,4,5,6)P$_5$ and Ins(3,4,5,6)P$_6$, and in turn InsP$_6$ (see Menneti et al., 1993), the levels of which change relatively slowly upon receptor activation (Pittet et al., 1989). As yet, there has been little progress in understanding the roles which these higher inositol phosphates may play, aside from two highly specialized functions. Ins(1,3,4,5,6)P$_5$ regulates the affinity of avian haemoglobin for oxygen (Isaacks & Harkness, 1980), while both Ins(1,3,4,5,6)P$_5$ and InsP$_6$ may be neurotransmitters (Vallejo et al., 1988).

The metabolism of Ins(1,4,5)P$_3$ therefore leads to the generation of three inositol monophosphates: Ins(1)P$_1$, Ins(3)P$_1$, and Ins(4)P$_1$. All of these are converted to free inositol by the action of a single enzyme, inositol monophosphatase (Ackermann et al., 1987; Gee et al., 1988a). This enzyme is Mg$^{2+}$-dependent and although not inhibited by inositol it is inhibited by phosphate (K$_i$ of 0.5 mM) (Gee et al., 1988a) and submillimolar concentrations of lithium (Hallcher & Sherman, 1980; Gee et al., 1988a).

The free inositol that is released replenishes the free inositol pool of the cell and is available for the resynthesis of the (poly)phosphoinositides. This complex pathway of Ins(1,4,5)P$_3$ metabolism therefore serves to terminate second-messenger action and efficiently conserves the
cellular myo-inositol pool for the resynthesis of the (poly)phosphoinositides.

1.7 Diacylglycerol and Protein Kinase C.

Diacylglycerol (DAG) is the other second messenger produced by the PLC-catalyzed hydrolysis of PtdIns(4,5)P$_2$. However, it can also be formed by the action of PLC on PtdIns(4)P and PtdIns. Unlike the aqueous-soluble product Ins(1,4,5)P$_3$ this metabolite, because of its lipophilic nature, remains in the plane of the membrane bilayer. The proposal that DAG was the intermediate between PtdIns(4,5)P$_2$ and protein kinase C (PKC) activation was first made in 1981 by Takai et al. Originally, PKC was discovered as a histone protein kinase from rat brain which was specific for serine and threonine (Inoue et al., 1977). It was subsequently shown that PKC was activated by limited proteolysis (Inoue et al., 1977), Ca$^{2+}$ and (phospho)lipids (Takai et al., 1979).

From purification studies (Huang et al., 1986), it became clear that PKC represented a group of at least three isoenzymes (alpha, beta and gamma). In more recent studies, nine distinct PKC enzymes have been purified from a variety of different species and tissues or cell lines (for review see Hug & Sarre, 1993), and the nucleotide sequences of their corresponding cDNA’s determined. The isoenzymes of PKC can be grouped into two main groups, the Ca$^{2+}$-dependent or conventional PKC's (cPKCs) and the Ca$^{2+}$-independent or novel PKC's (nPKC's) (Ohno et al., 1991). Four of the isoenzymes belong to the former group and five to the second group.

The requirements of PKC for activation vary slightly for each isoenzyme. Activation of the cPKCs is thought to require DAG as activator and phosphatidylserine (PtdSer) as a cofactor of activation, the presence of both reducing the Ca$^{2+}$ requirement of PKC from the millimolar range to the micromolar range (Lee & Bell, 1991, and refs. therein). This increase in sensitivity means that DAG can render PKC fully active without any actual increase in intracellular Ca$^{2+}$ (Kishimoto et al., 1980; Rasmussen, et al., 1985). As outlined above, members of the nPKC group do not require Ca$^{2+}$ for activation, but require DAG/PtdSer (Liyange et al., 1992).
of PKC, at least in vitro. Arachidonic acid (Ogita et al., 1992) is capable of activating a number of the cPKC's, even in a Ca\(^{2+}\)-independent manner.

PKC isoforms are thought to be cytosolic in nature. Activation is brought about when PKC translocates to the membrane and associates with PtdSer and DAG. The substrates for activated PKC appear to be numerous within the cell (for review see Hug and Sarre, 1993).

In 1982, Castagna et al. (1982) demonstrated the ability of phorbol esters to activate PKC in a similar manner to that of DAG, even at concentrations of up to 1000-fold less than that required for PKC activation by DAG. Use of phorbol esters has given insight into the many possible cellular functions with which DAG and PKC may be involved. These esters have been shown to elicit a large variety of cellular responses including, the promotion of tumours (Blumberg, 1980), cellular proliferation, (Dicker & Rozengurt, 1980), prostaglandin production (Edwards et al., 1985) and activation of platelets (Zucker et al., 1974).

Several inhibitors of PKC have been developed during recent years. The microbial product staurosporine was one of the first in a line of PKC inhibitors (Tamaoki et al., 1986). This compound's ability to inhibit PKC is by virtue of its being able to prevent ATP from binding to its binding site on PKC therefore preventing PKC from phosphorylating the target proteins. Staurosporine is however, fairly unspecific since it also prevents ATP binding to other kinases (e.g. PKA etc.) thereby inhibiting them also. More recently, a series of structural analogues of staurosporine has been developed, e.g. Ro 318220 (Davis et al., 1989). This compound works by the same mechanism as that of staurosporine (i.e. competing for the ATP binding site) however it is over 1000-fold more selective for PKC over other kinases (Davis et al., 1989) probably due to subtle structural differences in the ATP binding site between the different ATP-dependent kinases.

As mentioned earlier, DAG can come from a number of distinct sources (de novo synthesis, hydrolysis of PtdIns(4,5)P\(_2\) and other phospholipids including PtdCho and phosphatidylethanolamine (PtdEtN), or indeed from the dephosphorylation of PA). It has been known for some time that the DAG in cells consists of a number of different molecular species. Recent work by Pettit & Wakelam (1993) has shown that the diacyl structure of the major
Recent work by Pettit & Wakelam (1993) has shown that the diacyl structure of the major phospholipids found in the Swiss 3T3 cells, namely PtdCho, PtdEtN, PtdIns and phosphatidylserine (PtdSer) are significantly different, suggesting that the different species of DAG found in this cell type may be due to different phospholipid sources. Furthermore, this study also demonstrated that a number of the different species of DAG formed upon agonist stimulation in Swiss 3T3 cells accumulated in an agonist- and time-dependent manner, however, the temporal aspects of the accumulation varied between the individual species. This may possibly be due to differences in the phospholipid substrate utilized in the formation of DAG with time of stimulation. One tempting suggestion is that the different agonist-sensitive DAG species may interact with specific isoforms of PKC.

1.8 Phosphoinositide Synthesis.

PtdIns is synthesised either in the endoplasmic reticulum (Benjamins & Agranoff, 1969; Takenawa & Egawa, 1977) or in the plasma membrane of cells (Imai & Gershengorn, 1987). Phosphatidic acid (PA) combines with intracellular cytidine 5-trisphosphate (CTP) to produce CMP-phosphatidate (CMP-PA), a reaction catalyzed by the enzyme CTP-PA cytidyl transferase. CMP-PA subsequently combines with myo-inositol, in the presence of PtdIns synthase to generate PtdIns. Myo-inositol can originate from three sources: de novo synthesis from glucose via Ins(3)P$_1$ (see Chen & Charlampous, 1969; Eisenberg, 1967); from the extracellular environment via a facilitated transport mechanism (MiMitoris et al., 1980); or from inositol (poly)phosphate metabolism (see Shears, 1991). PA can also be formed de novo from glucose (via glycolysis forming glycerol 3-phosphate which combines with fatty acid groups formed from lipogenesis) however, it is also formed from the metabolism of DAG by the action of diacylglycerol kinase on DAG (Kanoh, et al., 1990) and from the action of PLD on phosphatidyl choline.

PtdIns can be transported rapidly from its site of synthesis to the membrane bilayer by means of a PtdIns transfer protein (George & Helmkamp, 1985; Somerharju et al., 1983). It is therefore readily accessible to the kinases responsible for converting PtdIns into its
phosphorylated derivatives PtdIns(4)P$_1$ and PtdIns(4,5)P$_2$. PtdIns(4,5)P$_2$ is located in the inner leaflet of the plasma membrane and is formed by a two stage phosphorylation of PtdIns which is first phosphorylated at the 4-position of its inositol headgroup by a specific kinase (PtdIns kinase) to form PtdIns(4)P; this is in turn further phosphorylated (PtdIns(4)P kinase) at the 5-position to give PtdIns(4,5)P$_2$ (see Berridge & Irvine, 1984). As well as the two kinases responsible for the synthesis for the stepwise phosphorylation of PtdIns to PtdIns(4,5)P$_2$ there are corresponding phosphomonoesterases which convert PtdIns(4,5)P$_2$ back to PtdIns. These kinases and phosphatases constitute two linked PtdIns/PtdIns(4,5)P$_2$ futile cycles whereby phosphates are constantly being added to, and removed from, the 4- and 5-positions of the inositol headgroup (see Berridge & Irvine, 1984).

The presence of a PtdIns 3-kinase has been reported in a number of tissues, allowing for the formation of PtdIns(3,4,5)P$_3$ from PtdIns(4,5)P$_2$. Sequential dephosphorylation of PtdIns(3,4,5)P$_3$ results in the formation of the lipids PtdIns(3,4)P$_2$ and PtdIns(3)P. PtdIns(3,4,5)P$_3$, PtdIns(3,4)P$_2$ and PtdIns(3)P however, appear to be resistant to hydrolysis by any of the PLC isoforms yet identified, and their precise function is unclear (see Downes & Carter, 1991). One possible role which has been proposed is an involvement in cell mitosis (see Downes & Carter, 1991). PtdIns 3-kinase has been isolated from bovine brain (Otsu et al., 1991) and is activated by nerve growth factor (Carter & Downes, 1992) suggesting it may be required for neuronal survival and development.

1.9 Lithium and Cell Signalling.

The psychotropic effect of lithium was discovered in 1949 by Cade who predicted that urate salts should prevent the state of hyperexcitability induced by uremia in guinea-pigs (Cade, 1949). He found that lithium urate produced a calming effect and quickly went on to discover that it was due to the lithium and not the urate. Furthermore, he showed that lithium salts produced a rapid improvement in patients suffering from mania. Lithium, at a plasma concentration of 0.6-1.5 mM, has since been shown to control the manic phase of manic
(bipolar) depression although this ion has little or no effect on unipolar depression. Used prophylactically in bipolar depression, lithium is able to prevent the swings of mood, from depression to euphoria, and thus is effective in controlling both the manic as well as the depressive phases of the illness (Rosenthal & Goodwin, 1982).

Since this discovery a great deal of effort has gone into trying to elucidate the mechanism by which lithium produces its therapeutic action, in order that other more selective and less toxic drugs may be developed. Over the intervening years a number of theories have been put forward for the mechanism of action of lithium. These include: interfering with G-protein coupling, interfering with ion transport, inhibition of adenylate cyclase, and disruption of phosphoinositide signalling (see below).

As discussed earlier, a large number of receptors transduce their signal to their intracellular effector via a guanine nucleotide-binding protein, or G-protein. Avissar et al. (1988), by measuring both agonist-dependent changes in GTP binding to G-proteins and guanine-nucleotide-dependent decreases in the affinity of the respective receptor for the agonist, has shown that lithium can inhibit the coupling of both muscarinic cholinergic and β-adrenergic receptors to PTX- and cholera toxin-sensitive G-proteins. The concentration of lithium which exerts this effect is well within the therapeutic range (0.6 mM). It has been suggested that this effect could also explain the data obtained on the effects of lithium upon cAMP and Ins(1,4,5)P₃ accumulation (see below).

The most important mechanism of entry of lithium into the cell is thought to be via Na⁺ channels in excitable tissues. The extrusion of this ion has been shown not to be significantly reduced by increasing the concentration of K⁺ (Smith, 1974), suggesting that its extrusion via the Na⁺/K⁺ ATPase is not of major importance. Lithium, therefore, tends to accumulate within the cell. Indeed in rat cortical synaptosomes lithium accumulates, such that with 0.3 to 1 mM external lithium, the intracellular concentration of this ion is 3-fold that of the external concentration (Schmalzing, 1986), this can result in a partial depolarization of the cell. It has been suggested that lithium may in some way interfere with the transport of other ions within
the cell and indeed there is evidence from work in rat anterior pituitary cells that lithium can block potassium channels (Kato et al., 1991), however with a $K_i$ of 16 mM this concentration is well above the therapeutic range and indeed may be involved in the toxic effects of lithium.

Lithium has also been demonstrated to interfere directly with second messenger systems. Palmer et al. (1972) has demonstrated that lithium in vitro at a concentration slightly higher than the therapeutic concentration (2 mM) reduced noradrenaline stimulated cAMP accumulation in rat cerebral cortex slices. This observation has been confirmed (Walker, 1974). Inhibition by lithium has subsequently been shown to be due to a direct action of lithium on the adenylate cyclase enzyme, since forskolin stimulated cAMP formation is also inhibited by lithium (Andersen & Geisler, 1984). This has also been shown for the purified adenylate cyclase in the absence of G-proteins (Mork & Geisler, 1987). The effect is, therefore, not necessarily dependent upon any uncoupling of adenylate cyclase linked receptors and G-protein as has been proposed by Avissar et al. (1988) (see above). It has been suggested that this direct effect of lithium on adenylate cyclase activity is possibly due to lithium competing with $Mg^{2+}$, a cofactor for this enzyme. Indeed, the inhibitory effect of lithium in vitro on forskolin and $Ca^{2+}$-stimulated adenylate cyclase activity in rat cerebral cortex is competitive with $Mg^{2+}$ (Mork & Geisler, 1987).

The first indications that lithium may interfere with (poly)phosphoinositide metabolism came from Allison & Stewart (1971). They demonstrated in vivo that plasma lithium concentrations in the therapeutic range increased rat cerebral InsP$_1$ and caused a stoichiometric similar reduction in the inositol content of the tissue. These results suggested that lithium was inhibiting the enzyme involved in the metabolism of InsP$_1$ to free inositol, the inositol monophosphatase. Subsequent studies by Hallicher and Sherman (1980) confirmed that lithium did indeed inhibit this enzyme, moreover the inhibition produced by lithium was unusual in that it was uncompetitive, i.e. the inhibitor lithium binds only to the enzyme/substrate or enzyme/product complex and not the enzyme alone (see Gani et al., 1993). The consequence of this is that the ability of lithium to inhibit the inositol monophosphatase enzyme increases as
for the purified enzyme (Gee et al., 1988a). It has also been demonstrated that lithium is an 
uncompetitive inhibitor of the Ins(1,4)P_2/Ins(1,3,4)P_3 1-phosphatase (see Gee et al., 1988b), 
although the K_i for lithium inhibition of this enzyme is significantly different from that of the 
inositol monophosphatase (9.63 mM and 0.46 mM for Ins(1,4)P_2 and Ins(1,3,4)P_3 compared 
with 1.0 mM and 0.28 mM for Ins(1)P_1 and Ins(4)P_1, respectively (Gee et al., 1988b). The 
monophosphatase enzyme is a dimer of two 30 kDa subunits (Gee et al., 1988a) and it has 
recently been cloned (Diehl et al., 1990).

The consequences of the inhibition of the monophosphatase enzyme on 
(poly)phosphoinositide metabolism have been extensively studied. In rat cerebral cortex slices 
lithium (1 mM) has been demonstrated to cause a linear increase in agonist-stimulated inositol 
monophosphate (InsP_1) accumulation. This results in a decrease in the free inositol within the 
cell which can be demonstrated indirectly by a dramatic increase in the levels of CMP-PA, the 
co-substrate for the enzyme PtdIns synthase (Kennedy et al., 1990). This decrease in cellular 
inositol levels is thought to result in a decrease in the synthesis of the inositol phospholipids, 
however, labelled PtdIns(4,5)P_2 levels in a number of preparations including GH3 cells 
(Downes & Stone, 1986) and adrenal glomerulosa cells (Balla et al., 1984) appear to be 
unaffected by lithium addition. The lack of any effect of lithium on PtdIns(4,5)P_2, the 
precursor of Ins(1,4,5)P_3, may be due to only a small proportion of the total PtdIns(4,5)P_2 
being in an agonist-sensitive pool. In rat cortical slices, after a lag period, a time dependent 
decrease in the levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is observed (Kennedy et al., 1989; 
see Chapter 3). This lag period is probably the time required for depletion of the agonist-
sensitive PtdIns(4,5)P_2 pool in this tissue, since addition of exogenous myo-inositol to lithium 
treated cortical slices can reduce the inhibitory effects of lithium on inositol polyphosphate 
accumulation (Kennedy et al., 1990).

Within the therapeutic concentration range lithium has few peripheral effects and indeed the 
effect of this ion appears to be fairly selective against mania. This selectivity would seem to be 
difficult to relate to the general effects which lithium may have if its antimanic properties were
mediated through either G-protein coupling, ion transport or cAMP formation. This together with the fact that the high concentrations of lithium required for the observed effects on ion transport and cAMP, which are in the toxic range, argue against any of these mechanisms being significantly involved in the control of manic depression.

Since the blockade of the monophosphatase by lithium blocks two sources of free inositol within the cell, that from recycling of the inositol phosphates and that from de novo synthesis, Berridge et al. (1989) have argued that cells or tissues which are sensitive to lithium are those that are unable to buffer their internal stores of inositol by uptake of external inositol. They have gone on to propose that the selectivity of lithium action in the brain could possibly be due to the fact that the brain is an enclosed system, surrounded by the blood-brain barrier. On administration of lithium, inositol is trapped in the form of InsPi, the cells only source of inositol is therefore via an uptake mechanism from the extracellular environment. As inositol levels in the cerebral interstitial fluid decrease, due to the poor transport of inositol through the blood-brain barrier from the plasma, the cells within the CNS, unlike those in the periphery, will be selectively affected by lithium. Berridge et al. (1989) have suggested that the cells within the CNS which are responsible for the condition of mania may have some dysfunction in their phosphoinositide signalling pathway, such that this pathway is hyperactive. Since the action of lithium, in inhibiting the monophosphatase enzyme, is stimulus dependent, i.e. dependent upon the concentration of the substrate, the effect of lithium upon these cells will be greater than the effect of this ion on normal cells.

Therefore the disruption of the (poly)phosphoinositide signalling pathway is the only one of the proposed mechanisms of action of lithium in mania which is able to explain the selectivity that lithium has against mania.
1.10 Aims of Thesis.

The studies described in this thesis are concerned with the examination of phosphoinositide signalling in brain, and model cells expressing recombinant receptors, and the mechanism by which the monovalent ion lithium disrupts this second messenger system.

A great number of studies have examined the effects of lithium on phosphoinositide signalling in cortical slice preparations in a number of rodent species (for review see Nahorski et al., 1991). Therefore the initial aim of this present study was to undertake preliminary experiments which would confirm the effects of lithium previously observed. Furthermore, the effects of lithium on phosphoinositide metabolism were to be examined in various brain regions to examine whether the action of lithium was a general phenomenon throughout the brain or had a specific effect in a certain brain region. For both these studies a number of techniques were to be employed which would characterize phosphoinositide metabolism by examining various aspects of this signalling system. These included the measurement of the levels of the inositol phosphate isomers, by anion-exchange chromatography, h.p.l.c. and radioreceptor assay (for Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄), CMP-phosphatidate (CMP-PA) and the inositol phospholipids.

A major aim of this thesis was to move away from examining the effects of lithium upon phosphoinositide metabolism in a complex system such as the cerebral cortex slice preparation and instead utilize a simpler model system. Cerebral cortex slices consist of a heterogeneous cell and receptor population which often make the interpretation of the data obtained difficult. Therefore, after the initial characterisation of phosphoinositide metabolism in rat cerebral cortex slices comparative experiments were to be performed on a simpler homogeneous system, the Chinese hamster ovary (CHO) cell line which expressed recombinant human M1 muscarinic receptors, to determine whether this model system would useful in examining the action of lithium. Use of this cell line should not only make the interpretation of the data more easy but also allow more informative experiments to be performed, including examining the effect of lithium upon Ins(1,4,5)P₃ induced Ca²⁺ release in intact Fura-2 loaded cells.

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Chapter 2

General Methods
METHODS.

2.1 PREPARATION OF BRAIN SLICES.

The methods describing the preparation of rat brain slices and assay of phosphoinositide responses are essentially those described previously (Brown et al., 1984; Batty et al., 1985; Batty & Nahorski, 1985). Male Wistar rats (250-300 g) were sacrificed by a blow to the head followed by decapitation. The brains were removed rapidly and dissected on ice (Glowinski & Iversen, 1966) with a total dissection time of approximately 4-5 min per brain. Tissue from cerebral cortex or other brain areas, including hippocampus and striatum, were usually pooled from several rats and transferred to a filter paper-covered plastic disc and cross-chopped at 90° into 350 x 350 μm slices using a McIlwain tissue chopper. The chopped tissue was dispersed into separate slices by vigorous agitation in 20-25 ml modified Krebs-Henseleit buffer, pH 7.4, at 37°C (see Appendix I for buffer composition). The tissue slices were then transferred to a 500 ml, flat-bottomed, screw-topped flask containing 200 ml of the aforementioned buffer. The slices were allowed to gravity pack, for approximately 30 s, the buffer was aspirated and replaced with fresh buffer. This process, which removes any released neurotransmitters and debris (e.g. inadequately chopped tissue), was repeated twice. Slices were then incubated for 60 min in Krebs-Henseleit buffer equilibrated previously with 95% / 5% (O2/CO2), pH 7.4, at 37°C in a shaking water bath. During this incubation period the buffer was replaced every 15 min with freshly gassed buffer.

2.2 LABELLING OF BRAIN SLICES WITH [3H]INOSITOL.

After the 60 min pre-incubation period the brain slices were washed in 150-200 ml of Krebs buffer and allowed to settle under gravity. 25 μl or 50 μl aliquots were then dispensed into 5 ml flat-bottomed polypropylene vials containing a volume of Krebs buffer (200-265 μl) which allowed a final volume of 300 μl to be achieved after the addition of slices, [3H]inositol and drugs. [3H]inositol (5.0 μCi per vial, unless otherwise stated) was added at this stage as 10 μl aliquots. The specific activity of the [3H]inositol used throughout these studies was 16-
20 Ci/mmol which represented a concentration of approximately 1.0 μM. After the addition of brain slices, the incubation vials were gassed with 95% / 5% (O₂/CO₂), capped and replaced in the shaking water bath at 37°C for a further 60 min with gassing at regular 15 min intervals.

2.3 PREPARATION OF [³H]INOSITOL.

In order to prevent the anionic contaminants present in commercially available [³H]inositol from interfering with assay measurements of [³H]InsP’s, a "cleaning up" step was employed. [³H]Inositol was routinely dried down under a steady stream of N₂ and redissolved in water. This volume was passed down a column containing 200 μl of Dowex AG1-X8 (200-400 mesh; formate form). Once the eluate had been collected, the column was washed with the require volume of water to give the desired concentration of [³H]inositol.

2.4 LABELLING OF BRAIN SLICES WITH [³H]CYTIDINE.

[³H]Cytidine labelling of brain slices was essentially identical to that described above for [³H]inositol labelling. One notable exception, however, was that [³H]cytidine did not require any "cleaning up" process and was instead simply added from the stock solution at a final concentration of 0.2 μCi/vial and incubated for 60 min (see Figs. 2.1 and 2.2).

2.5 TERMINATION AND ASSAYING OF [³H]INOSITOL PHOSPHATES.

The method used is basically that described by Downes et al. (1986). After the labelling period drugs were added, as described in the text, in volumes of 10 μl. The incubation of the individual samples was then continued for the appropriate length of time before incubations were terminated by addition of 300 μl of ice-cold 10% (v/v) perchloric acid (PCA). Samples were left to stand for 20 min on ice and then vigorously mixed before being centrifuged at 3,000 x g for 20 min. Aliquots (450 μl) of each supernatant were transferred to separate tubes containing 125 μl of 10 mM EDTA. The perchloric acid was then extracted by addition of 750 μl of a freshly prepared 1:1 (v/v) mixture of freon/tri-n-octylamine (freon = 1,1,2-
Figure 2.1  Effects of $[^3H]$cytidine concentration upon the extent of $[^3H]$CMP-PA labelling.

Rat cerebral cortex slices were preincubated and subsequently labelled for 1 hour with cytidine at a concentration of 0.1, 0.2, 0.4 and 1.0 $\mu$Ci / vial. Drugs were then added for a further 30 min. before reactions were terminated and the $[^3H]$CMP-PA extracted. Data represent the means ± SEM for three separate experiments each performed in triplicate.

- Basal
- CCh (1mM) + Li (5mM)
- Li (5mM)
Figure 2.2 Effects of the length of labelling period upon the accumulation of \([3^\text{H}]\text{CMP-PA}\) accumulation.

Rat cerebral cortex slices were preincubated and subsequently labelled for 30, 60 or 90 min with \([3^\text{H}]\text{cytidine}\) (0.2 \(\mu\text{Ci} / \text{vial})). Drugs were added for a further 30 min before reactions were terminated and the \([3^\text{H}]\text{CMP-PA}\) extracted. Data represent the means ± SEM for three experiments each performed in triplicate.

- Basal
- Carbachol (1mM)
- Li (5mM)
- Carbachol + Li

The concentration of \([3^\text{H}]\text{cytidine}\) was approximately 33.5 nM.
trichlorotrifluoroethane), followed by vigorous mixing of the samples. After centrifugation for a further 20 min at 3,000 x g three phases were obtained. The lower phase was freon plus unreacted tri-n-octylamine, the middle phase tri-n-octylamine perchlorate and the upper phase the neutralised sample plus all water-soluble components (Batty et al., 1989). A 350 μl sample of the upper phase was removed and adjusted to pH 7.0 by addition of 40 μl of 60 mM NaHCO₃.

2.6 SEPARATION OF INDIVIDUAL [3H]INOSITOL PHOSPHATES.

Following neutralisation of the samples, the [3H]InsP's were separated on columns containing 1 ml of a 50% (w/v) slurry of Dowex AG1-X8 analytical grade anion exchange resin (200-400 mesh; formate form) (Batty et al., 1985). Samples were added to the columns and allowed to elute through. The [3H] InsP's bound to the columns were separated by sequential elution using a system based on that described by Hubscher and Hawthorne (1957), Ellis et al. (1963) and Emilsson and Sundler (1984) with modifications described by Batty et al. (1985). Thus, 20 ml of water was added to each column to remove free [3H]inositol. [3H]Glycerophospho-inositol (GroPIns) was eluted by 15 ml of 25 mM HCOONH₄. [3H]InsP₁, [3H]InsP₂, [3H]InsP₃ and [3H]InsP₄ were sequentially eluted by 20 ml of 0.2 M HCOONH₄/0.1 M HCOOH, 15 ml of 0.5 M HCOONH₄/0.1 M HCOOH, 15 ml of 0.7 M HCOONH₄/0.1 M HCOOH and 15 ml of 1.0 M HCOONH₄/0.1 M HCOOH respectively.

Complete elution profiles were obtained for extracts from carbachol-stimulated cerebral cortical slices, prepared in the presence of lithium, by collecting and subsequent liquid scintillation counting of 2 ml samples of total fractions (Fig. 2.3). Once the elution profiles had been established for each batch of Dowex, the [3H]InsP's were routinely eluted with the volumes and concentrations of buffer mentioned above. A 2 ml sample from each fraction was then counted in 18 ml of scintillant.

Normally the Dowex columns were regenerated for future use. The elution of a further 10 ml of 2.0 M HCOONH₄/0.1 M HCOOH, followed by 40 ml of water, effectively returned the
Figure 2.3 Dowex column elution profile of [3H]InsP's extracted from CHO-M1 cells stimulated with carbachol in the presence of lithium.

CHO-M1 cells were stimulated for 30 min with carbachol in the presence of 5 mM lithium. After extraction and neutralization, as described in section 2.5, samples were applied to Dowex columns. The fractions designated Ins, GroPIns, InsP1, InsP2, InsP3 and InsP4 were eluted as described in section 2.6.
resin to a state in which it could be reused for similar experiments. The resin, however, was replaced periodically (after 5-6 experiments).

2.7 SEPARATION OF INDIVIDUAL [3H]INOSITOL PHOSPHATE ISOMERS BY H.P.L.C.

Samples for h.p.l.c. analysis were prepared identically to those for Dowex column, as described above (see 2.5). H.p.l.c. analyses of [3H]inositol phosphate isomers were performed by a modification of the method of Dean & Moyer (1987) as described by Batty et al. (1989). Separation was achieved with a Partisil (10 μm) SAX analytical column (Technicol) equipped with a pre-column packed with Whatman pellicular anion-exchange resin and eluted with gradients comprising water and NH₄H₂PO₄, adjusted to pH 3.7 with H₃PO₄. After sample injection (2 ml), free [3H]inositol was eluted by washing the column for 5-15 min with water, depending upon the extent of sample radiolabelling. [3H]Inositol phosphates were then separated by applying three consecutive gradients at a flow rate of 1 ml / min (see Appendix II for gradient). GroPIIns and InsP₁'s were resolved by applying a linear gradient of 0-60 mM NH₄H₂PO₄ over 30 min. GroPIInsP and InsP₂'s were then separated by isocratic elution at 190 mM NH₄H₂PO₄ for 15 min, followed by a linear increase in eluent concentration to 300 mM over a further 15 min. GroPIInsP₂ and InsP₃'s were separated by isocratic elution for 35 min at 500 mM NH₄H₂PO₄. Ins(1,3,4,5)P₄ was then displaced from the column by a 15 min wash at 1.4 M NH₄H₂PO₄ (Fig 2.4). For routine h.p.l.c. analysis of samples, fractions (0.5-1.0 min) were collected across the chromatographic windows appropriate for Ins, InsP₁'s, InsP₂'s, InsP₃'s and InsP₄ and 5 ml of scintillant (Flo-scint IV) was added to each sample before counting.

Identification of sample [3H]inositol phosphate isomers was based on co-elution with authentic ³H-labelled standards in separate runs. To overcome problems associated with variations in retention times either between columns or due to column aging, samples were
Figure 2.4 H.p.l.c. separation of the [3H]inositol phosphates present in an extract from carbachol-stimulated cerebral cortex slices labelled with [3H]inositol.

Samples (50 μl) of cerebral-cortex slices were labelled with 5 μCi of [3H]inositol for 60 min and then incubated for a further 10 min with carbachol (1mM). The samples were prepared as described in 2.5. before addition to the column. Note that, before fraction 0, [3H]inositol was eluted by washing the column with water for 15 min.
routinely spiked with 50-100 nM each of adenosine and guanosine mono-, di-, tri- and tetra-
phosphates before injection (Fig 2.4). Nucleotides were detected by continuous u.v.
monitoring of the column eluate at 254 nm. Typically InsP$_1$'s were eluted at retention times
intermediate to AMP and GMP, InsP$_2$'s between ADP and GDP (except [H]Ins(4,5)P$_2$
which ran 1-2 min after GDP), InsP$_3$'s between ATP and GTP, and InsP$_4$ with tetra-
phosphates, thus providing a means of standardizing each run (Irvine et al., 1985; Burgess et
al., 1985; Batty et al., 1989).

2.8 TERMINATION AND ASSAYING OF [H]CYTIDINE LABELLING EXPERIMENT.

After the labelling period drugs were added, as described in the text, in volumes of 10 μL.
The incubation of the individual samples was then continued for the appropriate length of time
before incubations were terminated by addition of 300 μL of ice-cold 1 M trichloroacetic acid
(TCA). Samples were allowed to extract for 10-15 min on ice and were then centrifuged at
3,000 x g for 20 min at 4°C. The supernatant was removed and discarded and 1 mL of 5% TCA
containing 1mM EDTA was added to the pellet. Samples were agitated gently and centrifuged
(3,000 x g, 20 min, 4°C). The supernatant was once again removed and discarded and the
slices washed with 1 mL of water. After a subsequent spin (3,000 x g, 20 min, 4°C) and
removal of the supernatant, 0.94 mL of a 2:1 (v/v) mixture of CH$_3$OH : CHCl$_3$ containing 100
mM HCl was added to the samples which were then shaken and left for 10-15min. After this
period 0.31 mL of CHCl$_3$ and 0.56 mL of 0.1M HCl were added before the samples were
vigorously mixed. After centrifugation at 3,000 x g for 10 min at 4°C two phases were
obtained. The upper phase, containing water soluble products, was removed and discarded. A
0.4 mL aliquot of the lower phase, containing the [H]CMP-phosphatidate ([H]CMP-PtdOH),
was removed, allowed to evaporate to dryness overnight and the radioactivity counted after
addition of 5 mL of scintillant.

Although validation of the $^3$H-containing lipids by thin layer chromatography was not carried
out, several other groups have reported that the only product isolated under these labelling
conditions is CMP-PA (Downes & Stone, 1986; Godfrey et al., 1989).

2.9 MEASUREMENT OF Ins(1,4,5)P<sub>3</sub> MASS.

Ins(1,4,5)P<sub>3</sub> mass was measured in both brain slices and cell lines using a modification of the method of Bradford & Rubin (1986) as described by Challiss et al. (1988). This assay measures the displacement of [3H]Ins(1,4,5)P<sub>3</sub> binding to a stereospecific membrane receptor prepared as a crude 'P<sub>3</sub>' membrane fraction from bovine adrenal cortex (Challiss et al., 1988; Palmer et al., 1989).

Ins(1,4,5)P<sub>3</sub> concentrations were calculated using a standard curve constructed using authentic Ins(1,4,5)P<sub>3</sub> (0.036 - 12 pmol) as standard and [3H]Ins(1,4,5)P<sub>3</sub> as the radioligand. Non-specific binding was defined in the presence of 10 μM Ins(1,4,5)P<sub>3</sub).

Samples for this assay were prepared as described in 2.5, for brain slices, and 2.18, for cell lines, with the exception that no preincubation with [3H]inositol was required. To 30 μl of sample or standard, was added 30 μl of assay buffer (100 mM Tris/HCl, 4 mM EDTA, pH 8.0), 30 μl of [3H]Ins(1,4,5)P<sub>3</sub> (7,000-9,000 d.p.m.) and finally 30 μl of binding protein (450-600 μg of protein). The samples were vortexed and left to equilibrate on ice for 30 min. Bound and free radioligand were separated by rapid vacuum filtration. Immediately before filtration the samples were diluted with 3 ml of ice-cold wash buffer (see Appendix I for composition) and applied to a pre-wetted Whatman GF/B filter under vacuum. The assay tube was then rinsed and the filter washed with two further 3 ml additions of wash-buffer. This procedure was semi-automated by use of a Brandel cell harvester. All procedures were conducted at < 4°C. Radioactivity on the filters was determined by liquid scintillation counting after allowing the filters to extract in scintillant overnight. Protein measurements were made by the method of Lowry et al. (1951).
2.10 MEASUREMENT OF Ins(1,3,4,5)P_4 MASS

Ins(1,3,4,5)P_4 mass was measured using a modification of the method of Donie & Reiser (1989) as described by Challiss & Nahorski (1990). As for the measurement of Ins(1,4,5)P_3 this assay measures the displacement of [32P]Ins(1,3,4,5)P_4 binding to a selective, high-affinity membrane binding site prepared from rat or pig cerebella.

Ins(1,3,4,5)P_4 concentrations were calculated using a standard curve constructed using authentic Ins(1,3,4,5)P_4 (0.036-120 pmol) as standard and [32P]Ins(1,3,4,5)P_4 as the radioligand. Non-specific binding was defined in the presence of 10 μM Ins(1,3,4,5)P_4.

To 40 μl aliquots of each sample was added 40 μl of assay buffer (100 mM sodium acetate, 100 mM KH_2PO_4, 4 mM EDTA, pH 5.0), 40 μl of [32P]Ins(1,3,4,5)P_4 (12,000-20,000 d.p.m.) and finally 40 μl of binding protein (200-300 μg of protein). The samples were vortexed and left to equilibrate on ice for 30 min. Bound and free radioligand were separated by rapid vacuum filtration. Immediately before filtration the samples were diluted with 3 ml of ice-cold wash buffer (see Appendix I for composition) and applied to a pre-wetted Whatman GF/B filter under vacuum. The assay tube was then rinsed and the filter washed with two further 3 ml additions of wash-buffer. All procedures were conducted at < 4°C. Radioactivity on the filters was determined by liquid scintillation counting after allowing the filters to extract in scintillant overnight. Protein measurements were made by the method of Lowry et al. (1951).

2.11 PREPARATION OF AN [3H]Ins(4,5)P_2 STANDARD

[3H]Ins(4,5)P_2 was prepared by mild alkaline phosphatase treatment of [3H]Ins(1,4,5)P_3 essentially as described previously by Hughes & Drummond (1987) (Fig. 2.5). Calf intestinal alkaline phosphatase (10 units) was dissolved in a solution of bovine serum albumin (BSA) (500 μl, 0.1%). A 50 μl aliquot of this stock solution was added to 1940 μl of 0.2 mM Tris/maleate buffer (pH 9.0) containing 2 mM MgCl_2, to which 0.05 μCi of [3H]Ins(1,4,5)P_3
Figure 2.5  H.p.l.c. separation of the products of mild alkaline phosphatase treatment of $[^3H]$Ins(1,4,5)P$_3$.

In order to produce an $[^3H]$Ins(4,5)P$_2$ standard $[^3H]$Ins(1,4,5)P$_3$ was treated with calf intestinal alkaline phosphatase, as described in 2.11.
was added. After 60 min, at 37°C, the reaction was terminated by addition of 0.2 ml of PCA (10%, v/v). The acid was extracted from the sample as described in 2.5. Neutralized aliquots of the sample were stored at -20°C until required.

2.12 MEASUREMENTS OF TOTAL [3H]INOSITOL PHOSPHOLIPIDS.

Tissue incubations and termination procedures were identical to those described in 2.2 and 2.5, therefore measurements of individual [3H]InsP's and [3H]inositol phospholipids could be obtained for the same sample. Once the supernatants for [3H]InsP measurements had been removed, the remaining pellet was washed with 1 ml of 7% (v/v) PCA containing 1 mM EDTA and centrifuged at 3,000 x g for 5 min at 4°C. The supernatant was removed and discarded, the pellet washed with 1 ml of water and the samples centrifuged (as above) (Griffin & Hawthorne, 1978). The supernatant was again removed and discarded. The labelled phospholipids were extracted as previously described by Downes and Wusteman (1983). Thus, 0.94 ml of a 2:1 (v/v) mixture of CH₂OH : CHCl₃ containing 100 mM HCl was added to the tissue pellet. The samples were then left to stand at room temperature for 10-15 min. The aqueous and organic phases were then partitioned by addition of 0.31 ml of CHCl₃ and 0.56 ml of 0.1 M HCl followed by centrifugation at 3,000 x g for 10 min, at 4°C. The upper aqueous phase was removed and discarded and 400 μl of the chloroform lower phase transferred to a 5 ml scintillation vial, allowed to evaporate to dryness overnight and the radioactivity counted after addition of 5 ml of scintillant.

2.13 MAINTENANCE OF CELLS IN CULTURE.

Chinese hamster ovary (CHO) cells expressing recombinant muscarinic M₁-receptors were kindly supplied by Dr N.J. Buckley (National Institute for Medical Research, London, U.K.). The cells (passage 25-60) were maintained in alpha-minimum essential media (Gibco) supplemented with newborn calf serum (10% v/v), streptomycin (100 μg / ml), penicillin (100 units / ml) and amphotericin B (Fungizone) (2.5 μg / ml).
SH-SY5Y human neuroblastoma cells were kindly supplied by Dr. J. Biedler (Sloan-Kettering Institute, New York, U.S.A.). The cells (passage 70-100) were maintained in minimum essential media (Gibco) supplemented with newborn calf serum (10% v/v), L-glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 units/ml) and amphotericin B (Fungizone) (2.5 μg/ml).

Cell lines, in 175 cm² tissue culture flasks containing 30 ml of culture media, were maintained at 37°C in 5% CO₂/95% humidified air and subcultured twice weekly (split ratio, 1:4).

Before use the cells were harvested in phosphate-buffered saline (PBS) (see Appendix I for composition) containing 500 μM EDTA. Following centrifugation at 500 x g for 2 min and removal of the supernatant, the cells were resuspended in a volume of either culture media or buffer ready for experimental use.

2.14 PHINMS SATURATION BINDING TO CHO-M₁ CHOLs.

Harvested CHO-M₁ cells were resuspended in Krebs-HEPES buffer, at 37°C, at an approximate protein concentration of 1 mg/ml. Aliquots (100 μl) of the cell suspension were incubated with [³H]NMS (0.03 to 3 nM final concentration) in a final volume of 1 ml for 60 min at 37°C. Specific [³H]NMS binding was defined as the difference between total binding and that remaining in the presence of 1 μM atropine.

Bound and free radioligand were separated by rapid vacuum filtration. Immediately before filtration the samples were diluted with 3 ml of ice-cold Krebs-HEPES buffer and applied to a pre-wetted Whatman GF/B filter under vacuum using a Brandel cell harvester. The assay tube was then rinsed and the filter washed with two further 3 ml additions of wash-buffer. All procedures were conducted at < 4°C. Radioactivity on the filters was determined by liquid scintillation counting after allowing the filters to extract in scintillant overnight. Protein measurements were made by the method of Lowry et al. (1951).
2.15 Pirenzepine Displacement of [3H]NMS Binding in CHO-M1 Cells.

Harvested CHO-M1 cells were resuspended in Krebs-HEPES buffer, at 37°C, at an approximate protein concentration of 1mg/ml. Aliquots (100 μl) of the cell suspension were incubated with [3H]NMS (0.5 nM final concentration) in a final volume of 1 ml in the presence of pirenzepine (0.003 - 100 μM final concentration) for 60 min at 37°C.

Bound and free radioligand were separated and quantified as described for [3H]NMS saturation binding in 2.13. Protein measurements were made by the method of Lowry et al. (1951).

The IC\textsubscript{50} value for pirenzepine in CHO-M1 cells was calculated from the IC\textsubscript{50} values using the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

2.16 [3H]inositol Labelling of Cells.

Harvested CHO-M1 cells were resuspended in culture media (approximately 1 flask of cells in 50 ml of media) containing [3H]inositol, (2-5 μCi / ml). Cultures were seeded into 24-well dishes (0.5 ml of media per well) 48 h prior to the experiment and were allowed to reach equilibrium labelling at 37°C in 5% CO\textsubscript{2} / 95% humidified air (see Fig. 2.6). The [3H]inositol was not cleaned as for the experiments involving brain slices (see 2.3), but was instead dried under a steady stream of N\textsubscript{2}, redissolved in 5 ml of media and this was in turn filtered into the remaining media through a 0.2 μm filter.

2.17 Termination and Assaying of [3H]inositol Phosphates in Cells.

After the labelling period, the media was removed and the cells were washed with 1 ml of Krebs-Hepes buffer at 37°C (see Appendix I for composition). The buffer was replaced with 1 ml of fresh buffer. The cells were allowed to stabilize for 15 min at 37°C before the buffer was removed and replaced with 100 μl of buffer containing drugs, as described in the text. The incubation of the individual samples was continued for the appropriate length of time before
Figure 2.6 Time-course of the incorporation of [³H]inositol into inositol phospholipids in CHO-M₁ cells.

Cells were labelled for 12, 24, 36 or 48 hours in either 1 μCi / ml (○) or 5 μCi / ml (●) [³H]inositol containing media. Data represent the mean ± SEM from three experiments each performed in triplicate.
incubations were terminated by addition of 100μl of ice-cold 10% (v/v) perchloric acid (PCA).
Samples were left to extract for 20 min on ice, after which a 200μl aliquot of each supernatant
was transferred to a 2 ml Eppendorf vial containing 40μl of 10 mM EDTA. The perchloric acid
was extracted as described in section 2.5. Thus, 250μl of a freshly prepared 1:1 (v/v) mixture
of freon/tri-n-octylamine was added to the samples, followed by vigorous mixing of the
samples. After centrifugation for a further 20 min at 3,000 × g three phases were obtained. A
200μl sample of the upper phase was removed and adjusted to pH 7.0 by addition of 20 μl of
60 mM NaHCO₃.

[3H]inositol phosphate isomers were subsequently separated by Dowex anion exchange
chromatography (see 2.6) or by h.p.l.c. (see 2.7).

