# LITHIUM ACTIONS ON INOSITOL LIPID CELL SIGNALLING

ELEANOR D. KENNEDY

Lithium ($Li^+$) is the major drug presently prescribed in the treatment of manic depression. Its site of action within the central nervous system is unknown although it has been known for several years to have profound effects upon inositol (poly)phosphate metabolism. The basis of the work presented here is to investigate the effects of $Li^+$ upon the accumulation of labelled and unlabelled inositol (poly)phosphates in muscarinic cholinergically stimulated rat cerebral cortical slices and cultured cell lines. Its effects on the accumulation of CMP-phosphatidic acid (CMP-PA), a precursor to (poly)phosphoinositide lipid resynthesis and upon the inhibition of inositol monophosphatase activity are also described.

It has been shown that the presence of $Li^+$ leads to reduced accumulation of, not only $Ins(1,3,4,5)P_4$ but also of $Ins(1,4,5)P_3$, the molecule responsible for the release of intracellular $Ca^{2+}$. The labelled $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ which accumulate under these conditions were separated in the absence of $Mg^{++}$ using an enzymic preparation from rat cerebral homogenate. This technique allows an effective and accurate separation which circumventss the use of h.p.l.c.

It has also been demonstrated that both $[^3H]InsP_1$ and $[^{14}C]CMP-PA$ increase with similar $EC_{50}$ values in the presence of increasing $Li^+$ concentrations and also with increasing time in the presence of $Li^+$. Furthermore, work has indicated that the preincubation of rat cerebral cortical slices with myo-inositol can, at least partially, reverse the accumulation of labelled CMP-PA. However, if the myo-inositol is added subsequent to the $Li^+$ block being established, incomplete reversal is only observed in the presence of very high concentrations of inositol. In addition, similar experiments were conducted to observe the effects of myo-inositol upon the $Li^+$-induced reduced accumulation of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ as measured by radio-receptor assay. Whilst the presence of 10mM myo-inositol prolonged the lag phase routinely observed between 5 and 10 mins, it could not prevent the fall in accumulation from occurring. Scyllo-inositol, a naturally occurring isomer of myo-inositol, was found to be completely ineffective in reducing the accumulation of $[^{14}C]CMP-PA$.

In other experiments, the effects of $Li^+$ upon the carbachol-stimulated inositol (poly)phosphate fractions were examined in CHO cells transfected with an $M_1$ muscarinic receptor subtype and also in SH-SY5Y cells and the results compared to those obtained in rat cerebral cortical slices.

Finally, an assessment of inhibition of the inositol monophosphatase activity was made in a variety of cell free systems. This inhibition was caused using either $Li^+$ or polyclonal antibodies raised to purified inositol monophosphatase.

In conclusion, the results obtained verify the profound effects which $Li^+$ can have upon inositol (poly)phosphate metabolism and suggest that whilst the primary intracellular target of $Li^+$ may indeed be the inositol monophosphatase it may also be causing other subtle alterations to the cell functioning. These possibilities and their implications are discussed.

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# CHAPTER 1


## Introduction

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## INTRODUCTION

The evolution of multicellular organisms has demanded the development of sophisticated mechanisms which allow cell-to-cell communication. External stimuli are detected by receptors which facilitate transduction and amplification of the message intracellularly by means of "second messengers". Consequently, the control of events like metabolism, secretion, contraction and proliferation can be tightly regulated. Several such paradigms have been understood for many years including the activation and inhibition of adenylate cyclase, the modulation of ion channel opening and protein kinase stimulation. In recent years, however, the interest surrounding inositol lipid metabolism as a putative transduction pathway has increased substantially into what is now a divergent field of investigation.

### History

In 1930, Anderson and Roberts demonstrated the existence of inositol - containing lipids in mycobacterial membranes (Anderson and Roberts, 1930). The first report of such lipids in eukaryotes, however, was not until 1942 when Folch and Woolley isolated a phospholipid fraction from bovine brain (Folch and Woolley, 1942). The actual components of this fraction were later shown to be phosphatidylinositol 4-phosphate (PtdIns(4)P), phosphatidylinositol $(4,5)P_2$ (PtdIns$(4,5)P_2$) 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 assumed that, at least in eukaryotic membranes, they constitute a minor

(<10%) component of the total phospholipid (see Fig. 1.1).

In the 1950's, Hokin and Hokin, investigating the lipid labelling that followed agonist stimulation of exocrine pancreas, reported that the greatest increases observed in incorporation were into phosphatidic acid (PA) and PtdIns (Hokin and Hokin, 1958). They referred to this phenomenon as the "PI response". Furthermore, closer investigation of this response revealed that, whilst the incorporation of labelled phosphate was large, 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 turnover of the inositol headgroup. This occurrence had been previously suspected when it was found that, in stimulated avian salt glands, PA was formed rapidly upon addition of the agonist but that once the agonist was removed, PA was not metabolized into its component moieties. Indeed, as the levels of PA decreased, those of PtdIns increased, implicating a simple headgroup exchange mechanism (for review see Hokin and Hokin, 1964). Although having established that PtdIns was present in cell membranes and that its levels appeared to alter in the presence of agonist, the implication that inositol lipids were involved in intracellular signalling took over a decade to develop (see for example Durell, 1969; de Robertis, 1971; Michell, 1975; Michell and Kirk, 1981). Several key features became apparent during this period which enhanced the view that the "PI response" was intrinsically associated with receptor function. The most important of these was the discovery that certain receptors, notably the muscarinic cholinergic and α-adrenergic receptors, appeared to promote more inositol lipid metabolism than others. Furthermore, this stimulation was not linked to any obvious cellular response. Instead, these receptors appeared to induce increases in the intracellular $Ca^{2+}$ concentration (Michell, 1975; Jones and Michell, 1975).

Durell's appraisal of the system was the first in which a role for a phospholipase C (PLC)-mediated hydrolysis of inositol lipids was implicated

Phosphatidylinositol
(PtdIns)
(monophosphoinositide)

Phosphatidylinositol 4-phosphate
(PtdIns(4)P)
(diphosphoinositide)

Phosphatidylinositol 4,5-bisphosphate
(PtdIns(4,5)P$_2$)
(triphosphoinositide)
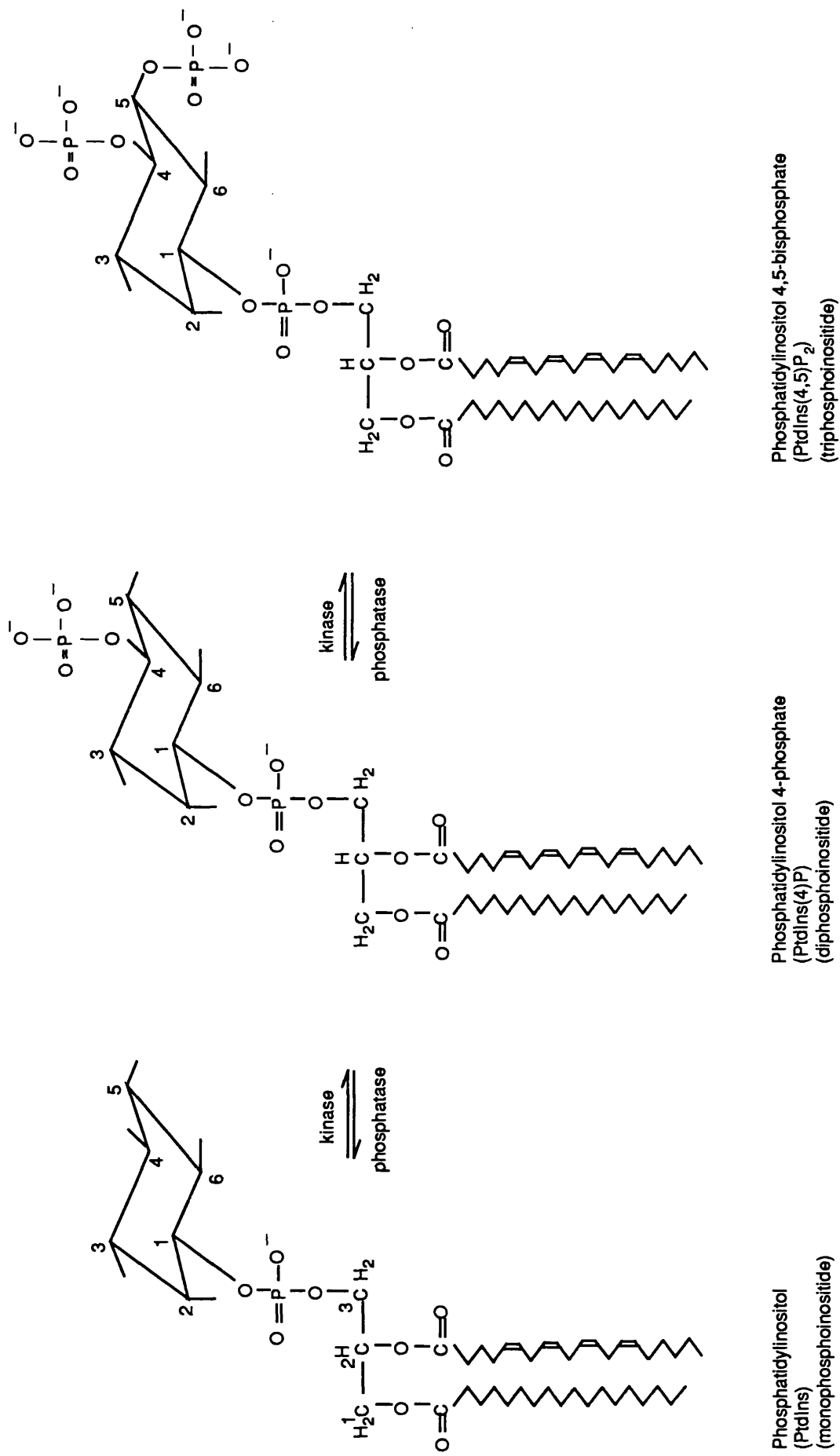
Fig. 1.1 - Structure of the (poly)phosphoinositide lipids in brain. A large proportion of these inositol-containing lipids possess stearate (C18:0) on the 1 - carbon and arachidonate (C20:4) on the 2 - carbon of the glycerol moiety. The only structural distinction between these lipids is the degree of phosphorylation on the 4- and 5-positions.

(Durell, 1969) although Hokin and Hokin had previously suggested that hydrolysis of PtdIns to 1,2-diacylglycerol (1,2-DAG) and inositol monophosphate ($InsP_1$) by a PtdIns-specific PLC could be the initiator of the intracellular signal (for review see Hokin, 1985). The realization that the initial target for PLC was, in fact, $PtdIns(4,5)P_2$ and not PtdIns, as had been thought, came in 1981 (Michell and Kirk, 1981; Kirk et al., 1981). This would lead to the production of not only 1,2-DAG but also of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). The seminal studies of Streb et al. (1983) indicated that in rat pancreatic acinar cells, it was this latter moiety that was responsible for intracellular $Ca^{2+}$ mobilization from a non-mitochondrial, intracellular source following receptor-activated inositol lipid hydrolysis whilst the 1,2-DAG metabolite was subsequently found to activate endogenous protein kinase C (PKC)(for reviews see Nishizuka, 1984 and 1986). Since 1981, the progress in the field of inositol lipid signal transduction has been rapid and its intricacies appear complex. Fig. 1.2 illustrates the cascade phenomenon that occurs upon agonist stimulation as it is presently understood.

The aim of this introduction is to discuss the constituent elements of this metabolic pathway from the receptor to the regeneration of free inositol with particular emphasis on the pathway in neuronal tissue. The known therapeutic and pharmacological aspects of $Li^+$ will also be described.

## Muscarinic cholinergic receptors

Receptors can be characterized as either intracellular or membrane-bound. Likewise, the agonists which stimulate these receptors can be divided into two broad groups, namely hormones which activate the former types (e.g. the steroids) or extracellular stimuli which interact with cell-surface receptors. This latter group includes peptide and amine hormones, neurotransmitters, growth hormones and antigens. Their intrinsic

Fig 1.2 - Cascade reaction of inositol (poly)phosphate synthesis and metabolism following agonist stimulation.

Ag = agonist; R = receptor; Gp = G-protein; PLC = phospholipase C; DAG = diacylglycerol; PKC = protein kinase C

(a) = 3-kinase    Mg++ dependent
(b) = 5-phosphatase    Mg++ dependent
(c) = 1-phosphatase    Mg++ dependent; inhibited by Li+
(d) = 4-phosphatase    Mg++ independent
(e) = 3-phosphatase    Mg++ independent
(f) = inositol monophosphatase    Mg++ dependent; inhibited by Li+

lipophobicity prevents them from entering their target cell and, thus, they must transfer their message across the cell membrane via the receptor. The process of signal transduction relies on the cell's ability to create a "cascade" effect. This means that a few molecules of signal, working through the appropriate receptor, can generate an enormous effect within the cell.

Muscarinic cholinergic receptors can be pharmacologically identified from other cholinergic receptors by their capacity to be selectively stimulated by muscarine. They are responsible for mediating the "slow" actions of the neurotransmitter, acetylcholine - for example, stimulation of secretion from cells, contraction of smooth muscle and relaxation of cardiac muscle. Although originally thought to consist of only two members, $M_1$ and $M_2$, on the basis of their affinity for the selective antagonist, pirenzepine (Hammer and Giachetti, 1982), molecular cloning has now identified an extended family with at least five members in the human genome (see Buckley, 1990). The receptors which these clones encode for are nominally termed $M_1$, $M_2$, $M_3$, $M_4$ and $M_5$ and all five have been successfully transfected into recipient cells and expressed (for review see Bonner, 1989). The assignment of different 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 $m_1$, $m_3$ and $m_5$ messenger RNA (mRNA) couple to the stimulation of (poly)phosphoinositide metabolism whilst $M_2$ and $M_4$ both appear to inhibit the adenylate cyclase system. Several exceptions to this generalization do exist, however. $M_2$ and $M_4$ receptors can couple to stimulation of (poly)phosphoinositide hydrolysis although this is noticeably less efficient than their coupling to adenylate cyclase inhibition (Ashkenazi et al. 1987; Peralta et al. 1988; Ashkenazi et al. 1989). Moreover, RAT-1 cells transfected with $M_1$ receptors couple to both stimulation of (poly)phosphoinositide hydrolysis and inhibition of adenylate cyclase (Pinkras-Kramarski et al. 1988; Stein et al. 1988).

Northern blot analysis and in situ hybridization studies have been

utilized to determine the distribution of muscarinic receptors in a variety of cell types (see Buckley, 1990; Brann et al. 1987; Buckley et al. 1988). Cerebral cortex, which is the tissue used in the majority of the experiments described here, is thought to express $M_1$, $M_3$ and $M_4$ receptors although the mRNA for the latter two appears to be much less abundant than for the $M_1$. The two other cell types which are predominantly used in the following chapters are Chinese hamster ovary (CHO) cells which have been stably transfected with the human $M_1$ receptor gene and SH-SY5Y cells which are of neuronal origin. According to Lambert et al. (1989), these cells express a homogeneous population of $M_3$ receptors.