2.18 LABELLING OF CHO-M₁ CELLS WITH [3H]CYTIDINE.

Harvested CHO-M₁ cells were resuspended in culture media (approximately 1/5 of a flask of
cells in 25 ml of media). Cultures were seeded into 24-well dishes (0.5 ml of media per well)
24 h prior to the experiment and maintained at 37°C in 5% CO₂/95% humidified air. For
[3H]cytidine labelling of the cells the media was removed and the cells were washed with 1 ml
of Krebs-Hepes buffer at 37°C (see Appendix I for composition). The buffer was then
replaced with 300μl of fresh buffer containing [3H]cytidine (0.2 μCi/300μl). The cells were
allowed to label for a period of 1 h.

2.19 TERMINATION AND ASSAYING OF [3H]CYTIDINE LABELLING EXPERIMENT
IN CELLS.

After the labelling period, the media was removed and the cells were washed with 1 ml of
Krebs-Hepes buffer at 37°C (see Appendix I for composition). The buffer was replaced with
1 ml of fresh buffer. The cells were allowed to stabilize for 15 min at 37°C before the buffer
was removed and replaced with 100μl of buffer containing drugs, as described in the text. The
incubation of the individual samples was continued for the appropriate length of time before
incubations were terminated by addition of 100 μl of ice-cold trichloroacetic acid (1M; TCA). Samples were left to extract for 20 min on ice. The supernatants were removed and the cells washed consecutively with 1 ml of 5% TCA containing 1 mM EDTA and 1 ml of water. [3H]CMP-PA was then extracted from the cells as described in section 2.8.

2.20 MEASUREMENTS OF TOTAL [3H]INOSITOL PHOSPHOLIPIDS IN CELLS.

As for brain slices, tissue incubations and termination procedures were identical to those described in sections 2.2 and 2.5., therefore, measurements of individual [3H]InoP's and [3H]inositol phospholipids could be obtained from the same sample. Once the supernatants for [3H]InoP measurements had been removed, the plated cells were washed consecutively with 1 ml of 7% (v/v) PCA containing 1 mM EDTA followed by 1 ml of water. The labelled phospholipids were then extracted and quantified as described in section 2.12.

2.21 MEASUREMENTS OF INDIVIDUAL [3H]INOSITOL PHOSPHOLIPIDS IN CELLS.

i. Extraction.

Extraction of the [3H]inositol phospholipids was carried out by the methods described in section 2.12.

ii. Deacylation.

The deacylation method is essentially that described by Wells and Dittmer (1965). Lipid extracts were redissolved in 1 ml of chloroform to which 0.2 ml of methanol and 0.4 ml of 0.5 M NaOH in methanol/water (19:1, v/v) was subsequently added. The samples were vigorously mixed and allowed to stand at room temperature for 20 min. After this, 1 ml of chloroform, 0.6 ml of methanol and 0.6 ml of water were added to each sample. The samples were again thoroughly mixed and centrifuged at 3,000 x g for 10 min after which 1 ml of the upper phase was removed for analysis of glycerophosphoinositol derivatives. Samples were neutralized by passing them through columns containing Dowex-50 (H+ form, 50-100 mesh). The resin was then washed with 1 ml of water and the pH of the samples adjusted to pH 7.0 by
Figure 2.7 Elution profile for glycerophosphoinositol derivatives produced from extracts of CHO-M1 cells.

Cells were incubated with [3H]inositol (5 μCi / ml) for 48 h prior to the experiment. The cells were then incubated for 30 min with either buffer alone (line only), carbachol (1mM) (open circle) or carbachol plus lithium (closed circle) before the reactions were terminated. After extraction and neutralisation the samples were deacetylated as described in 2.20 (i. & ii.). The samples were then applied to Dowex columns and eluted with the buffers described in 2.21.
addition of 25 μl of 60 mM NaHCO₃. A 1 ml aliquot of the lower phase was dried under N₂ and counted in order to determine the residual radioactivity associated with the CHCl₃ phase and thus establish the efficiency of deacylation (found to be routinely greater than 98%).

2.22 SEPARATION OF INDIVIDUAL DEACYLATION PRODUCTS.

Separation of glycerophosphoinositols (GroPIns) was achieved by the methods described by Creba et al. (1983) and Berriedge (1983). Samples were added to columns of the same composition as described in section 2.6. Free inositol was removed by addition of 20 ml of water. [³H]GroPIns, [³H]GroPInsP and [³H]GroPInsP₂ were eluted sequentially by the addition of 12 ml of 0.18 M HCOONH₄/0.1 M Na₂B₄O₇, 20 ml of 0.3 M HCOONH₄/0.1 M HCOOH and 15 ml of 0.75 M HCOONH₄/0.1 M HCOOH respectively. Complete elution profiles for the separation of the GroPIns(P₂) derivatives were established by collecting and counting of successive 2 ml aliquots of the column eluates, using extracts prepared from tissue samples originally labelled with [³H]inositol (Fig. 2.7). Once the elution profiles for the separation of deacylated products had been established, the glycerophosphoinositol derivatives were routinely eluted with the volumes and concentrations of buffers mentioned above and then a 2 ml fraction from each sample was counted in 18 ml of scintillant.

2.23 MEASUREMENT OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE MASS IN CHO-M₁ CELLS.

CHO-M₁ cells were prepared as in sections 2.16 and 2.17 with the exception that no [³H]inositol was added. Subsequent extraction of the unlabelled inositol phospholipids was performed as described in section 2.13. 400 μl of the lower chloroform phase containing the unlabelled lipids was evaporated to dryness under a steady stream of N₂. 1 M KOH (0.25 ml) was added to the residues and the tubes were tightly capped and heated at 100°C for 15 min. This process produces a complete alkaline hydrolysis of PtdIns(4,5)P₂ and yields three
products Ins(1,4,5)P3, Ins(2,4,5)P2 and Ins(4,5)P2 in the ratio of 66:20:14 (Chilvers et al., 1991; also see Fig. 2.8).

After cooling on ice, samples were neutralized by addition to columns containing 0.5 ml of a 50% slurry of Dowex 50 (200-400 mesh; H+ form), and the eluate collected. The columns were washed with 1.5 ml of water and the total eluate was then washed with 2 x 2 ml of a mixture of butanol:light petroleum ether (5:1, v/v). A 500 μl aliquot of the lower phase was then taken and lyophilized. The lyophilizate was dissolved in 30 μl of water and the Ins(1,4,5)P3 concentration of the sample was determined as described in section 2.9.

2.24 MEASUREMENT OF 45Ca2+ RELEASE FROM SH-SYSY CELLS.

Harvested SH-SYSY cells (see 2.13) were resuspended in 0.8 ml of intracellular buffer (ICB) at 20°C (see Appendix I for composition) and transferred to a cuvette. The cells were electrically permeabilized using a BioRad Gene Pulsar (6 pulses at 1.5 kV, 3 μF) (Wojcikiewicz et al., 1990). The cells were transferred to a 10 ml vial and centrifuged (500 x g for 2 min) before the supernatant was removed. The cells were then allowed to load with 45Ca2+ by resuspending the cells in ICB containing 1 μCi/ml of 45Ca2+ for a period of 15 min at a density of 1-2 mg of protein / ml (Strupish et al., 1988).

Aliquots of cells (100 μl) were added to 100 μl of ICB, containing either Ins(1,4,5)P3, Ins(4,5)P2 or Ins(1,4,5)P3 and Ins(4,5)P2 in combination, in polypropylene microfuge tubes. After an incubation period 2 min at 20°C, a time that has been previously shown to produce maximal 45Ca2+ release in this cell line (Safinya et al., 1991), 500 μl of a silicon oil mixture (9:11 of Dow Corning 550:556) was added and the cells were separated from the media by centrifugation (16,000 x g for 3 min). The media and oil were aspirated and 1 ml of scintillant was added to each of the samples before counting.
Figure 2.8  $[\text{H}]\text{InsP}_x$ products of alkaline hydrolysis of $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$.

A sample of $[\text{H}]\text{PtdIns}(4,5)\text{P}_2$ was hydrolysed as described in 2.24. The resultant $[\text{H}]\text{inositol phosphates}$ produced were separated by h.p.l.c. as described in 2.7.
2.25 FURA-2 MEASUREMENTS OF CHANGES IN INTRACELLULAR CALCIUM.

Changes in the intracellular Ca\(^{2+}\) concentration produced by agonist were examined in suspensions of CHO-M1 cells previously loaded with the Ca\(^{2+}\)-sensitive dye Fura-2 (Gryniewicz et al., 1985).

CHO-M1 cells were harvested, as described in 2.13, and resuspended in Krebs-Hepes buffer (20°C; 1 flask / 20 ml of buffer) which had been supplemented with sulfinpyrazone (250 μM final concentration) added to prevent leakage of fura-2 free acid from the loaded cells. 5 ml aliquots of the cell suspension were dispensed into 30 ml vials. Following centrifugation (500 x g for 2 min) and removal of the supernatants the cells to be loaded were resuspended in 3 ml of supplemented Krebs-Hepes buffer containing 3 μM Fura-2AM, the acetoxymethylester of the free acid, which diffuses into the cells where it is converted by intracellular esterases to the Ca\(^{2+}\)-sensitive fura-2 free acid (Williams et al., 1985). After a 30 min incubation period the cells were washed, resuspended in 3 ml of buffer and transferred to cuvettes for experimentation. The fluorescence was measured, once every second, at 340 and 380 nm excitation and 510 nm emission wavelengths in a Perkin Elmer fluorimeter. The ratio of the two emission wavelengths gave an index of the real changes in intracellular Ca\(^{2+}\) concentration. Autofluorescence measurements were made in cells prepared as above with the exception that no fura-2AM was present during the incubation period. All data shown are ratios from which autofluorescence has been subtracted.

2.26 DETERMINATION OF PROTEIN.

Measurement of protein concentration in sodium hydroxide digests of tissue samples was performed according to the method of Lowry et al. (1951). The reference standards were prepared using bovine serum albumin (stock 1mg/ml in water, stored at -20°C) and a standard curve was generated using 0-80 μg of protein per assay.

A 200 μl aliquot of sample, or standard, was added to 1 ml of a solution containing 2% Na\(_2\)CO\(_3\)/0.4% NaOH; 1% CuSO\(_4\); 2% sodium potassium tartrate in the ratio of 100:1:1
respectively. After 10 mins, 100 μl of a 25% v/v solution of Folin Ciocalteau reagent was added. After a further 30 min incubation, the samples were diluted with 1 ml of water and the $A_{750}$ was measured using a Beckman spectrophotometer with on-line calibration programming.
Chapter 3

Comparative effects of lithium
on the phosphoinositide cycle in rat
cerebral cortex, hippocampus and striatum.
3.1 Introduction.

The CNS has been shown to contain more pharmacologically distinct receptors coupled to phosphoinositide turnover than has been demonstrated in any other tissue (see Fisher & Agranoff, 1987). Activation of these receptors results in the formation of the second messengers Ins(1,4,5)P$_3$ and DAG, which release Ca$^{2+}$ and activate PKC respectively (Nahorski, 1988; Chuang, 1989; Fowler & Tiger, 1991; Fisher et al., 1992). With the great diversity of receptor subtypes and cell types within the brain it would be naïve therefore to imagine that phosphoinositide metabolism was identical in all the constitutive regions of the brain. Indeed, it has been demonstrated in rat brain, for example, that the activity of the Ins(1,4,5)P$_3$ metabolising enzymes, the 3-kinase and 5-phosphatase, differ depending upon the region of the brain in which they are expressed (Hancock et al., 1990). The activity of the 3-kinase is lower in hypothalamus and pons compared with other areas of the brain, whereas 5-phosphatase activity is lower in the cerebellum compared with cortex. These subtle differences in enzyme activities may therefore result in differences in phosphoinositide metabolism between these regions. Indeed, a number of studies have examined this possibility, although they have been limited to either separating total [3H]inositol phosphate fractions (i.e. [3H]InsP$_3$) (Fisher & Bartus, 1985; Gonzales & Crews, 1985; Rooney & Nahorski, 1986) or have only examined individual brain regions (Kelly et al., 1988; Stephens & Logan, 1989a,b), which makes it difficult to make comparisons between the various regions. A clear definitive study comparing phosphoinositide metabolism between brain regions has therefore not yet been undertaken.

More importantly for this present study is to examine whether the anti-manic agent lithium may have differential effects on phosphoinositide metabolism in the various brain regions. This is important in order to determine whether the effect of lithium is general throughout the brain or is selective for a specific region of the brain or group of neurons. The effects of lithium on phosphoinositide metabolism have been well characterised in rat cerebral cortex (see discussion and also Chapter 1). However, at present the precise location of lithium action in the brain
which is involved in this drug's anti-manic action is unknown. Recent attention has focused on 5-HT receptors (possibly 5-HT2) in the limbic region of the brain (Wood & Goodwin, 1987), although a great deal more work is required to determine the precise site of action of lithium. A selective action of lithium, if it existed, could arise from a number of mechanisms. Firstly, lithium may not have access to all areas of the CNS or may be poorly transported into certain cell types, therefore only the cells which come into contact with lithium will be affected. Secondly, the different cell types within the CNS may express various isoforms of the enzyme PI synthase with differing $K_m$ values for inositol. This has already been demonstrated, for example, for PI synthase purified from GH3 cells and rat liver, with a $K_m$ for inositol in GH3 cells in the region of 60 µM (Imai & Gershengorn, 1987) compared with that of 2.5 mM in rat liver (Takenawa & Egawa, 1977). The higher the affinity of the PI synthase enzyme for inositol the less of an effect lithium will have on phosphoinositide metabolism, since the small reduction in free inositol level within the cell (which is normally in the mM range) produced by the lithium blockade of the inositol monophosphatase enzyme, may not reduce inositol levels below the $K_m$ of the higher affinity form of the enzyme. Finally, some cells may either have greater inositol reserves or have a more effective uptake mechanism for inositol than others allowing them to continue to produce PtdIns, and more importantly PtdIns(4,5)P2, even after the inhibition of the inositol monophosphatase enzyme.

The work presented in this chapter, therefore, compares (poly)phosphoinositide signalling in cortex, hippocampus and striatum to determine whether regional variations in phosphoinositide metabolism exist. The effect of lithium upon the phosphoinositide metabolism in these region is also examined to determine whether the action of lithium is general throughout the brain or is selective for a specific region.
3.2 Results

3.2.1 [3H]InsP₁, [3H]InsP₂ and [3H]InsP₄ accumulations in [3H]inositol prelabelled hippocampal, striatal and cerebral cortical slices.

Initial studies involved the assessment of [3H]InsP₁, [3H]InsP₂ and [3H]InsP₄ accumulations in [3H]inositol prelabelled slices of hippocampus, striatum and cortex in response to a maximally-effective concentration of carbachol (1 mM) in the absence or presence of lithium (1 mM). Time-course data are shown in Figs. 3.1.1, 3.1.2 & 3.1.3. Addition of lithium per se did not cause any increase in basal accumulations of [3H]InsP₁, [3H]InsP₂ or [3H]InsP₄ in any brain region, at least over the 30 min time-course studied here (data not shown). Considerable variation in basal [3H]InsP₁ accumulation was observed, with the lowest levels being seen in striatal slices (4522 ± 560 d.p.m./25 µl of slices) and the highest (27664 ± 7589 d.p.m./25 µl of slices) in hippocampal slices. In contrast, less variation was observed in basal [3H]InsP₂ and [3H]InsP₄ accumulations in the different brain regions, although basal [3H]InsP₂ levels tended to be higher in striatal slices (1570 ± 165 d.p.m./25 µl of slices) compared to hippocampal and cortical slices (1083 ± 186 and 846 ±118 d.p.m./25 µl of slices, respectively.

The variability of brain region-specific [3H]InsP₁ accumulations could not be accounted for by variations in the extent of inositol phospholipid labelling. Total [3H]inositol phospholipids were similar between slice preparations of different regional origin (hippocampus 319710 ± 29440; striatum 351650 ± 6440; cortex 348750 ± 43200 d.p.m./25 µl of slices (mean ± s.e.m. for 3 separate experiments)). Furthermore, no significant change in total inositol phospholipid labelling was observed under any conditions, including incubation of slices in the presence of carbachol plus lithium for 30 min (data not shown).

Carbachol addition caused a significant increase in [3H]InsP₁ accumulation in striatal and cerebral cortical slices by 5 min, and in all regions by 10 min. For all brain regions the initial
Figure 3.1.1 Time-course of carbachol-stimulated [H]InsP₁, [H]InsP₂ and [H]InsP₄ accumulation in cerebral cortical slices.

Time-course of carbachol-stimulated [H]InsP₁, [H]InsP₂ and [H]InsP₄ accumulation in cerebral cortical slices in the presence (■) and absence (○) of lithium. Carbachol (1 mM) and lithium (1 mM) were added as indicated at time zero. Values are means ± s.e.m. for at least three experiments. Statistically significant differences (Student's t-test), at the indicated times, between carbachol and carbachol plus lithium conditions are indicated by: *p < 0.05; **p < 0.01; ***p < 0.001.

Time-course of carbachol-stimulated [H]InsP₁, [H]InsP₂ and [H]InsP₄ accumulation in cerebral cortical slices in the presence (■) and absence (○) of lithium. Carbachol (1 mM) and lithium (1 mM) were added as indicated at time zero. Values are means ± s.e.m. for at least three experiments. Statistically significant differences (Student's t-test), at the indicated times, between carbachol and carbachol plus lithium conditions are indicated by: *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 3.1.2 Time-course of carbachol-stimulated $[^3H]InsP_1$, $[^3H]InsP_2$ and $[^3H]InsP_4$ accumulation in hippocampal slices.

Time-course of carbachol-stimulated $[^3H]InsP_1$, $[^3H]InsP_2$ and $[^3H]InsP_4$ accumulation in hippocampal slices in the presence (●) and absence (○) of lithium. Carbachol (1 mM) and lithium (1 mM) were added as indicated at time zero. Values are mean ± s.e.m. for at least three experiments. Statistically significant differences (Student's t test), at the indicated times, between carbachol and carbachol plus lithium conditions are indicated by: *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 3.1.3

Time-course of carbachol-stimulated [3H]InsP₁, [3H]InsP₂ and [3H]InsP₄ accumulation in striatal slices.

Time-course of carbachol-stimulated [3H]InsP₁, [3H]InsP₂ and [3H]InsP₄ accumulation in striatal slices in the presence (▲) and absence (●) of lithium. Carbachol (1 mM) and lithium (1 mM) were added as indicated at time zero. Values are means ± s.e.m. for at least three experiments. Statistically significant differences (Student's t test), at the indicated times, between carbachol and carbachol plus lithium conditions are indicated by: *p < 0.05; **p < 0.01; ***p < 0.001.
increases in [PH]InsP\(_1\) accumulation reached plateau level after 5 min, addition of carbachol plus lithium evoked linear accumulations of [PH]InsP\(_1\) over the 30 min time-course in all regions (Fig. 3.1.1, 3.1.2 & 3.1.3). At the 30 min time point [PH]InsP\(_1\) accumulations in the presence of carbachol and lithium were comparable in all brain regions studied, however, because of the variable basal [PH]InsP\(_1\) observed there were marked differences in the extent of [PH]InsP\(_1\) accumulation when expressed relative to basal values (fold over basal: striatum 14.6; cortex, 3.1; hippocampus, 2.4).

In the absence of lithium, carbachol evoked 3-5 fold increases in [PH]InsP\(_2\) accumulations, with the new levels being maintained over the 30 min time-course of the study (Fig. 3.1.1. & 3.1.2.). In the presence of lithium, the initial carbachol-stimulated increases in [PH]InsP\(_2\) subsequently diminished such that no significant difference between carbachol-stimulated levels in the absence and presence of lithium were observed at the 20 and 30 min time points. In contrast, the lithium enhancement of the carbachol-stimulated [PH]InsP\(_2\) response in hippocampal slices was maintained throughout the 30 min period of study.

Carbachol addition caused a dramatic increases in [PH]InsP\(_4\) accumulation in all brain regions studied (fold over basal: hippocampus, 13.5; striatum, 7.2; cortex, 8.0). In all cases the maximal increase in [PH]InsP\(_4\) was achieved by 5 min after carbachol addition and the elevated levels were well maintained in hippocampal and cortical slices (Fig. 3.1.1. & 3.1.2.). In contrast, [PH]InsP\(_4\) accumulation in striatal slices decreased over the subsequent 25 min time-course, such that by 30 min post-carbachol addition [PH]InsP\(_4\) accumulation was only 3-fold above basal (Fig. 3.1.3.). [PH]InsP\(_4\) accumulations in response to carbachol challenge were initially unaffected by the presence of lithium, although the initial increase in [PH]InsP\(_4\) tended to be lower in striatal slices. For both hippocampal and cortical preparations, [PH]InsP\(_4\) accumulations were maintained over the initial 10 min following carbachol addition. However, [PH]InsP\(_4\) levels subsequently declined such that by 30 min post-carbachol addition [PH]InsP\(_4\)
accumulations were significantly decreased in the presence of lithium. The effect of lithium on
carbachol-stimulated [3H]InsP₄ accumulation in striatal slices was more difficult to gauge. The
time-dependent decline in [3H]InsP₄ accumulation following carbachol addition is greater in the
presence, compared to the absence, of lithium (Fig. 3.1.3.) with significant differences being
observed at the 10 min and 20 min time points.

3.2.2 Effects of lithium on carbachol-stimulated mass changes in Ins(1,4,5)P₃ and
Ins(1,3,4,5)P₄.

In addition to the studies involving [3H]inositol prelabelled brain slices, the regional effects of
carbachol and/or lithium on cellular concentrations of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were
also investigated by using radioreceptor mass assays. Time-course data for the effects of
lithium (1 mM) on carbachol-stimulated inositol polyphosphate responses in hippocampal and
striatal slice preparations are shown in Figs. 3.2.1 & 3.2.2. Data for cerebral cortex have been
omitted as previously published results are in close agreement with those obtained in the
present series of experiments (Kennedy et al., 1990).

Basal accumulations of Ins(1,4,5)P₃ were similar in hippocampal and cortical slices (22.9 ±
0.9 & 21.7 ± 1.0 pmol/mg of protein, respectively), and slightly higher than those found in
striatal slices (16.3 ± 0.7 pmol/mg of protein). Basal accumulations of Ins(1,3,4,5)P₄ were
similar in all slice preparations (hippocampus, 4.0 ± 0.6; striatum, 4.1 ± 0.6; cortex 3.6 ± 0.3
pmol/mg of protein). Incubation of slices in the presence of lithium (1 mM) for 30 min did not
significantly affect levels of either inositol polyphosphate (data not shown). Carbachol elicited
modest, but significant, increases in Ins(1,4,5)P₃ mass accumulations in all brain regions (30 -
40 %); in contrast, large increases in Ins(1,3,4,5)P₄ mass accumulations were observed in all
regions, with the increase at 5 min after carbachol addition being greatest in hippocampus (20-
fold over basal) and least in striatum (8-fold over basal).

In hippocampus, the carbachol-stimulated levels of both polyphosphates were maintained
throughout the 30 min time-course. In absence or presence of lithium the initial carbachol-
Figure 3.2.1

Time-course of carbachol-stimulated Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ mass accumulations in rat hippocampal slices.

Time-course of carbachol-stimulated Ins(1,4,5)P$_3$ (A.) and Ins(1,3,4,5)P$_4$ (B.) mass accumulations in the absence (0) and presence (●) of lithium. Carbachol (1 mM) and LiCl (1 mM) were added simultaneously at time zero. Data are mean ± s.e.m. for at least three separate experiments. Statistical significance (Student’s t test), at the indicated times between carbachol and carbachol plus lithium conditions are indicated: *p < 0.05, **p < 0.01, ***p < 0.001.
**Figure 3.2.2** Time-course of carbachol-stimulated Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ mass accumulations in rat striatal slices.

Time-course of carbachol-stimulated Ins(1,4,5)P₃ (A.) and Ins(1,3,4,5)P₄ (B.) mass accumulations in the absence (△) and presence (▲) of lithium. Carbachol (1 mM) and LiCl (1 mM) were added simultaneously at time zero. Data are mean ± s.e.m. for at least three separate experiments. Statistical significance (Student's t test), at the indicated times between carbachol and carbachol plus lithium conditions are indicated: *p < 0.05, **p < 0.01, ***p < 0.001.
stimulated increases in mass accumulations were similar (Fig. 3.2.1. A, B.), however, following a characteristic 5-10 min lag, lithium caused a subsequent profound decrease in the levels of both Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$, which decreased by >90% and 40% respectively by 30 min compared to preparations to which only agonist was added. These data are in excellent agreement with the time-course studies of the lithium effect in inositol polyphosphate mass accumulations reported previously in cerebral cortex slices (Kennedy et al., 1989, 1990).

In striatum, carbachol-stimulated Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ mass accumulations were both maximal after 5 min and declined thereafter. The data for [H]-InsP$_4$ and Ins(1,3,4,5)P$_4$ mass are therefore in close agreement with respect to the time-course and magnitude of the changes observed. Carbachol stimulation of striatal slices in the absence or presence of lithium resulted in initially similar increases in Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ accumulations. Lithium caused a greater subsequent decrease in mass levels of both inositol polyphosphates, although the lithium effect only reached significance at 20 min for Ins(1,4,5)P$_3$ and 15-30 min for Ins(1,3,4,5)P$_4$ (Fig. 3.2.2. A, B).

3.2.3 Concentration-dependence of the effects of lithium.

The concentration-dependence of the lithium action to enhance [H]-InsP$_4$, and inhibit Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ accumulations in hippocampal slices 20 min after carbachol addition is shown in Fig. 3.3. A. Lithium (10 mM) caused a 5-fold enhancement of carbachol-stimulated [H]-InsP$_4$ accumulation, whilst 50% of the maximal enhancement (EC$_{50}$) was achieved in the presence of 0.67 ± 0.14 mM lithium. Maximal inhibitory effects of lithium on carbachol-stimulated Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ accumulations were obtained with 2 mM lithium (88% and 75% respectively), with half-maximal inhibitions being observed at 0.22 ± 0.09 and 0.33 ± 0.13 mM lithium respectively (if curves were analyzed for lithium concentrations up to an assumed maximum of 2 mM). It is noteworthy that diminished inhibitory effects of lithium were observed at higher concentrations, though at 10 mM lithium
Concentration-dependent effect of lithium on [3H]InsP₁ accumulation and Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ mass accumulation in hippocampal slices.

Concentration-dependent effect of lithium on [3H]InsP₁ (A.), accumulation, and Ins(1,4,5)P₃ (B.) and Ins(1,3,4,5)P₄ (C.) mass accumulation in hippocampal slices. Hippocampal slices were incubated in the presence of carbachol (1 mM) and the indicated concentrations of lithium for 20 min. Values are means ± s.e.m. for at least three separate experiments. Basal values (incubation for 20 min in the absence of carbachol and lithium) were [3H]InsP₁, 11341 ± 1818 d.p.m./25 μl of slices; Ins(1,4,5)P₃, 19.5 ± 0.9 pmol/mg of protein; and Ins(1,3,4,5)P₄, 2.4 ± 0.3 pmol/mg of protein. Incubations of slices in the presence of 10 mM lithium, but absence of carbachol, for 20 min did not significantly alter any of these basal values (data not shown).
the inhibitory effects on Ins(1,4,5)P3 and Ins(1,3,4,5)P4 responses were still profound (57 % and 59 % respectively; Fig. 3.3, B,C). These effects of lithium are in good agreement with those reported previously for cerebral cortex preparations (Kennedy et al., 1989); it was not possible to obtain data for the inhibitory effects of lithium on inositol polyphosphate responses in striatal slices, because of the small differences observed between carbachol-stimulated accumulations in the absence and presence of lithium (Figs. 3.3, A & B).

3.2.4 Effects of lithium on carbachol-stimulated [3H]CMP-PA accumulation.

The effect of carbachol stimulation in the absence and presence of lithium on [3H]CMP-PA accumulation in regional brain slice preparations has also been studied (Table, 3.1.). Additions of carbachol or lithium alone evoked only small changes in [3H]CMP-PA in all regions, with a significant reduction only being observed in cerebral cortex (30 %; Table, 3.1.); in contrast, incubation of slices in the presence of lithium for 10 min increased [3H]CMP-PA accumulations, and this effect reached significance in striatal slices with a 60 % increase in [3H]CMP-PA (Table, 3.1.). Incubation in the presence of both carbachol and lithium caused prompt, time-dependent increases in [3H]CMP-PA accumulations, with significant increases being observed within 2 min of carbachol plus lithium addition in all brain regions. If basal labelling is taken into account, the greatest accumulation of [3H]CMP-PA was observed in striatal slices (5-fold over basal). As reported previously for cerebral cortex slices (Godfrey, 1989; Kennedy et al., 1990), agonist-stimulated [3H]CMP-PA accumulation could be reversed by supplementation of the incubation medium with myo-inositol. Data for the concentration-response relationship of myo-inositol reversal of [3H]CMP-PA accumulation in hippocampal and striatal slices are shown in Fig. 3.4. Complete reversal could be achieved in the presence of 30 mM myo-inositol, with a half-maximal reversal occurring at approximately 1 mM. Supplementation of incubation medium with 10 mM scyllo-inositol did not significantly affect carbachol plus lithium-stimulated [3H]CMP-PA accumulation (data not shown).
Table 3.1  Time-course of [3H]CMP-PA accumulation in hippocampal, striatal and cerebral cortical slices.

Slices were incubated in the presence of [3H]cytidine for 60 min. Additions of carbachol (CCH; 1 mM) and or lithium (LiCl; 5 mM) were made as indicated, and incubations were terminated by addition of 1 M trichloroacetic acid. [3H]CMP-PA was recovered as described in the Methods. Data are mean ± s.e.m. for at least three experiments. For all brain regions examined statistically significant increases in [3H]CMP-PA accumulation was observed at 2 min after addition of carbachol plus lithium and at all subsequent incubation times. In addition, significant differences between the 10 min control CMPPA accumulation and those observed in the presence of only carbachol ("p < 0.05) or lithium ("p < 0.05) are indicated.

Numbers in brackets represent n values.

<table>
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<tr>
<th>Addition</th>
<th>Time (min)</th>
<th>hippocampus</th>
<th>striatum</th>
<th>cerebral cortex</th>
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<tr>
<td>control</td>
<td>0</td>
<td>1026 ± 109</td>
<td>782 ± 152</td>
<td>1601 ± 311</td>
</tr>
<tr>
<td>+ CCH + LiCl</td>
<td>1</td>
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<td></td>
<td>2</td>
<td>1796 ± 197</td>
<td>1673 ± 104</td>
<td>2385 ± 123</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2709 ± 270</td>
<td>2964 ± 242</td>
<td>3730 ± 123</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3478 ± 241</td>
<td>4317 ± 313</td>
<td>5107 ± 232</td>
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<tr>
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<td>829 ± 156</td>
<td>1809 ± 154</td>
</tr>
<tr>
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<td>10</td>
<td>975 ± 113</td>
<td>813 ± 122</td>
<td>1265 ± 61</td>
</tr>
<tr>
<td>+ LiCl</td>
<td>10</td>
<td>1375 ± 99</td>
<td>1322 ± 74</td>
<td>1905 ± 176</td>
</tr>
</tbody>
</table>
Figure 3.4 Concentration-dependent reversal by myo-inositol of $[^3H]$CMP-PA accumulation in hippocampal and striatal slices.

Concentration-dependent reversal by myo-inositol of $[^3H]$CMP-PA accumulation in hippocampal (▲) and striatal (■) slices in the presence of carbachol (1 mM) plus lithium (5 mM). Slices were labelled with $[^3H]$cytidine as described in the Methods. Slices were incubated in the presence of the indicated concentrations of myo-inositol for 30 min before challenge with carbachol (1 mM) and lithium (5 mM) for 20 min. Data are mean ± s.e.m. for at least three experiments. Basal accumulations of $[^3H]$CMP-PA were 2,001 ± 218 and 1,381 ± 148 d.p.m./25 μl for hippocampal and striatal slices respectively.
5.2.5 Reversal of the effects of lithium on carbachol-stimulated Ins(1,4,5)P₃ accumulation by myo-inositol supplementation.

The ability of myo-inositol to prevent the inhibitory effects of lithium (1 mM) on carbachol-stimulated Ins(1,4,5)P₃ accumulation was also investigated (Fig. 3.5.). In striatal slices (Fig. 3.5. A.), lithium evoked a significant inhibition of carbachol-stimulated Ins(1,4,5)P₃ accumulation at 15 and 20 min after carbachol plus lithium addition, and this inhibitory effect was totally unaffected by the presence of 10 mM myo-inositol. In contrast, the inhibition of carbachol-stimulated Ins(1,4,5)P₃ accumulation by lithium observed 15 and 20 min after agonist addition in hippocampal slices was essentially prevented by myo-inositol supplementation (Fig. 3.5. B.). It should be noted that previously reported data has (Kennedy et al., 1990) demonstrated that myo-inositol (10 mM) delayed, but did not prevent, the inhibitory effect of lithium (1 mM) on carbachol-stimulated Ins(1,4,5)P₃ accumulation in cerebral cortex slices.
The effect of myo-inositol supplementation on inhibition of carbachol-stimulated Ins(1,4,5)P₃ mass accumulation in striatal and hippocampal slices.

Figure 3.5

Effect of myo-inositol supplementation on inhibition of carbachol-stimulated Ins(1,4,5)P₃ mass accumulation in striatal (A.) and hippocampal slices (B.). Slices were incubated in the absence (□,■) or presence (▲,▲) of 10 mM myo-inositol for 60 min. Additions of 1 mM carbachol alone (□,▲) or carbachol plus lithium (1 mM;■,□) were made as indicated and incubations were continued for the times indicated. Data are mean ± s.e.m. for at least three experiments. Incubation of slices in the presence of lithium and/or myo-inositol did not significantly affect basal levels of Ins(1,4,5)P₃ (○). Statistically significant differences (Student's t test) between carbachol plus lithium stimulated accumulations in the absence or presence of 10 mM myo-inositol are indicated: *p < 0.05, **p < 0.01.
3.3 Discussion.

There is increasing evidence to support the hypothesis that lithium exerts its subtle and selective neuronal effects in disease states such as manic-depression by reducing the supply of myo-inositol for phosphoinositide synthesis (Berridge et al., 1989; Nahorski et al., 1991). Central to this hypothesis is the uncompetitive nature of the inhibitory action of lithium upon inositol monophosphatase. This unusual type of inhibition provides a basis for a strong stimulus-dependency, whereby cells undergoing marked agonist-stimulation of phosphoinositide turnover will become selectively deprived of myo-inositol via the recycling and de novo synthetic pathways (Nahorski et al., 1991).

Most support for this mechanism has come from experiments utilizing cerebral cortex preparations to demonstrate that in the presence of an agonist that can mediate receptor-stimulated phosphoinositide turnover, lithium evokes a potent (sub-millimolar) enhancement of agonist-stimulated inositol monophosphate accumulation (Batty & Nahorski, 1990), a concomitant accumulation of CMP-PA (Godfrey, 1989; Kennedy et al., 1990), and time-dependent decreases in Ins(1,4,5)P3 and Ins(1,3,4,5)P4 accumulations (Batty & Nahorski, 1987; Whitworth & Kendall, 1988; Kennedy et al., 1989, 1990). Such observations have been interpreted to indicate that efficient regeneration of myo-inositol by the inositol polyphosphate degradation pathway is essential for maintaining adequate rates of inositol phospholipid resynthesis in cerebral cortex and the maintenance of a sufficient substrate supply for PLC (Nahorski et al., 1991).

Although some attempts have been made to compare phosphoinositide turnover in different areas of the brain, these studies have been restricted, in the main, to separating a total \[^{3}H\text{InsP}_5\] fraction (Gonzales & Crews, 1985; Fisher & Barus, 1985; Rooney & Nahorski, 1986) or have not specifically addressed the comparative actions of lithium (Kelly et al., 1988; Stephen & Logan, 1989, 1989b). Therefore, here it has been investigated whether preparations from different brain regions exhibit a similar susceptibility to lithium as have been reported for cerebral cortex, as differences related to receptor density/coupling and access to
myo-inositol or lithium could have implications for the selectivity of lithium action.

Added impetus for this investigation was given by a recent report by Lee et al. (1992) which appears to challenge, at least in part, the explanation of lithium action in brain proposed by Berridge et al. (1989) by providing experimental evidence that lithium (at concentrations only slightly greater than the therapeutic range) causes an increased accumulation of Ins(1,4,5)P3 and Ins(1,3,4,5)P4 in acetylcholine-stimulated guinea-pig cerebral cortex slices. Furthermore, in the same report it was shown that lithium could cause similar increases in acetylcholine-stimulated inositol polyphosphate accumulations in rat and mouse cerebral cortex slices if the incubation medium was supplemented with myo-inositol. Such findings suggest that an enhancement of agonist-stimulated inositol polyphosphate accumulation by lithium can be unmasked if cellular myo-inositol depletion is prevented. However, a mechanistic basis for such a lithium-mediated action remains unclear.

In this present study it has been demonstrated that upon challenge of prelabelled hippocampal, striatal or cortical slices with a maximally-effective concentration of carbachol (1 mM), [3H]InsP1 increased rapidly to a new maintained steady-state level, whereas carbachol addition in the presence of lithium (1 mM) evoked linear rates of [3H]InsP1 accumulation in all slice preparations. The concentration-dependence of the lithium effect was also similar, with submillimolar EC50's being determined for enhancement of [3H]InsP1 in all regions. In agreement with previous observations (Kennedy et al., 1989), the initial carbachol-stimulated increase in [3H]InsP2 accumulation was enhanced in the presence of lithium (1 mM) in cortical slices, with similar patterns being observed in hippocampal and striatal preparations. It is unlikely that such an effect is attributable to an inhibitory effect of lithium upon Ins(1,4)P2/Ins(1,3,4)P3 1-phosphatase, as this enzyme is less sensitive to inhibition by lithium (EC50 of about 5 mM with respect to Ins(1,4)P2 as substrate (Gee et al., 1988b) compared to inositol monophosphatase (Gee et al., 1988a). Furthermore, the enhancement of [3H]InsP2 accumulation does not temporally correlate with the lithium-evoked decrease in Ins(1,4,5)P3 and Ins(1,3,4,5)P4
accumulation. This phenomenon has been further investigated in cerebral cortex slices by using h.p.Lc. analysis to resolve the isomeric constituents of the [H]InsP$_2$ fraction (Jenkinson et al., 1992a; see Chapter 5); it has been demonstrated that Ins(1,4)P$_2$ is the quantitatively important bisphosphate isomer accounting for the majority of the total [H]InsP$_2$ fraction. It has been tentatively proposed that lithium may exert a novel action to enhance the flux through the PtdInsP$_2$/Ins(1,4,5)P$_3$/Ins(1,4)P$_2$ pathway; however, the precise manner in which this intriguing action might be brought about remains undefined (Jenkinson et al., 1992a).

Investigations of agonist-stimulated inositol polyphosphate accumulations has provided evidence for regional differences in response profiles. Data on regional [H]InsP$_3$ and [H]InsP$_4$ accumulations, gained from experiments utilizing [H]inositol-labelled slices, has been supplemented by the use of methods which allow mass levels of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ to be measured. Confidence in the findings is strengthened by the close agreement, both in terms of the magnitude and time-course of changes, between Ins(1,3,4,5)P$_4$ mass and [H]InsP$_4$ data (see below).

Carbachol stimulation of hippocampal or cortical slices resulted in sustained increases in [H]InsP$_4$, and Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ mass accumulations which were maximal within 5 min of agonist addition and did not significantly decline throughout the 30 min experimental period (Figs. 3.1.1, 3.1.2. & 3.3. B. & C.; see also Kennedy et al., 1990). In contrast, carbachol-stimulated inositol polyphosphate accumulations were not maintained in striatal slices, thus continued exposure to agonist per se resulted in decreased accumulations being observed after 15-20 min compared to the 5 min time point, reminiscent of the response observed in carbachol plus lithium treated cortical slices. This could be possibly due to cells within the striatum either having low inositol reserves compared with cells present in cortex or hippocampus or alternatively not being able to transport inositol into the cell at a sufficient rate. Recent characterization of brain regional muscarinic receptor populations using receptor subtype-specific antisera suggests that hippocampus, cortex and striatum all contain broadly

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similar m1/m3 receptor complements (Wall et al., 1991a, 1991b), although in situ hybridization studies provide evidence for a lower levels of m3 mRNA in striatum (Levey et al., 1991). Therefore the heterogeneity of muscarinic receptors underlying these differences in regional polyphosphate responses to carbachol may be responsible for such an effect.

The magnitude, time-dependency and concentration-response relationship of lithium effects on carbachol-stimulated Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulations in hippocampus were entirely consistent with previous published work using cerebral cortex slices (Kennedy et al., 1989). It should be noted that even at the highest concentration of lithium (10 mM) a highly significant inhibition of inositol polyphosphate responses was observed, lending no support to the recent report of Lee and colleagues (1992).

Another aspect of our efforts to establish whether regional variations occur in the effects of lithium on agonist-stimulated phosphoinositide turnover has been to investigate the accumulation of CMP-PA. Accumulation of this intermediary metabolite has been used as a sensitive indicator of a lithium-mediated perturbation of the phosphoinositide cycle (Godfrey, 1989; Hwang et al., 1990; Kennedy et al., 1990) (however see later). Basal labelling of CMP-PA showed some regional variation, being 2-fold greater in cerebral cortex compared to striatal slices. Carbachol (1 mM) addition had little effect on [3H]CMP-PA accumulation, although a small (30%) but significant decrease in CMP-PA labelling was observed in cortical slices. This is unlikely to be due to an increased flux through PtdIns synthase, however the mechanism underlying this decrease is unknown. Addition of lithium per se caused small increases in [3H]CMP-PA accumulation in all regions, with the greatest increase (63%) being noted in striatal slices. These data suggest that the regional brain slice preparations investigated can maintain adequate myo-inositol recycling in the presence of maximal agonist concentration. However, co-addition of carbachol and lithium caused a prompt [3H]CMP-PA accumulation in all regions studied (Table 3.1). Over the 10 min time-course investigated, the greatest increase in [3H]CMP-PA (relative to basal values) was seen in striatal (552 ± 45%) compared to hippocampal or cortical slices, suggesting an accentuated
dependence on myo-inositol availability from the inositol polyphosphate dephosphorylation pathway of this brain region.

The \([3H]\text{CMP-PA accumulation observed in cerebral cortex slices in the presence of agonist plus lithium can be reversed by supplementation of the incubation medium with myo-inositol, either prior to, or subsequent to agonist challenge (Kennedy et al., 1990). The concentration-dependence of myo-inositol reversal of \([3H]\text{CMP-PA accumulation appears to be similar in all regions studied, with prior supplementation of incubation medium with myo-inositol (10 mM) essentially preventing agonist plus lithium-stimulated \([3H]\text{CMP-PA accumulation, with half-maximal effects being observed at around 1 mM myo-inositol in hippocampal and striatal slices (Fig. 3.4.)}. These data are in close agreement with those for rat cerebral cortex reported previously by Godfrey (1989). Taken together, the \([3H]\text{CMP-PA data suggest that the cellular sub-populations present in hippocampus, striatum and cortex which are responsible for the observed responses possess a PtdIns synthase activity which is likely to exhibit a high }K_m\text{ for myo-inositol (in the mM range). Indeed, such a PtdIns synthase activity has been purified from rat brain (Ghalayini & Eichberg, 1985), exhibiting kinetics which would indicate that it is not saturated with myo-inositol, and is therefore sensitive to alterations in the cellular myo-inositol pool. Similarly, all regions require the extracellular myo-inositol concentration to be raised to high levels (> 10 mM) if uptake is to fully compensate for the cellular myo-inositol deficit caused by the presence of lithium.}

Although incubation of cerebral cortex slices in the presence of high concentrations of myo-inositol can reverse carbachol plus lithium-stimulated \([3H]\text{CMP-PA accumulation, the concurrent time-dependent decrease in }\text{Ins(1,4,5)P}_3\text{ and }\text{Ins(1,3,4,5)P}_4\text{ accumulation is attenuated, but not prevented (Kennedy et al., 1990). In the present study we have also investigated the effect of myo-inositol supplementation upon }\text{Ins(1,4,5)P}_3\text{ accumulations in hippocampal and striatal slices. In hippocampal slices, the inhibition of carbachol-stimulated }\text{Ins(1,4,5)P}_3\text{ by lithium (1 mM) was reversed by myo-inositol (10 mM), whereas the decay of }\text{Ins(1,4,5)P}_3\text{ with agonist alone, or the additional inhibitory effect of lithium, were unaffected.}
by myo-inositol supplementation in striatum, with cerebral cortex slices exhibiting an intermediate response as reported previously (Kennedy et al., 1990). It should also be noted that in contrast to data reported previously by Lee et al. (1992), none of our investigations with myo-inositol supplementation revealed a stimulatory action of lithium upon carbachol-stimulated inositol polyphosphate accumulation. In the light of the similarity between regions with respect to myo-inositol effects upon carbachol plus lithium-stimulated CMP-PA accumulation, this discrepancy in the inositol polyphosphate response is difficult to explain simply in terms of inositol phospholipid depletion (Nahorski et al., 1991), and suggest that the action of lithium to decrease agonist-stimulated inositol polyphosphate accumulation may not occur only through limitation of inositol phospholipid resynthesis.

A number of recent studies (Brami et al., 1991; Heacock et al., 1993) have demonstrated that the accumulation of CMP-PA in response to agonist and lithium may not be directly related to the accumulation of inositol phosphates as was initially thought (Godfrey, 1990). Heacock and coworkers (1993) have observed that some agonists, such as endothelin, in the presence of lithium can produce a significant increase in the accumulation of the inositol phosphates (Ins$_P$) without producing any significant increase in the accumulation of CMP-PA. Indeed, this study went on to demonstrate in brain slice preparations that with some agonists, such as carbachol, regional variations in the ratio of agonist-stimulated Ins$_P$ versus CMP-PA were possible. For example, carbachol produced a similar increase in CMP-PA and Ins$_P$ accumulation in hippocampal and cortical slices (as measured in % over basal), whereas in cerebellar slices carbachol produced a significantly larger increase in the accumulation of Ins$_P$ compared with the stimulated increase in CMP-PA accumulation.

In assuming a direct relationship between the receptor activated phosphoinositide turnover and CMP-PA accumulation one major assumption is made. It is assumed that the CMP-PA formed comes solely from DAG generated directly from PLC mediated hydrolysis of PtdIns$(4,5)P_2$. This may not however be the only mechanism of generating DAG in agonist-stimulated brain slices. Qian & Drewes (1990) have shown that the intrinsic agonist for muscarinic receptors in
**vivo**, acetylcholine, can produce a rapid accumulation of PA from phosphatidylcholine in synaptic membranes of canine cerebral cortex. Additional evidence has demonstrated that DAG was produced from phosphatidylcholine by the action of phospholipase D (PLD) to yield PA which was dephosphorylated by PA phosphatase giving DAG. It is therefore possible that part of the agonist-stimulated increases in CMP-PA levels observed above may come from this route, with the various agonists stimulation PLC and PLD to different degrees resulting in the responses observed by Bruni et al. (1991).

Although this hypothesis may explain why CMP-PA accumulation does not always correlate with inositol phosphate accumulation, it does not offer an explanation as to why exogenous inositol can reverse the effects of lithium upon agonist-stimulated CMP-PA accumulation in striatum without having any significant effect upon the lithium induced reduction in carbachol-stimulated Ins(1,4,5)P₃ mass in this preparation.

The work described in following chapter moves on from the work described here to examine in much greater detail a number of aspects of lithium action upon phosphoinositide metabolism in rat cerebral cortex.
Chapter 4

Lithium Disruption of Phosphoinositide Signalling in Rat Cerebral Cortex.
4.1 Introduction.

The ability of lithium to disrupt phosphoinositide signalling was first demonstrated by Allison and Stewart (1971) who initially demonstrated that in rats treated with lithium the levels of free inositol in the brain substantially decreased by up to 30% after treatment. Furthermore, Allison et al. (1976) later went on to suggest that this decrease in inositol levels was due to an inhibition of the inositol monophosphatase by lithium. This conclusion was reached by virtue of the fact that lithium produced a considerable increase in the levels of inositol monophosphate in rat brain and this increase was stoichiometrically similar to the decrease in the levels of inositol observed in this tissue. Hallcher and Sherman (1980) subsequently confirmed in partly purified enzyme preparations of inositol monophosphatase that lithium did indeed inhibit, and the inhibition was uncompetitive in nature (K_i value of 1.0 mM and 0.26 mM with respect to Ins(1)P_1 and Ins(4)P_1 (Gee et al., 1988a)). It has been subsequently demonstrated that lithium is also an uncompetitive inhibitor of the Ins(1,3,4)P_3/Ins(1,4)P_2 1-phosphatase (K_i value of 9.63 mM and 0.46 mM with respect to Ins(1,4)P_2 and Ins(1,3,4)P_3) (Gee et al., 1988b).

A number of studies have specifically examined the effects of lithium upon phosphoinositide metabolism in rat cerebral cortex (e.g. Batty & Nahorski, 1987; Kennedy et al., 1989), however only a few have attempted to examine the effects of this agent on the individual inositol phosphate isomers which constitute the crude InsP_3 fractions that generally have been examined in such studies. However, the complexity of Ins(1,4,5)P_3 metabolism in cerebral cortex necessitates a more complete analysis since subtle effects of lithium on the individual isomers are not always revealed and may be masked by the measurement of InsP_4 total fractions. For example, any lithium-induced decrease in the agonist-stimulated accumulation of Ins(1,4,5)P_3 may be masked by an increase in the levels of Ins(1,3,4)P_3 produced by the inhibition of one of the routes of metabolism of this isomer by lithium. Therefore in measuring the total InsP_3 fraction the profile of the response obtained would be a sum of these two
separate effects of lithium and the overall effect of lithium may depend upon the concentration used.

In this chapter, therefore, the concentration-dependent and temporal aspects of the effects of lithium upon phosphoinositide metabolism have been examined in rat cerebral cortex slices. An attempt is made to examine whether the disruption of phosphoinositide signalling by lithium is related to stimulus strength. A consequence of the uncompetitive nature of the inhibition of the inositol monophosphatase produced by lithium is that as the concentration of the substrate for this enzyme increases, so does the inhibition produced by lithium. Therefore, the effect of lithium upon phosphoinositide metabolism in the presence of increasing concentrations of agonist has been examined in order to determine whether such an effect can be observed in this preparation. The specificity of the inhibitory effect of lithium is also examined in relation to different agonists to determine whether the actions of lithium are agonist specific, as has been suggested previously (Whitworth & Kendall, 1988).
4.2 Results.

4.2.1 Time-course of the Effects of Lithium Upon Carbachol-stimulated Accumulation of Inositol (poly)phosphate Isomers in Rat Cerebral Cortex Slices.

Phosphoinositide metabolism was examined in [3H]inositol labelled rat cerebral cortex slices by examining the individual species of inositol phosphates produced upon agonist stimulation of this preparation. The effects of lithium upon the temporal aspects of the accumulation of the isomeric species was also examined.

The effect of lithium (1 mM) addition on carbachol-stimulated (1 mM) accumulation of the major inositol phosphate isomers generated upon agonist challenge was examined in rat cerebral cortex slices. In total nine inositol phosphates were examined, including: Ins(1/3)P₁, Ins(4)P₁, Ins(1,3)P₂, Ins(1,4)P₂, Ins(3,4)P₂, Ins(4,5)P₂, Ins(1,3,4)P₃, Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, in order to try and establish the pattern of Ins(1,4,5)P₃ metabolism in this tissue. For all the isomers examined lithium (1 mM) alone had no significant effect on basal values.

The addition of carbachol alone (1 mM) produced a rapid increase in the accumulation of [3H]Ins(1,4,5)P₃ which reached a maximum of 3.0-fold over basal (1304 ± 182 d.p.m./sample) by 10 min (Fig. 4.4.), before plateauing. Ins(1,4,5)P₃ levels remained at this elevated level throughout the remainder of the time-course examined. Lithium (1 mM) in the presence of carbachol had no significant effect on the initial rapid production of Ins(1,4,5)P₃ produced by carbachol alone, however after a lag period of approximately 10 min Ins(1,4,5)P₃ levels were significantly reduced (53.1% decrease) by lithium compared with carbachol-stimulated values (Fig 4.4.).

The immediate metabolites of Ins(1,4,5)P₃, Ins(1,4)P₂ and Ins(1,3,4,5)P₄, which are produced by the action of 5-phosphatase and 3-kinase activities respectively, are shown in Figs. 4.2. and 4.5. respectively.

Upon the addition of carbachol alone (1 mM) [3H]Ins(1,4)P₂ levels increased rapidly over the
initial 5 min of the time-course reaching a maximum of 8.0-fold over basal (2428 ± 85 d.p.m./sample) by 5 min. Levels of this bisphosphate then decreased slowly over the remainder of the time-course with levels reaching 6.0-fold over basal by 30 min. Lithium (1 mM) in the presence of carbachol enhanced the initial rapid increase in Ins(1,4)P₂ accumulation with levels reaching 10.9-fold over basal by 5 min. Ins(1,4)P₂ levels decreased over the subsequent 25 min of the time-course, such that by 30 min Ins(1,4)P₂ levels were 4.8-fold over basal values.

Carbachol alone (1 mM) produced a increase in the levels of Ins(1,3,4,5)P₄ with levels increasing rapidly reaching a maximum of 19.6-fold over basal (416 ± 37 d.p.m./sample) at 5 min, before plateauing. Levels did not significantly change throughout the remainder of the time-course. Lithium in the presence of carbachol had no effect on the initial 10 min of the carbachol-stimulated accumulation of Ins(1,3,4,5)P₄, however, after a lag period of 10-20 min (a similar period to that observed for the effect of lithium on Ins(1,4,5)P₃ levels) the levels of this tetrakisphosphate were reduced by 58.4 % compared with values carbachol-stimulated values (at 30 min).

The metabolism of Ins(1,3,4,5)P₄ in cortex is via a dephosphorylation to Ins(1,3,4)P₃, via the same 5-phosphatase involved in the metabolism of Ins(1,4,5)P₃. In the presence of carbachol alone (1 mM) [FH]Ins(1,3,4)P₃ levels increased rapidly reaching 29.9-fold over basal (75 ± 5 d.p.m./sample) at 5 min. Levels then slowly decreased reaching 22.9-fold over basal by 30 min. Lithium in the presence of carbachol enhanced the initial accumulation of Ins(1,3,4)P₃ observed in the presence of carbachol at 5 min (41.3-fold increase over basal in the presence of carbachol and lithium). In the continued presence of agonist and lithium Ins(1,3,4)P₃ levels subsequently decreased reaching 18.3-fold over basal by 30 min, i.e. below agonist-stimulated values at this time-point.

Ins(1,3,4)P₃ metabolism proceeds via either a 1- or a 4-phosphatase, resulting in the formation of Ins(3,4)P₂ and Ins(1,3)P₂ respectively. In the presence of carbachol alone Ins(3,4)P₂ levels slowly increased over the time-course examined, with levels reaching 10.9-
Figure 4.1. Time-Course of the Effects of Lithium on Carbachol-Stimulated Ins(1/3)P₁ and Ins(4)P₁ levels in Rat Cerebral Cortex Slices

[3H]Inositol labelled Cerebral cortex slices were stimulated with carbachol in the absence (0) or presence (●) of lithium (1 mM) for the times indicated and the levels of the isomers Ins(1/3)P₁ and Ins(4)P₁ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments. Basal values for Ins(1/3)P₁ and Ins(4)P₁ were 10165 ± 429 d.p.m. and 3307 ± 429 d.p.m. respectively. Statistically significant differences between (0) and (●) are shown as *
P < 0.05, **P < 0.01 and ***P < 0.001.
Figure 4.2. Time-Course of the Effects of Lithium on Carbachol-
Stimulated Ins(1,3)P₂ and Ins(1,4)P₂ levels in Rat Cerebral
Cortex Slices

[³H]Inositol labelled Cerebral cortex slices were stimulated with
carbachol in the absence () or presence (•) of lithium (1 mM) for the
times indicated and the levels of the isomers Ins(1,3)P₂ and Ins(1,4)P₂
were measured as described in the Methods. Data represent the means ±
S.E.M. of three experiments. Basal values for Ins(1,3)P₂ and Ins(1,4)P₂
were 64 ± 11 d.p.m. and 2428 ± 85 d.p.m. respectively.
Statistically significant differences between () and (•) are shown
as *P < 0.05, **P < 0.01 and ***P < 0.001.
Time-Course of the Effects of Lithium on Carbachol-
Stimulated Ins(3,4)P₂ and Ins(4,5)P₂ levels in Rat Cerebral
Cortex Slices

[3H]Inositol labelled Cerebral cortex slices were stimulated with
carbachol in the absence (○) or presence (●) of lithium (1 mM) for the
times indicated and the levels of the isomers Ins(3,4)P₂ and Ins(4,5)P₂
were measured as described in the Methods. Data represent the means ±
S.E.M. of three experiments. Basal values for Ins(3,4)P₂ and Ins(4,5)P₂
were 161 ± 22 d.p.m. and 51 ± 8 d.p.m. respectively.
Statistically significant differences between (○) and (●) are shown
as *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 4.3.
Figure 4.4. Time-Course of the Effects of Lithium on Carbachol-Stimulated Ins(1,3,4)P₃ and Ins(1,4,5)P₃ levels in Rat Cerebral Cortex Slices

[³H]Insitol labelled cerebral cortex slices were stimulated with carbachol in the absence (0) or presence (●) of lithium (1 mM) for the times indicated and the levels of the isomers Ins(1,3,4)P₃ and Ins(1,4,5)P₃ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments. Basal values for Ins(1,3,4)P₃ and Ins(1,4,5)P₃ were 75 ± 5 d.p.m. and 1304 ± 182 d.p.m. respectively.

Statistically significant differences between (0) and (●) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
Figure 4.5. Time-Course of the Effects of Lithium on Carbachol-Stimulated Ins(1,3,4,5)P$_4$ levels in Rat Cerebral Cortex Slices

[3H]Inositol labelled Cerebral cortex slices were stimulated with carbachol in the absence (0) or presence (●) of lithium (1 mM) for the times indicated and the levels of the isomer Ins(1,3,4,5)P$_4$ was measured as described in the Methods. Data represent the means ± s.e.m. of three experiments. Basal values for Ins(1,3,4,5)P$_4$ was 416 ± 37 d.p.m.

Statistically significant differences between (0) and (●) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
fold over basal values (161 ± 22 d.p.m./sample). The addition of lithium had no significant
effect on the agonist-stimulated accumulation of Ins(3,4)P2 over the time-course examined.

Carbachol alone (1 mM) produced a rapid increase in the levels of Ins(1,3)P2 which reached a
maximum of 7.1-fold over basal (64 ± 11 d.p.m./sample) by 10 min, before decreasing to 6.0-
fold over basal by 30 min. Lithium addition enhanced the initial increase in carbachol-
stimulated Ins(1,3)P2 levels observed at 10 min (10.1-fold increase over basal in the presence
of agonist and lithium), however this enhancement was not maintained and the levels of this
bisphosphate subsequently decreased to 5.5-fold over basal, i.e. lithium produced a 9.6 %
decrease in the levels of Ins(1,3)P2 compared with carbachol-stimulated values, at 30 min.

The effects of lithium on agonist stimulated levels of Ins(4,5)P2 were also examined. This
bisphosphate may represent a third route of metabolism for Ins(1,4,5)P3 in cerebral cortex
slices (see Shears, 1991; also see Chapter 5). Carbachol alone (1 mM) produced a slow
increase in Ins(4,5)P2 levels before reaching a maximum (14.2-fold over basal; basal value =
51 ± 8 d.p.m./sample) and plateauing at 20 min. The addition of carbachol and lithium
produced a linear accumulation of Ins(4,5)P2 over the time-course examined, with levels
reaching 149.2-fold over basal by 30 min in the presence of both agents.

The accumulation of the inositol monophosphates Ins(1/3)P1 and Ins(4)P1 in the presence of
carbachol and carbachol plus lithium are shown in figure 4.1. In the presence of carbachol
alone (1 mM) [3H]Ins(1/3)P1 levels increased over the first 5 min of the time-course after an
initial lag period of 1-2 min, levels reached a maximum of 4.0-fold over basal (10165 ± 429
d.p.m./sample) at 20 min. The addition of lithium (1 mM) produced a marked enhancement in
the agonist-stimulated accumulation of Ins(1/3)P1. In the presence of agonist and lithium,
Ins(1/3)P1 accumulation was linear over the time-course examined, after an initial lag period of
1-2 min, such that at 30 min Ins(1/3)P1 levels were 19.8-fold over basal values.

The accumulation of Ins(4)P1 was not identical to that of Ins(1/3)P1 as might have been
expected. With carbachol alone Ins(4)P1 levels increased over the initial 10 min before reaching
a plateau of 2.5-fold over basal (3307 ± 429 d.p.m./sample) values at 20 min. In the presence of lithium the accumulation of Ins(4)P1 increased over the time-course examined (maximum of 22.4-fold over basal at 30 min), however the accumulation did not appear to be linear over the 30 min time course.

No other inositol monophosphate isomers were detected.

4.2.2. Concentration-dependent Effects of Lithium On Carbachol-stimulated Inositol (poly)phosphate Accumulation in Rat Cerebral Cortex Slices.

The concentration-dependent effects of lithium (0.01-10 mM) upon the accumulation of the various inositol phosphate isomers in carbachol-stimulated rat cerebral cortex slices was examined. All incubations with agonist in the absence or presence of lithium were for 30 min. Lithium alone (10 mM) did not have any significant effect on the basal levels of any of the inositol phosphate isomers examined over the 30 min incubation period of the experiment.

Carbachol alone (1 mM) produced a 2.9-fold increase over basal (1013 ± 150 d.p.m./sample) in the levels of Ins(1,4,5)P3 at 20 min (Fig. 4.9), similar to that observed previously during time-course studies. Increasing concentrations of Liium (0.1-10 mM) produced a concentration-dependent decrease in the carbachol-stimulated levels of Ins(1,4,5)P3 (IC50 = 0.82 ± 0.21 mM), with a maximum inhibition (42.8% compared with carbachol-stimulated values (2995 ± 597 d.p.m./sample) occurring with 10 mM lithium.

Carbachol (1 mM) produced a 25.9-fold increase in Ins(1,3,4,5)P4 levels compared with basal values (268 ± 30 d.p.m./sample) at 30 min (Fig. 4.10). Lithium produced a concentration-dependent decrease in the levels of Ins(1,3,4,5)P4 with a similar IC50 value of 0.39 ± 0.15 mM to that for Ins(1,4,5)P3. Lithium at a concentration of 10 mM produced a maximum inhibition of carbachol-stimulated Ins(1,3,4,5)P4 levels of 64.6% compared with carbachol-stimulated values (6942 ± 1168 d.p.m./sample).

In the presence of carbachol alone (1 mM) Ins(1,4)P2 levels increased by 3.8-fold over basal (2940 ± 500 d.p.m./sample)(Fig. 4.7). Lithium (0.1-10 mM) had no effect on Ins(1,4)P2
accumulation except at the highest concentration (10 mM) were an increase to 3.0-fold over carbachol-stimulated values (11047 ± 1019 d.p.m./sample) was observed. The EC\textsubscript{50} of this effect could therefore not be calculated, however it is clearly greater than 3 mM.

Ins(4,5)P\textsubscript{2} levels increased 12.7-fold over basal (44 ± 6 d.p.m./sample) in the presence of carbachol alone (Fig. 4.8). Lithium produced a concentration-dependent increase in the levels of Ins(4,5)P\textsubscript{2} with an EC\textsubscript{50} value of 94 ± 3 \textmu M. A maximal increase was observed with 3 mM lithium, which produced a 12.1-fold increase over carbachol-stimulated values (558 ± 96 d.p.m./sample).

Carbachol alone produced a 26.3-fold increase over basal values (68 ± 4 d.p.m./sample) in the accumulation of Ins(1,3,4)P\textsubscript{3} (Fig. 4.9). Lithium had no significant effect on carbachol-stimulated levels of Ins(1,3,4)P\textsubscript{3} even at the highest concentration examined (10 mM).

The levels of Ins(3,4)P\textsubscript{2} were enhanced upon the addition of carbachol alone (Fig. 4.8). At 30 min this agonist produced a 16.7-fold increase over basal (175 ± 7 d.p.m./sample) in the accumulation of this isomer. Lithium did not produce a significant increase in the carbachol-stimulated levels of Ins(3,4)P\textsubscript{2}.

Carbachol alone produced a 47.2-fold increase over basal (87 ± 4 d.p.m./sample) in the accumulation of Ins(1,3)P\textsubscript{2} at 30 min (Fig. 4.7). Lithium (0.1-10 mM) had no significant effect on the carbachol-stimulated increase in Ins(1,3)P\textsubscript{2} accumulation at this 30 min time point.

Finally, the effects of lithium on the inositol monophosphates are shown in figure 4.6. Carbachol alone produced a 4.6-fold increase and a 2.0-fold increase over basal values (8229 ± 885 d.p.m./sample and 3482 ± 365 d.p.m./sample, respectively) in the accumulation of Ins(1/3)P\textsubscript{1} and Ins(4)P\textsubscript{1} respectively. Lithium produced a concentration-dependent increase in the agonist-stimulated accumulations of both isomers. Ins(1/3)P\textsubscript{1} levels were increased to a maximum of 6.5-fold over carbachol-stimulated values (37681 ± 3351 d.p.m./sample) in the presence of 10 mM lithium, with an EC\textsubscript{50} for this effect of 0.59 ± 0.21 mM. Ins(4)P\textsubscript{1} levels were increased to a maximum of 8.9-fold over carbachol-stimulated values (6858 ± 730 d.p.m./sample).
Figure 4.6. Concentration-dependent Effects of Lithium on Carbachol-Stimulated \textit{Ins}(1/3)\textit{P}_1 and \textit{Ins}(4)\textit{P}_1 levels in Rat Cerebral Cortex Slices

\[^{[3]H}]\text{Inositol\ labelled\ cerebral\ cortex\ slices\ were\ stimulated\ with\ carbachol\ (1\ mM)\ in\ the\ presence\ of\ increasing\ concentrations\ of\ lithium\ (0.01-10\ mM)\ for\ 30\ min\ and\ the\ levels\ of\ the\ isomers\ \textit{Ins}(1/3)\textit{P}_1\ and\ \textit{Ins}(4)\textit{P}_1\ were\ measured\ as\ described\ in\ the\ Methods.\ Data\ represent\ the\ means\ \pm\ s.e.m.\ of\ three\ experiments.\ Carbachol-stimulated\ values\ for\ \textit{Ins}(1/3)\textit{P}_1\ and\ \textit{Ins}(4)\textit{P}_1\ were\ 37681\ \pm\ 3351\ d.p.m.\ and\ 6858\ \pm\ 730\ d.p.m.\ respectively.\]
**Figure 4.7.** Concentration-dependent Effects of Lithium on Carbachol-Stimulated Ins(1,3)P₂ and Ins(1,4)P₂ levels in Rat Cerebral Cortex Slices

[³H]Inositol labelled cerebral cortex slices were stimulated with carbachol (1 mM) in the presence of increasing concentrations of lithium (0.01-10 mM) for 30 min and the levels of the isomers Ins(1,3)P₂ and Ins(1,4)P₂ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments. Carbachol-stimulated values for Ins(1,3)P₂ and Ins(1,4)P₂ were 4107 ± 785 d.p.m. and 11047 ± 1019 d.p.m. respectively.
[3H]Inositol labelled cerebral cortex slices were stimulated with carbachol (1 mM) in the presence of increasing concentrations of lithium (0.01-10 mM) for 30 min and the levels of the isomers Ins(3,4)P₂ and Ins(4,5)P₂ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments. Carbachol-stimulated values for Ins(3,4)P₂ and Ins(4,5)P₂ were 2098 ± 392 d.p.m. and 558 ± 96 d.p.m. respectively.
Figure 4.9. Concentration-dependent Effects of Lithium on Carbachol-Stimulated Ins(1,3,4)P₃ and Ins(1,4,5)P₃ levels in Rat Cerebral Cortex Slices

[³H]Inositol labelled cerebral cortex slices were stimulated with carbachol (1 mM) in the presence of increasing concentrations of lithium (0.01-10 mM) for 30 min and the levels of the isomers Ins(1,3,4)P₃ and Ins(1,4,5)P₃ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments. Carbachol-stimulated values for Ins(1,3,4)P₃ and Ins(1,4,5)P₃ were 1788 ± 164 d.p.m. and 2955 ± 597 d.p.m. respectively.
Figure 4.10. Concentration-dependent Effects of Lithium on Carbachol-Stimulated Ins(1,3,4,5)P$_4$ levels in Rat Cerebral Cortex Slices

$[^3]$H]Inositol labelled cerebral cortex slices were stimulated with carbachol (1 mM) in the presence of increasing concentrations of lithium (0.01-10 mM) for 30 min and the levels of the isomer Ins(1,3,4,5)P$_4$ was measured as described in the Methods. Data represent the means ± s.e.m. of three experiments. Carbachol-stimulated value for Ins(1,3,4,5)P$_4$ was 6942 ± 1168 d.p.m.
d.p.m./sample) in the presence of 10 mM lithium; the EC\textsubscript{50} for this effect being 0.37 ± 0.18 mM. Both of these values are comparable with the IC\textsubscript{50} value for lithium inhibition of the purified inositol monophosphatase enzyme (Gee et al., 1988a).

4.2.3 The Effect of Carbachol Concentration Upon Lithium Disruption of Phosphoinositide Signalling in Rat Cerebral Cortex Slices.

The effect of increasing concentrations of carbachol upon the ability of lithium to disrupt phosphoinositide signalling was examined in rat cerebral cortex slices to determine whether the actions of lithium were related to stimulus strength in this preparation. All incubations with agonist in the absence or presence of lithium were for 20 min. Lithium (1 mM) produced a significant increase in the basal levels of Ins(1/3)Pi (6325 ± 379 d.p.m.) and Ins(4)P\textsubscript{1} (5429 ± 652 d.p.m.) only.

Carbachol produced a concentration-dependent increase (EC\textsubscript{50} = 68.1 ± 1.2 µM) in the levels of Ins(1/3)P\textsubscript{1} which reached a maximum of 13.5-fold over basal (4207 ± 664 d.p.m./sample) in the presence of 3 mM carbachol (Fig. 4.11). The addition of carbachol plus lithium (1 mM) resulted in an increase in the maximal accumulation of Ins(1/3)P\textsubscript{1}, with 3 mM carbachol in the presence of lithium (1 mM) eliciting a 61.6-fold increase over basal. The EC\textsubscript{50} value for the carbachol-stimulated accumulation of Ins(1/3)P\textsubscript{1} in the presence of lithium was 54.7 ± 7.0 µM.

Carbachol produced a similar concentration-dependent increase in the levels Ins(4)P\textsubscript{1} (EC\textsubscript{50} = 53.0 ± 2.1 µM) which reached a maximum of 3.6-fold over basal (2599 ± 436 d.p.m./sample) in the presence of 3 mM carbachol (Fig. 4.11). The addition of carbachol and lithium (1 mM) resulted in an increase in the maximal accumulation of Ins(4)P\textsubscript{1}, with 3 mM carbachol in the presence of lithium (1 mM) eliciting a 32.3-fold increase over basal. The EC\textsubscript{50} value for the carbachol-stimulated accumulation of Ins(4)P\textsubscript{1} in the presence of lithium was 21.0 ± 5.6 µM.

Carbachol elicited Ins(1,4,5)P\textsubscript{3} accumulation in a concentration-dependent manner with an EC\textsubscript{50} values of 40 ± 2.6 µM (Fig. 4.12). A maximal increase in Ins(1,4,5)P\textsubscript{3} accumulation
Figure 4.11. The Effects of Carbachol Concentration Upon Lithium Disruption of Phosphoinositide Signalling In Rat Cerebral Cortex Slices

[3H]Inositol labelled cerebral cortex slices were stimulated with increasing concentrations of carbachol (0.03-3 mM) in the absence (○) or presence (●) of lithium (1 mM) for 20 min. The levels of the isomers Ins(1/3)P1 and Ins(4)P1 were measured as described in the Methods. Data represent the means ± s.e.m. for three experiments. Basal values for Ins(1/3)P1 and Ins(4)P1 were 4207 ± 664 d.p.m. and 2599 ± 436 d.p.m. respectively.
Figure 4.12. The Effects of Carbachol Concentration Upon Lithium Disruption of Phosphoinositide Signalling In Rat Cerebral Cortex Slices

[3H]Inositol labelled cerebral cortex slices were stimulated with increasing concentrations of carbachol (0.03-3 mM) in the absence (O) or presence (★) of lithium (1 mM) for 20 min. The levels of the isomers Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were measured as described in the Methods. Data represent the means ± s.e.m. for of three experiments. Basal values for Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were 1004 ± 148 d.p.m. and 352 ± 65 d.p.m. respectively.
was elicited by 3 mM carbachol which produced a 4.1-fold increase in Ins(1,4,5)P₃ levels compared with basal values (1004 ± 148 d.p.m./sample). The addition of carbachol and lithium resulted in a decrease in the maximal accumulation of Ins(1,4,5)P₃, with 3 mM carbachol in the presence of lithium (1 mM) eliciting only a 2.8-fold increase over basal values. The EC₅₀ value for the carbachol stimulated accumulation of Ins(1,4,5)P₃ in the presence of lithium was 57.7 ± 14.4 μM.

Carbachol also elicited Ins(1,3,4,5)P₄ accumulation in a concentration-dependent manner with an EC₅₀ values of 57.2 ± 2.2 μM. A maximal increase in Ins(1,3,4,5)P₄ accumulation was elicited by 3 mM carbachol which produced a 42.6-fold increase in Ins(1,3,4,5)P₄ levels compared with basal values (352 ± 65 d.p.m./sample). The addition of carbachol and lithium resulted in a decrease in the maximal accumulation of Ins(1,3,4,5)P₄, with 3 mM carbachol in the presence of lithium (1 mM) eliciting only a 14.4-fold increase over basal values. The EC₅₀ value for the carbachol-stimulated accumulation of Ins(1,4,5)P₃ in the presence of lithium was 27.3 ± 6.5 μM.

4.2.4. The Effects of Different Agonists Upon Lithium Disruption of Phosphoinositide Signalling in Rat Cerebral Cortex Slices.

The ability of a number of different agonists to stimulate phosphoinositide metabolism in [³H]inositol-labelled rat cerebral cortex slices was examined in the absence and presence of lithium. The effects of the agonists carbachol (CCh; 1 mM), noradrenaline (NA; 100 μM), KCl (30 mM final), histamine (Hist; 1 mM), 1-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPD; 300 μM), quisqualate (Quis; 10 μM) and 5-hydroxytryptamine (5-HT; 100 μM) on the levels of Ins(1,4,5)P₃, Ins(1,3,4,5)P₄ and the monophosphates Ins(1/3)P₁ and Ins(4)P₁ were all examined after 20 min incubations in the absence and presence of lithium.

In the absence of lithium histamine did not produce significant increases in the levels of Ins(1/3)P₁ compared with basal values (5633 ± 425 d.p.m./sample). However, CCh, NA, KCl, t-ACPD Quis and 5-HT all produced a significant increase in the levels of Ins(1/3)P₁,
Figure 4.13. The Effects of Different Agonists Upon Lithium Disruption of Phosphoinositide Signalling in Rat Cerebral Cortex Slices

[3H]Inositol labelled cerebral cortex slices were stimulated with a variety of agonists (Carbachol: CCh (1 mM); noradrenaline: NA (100 μM); KCl: K+ (30 mM final); histamine: Hist (1 mM); 1-aminocyclopentane-trans-1,3-dicarboxylic acid: tACPD (300 μM); quisqualate: Quis (10 μM); 5-hydroxytryptamine: 5-HT (100 μM)) in the absence (■) or presence (□) of lithium (1 mM) for 20 min. The levels of the isomers Ins(1/3)P1 and Ins(4)P1 were measured as described in the Methods. Data represent the means ± s.e.m. for three experiments. Statistically significant differences between (■) and (□) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
The Effects of Different Agonists Upon Lithium Disruption of Phosphoinositide Signalling in Rat Cerebral Cortex Slices

[3H]Inositol labelled cerebral cortex slices were stimulated with a variety of agonists (Carbachol: CCh (1 mM); noradrenaline: NA (100 µM); KCl: K+ (30 mM final); histamine: Hist (1 mM); 1-aminocyclopentane-trans-1,3-dicarboxylic acid: tACPD (300 µM); quisqualate: Quis (10 µM); 5-hydroxytryptamine: 5-HT (100 µM)) in the absence (■) or presence (□) of lithium (1 mM) for 20 min. The levels of the isomers Ins(1,4,5)P3 and Ins(1,3,4,5)P4 were measured as described in the Methods. Data represent the means ± s.e.m. for three experiments.

Statistically significant differences between (■) and (□) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
although to varying degrees (14.8-, 1.8-, 6.5-, 1.6-, 9.1-, 2.4-fold over basal, respectively). The addition of lithium (1 mM) resulted in an enhancement in the agonist-stimulated levels of Ins(1/3)P$_1$ of 4.1-, 3.0-, 2.9-, 2.0-, 4.3-, 3.0-, 2.2-fold for CCh, NA, KCl, Hist, t-ACPD, Quis and 5-HT respectively, compared with the relative agonist-stimulated levels.

The addition of CCh, NA, KCl, Hist, t-ACPD, Quis and 5-HT all produced an increase in the levels of Ins(4)P$_1$, although again to varying degrees (3.7-, 1.9-, 3.1-, 1.2-, 1.2-, 3.0- and 1.3-fold over basal) compared with basal values (3329 ± 199 d.p.m./sample). The addition of lithium resulted in an enhancement in the accumulation of Ins(4)P$_1$ produced by these agonists resulting in a 8.8-, 7.0-, 4.6-, 2.8-, 6.8-, 4.7- and 4.1-fold enhancement for CCh, NA, KCl, Hist, t-ACPD, Quis and 5-HT respectively compared with the relative agonist-stimulated levels.

CCh, NA, KCl and Quis all produced an increase in the levels of Ins(1,4,5)P$_3$ (4.7-, 1.7-, 2.0- and 1.5-fold over basal) compared with basal values (1619 ± 215 d.p.m./sample). The addition of lithium (1 mM) resulted in a decrease in the levels of CCh and KCl-stimulated Ins(1,4,5)P$_3$ levels (36.5 % and 34.9 % reduction compared with the relevant agonist-stimulated values) and a small increase in histamine-stimulated Ins(1,4,5)P$_3$ levels (1.3-fold increase compared with agonist-stimulated values), with no effect being observed in the Ins(1,4,5)P$_3$ levels stimulated by NA or Quis.

All the agonists examined produced an increase in the levels of Ins(1,3,4,5)P$_4$ with CCh, NA, KCl, Hist, t-ACPD, Quis and 5-HT producing a 59.7-, 2.0-, 5.8-, 1.1-, 3.9-, 16.4- and 4.6-fold increase in the levels of Ins(1,3,4,5)P$_4$ compared with basal values (407 ± 43 d.p.m./sample). The addition of lithium (1 mM) resulted in a decrease in CCh, t-ACPD, Quis and 5-HT-stimulated Ins(1,3,4,5)P$_4$ levels (59.4 %, 61.6 %, 42.3 % and 83.0% decrease compared with the relative agonist-stimulated accumulation of Ins(1,3,4,5)P$_4$).
4.2.5. The Effects of Lithium and Inositol Upon CMP-PA Accumulation Stimulated by Different Agonists.

The effects of lithium and inositol on CMP-PA accumulation stimulated by carbachol (CCh; 1 mM), quisqualate (Quis; 10 µM), noradrenaline (NA; 100 µM), 5-hydroxytryptamine (5-HT 100 µM) and KCl (30 mM final) were examined in [3H]inositol-labelled rat cerebral cortex slices.

The addition of CCh, Quis, NA, 5-HT or KCl alone had no significant effect on basal CMP-PA levels (1738 ± 192 d.p.m./sample). However, addition of agonist and lithium (5 mM) produced an increase in the accumulation of CMP-PA in the presence of each of the individual agonists examined, with CCh, Quis, NA, 5-HT and KCl producing 7.9-, 3.6-, 2.9-, 2.0- and 4.3-fold increases over basal values in the levels of CMP-PA after a 20 min incubation.

Pretreatment of the cerebral cortex slices with 10 mM inositol for 1 h prior to agonist and lithium addition resulted in a decrease in the basal and stimulated CMP-PA levels. Basal CMP-PA levels in the presence of inositol were reduced by 75.7 % compared to basal levels in untreated slices. In the presence of inositol, CCh, Quis or KCl plus lithium (5 mM) only produced a 3.6-, 1.1- and 2.7-fold increase in the accumulation of CMP-PA over basal values (1738 ± 192 d.p.m./sample), whereas NA and 5-HT levels in the presence of lithium and inositol were 51.2 % and 49.6 % lower than basal values.

4.3.6. The Effect of Atropine Upon Carbachol-stimulated InsP3 and InsP4 Levels in Rat Cerebral Cortex Slices.

In order to determine whether specific activity changes occur in the labelling of the total InsP3 and InsP4 fractions in rat cerebral cortex slices during agonist-stimulation of phosphoinositide turnover, the effect of receptor blockade by atropine (10 µM) upon carbachol stimulated (in the absence and presence of lithium) InsP3 and InsP4 levels was examined (see discussion).

Carbachol alone produced a maximal 1.9-fold increase in InsP3 levels, compared with basal values (2060 ± 195 d.p.m./sample), by 5 min. These levels of InsP3 were maintained.
Figure 4.15. The Effects of Lithium and Inositol Upon CMP-PA Accumulation Stimulated by Different Agonists.

[3H]Cytidine labelled cerebral cortex slices were incubated with a variety of agonists (Carbachol: CCh (1 mM); quisqualate: Quis (10 μM); noradrenaline: NA (100 μM); 5-hydroxytryptamine: 5-HT (100 μM); KCl: K+ (30 mM final)) in the absence (■) or presence of lithium (□) (5 mM) for 20 min. The effects of preincubation of the slices with myo-inositol (10 mM) for 1 hr prior to agonist plus lithium addition (in the continued presence of myo-inositol was also examined (■). Data represent the mean ± s.e.m. for three experiments.
Figure 4.16. Atropine Reversal of Carbachol-Stimulated InsP₃ and InsP₄ Accumulation in The Absence and Presence of Lithium in Rat Cerebral Cortex Slices.

[³H]Inositol labelled cerebral cortex slices were incubated with carbachol (1 mM) for the times indicated in the absence (○) or presence (●) of lithium (1 mM). After 30 min, 10 μM atropine was added in the continued absence (△) or presence (▲) of lithium. Data represent the mean ± s.e.m. for three experiments. A. InsP₃ and B. InsP₄ fractions.
throughout the remainder of the time-course. Lithium (1 mM) had no significant effect upon
carbachol-stimulated InsP₃ levels over the time-course examined. Addition of atropine (10 μM)
at 30 min resulted in a rapid decrease in carbachol-stimulated InsP₃ levels both in the absence
and presence of lithium, with a reduction of 53.2 % and 28.1 % respectively.

Carbachol alone produced a maximal 4.5-fold increase in InsP₄ levels, compared with basal
values (894 ± 98 d.p.m./sample), by 5 min. These levels were maintained throughout the
remainder of the time-course. Lithium (1 mM) addition had no effect on initial carbachol-
stimulated levels of InsP₄ observed at 5 min, however lithium did produce a 47.3 % reduction
in carbachol-stimulated InsP₄ levels by 30 min which was maintained throughout the time-
course examined. Addition of atropine (10 μM) at 30 min resulted in a rapid decrease in
carbachol-stimulated InsP₄ levels both in the absence and presence of lithium, reducing InsP₄
levels in both instances to basal values (894 ± 98 d.p.m./sample).
4.3. Discussion.

4.3.1. Phosphoinositide Metabolism In Rat Cerebral Cortex Slices.

The metabolism of Ins(1,4,5)P$_3$ in cerebral cortex slices is complex and is known to proceed via at least two distinct routes resulting in the formation of several distinct inositol phosphate isomeric species (see Chapter 1). The reactions by which inositol phosphates are metabolized have been analyzed mainly in cell free systems (Shears et al., 1987; Storey et al., 1984; Inhorn et al., 1987), and there is relatively little detailed information about the formation and metabolism of inositol polyphosphates in cerebral cortex. A number of studies have examined phosphoinositide metabolism in rat cerebral cortex slices however the majority have restricted the examination to measuring the total mono-, bis-, tris- and tetralcisphosphate fractions (Batty and Nahorski, 1985, 1989; Rooney & Nahorski, 1986; 1989) and have not attempted to resolve these fractions into their individual isomeric species. Only a few studies have endeavoured to examine the composition of the individual isomers formed upon agonist-stimulation of this preparation (Batty et al., 1989; Batty & Nahorski, 1992). Similarly, studies examining the effects of lithium on phosphoinositide metabolism in cerebral cortex have, on the whole, concerned themselves with examining the effects of this ion on the InsP$_{1,4}$ fractions (Whitworth & Kendall, 1988; Kennedy et al., 1989; Batty & Nahorski, 1989). The work described in this section, therefore, has attempted a fuller characterization of phosphoinositide metabolism in rat cerebral cortex slices and examined the disruptive effects which lithium has upon this system.

The accumulation of Ins(1,4,5)P$_3$ in this preparation in response to carbachol was similar to that observed in previous studies (Batty & Nahorski, 1985; Kennedy et al., 1989). The metabolism of Ins(1,4,5)P$_3$ to Ins(1,4)P$_2$ was extremely rapid, with the time-course of accumulation for Ins(1,4)P$_2$ closely following that of Ins(1,4,5)P$_3$. Ins(1,4)P$_2$ has been shown in a number of preparations to be dephosphorylated solely via a 1-phosphatase (the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase) forming Ins(4)P$_1$. Indeed, work in broken-cell preparations from cerebral cortex has demonstrated that [P$^3$H]Ins(1,4)P$_2$ is metabolized to form
only Ins(4)P₁ (Batty et al., 1989). However, more recent work by Batty & Nahorski (1992) in cerebral cortex slices has prompted the suggestion that Ins(1,4)P₂ may be also metabolized via a 4-phosphatase giving rise to Ins(1)P₁. In that study the effect of atropine addition upon carbachol-stimulated inositol phosphate isomer accumulation in the presence of lithium was examined. From analysis of the relative proportions of the individual inositol phosphate isomers produced after atropine addition they concluded that Ins(1,4)P₂ was metabolised to both Ins(1)P₁ and Ins(4)P₁ in this tissue. These studies therefore may call into doubt the validity of examining inositol phosphate metabolism in cell-free preparations.

Ins(1,4,5)P₃ has also been shown to be phosphorylated by a 3-kinase giving rise to the formation of the putative second messenger Ins(1,3,4,5)P₄ (Irvine et al., 1986). Although this route of metabolism is not present in all tissues which have been examined (Van Haastert et al., 1989; Harrison et al., 1990; Wood et al., 1989), such an activity has clearly been demonstrated in cerebral cortex (Batty et al., 1989; Batty & Nahorski, 1992). This present study confirms these results with a rapid accumulation in the levels of Ins(1,3,4,5)P₄ being observed in the presence of agonist.

The major route of metabolism of Ins(1,4,5)P₃ in this preparation appears to be via 3-kinase phosphorylation of this isomer, resulting in the formation of Ins(1,3,4,5)P₄. This can be deduced by examining the ratio of Ins(1/3)P₁ : Ins(4)P₁, which in the presence of lithium should reflect the relative proportion of Ins(1,4,5)P₃ metabolized via the 3-kinase and 5-phosphatase respectively (assuming that PtdIns(4,5)P₂ is the sole substrate for PI-PLC and that Ins(1,4)P₂ is not dephosphorylated to Ins(1)P₁). These data are therefore in agreement with results previously obtained in this tissue (Batty & Nahorski, 1992).