Like many of the receptors which are coupled to functional G-proteins, the amino acid sequence of the muscarinic receptor contains seven hydrophobic sequences which reputedly span the membrane bilayer. It appears that the sequence of all the muscarinic receptor subtypes for which mRNA has been isolated is well conserved. However, that for the $M_1$, $M_3$ and $M_5$ receptors bear the closest resemblance to each other. Similarly, the $m_2$ and $m_4$ mRNA sequences are obviously related but they exhibit less homology to the other three (Bonner, 1989).

## Muscarinic receptor - G-protein coupling

GTP-binding proteins are commonly found in the membrane as heterotrimers consisting of $\alpha$-, $\beta$- and $\gamma$- subunits. As with most of the components of the (poly)phosphoinositide signalling pathway, there appears to be a complicated, extended family of G-proteins. These include both stimulatory and inhibitory G-proteins which are associated with the adenylate cyclase signalling pathway, those which are associated with phototransduction and ion channel activity and others like $G_0$ and the low molecular weight G-proteins for which the functions are as yet unknown (for reviews see Gilman, 1987; Neer and Clapman, 1988; Birnbaumer, 1990).

The interaction of a receptor with a G-protein is driven by the binding of the appropriate ligand to the receptor. The α-subunits of G-proteins have intrinsic GTPase activity. When a G-protein couples to a receptor, this stimulates dissociation of the G-protein and GDP, which is bound when the G-protein is inactive. The complex then binds GTP and the α-subunit dissociates from the heterotrimeric complex. This couples the receptor stimulation to the next component in the transduction pathway. The half-life of the agonist - receptor - G-protein complex is very short and it has be calculated that one receptor can interact with several G-proteins over a period of a few seconds (Pederson and Ross, 1982; Hekman et al. 1984).

The G-protein associated with (poly)phosphoinositde metabolism has been the subject of some debate since its original conception (for review see Cockcroft, 1987). Putatively termed "$G_p$", there appears to be a degree of heterogeneity within this family of proteins also (see for example Brass et al. 1987; Ashkenazi et al. 1989). As it has yet to be purified and characterized, efforts to isolate the protein in association with the PtdIns-PLC have been made. Whilst early experiments were promising, the results indicated that, if they represented a physiological state and were not an artefact of the purification procedure, the $G_p$ may have a subunit structure radically different to that of the well-characterized G-proteins in other coupled systems (Baldassare and Fisher, 1986; Wang et al. 1988; Lapetina and Reep, 1987).


## G-protein - PLC coupling


The original proposal that G-proteins couple to PtdIns-PLC enzymes was in 1983 when Gomperts described their involvement in histamine release from rat mast cells (Gomperts, 1983). Non-hydrolyzable analogues of GTP, like guanine 5'-(3-0-thio)triphosphate (GTPγS), and $AlF_4^-$ have since proved to be invaluable in investigations involving G-proteins because they effect

long-term stimulation of the G-protein (Cockcroft and Gomperts, 1985; Bigay
et al. 1987). Using such pharmacological tools, a wide variety of cell
systems have been identified in which G-proteins appear to couple to
PtdIns-PLC, including platelets (Brass et al. 1986), pancreatic acinar cells
(Merritt et al. 1986), SH-SY5Y cells (Wojcikiewicz et al. 1990) and cerebral
cortex (Gonzales and Crews, 1985).


## PtdIns-PLC enzymes


The PLC enzymes appear to be a multifarious family of phosphodiesterases
which hydrolyze the glycerophosphate bond of intact phospholipids to generate
DAG and the aqueous-soluble headgroup (see Fig. 1.3). The vast majority of
known PLC activities hydrolyze all three (poly)phosphoinositide lipids
(Wilson et al. 1984) to yield DAG as a common product and $Ins(1)P$, $Ins(1,4)P_2$
or $Ins(1,4,5)P_3$ depending on the state of phosphorylation of the parent
lipid.

The PtdIns-PLC enzymes can be characterized into four main submembers of
a superfamily, namely $\alpha$, $\beta$, $\gamma$ and $\delta$ on the basis of their molecular weight,
purification, cloning and immunoreactivity. They also require $Ca^{2+}$ for
activity in vitro and it is now apparent that, at micromolar concentrations
of $Ca^{2+}$ the hydrolyses of $PtdIns(4)P$ and $PtdIns(4,5)P_2$ occur in preference to
that of PtdIns (Wilson et al. 1984; Nakanishi et al. 1985; Banno et al.
1986). This sensitivity to $Ca^{2+}$ can be altered by the pH of the assay and
the presence of monovalent ions (Allen and Michell, 1974). The nature of the
lipid substrate being utilized and the particular PLC isoenzyme under
investigation are also important variables to be considered when studying the
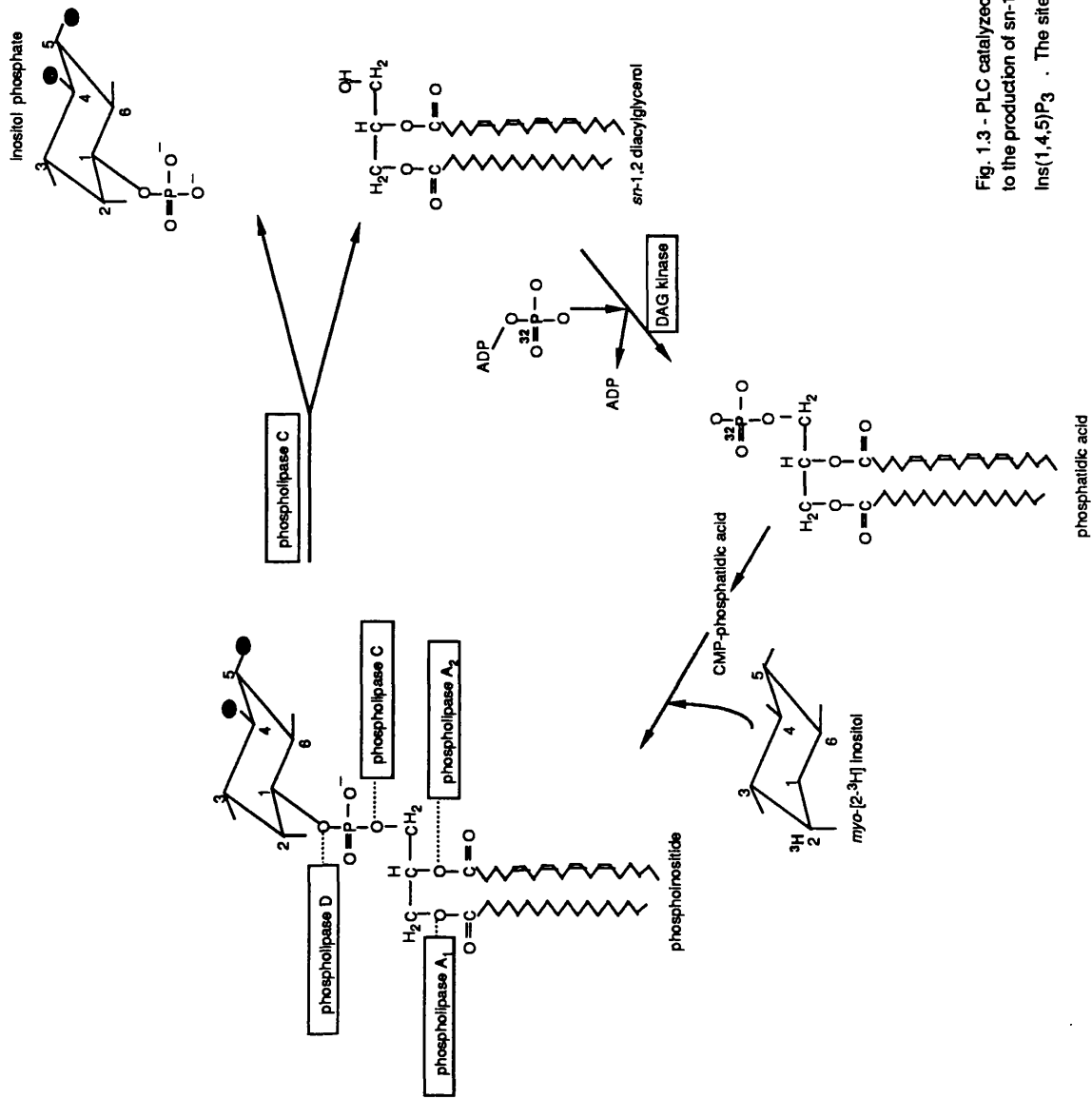$Ca^{2+}$ dependency (Nakanishi et al. 1985; Hofmann and Majerus, 1982).

Fig. 1.3 - PLC catalyzed hydrolysis of the (poly)phosphoinositide lipids. This enzymic cleavage always leads to the production of sn-1,2-diacylglycerol and,depending on the parent lipid, Ins(1)P, Ins(1,4)P$_2$ or Ins(1,4,5)P$_3$. The site of cleavage of phospholipases A, A$_2$ and D are also shown for comparison.

## (Poly)phosphoinositide lipid synthesis

PtdIns is a unique lipid in mammalian cells. It is the only parent lipid known which can be sequentially phosphorylated. Most commonly these phosphorylations occur at the 4- and 5- positions to yield PtdIns(4)P and PtdIns(4,5)$P_2$ although the recent discovery of three novel (poly)phosphoinositide lipids - PtdIns(3)P, PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ - means that even this statement may be over-simplified (Traynor-Kaplan et al. 1988; Whitman et al. 1988; Traynor-Kaplan et al. 1989).

PtdIns is synthesized at the level of the endoplasmic reticulum (Benjamins and Agranoff, 1969; Takenawa and Egawa, 1977) and is formed by the combination of phosphatidic acid (PA) and myo-inositol, both of which may be formed de novo from D-glucose. PA subsequently combines with intracellular cytidine 5'-triphosphate (CTP) in the presence of CTP-PA cytidyl transferase to produce CMP-phosphatidic acid (CMP-PA). CMP-PA then combines with myo-inositol to generate PtdIns.

The inositol-containing lipids found in animal tissues characteristically exhibit a predominantly 1-stearoyl, 2-arachidonoyl fatty acid composition on the sn-glycerol backbone (Holub and Kuksis, 1978). The composition of PA, however, although a close metabolic precursor of PtdIns, has very low levels of these two lipids in the 1- and 2- positions when formed de novo. In cells in which there is active inositide lipid hydrolysis, PA can be produced directly by the phosphorylation of DAG and, and as a result, PtdIns will retain its distinctive fatty acyl arrangement without PA undergoing extensive cycles of deacylation and reacylation. Indeed, results indicating that the PA formed in such a way is rich in both stearoyl and arachidonoyl moieties have been obtained for example in stimulated platelets (Broekman et al. 1981) and murine pancreatic tissue (Geison et al. 1976).

PtdIns can be rapidly transported from its site of synthesis by means of

the PtdIns transfer protein (George and Helmkamp, 1985; Somerharju et al. 1983). It is, therefore, readily accessible to the kinases which are responsible for converting PtdIns into its phosphorylated derivatives. PtdIns kinase has been implicated in playing a pivotal role in linking inositol lipid hydrolysis to tyrosine kinase activity (Sale et al. 1986; Sugimoto et al. 1984; Macara et al. 1984; Thompson et al. 1985). The two kinases - PtdIns kinase and PtdIns(4)P kinase - also have complimentary phosphatases which convert PtdIns(4,5)$P_2$ firstly into PtdIns(4)P and then to PtdIns. The purpose of these so-called "futile cycles" of phosphorylation and dephosphorylation is not fully understood.


## PtdIns(4,5)$P_2$ metabolism


The first evidence that Ins(1,4,5)$P_3$, the aqueous-soluble headgroup released following PLC-catalyzed hydrolysis of PtdIns(4,5)$P_2$, was responsible for $Ca^{2+}$ mobilization was made by Streb et al. (1983), who monitored the sequestration and release of $Ca^{2+}$ in permeabilized pancreatic cells and established that $Ca^{2+}$ was mobilized from an intracellular, non-mitochondrial site. The actual location has been debated but it is now believed that the receptor for Ins(1,4,5)$P_3$ is situated either on the endoplasmic reticulum (Supattapone et al. 1988) or on a specific organelle similar in structure to the endoplasmic reticulum (Volpe et al. 1988). The irregularities observed concerning the $Ca^{2+}$ dependency of this receptor-mediated $Ca^{2+}$ release in different tissue preparations have now been resolved with the identification of the $Ca^{2+}$-binding protein, calmedin (Danoff et al. 1988). This enzyme, which is present in neuronal tissues, appears to confer an inhibitory effect upon the Ins$P_3$ receptor by binding $Ca^{2+}$ and inhibiting further release.

The actual mechanism by which Ins(1,4,5)$P_3$ releases $Ca^{2+}$ has been the subject of a variety of different theories (see for example Volpe et al. 1988; Putney 1986; Irvine 1990). Irvine's proposal of "quantal" $Ca^{2+}$ release

is the only one which accomodates a role for $Ins(1,3,4,5)P_4$ however. This molecule, originally isolated from carbachol stimulated rat cerebral cortical slices (Batty et al. 1985) has been suggested to act synergistically with $Ins(1,4,5)P_3$ to initiate $Ca^{2+}$ influx from the extracellular medium (Irvine and Moor, 1986; Changya et al. 1989). More recent reports, however, have indicated that $Ins(1,3,4,5)P_4$ can release $Ca^{2+}$ by itself without the presence of $Ins(1,4,5)P_3$ (Joseph et al. 1989; Ely et al. 1990).

The other metabolites arising from the hydrolysis of $PtdIns(4,5)P_2$ will be discussed briefly below.


## Inositol (poly)phosphate metabolism


The enzymes responsible for the metabolism of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ have been extensively reviewed in recent years (see for example Shears, 1990). Therefore, only a short summary of the more relevant discoveries will be presented here.

The enzyme primarily responsible for the attenuation of the intracellular signal is the 5-phosphomonoesterase which converts $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ and $Ins(1,3,4,5)P_4$ to $Ins(1,3,4)P_3$, both of which are inactive with respect to releasing $Ca^{2+}$ (Streb et al. 1983; Strupish et al. 1988). The other molecule which appears to act as a substrate for the enzyme is the $Ins(cyc\ 1:2,4,5)P_3$. Although previously thought to be effective at mobilizing intracellular $Ca^{2+}$ (Irvine et al. 1986; Wilson et al. 1985), it now seems that it is at least substantially weaker than $Ins\ (1,4,5)P_3$ in this role (Willcocks et al. 1989).

Difficulties in the purification of the 5-phosphomonoesterase has led to speculation that it may be present as a variety of different isoenzymes and exhibit both tissue and species specific differences. 5-Phosphomonoesterase activity appears, at least in brain, to be present in at least two forms which differ in molecular weight and in the $K_m$ values for both $Ins(1,4,5)P_3$

and $Ins(1,3,4,5)P_4$. The studies carried out to determine if the enzyme is a substrate for protein kinase C (PKC) are discussed in Chapter 3.

The products of 5-phosphomonoesterase activity have, as stated previously, no $Ca^{2+}$ mobilizing activity. It has been proposed that both $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$ are, instead, important in regulating gene expression (Sylvia et al. 1988; Berridge and Irvine, 1984). Their metabolism, in turn, can be regulated via either an inositol 1-phosphatase or an inositol 4-phosphatase. The multiplicity of enzymes responsible for the metabolism of the individual inositol (poly)phosphates has not yet been fully elucidated and, therefore, it is not known if the 4-phosphatase, for example, exists as a single activity or if it is present as a family of enzymes. However, a great number of studies now indicate that less than 5% of the $Ins(1,4)P_2$ dephosphorylation is via a 4-phosphatase implying that the remainder is hydrolyzed by a 1-phosphatase (see for example Dean and Moyer,1987; Balla et al. 1988; Ragan et al. 1988). There have been reports that, in brain, there is an active 4-phosphatase activity (Takimoto et al. 1987; Ackermann et al. 1987) although this has not been substantiated in comparable studies by Ragan et al.(1988). In rat parotid acinar cells, certain conditions must be met before the $Ins(1,4)P_2$ will apparently be metabolized to Ins(1)P (Hughes and Putney, 1989). These include a high prevailing concentration of $Ins(1,4)P_2$ and also a relative degree of inhibition of the 1-phosphatase by $Li^+$ (Inhorn and Majerus, 1987) or ATP (Shears et al. 1987) or by competition for the same enzyme by $Ins(1,3,4)P_3$. The metabolism of this latter isomer and, indeed, of $Ins(3,4)P_2$ by a 4-phosphatase activity has been demonstrated in a variety of tissues (Bansal et al. 1987; Shears et al. 1987; Dean and Moyer, 1988).

Alternatively, $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$ can be metabolized by a 1-phosphatase activity. This single enzyme has been purified from a number of sources (see for example Inhorn and Majerus, 1987; Ragan et al. 1988). It has proved, however, to be one of the most controversial issues in inositol

(poly)phosphate metabolism with its apparent molecular weight, $Li^+$ sensitivity and kinetic parameters still under investigation (for discussion see Shears, 1990 and refs. therein).

In addition, a variety of other enzymes have been identified which complicate the metabolic scheme shown in Fig. 1.2 still further. A 3-phosphatase activity has been resolved by Bansal et al.(1987) which attacks $Ins(1,3)P_2$. Similarly, a 3-phosphatase enzyme which converts $Ins(1,3,4,5)P_4$ to $Ins(1,4,5)P_3$ and, thus, acts in concert with the 3-kinase, has been identified (Doughney et al. 1988; Hoer et al. 1988). Kinases have also been isolated which convert $Ins(1,3,4)P_3$ to $Ins(1,3,4,6)P_4$, $Ins(1,3,4,6)P_4$ to $Ins(1,3,4,5,6)P_5$ and $Ins(3,4,5,6)P_4$ to $Ins(1,3,4,5,6)P_5$ (see for review Shears, 1989). The inositol monophosphatase, on which $Li^+$ is known to have a most profound effect, is dealt with separately in Chapter 5.


## DAG and PKC


The other moiety which is produced by PLC-mediated hydrolysis of $PtdIns(4,5)P_2$ is DAG. This metabolite retains the characteristic 1-stearoyl, 2-arachidonoyl fatty acid composition discussed earlier. Because of its obvious lipophilicity, DAG, unlike the corresponding headgroup molecule, remains in the plane of the membrane bilayer.

The proposal that DAG was the intermediate agent between $PtdIns(4,5)P_2$ and PKC activation was first made in 1981 by Takai et al. Several years prior to this report, Nishizuka and co-workers had characterized PKC from brain as a serine- and threonine specific, proteolytically activated kinase that was independent of cyclic nucleotide stimulation (Inoue et al. 1977). Subsequent detailed studies have identified PKC as a diverse family of monomeric holoenzymes with a wide tissue distribution (for reviews see Nishizuka, 1984; 1986; 1988).

The requirements of PKC for activation vary slightly for each isoenzyme

although the presence of acidic phospholipids like phosphatidylserine and $Ca^{2+}$ appear to be universally essential. It is also now apparent that very small increases in the levels of DAG within the cell radically alter the affinity of PKC for $Ca^{2+}$ from approximately $10^{-5}$ - $10^{-4}$M to $10^{-7}$M. This increase in sensitivity means that DAG can render PKC fully active without any actual net increase in intracellular $Ca^{2+}$ (Kishimoto et al. 1980; Rasmussen et al. 1985). The apparent alteration in the susceptibility of the enzyme for $Ca^{2+}$ activation appears to occur when the cell is stimulated. Usually cytosolic and, presumably, inactive, PKC translocates to the membrane and associates with phosphatidylserine and DAG. In vitro, the list of cellular substrates for PKC appears to be endless and includes histone $H_1$, a property exploited in assays for PKC (Kikkawa et al. 1982; Ikebe et al. 1985; Sibley et al. 1984; Connolly et al. 1986; Aloyo et al. 1983).

As an important component of signal transduction, the discovery that phorbol esters, potent co-carcinogens isolated from Croton oil, could activate PKC was a major step forward in understanding (poly)phosphoinositide metabolism. Phorbol esters are now understood to elicit an array of biological responses including tumour promotion (Blumberg, 1980), cell proliferation (Dicker and Rozengurt, 1980), prostaglandin production (Edwards et al. 1985) and platelet activation (Zucker et al. 1974; White et al. 1974). In 1982, Castagna et al. demonstrated the ability of phorbol esters to act as DAG-mimetics in stimulating PKC, at concentrations up to 1000-fold less than that required for PKC activation by DAG. This correlation appears to hold both in vitro and in vivo (Castagna et al. 1982; Neidel et al. 1983; Yamanishi et al. 1983).

Phorbol esters are extremely lipophilic and, therefore, partition into cell membranes very easily. In a manner similar to that of DAG, phorbol esters enhance PKC translocation to the cell membrane but fortuitously by-pass all the stages of receptor-mediated (poly)phosphoinositide metabolism, allowing the involvment of PKC to be isolated from the other

factors associated with this mechanism.

Having identified pharmacologically useful activators of PKC, it was important also to be able to inhibit the enzyme. Many studies using polymyxin B, isoquinoline, sulfonamides and sphingosine have claimed a reasonable degree of PKC inhibition. Similarly, the description of the microbial product, staurosporine, by Tamaoki et al.(1986) has led to its use as a "selective" PKC inhibitor becoming more common. However, although staurosporine remains a useful tool in investigating phosphorylation reactions within the cell, its inhibitory properties are now known to extend over a wide range of protein kinases making results on its effects solely on PKC difficult to establish (see Ruegg and Burgess, 1989 and refs. therein).

DAG, the endogenous activator of PKC, can, theoretically at least, be produced from the esteric cleavage of PtdIns, PtdIns(4)P and PtdIns(4,5)$P_2$. Like other second messengers, DAG is rapidly metabolized. Most commonly, with regard to (poly)phosphoinositide metabolism, the concurrent appearance of PA indicated that the most probable route for this metabolism was via a DAG-kinase (Hokin and Hokin, 1963; Takenawa et al. 1982). Although the enzyme has not been widely studied in detail, the rapid and universal appearance of PA in cells which have been labelled and then stimulated with an appropriate agonist has generally been taken as evidence for the ubiquitous tissue distribution of this enzyme.

The PA which is formed in this way is assumed to be transported back to the E.R. to be reincorporated into PtdIns (for review see Michell, 1975). Firstly, it is converted into CMP-PA. This intermediate then combines with inositol to regenerate PtdIns. These two steps are catalyzed by CTP-PA cytidyltransferase and PtdIns synthetase respectively - enzymes which occur at the level of the E.R. (for review see Abdel-Latif, 1986). The pathway from DAG to PtdIns resynthesis is shown in Fig. 1.4.

sn 1,2 DIACYLGLYCEROL
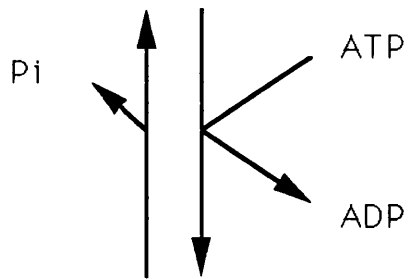


PHOSPHATIDIC ACID

CMP – PHOSPHATIDIC ACID

PtdIns $\rightleftharpoons$ PtdIns(4)P $\rightleftharpoons$ PtdIns(4,5)P$_2$
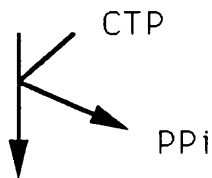
Fig. 1.4 – Resynthesis of the polyphosphoinositide lipids from sn 1,2 diacylglycerol
Abbreviations are as follows : ATP = adenosine triphosphate; ADP = adenosine diphosphate; CTP = cytidine triphosphate; CMP = cytidine monophosphate; Pi = inorganic phosphate; PPi = inorganic pyrophosphate.

## Inositol

Inositol, which combines with CMP-PA, is a six-carbon member of the vitamin B complex. It is found in practically all animal and plant cells and, like all essential n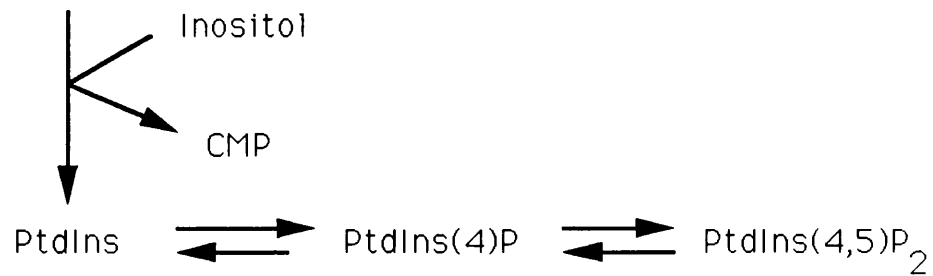utrients, deficiency can lead to abnormal cellular functions. In studies in mice, Woolley (1941) found that lack of inositol could cause inadequate growth, alopecia and death. As reviewed by Wells (1989), insufficient inositol can have profound effects upon mammalian development, most significantly on the reproductive and respiratory systems.

In the resynthesis of PtdIns, the inositol that combines with CMP-PA can originate from three sources:-

(1) de novo synthesis - Studies by Chen and Charlampous (1966) and Eisenberg (1967) indicated that inositol could be synthesized de novo from glucose. This pathway consists of two enzymic activities - myo-inositol 1-phosphate synthase and inositol monophosphatase and an intermediate which was found to be L-Ins(1)P. The synthetic activities in the tissues examined so far indicate that the level of inositol obtained in this way is fairly constant except in the testis which appears to have a very active de novo pathway (Eisenberg, 1967).

(2) dietary intake - Dietary inositol can be taken up into cells by a transport mechanism which appears to differ between tissues. Molitoris et al. (1980), for example, found the transport to be non-saturable in skeletal muscle whilst Caspary and Crane (1970) reported that a saturable mechanism, dependent upon a $Na^+$-electrochemical gradient, transported inositol in the small intestine. Work by Spector (1976) on inositol transport in brain slices demonstrated a transport system which was partially saturable and inhibited only 35% by dinitrophenol. Thus, in rabbit brain slices, this transport system appears to be at least partially energy dependent.

In in vivo situations, this type of transport will be used to provide a

constant intracellular concentration of inositol. In the cerebrospinal fluid
this concentration has been calculated to be around 0.47 mmole/l (see
Sherman, 1989). In vivo, however, inositol cannot transverse the blood brain
barrier and, thus, inositol homeostasis in the central nervous system is
probably maintained via other mechanisms. This will, in turn, render the
brain uniquely susceptible to inositol deficiency.

(3) via inositol (poly)phosphate metabolism - This is essential, particularly
in the central nervous system. The inositol formed by the metabolic pathway
is recycled into the production of the inositide lipids.


## (Poly)phosphoinositide metabolism in brain


Most of the studies which have investigated and described
polyphosphoinositide metabolism as it is currently understood have been
undertaken in peripheral tissues and cultured cell systems. However, the
large number of neurotransmitter receptor agonists which have been shown to
elicit a polyphosphoinositide response (see for example Brown et al. 1984;
Daum et al. 1984; Jacobson et al. 1985; Rooney and Nahorski, 1986; Kendall et
al. 1985), coupled to the high density of $Ins(1,4,5)P_3$ receptors (see Worley
et al. 1987; Joseph and Rice,1989) and PKC (see Worley et al. 1987) within
the brain has indicated that this signalling pathway may be important in the
central nervous system.

Original studies were carried out using synaptosomal preparations which
suggested that polyphosphoinositide metabolism was presynaptic and associated
with neurotransmitter release (Hawthorne and Pickard, 1979). However,
Nahorski et al. (1986) later described the possibility that such experiments
were contaminated with dendritic fragments. Recent evidence has pointed
towards a postsynaptic localization for receptor-mediated
(poly)phosphoinositide hydrolysis. Surgical and chemical lesioning studies

have enhanced this viewpoint. In 1980, Fisher et al. described experiments in which prior removal of the cholinergic innervation to rat hippocampus resulted in a marked reduction in the activity of the presynaptic marker, choline acetyltransferase. The muscarinic receptor-induced inositol polyphosphate accumulation was not affected, however. Moreover, lesion of the postsynaptic structures with ibotenate, a neurotoxin, resulted in the loss of both muscarinic receptor number and the phosphoinositide response (Fisher et al. 1981). These experiments indicate that the PtdIns response is primarily postsynaptic and neuronal. This does not appear to be exclusive, however, with studies indicating the occurence of (poly)phosphoinositide hydrolysis in both astrocytoma cells (Cohen et al. 1983) and primary cultures of astrocytes (Pearce et al. 1985). Furthermore, the ability to detect reproducible polyphosphoinositide responses in brain slices prepared from rat cerebral cortex has ensured that this signalling pathway has now been widely studied (see Batty, 1987; Rooney, 1987). The use of such a system is advantageous because it allows experimentation in a tissue where neuronal processes are kept intact and in which the physiological heterogeneity is preserved.


## Lithium


$Li^+$ is the smallest of the alkali metals yet it has remarkable effects both on behaviour and early embryonic development. It is presently the major drug used in controlling manic-depression (Rosenthal and Goodwin, 1982) although it also appears to be effective in the treatments of aggressive and self-mutilating behaviour (Wickham and Reed, 1987) and cluster headaches (Ekbom, 1981). $Li^+$ also has teratogenic effects on the embryos of many organisms (for review see Busa, 1988). In 1986, Kao et al. reported that injection of LiCl into the prospective ventral cells of the Xenopus embryo at the early blastula stage, led to the formation of two heads. This so-called

"Janus-twin" embryo is an important example of the far-reaching phenotypic effects that $Li^+$ can have. On the basis of similar experiments, Busa (1988) has suggested that, during normal frog embryogenesis, the role of (poly)phosphoinositide signalling is crucial to the development of the vegetal and animal poles.