A number of studies have suggested that the activity of the 3-kinase involved in the formation of Ins(1,3,4,5)P₄ from Ins(1,4,5)P₃ can be influenced by changes in the intracellular Ca²⁺ concentration. Early work by Biden et al. (1987) demonstrated that an increase in [Ca²⁺]ᵢ resulted in a marked increase in 3-kinase activity and that this increased activity was due an
increase in $V_{\text{max}}$ rather than $K_{\text{m}}$ (Biden & Wollheim, 1986). These findings therefore lead to the possibility that the rise in [Ca$^{2+}$] produced by Ins(1,4,5)P$_3$ may actually direct the metabolism of this isomer away from Ins(1,4)P$_2$ and towards Ins(1,3,4,5)P$_4$.

Similarly, it has also been shown by a number of groups that PKC can affect the activity of the 3-kinase (see Chapter 5 for fuller discussion). Imboden & Pattison (1987) provided the first evidence for such a role for PKC. In a malignant human T-cell line they demonstrated that phorbol ester treatment resulted in persistent activation of the partly purified 3-kinase. However, with the discovery that the 3-kinase was calpain sensitive it was necessary to undertake these experiments in the presence of calpain inhibitors, since during the preparation of cell-free extracts Ins(1,4,5)P$_3$ 3-kinase may be partially degraded to forms with altered catalytic and regulatory properties. Under such conditions Sim et al. (1990) found that phosphorylation of the 3-kinase by PKC resulted in a decrease in the $V_{\text{max}}$ of this enzyme.

These results therefore suggest that the metabolism of Ins(1,4,5)P$_3$ is under a degree of control in vivo.

Ins(1,3,4,5)P$_4$ is metabolized by the Ins(1,3,4,5)P$_4$/Ins(1,4,5)P$_3$ 5-phosphatase (Connolly et al., 1987) to Ins(1,3,4)P$_3$, which is in turn dephosphorylated by either a 1-phosphatase (the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase) (Inhorn & Majerus, 1987; Gee et al., 1988b) or a 4-phosphatase activity (Bansal et al., 1987). From a number of studies in various preparations including permeabilized adrenal glomerulosa cells (Balla et al., 1988), liver homogenates (Shears et al., 1987) and brain extract (Inhorn et al., 1987) it appears that the major route of metabolism of Ins(1,3,4)P$_3$ is to Ins(3,4)P$_2$. However, in this present study Ins(1,3)P$_2$ accumulation predominated upon agonist-stimulation, as has previously been demonstrated in bovine brain homogenates (Inhorn & Majerus, 1988).

The results obtain for the effects of lithium upon phosphoinositide metabolism in rat cerebral cortex in this study are comparable to those obtained previously in this preparation (Kennedy et al., 1989, 1990; Batty & Nahorski, 1989, 1992). For example, in relation to the effects of lithium upon the carbachol-stimulated accumulation of the inositol monophosphates Ins(1/3)P$_1$
and Ins(4)P$_1$ both the linear profile of accumulation of these isomers and the EC$_{50}$ for the effects of lithium were similar to results previously described for the total InsP$_1$ fraction from cerebral cortex slices (Kennedy et al., 1989; Rooney & Nahorski, 1989). The EC$_{50}$ for the accumulation of these isomers was also similar to the K$_i$ for lithium for the purified monophosphatase enzyme (K$_i$ values of 1.0 mM and 0.26 mM for Ins(1)P$_1$ and Ins(4)P$_1$, respectively; Gee et al., 1988a).

Similarly, lithium produced a time and concentration-dependent decrease in the levels of $[^{3}H]$Ins(1,4,5)P$_3$ and $[^{3}H]$Ins(1,3,4,5)P$_4$ which, at least for $[^{3}H]$Ins(1,3,4,5)P$_4$, was comparable with the previously observed effects of this ion upon the mass levels of this isomer (Kennedy et al., 1990). The inhibitory effects of lithium upon the agonist-stimulated accumulation of the inositol polyphosphates Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ were shown to have a similar IC$_{50}$ value to that observed for the accumulation of the inositol monophosphates. It has been proposed that these two effects of lithium are linked, with the inhibition of the inositol monophosphatase resulting in an increase in the levels of the monophosphates and, after a subsequent lag period due to the time required to cause depletion of intracellular inositol/inositol phospholipid reserves, a decrease in the levels of the inositol polyphosphates (Kennedy et al., 1989, 1990).

Lithium has been shown to produce a time-dependent inhibition of the carbachol-stimulated InsP$_4$ response, however, maximally effective concentrations of lithium result in only a 50-60 % inhibition of this agonist-stimulated InsP$_4$ accumulation (Whitworth & Kendall, 1988; Batty & Nahorski, 1989; Kennedy et al., 1989, 1990; Rooney & Nahorski, 1989). It has been unclear whether the inability of lithium to return carbachol-stimulated InsP$_4$ levels to basal values was a real effect or was merely due to a change in the specific activity of $[^{3}H]$InsP$_4$ radiolabelling in this tissue. Batty & Nahorski (1989) have previously demonstrated that upon agonist-stimulation of phosphoinositide metabolism in rat cerebral cortex slices the specific activity of $[^{3}H]$InsP$_3$ increased, resulting in an underestimation of the inhibitory effect of
lithium upon Ins(1,4,5)P₃ accumulation. Indeed with the development of Ins(1,4,5)P₃ mass assays it has been clearly demonstrated that in rat cerebral cortex lithium can completely inhibit carbachol-stimulated Ins(1,4,5)P₃ accumulation in a concentration-dependent manner (Jenkinson et al., 1993; also see chapter 3). This should be compared with the effects of lithium upon [³H]inositol labelled InsP₃ were maximally effective concentrations of lithium only result in a 40-50 % decrease in the levels of [³H]Ins(1,4,5)P₃ (see above).

The antagonist-reversal approach, originally used by Batty and Nahorski (1989), was employed in this study to examine whether specific activity changes occurred in [³H]InsP₄ labelling. This approach relies on an antagonist "blocking" the receptor and in turn "switching-off" agonist-sensitive phosphoinositide metabolism. Levels of the inositol phosphates formed during agonist-stimulation should then return to basal values as they are rapidly metabolised, unless a change in the specific activity of the metabolite has occurred.

Addition of atropine resulted in a decrease in the levels of agonist and agonist plus lithium-stimulated [³H]InsP₃ levels, however, levels in both instances did not return to basal values (53.2 % and 28.1 % reduction compared with carbachol-stimulated values, respectively). This failure of atropine addition to return InsP₃ levels to basal values demonstrated that a change in the specific activity of the radiolabelling of this inositol phosphate had occurred, in agreement with previous studies (Batty & Nahorski, 1989). In the same experiment the effect of atropine addition upon InsP₄ levels was also examined. Atropine addition resulted in a decrease in the agonist and agonist plus lithium-stimulated InsP₄ accumulation, however unlike InsP₃, InsP₄ levels in both instances approached basal values demonstrating that no change in the specific activity of InsP₄ radiolabelling had occur in this preparation. These results therefore demonstrate that there is indeed a lithium-insensitive component of the carbachol-stimulated InsP₄ response. The change in specific activity of the InsP₃ fraction, but not InsP₄, may be related to the relative sizes of the pools of both isomers, with the InsP₄ pool being much smaller and therefore possibly less susceptible to such changes.
The question then arises as to where this insensitive InsP₄ pool is derived from and indeed why it is insensitive to the actions of lithium. Since it has been shown from Ins(1,4,5)P₃ mass data that lithium can completely inhibit the accumulation of Ins(1,4,5)P₃ produced by agonist, the direct precursor for Ins(1,3,4,5)P₄, it is difficult to reconcile why Ins(1,3,4,5)P₄ levels should be resistant to higher concentrations of lithium (~ 10 mM). One possibility, however, may be that the Ins(1,3,4,5)P₄ fraction collected may be contaminated with another InsP₄ isomer which accumulates upon agonist addition but which is not significantly affected by lithium addition. One possible candidate could possibly be Ins(3,4,5,6)P₄. This isomer has been demonstrated to accumulate upon agonist-stimulation in f-met-leu-phe-stimulated (FMLP) HL60 cells (Pittet et al., 1989) and in bombesin-stimulated AR4-2J cells (Menniti, 1990). The effects of lithium upon this isomer are unclear, however the lithium-insensitive component could possibly be due to accumulation of this isomer.

As mentioned in section 4.1 lithium is now known to inhibit two of the enzymes in this system, the monophosphatase (Hallicher & Sherman, 1980; Gee et al., 1988a) and the Ins(1,4)P₂/Ins(1,3,4)P₃ 1-phosphatase (Inhorn et al., 1987; Gee et al., 1988b) albeit at different concentrations. At a concentration of 1 mM, which is utilized in this series of experiments, lithium should markedly inhibit the inositol monophosphatase however only partially inhibit, if at all, the Ins(1,4)P₂/Ins(1,3,4)P₃ 1-phosphatase (see Gee et al., 1988b). Indeed, examination of the concentration-response curves depicted in figures 4.6, 4.7 and 4.9 demonstrated that at 1 mM the monophosphatase was completely inhibited, whereas the Ins(1,4)P₂/Ins(1,3,4)P₃ 1-phosphatase was apparently not significantly affected since the agonist-stimulated levels of the substrates for this enzyme, Ins(1,4)P₂ and Ins(1,3,4)P₃, were not enhanced by this concentration of lithium albeit at the single time point examined (30 min.). However, considering the time-course data presented in figure 4.4 it is likely that a concentration-response curve for lithium at 5 min would produce a different curve from that shown in figure 4.9, with an increase in the accumulation of Ins(1,3,4)P₃ occurring with 1 mM lithium. Indeed, it is clear that lithium enhances the accumulation of Ins(1,4)P₂ and
Ins(1,3,4)P$_3$ at the 10 min and 5 min time-points, respectively. Similarly, lithium also
enhances the accumulation of agonist-stimulated levels of Ins(1,3)P$_2$ at the 10 min time-point.
This latter finding is entirely unexpected since the metabolism of Ins(1,3)P$_2$ is via a lithium
insensitive enzyme. Previous studies examining phosphoinositide metabolism in rat cerebral
cortex have also demonstrated such an effect of lithium, however, only on the total InsP$_2$
fraction (Kennedy et al., 1988; Jenkinson et al., 1993; see chapter 3).

The mechanism underlying this enhanced accumulation in agonist-stimulated levels of these
three isomers is unclear. One possibility might be that lithium may actually enhance the activity
of the 5-phosphatase involved in the dephosphorylation of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$.
An enhancement in the activity of this enzyme would result in an increase in the formation of
Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ which may only be short lived due to the decrease in the levels of
Ins(1,4,5)P$_3$ produced by the blockade of inositol recycling by lithium, this would result in the
short lived (5-10 min) enhancement of agonist-stimulated Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ levels
(and in turn Ins(1,3)P$_2$) observed. Connolly et al. (1986) have reported that the Ins(1,4,5)P$_3$/
Ins(1,3,4,5)P$_4$ 5-phosphatase from platelets can be phosphorylated by PKC in the presence of
phosphatidylycerine, Ca$^{2+}$ and DAG, resulting in an increase in the activity of this enzyme of
approximately 4-fold. This enhanced activity was found to be due to an increase in the $V_{\text{max}}$ of
Ins(1,4,5)P$_3$ dephosphorylation and was not due to an increase in the affinity of the enzyme
for Ins(1,4,5)P$_3$. These findings in platelets have since been confirmed by a number of groups
(Molina y Vedia & Lapetina, 1986; King & Rittenhouse, 1989). However, these results have
proved controversial with other groups finding that Ins(1,4,5)Py/Ins(1,3,4,5)P$_4$ 5-phosphatase
activity in other preparations, either soluble or particulate, was not significantly affected by
phorbol esters. Orellana et al. (1985) found that Ins(1,4,5)P$_3$ metabolism was unaffected in a
1321N1 astrocytoma cell membrane preparation after treatment with PMA. A similar result was
also obtained in PMA treated RINm5F cells (Biden et al., 1988). It therefore appears that this
effect of PKC upon the activity of the 5-phosphatase may be dependent upon cell type. If PKC
does enhance the activity of the Ins(1,4,5)P$_3$/Ins(1,3,4,5)P$_4$ 5-phosphatase in cerebral cortex
then it may be possible that lithium might enhance PKC activity indirectly by increasing the
agonist-stimulated levels of DAG. Indeed, it has been shown in a number of preparations that
lithium addition can result in an increase in agonist-stimulated DAG levels (Drummond &
Raeburn, 1984; Bocckino et al., 1985; Brami et al., 1991, 1993), although generally at higher
concentrations of lithium than those used in the present study.

Yet another unexpected observation was the lithium-sensitive accumulation of the inositol
bisphosphate isomer Ins(4,5)P$_2$ (for a fuller discussion see chapter 5). Although the route of
metabolism of this isomer is not clear it has been shown that it is a poor substrate for the
lithium-sensitive Ins(1,4,5)P$_3$/Ins(1,3,4,5)P$_4$ 5-phosphatase (Mitchell et al., 1989), and it is
therefore likely that its metabolism proceeds via either a distinct 5-phosphatase activity or
indeed a 4-phosphatase activity. In either case these data strongly suggest the presence of a
novel lithium-sensitive enzyme in the metabolism of the inositol phosphates. Indeed, a lithium-
sensitive accumulation of this isomer has previously been reported in TRH-stimulated GH$_3$
cells (Hughes & Drummond, 1987); the enhanced accumulation of this isomer produced by
lithium in these cells was in the order of 6-7-fold over agonist-stimulated values (compared
with 10-11-fold in cerebral cortex).

It appears therefore that the disruption of phosphoinositide metabolism by lithium in cerebral
cortex slices may not occur solely as a result of an inhibition of the monophosphatase but may
also involve more subtle effects on other enzymes involved in the metabolism of Ins(1,4,5)P$_3$.
However, the complexity of brain slices, in respect to both cellular and receptor subtype
composition, make interpretation of data obtained difficult. It is therefore important to find a
simpler model system in which a more detailed analysis of the action(s) of lithium upon
phosphoinositide metabolism can be undertaken. Such a model system is examined in
chapters 6 and 7.
4.3.2. The Effect of Carbachol Concentration Upon Lithium Disruption of Phosphoinositide Signalling in Rat Cerebral Cortex Slices.

The work presented thus far has examined the effects of lithium on phosphoinositide metabolism in rat cerebral cortex, however at concentrations of agonist that are far in excess of those likely to occur in vivo. Therefore, in this section the effects of lithium on phosphoinositide metabolism in the presence of lower concentrations of agonist have been examined to determine whether lithium could have a significant effect upon this system at physiological agonist concentrations.

As mentioned in chapter 1, lithium is an uncompetitive inhibitor of the inositol monophosphatase (Hallcher & Sherman, 1980; Gee et al., 1988). This unusual type of inhibition has a number of significant consequences. Since lithium only binds to and inhibits the substrate or product bound form of the enzyme the inhibition produced is enhanced by increasing substrate concentrations, therefore the inhibition is self-perpetuating (Gani et al., 1993). Indeed, it has been suggested by Berridge et al. (1989) that lithium may selectively target certain cortical neurons, in patients with manic depression, possibly because phosphoinositide metabolism in these neurons is hyperactive and with the increase in the flux through the cycle the substrate for the monophosphatase would be increased, therefore selectively enhancing the inhibition of inositol monophosphate metabolism in these cells. However, this is only a hypothesis suggesting what may occur in vivo, translated from in vitro experiments on purified enzyme preparations (see Gani et al., 1993).

This study has examined the effects of lithium on inositol (poly)phosphate accumulation stimulated by increasing concentrations of carbachol. As the concentration of carbachol was successively increased there was a concomitant increase in the production of Ins(1,4,5)P₃, as shown in figure 4.12. Ins(1,4,5)P₃ was in turn sequentially dephosphorylated to form the inositol monophosphates Ins(1/3)P₁ and Ins(4)P₁. Therefore, as mentioned above, as the production of Ins(1,4,5)P₃ increased so too did the levels of the inositol monophosphates, as shown in figure 4.11. If the inhibition of the monophosphatase increases as substrate...
concentrations increase then it would be expected that at higher concentrations of carbachol the
effect of lithium upon the accumulation of carbachol-stimulated inositol monophosphates
would be greater than that observed at lower concentrations of agonist. An increase in the
accumulation of the monophosphates over and above that observed in the presence of carbachol
alone would also be expected. However, even if the inhibition was a simple non-competitive
inhibition, since there is a greater production of the inositol monophosphates observed at
higher carbachol concentrations this would naturally lead to an enhancement in the
accumulation of these inositol phosphate isomers in the presence of an inhibitor compared to in
its absence. It is therefore important not to consider the absolute increase in the accumulation of
the inositol monophosphates but to examine the relative percentage or fold increase in the
presence of inhibitor compared to the absence of inhibitor at low and high carbachol
concentrations to determine whether the accumulation of the inositol monophosphates is greater
at higher carbachol concentrations due to an increase in the inhibitory effect of lithium upon the
monophosphatase, rather than merely an increase in the production of the inositol
monophosphates.

Examination of the data shown in figure 4.11 reveals that for Ins(1/3)P₁ accumulation in the
presence of low (0.03 mM) and high (1 mM) concentrations of agonist the fold increase in the
accumulation of this isomer in the presence and absence of lithium at both concentrations was
similar (6.6-fold and 5.2-fold at 0.03 mM and 1.0 mM, respectively). Similarly, for Ins(4)P₁
at low and high carbachol concentrations there was no significant difference in the fold
accumulations in the absence and presence of lithium (16.0-fold and 12.2-fold at 0.03 mM and
1.0 mM, respectively). These data therefore do not demonstrate that the inhibition of the
monophosphatase, produced by lithium, is enhanced by an increase in the substrate
concentration. One possible reason for the failure to observe such an effect may be that the
concentration range of substrate, i.e. Ins(1/3)P₁ and Ins(4)P₁, which are present in the cell
even at low carbachol concentrations may be enough to produce a maximal inhibitory effect of
the monophosphatase by 1 mM lithium, such that further increases in the substrate
concentration has no significant effect upon the inhibition produced.

Figure 4.12 demonstrates the inhibitory effects of lithium (1 mM) on carbachol-stimulated (0.03 - 3.0 mM) levels of Ins(1,4,5)P3 and Ins(1,3,4,5)P4. At the lower concentrations of carbachol (~ 0.1 mM) lithium has no significant effect on the accumulation of either of these inositol polyphosphates. However, at carbachol concentrations greater than this lithium significantly reduces the agonist-stimulated accumulation of Ins(1,3,4,5)P4. Ins(1,4,5)P3 levels do not appear to be as sensitive to the effects of lithium, and the accumulation of this inositol polyphosphate isomer is only affected by lithium when carbachol concentrations are greater than 0.1 mM. The Ins(1,4,5)P3 data are complicated however due to the large specific activity changes in the radiolabelling of [3H]Ins(1,4,5)P3 which are known to occur (Batty & Nahorski, 1989; also see above). These data therefore demonstrates that the effect of lithium upon inositol polyphosphate accumulation occurs in the presence of sub-maximal concentrations of agonist, in line with physiological concentrations, and does not require supramaximal stimulation of phosphoinositide metabolism.

Although in this study no effect of lithium was observed upon Ins(1,3,4,5)P4 levels stimulated by low carbachol concentration this does not necessarily mean that lithium has no effect on Ins(1,3,4,5)P4 levels produced at low concentrations of agonist. Since only one time point has been examined in this study it may be possible that lithium does have a significant effect upon Ins(1,3,4,5)P4 accumulation at low carbachol concentrations, however more time may be required for these effects of lithium to become apparent. Indeed, as lithium produces a significant increase in the levels of the inositol monophosphates stimulated by low concentrations of carbachol, it is possible that more time may be required in order to deplete the inositol reserves and inositol phospholipid pools within the susceptible cells when the slices are being stimulated submaximally. Although there is an initial accumulation of inositol monophosphates it is possible that the lag period observed for the inhibition of the accumulation of Ins(1,4,5)P3 and Ins(1,3,4,5)P3 may be greater than that observed for higher concentrations of agonist.
In summary, lithium can significantly disrupt phosphoinositide metabolism stimulated with concentrations of agonist closer to the physiological range.

4.3.3. Effects of Lithium Upon Phosphoinositide Metabolism Stimulated With Different Agonists.

The ability of lithium to disrupt phosphoinositide metabolism stimulated by a number of different agonists was examined to determine whether lithium could selectively, or more potently, disrupt this signalling pathway when stimulated by a certain agonist. A similar study by Whitworth and Kendall (1988), which also examined the effects of lithium upon phosphoinositide metabolism stimulated by a variety of agonists, found that lithium selectively inhibited the carbachol-stimulated accumulation of Ins(1,3,4,5)P₄, however had little or no effect upon the accumulation of this isomer by any other agonist. These data were taken to suggest that lithium selectively disrupts muscarinic receptor-stimulated phosphoinositide metabolism in mouse cerebral cortex. However, another possible reason for this apparent selectivity of lithium could be due to the ability of each of the agonists examined to stimulate phosphoinositide metabolism to markedly different extents (see above). Indeed, it is clear from the data shown in figure 4.13 that by examining the accumulation of inositol monophosphates produced by the stimulation of phosphoinositide metabolism by a number of agonists that carbachol clearly produces the largest accumulation of these isomers, demonstrating that this agonist produces the largest stimulation of this signalling pathway compared to the other agonists examined, similar to previously reported results in this tissue (Whitworth & Kendall, 1988; Heacock et al., 1993). The data could therefore be explained by the arguments presented in section 4.3.2, in that the agonists which produce the greatest flux through the cycle will be affected to a greater degree by lithium, since this ion will more profoundly inhibit the monophosphatase in the presence of the higher concentration of substrate. Alternatively, there may not be an effect of lithium at one time-point, however, with a long enough stimulation, even with a weak agonist such as histamine, it may be possible to observe an effect of lithium, after sufficient time has passed for the levels of inositol and inositol phospholipids to be
The data in this present study are not in agreement with the selective effects of lithium on agonist-stimulated Ins(1,3,4,5)P$_4$ levels that were described by Whitworth & Kendall (1988). The effects of lithium do not appear to be dependent upon the agonist examined, with lithium producing a decrease in the Ins(1,3,4,5)P$_4$ levels stimulated by carbachol, t-ACPD, quisqualate and 5-hydroxytryptamine.

Similar to the results obtained in section 4.3.2, the inhibition of the inositol monophosphatase by lithium did not appear to increase with an increase in the flux of inositol phosphates passing through the system (as measured by fold / percentage increase in the accumulation of the inositol monophosphates in the presence of lithium compared with the absence for each agonist).

The effects of a number of different agonists (carbachol, quisqualate, noradrenaline, 5-hydroxytryptamine and KCl) upon the lithium-induced accumulations in CMP-PA were also examined. None of the agonists examined produced an increase in the accumulation of CMP-PA in the absence of lithium. However, in the presence of this ion all produced a significant increase in the levels CMP-PA. As would be expected, the relative accumulations of CMP-PA produced by each agonist were proportionately similar to the accumulation of the sum of the inositol monophosphates produced by each agonist, since the inhibition of the monophosphatase by lithium has a direct effect on both these intermediates in the cycle. These data are in disagreement with similar studies presented in a recent report by Heacock et al. (1993). In that study it was found that there was a nonequivalence in the accumulation of the total inositol phosphate fraction and CMP-PA produced by a variety of agonists in rat cerebral cortex slices and other brain regions. Although it found that carbachol produced a similar fold increase over basal in the accumulation of both the inositol phosphates and CMP-PA, quisqualate was found to produce a significantly larger increase in the accumulation of the inositol phosphates compared to that observed for CMP-PA. This study (Heacock et al., 1993) therefore concluded that the use of CMP-PA measurements as a measure of phosphoinositide metabolism was not as valid as was initially suggested (Godfrey, 1989). However, the data...
obtained from this present study suggest that the accumulation of the inositol phosphates and CMP-PA are equivalent at least in cerebral cortex and that this is true for a variety of different agonists.

In summary, it has been shown that lithium can profoundly disrupt phosphoinositide metabolism in rat cerebral cortex slices. This disruptive effect is not dependent upon the agonist under examination and is significant at physiologically relevant concentrations of agonist. The major disruptive effects of lithium appear to be due to an inhibition of the inositol monophosphatase, however, more subtle effects upon the accumulation of the inositol bisphosphate isomer Ins(4,5)P$_2$ were also observed. The following chapter examines in more detail the effects which lithium has upon this isomer.
Chapter 5

Evidence for lithium-sensitive
inositol 4,5-bisphosphate accumulation in
cholinoceptor-stimulated cerebral-cortex slices
5.1 Introduction

Phosphodiesteric cleavage of PtdInsP₂ by PLC produces Ins(1,4,5)P₃ and DAG which release Ca²⁺ from intracellular stores and activate PKC respectively (Nahorski, 1988; Downes & MacPhee, 1990; Shears, 1991; Fisher et al., 1992). It is now known that the metabolism of Ins(1,4,5)P₃ is rapid and can follow two possible pathways (see Shears, 1991). The simple dephosphorylation route proceeds by a 5-phosphatase, to Ins(1,4)P₂, and

Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase to Ins(4)P₁. Alternatively, ATP-dependent phosphorylation of Ins(1,4,5)P₃ by 3-kinase generates the putative second messenger

Ins(1,3,4,5)P₄ which then undergoes sequential dephosphorylation via a 5-phosphatase, to

Ins(1,3,4)P₂, and either a 4-phosphatase or an Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase route to produce Ins(1,3)P₂ and Ins(3,4)P₂ respectively. Subsequent dephosphorylation produces Ins(1)P₁ and Ins(3)P₁, which along with Ins(4)P₁ are metabolized by the inositol monophosphatase enzyme to release myo-inositol (Shears, 1991).

As discussed earlier, two of the enzymes involved in this complex metabolism have been shown to be lithium sensitive, namely the inositol monophosphatase and the

Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase (Halleher & Sherman, 1980; Gee et al., 1988a, 1988b). Both enzymes are uncompetitively inhibited by lithium however the inhibition of the inositol monophosphatase is more potent (Kᵢ value of 1.0 mM and 0.26 mM for Ins(1)P₁ and Ins(4)P₁, respectively) (Halleher & Sherman, 1980; Gee et al., 1988a) compared to that for the

Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase (Kᵢ value of 9.63 mM and 0.46 mM for Ins(1,4)P₂ and Ins(1,3,4)P₃, respectively) (Gee et al., 1988b).

Whilst attempting to analyze more fully the inositol phosphate metabolite profile in lithium-treated cerebral cortex slices (see Chapter 4), the accumulation of an unknown labelled bisphosphate which had previously been reported in this tissue as a very minor product after muscarinic-receptor stimulation and had been tentatively identified as Ins(4,5)P₂ (Batty et al.,
1989) was observed. Agonist-stimulated accumulation of this inositol bisphosphate was dramatically enhanced by the addition of therapeutically relevant concentrations of lithium. The accumulation of this metabolite suggests the existence of an additional lithium-sensitive enzyme involved in the metabolism of this bisphosphate (Ins(4,5)P₂) and adds greater complexity to the present scheme of inositol phosphate metabolism in cerebral tissue with the possibility that this inositol phosphate isomer may represent a metabolite in a third alternative route of metabolism for Ins(1,4,5)P₃.

5.2 Methods.

5.2.1 Ins(1,4,5)P₃ and Ins(4,5)P₂ metabolism in rat cortical homogenates and permeabilised CHO-m1 cells.

The metabolism of [³H]Ins(1,4,5)P₃ and Ins(4,5)P₂ were investigated in rat cortical homogenates and in electrically permeabilised CHO-m1 cells.

The cerebral cortex from a male Wistar rat was dissected on ice, as described previously (2.1), and was then homogenized (3 x 15 sec bursts with a Polytron tissue homogenizer) in 10 ml of ICB (see Appendix I for composition). The resulting homogenate was diluted 5-fold with buffer and was stored on ice for use that day. CHO-m1 cells were harvested, as described previously (2.14), and following permeabilization (see 2.25) were resuspended at a concentration of 3-4 mg/ml and were allowed to recover for 20 min at 20°C.

100 μl aliquots of cortical homogenate or cells in suspension were added to 100 μl of buffer containing either 5 μM Ins(1,4,5)P₃ and 50,000 d.p.m. of [³H]Ins(1,4,5)P₃ or 1 μM Ins(4,5)P₂ and 50,000 d.p.m. of [³H]Ins(4,5)P₂ (prepared from [³H]Ins(1,4,5)P₃ as described in Chapter 2) in the absence or presence of either lithium (1 mM) or L-chiro-Ins(1,4,6)PS₃ (100 μM). The reactions were terminated by addition of 200 μl of PCA after the indicated period of time (see text). Samples were neutralized as described in section 2.18; the [³H]inositol phosphates formed were separated by h.p.l.c. (section 2.7) and counted.
5.3 Results.

5.3.1 Effect of lithium on inositol bisphosphate isomer accumulation.

In agreement with previous studies in carbachol-stimulated cerebral-cortex slices (Batty et al., 1989), four labelled bisphosphate products could be separated by h.p.l.c. (Fig. 5.1). Identification of three of the metabolites as Ins(1,3)P2, Ins(1,4)P2 and Ins(3,4)P2 was previously achieved by co-elution of standards and analysis of appropriate alditols (Batty et al., 1989). The late-running minor peak had been tentatively identified as Ins(4,5)P2 (Batty et al., 1989), and in these present experiments support for this identification came from an identical elution of the alkaline phosphatase product of authentic [3H]Ins(1,4,5)P3 (Fig. 5.1) and analysis of the alditol products of this unknown isomer (see later). The accumulation of each of the inositol bisphosphate products (Ins(1,3)P2, Ins(1,4)P2, Ins(3,4)P2 and Ins(4,5)P2) was increased by lithium upon muscarinic-receptor stimulation (47-, 4-, 12-, and 23-fold respectively after 30 min), albeit from very different carbachol-stimulated levels (carbachol stimulated values at 30 min: Ins(1,3)P2, 4107 ± 785 d.p.m.; Ins(1,4)P2, 11047 ± 1019 d.p.m.; Ins(3,4)P2, 2098 ± 392 d.p.m.; Ins(4,5)P2, 558 ± 96 d.p.m.) (Fig. 5.2).

Lithium (10 μM - 10 mM) had either no effect, or a relatively weak effect, on Ins(1,3)P2, Ins(1,4)P2 and Ins(3,4)P2 accumulations. However, this univalent ion produced an 11-fold increase in carbachol-stimulated Ins(4,5)P2 accumulation, and this enhancement was observed at low concentrations of lithium (EC50 = 94 ± 3 μM).

The results in Fig. 5.3 demonstrate the time-course of accumulation of inositol bisphosphate isomers in carbachol-stimulated (1 mM) cerebral-cortex slices in the presence and absence of lithium (1 mM). Carbachol-stimulated Ins(1,3)P2, Ins(1,4)P2 and Ins(4,5)P2 accumulation in the absence of lithium was rapid, reaching a new steady state by 5-10 min. Although Ins(3,4)P2 appeared to increase throughout the 30 min incubation, its relatively low accumulation did not suggest a clear kinetic difference from the other bisphosphate isomers.

In the presence of lithium (1 mM) a significant enhancement of carbachol-stimulated
Figure 5.1  H.p.l.c. separation of [³H]inositol bisphosphates.

H.P.L.C. separation of the [³H]inositol bisphosphates present in an extract from carbachol-stimulated (1 mM) cerebral-cortex slices in the absence (0) and presence (O) of 1 mM lithium (30 min incubation). The dotted line demonstrates the co-elution of [³H]Ins(4,5)P₂ prepared by alkaline phosphatase treatment of [³H]Ins(1,4,5)P₃. Positions of other bisphosphate isomer peaks are indicated.
Figure 5.2 Effect of lithium on carbachol-stimulated inositol bisphosphate isomer accumulation in cerebral-cortex slices.

[3H]inositol bisphosphate isomer accumulation in carbachol-stimulated (1 mM) rat cerebral-cortex slices in the presence of increasing concentrations of lithium (0.01-10 mM). All incubations were for 30 min. Values are means ± s.e.m. of data normalized from three separate experiments. Carbachol-stimulated values were (in d.p.m.) as follows: Ins(1,3)P2, 4107 ± 785; Ins(1,4)P2, 11047 ± 1019; Ins(3,4)P2, 2098 ± 392; Ins(4,5)P2, 558 ± 96.
Figure 5.3  Time-courses of carbachol-stimulated inositol bisphosphate isomer accumulation in cerebral-cortex slices.

[3H]Inositol bisphosphate accumulation in carbachol-stimulated (1 mM) rat cerebral-cortex slices in the absence (○) and presence (●) of lithium (1 mM). Values are means ± s.e.m. of data normalized from three separate experiments. Basal values of [3H]inositol bisphosphates were (in d.p.m.) as follows:

Ins(1,3)P2, 64 ± 11; Ins(1,4)P2, 2428 ± 85; Ins(3,4)P2, 104 ± 27; Ins(4,5)P2, 51 ± 8.
Ins(1,3)P$_2$ at 5 and 10 min and in Ins(1,4)P$_2$ accumulation at 10 min was observed. However, at the 20 and 30 min time points the accumulations of these bisphosphates were not significantly different in the absence and presence of lithium. Ins(3,4)P$_2$ accumulation was not influenced by this ion at any of the incubation times investigated. However, lithium produced a dramatic enhancement in Ins(4,5)P$_2$ accumulation at all time points studied, such that at 30 min Ins(4,5)P$_2$ made up 34.0 ± 3.6 % of the total inositol bisphosphate fraction. Ins(4,5)P$_2$ accumulation appeared to be linear with time in the presence of lithium, indicative of an inhibition of its metabolism.

5.3.2 Kinetic analysis of inositol phosphate metabolism.

Attempts to detect metabolism of Ins(4,5)P by cerebral-cortical homogenates were not successful. Negligible loss of [3H]Ins(4,5)P$_2$ (in the presence of 1 μM Ins(4,5)P$_2$) was observed even after long incubations in an intracellular buffer with a variety of concentrations of cerebral homogenates and permeabilised CHO cells. However, metabolism of Ins(4,5)P$_2$ was easily detected by a kinetic decay approach previously employed by Batty & Nahorski, (1987). In brief inositol phosphate accumulation was stimulated to an elevated steady-state by carbachol (1 mM); the non-selective muscarinic antagonist atropine (10 μM) was then added to suppress stimulated synthesis, and the decay in the inositol phosphate isomer accumulation provided a measure of the rate of metabolism. The results in Figs. 5.4.1 & 5.4.2 demonstrate the time-course of accumulation of inositol phosphate isomers in the absence and presence of lithium (1 mM) in carbachol-stimulated (1 mM) cerebral-cortex slices. In the absence of lithium all isomers examined reached steady-state levels by 10 min, at which time atropine (10 μM) was added and the decay kinetics of the individual isomers was examined. As expected, Ins(1,4,5)P$_3$ levels fell dramatically reaching new steady-state, slightly higher than basal, within 1 min (see below). Other accumulated inositol phosphate isomers decayed to basal more slowly.

When incubations were performed in the presence of lithium (1 mM) a sustained enhancement
Reversibility of carbachol-stimulated inositol phosphate accumulations after atropine addition in cerebral cortex slices.

[3H]Inositol phosphate isomer accumulation in carbachol-stimulated (1 mM) rat cerebral-cortex slices in the absence (△, ▲) and presence of lithium (⊙, ●) (1 mM). The non-selective muscarinic antagonist atropine (10 μM) was added at 10 min (△, ▲), and the decay rates for each inositol phosphate isomer are shown. Values are means ± s.e.m. of data normalized from three separate experiments. Basal values of [3H]inositol phosphates were (in d.p.m.) as follows: Ins(1)P₁, 5846 ± 1442; Ins(4)P₁, 2902 ± 236; Ins(1,3)P₂, 73 ± 17; Ins(1,4)P₂, 2150 ± 324.
Figure 5.4 B. Reversibility of carbachol-stimulated inositol phosphate accumulations after atropine addition in cerebral cortex slices.

[3H]inositol phosphate isomer accumulation in carbachol-stimulated (1 mM) rat cerebral-cortex slices in the absence (Δ, ▲) and presence of lithium (0, ) (1 mM). The non-selective muscarinic antagonist atropine (10 μM) was added at 10 min (Δ, ▲), and the decay rates for each inositol phosphate isomer are shown. Values are means ± s.e.m. of data normalized from three separate experiments. Basal values of [3H]inositol phosphates were (in d.p.m.) as follows: Ins(4,5)P₂, 33 ± 8; Ins(1,3,4)P₃, 146 ± 41; Ins(1,4,5)P₃, 1840 ± 410; Ins(1,3,4,5)P₄, 429 ± 151.
in carbachol-stimulated Ins(1)P₃, Ins(4)P₁ and Ins(4,5)P₂, and a decrease in Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ was observed, as shown in Figs. 5.4.1 & 5.4.2. Lithium also altered the decay kinetics of several isomers. The decay of both inositol monophosphate isomers was substantially decreased, although not totally arrested, by lithium (1 mM). The metabolism of Ins(1,3)P₂ was essentially unaffected, whereas that of Ins(1,4)P₂ was slightly delayed, probably owing to a weak inhibitory effect of lithium on the dephosphorylation of Ins(1,4)P₂ by Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase. Ins(4,5)P₂ metabolism was clearly suppressed by lithium (1 mM), and there was a delay of approximately 4 min after atropine blockade before any decrease in the accumulation of this isomer was observed. However, a clear loss at 10 min suggests that the metabolism of Ins(4,5)P₂ is not completely blocked by this concentration of lithium (1 mM).

Ins(1,4,5)P₃ metabolism was rapid and unaffected by lithium (1 mM) with Ins(1,4,5)P₃ reaching a new lower steady-state within 1 min after addition of atropine. It should be noted, however that in both the absence and presence of lithium, addition of atropine resulted in the decay of Ins(1,4,5)P₃ to new steady-state levels slightly greater than basal. It seems probable that this reflects changes in the specific-radioactivity of [³²P]Ins(1,4,5)P₃ after carbachol stimulation of the cerebral cortex slices. This phenomenon has previously been described in cortex slice experiments (Batty & Nahorski, 1987). This specific-radioactivity change is only a significant feature with the immediate product of receptor-stimulated PtdInsP₂ hydrolysis, Ins(1,4,5)P₃. Both Ins(1,3,4)P₃ and Ins(1,3,4,5)P₄ decayed to basal values and were unaffected by lithium (1 mM).

5.3.3 Chemical degradation of the unknown inositol phosphate and identification of the resultant alkali.

The unknown InsP₂, believed to be Ins(4,5)P₂, has chromatographic properties consistent with Ins(4,5)P₂ (see section 5.3.1), which is the latest eluting InsP₂ known in this h.p.l.c. system (see Batty et al., 1989; Wreggett & Irvine, 1989 and Stephens et al., 1989). However,
the elution position of all InsP2 isomers is not known, therefore further identification was sought. The initial strategy was to submit the unknown bisphosphate to extensive degradation by periodate; an internal spike of [3H]Ins(1,3,4,5)P4 was included, which is resistant to periodate, and so yields inositol making it possible to check the percentage recovery (Batty et al., 1985; there is no InsP2 which gives inositol). On analysis, only inositol (90 % recovery) was detected, with no other polyols, demonstrating that the InsP2 does not produce a radiolabelled polyol on extensive exposure to periodate. The only three inositol bisphosphates which are completely destroyed by periodate are para-isomers, Ins(1,4)P2, Ins(3,6)P2 and Ins(2,5)P2 (Tomlinson & Ballou, 1961). The first two of these (an enantiomeric pair) are eliminated by the h.p.l.c. behaviour of the InsP2 in question, so only Ins(2,5)P2 remains. However, since the myo-inositol ring is labelled in the 2-OH position, there are three other isomers, Ins(4,5)P2 and Ins(5,6)P2 (L-enantiomer of D-Ins(4,5)P2) and Ins(4,6)P2 which, although degraded only as far as erythritol or xylitol, will yield non-radiolabelled erythritol or xylitol respectively, i.e. they will lose their radiolabel. Thus in summary, the absence of radiolabelled non-cyclic polyols narrows the possibilities to Ins(2,5)P2, D- or L-Ins(4,5)P2 and Ins(4,6)P2.

The elution position of Ins(2,5)P2 is unknown. However, this possibility has been eliminated by taking advantage of the fact that as the 2-phosphate is cis to the 1 and 3 phosphates; under conditions where cis-cis phosphate migration (but not trans migration) occurs (Pizer & Ballou, 1959) one should see the appearance of both Ins(1,5)P2 and Ins(3,5)P2 (L-enantiomer of D-Ins(1,5)P2), whose elution position, between Ins(1,4)P2 and Ins(3,4)P2 is known (Stephens & Downes, 1990). Fig. 5.5 shows the results of such an analysis. An internal spike of [14C]Ins(1,4)P2 yields two extra peaks, one (the later eluting) co-elutes with [3H]Ins(3,4)P2 in a separate analysis (not shown), and the other eluting between Ins(1,4)P2 and Ins(3,4)P2 is presumably Ins(2,4)P2. However, although there are some very small [3H] peaks appearing (of unknown origin or identity), there is clearly no formation of D- or L-Ins(1,5)P2 which, by
Figure 5.5  H.p.l.c. elution of the products of unknown inositol bisphosphate after incubation under conditions suitable for \textit{cis-cis} phosphate migration.

The elution profile of the products of the unknown inositol bisphosphate under conditions of suitable for \textit{cis-cis} phosphate migration reveal that the unknown isomer (○) is not Ins(2,5)P$_2$. The elution positions of Ins(1,4)P$_2$, Ins(2,4)P$_2$ and Ins(3,4)P$_2$ are also shown (●).
comparison with the Ins(1,4)P₂, should be about 45% of the starting material were that to be Ins(2,5)P₂. Thus this isomer can be eliminated, and the unknown InsP₂ must be D- or L-Ins(4,5)P₂ or Ins(4,6)P₂; in fact, the absence of any extra InsP₂ being generated tells us that there are no phosphates on the 1, 2 or 3 positions. (see also Tomlinson & Ballou, 1961).

Distinguishing InsP enantiomers, even though InsP is destroyed by periodate, has been achieved by partial periodate degradation, followed by D/L analysis of the resultant polyols by L-iditol dehydrogenase (Stephens, 1990). The useful compound with respect to D or L-Ins(4,5)P₂ would be glucitol (since both D- and L-iditol would be derived form either D- or L-Ins(4,5)P₂). Also, the polyol pattern produced by partial periodate degradation would enable the elimination of Ins(4,5)P₂. Unfortunately this partial periodation did not yield any detectable (1%) non-cyclic polyols. Periodate oxidation of a cyclitol ring proceeds more slowly than a non-cyclic alditol, so only a small quantity of the non-cyclic compound will accumulate (Stephens, 1990). Anything which slows up the oxidation of the cyclitol ring will decrease this yield further and it is suspected that the flattening of the cyclitol ring caused by the presence of the 4- and 5-position phosphates (Brown, 1969) has had this affect, and consequently the non-cyclic alditols never accumulate. Carrying out the oxidation at pH < 2, so that the charge on the phosphates is minimised (Brown, 1969) did not help. Under all conditions and times the only product found after reduction and dephosphorylation was inositol. Thus it is not possible to tell whether the accumulating bisphosphate in cortical slices is D- or L-Ins(4,5)P₂ or Ins(4,6)P₂, but consideration of likely radiolabelled precursor leads to the suggestion that D-Ins(4,5)P₂ is by far the most likely.

5.3.4 Effect of PKC-activated phospholipase D (PLD) on Ins(4,5)P₅ accumulation.

The possibility that Ins(4,5)P₂ is the product of PLD hydrolysis of PtdInsP₂ was examined indirectly. The present evidence implicates the possibility for a role of PKC in the activation of PLD (Billah & Anthes, 1990) and therefore the effects of the active phorbol 12,13-dibutyrate (PDBu) and the PKC inhibitor Ro 318220 (Cook et al., 1991) were examined (Table. 5.1.) on
Table 5.1. Effects of phorbol 12,13-dibutyrate or Ro 310220 on carbachol-stimulated Ins(4,5)P$_2$, Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ accumulations in rat cerebral cortex slices.

Cerebral cortex slices were pre-incubated and labelled with $[^3]$H]inositol as described in the methods section. Phorbol 12,13-dibutyrate (PDBu; 5µM) or Ro 310220 (5µM) were added 10 min prior to LiCl (10mM) and/or carbachol (10mM) addition for 15 min. Values are means ± s.e.m. for three experiments performed in triplicate and are expressed as d.p.m./sample.