In non-developmental studies, however, injections of LiCl directly into the cell under investigation are not normally feasible. It is important, therefore, to assess briefly the transport of $Li^+$ _in vivo_. Many of the preliminary studies were carried out in the red blood cell because of their accessibility and the fact that electrolyte transport by the membrane is similar to that of a neuron. Initially, two potential pathways for $Li^+$ transport into cells were proposed - the $Na^+$-$K^+$ ATPase and the $Na^+$ channel (for review see Ehrlich and Diamond, 1980). However, Smith (1974) demonstrated that $Li^+$ could not be competitively inhibited by $K^+$ and so it was suggested that the former did not contribute significantly to $Li^+$ transport, at least in skeletal muscle.

In 1977, Richelson indicated that the cell-to-extracellular ratio of $Li^+$ was approximately 4 in cultured neuroblastoma cells. This is much greater than the corresponding ratio in other systems examined (Richelson, 1977) indicating perhaps that either the influx or efflux of $Li^+$ from the central nervous system is different from other tissues. Although the values observed are lower than would be expected if passive $Li^+$ entry occurred, current theories revolve around the hypothesis that $Li^+$ selectively enters the cells of the central nervous system and cannot exit the cells as efficiently as it can other cells (see Renshaw and Wicklund, 1988 however). It can be seen, therefore, that $Li^+$, as a non-physiological ion, can enter the cells which, in manic-depressive patients are believed to be abnormally active (for reviews see Berridge _et al_. 1990; Drummond, 1987). Its primary intracellular target, the inositol monophosphatase, will be discussed in Chapter 5.

In 1982, Berridge _et al_. proposed that $Li^+$ inhibition of the inositol

monophosphatase was central to its therapeutic uses. By inhibiting the production of free inositol from inositol (poly)phosphate metabolism, the cycle of (poly)phosphoinositide lipid hydrolysis would be attenuated under conditions of chronic stimulation. This would arise because, although CMP-PA would continue to accumulate, the inositol essential for the resynthesis of the lipids would become depleted. As stated previously, the central nervous system relies predominantly upon inositol from either de novo synthesis or via inositol (poly)phosphate metabolism. In the presence of $Li^+$, neither of these two pathways operate efficiently and, because the central nervous system does not have access to extracellular inositol, the brain is selectively affected by such treatment. The suggestion, therefore, was that $Li^+$ would slow down the cycle of (poly)phosphoinositide metabolism in cells which were hyperactive and thus cause a cessation of manic-depressive behaviour. This has yet to be proved. However, it has led to $Li^+$ being adopted as a very useful pharmacological tool in studies of inositol (poly)phosphate metabolism. Because the major site of inhibition is at the last dephosphorylation step, addition of $Li^+$ to assays can amplify the signal and allow particularly the inositol monophosphate fraction to accumulate markedly. This then facilitates the corresponding measurements.

The aims of the research presented here are:

1. To investigate the effects of $Li^+$ upon both inositol (poly)phosphate and CMP-PA accumulation in rat cerebral cortical slices.

2. To compare these results with the effects of $Li^+$ in cultured cell lines.

3. To study the inhibition of the inositol monophosphatase activity by $Li^+$ in cell-free preparations.

C H A P T E R  2.1


General Methods

CHAPTER 2.1


GENERAL METHODS


Introduction


The methods discussed in this chapter revolve around the central issue of

separating the various inositol (poly)phosphate fractions which accumulate

following agonist stimulation. These include characterization using both

anion exchange chromatography and high performance liquid chromatography

(h.p.l.c.). In addition, two other novel methods have been used. One

facilitates resolution of the labelled inositol trisphosphate fraction into

its component isomers without the use of h.p.l.c. and the other enables the

concentration of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ to be calculated using

radioreceptor assays.


EXPERIMENTS INVOLVING RAT CEREBRAL CORTEX SLICES


a) Tissue source


Male Wistar rats (150-250g) were stunned by a blow on the back of the

neck and rapidly decapitated. This method of sacrifice is quick and less

bloody than other approaches and, whilst tissue in direct contact with

stagnated or deoxygenated blood may be affected, decapitation tends to yield

superior results. The skull was then opened by incision along the suture

joints, the brain excised with a micro spatula and transferred onto ice.

A vertical slice was made from the brain, when placed superior view

uppermost and anterior view forward, of approximately 1-2mM in thickness to

remove the olfactory bulbs. A second slice of 2-3mM width was subsequently removed which consists primarily of parietal cortex surrounding an inner core of striata. These two tissues were separated by gently trimming the former tissue from the outer edge of the section thus leaving the pair of striata with the central septum intact. Using a fine scalpel blade, the brain was further dissected down the central sulcus and peeled back exposing the hippocampal tissue. This, in turn, could be unfolded from the cortex leaving the cortical tissue in its entirety.

The cortical tissue from these procedures was then spaced evenly on a filter paper-covered plastic cutting platform and cross-chopped rapidly on a McIlwain tissue chopper at 350μm x 350μm. This whole process was timed to take between 5 and 10 mins. The slices of cortex were then scraped into a 20ml sterilin vial containing oxygenated Krebs-Henseliet buffer at 37°C dispersed by vigourous shaking. The concentration of various salts in the Krebs-Henseleit buffer will be discussed in more detail in part (i). The 20mls of buffer containing the brain slices were then tipped immediately into a 500ml capacity Duran bottle containing a further 100-200mls of the aforementioned buffer. The slices were allowed to gravity pack for approximately 30 sec., the buffer was aspirated and the preparation replenished with fresh buffer. This washing process, which removes all broken slices and the majority of the contents of broken cells both neuronal and glial in origin, was repeated twice. Following this the buffer was replaced and the bottle placed in a shaking water bath at 37°C. The above process was then repeated at regular 10-15 min intervals and the slices gassed with 95% $O_2$ : 5% $CO_2$ for a further 60 min.

After one hour the slices were allowed to sediment as before and the buffer once more removed. 50μl aliquots were then dispensed into flat-bottomed vials containing a final volume of 300μl in all the experiments which will be described, 210μl of which was the aforementioned Krebs-Henseliet buffer. Each sample was then gassed and replaced into the

shaking water bath for a further 60 min again with gassing at regular 10-15 min intervals. If samples were to be labelled, the appropriate radioactivity was added at this stage for 30 mins if a "batch" elution was required or for 60 mins if either individual inositol phosphate fraction or CMP-PA accumulation were to be studied (see Brown et al. 1984; Batty, 1987; Rooney,1987).

## b) [$^3$H]Inositol labelling

[$^3$H]Inositol was dried down under a steady stream of N$_2$ and then redissolved into Krebs-Henseleit buffer. This volume was then passed down a small, plastic bead-plugged column constructed in a 1ml Gilson-pipette tip containing 0.5ml AG 1-X8 Dowex (usually 200-400 mesh; formate form). All Dowex preparations were routinely of a 50% (w/v) slurry with water. This process effectively "cleans-up" inositol by removing any polar impurities which may lead to high background values being observed at later stages in the experiment. The final concentration of inositol obtained in each vial was 0.5μCi for experiments in which a "batch" elution was taken in order to examine changes in total inositol polyphosphate accumulation or 5μCi for experiments in which individual inositol (poly)phosphate fractions were investigated. The former samples were labelled for just 30 mins whilst the latter were labelled for 60 mins.

## c) [$^{14}$C]Cytidine labelling

[$^{14}$C]Cytidine labelling did not require any "cleaning" process like [$^3$H]inositol and was simply added from the stock solution at a final concentration of 0.1μCi/vial for 60 mins.

After the addition of label the samples were gassed every 10-15 mins as mentioned above, and after the correct labelling period, additions of agonist

(usually carbachol at a final concentration of 1mM), arecoline, lithium etc., always in a final volume of 10µl, were made simultaneously . The incubation of individual samples was then continued for the appropriate length of time.

d) Termination and "batch" elution of [$^3$H]inositol labelled samples

Reactions were terminated by the addition of 0.94ml 2:1 (v/v) CH$_3$OH : CHCl$_3$ and then the further addition of 0.31ml CHCl$_3$ and 0.3ml water helped separate the phases. Samples were left at room temperature for 10 mins and the aqueous : non aqueous phases partitioned by centrifugation at 3000g for a further 10 mins. After this type of termination, the upper phase contains the [$^3$H]inositol phosphates and, indeed, in the presence of lithium greater than 90% of this fraction will be accounted for by [$^3$H]InsP$_1$. The lower phase contains labelled lipid, 90% of which is [$^3$H]PtdIns. In order to measure [$^3$H]InsPs, 0.75ml of the upper phase was removed , diluted to 3mls in water and the sample assayed in a glass column containing 0.5ml Dowex (100-200 mesh; chloride form). To elute the total [$^3$H]InsPs bound, three sequential elutions must be performed. Firstly, 20ml water was added to facilitate removal of all excess, unincorporated [$^3$H]inositol. [$^3$H]GroPtdIns was then removed by washing the column with 20mls 0.025M ammonium formate (NH$_4$COOH) and finally the [$^3$H]inositol phosphate fraction was eluted with 2mls 1.0M HCl. This 2ml fraction was counted with 10mls scintillation fluid and radioactivity determined by liquid scintillation spectroscopy.

e) Termination and elution of individual [$^3$H]inositol phosphate fractions

Reactions were terminated with 300µl ice-cold 1M trichloroacetic acid (TCA). At all subsequent steps of the extraction procedure, the temperature was maintained at 0 - 4°C where possible until the samples were neutralized.

After 10-20 min on ice, samples were spun for a further 20 min at 3000g to pellet the tissue fragments. 500$\mu$l aliquots were subsequently removed from each sample supernatant and, unless otherwise specified, triplicates were pooled at this stage. The acid extraction was completed by the addition of 5 x 2 volumes of water-saturated diethyl ether. The water-saturation of the solvent helps to prevent against large volume changes which would occur otherwise. With each addition of the water-saturated diethyl ether, samples were vortexed vigourously and the upper phase aspirated. After this extraction procedure samples were routinely pH 4-6 depending on how efficiently the extraction was completed. Samples were then neutralized with 25mM $NaHCO_3$ except in studies involving the enzymic separation of the two prominent inositol trisphosphate isomers present in carbachol-stimulated rat cerebral cortical tissue. This issue will be discussed fully in Section 2.2 which deals exclusively with the development of a technique to split the trisphosphate fraction into the $Ins(1,4,5)P_3$ and $Ins(1,4,5)P_3$ components without the use of high performance liquid chromatography.

In a manner similar to the "batch" elution of $[^3H]$ labelled samples, samples neutralized previously were loaded onto a water-equilibrated column consisting 1.0 ml Dowex (200-400 mesh, formate form). In all the experiments carried out using this method, the entire sample volume was loaded. As before, unincorporated $[^3H]$inositol was eluted with 20mls water. Using a system based on that described by Hubsher and Hawthorne (1957) and later by Ellis et al. (1963), ammonium formate solutions of increasing molarity were used to perfect a sequential column elution of $[^3H]GroPIns$, $[^3H]InsP_1$, $[^3H]InsP_2$, $[^3H]InsP_3$ and $[^3H]InsP_4$. However, it was discovered that the efficiency of elution varies slightly with each batch of Dowex (200-400 mesh, formate form) and thus it was essential to set up extensive elution profiles for the anion exchange resin using 2ml aliquots of the ammonium formate solutions mentioned above. By so doing, the possibility of cross-contamination of one fraction into another - a complication that most

frequently affects the tris- and tetrakisphosphate fractions is eliminated. An example of two elution profiles are shown in Fig. 2.1 for comparison.

In direct contrast to previous work from this laboratory (Batty et al. 1985), all batches of Dowex (200-400 mesh, formate form) were found to elute $[^3H]InsP_3$ with less cross-contamination with a 0.7M $NH_4COOH$/0.1M HCOOH than with the 0.8M $NH_4COOH$/0.1M HCOOH quoted by this group. In all subsequent experiments the following ammonium formate/formic acid solutions were used to elute tritiated inositol phosphate fractions: 0.2M $NH_4COOH$ ($[^3H]InsP_1$); 0.5M $NH_4COOH$/0.1M HCOOH ($[^3H]InsP_2$); 0.7M $NH_4COOH$/0.1M HCOOH ($[^3H]InsP_3$) and 1.0M $NH_4COOH$/0.1M HCOOH ($[^3H]InsP_4$).

The actual volumes used to elute each fraction varied however according to the resin. These changes amounted only a few millilitres either way of that specified by Batty et al. (1985). Typically, the following volumes would be used for elution: 20 mls water ($[^3H]Ins$); 12 mls 0.025M $NH_4COOH$ ($[^3H]GroPtdIns$); 14 mls 0.02M $NH_4COOH$ ($[^3H]InsP_1$); 12 mls 0.05M $NH_4COOH$ ($[^3H]InsP_2$); 12 mls 0.7M $NH_4COOH$ ($[^3H]InsP_3$) and 14 mls 1.0M $NH_4COOH$/0.1M HCOOH ($[^3H]InsP_4$). However, because these values form only a rough guideline, all the experiments in which individual $[^3H]$inositol phosphate fractions are studied are expressed as DPM/total volume eluted rather than DPM/2ml aliquot, a solution which will circumvent the problem of resin variation.

Following these elutions in bulk, 2mls aliquots were removed and counted in the presence of 18mls liquid scintillation fluid. In most instances the chloride and formate form Dowex columns used in the "batch" and individual inositol phosphate fraction elutions respectively were regenerated for future use. The elution of a further 10mls of 1.0M HCl from the chloride columns or of 10 mls 2.0M $NH_4COOH$/0.1M HCOOH from the formate columns effectively returned the resin to a state in which it could be reused for similar experiments. However, although increasing the use of these columns, anomalies in elution pattern can be observed if the columns are regenerated

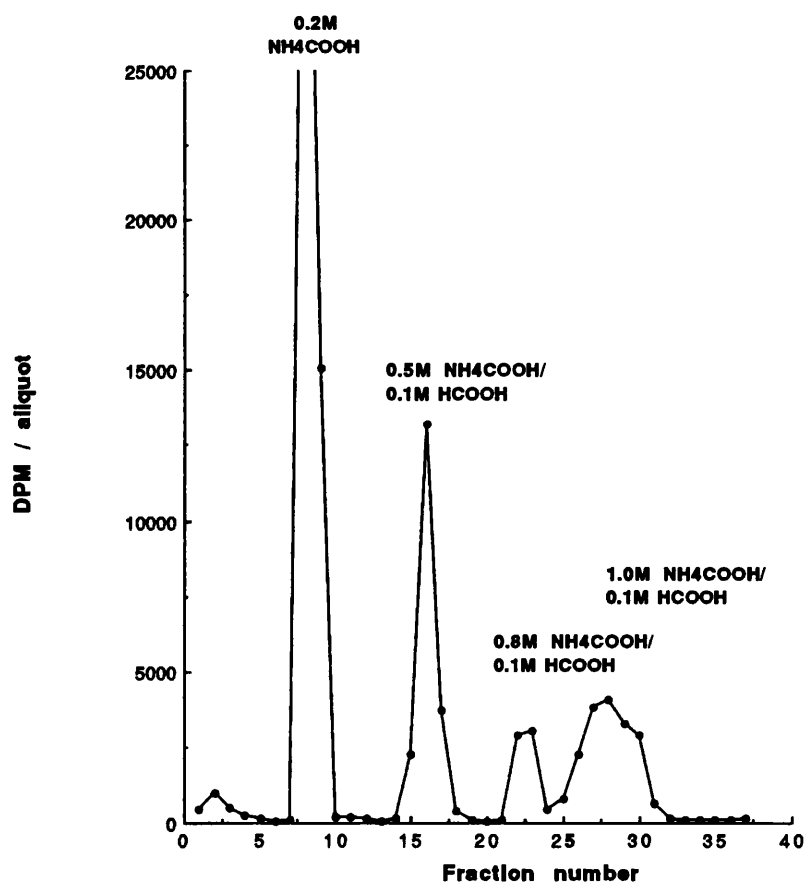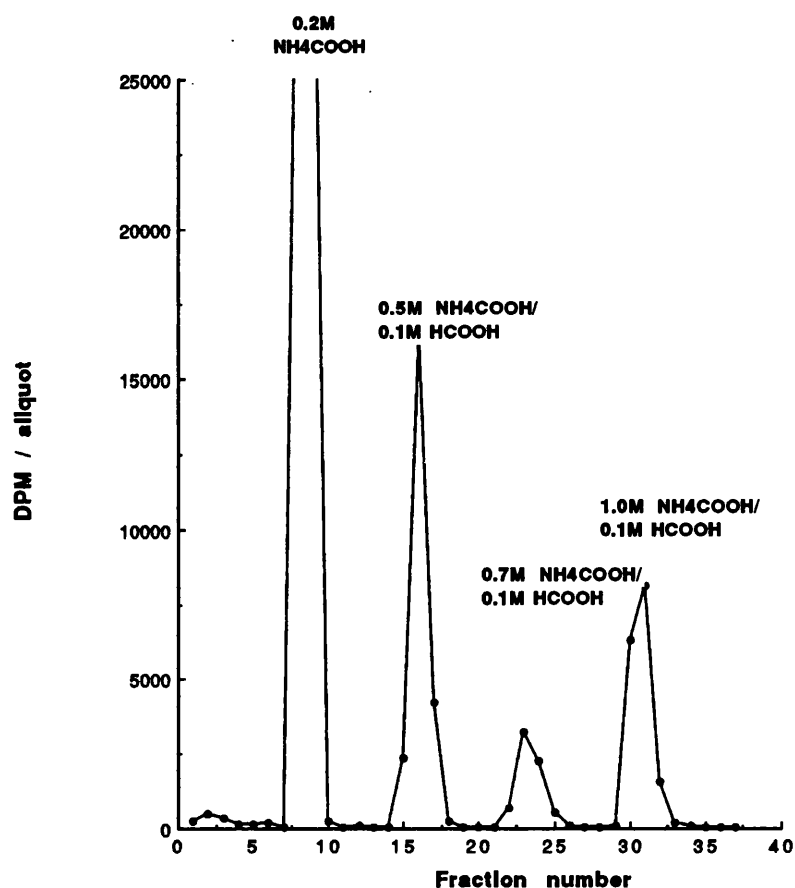**Fig. 2.1** - Elution profiles of the tritiated inositol phosphates from carbachol stimulated rat cerebral cortical slices.
The elution of $InsP_1$, $InsP_2$ and $InsP_3$ are identical in both panels. However, the lower panel illustrates the cross-contamination which can occur when the $InsP_3$ fraction is eluted with 0.8M NH4COOH/0.1M HCOOH compared to 0.7M NH4COOH/0.1M HCOOH.

more than 5-8 times. To safeguard against this, the resin was replaced
periodically.


f) Termination and assay of [$^{14}$C]cytidine labelled experiment


As with [$^3$H]inositol labelled experiments in which individual inositol
phosphate fractions were investigated, reactions labelled with [$^{14}$C]cytidine
were terminated by the addition of an equal volume of ice-cold 1M TCA and the
samples allowed to extract for 10-20 mins at 4°C. Following centrifugation,
the supernatant was discarded and 1ml 5% TCA containing 1mM EDTA added to the
pellet. Samples were then agitated gently to remove the slices from the
bottom of the individual vials. The above procedure was repeated and the
slices washed with 1ml water. After the subsequent spin and removal of the
supernatant, 0.94ml 2:1 (v/v) $CH_3OH$ : $CHCl_3$ containing 100ml HCl was added,
the samples shaken and left for 10-15 min. At the end of this period, 0.31ml
$CHCl_3$ and 0.56ml 0.1M HCl were added to this, vials vortexed vigorously and
spun for 10 mins at 3000g. 0.4ml was then carefully removed from the lower
phase, allowed to evaporate to dryness overnight and the radioactivity
counted in 5mls scintillation fluid. Triplicate samples in this type of
experiment were not pooled. Although thin layer chromatography validation of
the [$^{14}$C]-containing lipids was not carried out, several other groups have
reported that the only product isolated under these labelling conditions is
CMP-PA (see Chapter 3 for a fuller discussion).


g) Measurement of Ins(1,4,5)P$_3$ concentration by a radio-receptor assay


Most of the studies involving inositol (poly)phosphate metabolism utilise
radiolabelled tissue or cell extracts. Often, changes in the specific
radioactivity can occur which lead to anomalous results because they do not
represent changes in the mass levels of the fraction in question (Verhoeven

<u>et al</u>. 1987; Challiss <u>et al</u>. 1988). It is therefore advantageous to be able

to measure directly the concentration of the inositol (poly)phosphates.

Recently a technique involving a radioreceptor binding assay has been

developed which effectively measures the mass $Ins(1,4,5)P_3$ using membrane

prepared from bovine adrenal cortex (Challiss <u>et al</u>. 1988). The assay used

in the work described here has since been characterized and validated

(Challiss <u>et al</u>. 1990). Other methods have been described which measure

$Ins(1,4,5)P_3$ mass (Meek, 1986; Bradford and Rubin, 1986; Tarver <u>et al</u>. 1987;

Mayr, 1988; Palmer <u>et al</u>. 1989) but because of the simplicity of the method

of Challiss <u>et al</u>. (1988) and verification of its usefulness in the systems

which will be discussed here, this method was favoured over the others

available. The method used is described in detail by Challiss <u>et al</u>. (1988).

All measurements discussed in this thesis were carried out by Dr John

Challiss.


h)  <u>Measurement of $Ins(1,3,4,5)P_3$ concentration by a radio-receptor assay</u>


Like $Ins(1,4,5)P_3$, the $Ins(1,3,4,5)P_4$ isomer is thought to play a pivotal

role in $Ca^{2+}$ signalling in stimulated cells (Irvine, 1990) and, as such, it

would be beneficial to be able to measure the mass amount of this metabolite

intracellularly. In 1989, Donié and Reiser described a novel radio-receptor

binding assay for the quantitation of $Ins(1,3,4,5)P_4$. They utilized a

preparation from porcine cerebellum which displayed high affinity sites for

$[^3H]Ins(1,3,4,5)P_4$. This technique appeared to be very specific. Of all of

the inositol (poly)phosphates considered, only $Ins(1,3,4,5,6)P_5$ demonstrated

any cross-reactivity. However, the $EC_{50}$ volume for this compound was two

orders of magnitude less potent at binding and thus, it was assumed that,

under the conditions used, primarily $Ins(1,3,4,5)P_4$ was bound.

In a modification of this method, Challiss and Nahorski (1990) opted to

use rat cerebella as the source of binding protein and $[^{32}P]Ins(1,3,4,5)P_4$

instead of the tritiated isotope. All measurements discussed in this thesis were carried out by Dr John Challiss.


i) Krebs-Henseleit buffer


The importance of the concentrations of the constituent salts in the buffer used throughout the experiments described here was investigated in a brief study. Several preliminary experiments on inositol (poly)phosphate responses in rat cerebral cortical slices indicated that a significant effect upon their accumulation could be observed when the levels of extracellular $Ca^{2+}$ was altered within the physiological range. Few studies had been reported on the $Ca^{2+}$ dependency of PtdIns metabolism in rat cerebral cortical slices until Kendall and Nahorski (1984) showed that, whilst omission of $Ca^{2+}$ from the extracellular medium severely restricted the accumulation of labelled inositol phosphate, there was, in fact, an enhancement of the incorporation of tritiated inositol into the PtdIns fraction. They indicated that [$^3$H]inositol phosphate accumulation in mAChR-stimulated rat cerebral cortical slices was not affected when the $Ca^{2+}$ concentration was dropped to 10µM (essentially "$Ca^{2+}$-free") but that histamine and, to a lesser extent, noradrenaline - and 5-hydroxytryptamine-induced breakdown was still suppressed.

Clearly, $Ca^{2+}$ concentrations used experimentally will dictate the levels of [$^3$H]inositol incorporated into the inositol (poly)phosphate and (poly)phosphoinositide fractions and that the $Ca^{2+}$ concentrations employed by different groups do, indeed, vary considerably, may account for the differences in the levels of agonist stimulated [$^3$H]inositol phosphate reported. For example, Gurwitz and Sokolovsky (1987) used buffer containing 0.8mM $CaCl_2$ in their analysis of PtdIns hydrolysis in rat cerebral cortex and heart atrium slices; Fisher and Snyder (1987) 2.2mM $CaCl_2$-containing buffer for guinea pig brain regional studies and Howerton and Rutledge (1988) 2.54mM

$CaCl_2$ Krebs bicarbonate for incubation of cerebral cortex. These differences may be having pronounced effects on the results obtained by different groups. As a result, it was important to determine which $Ca^{2+}$ concentration would optimise [$^3$H]inositol phosphate production and should, therefore, be used in all future experiments.

The buffer used in the incubation of the slices in these experiments had the following salt concentrations: NaCl 119 mmol/l; KCl 4.7 mmol/l; $MgSO_4.7H_2O$ 1.2 mmol/l; $KH_2PO_4$ 1.2 mmol/l; $NaHCO_3$ 25 mmol/l and glucose 11.7 mmol/l. To this, two different $CaCl_2$ additions were made such that one buffer had approximately 1.2 mmol/l whilst the other had 2.4 mmol/l. Simple "batch" elutions were taken after the slices had been labelled for 30 mins and stimulated in the presence of either millimolar carbachol or noradrenaline for appropriate lengths of time. These samples were stimulated in either the presence or absence of 5mM $Li^+$.

Fig. 2.2 illustrates the effect of incubating the slices in two different Krebs-Henseleit buffers - one with a final concentration of 2.4 mmol/l $CaCl_2$ and the other with 1.2 mmol/l $CaCl_2$. The graph clearly demonstrates that with increasing concentrations of carbachol, the total [$^3$H]inositol phosphate fraction extracted in the presence of 5mM $Li^+$ is significantly greater in the presence of 1.2 mmol/l $Ca^{2+}$ in comparison to 2.4 mmol/l. Even at very low concentrations of carbachol after 45 mins stimulation, the [$^3$H]inositol phosphate formation is almost 4-fold greater with lower $Ca^{2+}$ concentrations. This level of increase - between 3.5 and 4.0-fold - is maintained throughout the experiment such that, at carbachol concentrations of 1mM, which is the concentration routinely used in other experiments, the levels of [$^3$H]inositol phosphates are much more easily detected if the Krebs-Henseleit buffer contained 1.2 mmol/l $Ca^{2+}$ than if it had 2.4 mmol/l. A similar situation was observed when identical experiments were performed using noradrenaline instead of carbachol as the agonist (Fig. 2.3). $\alpha_1$-Adrenergic receptor stimulation does not lead to such large changes in the total [$^3$H]inositol

Fig. 2.2 - Brain slices labelled with myo-[2-H]Inositol for 30 min. In buffer containing either 2.4mmol/l ( ● ) or 1.3mmol/l ( ○ ) $Ca^{++}$ were stimulated with various concentrations of carbachol for 45 min. In the presence of 5mmol/l $Li^{+}$. Data represents the mean ± S.E.M. for at least 3 separate determinations.