<table>
<thead>
<tr>
<th>InsP species</th>
<th>$+/-$ Lithium</th>
<th>$-/-$</th>
<th>$+/+$</th>
<th>$-/$</th>
<th>$+/+$</th>
<th>$-/+$</th>
<th>$+/+$</th>
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<tbody>
<tr>
<td>Control</td>
<td>$31 ± 7$</td>
<td>$42 ± 9$</td>
<td>$1513 ± 20$</td>
<td>$1320 ± 274$</td>
<td>$428 ± 16$</td>
<td>$262 ± 48$</td>
<td></td>
</tr>
<tr>
<td>PDBu</td>
<td>$28 ± 5$</td>
<td>$55 ± 11$</td>
<td>$1729 ± 218$</td>
<td>$1412 ± 312$</td>
<td>$389 ± 25$</td>
<td>$253 ± 70$</td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>$839 ± 116$</td>
<td>$5109 ± 220$</td>
<td>$4498 ± 456$</td>
<td>$4210 ± 215$</td>
<td>$12051 ± 1646$</td>
<td>$6253 ± 645$</td>
<td></td>
</tr>
<tr>
<td>Carbachol + PDBu</td>
<td>$1190 ± 51$</td>
<td>$6464 ± 599$</td>
<td>$4640 ± 321$</td>
<td>$3845 ± 568$</td>
<td>$16626 ± 2223$</td>
<td>$7116 ± 981$</td>
<td></td>
</tr>
<tr>
<td>Carbachol + Ro 310220</td>
<td>$882 ± 118$</td>
<td>$4098 ± 264$</td>
<td>$4959 ± 264$</td>
<td>$3879 ± 398$</td>
<td>$12348 ± 1300$</td>
<td>$5345 ± 485$</td>
<td></td>
</tr>
</tbody>
</table>
carbachol-stimulated accumulations of \( \text{Ins}(4,5)P_2 \) and the possible precursors, \( \text{Ins}(1,4,5)P_3 \) and \( \text{Ins}(1,3,4,5)P_4 \). Basal and agonist-stimulated levels of \( \text{Ins}(4,5)P_2 \), \( \text{Ins}(1,4,5)P_3 \) and \( \text{Ins}(1,3,4,5)P_4 \), in both the absence and presence of lithium (1 mM), were unaffected by either activation or inhibition of PKC by PDBu or Ro 318220 respectively. These results are not consistent with PKC-activated PLD involvement in the production of \( \text{Ins}(4,5)P_2 \) in cerebral-cortex slices.

5.3.5 Effect of PKC activation and inhibition upon \( \text{Ins}(1,4,5)P_3 \) and \( \text{Ins}(1,3,4,5)P_4 \)

A number of conflicting reports have suggested that PKC may either stimulate or inhibit 3-kinase activity in a number of cell types, resulting in an increase in the formation of \( \text{Ins}(1,3,4,5)P_4 \). In this present study in cerebral cortex slices, neither activation or inhibition of PKC (by PDBu or Ro 318220, respectively) had any significant effect on carbachol-stimulated (1 mM) accumulation of either \( \text{Ins}(1,4,5)P_3 \) or \( \text{Ins}(1,3,4,5)P_4 \), either in the absence or presence of lithium. Similarly, PDBu alone had no effect on basal \( \text{Ins}(1,4,5)P_3 \) or \( \text{Ins}(1,3,4,5)P_4 \) levels. These results are not consistent, therefore, with a PKC-induced enhancement of 3-kinase activity in this preparation.

5.3.6 The effect of various agonists on \( \text{Ins}(4,5)P_2 \) accumulation

In all the experiments discussed so far the effects of lithium upon carbachol-stimulated \( \text{Ins}(4,5)P_2 \) accumulation have been discussed. Here the ability of a variety of agonists to stimulate \( \text{Ins}(4,5)P_2 \) accumulation was examined, both in the absence and presence of lithium, to determine whether \( \text{Ins}(4,5)P_2 \) accumulation is dependent upon stimulation by a particular agonist or whether its accumulation is a general feature of all agonists which stimulate the phosphoinositide cycle.

The effects of seven agonists upon \( \text{Ins}(4,5)P_2 \) accumulation were examined; these included
Figure 5.6 The effect of a variety of agonists upon Ins(4,5)P₂ accumulation in rat cortical slices.

A. The ability of the different agonists examined (Carbachol: CCh (1 mM); noradrenaline: NA (100 μM); KCl: K⁺ (30 mM final); histamine: Hist (1 mM); 1-aminocyclopentane-trans-1,3-dicarboxylic acid: tACPD (300 μM); quisqualate: Quis (10 μM); 5-hydroxytryptamine: 5-HT (100 μM)) to raise Ins(4,5)P₂ levels in rat cortical slices after 20 min incubation in the absence (■) or presence of lithium (□ ; 1 mM). B. A correlation between the ability of the different agonists examined to stimulate Ins(4,5)P₂ accumulation versus the extent to which they stimulate phosphoinositide signalling (as measured by total InsP₁ accumulation in the presence of lithium (1 mM). Data represent mean ± s.e.m. for three experiments.
carbachol (1 mM), noradrenaline (10 μM), KCl (30 mM; final concentration), histamine (1 mM), t-ACPD (1-aminocyclopentane dicarboxylic acid; 300 μM), quisqualate (10 μM) and 5-hydroxytryptamine (5-HT; 100 μM). All produced significant increases in Ins(4,5)P2 accumulation over basal in the absence of lithium, with carbachol producing the greatest (19.6-fold over basal) and histamine the least (1.2-fold over basal). The accumulation of Ins(4,5)P2 produced by each agonist was enhanced by lithium, with this ion producing the greatest increase, compared with basal values, in carbachol stimulated slices (139.7-fold) and least in histamine stimulated slices (2.5-fold) (Fig. 5.6 A.).

Figure 5.6 B. shows a comparison of Ins(4,5)P2 accumulation produced by the various agonists in the presence of lithium against the extent to which each agonist stimulates the phosphoinositide cycle (as determined by InsPi accumulation in the presence of lithium (1 mM)). Interestingly, Ins(4,5)P2 accumulation produced by the various agonists was directly proportional to the degree of stimulation of phosphoinositide metabolism produced by that agonist.

It is therefore clear that the accumulation of Ins(4,5)P2 is not agonist specific (or receptor specific) but instead is stimulated by a variety of agonists and the degree to which Ins(4,5)P2 accumulates is solely dependent upon the extent to which phospholipase C activity is stimulated by that agonist.

5.3.7 Ins(4,5)P2 accumulation in CHO-m1 cells.

During studies examining phosphoinositide metabolism in Chinese hamster ovary (CHO) cells expressing stably transfected m1-muscarinic receptors (discussed in Chapters 6. & 7.) it was observed that carbachol-stimulation produced a significant increase in the accumulation of Ins(4,5)P2 in this cell line. In addition agonist-stimulated levels of Ins(4,5)P2 were greatly enhanced by lithium in a concentration-dependent manner, as shown in Fig. 5.7.

Carbachol (1 mM) alone produced a time-dependent increase in Ins(4,5)P2 accumulation which rose at a steady-state over the time-course examined (Fig. 5.7 B., such that by 30 min
Figure 5.7 Effect of lithium on carbachol-stimulated $[^3H]$Ins(4,5)P$_2$ accumulation in CHO-m1 cells.

Carbachol-stimulated (1 mM) $[^3H]$Ins(4,5)P$_2$ accumulation was examined in CHO-m1 cells in the absence (Ο) or presence (●) of lithium (5 mM) at the time points indicated (A.). The effect of increasing concentrations of lithium (0.1-10 mM) on carbachol-stimulated Ins(4,5)P$_2$ accumulation at a 20 min time point is also shown (B.). Data represent means ± s.e.m. for three separate experiments. Basal values are described in the text.
levels were 102-fold greater than basal values (Li; 47.1 ± 8.2 d.p.m.). Lithium alone (5 mM) had no significant effect on basal Ins(4,5)P2 levels (+ Li; 55.0 ± 11.3 d.p.m.). Carbachol plus lithium however produced a dramatic linear increase in Ins(4,5)P2 levels reaching 554-fold over basal by 30 min.

The concentration-dependency of the effect of lithium on carbachol-stimulated Ins(4,5)P2 accumulation is shown in Fig. 5.7 A. Lithium (0.1-10 mM) produced a concentration-dependent increase in agonist-stimulated Ins(4,5)P2 accumulation after a 20 min incubation; the EC50 for this effect being 0.36 ± 0.03 mM. Maximal enhancement was produced by 3 mM lithium which produced a 3.9-fold increase over carbachol-stimulated levels (1438 ± 67 d.p.m.).

Both the time and concentration-dependency of the effect of lithium upon Ins(4,5)P2 accumulation in CHO-m1 cells are therefore qualitatively comparable with those discussed above for rat cortical slices.

5.3.8 Ins(1,4,5)P3 metabolism studies in rat cortical homogenates and in permeabilised CHO-m1 cells.

In order to determine whether Ins(1,4,5)P3 was the precursor of Ins(4,5)P2 in rat cerebral cortex slices, [3H]Ins(1,4,5)P3 was incubated for varying periods of time with rat cortical homogenates before the reactions were stopped and the resulting [3H]inositol phosphates assayed.

This approach lead to similar results as those previously published for rat cortical homogenates (Batty et al., 1989). In the absence of ATP [3H]Ins(1,4,5)P3 was rapidly metabolised by a 5-phosphatase to give [3H]Ins(1,4)P2, which in turn was dephosphorylated by the Ins(1,4)P2/Ins(1,3,4)P3 1-phosphatase to give [3H]Ins(4)P2. Incubations in the presence of lithium (1 mM), which from previous results would be expected to enhance any [3H]Ins(4,5)P2 accumulation which may occur, only produced an increase in the accumulation
Figure 5.8 [3H]Ins(1,4,5)P$_3$ metabolism by permeabilised CHO-m1 cells.

The metabolism of [3H]Ins(1,4,5)P$_3$ by permeabilised CHO-m1 cells in the absence (0) and presence (●) of L-chiro-Ins(1,4,6)PS$_3$ (100 µM). The levels of the various inositol phosphate products of Ins(1,4,5)P$_3$ (A.) metabolism, Ins(4)P$_1$ (B.) and Ins(1,4)P$_2$ (C.), at the time points indicated are also shown. Data represent one experiment.
of [3H]Ins(4)P and to a smaller extent [3H]Ins(1,4)P2. However, under both of these conditions no detectible levels of [3H]Ins(4,5)P2 were observed.

Since disruption of the structured intracellular environment may have lead to the lack of Ins(4,5)P2 production observed in the cortical homogenate studies (see discussion) it was decided to examine [3H]Ins(1,4,5)P3 metabolism in a more intact system, namely permeabilised CHO-m1 cells. As mentioned earlier, carbachol-stimulation of phosphoinositide metabolism in CHO-m1 cells produces a moderate increase in Ins(4,5)P2 levels which, like cortical slices, is dramatically enhanced by lithium. This cell type is therefore ideal for the present study.

Fig. 5.8 shows the results obtained for [3H]Ins(1,4,5)P3 metabolism by permeabilized CHO-m1 cells. Results were similar to those obtained in cortical homogenates with [3H]Ins(1,4,5)P3 being sequentially metabolised to [3H]Ins(1,4)P2 and [3H]Ins(4)P, similar to previous results obtained for [3H]Ins(1,4,5)P3 metabolism in permeabilised SH-SY5Y cells (Safirany et al., 1991). The inclusion of L-chiro-Ins(1,4,6)P3 in the incubation was made in order to try and divert the metabolism away from dephosphorylation by the 5-phosphatase; L-chiro-Ins(1,4,6)P3 has been shown in a human erythrocyte ghost preparation to be a relatively potent ($K_i \approx 200 \text{ mM}$) and selective inhibitor of this enzyme (Safirany et al., 1992). It was hoped that diverting metabolism from this major metabolic route may allow some metabolism of Ins(1,4,5)P3 to Ins(4,5)P2 to be observed. Unfortunately the only effect of this inhibitor was to reduce Ins(1,4,5)P3 metabolism and in turn cause a reduction in production of Ins(1,4)P2 and Ins(4)P compared with untreated cells. The addition of lithium (1 mM) to the cells caused a similar increase in [3H]Ins(4)P and [3H]Ins(1,4)P2 as that observed in cortical homogenate studies.

Under all the conditions examined no detectable levels of [3H]Ins(4,5)P2 were observed in CHO-m1 cells, giving no support here to the hypothesis that Ins(4,5)P2 is a metabolite of

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Ins(1,4,5)P$_3$ in mammalian cells.

3.3.9 The effect of Ins(4,5)P$_2$ on Ca$^{2+}$ release in the neuroblastoma cell line SH-SY5Y.

Due to the difficulty of measuring the effect of Ins(4,5)P$_2$ on intracellular Ca$^{2+}$ release in a heterogeneous cell population such as rat cortical slices the Ca$^{2+}$ mobilization properties of this bisphosphate were examined in the neuroblastoma cell line SH-SY5Y.

The results from initial studies comparing the ability of Ins(1,4,5)P$_3$ and Ins(4,5)P$_2$ in releasing $^{45}$Ca$^{2+}$ from permeabilised SH-SY5Y cells are shown in Fig. 5.9A. Ins(1,4,5)P$_3$ produced a concentration-dependent increase in $^{45}$Ca$^{2+}$ release with an EC$_{50}$ value of 0.27 ± 0.04 μM; in excellent agreement with previously reported results (Safrany et al., 1991). A maximal release of 59.0 ± 3.7% was observed with Ins(1,4,5)P$_3$ concentrations greater than 1 μM.

Similarly, Ins(4,5)P$_2$ produced a concentration-dependent increase in $^{45}$Ca$^{2+}$ release although with an EC$_{50}$ value of 21.3 ± 1.7 μM, some 79-fold less potent than Ins(1,4,5)P$_3$. Ins(4,5)P$_2$ was shown to be a full agonist in relation to the release of $^{45}$Ca$^{2+}$, with concentrations greater than 100 μM producing a statistically similar $^{45}$Ca$^{2+}$ release (46.7 ± 8.4%) compared with that of Ins(1,4,5)P$_3$.

To examine whether Ins(4,5)P$_2$ may have a role in antagonising the action of Ins(1,4,5)P$_3$ in releasing $^{45}$Ca$^{2+}$, by virtue of it competing with Ins(1,4,5)P$_3$ for its receptor, the effect of increasing concentrations of Ins(4,5)P$_2$ (1.0 - 100 μM) on Ins(1,4,5)P$_3$ (1 μM) induced $^{45}$Ca$^{2+}$ release was examined. Ins(1,4,5)P$_3$ (1 μM) alone produced a 65 ± 6% release of $^{45}$Ca$^{2+}$. Ins(4,5)P$_2$ at the concentrations examined had no significant effect on $^{45}$Ca$^{2+}$ release induced by Ins(1,4,5)P$_3$ (Fig. 5.9B).
Figure 5.9 The Ca\(^{2+}\) mobilization properties of Ins(4,5)P\(_2\) in permeabilised SH-SY5Y cells.

A. The concentration-dependancy of the ability of the inositol phosphates Ins(4,5)P\(_2\) (●) and Ins(1,4,5)P\(_3\) (○) to release \(^{45}\)Ca\(^{2+}\) from intracellular calcium stores in prelabelled SH-SY5Y cells (see Methods). B. The effect of increasing concentrations of Ins(4,5)P\(_2\) (1.0 - 300 \(\mu\)M) on Ins(1,4,5)P\(_3\) (1 \(\mu\)M) evoked \(^{45}\)Ca\(^{2+}\) release. Data represent the mean ± s.e.m for three separate experiments.
5.4 Discussion.

There is now substantial evidence, from both the accumulation of metabolites in mammalian cells and the products resulting from homogenate incubations, that Ins(1,4,5)P₃ can be phosphorylated by a 3-kinase to Ins(1,3,4,5)P₄ or dephosphorylated by a 5-phosphatase to Ins(1,4)P₂ (see Downes & MacPhee, 1990 and Shears, 1991). Although the presence of substantial Ins(1,4,5)P₃ 1-phosphatase activity in the slime mould Dictyostelium discoideum (Van Lookeren Campagne, 1988) generates the unusual inositol bisphosphate isomer Ins(4,5)P₂, there is little evidence to date that this latter metabolite accumulates in agonist-stimulated mammalian cells. Hughes and Drummond (1987) first reported a late-running [³H]inositol bisphosphate h.p.l.c. peak in thyrotropin-releasing hormone (TRH)-stimulated GH₃ cells that was somewhat enhanced by lithium (10 mM). Batty and coworkers (1989) also observed accumulation of a minor late-running bisphosphate peak in carbachol-stimulated cerebral-cortex slices. Its tentative identification as Ins(4,5)P₂ in that study has been supported here by the co-elution of the alkaline phosphatase product of Ins(1,4,5)P₃. Further analysis of this product by examining the corresponding alditols (see section 5.3.3) cannot unequivocally identify Ins(4,5)P₂. However, at present its elution profile and co-elution with standard strongly suggest its identity as Ins(4,5)P₂. The major observation of this study is that lithium greatly enhances the accumulation of this unusual metabolite, such that at 30 min it represents almost 35 % of the total bisphosphate fraction. This should not, however, be compared quantitatively, since agonist-stimulated accumulations of the other major bisphosphate isomers, Ins(1,3)P₂ and Ins(1,4)P₂, are relatively lithium-insensitive and their rate of turnover at steady state could greatly exceed that for Ins(4,5)P₂ (see below).

The enhanced and linear accumulation of Ins(4,5)P₂ with time in the presence of low concentrations of lithium is reminiscent of the effects of this ion on inositol monophosphate accumulation (Kennedy et al., 1989 and Batty & Nahorski, 1987) (Fig. 5.3), which results from the uncompetitive inhibition of the inositol monophosphatase enzyme (Hallcher &
Sherman, 1980 and Gee et al., 1988a). Thus it is tempting to suggest that Ins(4,5)P₂ is generated in cerebral tissue after receptor activation and that it is metabolized by an enzyme that is potently inhibited by lithium. Indeed, the EC₅₀ for lithium enhancing its accumulation by carbachol (94 ± 3 μM) could suggest a more potent inhibition than on the monophosphatase (however, see below). Alternatively, lithium may enhance Ins(4,5)P₂ formation from putative precursors, assuming that the metabolism of Ins(4,5)P₂ is rate limiting. This should be considered in the light of the unexpected agonist-stimulated accumulations of Ins(1,4)P₂ and Ins(1,3)P₂ observed at 5-10 min in the presence of lithium (1 mM). From our knowledge of the specificity and lithium-sensitivity of the Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase (Shears, 1991), this concentration of lithium should only minimally affect the accumulation of the former and be totally ineffective against the latter isomer. Thus the possibility remains that lithium enhances dephosphorylation of Ins(1,4,5)P₃ and/or Ins(1,3,4,5)P₄, resulting in the depletion of these inositol phosphate second messengers and enhancement of the three bisphosphate isomers. However, there is no support for this hypothesis from the atropine reversal experiments, since the rate of metabolism of either Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ was not influenced by lithium. This hypothesis would also be inconsistent with the clear effects of lithium on inositol depletion and suppression of phosphoinositide synthesis (Berridge et al., 1989 and Nahorski et al., 1991) being the major determinants of reduced Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ accumulation.

After receptor blockade, the steady-state levels of Ins(4,5)P₂ in the absence of lithium quickly decays, suggesting that in the intact cell metabolism occurs rapidly. The failure to observe such metabolism with cortical homogenates may suggest strict control of hydrolysis in the intact cell. The linear accumulation of this bisphosphate with lithium is more likely a consequence of inhibition of its metabolism. Indeed, receptor blockade in the presence of lithium, which produced a rapid and dramatic decrease in Ins(1,3)P₂ and Ins(1,4)P₂, results in no decay of Ins(4,5)P₂ within the first 4 min after atropine addition. However, by 10 min a significant decrease in Ins(4,5)P₂ accumulation was observed, indicating that the enzyme(s)
involved in metabolizing Ins(4,5)P$_2$ are not fully inhibited. This contrasts somewhat with the results mentioned above, in which half-maximal enhancement of carbachol-stimulated Ins(4,5)P$_2$ accumulation by lithium occurred at 94 ± 3 μM and appeared to be maximal at 1 mM. This anomaly would be consistent if Ins(4,5)P$_2$ was being produced from Ins(1,4,5)P$_3$ via a lithium-sensitive 1-phosphatase similar to that found in Dictyostelium discoideum (Van Lookeren Campagne, 1988), though with a somewhat higher K$_i$ for lithium. This would also be consistent with the bell-shaped lithium-concentration / response curve obtained for carbachol-stimulated Ins(4,5)P$_2$ accumulation and the inability of 1 mM lithium to block Ins(4,5)P$_2$ metabolism completely, as was observed in the experiment examining the kinetics of the decay of this isomer. Finally, in the presence of lithium (1 mM) the metabolism of Ins(1)P$_1$ and Ins(4)P$_1$ was very slow and less than that for Ins(4,5)P$_2$, suggesting that the blockade of Ins(1)P$_1$ and Ins(4)P$_1$ metabolism by lithium (EC$_{50}$ ~ 0.5 mM) is more potent than that for Ins(4,5)P$_2$. This suggests an IC$_{50}$ for lithium inhibition of the enzyme(s) metabolizing Ins(4,5)P$_2$ of greater than 0.5 mM, rather than the 90 μM that could result from a truncated concentration-response curve incurred by inhibitory effects of higher lithium concentrations on Ins(4,5)P$_2$ synthesis. These data would also suggest that there may be a dramatic flux through Ins(4,5)P$_2$ in cerebral cortex under agonist-stimulated conditions and that this is underestimated because of the inability to dissect pharmacologically the suppression component of its metabolism with lithium.

The route of dephosphorylation of Ins(4,5)P$_2$ is unknown. Ins(4,5)P$_2$ has been shown to be a substrate, albeit a poor one, for the Ins(1,4,5)P$_3$/Ins(1,3,4,5)P$_4$ 5-phosphatase (Mitchell et al., 1989). Whether Ins(4,5)P$_2$ is dephosphorylated to Ins(4)P$_2$ or Ins(5)P$_1$ by a 5- or a 4-phosphatase activity, respectively, is still to be answered. However, significant Ins(5)P$_1$ accumulation has been reported in cerebral cortex of rats treated with lithium and the muscarinic agonist pilocarpine in vivo (Ackermann et al., 1987).

Two possible routes should be considered as a source of Ins(4,5)P$_2$. Firstly, PLD could
hydrolyse PtdInsP$_2$ to form Ins(4,5)P$_2$ (and phosphatidic acid) directly, by-passing the production of the second messenger Ins(1,4,5)P$_3$. It has been shown in [3H]glycerol labelled canine kidney cells that on addition of the agonist bradykinin there is a rapid formation of [3H]PtdEtOH accompanied by a decrease in [3H]PtdIns. This suggests that in this mammalian cell type there is a PtdIns selective PLD activity (Huang et al., 1992). An extension of this study, which employed a cell free assay system, demonstrated that this PtdIns selective PLD was different from PtdCho selective PLD in a number of ways, including subcellular location, substrate specificity and co-factor requirements. Such a PtdIns selective PLD activity has also been demonstrated in bovine and human serum (Hoener & Brodbeck, 1992). It has not yet been shown, however, whether this PtdIns selective PLD activity has any role in PtdInsP$_2$ metabolism, as it does in PtdIns metabolism. However such an activity directed against PtdInsP$_2$ could result in the formation of Ins(4,5)P$_2$. PLD has been shown to be stimulated by activators of PKC, such as phorbol esters (for review see Billah & Anthes, 1990), suggesting that agonists may use such a route of activation. However, recent studies do not indicate the presence of a carbachol-stimulated PLD activity in rat cerebral-cortex slices (Llafi & Fain, 1992). In the present study PKC-stimulation of PLD produced by PDBu had no effect on either basal or carbachol-stimulated levels of Ins(4,5)P$_2$ in either the absence or presence of lithium. Similarly, the selective PKC inhibitor Ro 318220, which has been shown to inhibit PKC-stimulated PLD activity in Swiss 3T3 cells (Cook et al., 1991), did not produce a significant decrease in carbachol-stimulated Ins(4,5)P$_2$ accumulation. These results therefore suggest that at least a PKC-activated PLD is probably not involved in the formation of Ins(4,5)P$_2$, although this does not necessarily rule out a role for PLD in the formation of Ins(4,5)P$_2$.

Alternatively, either the metabolism of Ins(1,4,5)P$_3$ by a 1-phosphatase, or indeed Ins(1,3,4,5)P$_4$ by a 1-phosphatase and a 3-phosphatase, could produce Ins(4,5)P$_2$. At first sight this route does not appear likely to account for the relatively large amount of Ins(4,5)P$_2$ accumulating, since this bisphosphate is not produced when Ins(1,4,5)P$_3$ is metabolized by
crude cortical homogenates (also see Batty et al., 1989) or permeabilised CHO-m1 cells (see section 5.3.7). It must be remembered however that in both of these model systems the structure of the cell, even in the latter, is greatly disturbed. It may well be that in the intact cell the enzymes are compartmentalized such that any 1-phosphatase activity that may be involved in the metabolism of Ins(1,4,5)P$_3$ to Ins(4,5)P$_2$ is not swamped by that of the overwhelming activity of the 5-phosphatase which was observed in Ins(1,4,5)P$_3$ metabolism both in cortical homogenates and in permeabilised CHO-m1 cells. Compartmentation, therefore, of 1-, 3- and the overwhelming 5-phosphatase activities within the cell could account for the production of Ins(4,5)P$_2$ from either Ins(1,4,5)P$_3$ or Ins(1,3,4,5)P$_4$ and as discussed above, a less sensitive effect of lithium on such enzyme(s) compared to the enzyme(s) involved in the metabolism of Ins(4,5)P$_2$ would be totally consistent with all the results discussed above.

With the difficulty in dissecting the routes of formation and degradation of Ins(4,5)P$_2$ in either tissue homogenates or in permeabilised cell preparations it appears the only way to determine unequivocally whether Ins(4,5)P$_2$ is formed from Ins(1,4,5)P$_3$ is to purify the 1-phosphatase from cerebral cortex and examine the metabolism of [PH]Ins(1,4,5)P$_3$ in vitro. Such an approach may also be useful in determining the route of metabolism of Ins(4,5)P$_2$, i.e. whether it is via a 4- or a 5-phosphatase. With this approach it would also be possible to determine the lithium sensitivities of the individual enzymes involved.

Table 5.1 describes the effects of activation and inhibition of PKC, by PDBu and Ro 318220 respectively on carbachol stimulated Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ levels in rat cerebral cortex slices. It has previously been shown that both an increase in intracellular Ca$^{2+}$ and activation of protein kinase C can regulate 3-kinase activity in a number of cell types. Increases in intracellular Ca$^{2+}$ levels stimulated by agonist addition result in an increase in the activity of the 3-kinase, through an interaction of Ca$^{2+}$ with calmodulin (Biden et al., 1987). Initial evidence for PKC-activation of 3-kinase was obtained from malignant human T-cells stimulated with either phorbol esters or monoclonal antibodies (Imboden & Pattison, 1987). The 3-kinase which was partly purified from these cells exhibited a persistent activation.
Similar experiments confirmed these effects in insulinoma (Biden et al., 1988) and platelets (King & Rittenhouse, 1989). However, these studies on PKC-activation of the 3-kinase all predate the recognition that the 3-kinase is a calpain-sensitive enzyme (Choi et al., 1990). Unless calpain inhibitors are present during the preparation of the cell-free extracts, Ins(1,4,5)P3 3-kinase may be partially degraded to forms with altered catalytic and regulatory properties. Studies with purified, intact 3-kinase and PKC have demonstrated that the resultant phosphorylation of the 3-kinase results in a decrease in the $V_{\text{max}}$ of the 3-kinase by 75% (Sim et al., 1990). The above observations give rise to the possibility that the 3-kinase may be differentially regulated during agonist stimulation, stimulated at first by Ca$^{2+}$ and subsequently inhibited by PKC. In this present study no significant effect of PKC activation or inhibition was observed on the carbachol-stimulated accumulation of Ins(1,3,4,5)P$_4$ or Ins(1,4,5)P$_3$, suggesting that the activity of the 3-kinase in cerebral cortex slices is not enhanced or inhibited by activation of PKC under agonist-stimulated conditions. As mentioned previously, agonist addition causes an increase in the intracellular Ca$^{2+}$ concentration of the cell, which itself may interfere with 3-kinase activity, enhancing the activity of this enzyme and possibly masking a more subtle effect which PKC activation may have on this enzyme. Therefore, the effect of activating PKC with PDBu upon unstimulated Ins(1,3,4,5)P$_4$ and Ins(1,4,5)P$_3$ levels were examine in this tissue. Neither PDBu or Ro 318220 had any effect on basal levels of Ins(1,3,4,5)P$_4$ or Ins(1,4,5)P$_3$. These results therefore suggest that PKC may not have a major role in the control of 3-kinase activity in rat cerebral cortex.

Ins(4,5)P$_2$ was shown to accumulate in the presence of a number of different agonists, including excitatory amino acids, depolarising stimuli, and muscarinic agonists. The degree of Ins(4,5)P$_2$ accumulation produced by each agonist in the presence of lithium was directly related to the degree of stimulation of PLC produced by each respective agonist (as measured by levels of Ins(1)P$_1$ accumulation in the presence of agonist plus lithium (1 mM)). This data is interesting in that it demonstrates that the accumulation is not agonist specific but is stimulated by a large variety of agonists and that the greater the degree of stimulation of the cycle (i.e. the
greater the flux through the cycle), the greater is the accumulation of Ins(4,5)P2 (Fig. 5.6 B.). This latter point may be related to the fact that the lithium-sensitive enzyme involved in the metabolism of Ins(4,5)P2 may be uncompetitively inhibited by lithium. As previously mentioned (see Chapter 1) with this type of inhibition the greater the levels of substrate present the greater is the inhibition of the enzyme produced by lithium. This may explain why Ins(4,5)P2 accumulation is greater in the presence of agonists which stimulate a greater flux through the cycle.

The significance of agonist-stimulated Ins(4,5)P2 accumulation is not known. One obvious question to ask is does Ins(4,5)P2 have any role in the release of Ca\(^{2+}\) from intracellular stores? Ins(4,5)P2 has been shown here, and previously (Irvine et al., 1984), to release Ca\(^{2+}\) from intracellular stores in a similar manner to Ins(1,4,5)P3, albeit with a potency which is ~ 100-fold less than that for Ins(1,4,5)P3. Any calculation of the accumulated mass of Ins(4,5)P2 in order to determine the likelihood that concentrations of this isomer would reach levels high enough to release Ca\(^{2+}\) is complicated by (i) changes in the specific radioactivity of the labelled Ins(1,4,5)P3 and (ii) the inability to assess the true accumulation in a heterogeneous cell population such as cortical slices. However, Ins(4,5)P2 would seem unlikely to play a major in Ca\(^{2+}\) mobilization, in view of its rate of accumulation and the sustained increase in Ins(1,4,5)P3 seen in cerebral-cortex slices. On the other hand does Ins(4,5)P2 have any role in modulating Ins(1,4,5)P3-induced Ca\(^{2+}\) release? Since both molecules, Ins(1,4,5)P3 and Ins(4,5)P2, compete for the same receptor / channel complex in order to release intracellular Ca\(^{2+}\) from stores it may be that Ins(4,5)P2 in some way modulates the effect of Ins(1,4,5)P3 on releasing Ca\(^{2+}\). This hypothesis was unlikely and it has been shown (see Fig. 5.9 B.) that at even high concentrations of Ins(4,5)P2 that this bisphosphate isomer has no effect of Ins(1,4,5)P3 induced Ca\(^{2+}\) release (Fig. 5.9), at least over the time point examined.

Another inositol bisphosphate, Ins(1,4)P2, has been tentatively suggested to have a role in the activation of a DNA polymerase (Sylvia et al., 1988). It may be therefore that other possible
roles for Ins(4,5)P$_2$ await further investigation. However, the present results reveal that, in the presence of lithium and agonist Ins(4,5)P$_2$ accumulates linearly with time and this at the very least suggests further complexity to our present appreciation of inositol polyphosphate metabolism in brain.
Chapter 6

Characterisation of inositol (poly)phosphate metabolism in Chinese hamster ovary cells stably transfected with the M₁-muscarinic receptor subtype.
6.1. Introduction.

With the great volume of work now amassed on phosphoinositide metabolism in cerebral cortical slice preparations, and the therapeutic relevance, cerebral cortex was the tissue of preference for examining the effects which lithium may have upon this signalling system. Indeed, the work described in Chapters 3, 4 and 5 demonstrate the usefulness of this system in examining the subtle and complex effects of lithium; however this work also highlights some of the weaknesses of using such a heterogeneous system.

Although it could be argued that the cortical slice preparation is a relatively intact system (with synaptic connections remaining intact to a greater or lesser degree) which should reflect the effect of lithium in vivo, it can also be argued that cross-chopping involved in the preparation of the slices may disrupt the integrity of the structure of the tissue. Indeed, it is thought that during the preparation up to 50% of the cells within the slice are destroyed. Furthermore, washing of the slices may also reduce the concentration of free inositol within the slice. The data obtained from such a tissue can also be difficult to interpret due to the heterogeneity of cell types found within the preparation. It may well be that some cells within the preparation are affected by lithium, whereas others are not, with the overall observed effect being a sum of a spectrum of responses. This may lead to an underestimate of the effect of lithium. Indeed, it has been extremely difficult to show any effect of lithium upon agonist-influenced PtdIns(4,5)P$_2$ levels in brain slices and this may be due to only a small proportion of the cells being affected by agonist and / or lithium with the effect being masked by the lack of response of the other cells.

Such limitations of the cerebral cortical slice preparation have led us to look for a simpler homogeneous model cell system which should give clearer, more interpretable data. The advantages of such a cell system are numerous. The population of cells are homogeneous. The results will not, therefore, be complicated by the presence of other cells, as in the case of cerebral cortex slice work. The handling of the cell can also be designed to be much less harsh and indeed experiments can be carried out on plated down cells. One disadvantage, however is
that the cells are immortal and grow continuously and may therefore not be an ideal model of normal cells in situ.

The cell-line chosen for the purpose of these experiments was the Chinese hamster ovary cell-line (CHO cell-line). These cells also have the advantage that they do not contain any endogenous muscarinic receptors and it is possible to stably transfected them with various subtypes of this receptor class. The cell-line used in the following two Chapters is the CHO-M1 cell (i.e. CHO cell stably transfected with the M1 subtype of the muscarinic receptor class). This cell-line, therefore, has the further advantage of only having a single receptor population, unlike cerebral cortex slices where the phosphoinositide metabolism is mediated via up to three different subtypes: M1, M3 and M5, adding yet more complexity to the response.

The work in this Chapter examines the metabolism of the inositol phosphates in agonist-stimulated CHO-M1 cells and the effects of lithium upon this metabolism.
6.2. Results.

6.2.1. Characterisation of Muscarinic Receptors in CHO-M1 cells.

As mentioned earlier Chinese hamster ovary (CHO) cells stably transfected with the muscarinic M_{1}-receptor subtype were obtained from Dr. N. J. Buckley (N.I.M.R., Mill Hill, London). Before experimentation the muscarinic binding characteristics of the cells were briefly examined in order to confirm that the cells did only express one population of muscarinic receptors and that these indeed were of the M_{1}-subtype.

([H]N-methyl scopolamine (NMS) saturation binding was initially carried out to determine the B_{max} and K_{D} of [H]NMS binding in these cells. The results, depicted in figure 6.1. (A), gave a B_{max} of 816 fmol/mg of protein with a K_{D} of 0.17 ± 0.03 nM for [H]NMS.

Subsequently, pirenzepine displacement of [H]NMS binding was carried out on these cells. The data, shown in figure 6.1. (B), gave a Hill slope of 1.10 ± 0.08 and a K_{i} for pirenzepine of 9.4 ± 0.2 x 10^{-9} M, a value which would suggest that the receptor population was of the muscarinic M_{1}-subtype (see Buckley et al., 1989).

These data are consistent with the view that these cells express a single population of receptors and that these are of the M_{1}-subtype of muscarinic receptors. These data have since been confirmed in these cells by a more exhaustive examination of the rank order of potency of antagonists with respect to inhibition of [H]NMS binding (Burford, N.T., personal communication).

6.2.2. Time-course of the Effects of Lithium Upon Carbachol-Stimulated Accumulations of Inositol (only)phosphate Isomers in CHO-M1 Cells.

The effect of lithium (5 mM) addition on the carbachol-stimulated (1 mM) accumulation of several inositol phosphate isomers was examined in CHO-M1 cells which had been labelled to isotopic equilibrium with [H]inositol. Nine inositol phosphate isomers were examined, including: Ins(1/3)P, Ins(4)P, Ins(1,3)P, Ins(1,4)P, Ins(3,4)P, Ins(4,5)P, Ins(1,3)P, Ins(1,4)P, Ins(3,4)P, Ins(4,5)P.
Figure 6.1. Binding Characteristics of CHO-M1 cells.

A. [3H]NMS saturation analysis of CHO-M1 cells. Equilibrium dissociation constants (K_D) and maximal binding capacities (B_max) of CHO cells transfected with m1 DNA are described in the text. Data represent a single representative experiment.

B. Displacement of specific [3H]NMS binding to CHO-M1 cells by the selective M1-receptor antagonist pirenzepine. Concentrations of [3H]NMS were between 0.5-0.6 nM. Data represent mean ± s.e.m. for three experiments.
Ins(1,4,5)P₃, Ins(1,3,4)P₃, and Ins(1,3,4,5)P₄ in order to establish the pattern of
Ins(1,4,5)P₃ metabolism in this cell type. For all the isomers examined lithium (5 mM) alone
had no significant effect on basal values.

The addition of carbachol alone (1 mM) produced a rapid increase in the accumulation of
[³H]Ins(1,4,5)P₃ which reached a maximum of 18.8-fold over basal (330 ± 21 d.p.m./well)
at 10 s before falling to 5.9-fold over basal by 1 min (Fig. 6.5.). Subsequently, a slower rise
was observed which reached a plateau of 9.7-fold over basal between 10-20 min. Lithium (5
mM) in the presence of carbachol (1 mM) had no effect on the initial rapid production of
Ins(1,4,5)P₃ observed with carbachol alone, however the secondary plateau phase of the
response was significantly reduced after 10 min in the presence of lithium, and by 30 min
Ins(1,4,5)P₃ levels were reduced by 71.5 % compared with carbachol-stimulated values
(Fig. 6.5.).

The immediate metabolites of Ins(1,4,5)P₃, Ins(1,4)P₂ and Ins(1,3,4,5)P₄, which are
produced by the action of 5-phosphatase and 3-kinase activities respectively, are shown in
figures 6.3. & 6.5. respectively.

Upon addition of carbachol alone (1 mM) [³H]Ins(1,4)P₂ levels showed a similar profile to
that of Ins(1,4,5)P₃ production, at least over the time-course examined. Ins(1,4)P₂ levels
increased rapidly and reached a maximum of 6.9-fold over basal (935 ± 85 d.p.m./well) at 10 s
before falling to 3.2-fold over basal by 5 min (Fig. 6.3.). This was followed by a slower rise
in Ins(1,4)P₂ levels which reached a maximum of 5.9-fold over basal by 30 min. Lithium
(5 mM) in the presence of carbachol (1 mM) had no effect on the initial rapid production of
Ins(1,4)P₂ observed with carbachol alone over the first 10 s, however the secondary plateau
phase was dramatically altered. In the presence of lithium Ins(1,4)P₂ levels increased rapidly
between 1 and 10 min, in a linear manner, reaching a maximum of 13.2-fold over basal at 10
min. Ins(1,4)P₂ levels subsequently fell rapidly over the remainder of the time-course, such
that by 30 min levels had fallen to 3.9-fold over basal and were reduced relative to those
observed in the presence of carbachol alone.
In the presence of carbachol (1 mM) alone \[^{3}H\]Ins(1,3,4,5)P\(_4\) levels increased over the first 20 min of the time-course before reaching a maximum and plateauning at 16.7-fold over basal (Fig. 6.5.). Lithium (5 mM) in the presence of carbachol had no effect over the initial 5 min when compared with the response to carbachol alone. However, lithium produced a significant reduction in the carbachol-stimulated levels of Ins(1,3,4,5)P\(_4\) by 10 min, and by 30 min Ins(1,3,4,5)P\(_4\) levels were reduced by 73.4 % compared with carbachol-stimulated values (Fig. 6.5.).

Ins(1,3,4,5)P\(_4\) is dephosphorylated to Ins(1,3,4)P\(_3\), via the same 5-phosphatase as Ins(1,4,5)P\(_3\). In the presence of carbachol alone (1 mM) \[^{3}H\]Ins(1,3,4)P\(_3\) levels increased rapidly reaching 11.3-fold over basal (164 ± 5 d.p.m./well) at 1 min (Fig. 6.4.). Levels continued to increase slowly over the remainder of the time-course, and at 30 min Ins(1,3,4)P\(_3\) levels were 17.2-fold over basal. Lithium (5 mM) in the presence of carbachol (1 mM) had no effect on the initial rapid increase in Ins(1,3,4)P\(_3\) levels observed with carbachol alone, however later time points were significantly different. In the presence of lithium, carbachol-stimulated Ins(1,3,4)P\(_3\) levels increased rapidly between 1 and 10 min reaching a maximum of 39.2-fold over basal at 10 min. Ins(1,3,4)P\(_3\) levels subsequently fell rapidly over the remainder of the time-course, such that by 30 min levels had fallen to 13.8-fold over basal.

The metabolism of Ins(1,3,4)P\(_3\) has been shown in a number of cell lines to proceed via 1-phosphatase and also 4-phosphatase activities, resulting in the production of Ins(3,4)P\(_2\) and Ins(1,3)P\(_2\), respectively. However, no detectable levels of Ins(1,3)P\(_2\) could be found in CHO-M1 cells either in the presence of agonist or agonist plus lithium. It therefore appears that in this cell line Ins(1,3,4)P\(_3\) may be metabolised solely via a 1-phosphatase activity to give Ins(3,4)P\(_2\) or that Ins(1,3)P\(_2\) is so rapidly metabolised such that no accumulation of this metabolite is observed.

In the presence of carbachol (1 mM) alone \[^{3}H\]Ins(3,4)P\(_2\) levels increased over the initial 10 min of the time-course before reaching a maximum and plateauning at 63.6-fold over basal.
Figure 6.2. Time-Course of the Effects of Lithium on Carbachol-Stimulated Ins(1/3)P$_1$ and Ins(4)P$_1$ levels in CHO-M1 cells.

[3H]Inositol labelled CHO-M1 cells were stimulated with carbachol in the absence (O) or presence (●) of lithium (5 mM) for the times indicated and the levels of the isomers Ins(1/3)P$_1$ and Ins(4)P$_1$ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.

Statistically significant differences between (O) and (●) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
Figure 6.3. Time-Course of the Effects of Lithium on Carbachol-Stimulated Ins(1,4)P$_2$ and Ins(3,4)P$_2$ levels in CHO-M1 cells.

$[^3]$H]Insitol labelled CHO-M1 cells were stimulated with carbachol in the absence (0) or presence (•) of lithium (5 mM) for the times indicated and the levels of the isomers Ins(1,4)P$_2$ and Ins(3,4)P$_2$ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.

Statistically significant differences between (0) and (•) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
Figure 6.4. Time-Course of the Effects of Lithium on Carbachol-Stimulated Ins(4,5)P₂ and Ins(1,3,4)P₃ levels in CHO-M1 cells.

[²H]Inositol labelled CHO-M1 cells were stimulated with carbachol in the absence (○) or presence (●) of lithium (5 mM) for the times indicated and the levels of the isomers Ins(4,5)P₂ and Ins(1,3,4)P₃ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.

Statistically significant differences between (○) and (●) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
**Figure 6.5.** Time-Course of the Effects of Lithium on Carbachol-Stimulated Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ levels in CHO-M1 cells.

[³H]inositol labelled CHO-M1 cells were stimulated with carbachol in the absence (○) or presence (●) of lithium (5 mM) for the times indicated and the levels of the isomers Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.

Statistically significant differences between (○) and (●) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
(27 ± 4) between 10 and 20 min (Fig.6.3). Lithium (5 mM) in the presence of carbachol had no effect over the initial minute of the time-course, when compared with the effect of carbachol alone, however lithium produced a significant reduction in the agonist-stimulated levels of Ins(3,4)P2 (54.0 % reduction compared with carbachol control values at 10 min) which was maintained throughout the remainder of the time-course.

The effect of lithium on agonist-stimulated levels of Ins(4,5)P2 was also examined. The precursor for this bisphosphate is unknown, however it has been suggested that this isomer may be a third route of metabolism for Ins(1,4,5)P3 (Shears, 1991; also see Chapter 5).

Carbachol alone (1 mM) produces a slow, steady increase in the levels of [3H]Ins(4,5)P2 within the cell, reaching a maximum of 13.0-fold over basal (37 ± 9 d.p.m./well) at 30 min (Fig. 6.4.). In the presence of lithium (5 mM) and agonist the accumulation of Ins(4,5)P2 was not significantly different over the initial minute to that observed with agonist alone. However, lithium produced a significant increase in the accumulation of Ins(4,5)P2 accumulation over the remainder of the time-course, such that at 30 min lithium had produced a 5.4-fold greater increase in Ins(4,5)P2 levels compared with carbachol-stimulated values (2835± 189 d.p.m./well).

The accumulation of the inositol monophosphates Ins(1/3)P1 and Ins(4)P1, in the presence of carbachol and carbachol plus lithium are shown in Figure 6.2. In the presence of carbachol alone (1 mM) [3H]Ins(1/3)P1 levels increased steadily over the initial 10 to 20 min before plateauing at a maximum of 12.5-fold over basal (1350 ±70 d.p.m./well) at 20 min. The addition of lithium (5 mM) produced a marked enhancement in the accumulation of agonist-stimulated Ins(1/3)P1 levels. In the presence of agonist and lithium, Ins(1/3)P1 accumulated linearly over the time-course examined, such that at 30 min Ins(1/3)P1 levels were 68-fold over basal, or 5.2-fold over carbachol-stimulated values (17653 ± 81 d.p.m./well).

The accumulation of Ins(4)P1 was different from that of Ins(1/3)P1 as might have been expected (Fig. 6.2.). With carbachol alone (1 mM) Ins(4)P1 levels increased rapidly over the
initial minute before plateauing at a maximum of 25.3-fold over basal (480 ± 33 d.p.m./well at 20 min). In the presence of lithium the accumulation of Ins(4)P₁ over the initial 10 min of the time-course was rapid and linear. At 10 min the accumulation of Ins(4)P₁ plateaued at a maximum of 100-fold over basal or 4.6-fold over carbachol-stimulated values (13556 ± 394 d.p.m./well).

No detectable levels of any other inositol monophosphate isomer were detected.

6.2.3. Concentration-dependency of the Effects of Lithium on Carbachol-stimulated Inositol (Poly)phosphate Accumulation in CHO-M1 Cells.

The effects of increasing concentrations of lithium (0.1-10 mM) upon carbachol-stimulated inositol phosphate isomer accumulations were examined in CHO-M1 cells. All incubations with agonist ± lithium were for 20 min. Lithium alone (at concentrations of up to 10 mM) did not have any significant effect on the basal levels of any of the inositol phosphate isomers examined over the 20 min incubation period of the experiment.

Carbachol alone (1 mM) produced a 5.1-fold increase over basal (1165 d.p.m./well) in the levels of [3H]Ins(1,4,5)P₃ at 20 min (Fig. 6.9.), similar to that observed previously during time-course studies. Increasing concentrations of lithium (0.1-10 mM) produced a concentration-dependent decrease in the carbachol-stimulated levels of Ins(1,4,5)P₃ (IC₅₀ = 0.39 ± 0.03 mM), with a maximum inhibition (87.1 % compared with carbachol-stimulated values; 5965 ± 89 d.p.m./well) occurring with 10 mM lithium.

Carbachol (1 mM), as shown in figure 6.9., produced a 4.4-fold increase in [3H]Ins(1,3,4,5)P₄ compared with basal values (2955 ± 472 d.p.m./well) at 20 min. Lithium produced a concentration-dependent decrease in the levels of Ins(1,3,4,5)P₄ with a similar IC₅₀ value of 0.59 ± 0.27 mM to that for Ins(1,4,5)P₃. Lithium at a concentration of 10 mM produced a maximum inhibition of carbachol-stimulated Ins(1,3,4,5)P₄ levels of 88.2 % compared with carbachol-stimulated values (12922 ± 917 d.p.m./well).

In the presence of carbachol alone (1 mM) Ins(1,4)P₂ levels only increased by 1.7-fold over
basal (2974 ± 106 d.p.m./well) (Fig. 6.7.). Lithium (0.1-10 mM) had no effect on Ins(1,4)P₂ accumulation except at the highest concentration (10 mM) where a small increase to 3.5-fold over basal was observed. The EC₅₀ of this effect could therefore not be calculated, however it is likely to be greater than 3 mM.

[³H]Ins(4,5)P₂ levels increased 47.3-fold over a very low basal accumulation (30 ± 5 d.p.m./well) in the presence of carbachol (1 mM) alone (Fig. 6.8.). Lithium produced a concentration dependent increase in the levels of Ins(4,5)P₂ with an EC₅₀ of 0.33 ± 0.07 mM. A maximal increase was observed with 3 mM lithium, which produced a 3.9-fold increase over carbachol-stimulated values (1438 ± 67 d.p.m./well).

Carbachol alone (1 mM) produced a 11.6-fold increase over basal values (222 ± 8 d.p.m./well) in the accumulation of [³H]Ins(1,3,4)P₃ (Fig. 6.8.). Lithium had no effect on carbachol-stimulated levels of Ins(1,3,4)P₃ except at the highest concentration examined (10 mM) where a small 3.3-fold increase over the carbachol-stimulated values (2574 ± 103 d.p.m./well) was observed. Therefore, the EC₅₀ for this effect is greater than 3 mM, similar to that for Ins(1,4)P₂. This is not surprising since both isomers can be metabolised by the same 1-phosphatase within the cell (see Shears, 1991).

The levels of the metabolite of Ins(1,3,4)P₃, Ins(3,4)P₂, were enhanced upon the addition of carbachol alone (1 mM) (Fig. 6.7.). Such that at 20 min this agonist produced a 16.7-fold increase over basal (323 ± 18 d.p.m./well) in the accumulation of this isomer. Lithium produced a concentration-dependent decrease in the carbachol-stimulated levels of Ins(3,4)P₂ (IC₅₀ = 1.40 ± 0.05 mM) which was maximal with 10 mM lithium (79.8 % decrease compared with carbachol-stimulated values; 5423 ± 48 d.p.m./well).

Finally, the effects of lithium on the inositol monophosphates are shown in figure 6.6. Carbachol alone (1 mM) produced a 9.6-fold increase and a 5.2-fold increase over basal values (2370 ± 333 d.p.m./well and 3422 ± 510 d.p.m./well, respectively) in the accumulation of Ins(1/3)P₁ and Ins(6)P₁ respectively. Lithium produced a concentration-dependent increase in the agonist-stimulated accumulation of both isomers. Ins(1/3)P₁ levels were increased to a
Figure 6.6. Concentration-dependent Effects of Lithium on Carbachol-Stimulated $\text{Ins}(1/3)P_1$ and $\text{Ins}(4)P_1$ levels in CHO-M1 cells.

$[^{3}\text{H}]$Inositol labelled CHO-M1 cells were stimulated with carbachol in the presence of increasing concentrations of lithium (0.1-10 mM) for 20 min and the levels of the isomers $\text{Ins}(1/3)P_1$ and $\text{Ins}(4)P_1$ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.
Figure 6.7. Concentration-dependent Effects of Lithium on Carbachol-Stimulated Ins(1,4)P$_2$ and Ins(3,4)P$_2$ levels in CHO-M1 cells.

[$^3$H]Inositol labelled CHO-M1 cells were stimulated with carbachol in the presence of increasing concentrations of lithium (0.1-10 mM) for 20 min and the levels of the isomers Ins(1,4)P$_2$ and Ins(3,4)P$_2$ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.
**Figure 6.8.** Concentration-dependent Effects of Lithium on Carbachol-Stimulated Ins(4,5)P$_2$ and Ins(1,3,4)P$_3$ levels in CHO-M1 cells.

[3H]Inositol labelled CHO-M1 cells were stimulated with carbachol in the presence of increasing concentrations of lithium (0.1-10 mM) for 20 min and the levels of the isomers Ins(4,5)P$_2$ and Ins(1,3,4)P$_3$ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.
**Figure 6.9.** Concentration-dependent Effects of Lithium on Carbachol-Stimulated Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ levels in CHO-M1 cells.

[³H]inositol labelled CHO-M1 cells were stimulated with carbachol in the presence of increasing concentrations of lithium (0.1-10 mM) for 20 min and the levels of the isomers Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ were measured as described in the Methods. Data represent the means ± s.e.m. of three experiments.
maximum of 4.3-fold over carbachol controls (22756 ± 1877 d.p.m./well) with 10 mM lithium, with an EC\textsubscript{50} for this effect of 0.39 ± 0.06 mM. Ins(4)P\textsubscript{1} levels were increased to a maximum of 3.7-fold over carbachol control values (17756 ± 928 d.p.m./well) with 10 mM lithium. The EC\textsubscript{50} for this effect was 0.63 ± 0.27 mM. Both of these values were comparable with the EC\textsubscript{50} values observed for the effect of lithium on monophosphate accumulation in rat cerebral cortex slices (see Chapter 4) and the IC\textsubscript{50} value for lithium inhibition of the inositol monophosphatase enzyme.
6.3 Discussion.

It was initially suggested that the metabolism of Ins(1,4,5)P₃ in cells was via a simple dephosphorylation route, whereby Ins(1,4,5)P₃ was sequentially dephosphorylated to Ins(1,4)P₂ and then Ins(1)P₁ before being metabolised to release free inositol (Storey et al., 1984). Later studies, however have revealed that this route is not the only means most cells employ and the metabolism of Ins(1,4,5)P₃ is much more complex, proceeding via a number of distinct routes (see Shears, 1991; also see introduction). As described here, this is as true for CHO-M1 cells as it is for cerebral cortex.

The production of Ins(1,4,5)P₃ in CHO-M1 cells appears to be subtly different from that observed in cerebral cortex slices. Unlike the rapid and sustained increase observed in cerebral cortex (Kennedy et al., 1990; Batty et al., 1989; also see chapter 3) the Ins(1,4,5)P₃ response to carbachol in CHO-M1 cells is biphasic, composed of an initial rapid increase which peaks at 10 s before falling and a secondary slow rising plateau phase which is achieved over the second half of the time-course. This response is therefore more characteristic of the agonist-stimulated Ins(1,4,5)P₃ response previously observed in carbachol-stimulated SH-SY5Y cells (Lambert & Nahorski, 1990), bombesin-stimulated AR4-2J cells (Menniti et al., 1991) and carbachol-stimulated CHO-M3 cells (Tobin et al., 1992). The lack of ability to sustain the initial peak response has been attributed, at least in CHO-M3 cells, to a rapid desensitization of the response, possibly due to phosphorylation of the muscarinic receptor (Tobin et al., 1992). Indeed, it has been demonstrated that in CHO-M3 cells the muscarinic receptor is rapidly phosphorylated on one or more serine residues by a novel non-BARK receptor kinase (Tobin et al., personal communication). However, other theories may explain this rapid but transient Ins(1,4,5)P₃ response in these cells (see Chapter 7).

The metabolism in of Ins(1,4,5)P₃ in CHO-M1 cells appears to be via both 5-phosphatase and 3-kinase routes, since substantial levels of both Ins(1,4)P₂ and Ins(1,3,4,5)P₄ are found within these cells, following agonist-stimulation. The ratio of Ins(1/3)P₁ : Ins(4)P₁ in the presence of lithium should reflect the proportion of Ins(1,4,5)P₃ metabolised via 3-kinase and
5-phosphatase respectively, assuming PtdIns(4,5)P₂ is the sole substrate for PI-PLC and that Ins(1,4)P₂ is not dephosphorylated to Ins(1)P. Ins(1,4,5)P₃ appears to be metabolised equally by 3-kinase and 5-phosphatase activities at least over the initial 10 min of the time course, after which Ins(4)P₁ levels plateau whereas Ins(1/3)P₁ levels continue to increase linearly (see later). This is different from what has been observed in cerebral cortex were a substantially larger proportion of Ins(1,4,5)P₃ is metabolised via the phosphorylation route to Ins(1,3,4,5)P₄ (see chapter 4).

The 5-phosphatase involved in the dephosphorylation of Ins(1,4,5)P₃ to Ins(1,4)P₂ has been shown to consist of two activities, one of which is particulate and the other soluble (see Erneux & Takazawa, 1991). Both activities rapidly dephosphorylate Ins(1,4,5)P₁ and this can clearly be seen in the profile of the time-course of carbachol-stimulated Ins(1,4)P₂ accumulation. With agonist alone there is an initial peak in the levels of Ins(1,4)P₂ at 10 s, similar to that observed with Ins(1,4,5)P₃. The levels of this bisphosphate then fall before increasing again to a slow rising plateau. The close relationship between the profiles of Ins(1,4,5)P₃ and Ins(1,4)P₂ together with the similar temporal aspects of the responses clearly suggest that this constitutes a rapid dephosphorylation that is mediated by the 5-phosphatase in these cells. Ins(1,4)P₂ is the most abundant isomer formed upon receptor-stimulation in this cell type, similar to a number of other preparations including cerebral cortex (Batty et al., 1989), GH₃ cells (Hughes & Drummond, 1987; Dean & Moyer, 1987, 1988) and adrenal glomerulosa cells (Ball et al., 1988).

Agonist-stimulated levels of Ins(1,3,4,5)P₄ show a very different profile to those observed for either Ins(1,4,5)P₃ or Ins(1,4)P₂. Ins(1,3,4,5)P₄ levels increase gradually over the time-course before reaching a plateau at 20 min. The metabolism of Ins(1,3,4,5)P₄ is via a 5-phosphatase to Ins(1,3,4)P₃ (Hansen et al., 1987; Erneux et al., 1989). The 5-phosphatase involved in this dephosphorylation being the same enzyme which is involved in the metabolism of Ins(1,4,5)P₃ to Ins(1,4)P₂ (Hansen et al., 1987; Erneux et al., 1989). It is therefore
possible that at high concentrations Ins(1,3,4,5)P_4 may compete with the metabolism of Ins(1,4,5)P_3, perhaps prolonging the Ins(1,4,5)P_3 signal (see Erneux & Takazawa, 1991).

The 5-phosphatase has also been shown not to be inhibited by the metabolites it produces (Ins(1,4)P_2 and Ins(1,3,4)P_3) (Downes et al., 1982; Connolly et al., 1985; Hughes & Shears, 1990). The accumulation of Ins(1,3,4)P_3 as would be expected, therefore, has a similar profile to that of its precursor Ins(1,3,4,5)P_4.

The metabolism of Ins(1,3,4)P_3 appears to be unusual in these cells compared with other cell types in which inositol (poly)phosphate metabolism has been examined. For example, it has been shown that the metabolism of Ins(1,3,4)P_3 can proceed via two completely separate routes involving either a 1-phosphatase or a 4-phosphatase (Inhorn et al., 1987; Bansal et al., 1987). Thus the levels of both Ins(3,4)P_2 and Ins(1,3)P_2 increase upon receptor activation in a number of tissues (Balla et al., 1988; Guse et al., 1989; Hughes & Putney, 1989). Although substantial levels of Ins(3,4)P_2 are present in agonist-stimulated CHO-M1 cells, no agonist-stimulated accumulation of the Ins(1,3)P_2 isomer were observed using h.p.l.c. separation of the inositol phosphate isomers. This could suggest that the 4-phosphatase enzyme is either not present in CHO-M1 cells or that the activity/affinity of this enzyme for Ins(1,3,4)P_3 compared to that of the 1-phosphatase is low. Another more trivial explanation is that the Ins(1,3)P_2 and Ins(1,4)P_2 isomers are either not being fully resolved by the h.p.l.c. system being employed or that they are not being correctly identified. The elution time of Ins(1,4)P_2 has been confirmed using a [3H]Ins(1,4)P_2 standard. Also Ins(1,3)P_2 obtained from cerebral cortex slice preparations has been shown to elute at an earlier time (see Chapter 5), it therefore appears from this work that CHO-M1 cells do not produce Ins(1,3)P_2 as a metabolite of Ins(1,3,4)P_3.

Previous work in homogenates of liver (Shears et al., 1987), rat brain (Erneux et al., 1987) and GH4C1 cells (Irvine et al., 1987) suggests that Ins(1,3,4)P_3 is degraded predominantly to Ins(3,4)P_2. Indeed, studies in both GH3 cells (Hughes & Drummond, 1987; Dean & Moyer, 1987) and polymorphonuclear leucocytes (Dillon et al., 1987) have examined the individual
inositol phosphates isomers formed after agonist-stimulation and have failed to observe an increase in the levels of Ins(1,3)P$_2$. However, at least in one of these studies (Dean & Moyer, 1987), this may have been due to a misinterpretation of the h.p.l.c. data obtained, since the data clearly shows an early running bisphosphate peak which could relate to an Ins(1,3)P$_2$ fraction.

The above data, therefore suggest that Ins(1,3,4)P$_3$ is metabolised solely via the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase to Ins(3,4)P$_2$. Looking at the profile for the agonist-stimulated accumulation of Ins(3,4)P$_2$ the accumulation of this bisphosphate is very similar to that observed for Ins(1,3,4,5)P$_4$. In maximally stimulated cells the level of Ins(1,3,4)P$_3$ are close to the $K_m$ of this enzyme, therefore the 1-phosphatase is not saturated during agonist-stimulation (Tarver et al., 1987; Hughes et al., 1989).

As mentioned earlier, the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase not only removes the 1-phosphate from Ins(1,3,4)P$_3$, but also is involved in the dephosphorylation of Ins(1,4)P$_2$ to Ins(4)P$_1$ (Inhorn & Majerus, 1987; Gee et al., 1988b). Ins(4)P$_1$ levels increase gradually over the time-course before plateauing between 20-30 min. The profile observed for agonist-stimulated levels of Ins(1,4,5)P$_3$ and Ins(1,4)P$_2$ are not seen in the accumulation of this monophosphate. There is a disagreement as to whether Ins(1,4)P$_2$ can be dephosphorylated by a 4-phosphatase to generate Ins(1)P$_1$. A number of studies have demonstrated that Ins(1,4)P$_2$ is dephosphorylated solely by the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase to form only Ins(4)P$_1$ (Morris et al., 1988; Inhorn & Majerus, 1988). However, recently indirect evidence has been put forward to suggest that in cerebral cortex slices Ins(1,4)P$_2$ may indeed be metabolized either via a 1-phosphatase or a 4-phosphatase, possibly leading to another route of formation for Ins(1)P$_1$ (Batty et al., 1992). However, further work is required to confirm this study.

Therefore in summary, in CHO-M1 cells Ins(1,4,5)P$_3$ metabolism is rapid and appears proceed via two separate routes similar to that described previously for a number of cell types.
including angiotensin II-stimulated adrenal glomerulosa cells (Balla et al., 1988) and GH3 cells
Hughes & Drummond, 1987; Dean & Moyer, 1988). The first is a simple dephosphorylation
pathway via Ins(1,4,5)P3 to Ins(4)P1 (and perhaps Ins(1)P1). The second route is more complex
with Ins(1,4,5)P3 first being phosphorylated to Ins(1,3,4,5)P4 before being sequentially
dephosphorylated through Ins(1,3,4)P3, to produce Ins(3,4)P2 and then Ins(3)P1. The
accumulation of Ins(4,5)P2 also opens up the possibility of a further third possible route of
metabolism of Ins(1,4,5)P3 in this cell type (see later; also see Chapter 5), however the source
of this inositol phosphate isomer still remains to be established.

The co-addition of lithium with agonist in this study has demonstrated the profound effects
that this ion has upon inositol (poly)phosphate metabolism in this cell type. It is important for
this study to examine these effects to establish whether the CHO-M1 cell-line is suitable as a
model of phosphoinositide metabolism in brain, in that lithium produces similar effects to those
observed upon phosphoinositide metabolism in cerebral cortical slices and that these effects
occur over similar concentration ranges.

The addition of lithium (5 mM) to agonist-stimulated cells produced no significant effect on
the initial peak Ins(1,4,5)P3 response, however after a lag period of approximately 10 min a
significant decrease in the stimulated levels of Ins(1,4,5)P3 was observed. This delay in the
onset of the action of lithium has also been noted in carbachol-stimulated cerebral cortex
(Kennedy et al., 1990) and in angiotensin II-stimulated adrenal glomerulosa cells (Balla et al.,
1988) where a similar 10-15 min lag period has been observed. However, no such effect was
observed in a similar study in TRH-stimulated GH3 cells where lithium had no significant effect
upon agonist-stimulated Ins(1,4,5)P3 accumulation (Hughes & Drummond, 1987), at least
over the time-course examined. It has been suggested that the inhibition of Ins(1,4,5)P3
accumulation is due to lithium inhibiting the inositol monophosphatase enzyme resulting in a
decrease in the recycling of inositol and thus affecting the free inositol content of the cell and in
turn a decrease in the ability to synthesize of the immediate precursor of Ins(1,4,5)P3,
PtdIns(4,5)P2. The lag period is thus suggested to be the time required to deplete inositol and
inositol phospholipid reserves within the cell and thus affect the availability of PtdIns(4,5)P$_2$ for hydrolysis by PI-PLC. If this hypothesis is correct then the IC$_{50}$ for the effect of lithium on Ins(1,4,5)P$_3$ should be in approximate agreement with that for the inhibition of the inositol monophosphatase and the accumulation of the inositol monophosphates. From the data depicted in figure 6.9, the IC$_{50}$ for the lithium inhibition of Ins(1,4,5)P$_3$ was 0.39 ± 0.03 mM, similar to that obtained for the accumulation of the monophosphates (EC$_{50}$ = 0.39 ± 0.06 for Ins(1/3)P$_1$ and 0.63 ± 0.27 for Ins(4)P$_1$; see below). These data are therefore in agreement with such a hypothesis. It seems likely, therefore, that the reduction in the accumulation of Ins(1,4,5)P$_3$ is probably due to a decrease in the production of Ins(1,4,5)P$_3$. The lack of effect of lithium upon agonist-stimulated Ins(1,4,5)P$_3$ levels in GH$_3$ cells may therefore reflect a larger inositol reserve within these cells making them less susceptible to the actions of lithium.

It has also been suggested that lithium addition may also result in a decrease in the levels of these inositol polyphosphate isomers indirectly by stimulating the dephosphorylation of Ins(1,4,5)P$_3$ (also see Chapter 4). Indeed, it has been shown by treatment of intact platelets with phorbol esters and subsequently assaying 5-phosphatase in cell-free extracts (King & Rittenhouse, 1989), and more convincingly in reconstitution experiments (Connolly et al., 1986) that 5-phosphatase activity is enhanced by PKC. However, such an effect upon the 5-phosphatase has not been demonstrated in any other cell type (e.g. Biden et al., 1987; Kennedy et al., 1988) Despite this, it has been suggested that lithium may indirectly activate PKC by producing an increase in agonist-stimulated DAG levels within the cell, thereby resulting in a stimulation of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_3$ dephosphorylation (Kennedy et al., 1990). Indeed, it has been shown that lithium can significantly enhance agonist-stimulated DAG levels in GH$_3$ cells (Drummond & Raeburn, 1984), rat hepatocytes (Bocckino, et al., 1985) and NG108-15 cells (Brami et al., 1991, 1993). This may also be an explanation for the enhanced accumulation of agonist-stimulated levels of Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ produced by lithium in cerebral cortex slices (see chapter 4). It is difficult to distinguish such an effect of lithium in this study on CHO-M1 cells since the higher concentration of lithium used (5 mM)
should significantly affect Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase activity.

Lithium produced a similar time-dependent decrease in the agonist-stimulated levels of
Ins(1,3,4,5)P₄, reflecting the decrease in the levels of the precursor of this isomer
Ins(1,4,5)P₃. As described previously for cerebral cortex slices, Ins(1,3,4,5)P₄ levels are only
reduced by up to 60-70% by even a maximal concentration of lithium (10 mM) (Whitworth &
Kendall, 1988; Kennedy et al., 1990; see chapter 4). At these concentrations of lithium
carbachol-stimulated Ins(1,4,5)P₃ levels are returned to basal values. The question therefore
arises as to where the remaining 30-40% lithium-insensitive Ins(1,3,4,5)P₄ pool is generated
from? One possibility is that the Ins(1,3,4,5)P₄ fraction collected may be contaminated with
another InsP₄ isomer which accumulates rapidly upon agonist addition but which is not
significantly affected by lithium addition. One possible candidate could be Ins(3,4,5,6)P₄. This
isomer has been demonstrated to accumulate rapidly upon agonist-stimulation in FMLP-
stimulated HL60 cells (Pittet et al., 1989) and in bombesin-stimulated AR4-2J cells (Menniti,
1990). The effects of lithium upon this isomer are unclear, however the lithium-insensitive
component could possibly be due to accumulation of this isomer in CHO-M1 cells.

The effects of lithium upon agonist-stimulated Ins(1,4)P₂ levels are more complex. Although
the 5-phosphatase involved in the production of Ins(1,4)P₂ is not lithium sensitive (see Shears,
1991), the Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase has been shown to be lithium sensitive
(Inhorn & Majerus, 1987; Gee et al., 1988b). The purified 1-phosphatase enzyme has a Kᵢ
value for lithium of approximately 9.63 mM for Ins(1,4)P₂ and 0.46 mM for Ins(1,3,4)P₃
(Inhorn & Majerus, 1987; Gee et al., 1988b). The data shown in figure 6.7 would suggest an
EC₅₀ value for lithium for the accumulation of Ins(1,4)P₂, and hence inhibition of the 1-
phosphatase, greater than 3 mM, albeit at a single time point of 20 min. In the presence of
agonist and lithium (5 mM) therefore Ins(1,4)P₂ levels would be expected to rise. Figure 6.3.
does this to be the case. Ins(1,4)P₂ levels increase over the initial 10 min of the time-course,
such that by this time carbachol-stimulated levels in the presence of lithium are 3.4-fold greater
than those in the absence of lithium. After this initial increase, Ins(1,4)P$_2$ levels fall probably due to the decreasing Ins(1,4,5)P$_3$ production. Indeed by 30 min agonist-stimulated Ins(1,4)P$_2$ levels are significantly lower in the presence of lithium compared to in its absence. A strikingly similar action of lithium on agonist-stimulated levels of Ins(1,4)P$_2$ have also been observed in adrenal glomerulosa cells (Balla et al., 1988).

An almost identical response to the addition of lithium was observed in the agonist-stimulated accumulation of Ins(1,3,4)P$_3$, the 5-phosphatase metabolite of Ins(1,3,4,5)P$_4$. In the presence of agonist and lithium Ins(1,3,4)P$_3$ levels increased over the initial 10 min of the time course to a maximum of 2.3-fold over carbachol control values at 10 min before falling to below carbachol control values by 30 min. This is not surprising since Ins(1,3,4)P$_3$ is dephosphorylated by the same Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase as Ins(1,4)P$_2$ (Inhorn et al., 1987). The EC$_{50}$ for accumulation of agonist-stimulated Ins(1,4)P$_2$ levels produced by lithium was similar to that obtained for Ins(1,3,4)P$_3$, i.e. greater than 3 mM.

The effect of lithium upon both Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ accumulation would be expected to be greater at the earlier 5 and 10 min time points; this can be inferred from figures 6.3 and 6.4 respectively where the effect of lithium is greater at these time points than at 20 min.

The inhibition of the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase is also reflected in the accumulation of the products of this enzyme. The accumulation of carbachol-stimulated Ins(3,4)P$_2$ was reduced in the presence of lithium (54.0 % inhibition at 30 min). The 4-phosphatase involved in Ins(3,4)P$_2$ metabolism is not inhibited by lithium, therefore, because of the decrease in the rate of Ins(3,4)P$_2$ production, the levels of this bisphosphate are lower in the presence of agonist and lithium compared with agonist alone. This is comparable with the effects of lithium on this isomer in angiotensin II-stimulated adrenal glomerulosa cells (Balla et al., 1988), however no such effect was observed in a similar study in GH3 cells (Hughes & Drummond, 1987) or cerebral cortex slices (Jenkinson et al., 1992; see chapter 4).

One point of interest is in relation to the lack of any observed accumulation of Ins(1,3)P$_2$ in
these cells (see above). As mentioned previously this could possibly be due to a highly active 1-phosphatase "swamping" a poorly active 4-phosphatase resulting in the preferential formation of Ins(3,4)P$_2$ from Ins(1,3,4)P$_3$. The results presented here could be interpreted as arguing against this point since the addition of lithium at the highest concentration (10 mM) should inhibit the 1-phosphatase sufficiently to allow any 4-phosphatase activity, as measured by the production of Ins(1,3)P$_2$, to be observed. However if the metabolism of Ins(1,3)P$_2$ is rapid there may be no accumulation of this isomer. The lack of Ins(1,3)P$_2$ in these cells in the presence of lithium is therefore difficult to explain.

The metabolism of the inositol monophosphates is via a single enzyme, the inositol monophosphatase (Ackerman et al., 1987; Gee et al., 1988a). In most other tissues this enzyme is uncompetitively inhibited by lithium in the sub-millimolar range (IC$_{50}$ for agonist-stimulated InsP$_1$ accumulation ~ 0.5 mM in rat cortex (Kennedy et al., 1989; Whitworth & Kendall, 1988; Rooney & Nahorski, 1989)). The EC$_{50}$ for the accumulation of the inositol monophosphates in this study (0.39 ± 0.06 mM and 0.63 ± 0.27 mM for the accumulation of Ins(1,3)P$_1$ and Ins(4)P$_1$ respectively) would suggest that the monophosphatase enzyme in CHO-M1 cell has a similar K$_i$ for lithium in the region of 0.4-0.6 mM. Ins(4)P$_1$ metabolism is therefore complicated by the fact that both the enzymes involved in its production and dephosphorylation are lithium-sensitive, albeit with different K$_i$ values for lithium. In the presence of lithium, carbachol-stimulated Ins(4)P$_1$ levels increase over the initial 10 min of the time-course to 4.6-fold over carbachol control values. However, unlike the linear accumulation of the inositol monophosphates observed in cerebral cortex slices, the levels of Ins(4)P$_1$ plateaued after 10 min. This is likely to be related to the decrease in the production of Ins(1,4,5)P$_3$ resulting in a subsequent decrease in the levels of Ins(1,4)P$_2$ and hence Ins(4)P$_1$. However, in angiotensin II-stimulated adrenal glomerulosa cells although the Ins(1,4,5)P$_3$ and Ins(1,4)P$_2$ profiles in the presence of lithium were similar to those observed in CHO-M1 cells (i.e. Ins(1,4,5)P$_3$ levels return to basal by 30 min) no plateauing of the linear increase in the
accumulation of the Ins(4)P₁ isomer was observed over the time-course examined (Balla et al., 1988).

In contrast Ins(1/3)P₁ levels in the presence of agonist and lithium accumulated in a linear manner over the time-course examined. This was similar to the accumulation of this isomer in a number of other preparations including GH₃ cells (Hughes & Drummond, 1987) and adrenal glomerulosa cells (Balla et al., 1988). The difference observed in the agonist-stimulated accumulation of Ins(1/3)P₁ and Ins(4)P₁ in the presence of lithium may be due to Ins(1,3,4,5)P₄ buffering the levels of Ins(1/3)P₁ such that levels accumulate slowly but steadily over the time course. Alternatively, the addition of lithium to the stimulated cells may produce such a reduction in the level of PtdIns(4,5)P₂, the preferred substrate of PLC, that PI-PLC may start to hydrolyse PtdIns (and PtdIns(4)P) generating Ins(1)P₁ directly, bypassing Ins(1,4,5)P₃ and the rest of the cycle. Although PLC can hydrolyse PtdIns in reconstitution studies (Rhee et al., 1988; Meldrum et al., 1991) it has not been conclusively shown in intact cells. However, there is indirect evidence for such an action of PLC from a number of independent studies (Wilson et al., 1985; Imai & Gershengorn, 1986; Biden et al., 1992; see Chapter 7 for a fuller discussion).

Finally, detectable levels of the bisphosphate isomer Ins(4,5)P₂ can be found in CHO-M1 cells. The source of this bisphosphate is still undetermined (see Chapter 5), however it may constitute an alternative third route of metabolism for Ins(1,4,5)P₃ in these cells as it has been found to be in the slime mould Dictyostelium discoideum (Van Lookeren Campagne et al., 1988). In carbachol-stimulated CHO-M1 cells levels of this isomer increased slowly over the time-course examined, reaching 13.0-fold over basal by 30 min. As observed in brain slice experiments, the agonist-stimulated accumulation of this bisphosphate isomer was substantially enhanced by the addition of lithium (5 mM) in CHO-M1 cells with the accumulation of Ins(4,5)P₂ in the presence of lithium being linear with time. With the substantial decrease in agonist-stimulated Ins(1,4,5)P₃ levels produced by lithium a linear increase in the levels of this bisphosphate isomer would be unexpected if the precursor of this isomer was indeed
Ins(1,4,5)P$_3$. An alternatively source of Ins(4,5)P$_2$, as was discussed in chapter 5, could however be via PLD hydrolysis of PtdIns(4,5)P$_2$ with PLD being activated, as it is in number of other tissues (Billah & Anthes, 1990), by the increase in intracellular Ca$^{2+}$ produced by the increase in Ins(1,4,5)P$_3$ levels.

The enhancement in the carbachol-stimulated accumulation of Ins(4,5)P$_2$ by lithium appears to be concentration-dependent with respect to lithium (EC$_{50}$ = 0.33 ± 0.07 mM) adding further weight to the suggestion that the metabolism of Ins(4,5)P$_2$ is via a novel lithium sensitive enzyme, possibly a 4- or 5-phosphatase (Jenkinson et al., 1992). Previous results from cerebral cortex slices experiments have also suggested that the enzyme involved in the production of Ins(4,5)P$_2$ may be lithium-sensitive, although at higher concentrations than those required to inhibit Ins(4,5)P$_2$ dephosphorylation, since the concentration-response curve for the accumulation of Ins(4,5)P$_2$ to lithium was bell-shaped. No bell-shaped curve was obtained in CHO-M1 cells suggesting that the enzyme involved in Ins(4,5)P$_2$ production is either not lithium sensitive in CHO-M1 cells or is poorly inhibited by this ion.

In conclusion it would appear that the metabolism of the inositol (poly)phosphates in CHO-M1 cells is similar in a number of respects to that observed in cerebral cortex slices, although by no means identical. The lithium sensitivity of both the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase and the inositol monophosphatase were found to be similar to that found in brain and lithium appeared to have a similar disruptive effect upon phosphoinositide metabolism in CHO-M1 cells to that found in cerebral cortex slices (i.e. similar concentration and temporal effects of lithium upon agonist-stimulated inositol polyphosphate levels). This preliminary study, therefore, suggests that the CHO-M1 cell-line may be useful in examining the effects of lithium of phosphoinositide signalling. This possibility is further examined in the following chapter.
Chapter 7

Disruption of Phosphoinositide Signalling by Lithium in CHO Cells Expressing Recombinant M1 Muscarinic Receptors.
7.1. Introduction.

The work presented in this chapter examines the effect of lithium upon phosphoinositide metabolism in the Chinese hamster ovary (CHO) cell line which has been transfected to express the M1 muscarinic receptor subtype.

The metabolism of Ins(1,4,5)P$_3$ has been shown in cerebral cortex to be via two routes (see Shears, 1991). Ins(1,4,5)P$_3$ can be dephosphorylated to Ins(1,4)P$_2$ or phosphorylated by a 3-kinase to form Ins(1,3,4,5)P$_4$. This second phosphorylation route to Ins(1,3,4,5)P$_4$ is a major route of metabolism of Ins(1,4,5)P$_3$ in rat cerebral cortex slices however a number of cell types including Dictyostelium discoideum (van Haastert et al., 1989), ram sperm (Harrison et al., 1990) and squid photoreceptors (Wood et al., 1989) have been shown not to produce Ins(1,3,4,5)P$_4$ due to a lack of 3-kinase activity, making them unattractive as models of cerebral cortical phosphoinositide metabolism. As discussed in Chapter 6 the metabolism of the Ins(1,4,5)P$_3$ in CHO cells results in the formation of both Ins(1,4)P$_2$ and Ins(1,3,4,5)P$_4$, therefore possibly making this cell line a reasonable model for phosphoinositide metabolism in cerebral cortex.

In this chapter the study moves on from these preliminary experiments and attempt to more fully characterise the effects of lithium upon phosphoinositide signalling in this system by examining the effect of this ion on the other intermediates of this signalling pathway. As discussed in Chapters 3 and 4, addition of lithium to carbachol-stimulated cerebral cortex slices results in a time-dependent linear increase in the levels of InsP$_1$ (Ins(1/3)P$_1$ and Ins(4)P$_1$) (Kennedy et al., 1989) by virtue of lithium acting as an uncompetitive inhibitor of the inositol monophosphatase enzyme (Gee et al., 1988a.). This was interpreted as being the result of a decrease in the free inositol levels within the cells which was demonstrated indirectly in this preparation by an increase in the levels of CMP-PA (Godfrey, 1989; Kennedy et al., 1990), the co-substrate with inositol for the enzyme PtdIns synthase. Lithium also causes a decrease in agonist-stimulated levels of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ (see Chapter 3) in
cortical slices after a lag period of 10-15 min (Kennedy et al., 1989, 1990). The hypothesis proposed to reconcile these effects of lithium is that the inhibition of the inositol monophosphatase by lithium results in a decrease in the levels of free inositol within the cell (normally regenerated from the metabolism of the polyphosphates)(although also see Chapter 1). Therefore if the concentration of inositol within most cells is close to the $K_m$ for inositol of the enzyme PtdIns synthase (see Nahorski et al., 1991), the drop in inositol levels will result in a decrease in the rate of formation of the inositol phospholipids, PtdIns, PtdIns(4)P and the precursor for Ins(1,4,5)P$_3$, PtdIns(4,5)P$_2$, under conditions of continuous stimulation. It is therefore suggested that the time required for the lithium-induced decrease in agonist-stimulated Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ formation is a reflection of the time required for the depletion of inositol within the cell and in turn the inositol phospholipids, especially PtdIns(4,5)P$_2$. This hypothesis adequately explains all the observations thus far observed in Chapter 4. However, one anomaly does exist which questions the validity of this hypothesis.

Although it can shown that upon addition of lithium to agonist-stimulated cells that inositol, and after a subsequent lag period, Ins(1,4,5)P$_3$ levels decrease it has not been possible to demonstrate a significant effect of lithium upon agonist-stimulated levels of PtdIns(4,5)P$_2$. A number of studies in thyrotropin-releasing hormone (TRH)-stimulated GH$_3$ cells (Drummond, 1987), angiotensin II-stimulated adrenal glomerulosa cells (Balla et al., 1988) and carbachol-stimulated parotid gland slices (Downes & Stone, 1986) have failed to show this hypothesised effect of lithium upon agonist-stimulated PtdIns(4,5)P$_2$ levels. However, a number of studies have demonstrated that lithium can cause a decrease in agonist-stimulated levels of PtdIns (Downes & Stone, 1986; Drummond, 1987; Balla et al., 1988) and indeed PtdIns(4)P (Balla et al., 1988). Several reasons have been put forward to explain this apparent flaw in an otherwise elegant hypothesis. Firstly, it is possible that only a small fraction of the total PtdIns(4,5)P$_2$ pool within the cell is agonist-sensitive and therefore available for the generation of Ins(1,4,5)P$_3$. Indeed, in the presence of agonist PtdIns(4,5)P$_2$ levels in both adrenal
glomerulosa cells (Balla et al., 1988) and rat parotid gland (Downes & Wusteman, 1983) are reduced by 50-60% suggesting that there is an agonist-insensitive pool of PtdIns(4,5)P$_2$ (~40%). Any changes in the levels of PtdIns(4,5)P$_2$ may therefore be small and possibly not detectable by the methods used. Indeed it is known that of the total inositol phospholipid present within the cell PtdIns(4,5)P$_2$ only constitutes ~5% of this pool of lipids, whereas PtdIns constitutes ~85%, at least in CHO cells (see Results section of this chapter for exact percentages). A second reason for the failure to observe any effect of lithium upon PtdIns(4,5)P$_2$ levels is particularly pertinent when considering brain slice preparations. Since a brain slice is a heterogeneous population of vastly differing cell types (both neuronal and glial) it may be that differences in the response to either agonist or lithium, or a difference in the $K_m$ for the PtdIns synthase enzyme between cell types, may mask the effect of lithium upon agonist-stimulated PtdIns(4,5)P$_2$ levels which may only occur in a small population of cells within the preparation.

Therefore, by employing a homogeneous cell population, the CHO-M1 cell type, some of these problems may be overcome and the results obtained may be more definitive. The lithium sensitivity of the agonist-stimulated Ins(1,4,5)P$_3$ response observed in CHO-M1 cells (see Chapter 6) suggests that the PtdIns(4,5)P$_2$ levels within these cells do decrease upon addition of agonist and lithium. Indeed, in GH$_3$ cells stimulated with TRH only a very small increase in the levels of CMP-PA were observed, with no reduction in agonist-stimulated levels of Ins(1,4,5)P$_3$ (Hughes & Drummond, 1987) and as mentioned above no effect on PtdIns(4,5)P$_2$ levels was observed. It therefore appears that in this cell type inositol uptake into the cells is great enough to compensate for the loss of inositol obtained from the recycling of the inositol polyphosphates. Alternatively, the $K_m$ of inositol for the PtdIns synthase within GH$_3$ cells may be low, i.e. in the micromolar range, therefore a small decrease may not affect the synthesis of the inositol phospholipids, unlike brain slices where the $K_m$ for the PtdIns synthase for inositol may be higher, i.e. in the mM range, similar to the concentration of inositol in this preparation (2-4 mM in incubated slices (Heacock et al., 1993)) therefore any
small decrease in the levels of inositol would result in a decrease in the formation of PtdIns and
hence PtdIns(4,5)P₂. Similarly in one of the other tissues where PtdIns(4,5)P₂ levels has been
examined, namely parotid gland, CMP-PA accumulation was not immediate, as it is in cerebral
cortex, but instead only started to accumulate after a lag of 15 min (Downes & Stone, 1986),
suggesting that these cells have a substantial inositol reserve which may be able to buffer the
levels of PtdIns(4,5)P₂ at least over the time-course examined in the above study (Downes &
Stone, 1986). CHO-M1 cells therefore may be a more suitable model when examining the
effects of lithium on phosphoinositide synthesis.

The work presented in this chapter will therefore hopefully clarify the mechanism by which
lithium interferes with phosphoinositide signalling and produces a reduction in the levels of the
second messenger Ins(1,4,5)P₃.
7.2 Results.

7.2.1 The effect of carbachol and lithium upon Ins(1,4,5)P3 levels in CHO-M1 cells.

The effects of carbachol and lithium upon Ins(1,4,5)P3 levels were examined in CHO cells expressing the M1 muscarinic receptor subtype (Bmax = 816 fmol / mg protein) used previously in the studies described in Chapter 6.

The concentration-dependency of the carbachol-stimulated accumulation of Ins(1,4,5)P3 mass was examined both at the peak (10s) and the plateau (20 min) of the biphasic response (Fig. 7.1.) observed in Chapter 6. Carbachol (0.1-1000 μM) produced a concentration-dependent increase in the accumulation of Ins(1,4,5)P3 both at 10 s and 20 min, with EC50 values of 27.6 ± 7.1 μM and 6.3 ± 1.3 μM, respectively. The agonist-stimulated levels of Ins(1,4,5)P3 observed with maximal carbachol concentrations was significantly greater at the 10 s time point compared with the 20 min time point (597.3 ± 7.1 pmol/mg protein compared with 391.2 ± 23.0 pmol/mg protein, respectively at 100 μM carbachol).