Fig. 2.3 - Effect of extracellular $Ca^{++}$ on noradrenaline stimulated [$^3$H] inositol phosphate accumulation in rat cerebral cortical slices.
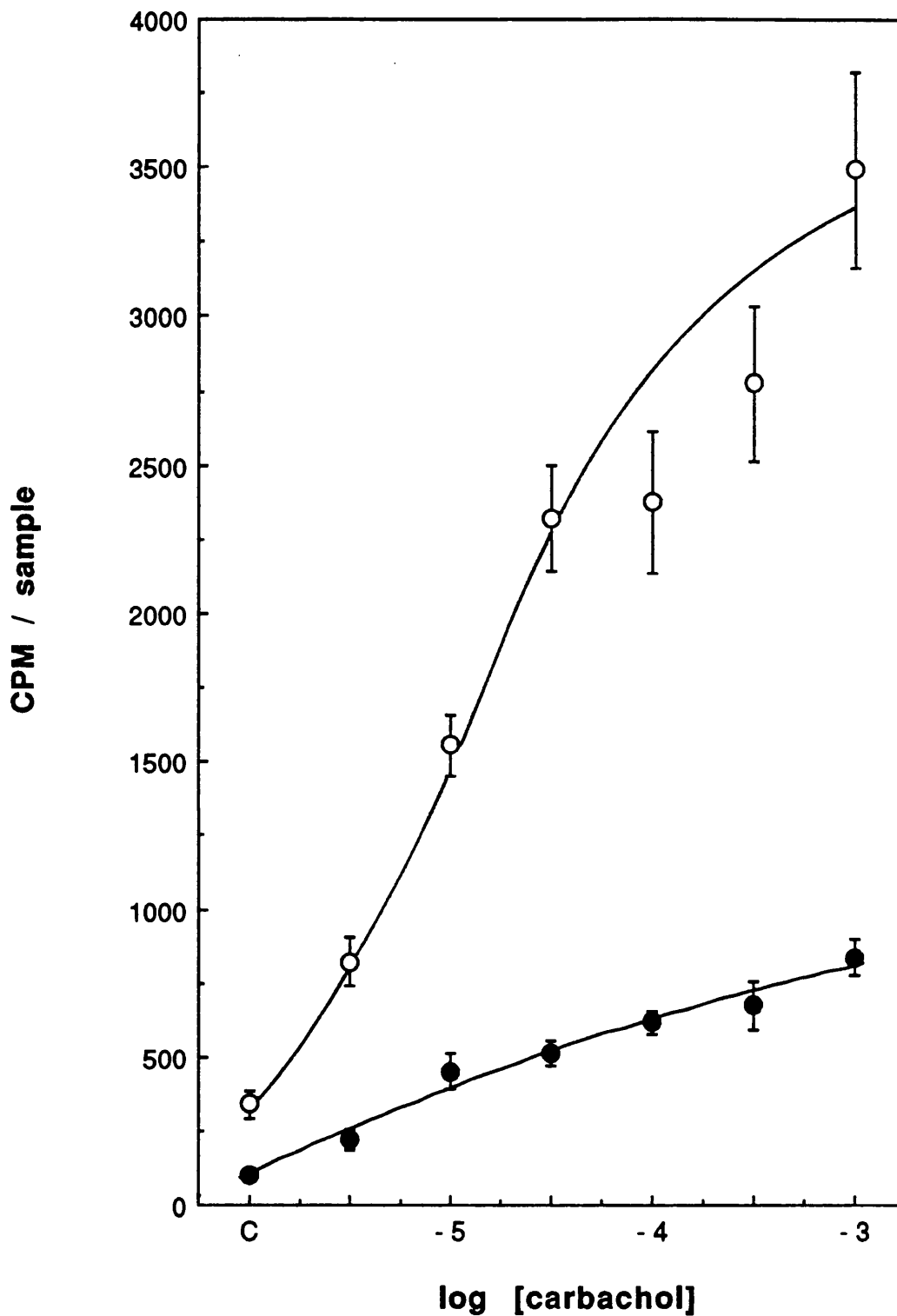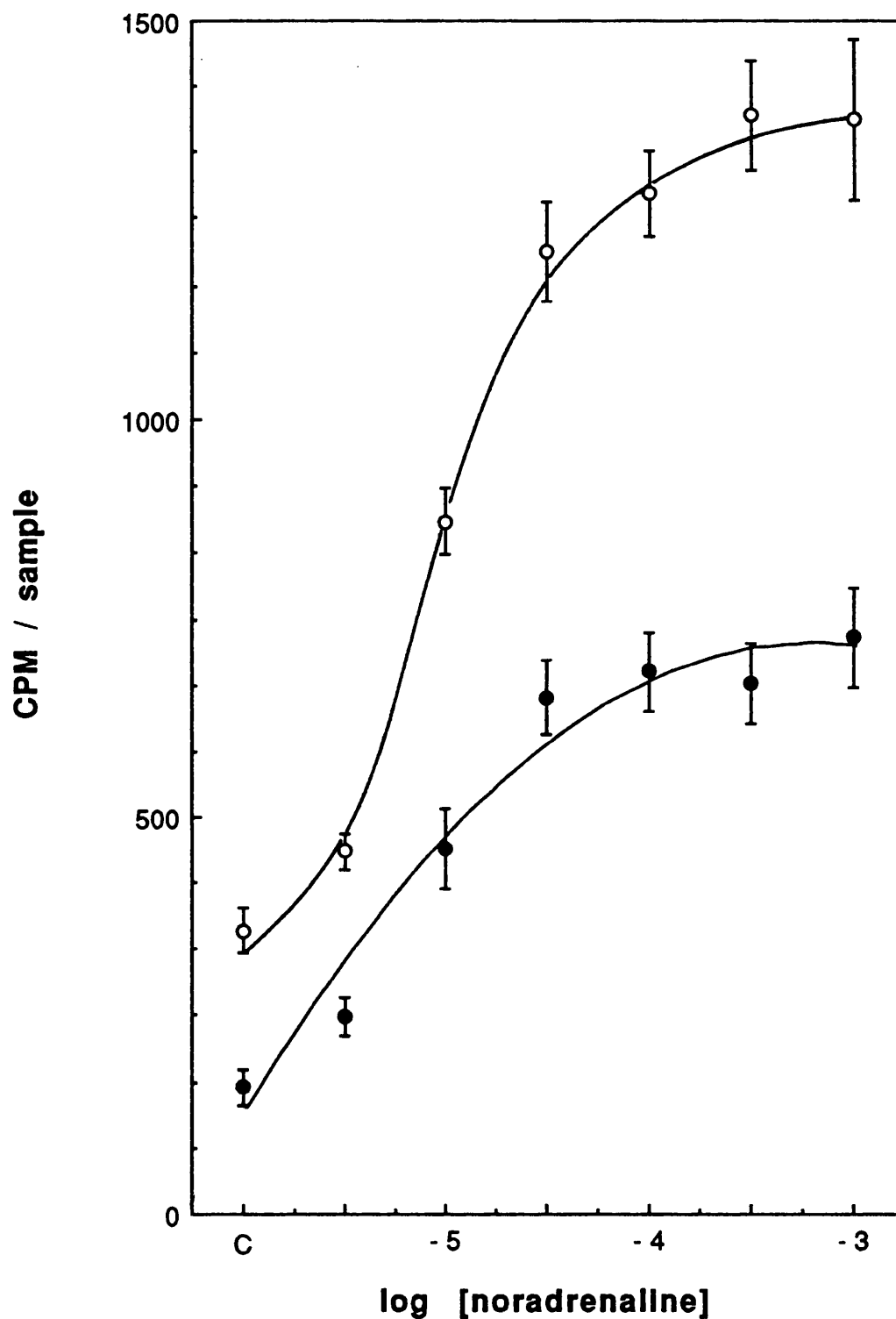Brain slices labelled with myo-[2-H]-inositol for 30 min. in buffer containing either 2.4 mmol/l (●) or 1.3 mmol/l (o) Ca and stimulated with agonist for 45 min. in the presence of 5 mmol/l Li$^+$. Data represents the mean ± S.E.M. for at least 3 separate determinations.

phosphate production as muscarinic receptor stimulation. Nevertheless, a similar pattern emerged with [$^3$H]inositol phosphate formation approximately 1.8-2.0 times greater in the presence of 1.2 mmol/l $Ca^{2+}$ compared to 2.4 mmol/l $Ca^{2+}$. Interestingly, in both instances, experiments in the presence of carbachol or noradrenaline where external $Ca^{2+}$ concentrations were reduced still further to 0.6 mmol/l, there was no comparable increment in the levels of detectable [$^3$H]inositol phosphates. Indeed, in all samples assayed, the total [$^3$H]inositol phosphate accumulation in the presence of 0.6 mmol/l $Ca^{2+}$ were essentially identical to that seen in the presence of 1.2 mmol/l $Ca^{2+}$ (data not shown). These results, obtained from [$^3$H]inositol labelled rat cerebral cortical slices, are in direct contrast to results from similar experiments carried out using [$^3$H]inositol labelled bovine tracheal smooth muscle slices (Baird et al. 1989). In these latter experiments 0.6 mmol/l $Ca^{2+}$ in the incubating buffer caused a further enhancement of the total [$^3$H]inositol phosphates separated compared to those extracted in the presence of 1.2 mmol/l $Ca^{2+}$. Indeed, only about 70% of the total [$^3$H]inositol phosphate formation in the presence of 0.6 mmol/l $Ca^{2+}$ was recorded when the bovine tracheal smooth muscle slices had been incubated with 1.2 mmol/l $Ca^{2+}$.

In Fig. 2.4, the time course of accumulation of total [$^3$H]inositol phosphates was considered in rat cerebral cortical slices stimulated with carbachol at a final concentration of 1mM either in the presence or absence of 5mM $Li^+$. As expected, the total [$^3$H]inositol phosphate fraction extracted is significantly decreased in the absence of $Li^+$ but, interestingly, the effect of the two $Ca^{2+}$ concentrations upon their levels were maintained even in the absence of $Li^+$ with 2.4 mmol/l $Ca^{2+}$ effectively reducing their accumulation by a constant factor throughout the time course.

These results illustrate that the prevailing $Ca^{2+}$ concentration in the incubating and labelling medium greatly influences the total [$^3$H]inositol phosphate formation. Relatively small changes in the extracellular $Ca^{2+}$ can have profound effects. In 1974, Schaers work indicated that of the total
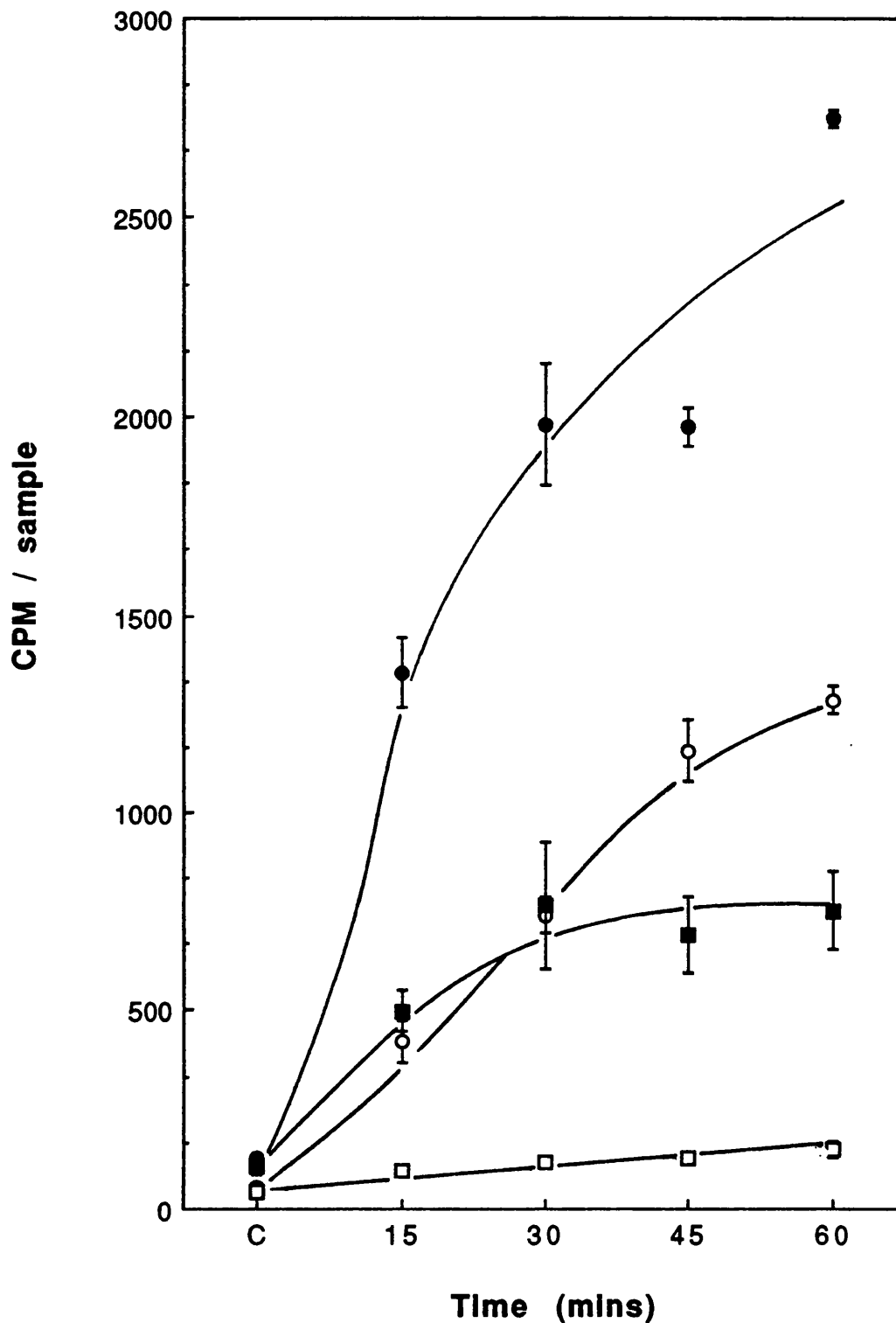
Fig. 2.4 - Effect of the extracellular $Ca^{++}$concentration upon total inositol phosphate accumulation in rat cerebral cortical slices labelled with myo-[2-H]inositol. The tissue was incubated in buffer containing either 2.4mmol/l (o,□) or 1.3mmol/l (●,■) $Ca^{++}$and subsequently stimulated with carbachol in either the presence (o,●) or absence (□,■) of 5mmol/l $Li^+$for the appropriate length of time. Data represents the mean ± S.E.M. for 3 separate determinations.

$Ca^{2+}$ content, only 80% of that added will be present as the free $Ca^{2+}$ ion (Schaer, 1974). The remaining 20% is complexed in soluble carbonate and bicarbonate molecules. However, even taking this into consideration, the resulting free $Ca^{2+}$ concentration is still well within the range found in "physiological buffers".

The effect of $Ca^{2+}$ on [³H]inositol phosphate formation is probably due to the labelling of their (poly)phosphoinositide lipid precursors than to a $Ca^{2+}$-induced enhancement of total inositol phosphate metabolism (Baird et al. 1989; Kendall and Nahorski, 1984; Neylon and Summers, 1987; see also Kukita et al. 1986 and Sasaguri et al. 1985 for two reports which indicate that $Ca^{2+}$ does enhance metabolism of the inositol phosphate fraction). $Ca^{2+}$ is known to have an inhibitory role at two essential points in (poly)phosphoinositide lipid resynthesis. Firstly, it inhibits the CMP-PA-inositol phosphatidate transferase reaction and secondly, it is thought to prevent incorporation of inositol through a headgroup exchange mechanism (Takenawa and Egawa, 1977; Egawa et al. 1981). As the concentration of external $Ca^{2+}$ increases both these processes wil be prohibited and the levels of (poly)phosphoinositide lipids will be reduced.

The data obtained indicates that, for both carbachol and noradrenaline, buffer containing a final concentration of 1.2 mmol/l $Ca^{2+}$ is the most advantageous for incubating and labelling rat cerebral cortical slices in. As a result, in all the following experiments, a Krebs-Henseleit buffer with a final $Ca^{2+}$ concentration of 1.2 mmol/l was routinely employed in order to detect optimal [³H]inositol phosphate production.

## j) Maintenance of cultured cell lines

Chinese hamster ovary (CHO) cells transfected with a stable $M_1$ receptor subtype were kindly donated by Merck, Sharp and Dohme Research Laboratories at Rahway, PA, USA. They were maintained in minimum essential medium which contained the following supplements: $NaCO_3$ (2g/1), fetal calf serum (10% v/v), glutamine (2mM), penicillin (100 units/ml) and streptomycin (100 ug/ml). "Geniticin" was also present (0.5 mg/ml). The cells, originally passage 8, were split 1:4 twice weekly.

SH-SY5Y human neuroblastoma stock cultures were kindly donated by Dr. J. Biedler, Sloan-Kettering Institute, New York, USA. They were maintained again in minimum essential medium supplemented with glutamine (2mM), fetal calf serum (10% v/v), streptomycin (100 ug/ml), penicillin (100 units/ml) and fungizone (2.5 μg/ml). Cells were split 1:6 weekly and fed twice weekly. Cultures were kept in 175$cm^2$ tissue culture flask in the presence of 80ml of the appropriate media at 37°C in 5% $CO_2$/humidified air.