The addition of carbachol (1mM) produced a rapid increase in Ins(1,4,5)P3 mass levels which reached a maximum of 21.7-fold over basal at 10 s before falling to 8.2-fold over basal by 1min. Subsequently, a slower rise was observed which reached a plateau of 18.3-fold over basal between 10-20 min after agonist addition. Both the peak and plateau response to carbachol were prevented by addition of the muscarinic antagonist atropine (10 μM) (data not shown). Lithium alone (5 mM) had no significant effect upon basal levels (Basal values in pmol / mg protein: - Li+ = 28.8 ± 4.3 ; + Li+ = 24.4 ± 4.8). Lithium (5mM) in the presence of carbachol (1mM) had no significant effect on the initial rapid production of Ins(1,4,5)P3 observed with carbachol alone (Fig. 7.2. A.), however the secondary plateau phase of the response was significantly reduced after 10 min by the addition of lithium, and by 30 min Ins(1,4,5)P3 levels were reduced by 77.4 % compared with carbachol control values (Fig. 7.2.B.).

Figure 7.3.A. shows the effects of lithium upon agonist-stimulated Ins(1,4,5)P3
Figure 7.1. Concentration-dependency of the Effects of Carbachol on Peak and Plateau Ins(1,4,5)P$_3$ Responses.

The effect of increasing concentrations of carbachol (0.1 - 1000 μM) on the accumulation of Ins(1,4,5)P$_3$ were examined at 10 s (●) and 20 min (○) time points (peak and plateau of the response respectively). Data represent the means ± S.E.M. for three experiments each performed in triplicate.
Figure 7.2. Effect of Lithium Upon The Time-Course of Carbachol-stimulated Ins(1,4,5)P₃ Mass Accumulation in CHO-M1 Cells.

The effect of lithium (5 mM) on mass Ins(1,4,5)P₃ accumulation in CHO-M1 cells stimulated with carbachol (1 mM) was examined. Cells were incubated with carbachol in the absence (○) or presence (●) of lithium for the indicated time periods. A. Expanded time-course of that shown in B. Data represent means ± S.E.M. for at least three experiments each performed in triplicate. Statistically significant differences in the effects of lithium on the agonist-stimulated groups are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
accumulation in CHO-M1 cells at 20 min. Lithium (0.1-10 mM) produced a concentration-
dependent decrease in the carbachol-stimulated (1mM) levels of Ins(1,4,5)P$_3$ with an IC$_{50}$
value of 0.51 ± 0.03 mM. Addition of 10mM lithium produced an 87.8 % inhibition of
carbachol-stimulated Ins(1,4,5)P$_3$ accumulation at 20 min.

7.2.2. Reversal of the effects of lithium upon Ins(1,4,5)P$_3$ mass levels by myo-inositol in
CHO-M1 cells.

Figure 7.3.B. shows the effects of myo-inositol addition upon agonist-stimulated
Ins(1,4,5)P$_3$ accumulation in the presence of lithium at 20 min. Cells were either preincubated
in normal buffer or buffer supplemented with myo-inositol (0.03-30 mM) for 30 min. Cells
were subsequently incubated with carbachol (1mM) and myo-inositol (0.03-30mM). Inositol
had no significant effect on basal or carbachol (1mM) stimulated values. Inositol did however
produced a concentration-dependent reversal of the lithium effect on Ins(1,4,5)P$_3$ mass (EC$_{50}$
value of 4.1 ± 0.1 mM) such that in the presence of 30mM myo-inositol Ins(1,4,5)P$_3$ levels
were not significantly different from those produced by carbachol (1mM) alone.

The effects of inositol on the time-dependent inhibition of Ins(1,4,5)P$_3$ mass levels are shown
in figures 7.4.A & B. Cells were either preincubated in normal buffer or buffer supplemented
with myo-inositol (10mM) for 30 min. Cells were subsequently incubated with either carbachol
alone (1mM), carbachol plus lithium (5mM) or carbachol plus lithium in the presence of myo-
inositol (10mM) for the times indicated. As described above carbachol alone produced a
characteristic peak-plateau response with only the plateau phase being inhibited by lithium
(5mM). In the presence of myo-inositol (10mM) the effect of lithium upon the plateau phase
was significantly reduced such that by 30 min in the presence of carbachol and lithium the
addition of myo-inositol (10mM) produced a 16.0 % reduction from carbachol control values
whereas carbachol plus lithium in the absence of myo-inositol produced a 79.5 % decrease in
Ins(1,4,5)P$_3$ mass levels (Fig. 7.4.B.). Myo-inositol (10mM) alone had no significant effect
Figure 7.3. Concentration-dependency Effect of Lithium and Inositol Upon Carbachol-stimulated Ins(1,4,5)P₃ Accumulation in CHO-M1 Cells.

A. CHO-M1 cells were incubated with carbachol (1 mM) and the indicated concentrations of lithium (0.1 - 10 mM) for 20 min. B. CHO-M1 cells were incubated with carbachol (1 mM) of carbachol plus lithium (5 mM) for 20 min. The effect the indicated concentrations of inositol (0.1 - 30 mM; added 30 min prior to drug addition) on carbachol plus lithium stimulated Ins(1,4,5)P₃ levels was examined after an incubation period of 20 min with drugs. Data represents means ± S.E.M. for at least three experiments each performed in triplicate.
Figure 7.4. The Effect of Inositol on the Time-dependent Inhibition of Carbachol-stimulated Ins(1,4,5)P₃ Levels by Lithium In CHO-M1 Cells.

The effects of inositol (10 mM) addition (30 min prior to drug addition) on the time-dependent inhibition of carbachol (1 mM) stimulated Ins(1,4,5)P₃ accumulation by lithium (5 mM) was examined in CHO-M1 cells. CHO-M1 cells were incubated with carbachol (○), carbachol plus lithium (●) or carbachol plus lithium plus inositol (▲) for the times indicated. A. Expanded time-course of that shown in B. Data represent means ± S.E.M. of three experiments each performed in triplicate.
on basal Ins(1,4,5)P3 levels or on the peak responses to carbachol either in the absence or presence of lithium (Fig. 7.4.A.).

7.2.3. The effects of lithium and myo-inositol upon carbachol-stimulated [3H]CMP-PA accumulation in CHO-M1 cells.

The addition of either carbachol (1mM) or lithium (5mM) alone produced in both instances a small but significant 1.3-fold increase in [3H]CMP-PA levels compared with basal values after a 30 min incubation (Basal value = 1408 ± 88 d.p.m. at 30 min compared with a basal value of 418 ± 53 at t0). Carbachol (1mM) in the presence of lithium (5mM) produced a rapid increase in the accumulation of [3H]CMP-PA after a delay of approximately 2 min. [3H]CMP-PA levels increased 10.5-fold over basal before plateauing at approximately 10 min. The levels remained at this value throughout the remainder of the time-course examined (Fig. 7.5.A.).

Figure 7.6.A. shows the effects of lithium upon agonist-stimulated [3H]CMP-PA accumulation at 20 min after agonist addition. Lithium (0.03-10 mM) produced a concentration-dependent increase in carbachol-stimulated [3H]CMP-PA levels (8.3-fold increase over basal in the presence of 10mM lithium) with an EC50 value of 0.49 ± 0.03 mM for lithium.

The inositol reversibility of the lithium induced accumulation of carbachol-stimulated [3H]CMP-PA is shown in Figure 7.6.B. Cells were preincubated for 30 min in buffer containing myo-inositol (0.1-30 mM). Subsequently, cells were incubated with carbachol (1mM) and lithium (5mM) in the presence of myo-inositol (0.1-30 mM; as above) for 20 min. Myo-inositol alone (10mM) had no significant effect upon basal [3H]CMP-PA values (Basal values at 30 min: -Ins = 1896 ± 102 d.p.m.; + Ins = 1789 ± 120 d.p.m.). Myo-inositol addition produced a concentration-dependent decrease of carbachol/lithium-stimulated [3H]CMP-PA accumulation with an IC50 value of 2.2 ± 0.1 mM. Addition of 10mM myo-inositol completely reversed the effects of lithium on agonist-stimulated [3H]CMP-PA levels, returning levels to basal values.
Figure 7.5. Effects of Lithium Upon Carbachol-Stimulated [3H]CMP-PA Accumulation In CHO-M1 cells.

The effects of lithium (5 mM) on carbachol (1 mM) stimulated [3H]CMP-PA accumulation in CHO-M1 cells was examined. For basal and carbachol-stimulated values see section 7.2.4. Data represent the means ± S.E.M. for three experiments each performed in triplicate.
Figure 7.6. The Effects of Lithium and Inositol Upon Carbachol-stimulated [3H]CMP-PA Accumulation in CHO-M1 Cells.

A. The concentration-dependency of the effect of lithium (0.03 - 10 mM) upon carbachol-stimulated [3H]CMP-PA accumulation was examined in CHO-M1 cells after an incubation period of 20 min with drugs. B. The concentration-dependency of the effects of inositol (0.1 - 30 mM; added 30 min prior to drug addition) upon carbachol plus lithium stimulated [3H]CMP-PA accumulation was examined in CHO-M1 cells after an incubation period of 20 min with carbachol and lithium. Data represents means ± S.E.M. for three experiments each performed in triplicate.
7.2.4. Time-course of the effects of carbachol and lithium upon inositol phospholipid levels in CHO-M1 cells.

CHO-M1 cells were labelled for 48 hrs in order to achieve equilibrium labelling of the inositol phospholipids. Under such labelling conditions the relative labelling of the individual inositol phospholipids PtdIns, PtdIns(4)P and PtdIns(4,5)P2 should correlate with the relative mass of each lipid. The relative levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P2 in unstimulated CHO-M1 cells was approximately 88%, 7% and 5% respectively.

The time-course of the effect of carbachol-stimulation, in the absence and presence of lithium (5mM), upon the levels of the individual phosphoinositides PtdIns, PtdIns(4)P and PtdIns(4,5)P2 was examined in CHO-M1 cells and is shown in figure 7.7.

Carbachol alone (1mM) produced a time-dependent decrease in PtdIns levels which reached a plateau after 20 min in these cells (Fig. 7.7.A.). At 30 min carbachol alone produced a 36.2% decrease in PI levels compared with basal values (Basal value = 98460 ± 1450 d.p.m./well). Lithium (5mM) alone had no significant effect on PI levels over the time-course examined. Lithium (5mM) in the presence of carbachol (1mM) caused a significantly greater decrease in PtdIns levels compared with carbachol alone, such that by 30 min PtdIns levels were reduced by 73.2% compared with basal (Fig. 7.7.A.).

PtdIns(4)P basal levels were lower than those for PtdIns (Basal value = 8092 ± 183 d.p.m./well; 8.2% of PtdIns basal). Carbachol alone (1mM) produced a rapid decrease of 58.6% in PtdIns(4)P levels compared with basal within the first 10 s after agonist addition (Fig. 7.7.B.). This level was maintained throughout the remainder of the time-course. Lithium alone (5mM) had no significant effect on PtdIns(4)P levels. Lithium (5mM) in the presence of carbachol (1mM) had no significant effect over the initial 10 min of the time-course when compared with the response of carbachol alone. However, at later time points lithium enhanced the decrease in PtdIns(4)P levels produced by carbachol alone, such that at 30 min carbachol in the presence of lithium produced a 77.1% decrease in PtdInsP levels compared with basal
Figure 7.7. Time-course of the Effects of Lithium Upon Inositol Phospholipid Levels in CHO-M1 Cells.

The effects of lithium (5 mM) upon carbachol-stimulated levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P2 were examined in CHO-M1 cells. Cells were incubated with carbachol in the absence (○) or presence (●) of lithium for the indicated periods of time. Data represents means ± S.E.M. for three experiments each performed in triplicate. Statistically significant differences in the effects of lithium on the agonist-stimulated groups are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
The images depict graphs showing the relationship between D.P.M./well and time (min) for different phosphoinositides (PtdIns, PtdIns(4)P, PtdIns(4,5)P2). Each graph includes data points at various time intervals (0, 5, 10, 15, 20, 25, 30 minutes) and indicates statistical significance with asterisks (*, **, ***). The graphs suggest a decline or increase in D.P.M./well over time, with some peaks and troughs, reflecting the dynamic nature of phosphoinositide metabolism or signaling. The data points are represented by different symbols (○, ●), and the error bars indicate variability or standard deviation.
values (Fig. 7.7.B.).

PtdIns(4,5)P₂ basal levels were lower than those of either PtdIns or PtdIns(4)P (Basal value = 4402 ± 598 d.p.m./well; 4.5 % of PtdIns basal). Carbachol alone produced a qualitatively similar initial decrease in PtdIns(4,5)P₂ values within the first 5 min to that described for PtdIns(4)P levels although the decrease was greater (78.7 % compared with basal value at 10 s) (Fig. 7.7.C.). Over the remainder of the time-course PtdIns(4,5)P₂ levels increased slowly such that by 30 min levels had recovered to 61.7 % of basal values. Lithium (5mM) in the presence of carbachol (1mM) had no effect on the initial rapid decrease in PtdIns(4,5)P₂ levels produced by carbachol, however it did prevent PtdIns(4,5)P₂ levels recovering such that PtdIns(4,5)P₂ levels in the presence of carbachol and lithium were only 30.4 % of basal values at 30 min (Fig. 7.7.C.).

7.2.5. The concentration-dependent effects of lithium and myo-inositol upon inositol phospholipid levels in carbachol-stimulated CHO-M1 cells.

The concentration-dependency of the effect of lithium on inositol phospholipid levels is shown in figure 7.8.

Carbachol (1mM) alone produced a 53.1 %, 51.6 % and 42.4 % decrease in [³H]PtdIns(4,5)P₂, [³H]PtdIns(4)P and [³H]PtdIns levels respectively in CHO-M1 cells compared with basal values (Basal values (in d.p.m.): PtdIns(4,5)P₂ = 9082 ± 249; PtdIns(4)P = 16072 ± 301; PtdIns = 211044 ± 21751)(Fig. 7.8.). Lithium alone (10mM) had no significant effect upon basal phosphoinositide levels. Lithium (0.1-10 mM) produced a concentration-dependent decrease in the levels of [³H]PtdIns(4,5)P₂, [³H]PtdIns(4)P and [³H]PtdIns with IC₅₀ values of 1.04 ± 0.04 mM, 0.51 ± 0.03 mM and 0.46 ± 0.03 mM respectively. Addition of 10 mM lithium in the presence of carbachol (1mM) produced a 74.0 %, 76.4 % and 75.3 % reduction in [³H]PtdIns(4,5)P₂, [³H]PtdIns(4)P and [³H]PtdIns levels.
The effects of increasing concentrations of lithium (0.1 - 10 mM) upon carbachol-stimulated levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ were examined after a 20 min incubation with both agonists. For basal values see section 7.2.6. Data represent means ± S.E.M. for three experiments each performed in triplicate.
Figure 7.9. The Concentration-dependent Effects of Inositol Upon Carbachol-stimulated PtdIns(4,5)P\(_2\) Mass levels in CHO-M1 Cells.

The effects of increasing concentrations of inositol (0.3 - 30 mM; added 30 min prior to drug additions) upon carbachol (1 mM) plus lithium (5 mM) stimulated PtdIns(4,5)P\(_2\) mass levels was examined after a 20 min incubation with drugs. For basal values see section 7.2.6. Data represent means ± S.E.M. for three experiments each performed in triplicate.
respectively compared with basal values (compare with carbachol-induced values shown above).

The inositol reversibility of the effect of lithium upon carbachol-induced PtdIns(4,5)P$_2$ mass levels is shown in Figure 7.9. PtdIns(4,5)P$_2$ mass was measured rather than measurement of the labelled lipid since addition of unlabelled inositol to the cells may result in large changes in the specific activity of the labelled inositol phospholipids, possibly resulting in inaccurate data. Carbachol alone (1mM) produced a 40.1% decrease in the mass levels of PtdIns(4,5)P$_2$ compared with basal values after 20 min (Basal value = 399 ± 24 pmol/mg protein at 20 min). Preincubation with myo-inositol (10mM) for 30 min (as described previously) followed by carbachol-stimulation in the continued presence of myo-inositol (10mM) for 20 min had no effect upon carbachol-stimulated PtdIns(4,5)P$_2$ mass levels. Carbachol (1mM) in the presence of lithium (5mM) produced a 61.6% decrease in the mass levels of PtdIns(4,5)P$_2$ after 20 min incubation compared with basal values. This was a significantly greater reduction than that observed with carbachol alone (1mM). Preincubation for 30 min followed by incubation with myo-inositol (0.3-30 mM) produced a concentration-dependent increase in carbachol/lithium-stimulated PtdIns(4,5)P$_2$ mass levels at 20 min, such that in the presence of 30mM myo-inositol the effects of lithium (5mM) was completely reversed (the relatively small window afforded by this experiment prevented accurate definition of the concentration-dependency of the reversal by inositol however an EC$_{50}$ value of approximately 3-5 mM was calculated); the values for PtdIns(4,5)P$_2$ mass in the presence of carbachol and lithium not being significantly different from those of carbachol alone.

7.2.6. The Effect of a Rechallenge with Carbachol upon the Inhibitory Effects of Lithium on Ins(1,4,5)P$_3$ Accumulation in CHO-M1 cells.

The effect of a second carbachol-stimulation of Ins(1,4,5)P$_3$ mass accumulation was examined in the absence and presence of lithium in CHO-M1 cells, the rationale being to
examine whether the effect of lithium on carbachol-stimulated Ins(1,4,5)P₃ accumulation would be greater after rechallenging with agonist.

Cells were initially exposed to either carbachol alone or carbachol plus lithium. Cells were subsequently washed with Krebs-HEPES buffer (5 x 1ml) either containing or not containing lithium (5 mM). After a 20 min recovery period the cells were then rechallenged with either carbachol (5 mM) alone or carbachol plus lithium (5 mM). Therefore, the three treatments examined were: carbachol (- lithium), wash (- lithium), carbachol (- lithium); carbachol (- lithium), wash (- lithium), carbachol (+ lithium); and carbachol (+ lithium), wash (+ lithium), carbachol (+ lithium).

The time-course of the initial carbachol-stimulated accumulation of Ins(1,4,5)P₃ (0-30 min) is described in more detail in 7.2.1 and is shown in figure 7.10, and more clearly in figure 7.2. The cells were washed with buffer for 20 min. This period of time was sufficient for Ins(1,4,5)P₃ levels to return to basal values (CCh (wash -Li) 33.4 ± 2.4; CCh+Li (wash -Li) 31.1 ± 6.8; and CCh+Li (wash +Li) 34.0 ± 3.2, pmol/ mg protein). Upon rechallenge with carbachol the response obtained was statistically similar to the initial carbachol response with a typical "peak/plateau" response being obtained. The peak response was maximal at 10 s after agonist addition and the magnitude of this response was similar to the initial (first) peak response (19.2-fold at 10 s for second stimulation). Ins(1,4,5)P₃ levels subsequently fell to a minimum of 8.7-fold over basal at 1 min before increasing over the remainder of the time-course, producing the plateau phase (13.4-fold over basal after 20 min).

Cells initially treated with carbachol and washed with buffer (- lithium) for 20 min were also subsequently challenged with carbachol in the presence of lithium to determine whether an initial exposure to agonist might affect the action of lithium on Ins(1,4,5)P₃ accumulation. This was found not to be the case. Upon rechallenge with carbachol, in the presence of lithium, the agonist produced a peak Ins(1,4,5)P₃ response identical to that obtained by a second stimulation with carbachol alone (20.9-fold over basal). Ins(1,4,5)P₃ levels subsequently fell to a minimum of 10.4-fold over basal at 1 min. Levels of Ins(1,4,5)P₃ increased slightly (11.6-
The Effects Of Rechallenge with Carbachol upon the Inhibitory Effects of Lithium on Ins(1,4,5)P3 Accumulation in CHO-M1 cells.

The effects of a second stimulation with either carbachol or carbachol plus lithium on Ins(1,4,5)P3 accumulation was examined in CHO-M1 cells which had been previously stimulated with one or both agents. Cells were incubated with either (I.) carbachol (1 mM) for 30 min; washed for 20 min in lithium free buffer; rechallenged with carbachol (1 mM) alone (●) for the indicated times or (ii.) carbachol (1 mM) for 30 min; washed for 20 min in lithium free buffer; rechallenged with carbachol (1 mM) plus lithium (5 mM) (▲) for the indicated times or (iii.) carbachol (1 mM) plus lithium (5 mM) for 30 min (○); washed for 20 min in lithium containing buffer; rechallenged with carbachol (1 mM) plus lithium (5 mM) (▲) for the indicated times. Data represent means ± S.E.M. for three experiments each performed in triplicate. Statistically significant differences in the effects of lithium on the secondary agonist plus lithium-stimulated groups (i.e. (▲) and (▲)) are shown as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. 
fold over basal) before gradually falling after 5 min, and levels were not significantly different from basal values 20 min after agonist addition. The effect of lithium on the plateau phase of the response was therefore more rapid than that observed during the initial stimulation of the cells with carbachol and lithium.

Finally, the effect of rechallenging cells with agonist and lithium after an initial exposure to agonist and lithium followed by a wash period with lithium containing buffer was examined. Prior to the second stimulation basal levels of Ins(1,4,5)P₃ were not significantly different from the initial basal values (34.0 ± 3.2 pmol/mg protein compared with 24.1 ± 4.3 pmol/mg protein). Upon rechallenge with carbachol and lithium Ins(1,4,5)P₃ levels increased rapidly giving a characteristic peak Ins(1,4,5)P₃ response at 10 s (20.8-fold over basal values). Ins(1,4,5)P₃ levels then fell rapidly approaching basal levels by 10 min. No subsequent increase in Ins(1,4,5)P₃ was observed with this treatment. A statistically significant difference in the levels of Ins(1,4,5)P₃ was observed at 1, 5 and 10 min time points between carbachol plus lithium treated cells which had initially been stimulated with carbachol alone (washed with -lithium buffer) and those stimulated with carbachol plus lithium (washed with + lithium buffer). The plateau phase was therefore effectively abolished with this treatment.

7.2.7. The Effect of a Rechallenge with Carbachol on the Action of Lithium Upon [³H]CMP-PA Accumulation in CHO-M1 cells.

The initial carbachol-stimulated increase in the levels of [³H]CMP-PA (0-30 min) observed in the presence of lithium was identical to that described in section 7.2.4. and is shown in figure 7.11 and more clearly in figure 7.5. The cells were subsequently washed with buffer containing lithium (5 mM) for 20 min. During this wash period [³H]CMP-PA levels decreased significantly from those observed at 30 min in the presence of agonist and lithium (5 mM) (19.1% decrease compared with maximum response at 30 min). Upon a second challenge with agonist and lithium [³H]CMP-PA levels did not significantly change and remained at a constant
Figure 7.11. The Effects Of Rechallenge With Carbachol on the Effect of Lithium Upon [3H]CMP-PA Accumulation In CHO-M1 Cells.

CHO-M1 cells were stimulated with carbachol (1 mM) and lithium (5 mM) for 30 min the cells were subsequently washed for 20 min with lithium containing buffer (5 mM) before cells were rechallenged with carbachol plus lithium for the indicated periods of time. Data represent means ± S.E.M. for three experiments each performed in triplicate.
level throughout the remainder of the time-course (27.7-fold over basal (t0) at 80 min). During this time-course basal levels slowly increased (418 ± 52 d.p.m. at t0 to 1408 ± 88 d.p.m. at 80 min). Lithium alone had no effect on basal values over the time-course examined.

7.2.8. The Effect of The Wash Period Upon Agonist-Stimulated Phosphoinositide Levels In CHO-M1 Cells.

The effect of the wash period upon agonist-stimulated levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P2 in the absence and presence of lithium was examined in CHO-M1 cells to determine whether these cells could regenerate phosphoinositide levels during this period.

The addition of carbachol alone produced 38.3%, 39.9% and 30.7% decreases in PtdIns, PtdIns(4)P and PtdIns(4,5)P2 levels respectively at 30 min (Basal levels: PtdIns= 305238 ± 8916; PtdIns(4)P= 24577 ± 2137; PtdIns(4,5)P2= 12492 ± 800)(Fig. 7.12). After a wash period of 20 min in Krebs-HEPES buffer (- lithium) the levels of all three inositol phospholipids had recovered to values which were not significantly different from unstimulated values (PtdIns= 268038 ±11448 d.p.m.; PtdIns(4)P= 19980 ± 1762 d.p.m.; PtdIns(4,5)P2= 11016 ± 512 d.p.m.).

The addition of carbachol and lithium (5 mM) produced 71.1%, 65.7% and 64.2% decreases in the levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P2 at 30 min. After a wash period of 20 min with buffer containing lithium (5 mM) the levels of PtdIns had not significantly recovered from agonist plus lithium-stimulated values. PtdIns(4)P levels had partially recovered (44.8% of unstimulated values) and the levels of the precursor of Ins(1,4,5)P3, PtdIns(4,5)P2, had fully recovered to unstimulated values (Fig. 7.12).
Figure 7.12. The Effect of The Wash Period Upon Agonist-stimulated Phosphoinositide Levels in CHO-M1 Cells.

The levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P$_2$ were examined in cells stimulated with carbachol in the absence or presence of lithium for 30 min which were subsequently washed for 20 min in buffer not containing or containing lithium. Data represent means ± S.E.M. for three experiments each performed in triplicate. Statistically significant differences between basal values and stimulated values are shown as *p < 0.05, **p < 0.01 and ***p < 0.001. Statistically significant differences between other groups are shown as +p < 0.05, ++p < 0.01 and +++p < 0.001.
7.2.9. The Effect of Rechallenge with Carbachol upon the Action of Lithium on Inositol Phospholipid Levels in CHO-M1 cells.

The effects of a second period of agonist stimulation on the levels of the inositol phospholipids PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 was examined in CHO-M1 cells in the absence and presence of lithium (5 mM) to examine whether the effect of lithium would be enhanced by sequential agonist challenges. The drug treatments were as described in section 7.2.7.

The initial challenge with carbachol (0-30 min) in the absence or presence of lithium produced identical changes in the levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 as described in section 7.2.7 and are shown in figures 7.13 and 7.14, and more clearly in figure 7.7. The cells were subsequently washed for 20 min with buffer alone or buffer containing lithium (5 mM). During this wash period the levels of the various inositol phospholipids examined, PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, changed as described in section 7.2.9. (see Fig. 7.12).

All values described below are related to the relative basal values for PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 obtained after the 20 min wash period (i.e. at the 50 min time point). On rechallenge with agonist alone PtdIns levels demonstrated a similar profile over the time-course as was observed for the initial challenge with agonist (Fig. 7.13). PtdIns levels initially increased over the first minute after rechallenge with agonist (1.1-fold increase over the relative unstimulated values) before falling and reaching a new steady-state level which was not statistically different from that observed with the initial agonist challenge (27.0% decrease compared with relative unstimulated values). Upon addition of agonist and lithium, after an initial challenge with agonist alone, PtdIns levels did not increase over the initial minute, instead levels decreased slowly to a new steady state (74.0% decrease compared to the relative unstimulated values) which, although significantly lower than that produced with agonist alone, was not significantly different to that observed with agonist and lithium during the initial challenge with agonist and lithium (Fig. 7.13). A rechallenge with carbachol and lithium, after
an initial challenge with both agents, resulted in a rapid and marked decrease in the levels of PtdIns (50.2% decrease compared with unstimulated values by 1 min). This new level was lower than that observed for either of the other two drug regimes.

Addition of agonist alone for a second time produced a rapid decrease in the levels of PtdIns(4)P which reached a minimum of 74.7% below the relative unstimulated values within 10 s (Fig. 7.13). PtdIns(4)P levels then slowly increased over the remainder of the time-course before reaching a plateau which was 39.3% below unstimulated values by 10 min. This was a similar profile to that obtained during the initial stimulation (Fig. 7.13). Addition of carbachol and lithium, after an initial challenge with carbachol alone, produced an initial drop within the first 10 s in PtdIns(4)P levels which was similar to that observed with a second stimulation with carbachol alone (64.0% below the relative unstimulated values). PtdIns(4)P levels subsequently increased over the next 20 min (51.3% below unstimulated values) of the time-course before decreasing again by 20 min. Rechallenge with agonist and lithium, after an initial challenge with these agents, produced a rapid decrease in PtdIns(4)P levels within 10 s (52.4% below the relative unstimulated values), however, the levels of this inositol phospholipid did not subsequently increased and remained at this new steady-state level throughout the remainder of the time-course examined (Fig. 7.13).

Addition of carbachol alone for a second time produced a rapid decrease in the levels of PtdIns(4,5)P2, similar to that observed during the initial stimulation with carbachol (70.8% decrease compared with the relative unstimulated values)(Fig. 7.14). PtdIns(4,5)P2 levels subsequently increased over the remainder of the time-course reaching a plateau (26.9% below unstimulated values) between 10-20 min. The addition of carbachol and lithium, after an initial stimulation with carbachol alone, produced a similar rapid decrease in the levels of PtdIns(4,5)P2 (61.6% below the relative unstimulated values at 10 s)(Fig 7.14). Levels subsequently increased over the next 10 min (37.6% below unstimulated values) before decreasing again after 20 min (42.8% below unstimulated values). The addition of agonist and lithium, after an initial stimulation with carbachol and lithium, produced a rapid decrease in the
The Effects of Rechallenge with Carbachol upon the Effects of Lithium on PtdIns and PtdIns(4)P Levels in CHO-M1 cells.

The effects of a second stimulation with either carbachol or carbachol plus lithium on PtdIns and PtdIns(4)P levels was examined in CHO-M1 cells which had been previously stimulated with one or both agents. Cells were incubated with either (i.) carbachol (1 mM) for 30 min; washed for 20 min in lithium-free buffer; rechallenged with carbachol (1 mM) alone (○) for the indicated times or (ii.) carbachol (1 mM) for 30 min; washed for 20 min in lithium-free buffer; rechallenged with carbachol (1 mM) plus lithium (5 mM) (△) for the indicated times or (iii.) carbachol (1 mM) plus lithium (5 mM) for 30 min (■); washed for 20 min in lithium-containing buffer; rechallenged with carbachol (1 mM) plus lithium (5 mM) (●) for the indicated times. Data represent means ± S.E.M. for three experiments each performed in triplicate.
Figure 7.14. The Effects Of Rechallenge with Carbachol upon the Effects of Lithium on PtdIns(4,5)P2 Levels in CHO-M1 cells.

The effects of a second stimulation with either carbachol or carbachol plus lithium on PtdIns(4,5)P2 levels was examined in CHO-M1 cells which had been previously stimulated with one or both agents. Cells were incubated with either (i.) carbachol (1 mM) for 30 min; washed for 20 min in lithium free buffer; rechallenged with carbachol (1 mM) alone (○) for the indicated times or (ii.) carbachol (1 mM) for 30 min; washed for 20 min in lithium free buffer; rechallenged with carbachol (1 mM) plus lithium (5 mM) (△) for the indicated times or (iii.) carbachol (1 mM) plus lithium (5 mM) for 30 min (●); washed for 20 min in lithium containing buffer; rechallenged with carbachol (1 mM) plus lithium (5 mM) (△) for the indicated times. Data represent means ± S.E.M. for three experiments each performed in triplicate.
levels of PtdIns(4,5)P2 which leached a minimum of 66.2% below unstimulated values by 1 min. Levels did not recover and remained at this new steady-state level throughout the remainder of the time-course examined (Fig. 7.14).

7.2.10. The Effect of Lithium on Carbachol-stimulated Changes in Intracellular Ca²⁺ Levels in Fura-2 Loaded CHO-M1 cells.

The effect of carbachol and lithium upon intracellular Ca²⁺ release were examined in populations of CHO-M1 cells both in suspensions of cells and in cells on cover-slips. Initial experiments were performed on cells in suspension, however, due to leakage of Fura-2 from the cells which made accurate [Ca²⁺]ᵢ measurements impossible these experiments were discontinued and the remainder of the experiments were performed on cells plated-down onto coverslips where Fura-2 leakage did not appear to be a significant problem.

Loading of CHO-M1 cells with Fura-2AM was found to be a significant problem. Although Fura-2AM appeared to enter the cells quite readily there appeared to be little metabolism of this esterified form of Fura-2, such that the levels of the free acid within the cells were relatively low compared with that observed in other cell types such as SH-SY5Y neuroblastoma. Manipulation of the conditions resulted in a small Ca²⁺ signal, however the results obtained were far from perfect.

Carbachol (1 mM) produced an initial Ca²⁺ "spike" which reached a maximum after 10 s before the [Ca²⁺]ᵢ decreased to a new steady-state plateau level between 1-2 min which was maintained for the remainder of the time-course (Fig. 7.15.). The addition of carbachol plus lithium (5 mM) appeared to produce an identical response to that observed with carbachol alone, at least over the limited number of experiments performed here (n = 2) (Fig. 7.15.). The plateau phase was shown to be "real" and not an artifact since addition of atropine (10 μM) resulted in a decrease in the ratio to basal values, albeit unusually slowly (Fig. 7.15).

These preliminary data therefore lend no support to the possibility that the reduction in agonist-
Figure 7.15. Effect of Lithium Upon Carbachol-stimulated Intracellular Ca\(^{2+}\) Release in Fura-2 Loaded CHO-M1 Cells.

Fura-2 loaded CHO-M1 cells were stimulated with carbachol (1 mM) in the absence (A.) and presence (B.) of lithium and the rise in \([\text{Ca}^{2+}]_i\) was measured as described in the Methods. The effects of atropine (10 \(\mu\)M) addition upon the plateau phase of the carbachol plus lithium-stimulated \([\text{Ca}^{2+}]_i\) response was also examined (C.). Data are from a single representative experiment which was repeated at least on one other occasion.
stimulated Ins(1,4,5)P$_3$ levels observed on addition of lithium may be translated into a decrease in the agonist-stimulated Ca$^{2+}$ signal. However, with such small changes in ratio, as have been observed in this study, it may not be possible to detect the small changes in [Ca$^{2+}$]$_i$ that lithium may produce. A great deal more work is required in order to determine whether lithium does have a significant effect upon agonist-stimulated Ca$^{2+}$ release in this cell line (see Chapter 8 for fuller discussion).

It has been previously demonstrated, in TRH-stimulated GH$_3$ cells, that chronic lithium treatment (for a period of 21 days) can result in a significant decrease in agonist-stimulated Ins(1,4,5)P$_3$ mass levels and in turn agonist-stimulated [Ca$^{2+}$]$_i$ (Varney et al., 1992). The functional consequence of the decrease in TRH-stimulated Ins(1,4,5)P$_3$ levels and [Ca$^{2+}$]$_i$ has been shown to be a significant decrease in TRH-stimulated secretion of prolactin from that cell type (Varney et al., 1992). This study therefore demonstrates that disruption of phosphoinositide signalling by lithium does significantly interfere with cellular function.
7.3. Discussion.

A large number of studies have examined phosphoinositide metabolism in a number of different preparations, the popular approach being to examine the labelled water soluble products of the cycle, namely the inositol phosphates (see Nahorski et al., 1991 and refs. therein). Indeed with this approach it has been possible to examine the various routes of metabolism of the second messenger Ins(1,4,5)P₃ in great detail in a number of cell types. However, this technique is limited due to problems of changes in the specific activity of the labelling of the inositol phosphates, especially Ins(1,4,5)P₃, which leads to confusing and inaccurate results (Batty & Nahorski, 1984; also see chapter 4). This method also only looks at one aspect of phosphoinositide signalling making the results obtained sometimes difficult to interpret when examined in the context of the whole signalling system. Only relatively few studies have concerned themselves with other components of this complex pathway, however to date no single study has examined all the individual components that constitute this signalling pathway.

The work described in this chapter expands the basic characterisation of phosphoinositide metabolism described previously chapter 6 and employs a number of other techniques in order to examine the changes in mass levels of the second messenger Ins(1,4,5)P₃, the measurement of the inositol containing lipids PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ and also one of the precursors of PtdIns, CMP-PA. The aim was therefore to produce a full characterisation of phosphoinositide signalling in this simple cellular system and at the same time examine the effects of lithium upon this signalling pathway.

As described in section 7.2.1, stimulation of CHO-M1 cells with a maximally effective concentration of the muscarinic agonist carbacochol results in a biphasic elevation in the mass levels of Ins(1,4,5)P₃. The first phase comprised of a rapid increase in Ins(1,4,5)P₃ levels which peaked within 10 s of agonist application and then fell to a new low by 1 min before subsequently increasing and reaching a plateau between 5 and 10 min. This profile was similar
to that described previously for muscarinic receptor activation in CHO-M3 cells (Tobin et al., 1992), the human neuroblastoma cell line SH-SY5Y (Lambert & Nahorski, 1990), and for bombesin and cholecystokinin stimulation of the rat pancreatoma cell line AR4-2J (Menneti et al., 1991). In comparison carbachol-stimulation of rat cortical slices produces a monophasic increase in Ins(1,4,5)P₃ levels which rapidly reaches a plateau (Kennedy et al., 1989), as opposed to the biphasic response observed with CHO-M1 cells. The differences in the profile of the carbachol-stimulated Ins(1,4,5)P₃ response obtained in these two preparations may be due to a variety of reasons. Firstly, as mentioned in chapter 7 the initial transient Ins(1,4,5)P₃ accumulation observed in CHO-M1 cells may be indicative of a rapid desensitisation of the production of Ins(1,4,5)P₃ (Tobin et al., 1992). Alternatively, this peak response may be due to the inability of CHO-M1 cells to maintain a constant level of PtdIns(4,5)P₂ under conditions of continued maximal stimulation (see later), whereas cerebral cortical slices may maintain the levels of this inositol phospholipid under these conditions. Although the initial peak of Ins(1,4,5)P₃ mass was apparently unaffected by the addition of lithium the secondary plateau phase was reduced in a concentration-dependent manner. The EC₅₀ for this inhibition of carbachol-stimulated Ins(1,4,5)P₃ accumulation was similar to that observed in rat cerebral cortex slices (see Chapter 3). This effect of lithium was significant only after 10 min, with the effect increasing with time over the remainder of the time-course examined. The presence of this lag period before lithium has any significant effect is characteristic of the effect that this ion has on carbachol-stimulated Ins(1,4,5)P₃ levels in preparations such as rat (Kennedy et al., 1989, 1990) and mouse cerebral cortex slices (Whitworth & Kendall, 1988) and bovine adrenal glomerulosa cells (Balla et al., 1988). Maximal concentrations of lithium (3 mM) return carbachol-stimulated Ins(1,4,5)P₃ levels in rat cerebral cortex towards basal after a similar lag period of 10 min (Kennedy et al., 1990; also see Chapter 3). It has been proposed that uncompetitive inhibition of inositol monophosphatase by lithium results in a decrease in the recycling of inositol, the precursor of the inositol phospholipids (see Nahorski et al., 1991). This in turn results in a depletion of the agonist-sensitive inositol phospholipid pool, and it has
been suggested that the lag period observed for the onset of lithium inhibition of agonist-stimulated Ins(1,4,5)P₃ accumulation is indicative of the time required for this depletion. The time required for lithium to produce a decrease in the levels of Ins(1,4,5)P₃ varies between different tissues and cell types. Indeed in GH₃ cells lithium appears to have no significant effect on TRH-stimulated Ins(1,4,5)P₃ levels (Hughes & Drummond, 1987) except when cells are inositol depleted for substantial periods of time (7-21 days)(Varney et al., 1992). Lithium also fails to produce a large increase in the agonist-stimulated levels of CMP-PA unlike those observed in CHO-cells or cerebral cortex slices (Drummond, 1987; also see later). This suggests that the inositol "reserves" within GH₃ cells are large and subsequently over short time-courses the production of the inositol phospholipids is unaffected by lithium. Conversely, this also suggests that inositol levels are readily depleted in both CHO-M1 cells and cerebral cortical slices. Indeed this hypothesis is reinforced in CHO-M1 cells by the observation that the effects of lithium in agonist-stimulated Ins(1,4,5)P₃ levels can be reversed in a concentration-dependent manner by the addition of myo-inositol. In rat cortical slices preincubation with high concentrations of inositol also causes an attenuation of the effects of lithium, though it does not wholly reverse it. This latter observation may be taken to suggest that the uptake mechanism for inositol by the agonist-sensitive cells in cerebral cortex slices is limiting. In CHO-M1 cells, as shown in figures 7.3., inositol addition reverses the effects of lithium over the entire time-course examined. These results are therefore in agreement with the results obtained in previous studies in other systems (see Nahorski et al., 1991).

The addition of agonist alone to CHO-M1 cells produced only a small increase in the levels of CMP-PA, however in the presence of lithium a rapid accumulation in CMP-PA was observed after a short lag period of 1-2 min. CMP-PA levels increased rapidly over the first 10 min after agonist application before plateauing. These results are again similar to previous results obtained in other preparations including rat cerebral cortex slices (Godfrey, 1989; Kennedy et al., 1990). Since the experiments were performed in inositol free buffer the short lag period observed before the rapid accumulation of CMP-PA levels probably reflects a small myo-
inositol reserve within the cell such that inositol is not the rate limiting factor of the enzyme PtdIns synthase over the initial 1-2 minutes of the time-course. Once the reserves within the cell have fallen below a concentration sufficient to saturate the enzyme the levels of the co-substrate CMP-PA rapidly increase and there is a concomitant decrease in the rate of synthesis of PtdIns. The rapid increase in CMP-PA accumulation is clearly shown to be due to inositol depletion in these cells by the fact that this effect of lithium can be reversed in a concentration-dependent manner by addition of exogenous myo-inositol. The accumulation of CMP-PA produced by lithium (EC50= 2.2 ± 0.1 mM) is similar to that for the accumulation of InsP1 in these cells (EC50= 0.4 ± 0.1 mM and 0.6 ± 0.3 mM for Ins(1/3)P1 and Ins(4)P1 respectively) as would be expected if the increase in CMP-PA was due to an inhibition of the monophosphatase and a resultant decrease in inositol recycling. As mentioned earlier CMP-PA levels plateau approximately 10 min after agonist addition in the presence of lithium. This may be related to the decrease in the production of Ins(1,4,5)P3 mass produced by lithium, which starts to become significant after 10 min. Indeed if Ins(1,4,5)P3 production is substantially reduced after 10 min in the presence of agonist and lithium this would also result in a decrease in the production of DAG, the precursor of CMP-PA. Therefore a plateauing of CMP-PA levels is not unexpected.

The relative levels of the [3H]inositol labelled inositol phospholipids PtdIns, PtdIns(4)P and PtdIns(4,5)P2 were also examined under identical conditions to those for Ins(1,4,5)P3 mass measurements. A number of previous studies, that have examined the action of lithium on phosphoinositide signalling, have examined the changes in agonist-stimulated levels of these three phospholipids upon addition of lithium. Drummond and Raeburn (1984) were the first to demonstrated that in TRH-stimulated GH3 cells addition of lithium resulted in a decrease in the agonist-stimulated levels of PtdIns (30 % decrease compared to unstimulated values) and PtdIns(4)P (20 % decrease compared with unstimulated values), however they were unable to show a significant decrease in TRH-stimulated PtdIns(4,5)P2 levels. This inability of lithium to produce a decrease in agonist-stimulated PtdIns(4,5)P2 levels has since been confirmed in
adrenal glomerulosa cells (Balla et al., 1988), parotid gland (Downes & Stone, 1986) and platelets (Huang & Detwiler, 1986). These results are therefore inconsistent with the inositol depletion hypothesis described above, where the agonist-stimulated levels of Ins(1,4,5)P3 are decreased by lithium due to a decrease in the levels of its precursor PtdIns(4,5)P2. This has been highlighted as a possible flaw in an otherwise elegant and simple hypothesis by some workers. However, a number of reasons may be put forward to explain this apparent discrepancy. The agonist-sensitive pool of PtdIns(4,5)P2 may only be a small part of the total PtdIns(4,5)P2 pool, which itself only constitutes a minor component of the membrane inositol phospholipids therefore the changes observed may be masked and difficult to quantify. Indeed, Imai & Gershengorn (1987) have demonstrated the existence of two separate pools of PtdIns, each pool synthesized in distinct compartments within the cell. From their work in TRH-stimulated GH3 cells they have shown that PtdIns can be synthesized both in the endoplasmic reticulum of the cell, from which it is transported via a transfer protein to the plasma membrane (George & Helmkamp, 1985; Somerharju et al., 1983), or in the plasma membrane itself. It is not known whether a different PtdIns synthase is involved in both compartments, however, it has been suggested that the plasma membrane PtdIns synthase may be responsible for PtdIns synthesis that occurs during cell stimulation (Imai & Gershengorn, 1987). Furthermore, it has been shown that a significant portion of the PtdIns(4,5)P2 pool is utilized in the generation of the second messenger PtdIns(3,4,5)P3. Measurement of PtdIns(3,4,5)P3 levels in human neutrophils suggest that approximately 15-20 % of the PtdIns(4,5)P2 pool may be involved in the synthesis of this inositol phospholipid (Stephens et al., 1991). Therefore, the methods used to examine changes in [3H]PtdIns(4,5)P2 levels may not be sensitive enough to pick up any small changes in the levels of this phospholipid that may occur after lithium addition.

In this present study application of agonist resulted in a rapid decrease in the levels of PtdIns(4)P and PtdIns(4,5)P2 over the initial 10 s before stabilizing. PtdIns levels however did not show this rapid fall, but instead gradually decreased at a steady rate over the time-course examined. This slow decrease may suggest a role for PtdIns in "buffering" the levels of
PtdIns(4)P and PtdIns(4,5)P$_2$ such that under conditions of maximal stimulation for long time periods PtdIns(4,5)P$_2$ levels are maintained (see later). Indeed, during the latter half of the time-course examined PtdIns(4,5)P$_2$ levels significantly recovered even in the continued presence of agonist whilst PtdIns levels decreased and PtdIns(4)P levels were unchanged. Therefore under these conditions it would appear that the synthesis of PtdIns(4,5)P$_2$ from PtdIns and PtdIns(4)P is at the expense of PtdIns levels, as might be expected, in order to maintain the potential for producing the signalling molecule Ins(1,4,5)P$_3$. This increase in PtdIns(4,5)P$_2$ levels in the continued presence of agonist is not unusual and has been described previously in both angiotensin II-stimulated adrenal glomerulosa cells (Balla et al., 1988) and thrombin-stimulated platelets (Huang & Detwiler, 1986). In both these cells PtdIns(4,5)P$_2$ levels returned to pre-stimulated levels within 10-20 mins suggesting that the activity of the kinases involved in the phosphorylation of PtdIns and PtdIns(4)P to form PtdIns(4,5)P$_2$ are highly active in these tissues or possibly that the receptors involved may desensitise rapidly. The PtdIns(4,5)P$_2$ levels in CHO-M1 cells do not return to pre-stimulated levels over the time course examined, possibly suggesting that the PtdIns 4-kinase and/or PtdIns(4)P 5-kinase have a lower activity in this cell type compared to that observed in adrenal glomerulosa cells or platelets.

The addition of lithium plus agonist caused a significant reduction in the levels of PtdIns after 5 min as would be expected if lithium was preventing the recycling of the precursor of PtdIns, namely inositol. Indeed, from the studies examining CMP-PA accumulation it appears that inositol levels become rate limiting for PtdIns synthase after 1-2 min, correlating well with the slightly later fall in the levels of PtdIns observed at 5 min. Lithium also caused a significant decrease in the agonist-stimulated levels of PtdIns(4)P, however, only after 20 min. This lag period may reflect the time required to deplete PtdIns significantly such that PtdIns levels fall below levels sufficient for maximal PtdIns 4-kinase activity. Levels of PtdIns do appear to plateau after 20 min reaching a new steady state under these conditions.

The most surprising result was that in CHO-M1 cells lithium produced a significant decrease
in the agonist-stimulated levels of PtdIns(4,5)P₂ after a lag period of 10 min. The lag period is therefore identical to the lag period observed for the decrease in agonist-stimulated
Ins(1,4,5)P₃ produced by lithium. This clearly indicates the possibility that a decrease in the levels of the precursor of Ins(1,4,5)P₃, PtdIns(4,5)P₂, results in a decrease in the production of this second messenger. Further evidence comes from the sensitivity of both carbachol-stimulated Ins(1,4,5)P₃ mass and PtdIns(4,5)P₂ levels to lithium. The carbachol-stimulated levels of both Ins(1,4,5)P₃ and PtdIns(4,5)P₂ are reduced in a concentration-dependent manner by lithium with IC₅₀ values for these effects of 0.5 ± 0.1 mM and 1.0 ± 0.1 mM respectively. The inhibition of Ins(1,4,5)P₃ accumulation by lithium is therefore significantly more potent than that observed for PtdIns(4,5)P₂, this apparent discrepancy could be explained if it were shown that only a small change in the levels of PtdIns(4,5)P₂ were required for the complete inhibition of the production of Ins(1,4,5)P₃, thus the concentration-inhibition curve for lithium inhibition of Ins(1,4,5)P₃ would lie to the left of that for PtdIns(4,5)P₂, as is observed here.

The question arises as to why a lithium-sensitive decrease in the agonist-stimulated levels of PtdIns(4,5)P₂ is observed here in CHO-M1 cells but not in the previous studies described above for adrenal glomerulosa cells (Balia et al., 1988) or platelets (Huang & Detwiler, 1986). As mentioned above, the agonist-stimulated levels of PtdIns(4,5)P₂ recover rapidly to basal values in these other cell types compared to the recovery of PtdIns(4,5)P₂ levels in CHO-M1 cells. If this is possibly due to the PtdIns 4-kinase and PtdIns(4)P 5-kinase having a higher activity in these cell types it may be that they also have a higher affinity for PtdIns and PdIns(4)P, therefore maintaining the levels of PtdIns(4,5)P₂ even in the presence of lithium. However, this is difficult to reconcile with the decrease in the agonist-stimulated Ins(1,4,5)P₃ levels produced by lithium. It is therefore more likely that lithium does produce a significant decrease in the agonist-stimulated levels of PtdIns(4,5)P₂ in these cells resulting in a concomitant decrease in Ins(1,4,5)P₃ production, but that the decrease has not been observed
due to the insensitivity of the methods used to examine the levels of this inositol phospholipid.

The reduction in the mass levels of PtdIns(4,5)P₂ produced by lithium in CHO-M1 cells (which are consistent with the changes observed in labelled studies) can be reversed completely in a concentration-dependent manner by the addition of exogenous inositol, the EC₅₀ value being similar to that for the reversal of the effects of lithium upon agonist stimulated levels of both Ins(1,4,5)P₃ mass and CMP-PA in these cells. Inositol addition appears therefore to bypass the block in inositol recycling produced by lithium allowing the cells to maintain inositol phospholipid levels.

The recovery of (poly)phosphoinositide levels after the removal of the agonist is shown in figure 7.12. Cells were either treated with carbachol or carbachol plus lithium for 30 min followed by a subsequent 20 min wash period with buffer either containing or not containing lithium, respectively. Carbachol produced a decrease in the levels of all three inositol phospholipids after a 30 min incubation, similar to that described above. However, after a wash period of 20 min the levels of all the (poly)phosphoinositides had recovered to the initial unstimulated values. Therefore the decrease in the levels of the phospholipids was probably due to the inability of the enzymes involved in the recycling of inositol to maintain adequate levels of inositol during continual maximal stimulation. These data also further suggest that inositol levels within this cell type are low under the experimental conditions employed here.

Carbachol plus lithium produced a more profound decrease in the levels of the (poly)phosphoinositides (see above) compared with carbachol alone. After the removal of the agonist by washing the cells in lithium containing buffer for 20 min the levels of the (poly)phosphoinositides were very different to those described previously for carbachol alone. PtdIns levels did not change after this wash period and PtdIns(4)P levels only recovered slightly. However, PtdIns(4,5)P₂ levels recovered fully and indeed the levels of this inositol phospholipid were slightly greater after this wash period than initial unstimulated values. These data differ significantly from those obtained by Downes & Stone (1986) in parotid gland. That study clearly showed that after atropine addition agonist-stimulated levels of PtdIns(4,5)P₂
either in the absence or presence of lithium returned to unstimulated levels after 5 min, with no
differences in the rates of recovery being observed (Downes & Stone, 1986). It appears that in
CHO-M1 cells, even though the levels of the precursors of PtdIns(4,5)P₂, PtdIns and
PtdIns(4)P, may be minimal, the cell still manages to recover and maintain the levels of this
lipid. This obviously reflects the importance that the cell places upon PtdIns(4,5)P₂ that it is
maintained at the expense of the other inositol phospholipids.

So far the work described in this chapter has only examined the effects of lithium upon a
single sustained stimulation of phosphoinositide metabolism. However, in vivo signalling
between neurons / cells is much more complex occurring via numerous impulses which
depending upon their nature and role may only last a fraction of a second each. It would
therefore be more realistic to look at the effects of lithium upon multiple stimulations of this
signalling system to determine whether the effects of lithium are potentiated or reduced by the
protocol employed. We have therefore examined the effect of lithium on an initial and second
agonist stimulation of the phosphoinositide system and compared the responses to determine
whether the effects of lithium are altered by re-stimulation.

A second stimulation of CHO-M1 cells with carbachol produced an Ins(1,4,5)P₃ response
with an almost identical profile to that produced by the first / initial stimulation. This is not
surprising taking into account the data in figure 7.12 (see above), which demonstrate that after
an initial 30 min stimulation with carbachol followed by a 20 min wash period the levels of all
the (poly)phosphoinositides recover to pre-stimulated values. Therefore, a second stimulation
should not be significantly different unless some other change in the system has occurred due
to the initial stimulation, such as receptor desensitization. Indeed, carbachol has been shown to
produce a small down-regulation of the M₃ receptor number in CHO-M3 cells which was
significant after a 30 min initial stimulation with carbachol (Tobin et al., 1992), however it was
also demonstrated that a 20 min wash period was sufficient to restore agonist-stimulated
Ins(1,4,5)P₃ accumulation. The profile of the inositol phospholipids PtdIns, PtdIns(4)P and
PtdIns(4,5)P₂ during this second stimulation with carbachol were also identical to those
observed during the initial stimulation. These results demonstrate that in the absence of lithium the cell can quite adequately maintain the levels of the (poly)phosphoinositides even after successive stimulations.

The effects of a second stimulation by carbachol in the presence of lithium after an initial stimulation with carbachol alone produced a similar Ins\((1,4,5)P_3\) response to that produced by an initial stimulation in the presence of both agents. The inositol phospholipids PtdIns and PtdIns\((4)P\) follow a similar profile as observed with the initial stimulation, however the levels of PtdIns\((4,5)P_2\) do differ slightly in that towards the end of the time-course PtdIns\((4,5)P_2\) levels remained decreased, whereas before they continued to recover.

A secondary stimulation with carbachol and lithium after an initial stimulation with both agents (followed by a wash with lithium containing buffer) produced a markedly different response to that produced by an initial stimulation with these agents. The secondary response consisted of an initial peak with no subsequent plateau phase. The initial levels of PtdIns\((4,5)P_2\) were similar to those for cells which had been stimulated for the first time with carbachol alone however the levels of the other (poly)phosphoinositides were substantially lower. With the second stimulation with carbachol and lithium the levels of PtdIns\((4,5)P_2\) rapidly decreased and did not recover over the remainder of the time-course examined. This suggests that unlike the other conditions examined, where PtdIns\((4,5)P_2\) levels are sustained by the "buffering" effect of PtdIns\((4)P\) and PtdIns, with successive stimulations in the presence of lithium the cells are unable to resynthesize PtdIns\((4,5)P_2\) due to the downstream blockade of its synthesis at the level of the inositol monophosphatase. An important point to note is that the inhibition of the monophosphatase by lithium was still present even during the wash period; the high levels of inositol monophosphate possibly enhanced the effect of lithium such that the inhibition was maintained throughout this period (since the inhibition produced by lithium increases with an increase in the substrate concentration). This is most clearly demonstrated by the levels of CMP-PA which increase upon the initial application of carbachol and lithium and only decrease slightly during the wash period, suggesting that only a small, of
any, recycling of inositol is occurring.

It has therefore been demonstrated that the effect of lithium is more profound upon a secondary stimulation than it is upon the initial agonist response. Furthermore, it is possible that with further successive stimulations in the presence of lithium the cell may not be able to maintain the levels of PtdIns(4,5)P₂, and agonist-stimulated Ins(1,4,5)P₃ generation would be completely inhibited due to a complete depletion of cellular inositol and the inositol phospholipids produced by the lithium inhibition of the inositol monophosphatase enzyme.

The data presented here for phosphoinositide metabolism in CHO-M1 cells provides a detailed analysis of the effects of lithium upon this signalling system and clearly confirms that the inositol depletion hypothesis of lithium action is correct, at least in this cell type. As mentioned earlier, the uncompetitive inhibition of the inositol monophosphatase by lithium results in a depletion in the levels of cellular inositol and in turn the (poly)phosphoinositides. During continuous stimulation, such as shown here, repetitive stimulation this depletion of the inositol phospholipids finally results in a decrease in the agonist-stimulated levels of the second messenger Ins(1,4,5)P₃. Whether the examination of phosphoinositide metabolism in the CHO-M1 cell type is a satisfactory and representative model of phosphoinositide signalling in the CNS may be questioned; the advantages and disadvantages of using such a model cell line are discussed in the following chapter.
Chapter 8

Conclusions And Further Perspectives.
Conclusion and Further Perspectives.

In this final chapter a brief summary of the major conclusions drawn from the work presented in the previous chapters is given. The possibilities for further work in a number of areas are also discussed.

The work described in the thesis has been performed mainly in two preparations, the rat cerebral cortex slice preparation and the Chinese hamster ovary (CHO) cell line expressing the M1-muscarinic receptor subtype. The rationale behind undertaking parallel studies on both preparations was to determine whether the CHO-M1 cell line would be a viable alternative to the cerebral cortical slice preparation as a simple cellular model system in which to interrogate a number of aspects of the actions of lithium upon phosphoinositide metabolism. The use of a such a model cell line could have a number of advantages over the cortical slice preparation, with the work described in chapters 6 and 7 clearly demonstrating the benefits of such a homogeneous cell population. The results presented in these chapters have clarified many questions which until now could not have been addressed by the use of heterogeneous cell preparations such as the rat cerebral cortical slice. However, it may be questioned whether the use of a fibroblast cell line such as the CHO cell in the modelling of a neuronal system is indeed justified and therefore these studies provide information on what is possible rather than what may actually occur with susceptible cells in the slice preparation.

CHO cells and neuronal cells such as those present in the CNS do differ in a number of respects. Neuronal cells, whether in culture or in vivo, form synapses between cells whereas CHO cells clearly do not. Neuronal cells are also "excitable" cells possessing voltage-gated Ca²⁺ channels through which extracellular Ca²⁺ can enter upon depolarization of the cell in response to a stimulus. Indeed, this influx of Ca²⁺ may have a modulatory role in controlling the activity of PI-PLC in these cells (see Eberhard & Holz, 1988) or influence the activity of Ins(1,4,5)P₃ metabolizing enzymes (see Shears, 1991). CHO cells, as mentioned previously, are a fibroblast cell line and as such are a non-excitable tissue. This difference may, therefore,
reflect upon the phosphoinositide response obtained in each preparation.

One other major difference between CHO cells and the neuronal cells present in cerebral cortical slices is that in the cortical slice preparation the neuronal cells are surrounded by other cell types, such as glial cells. The presence of these cells may have a significant effect upon the responses obtained, since it has been suggested that these cells may act as reservoirs for substances such as inositol which the neuronal cells can "tap" in times of need.

Although there are a number of significant differences in morphology between CHO cells and neuronal cells there are also a number of striking similarities. Examination of phosphoinositide metabolism in both cell types reveals a number of similarities, with both the profiles of inositol phosphate metabolites and CMP-PA accumulation, although not being identical, bearing a significant resemblance to those observed in cerebral cortex slices. CHO-M1 cells and cerebral cortex slices both appear to become inositol depleted rather rapidly (within 5 min) compared to other cell types, such as GH3 cells, which have been used in the past as appropriate models of neuronal phosphoinositide metabolism. As mentioned in chapter 6 one advantage of using such a homogeneous model cell line, which only expresses one muscarinic receptor subtype, is that the responses obtained are more easy to interpret than those obtained from cerebral cortex slices, since it is known that only one cell-type and one receptor is involved in the response.

The use of such a cell line also has other advantages including the possibility of performing more elegant and searching experiments which cannot be performed on cerebral cortex slices for the reasons eluded to above, and also due to the fact that the slices cannot be maintained over long time-courses (i.e. > 2-3 hours). One major advantage of CHO cells is that they can be grown in media containing [3H]inositol, and can therefore be labelled to isotopic equilibrium, not a possibility with brain slices again due to their limited in vitro viability. Equilibrium labelling should result in a measurement of the individual inositol phosphate isomers which reflects the relate mass / concentration of that isomer at least over early time points and largely eliminates the problem of specific activity changes in the radiolabelling (see chapter 4).

Preliminary experiments described in chapter 7 have shown that it may be possible to examine
the effect of lithium upon intracellular Ca\(^{2+}\) release, however, these cells appear to have a poor cytoplasmic esterase activity resulting in a slow rate of metabolism of Fura-2AM to release the free acid form. These problems should be overcome by manipulation of experimental conditions such as loading time, recovery time, Fura-2AM concentration, cell density and possibly by adding agents which either facilitate Fura-2AM entry into the cell (Pluronic) or prevent the extrusion of the free acid form of the dye, Fura-2 (sulfinpyrazone). The use of this cell line should, therefore, open up new avenues of research enabling the examination of downstream effects of lithium. This is important in determining whether the effect of lithium upon phosphoinositide metabolism can have any significant effect upon the physiological processes and responses with which this signalling system is involved.

One important question which has not been addressed in this thesis, but bears considerably upon the interpretation of the data, is whether Ins(1)P\(_1\) and Ins(1,4)P\(_2\) are formed solely from the metabolism of Ins(1,4,5)P\(_3\) and not from a direct action of PI-PLC upon PtdIns and PtdIns(4)P respectively. PI-PLC activity directed against PtdIns or PtdIns(4)P could allow the cell another means of generating DAG without the production of Ins(1,4,5)P\(_3\). Indeed it has been shown that when purified and reconstituted into phospholipid vesicles PLC isoforms under the appropriate conditions can hydrolyse PtdIns and PtdIns(4)P as well as PtdIns(4,5)P\(_2\) releasing Ins(1)P\(_1\), Ins(1,4)P\(_2\) and Ins(1,4,5)P\(_3\) respectively (Rhee \textit{et al.}, 1989; Meldrum \textit{et al.}, 1991). It is thought that the preferred substrate \textit{in vivo} is PtdIns(4,5)P\(_2\), however since Ins(1)P\(_1\) and Ins(1,4)P\(_2\) are also products of Ins(1,4,5)P\(_3\) metabolism (Berridge & Irvine, 1989; Downes & MacPhee, 1990) it has proved difficult to establish unequivocally the substrate specificity of receptor-stimulated PLC activity in whole cells. Indeed, it has been suggested that factors that activate phosphoinositide hydrolysis by causing an influx of Ca\(^{2+}\) may actually shift the substrate-specificity of PLC activity away from PtdIns(4,5)P\(_2\) towards PtdIns and PtdIns(4)P (Eberhard & Holz, 1988; Baird & Nahorski, 1990). In the study by Baird & Nahorski (1990) it was found that agents such as ionomycin, which stimulate PI-PLC activity by virtue of increasing the [Ca\(^{2+}\)]\(_i\), generated a more marked increase in the
accumulation of InsP₂ than InsP₃ compared with muscarinic-receptor agonists. This had also been previously observed in GH₃ cells and was taken to suggest that the increase in [Ca²⁺]ᵢ may enhance the activity of the PtdIns(4,5)P₂ phosphomonoesterase and/or the hydrolysis of PtdIns(4)P (compared with PtdIns(4,5)P₂) (Kolesnick & Gershengorn, 1984).

A number of studies have addressed this problem directly, however no consensus has been reached from the results obtained to date. A number groups have concluded that Ins(1,4,5)P₃ metabolism could account for all Ins(1)P₁ formed and a similar number have reached the opposite conclusion. One of the first studies undertaken was by Downes & Wustman (1983) who examined the rates of formation and degradation of labelled inositol phospholipids and inositol phosphates in rat parotid gland and concluded that PLC did not hydrolyse PtdIns, but instead the activity of this enzyme was directed against the polyphosphoinositides PtdIns(4)P and PtdIns(4,5)P₂. More recent detailed work by Hughes and Putney (1989) in the same tissue examined the kinetics of [³H]inositol phosphate metabolism in agonist-stimulated tissue. This study examined the turnover rates of Ins(1,4,5)P₃ and its metabolites Ins(1,4)P₂ and Ins(1,3,4)P₃ after the addition of atropine to muscarinic receptor stimulated cells. The flux through both these isomers did not exceed the calculated rate of breakdown of Ins(1,4,5)P₃, providing no evidence for PLC hydrolysis of PtdIns(4)P. Similarly the rates of formation of the inositol monophosphates did not exceed the rate of metabolism of Ins(1,4,5)P₃ in these cells providing no evidence for PLC hydrolysis of PtdIns. Hughes & Putney (1989) therefore concluded that agonist-stimulated PLC selectively hydrolysed PtdIns(4,5)P₂ giving rise only to Ins(1,4,5)P₃. A similar examination of Ins(1,4,5)P₃ metabolism in SK-N-SH neuroblastoma cells (Fisher et al., 1990) provided further evidence to suggest that the major substrate for PLC in vivo was indeed PtdIns(4,5)P₂.

In contrast to the above a number of studies have concluded that PI-PLC may also hydrolyse PtdIns and/or PtdIns(4)P. Early work by Wilson et al. (1985) examined the changes in the specific activity of PtdIns(4)P and PtdIns(4,5)P₂ in thrombin-stimulated platelets. This study
demonstrated that the specific activity of PtdIns(4)P was similar in stimulated and unstimulated cells indicating that there was little increase in the conversion of PtdIns to PtdIns(4)P during thrombin stimulation of these cells. The results were therefore inconsistent with a rapid flux of PtdIns via PtdIns(4)P to PtdIns(4,5)P2. However, approximately 50% of PtdIns was degraded upon agonist stimulation in these cells. These results suggested therefore that PtdIns was hydrolysed by PLC in this cell type. Indeed, Imai & Gershengom (1986) later went on to demonstrate, in labelled GH3 cells, that PtdIns(4,5)P2 turnover was a transient event, lasting for only approximately 3 min, whilst PtdIns turnover was persistent over the time-course examined (30 min). The data from this study therefore suggest that agonist-stimulation of PLC results in the initial hydrolysis of PtdIns(4,5)P2 followed by a more persistent hydrolysis of PtdIns(4)P and PtdIns. The possibility arises that PLC may have a preference for PtdIns(4,5)P2 in vivo, as has been demonstrated in vitro. However as the levels of PtdIns(4,5)P2 decrease, PLC starts to hydrolyse PtdIns(4)P and PtdIns. More recently, Anderson et al. (1993) have demonstrated that in adult rat left atria the rate of turnover of Ins(1,4,5)P3 was less than that observed for the accumulation of the inositol monophosphates. Moreover, neomycin, which by virtue of its greater affinity for more negatively charged phospholipids is a selective inhibitor of PtdIns(4,5)P2 hydrolysis (Taylor et al., 1988) and therefore Ins(1,4,5)P3 formation, inhibited the accumulation of inositol phosphates in noradrenaline-stimulated isolated neonatal cardiomyocytes but did not inhibit the accumulation in left atria. These data suggest that the inositol phosphates which accumulate in adult rat left atria are not derived from Ins(1,4,5)P3. A similar study employing neomycin was reported by Biden et al. (1992). They demonstrated that in carbachol-stimulated isolated pancreatic islets, which had been pretreated with neomycin, the rise in Ins(4)P1 was inhibited by approximately 85%, compared with untreated controls, whereas the increase in Ins(1,3)P1 was not significantly affected. This could not be explained by a selective inhibition of the 5-phosphatase route, since the increases in the metabolites Ins(1,3,4)P3, Ins(3,4)P2 and Ins(1,3)P2, which are generated via the 3-kinase pathway, were also markedly attenuated. Again the suggestion
was that PtdIns may be target for PLC catalysed hydrolysis. Therefore at present with the conflicting data described above it is still unclear as to whether PLC catalyses PtdIns and PtdIns(4)P as well as PtdIns(4,5)P_2 in vivo upon receptor activation. Data presented in this thesis however may add further indirect evidence to the hypothesis that PLC can indeed hydrolyse PtdIns forming Ins(1)P_1 directly. In chapter 6 it was shown that carbachol in the presence of lithium could produce a time-dependent increase in the accumulation of the inositol monophosphate isomers Ins(1,3)P_1 and Ins(4)P_1. The profiles of the accumulation of these two inositol monophosphates are however significantly different. Ins(1)P_1 accumulated in a linear manner over the 30 min time-course examined, whereas Ins(4)P_1 levels increased over the initial 10 min before reaching a plateau which was maintained throughout the remainder of the time-course. Lithium was shown to have an inhibitory effect upon carbachol-stimulated Ins(1,4,5)P_3 accumulation after a lag period of 10 min. Therefore by 10 min Ins(1,4,5)P_3 production began to decrease. The possibility arises therefore that both the plateauing of Ins(4)P_1 levels and the decrease in the production of Ins(1,4,5)P_3 are linked, with the decrease in Ins(1,4,5)P_3 levels resulting in plateauing in Ins(4)P_1 levels. If this is true then the question arises as to why Ins(1,3)P_1 levels continue to increase in a linear manner, especially since Ins(1,4,5)P_3 production is decreasing and the levels of Ins(1,3,4,5)P_4, a potential source of Ins(1,3)P_1, are also substantially reduced. One possible suggestion is that the Ins(1)P_1 may be formed by PLC hydrolysis of PtdIns the levels of which remained at sufficient levels throughout the time-course. Examination of the accumulation of the inositol monophosphates from extracts of cerebral cortex slices stimulated with carbachol in the presence of lithium reveals that although Ins(1)P_1 accumulation is linear Ins(4)P_1 appears not to be, with the accumulation slowly decreasing with time, such that the initial rate of accumulation is not maintained throughout the entire time-course. One inconsistency in this hypothesis however is that the CMP-PA accumulation observed in CHO-M1 cells was not linear but instead followed a similar profile to that observed for Ins(4)P_1.
accumulation. It is conceivable that this may relate to some compartmentation of the DAG formed from PtdIns(4,5)P$_2$ from that produced by PI-PLC hydrolysis of PtdIns.

CHO-M1 cells may therefore be a useful tool for examining the possibility of PI-PLC hydrolysis of PtdIns. Indeed one possible approach in answering this question may be to perform dual labelling experiments with this cell type. Cells could be initially labelled to equilibrium with [14C]inositol and subsequently stimulated with agonist in the presence of lithium and [3H]inositol. If [3H]Ins(1)P$_1$ was observed before any 3H labelling of either PtdIns(4,5)P$_2$ or Ins(1,4,5)P$_3$ was observed then this would be conclusive evidence that Ins(1)P$_1$ could be formed directly from PtdIns by the action of PLC. The inhibition of PtdIns(4,5)P$_2$ hydrolysis, by the inclusion of neomycin, may facilitate such investigations or provide further confirmatory evidence.

It is well established that lithium can uncompetitively inhibit both the inositol monophosphatase and the Ins(1,3,4)P$_3$/Ins(1,4)P$_2$ 1-phosphatase, albeit with different $K_i$ values (see Shears 1991). However, the data presented in chapter 5 suggest the possibility of a further lithium-sensitive enzyme that may be involved in the metabolism of the inositol bisphosphate Ins(4,5)P$_2$. The agonist-stimulated accumulation of this isomer was shown to increase markedly upon addition of lithium. From in vitro work undertaken on purified enzymes it would appear that Ins(4,5)P$_2$ is a poor substrate for either of the well characterized lithium-sensitive enzymes mentioned above (see Shears, 1991). Studies examining the metabolism of Ins(4,5)P$_2$ by cellular homogenates were unsuccessful, resulting in no accumulation of any dephosphorylation or indeed phosphorylation product. It is suspected that this lack of observed metabolism in these preparations possibly reflects a requirement for cellular integrity. The future approach therefore must be to isolate both 4- and 5-phosphatase activities from the cells and screen the purified enzymes both for their activity against Ins(4,5)P$_2$ and the effect of lithium on any such activity.

Similarly, lithium, at a concentration (1 mM) which, at the single time point examined, had no significant effect on carbachol-stimulated Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ accumulation, produced
an initial enhancement in the agonist-stimulated accumulation of Ins(1,4)P₂ and Ins(1,3,4)P₃.

This concentration of lithium, although less than the Kᵢ for Ins(1,4)P₂ for this enzyme (Kᵢ = 9.6 mM; Gee et al., 1988b), was significantly greater than the Kᵢ for Ins(1,3,4)P₃ for this enzyme (Kᵢ = 0.46 mM; Gee et al., 1988b). Therefore, a rise in Ins(1,3,4)P₃ levels may have been expected. However, as mentioned above, concentration-response curves generated to lithium demonstrated that 1 mM lithium had no significant effect upon the accumulation of these isomers. This lack of affect may be related to the time point chosen to examine the concentration-dependent effects of lithium, since lithium clearly had a marked affect upon the accumulation of both isomers at earlier time points. The data is therefore difficult to interpret without knowing what the concentration-dependent effects of accumulation of these isomers with respect to lithium are at the earlier time points where lithium appears to have a significant effect.

However the possibility arises that the enhanced accumulation of Ins(1,4)P₂ and Ins(1,3,4)P₃ could be due to an action of lithium in enhancing Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ 5-phosphatase activity in this tissue, as has been previously observed in platelets (Connolly et al., 1987). Again, the only conclusive method of examining this possibility would be to purify the enzymes which may be involved in this effect.

A great deal of work has now been published on the disruptive effects which lithium has upon phosphoinositide metabolism in a variety of preparations, however, it is still uncertain whether disruption of phosphoinositide metabolism by this ion has any role in its therapeutic action in manic-depressive disorders. As mentioned previously, it has been suggested that the condition of bipolar depression may be due to hyperactive phosphoinositide signalling in a small group of neurons within the brain and that the action of lithium is to selectively target these neurons and, by depleting inositol reserves within the cells, return the signalling to a normal level. Unfortunately, it has still not been demonstrated which area(s) of the brain or group of neurons may be involved in the condition of bipolar depression. Several recent reports have demonstrated that other disease states can arise from cell-surface receptor mutations which
result in a hyperactivity of the signalling systems which they initiate. For example, it has been demonstrated that somatic mutations in the thyrotropin receptor gene result in an increase in the activity of the adenylyl cyclase system, which these receptors are linked to, resulting in hyperfunctioning thyroid adenomas (Parma et al., 1993). Similarly, a mutation of the luteinizing hormone receptor has also been shown to enhance adenylyl cyclase and be involved in familial male precocious puberty (Shenker et al., 1993). It is therefore possible that the aetiology underlying a disease state such as bipolar depression may be related to some signalling dysfunction at the level of receptor / G-protein / effector coupling. However, it is difficult to reconcile such a simple model of hyperactivity within a signalling system, such as the phosphoinositide system, with the complex patterns of mood observed in bipolar depression, with the characteristic periodic swings from mania to depression. It is therefore likely that a more complex mechanism underlies this condition, with an alteration in phosphoinositide metabolism having a central role.

One possible way of determining the importance of the inhibition of the monophosphatase by lithium in the therapeutic action of this agent would be to develop selective inhibitors of this enzyme. It should then be possible to examine the effects of such inhibitors and determine whether inhibition of this enzyme has any therapeutic activity in relation to the disease state of mania. Indeed, some progress has been made in the design of such agents (Baker et al., 1991; Atack et al., 1993). However, the inhibitors available at present are competitive in nature. Whether such a competitive inhibitor could have as a profound/selective effect upon phosphoinositide metabolism as that observed with the uncompetitive inhibition produced by lithium is unclear at present. However, recent studies examining the competitive inhibition of the monophosphatase by beryllium (Faraci et al., 1993) suggest that such an inhibition of this enzyme will have little effect on agonist-stimulated inositol phosphate accumulation. Beryllium was demonstrated to have no significant effect upon carbachol-stimulated inositol phosphate accumulation in either rat cerebral cortex slices or permeabilized SK-N-SH cells.

The main findings of this thesis are that lithium, at therapeutic concentrations, produces a disruption in phosphoinositide metabolism due to the uncompetitive inhibition of the inositol...
monophosphatase by this monovalent ion. The inhibition of this enzyme deprives the cell of two important sources of inositol, that from the recycling of the inositol phosphates and that from de novo synthesis. In agonist-stimulated cells the inositol levels become depleted (at least in cerebral cortex slices and CHO-M1 cells). As inositol concentrations fall below the $K_m$ for PtdIns synthase the synthesis of the (poly)phosphoinositides decreases, resulting in a fall in the agonist-stimulated levels of PtdIns(4,5)P$_2$, the precursor of the second messenger Ins(1,4,5)P$_3$. The agonist-stimulated levels of Ins(1,4,5)P$_3$ subsequently decrease after a lag period which is related to the time required to deplete inositol and inositol phospholipid reserves. This scheme has been shown to be true for both a single sustained stimulus or indeed multiple stimulations of this signalling pathway in CHO-M1 cells. The data are therefore in line with, and add weight to, the current hypothesis of the mechanism of action of lithium in disruption of the phosphoinositide signalling system (see Nahorski et al., 1991). More subtle actions of lithium upon Ins(4,5)P$_2$, Ins(1,4)P$_2$ and Ins(1,3,4)P$_3$ accumulation in rat cerebral cortex may also play a role in the action of lithium in mania, however, this possibility requires further examination.

Recent data suggest that there may be a species variation in the cellular inositol content of cerebral tissue. Studies by Dixon et al. (1992) in monkey cerebral cortex slices and by Lee et al. (1992) in rat, mouse and guinea-pig cortical slices have demonstrated that although lithium can attenuate agonist-stimulated Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ levels in the cerebral cortex slices from rat and mouse, no significant attenuation of lithium upon these isomers was observed in guinea-pig or monkey cerebral cortex slices. Indeed, lithium addition, albeit at high concentrations (10 mM), resulted in an increase in the agonist-stimulated levels of Ins(1,4,5)P$_3$ in both these tissues. These data have therefore questioned the validity of the inositol depletion hypothesis of lithium action in vivo.

The majority of the evidence for this hypothesis has come from biochemical experiments performed in vivo and employing unphysiological concentrations of agonist. However, there is also support for the lithium depletion hypothesis from recent in vivo studies in cerebral cortex.
The examination of pilocarpine-stimulated InsP$_3$ accumulation in rat cerebral cortex has clearly demonstrated that lithium can attenuate the pilocarpine-induced accumulation of this isomer (after a lag period of approximately 60 min). These data therefore demonstrate that the reduction in cerebral inositol levels that have been observed in the presence of lithium (Allison & Stewart, 1971) are sufficient to produce a decrease in the agonist-stimulated levels of the inositol polyphosphates in this tissue in vivo.

An important question, however, is whether the characterized effects of lithium upon phosphoinositide signalling observed in this study are related to the anti-manic properties of this agent. The development of novel selective inhibitors of the monophosphatase, as discussed earlier, may help to answer this question. However, at present the evidence in favour of this action of lithium appears to be overwhelming.
Appendices
APPENDIX I

Buffers:

Krebs-Henseleit Buffer:

<table>
<thead>
<tr>
<th></th>
<th>(mM)</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.6</td>
<td>6.94</td>
</tr>
<tr>
<td>KCl</td>
<td>4.7</td>
<td>0.35</td>
</tr>
<tr>
<td>MgSO₄·6H₂O</td>
<td>1.2</td>
<td>0.29</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.2</td>
<td>0.16</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.0</td>
<td>2.10</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.7</td>
<td>2.10</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.3</td>
<td>0.19</td>
</tr>
</tbody>
</table>

The buffer was gassed for 15 min prior to use with O₂/CO₂ (95% : 5%) to bring the pH to 7.4.

Krebs-HEPES Buffer:

<table>
<thead>
<tr>
<th></th>
<th>(mM)</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.6</td>
<td>6.74</td>
</tr>
<tr>
<td>KCl</td>
<td>4.7</td>
<td>0.35</td>
</tr>
<tr>
<td>MgSO₄·6H₂O</td>
<td>1.2</td>
<td>0.29</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.2</td>
<td>0.16</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>4.2</td>
<td>0.35</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.7</td>
<td>2.10</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.3</td>
<td>0.19</td>
</tr>
<tr>
<td>HEPES (free acid)</td>
<td>10.0</td>
<td>2.38</td>
</tr>
</tbody>
</table>

The pH of the buffer was adjusted to 7.4 at 37°C by addition of NaOH.
**Intracellular Buffer (ICB):**

<table>
<thead>
<tr>
<th>Component</th>
<th>(mM)</th>
<th>(g/500ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>120</td>
<td>4.48</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2</td>
<td>0.14</td>
</tr>
<tr>
<td>(NaH$_2$COONa)$_2$.6H$_2$O</td>
<td>5</td>
<td>0.67</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>6</td>
<td>0.61</td>
</tr>
<tr>
<td>HEPES (free acid)</td>
<td>20</td>
<td>2.38</td>
</tr>
</tbody>
</table>

After preparation of the above buffer 50ml was made up containing 5mM ATP final concentration (0.136g/50ml of ICB). The pH was then corrected to 6.9 with 20% (w/v) KOH and 125µl of EGTA (10mM / 20mM KOH; pH 7.2) was added to drive the free [Ca$^{2+}$] to below 100nM.

**Ins(1,4,5)P$_3$ Mass Assay Wash Buffer:**

<table>
<thead>
<tr>
<th>Component</th>
<th>(mM)</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>25</td>
<td>3.03</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>5</td>
<td>0.42</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.2</td>
<td>0.37</td>
</tr>
</tbody>
</table>

The pH of the buffer was adjusted to 8.0 at 4°C by the addition of HCl.
Ins(1,3,4,5)P$_4$ Mass Assay Wash Buffer:

<table>
<thead>
<tr>
<th></th>
<th>(mM)</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Acetate</td>
<td>25</td>
<td>3.40</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>25</td>
<td>3.40</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>5</td>
<td>0.42</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>0.29</td>
</tr>
</tbody>
</table>

The pH of the buffer was adjusted to 5.0 at 40°C by the addition of HCl.

Phosphate Buffered Saline:

<table>
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<th>(mM)</th>
<th>(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137</td>
<td>8.00</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7</td>
<td>0.20</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>8.1</td>
<td>1.15</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.5</td>
<td>0.20</td>
</tr>
</tbody>
</table>

When used for harvesting cells 0.5mM EDTA (0.15 g/l) was added to above buffer.
**APPENDIX II.**

**H.p.l.c. Gradient For Separation Of Inositol Phosphate Isomers.**

This gradient is for a three solvent system comprising of:

A: $\text{H}_2\text{O}$

B$_1$: 0.5 M $\text{NH}_4\text{H}_2\text{PO}_4$

B$_2$: 1.4 M $\text{NH}_4\text{H}_2\text{PO}_4$

The pH of both solution B$_1$ and B$_2$ was adjusted to 3.7 by the addition of $\text{H}_3\text{PO}_4$. The values are expressed as percentage contribution by B$_1$ and B$_2$ at each time point. The flow rate was 1ml/min.

<table>
<thead>
<tr>
<th>T</th>
<th>% B$_1$</th>
<th>% B$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>30.1</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>60.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>95</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>95.1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>110</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>110.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
APPENDIX III

Materials

[2-3H]Inositol (16-20 Ci / mmol), [5-3H]cytidine (20-40 Ci / mmol), [1-3H]Ins(1,4,5)P$_3$ (17-20 Ci / mmol) and [5-32P]Ins(1,3,4,5)P$_3$ (120-160 Ci / mmol) were purchased from either NEN (DuPont) or Amersham International PLC. Tissue culture media was obtained from Gibco Ltd. (U.K.). Chemicals were purchased from Fisons, Aldrich, BDH or Sigma. Ins(4,5)P$_2$ was obtained from Boehringer-Mannheim, Ro318220 was a gift from Roche Products Ltd. (U.K.) and all other drugs were from Sigma. Analytical grade Dowex anion exchange resin was obtained from Bio-rad and liquid scintillation cocktails were from Canberra-Packard.
APPENDIX IV.

Abbreviations.

Inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphates are based on D-myoinositol unless otherwise stated. InsP₁, InsP₂, InsP₃, InsP₄, InsP₅ and InsP₆ refer to myoinositol mono, bis, tris, tetrakis, pentakis and hexakis phosphates, where appropriate the position of the locants around the inositol ring are given in parentheses to donate a specific isomer. The use of : indicates a cyclic phosphate, associated with the two positions. Similarly, the (poly)phosphoinositide lipid abbreviations are as follows:

PI Phosphoinositide
PtdIns Phosphatidylinositol
PtdIns(3)P Phosphatidylinositol 3-phosphate
PtdIns(4)P Phosphatidylinositol 4-phosphate
PtdIns(3,4)P₂ Phosphatidylinositol 3,4-bisphosphate
PtdIns(4,5)P₂ Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P₃ Phosphatidylinositol 3,4,5-trisphosphate

ADP adenosine 5'-diphosphate
AMP adenosine 5'-monophosphate
AP₄ adenosine 5'-tetraphosphate
ATP adenosine 5'-triphosphate
βARK β-adrenergic receptor kinase
Bₘₐₓ Maximal density of binding sites
Ca²⁺ Calcium, free ion
[Ca²⁺]ᵢ Intracellular Ca²⁺ concentration
CCh  Carbachol
CDNA  Complementary DNA
CHO cells Chinese hamster ovary cells
CMP  Cytidine monophosphate
CMP-PA  CMP phosphatidic acid
CNS  Central nervous system
cAMP  adenosine 3':5'-cyclic monophosphate
cGMP  guanosine 3':5'-cyclic monophosphate
DAG  sn-1,2-diacylglycerol
EC50  Concentration of an agent producing 50 % of maximal response
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
EGTA  Ethyleneglycol-bis-(B-aminoethyl ether) N,N,N',N'-tetraacetic acid
G-protein  Guanine nucleotide-binding protein
GDP  Guanosine 5'-diphosphate
GMP  Guanosine 5'-monophosphate
GP4  Guanosine 5'-tetraphosphate
GTP  Guanosine 5'-triphosphate
GroPtdIns  Glycerophosphorylinositol
HEPES  N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
HPLC  High performance liquid chromatography
IC50  Concentration of agent inhibiting a response by 50 %
ICB  Intracellular buffer
Kd  Dissociation constant
KM  Michaelis-Menten constant
Li+  Lithium, free ion
NGF  Nerve growth factor
PA   Phosphatidic acid
PBS  Phosphate buffered saline
PCA  Perchloric acid
PDGF Platelet derived growth factor
PI-PLC Phosphoinositide-specific phospholipase C
PLC  Phospholipase C
S.E.M. Standard error of the mean
TCA  Trichloroacetic acid
TRH  Thyrotropin releasing factor
$V_{\text{max}}$ Maximum velocity of an enzyme catalysed reaction
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605-607.


Diehl, R.E., Whitting, P. Potter, J., Gee, N.S., Ragan, C.I., Linemeyer, D., Schoepfer, R.,

11546-11552.


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Publications
PUBLICATIONS

At the time of writing the following publications had resulted from work described in this thesis:

Full papers:

Jenkinson, S., Challiss, R.A.J. & Nahorski, S.R.


Review papers:


Challiss, R.A.J., Jenkinson, S., Mistry, R., Batty, I.H. & Nahorski, S.R.
Review papers (cont.):

Jenkinson, S.
Separation of labelled inositol phosphate isomers by high pressure liquid chromatography.

Abstracts:

Quantitation of the lithium-sensitive component of the muscarinic receptor-stimulated inositol 1,3,4,5-tetrakisphosphate response in rat cerebral cortex. (1992) Biochem. Soc. Trans., 20, 137S.


Inhibition of agonist-stimulated inositol polyphosphate accumulations by lithium in cerebral cortex. (1992) 8th International Conference On Second Messengers & Phosphoproteins, Glasgow, U.K.
Abstracts (cont.):


Jenkinson, S., Challiss, R.A.J. & Nahorski, S.R.