## k) [$^3$H]Inositol labelling

For both cell lines, flasks were labelled 48 hrs prior to use with 4μCi/ml of [$^3$H]inositol. The [$^3$H]inositol was not cleaned as for the experiments involving rat cerebral cortical slices but instead dried down under a steady stream of $N_2$. This was redissolved in about 5ml of media which was in turn filtered into the remaining media through a 0.45μ millipore filter. The labelled media was the replaced over the monolayer. This procedure was identical for both the cell lines used in this type of experiment.

Before the cells were scraped in order to remove them from the surface of the flask on which they were growing, the labelled media was discarded and

each monolayer washed rapidly with Krebs Henseleit buffer. Each flask was then scraped into approximately 10ml of fresh Krebs Henseleit buffer. For short term incubations it is not necessary to include [$^3$H]inositol in this buffer. However, because the experiments discussed in the following chapters extend to at least 30 min it is essential to include [$^3$H]inositol in the Krebs Henseleit buffer into which the cells are scraped, at a final concentration of 4µCi/ml. This helps to prevent changes in the specific radioactivity The inositol was cleaned prior to use on a small anion exchange chromatography column as described previously.

Aliquots of cells in suspension in the labelled buffer are then incubated in flat bottomed vials at 37°C in a shaking water bath. The appropriate additions of agonist and Li$^+$ are made as soon as possible after the cells have been dispensed. Without the hour-long preincubation and labelling stages required for rat cerebral cortical slices, the additions can be made 10 min-15 min after aliquoting.

## 1) Termination and elution of individual [$^3$H]inositol phosphate fractions

The termination and extraction procedures used stop the reactions and isolate the [$^3$H]inositol (poly)phosphate fractions from labelled cell suspensions are as described for the characterization of the labelled inositol (poly)phosphates from rat cerebral cortical slices. In every labelled experiment, the individual inositol (poly)phosphate fractions were studied.

## m) Freeze thawing

The easiest method of determining enzymic activity in cells is to lyse the cells rapidly to release their intracellular contents. This was useful in studies of Li$^+$ and antibody inhibition of the inositol monophosphatase.

The media in which the cells were growing, in this case, was not removed. Instead the cells were scraped off __into__ the existing media and spun at approximately 1000 r.p.m. for 5 mins. The supernatant was then discarded and the pellet resuspended in cold Tris-buffered saline (TBS) (0.9%) at pH 7.4. This was subsequently respun and the second pellet stored at -70°C in a very small volume of TBS.

In order to lyse the cells, the aliquots were removed from the -70°C freezer and placed in a water bath preheated to 37°C. When thawed, the cells were gently pipetted up and down with a Gilson pipette to disrupt any cell aggregations which may occur during the thawing process. The aliquots were placed in dry ice, allowed to freeze thoroughly and then replaced in the water bath at 37°C. This entire procedure was carried out two or three times to ensure the majority of cells were lysed. The cell lysates were then spun down in a microcentrifuge and the supernatants removed. These supernatants contain inositol monophosphatase enzymic activity.

n)  Inhibition of the inositol monophosphatase enzyme

This assay, which was formulated by Ragan __et al__. (1988), exploits the production of free inositol by the enzyme. In the presence of active inositol monophosphatase enzyme, $InsP_1$ is converted to free inositol. In the presence of an inhibitory agent e.g. $Li^+$ or antibodies raised to purified inositol monophosphatase, the amount of free inositol will decrease.

The basic protocol involves the following components:

|  |  |  |  |
|---|---|---|---|
| 30μl | 10 mM | OPH | |
| 30μl | 100 mM | EGTA | |
| 60μl | 0.1 mM | Ins(1)P | |
| 10μl | $[^{14}C]$Ins(1)P | (12.5 nCi) | |
| xμl | cell supernatant or brain homogenate | | |
| (180-x)μl | monophosphatase assay buffer | | |

The OPH and EGTA are responsible for chelating metal ions which may otherwise affect the assay. The labelled Ins(1)P represents approximately 27000 d.p.m. The central issue of the experiment is to add sufficient cell supernatant or brain homogenate to convert approximately one third of the d.p.m. added into free inositol. This is because the rate of conversion remains linear over this level of substrate. The assay can incorporate appropriate volumes of either $Li^+$ or antisera. This extra addition is simply deducted from the monophosphatase assay buffer volume so that the final volume is always 300μl. This buffer contained 250mM KCl, 50mM Tris/HCl, 3mM $MgCl_2$, pH7.8 and all dilutions and additions, where possible, were made in this buffer.

The assays, carried out in flat-bottomed vials were subsequently incubated in a shaking water bath at 37°C for approximately 15 mins. Reactions were then terminated by the addition of 50μl 10% (w/v) TCA and 50μl 1M TRIS which had not been pH'ed. To this 2ml water was then added.

In order to determine the amount of Ins(1)P converted to free inositol, the terminated reaction is tipped onto a water-equilibrated formate form Dowex packed column and the sample tubes washed with a further 3ml water. This was allowed to elute. Subsequent to this, 5ml water is added and also allowed to elute. The eluate collected which has a final volume of approximately 10ml, is counted by liquid scintillation spectroscopy in the presence of 10ml scintillation cocktail. Using this assay, concentration dependent effects of either $Li^+$ or the polyclonal antibodies can be set up as will be discussed in Chapter 5.

In order to determine if the antibodies used were interacting with a particular sequence of the enzymes primary structure, or if it was part of its secondary struture, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques were carried out.

o)  SDS-PAGE


In 1967 Shapiro et al. reported that the molecular weight of most
proteins could be determined by measuring their mobility of polyacrylamide
gels which contained the detergent SDS.  Two years later this technique was
modified by Weber and Osborn (1969).  It has now developed into the standard
method for determination of the molecular weights of single polypeptide
chain.  When run against proteins of known molecular weight, the molecular
weight of other proteins can be calculated with an acuracy of between 5% and
15%  simply by determination of their mobility SDS PAGE gels.

The mini-gel apparatus used was cleaned thoroughly in water and then in
ethanol and assembled for use.  The bottom section of the gel - the so called
"running gel" contained the following:

| | | |
|---|---|---|
| 3mls  acrylamide | 2.5ml | running gel buffer |
| 32μl  APS         * | 100μl | SDS |
| 10μl  TEMED | 4.32ml | $H_2O$ |


Approximately 3.75ml of this was added to each gel apparatus and allowed to
set.  The "stacking gel, which was added to the top of this, consisted of:

| | | |
|---|---|---|
| 400μl  acrylamide | 1.0ml | stacking gel buffer |
| 30μl  APS | 40μl | SDS |
| 10μl  TEMED | 2.5ml | $H_2O$ |


Approximately 1.0ml was added to the top of the running gel and a comb
placed immediately into the stacking gel.  Once this had set, the comb was
gently removed.  The indentations it forms in the gel become the wells into
which the samples to be run are injected.

The samples which are run are first boiled at 100°C for between 5 and 15
mins in the presence of gel loading buffer.  Approximately 20μg protein/10μl

aliquot can then be loaded into the appropriate well. Each gel is then run in a gel tank containing running buffer at 25 mA. Usually, the gels take between one and two hours to run.

If the gel is to be stained for protein, it is then removed and placed in Coomassie Blue stain for about 20 mins. This stains the gel uniformly. In order to detect the proteins the gel is subsequently placed in destain for 30 mins and fixed overnight.

## p) Western transfers

Western blotting is a procedure developed to transfer proteins, in this case from an SDS gel onto nitrocellulose paper. In the experiments discussed here, blotting was carried out for about 20 mins at 300mA and then an extensive range of washing and fixing steps were carried out.

1) Firstly, the nitro cellulose blots were incubated at room temperature overnight in Tris buffered saline (TBS) which contains 1% milk powder. The proteins in the milk powder effectively block all the sites on the nitrocellulose paper to which the antibody could bind non-specifically.

2) This solution was then replaced with TBS/milk powder solution containing 0.1% Tween 20 and the blots incubated, again at room temperature, for 1 hr.

3) After this, each blot was incubated in 10ml TBS/milk powder/Tween 20 solution containing either 1:200 or a 1:1000 dilution of the appropriate antibody. In this case the 4th bleeds from the C-19 and C-20 rabbits were used. This incubation was carried out for 2hrs at room temperature.

4) These solutions were subsequently removed and each blot washed for 3 mins with RIPEA.

5) This was immediately followed by three washes each of 3 min in length in TBS containing 0.1% Tween.

6) 10ml TBS/milk powder/Tween 20 containing a 1:500 dilution of peroxidase-linked swine anti-rabbit immunoglobulin was then added and the blots left for 2 hrs at room temperature.

7) The blots were then rinsed once with RIPEA and three times with TBS/0.1% Tween 20 as described in steps 4 and 5 above.

8) Blots were subsequently rinsed briefly in 50mM sodium acetate (pH 5.0).

9) After this, some freshly prepared 50mM sodium acetate containing 30% $H_2O_2$ and 1% AEC in acetone was added. The pinkish colour which appears at sites on the nitrocellulose paper where antibody has bound, is allowed to develop for between 2 and 20 mins.

10) Reactions were terminated with 1% (w/v) TCA. Any pink bands will immediately turn black.

11) Blots can then be washed thoroughly in water, photographed and stored in a dark place.

The materials used in the processes discussed in this chapter are listed in the Appendix along with details of the sources and suppliers. All the buffers used in sections (o) and (p) above are also described.

C H A P T E R   2.2

Development of an Enzymic Method to Separate [³H]Inositol (1,4,5)- and

(1,3,4)- Trisphosphate Isomers in Tissue Extracts

CHAPTER 2.2

DEVELOPMENT OF AN ENZYMIC METHOD TO SEPARATE [$^3$H]INOSITOL(1,4,5)-
AND (1,3,4)-TRISPHOSPHATE ISOMERS IN TISSUE EXTRACTS

Introduction

It is now known that the metabolism of labelled inositol phosphates

in vitro is a complex procedure in which the $Ca^{2+}$-mobilizing Ins(1,4,5)P$_3$ can

be either dephosphorylated to give the corresponding bisphosphate or

phosphorylated via a 3-kinase catalyzed reaction to form Ins(1,3,4,5)P$_4$. The

enzymic degradation of each of these metabolites has been discussed

previously. However, in rat cerebral cortical slices labelled for 60 mins

with [$^3$H]myo-inositol and stimulated with carbachol, only two trisphosphate

isomers are prevalent, namely Ins(1,4,5)P$_3$ and Ins(1,3,4)P$_3$ (Batty et al.

1985; Batty and Nahorski, 1987). In 1988, Strupish et al. demonstrated that

in permeabilized GH$_3$ rat pituitary cells and Swiss 3T3 cells, Ins(1,3,4)P$_3$

does not release $Ca^{2+}$ - contrary to earlier reports which implied that it

could, albeit weakly (Irvine et al. 1986). In experiments in which the total

inositol trisphosphate fraction is eluted by Dowex anion exchange

chromatography, the contribution made by the Ins(1,4,5)P$_3$ and the

Ins(1,3,4)P$_3$ isomers are unknown. Obviously, since only one of the two is

active in releasing $Ca^{2+}$, it would be beneficial to be able to separate them.

However, the only reliable method of isolating one from the other was, until

recently, by high performance liquid chromatographic (h.p.l.c.) techniques -

a procedure which is both laborious and expensive. This method is hindered

further by its unsuitability to assay multiple samples quickly. This short

study examines the development of a method which separates the

[$^3$H]Ins(1,4,5)P$_3$ and [$^3$H]Ins(1,3,4)P$_3$ which accumulate in cholinergically

stimulated rat cerebral cortex by exploiting the $Mg^{++}$ dependency of the

enzymes responsible for the metabolism of these two trisphosphate isomers.

It had previously been described that in the absence of $Mg^{++}$ the Ins(1,3,4)$P_3$ isomer can be selectively dephosphorylated via the Ins(1,3)$P_2$ isomer (Inhorn et al. 1987; Batty et al. 1989). The enzymes responsible for the hydrolysis of the Ins(1,4)$P_2$ and Ins(1,3,4)$P_3$ to Ins(3,4)$P_2$ are $Mg^{++}$-dependent and cannot, therefore, proceed in the presence of a divalent cation chelator like EGTA. A simple separation of the trisphosphate fraction on Dowex formate columns following incubation with a crude rat brain extract in the absence of $Mg^{++}$ is described which provides accurate assessments of the $[^3H]$Ins(1,4,5)$P_3$ and $[^3H]$Ins(1,3,4)$P_3$ in stimulated cerebral cortical tissue. These measurements are compared with parallel analysis of the samples by h.p.l.c.


## Materials and methods


The tissue preparations for the rat cerebral cortical slices is as described in Chapter 2.1. The slices were stimulated with millimolar carbachol in the absence of $Li^+$ and the reactions terminated with ice-cold TCA (1M) as before. Following the water-saturated diethyl ether extraction instead of neutralizing the samples with NaHCO$_3$ as before, samples were adjusted to pH 7.0-7.5 with the following buffer: HEPES (25mM), EDTA (5mM), KCl (100mM). The values shown in brackets represent the final concentration of these salts following neutralization. Routinely the unneutralized samples had a final volume of 1.5ml from the 3 x 0.5ml supernatants removed from triplicate samples and pooled. The HEPES/EDTA/KCl buffer was added such that the final volume of each neutralized sample was 2ml. This volume then allowed three separate determinations of labelled trisphosphate content whilst not diluting the sample to such a degree that detection of the two isomers would be difficult.

Two other tissues were also prepared for trisphosphate fraction analysis:

## 1. Bovine tracheal smooth muscle

Bovine cervical trachealis muscle was prepared and cross-chopped ($300\mu m$ x $300\mu m$) according to the method of Hall and Hill (1988). Following a 60 mins preincubation period in Krebs Henseleit buffer at $37°C$ as described for the rat cerebral cortical slices preparation, slices were labelled in bulk with $50\mu Ci$ of [$^3$H]myo-inositol/ml packed slices for 60 mins and then stimulated for 30 mins with $10^{-4}M$ carbachol the absence of $Li^+$ (Chilvers et al. 1988). Reactions were terminated with 1M TCA as before but extracted with tri-n-octylamine/1,1,2-trichloro- 1,2,2-trifluoroethane (50:50, v/v) as described by Hawkins et al. (1986). Samples were adjusted to a neutral pH with the HEPES/EDTA/KCl buffer detailed above.

## 2. Rat parotid gland

Parotid glands from male Wistar rats (150-250g) were dissected and cross cut at a $60°$ angle ($150\mu m$ x $150\mu m$ x $150\mu m$) (Hanley et al. 1980). As with the other tissues involved in this study, after extensive washing, slices were preincubated for 60 mins with buffer replacement and oxygenation at regular time intervals. Parotid gland slices were subsequently labelled in bulk with $100\mu l$/ml of packed tissue for 90 mins at $37°C$. Slices were gassed regularly but, as with the bovine tracheal smooth muscle slices, there was no accompanying buffer replacement. The preparation was then stimulated with millimolar carbachol in the presence of 10mM $Li^+$ for a further 30 mins. Reactions were terminated with ice-cold 1M TCA, treated with 5 x 2 vol. of water saturated diethyl ether and adjusted to pH 7.0-7.5 with the HEPES/EDTA/KCl buffer as before.

## Prepration of standards

$[^3H]Ins(1,3,4)P_3$ was prepared by the dephosphorylation of authentic $[^3H]Ins(1,3,4,5)P_4$ with human erythrocyte membranes (Downes et al. 1986). Approximately 0.5µCi $[^3H]Ins(1,3,4,5)P_4$ was incubated with human erythrocyte membranes in 75mM HEPES, 5mM $MgCl_2$ (pH 7.2) for 120 mins at 37°C. The reaction was terminated with ice-cold TCA (1m) and the membranes precipitated by centrifugation. Pooled TCA extracts were washed with 5 x 2 vol. water saturated diethyl ether and neutralized with 25mM $NaHCO_3$.

The sample was subsequently loaded onto a 1ml Dowex formate column and the $[^3H]InsP_3$ fraction collected as 10 x 1ml fractions. The peak fractions were pooled, diluted 1 in 10 and applied to a QMA-Sep-Pak column. This had been prewashed with 10ml water, 20ml 1.0M triethylamine bicarbonate (TEAB, pH7.4) and 10ml water. The sample was applied and the column washed again with 10ml water. Inorganic phosphate was then removed in a 20ml wash of 0.15M TEAB and finally the $[^3H]Ins(1,3,4)P_3$ removed with 20ml 1.0M TEAB. Again the peak fractions were collected and freeze-dried in the presence of 10µl of mannitol (10mg/ml). This prevents absorption of the sample into the plastic of the tube used during the freeze drying process. The sample was then redissolved with 50% v/v methanol, redried and redissolved again in 100% methanol. This being more volatile caused a more rapid loss of the salt. Samples were subsequently stored in water containing 30% ethanol at 4°C until required $[^3H]Ins(1,4,5)P_3$ was supplied by NEN England Nuclear and was shown to be >98% pure by h.p.l.c.

## Assay Conditions for Selective Hydrolysis of $[^3H]Ins(1,3,4)P_3$

Four cerebral cortices were dissected from male Wistar rats and homogenized (3 x 15 sec bursts with a Polytron tissue homogenizer) at 4°C in

the following buffer: KCl (100mM) HEPES (25mM), NaCl (20mM), $MgCl_2$ (2mM) pH
7.4. The values shown in brackets represent the final concentrations of
these salts. Routinely cerebral cortical preparations were homogenized at a
concentration of 25% (w/v). Homogenates were then centrifuged at 100,000g
for 90 mins at 4°C and the supernatant used as the enzyme source.
Preliminary data was obtained which suggested that whole rat brain could
replace cerebral cortex in this preparation with identical results.
Interestingly, however, when fresh cerebral cortical preparations were
compared to supernatant which had been stored at -20°C activity was observed
to decrease with time, such that if 'Day 1' was considered arbitrarily to
cause 100% hydrolysis using a freshly prepared supernatant, by 'Day 7' the
values obtained would constitute only 75% hydrolysis and within three weeks
the levels of hydrolysis would only be between 10% and 15%. In comparison, a
single experiment using a whole brain supernatant which had been stored for
two weeks indicated that the levels of hydrolysis were maintained and that
90% - 95% of the trisphosphate fraction was hydrolysed over a 60 mins
incubation period. As a result, although the reason for this phenomenon
remained unknown, experiments were routinely carried out using cerebral
cortical or whole brain supernatants made fresh on the day of use.

The supernatant thus prepared, neutralized tissue extracts or standards
prepared as above were incubated with the supernatant in such preparations as
to give a final concentration of supernatant of 5% (w/v). For control
incubations an aliquot of the supernatant was heat-inactivated by boiling at
100°C for 15-20 mins. Reactions were terminated at the appropriate time with
ice-cold TCA to give a final concentration of 1M. The samples were then
extracted with water-saturated diethyl ether and adjusted to pH7 with 5mM
$NaHCO_3$.

The labelled inositol phosphates were separated by Dowex anion exchange
chromatography. [³H]Ins(1,4,5)P$_3$ and [³H]Ins(1,3,4)P$_3$ isomers were also
separated in corresponding studies by h.p.l.c. using a Partisil SAX column

and ammonium phosphate elution gradient as described by Challiss et al. (1988) and Batty et al. (1989).

## Results and Discussion

Studies by Batty and Nahorski (1989) have already established that the presence of EDTA in molar excess of the $Mg^{++}$ concentration in the buffer causes $[^3H]Ins(1,3,4)P_3$ to yield $[^3H]Ins(1,3)P_2$ exclusively. Under identical conditions neither $[^3H]Ins(1,4,5)P_3$ nor $[^3H]Ins(1,3,4,5)P_4$ undergo significant hydrolysis. As a result, assaying the labelled inositol phosphate production in carbachol stimulated rat cerebral cortical slice studies, in the absence of $Mg^{++}$, should result in the preferential degradation of only the $Ins(1,3,4)P_3$ isomer.

Table 2.1 shows the hydrolysis that was obtained for five different samples which contained known mixtures of prepared standards of the two trisphosphate isomers found cholinergically stimulated rat cerebral cortical slice preparations. The observed hydrolysis in the absence of $Mg^{++}$ agrees closely with the percentage of $[^3H]Ins(1,4,5)P_3$ in the sample. Furthermore, the actual d.p.m. lost from the measurable $InsP_3$ fraction corresponds with the d.p.m. gained in the $[^3H]InsP_1$ and $[^3H]InsP_2$ fractions together. The identity of the isomeric species which constituted this rise in the $[^3H]InsP_2$ and $[^3H]InsP_1$ fractions, although never identified by h.p.l.c., are believed to be $Ins(1,3)P_2$ and $Ins(1)P$ respectively. Because the inositol monophosphatase enzyme is dependent upon $Mg^{++}$ for activity the hydrolysis in the absence of $Mg^{++}$ stops at the level of this $[^3H]InsP_1$ fraction. This cannot be dephosphorylated to yield free inositol and thus it might be expected that the gain in $[^3H]InsP_1$ plus the gain in $[^3H]InsP_2$ would equal the loss in the total $[^3H]InsP_3$ fraction.

However, it was important to examine tissue extracts to ascertain if this

| Sample | Theoretical Percentage | Actual Percentage | Observed Hydrolysis (%) | Loss in $IP_3$ (dpm) | Gain in $IP_1$ (dpm) | Gain in $IP_2$ (dpm) |
|---|---|---|---|---|---|---|
| 1 | 100% 134 0% 145 | 100% 134 0% 145 | 92.9 ± 7.0 | 5740 | 5020 | 650 |
| 2 | 50% 134 50% 145 | 51.5% 134 48.5% 145 | 43.9 ± 1.5 | 4800 | 3850 | 360 |
| 3 | 75% 134 25% 145 | 75.1% 134 24.9% 145 | 77.6 ± 3.3 | 6600 | 5700 | 840 |
| 4 | 25% 134 75% 145 | 24.8% 134 75.2% 145 | 27.8 ± 1.2 | 3200 | 1960 | 1150 |
| 5 | 0% 134 100% 145 | 0% 134 100% 145 | 3.7 ± 1.1 | 350 | 220 | 120 |

Table 2.1 - Authentic tritiated standards of $Ins(1,3,4)P_3$ and $Ins(1,4,5)P_3$ were prepared in a variety of mixtures and treated with the supernatant prepared from rat cerebral cortical homogenate for a period of 30 min. at 37 C. Data represents the mean ± S.E.M. from at least 3 separate determinations. 134 = $Ins(1,3,4)P_3$ and 145 = $Ins(1,4,5)P_3$ .

method would be applicable to such systems. In the parotid gland, mAChR stimulation results in the formation of both $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$. Although the kinetics are somewhat slower, the $Ins(1,3,4)P_3$ isomer accumulates much more markedly than $Ins(1,4,5)P_3$ (Irvine et al. 1985). This is in direct contrast to cerebral cortex which accumulates inositol trisphosphates rapidly upon addition of agonist accumulating slightly more $Ins(1,4,5)P_3$ than $Ins(1,3,4)P_3$. Fig 2.5 illustrates the hydrolysis of the $[^3H]Ins(1,3,4)P_3$ isomer in rat parotid gland slices which had been stimulated with carbachol in the presence of 10mM $Li^+$. H.P.L.C. analysis revealed that approximately 90% of the total labelled trisphosphate fraction appeared as the $Ins(1,3,4)P_3$. After incubation with the cerebral homogenate, 87% of the total $InsP_3$ fraction had been lost i.e. the $Ins(1,3,4)P_3$ had been hydrolyzed. In comparison, identical extract incubated with active supernatant and separated on Dowex anion exchange chromatography columns demonstrated that almost 79% of the fraction was hydrolyzed after 30 mins which is in close agreement with the data obtained by h.p.l.c. A time course of the hydrolysis also indicated that by 30 mins over 90% of the hydrolysis which does occur was complete with only a slight increase occurring after this period. The hydrolytic procedure is, therefore, very rapid when conducted at 37°C. The control samples which were treated with heat-inactivated supernatant did not differ significantly throughout the 60 mins experimental period.

Similarly, bovine tracheal smooth muscle slices stimulated with submillimolar carbachol appear to accumulate more $Ins(1,3,4)P_3$ than $Ins(1,4,5)P_3$ over a 30 mins time period (Chilvers et al. 1990). In a comparison of the percentage hydrolysis which was measured by h.p.l.c. and by Dowex anion exchange chromatography, over 80% of the total fraction was observed to be $Ins(1,3,4)P_3$ (Table 2.2). The similarity between the figures obtained by these two methods is striking and enhances the validity of this enzymic method as a potential complement to h.p.l.c.

Rat cerebral cortical tissue stimulated maximally with carbachol
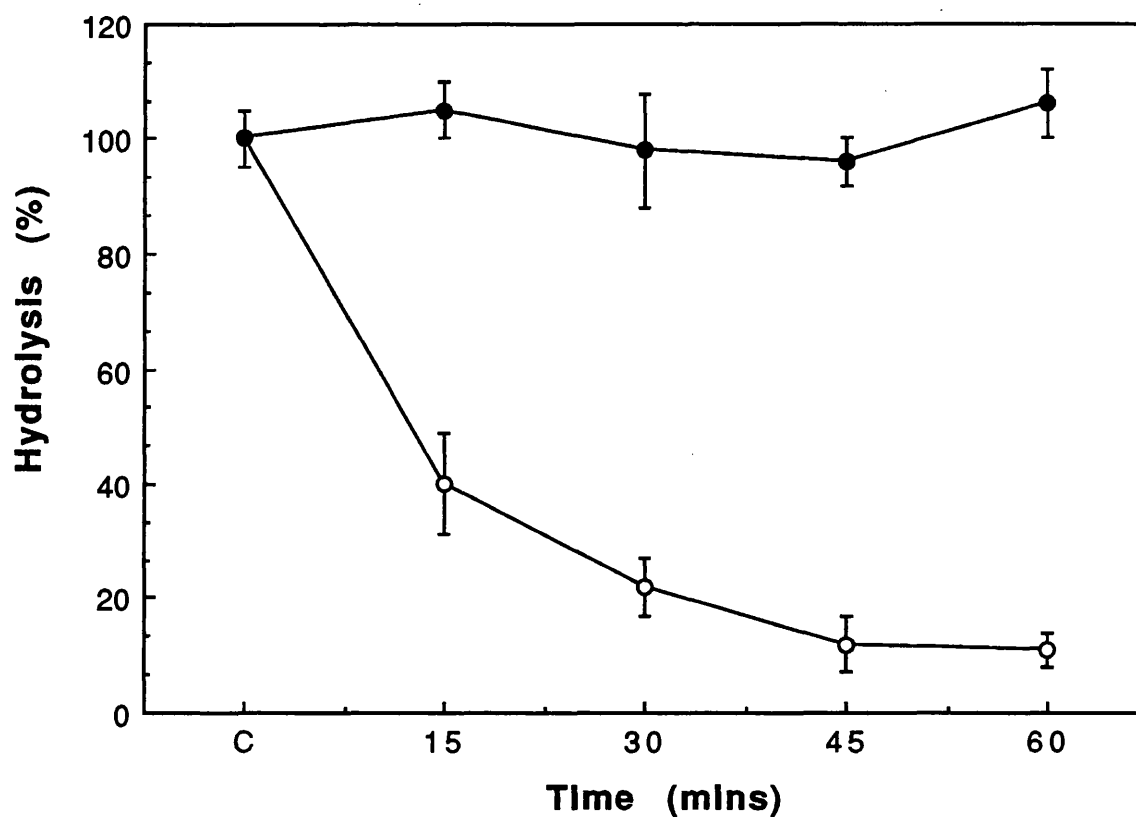
|  | H.P.L.C. | Enzymic method |
|---|---|---|
| Control | 100% | 100% |
| 30 min. | 12.8% | 21.2% |
| Hydrolysis | 87.2% | 78.8% |

Fig. 2.5 - Hydrolysis of the labelled inositol trisphosphate fraction from rat parotid gland slices.

Rat parotid gland extract was prepared according to the protocol described in Chapter 2. Aliquots of this were run in parallel determinations by h.p.l.c. and by the enzymic method. All samples were incubated for 30 min. at $37^{\circ}C$ - ( O )= active enzyme; ( ● ) =Inactived enzyme. Data represents values obtained from at least 2 separate determinations.

## H.P.L.C. analysis

| | $[^3H]Ins(1,3,4)P_3$ | $[^3H]Ins(1,4,5)P_3$ | $[^3H]Ins(1,3,4)P_3$ (%) |
|---|---|---|---|
| Control | 14 ± 4 | 253 ± 58 | 4.3 ± 2.3 |
| Carbachol (0.1mM) | 6049 ± 72 | 1208 ± 24 | 83.4 ± 0.8 |

## Enzymic analysis

| | $[^3H]InsP_3$ (inactive enzyme) | $[^3H]InsP_3$ (active enzyme) | $[^3H]Ins(1,3,4)P_3$ (%) |
|---|---|---|---|
| Control | 324 ± 206 | 393 ± 73 | 0 |
| Carbachol (0.1mM) | 6441 ± 668 | 1257 ± 186 | 80.7± 1.0 |

Table 2.2 - Determination of $[^3H]Ins(1,3,4)P_3$ and $[^3H]Ins(1,4,5)P_3$ in bovine tracheal smooth muscle slices - a comparison of h.p.l.c. and enzymic treatment. 100ul aliquots of myo-$[^3H]$inositol labelled smooth muscle slices were stimulated for 30 min. with 0.1mM carbachol or buffer and neutralized tissue extracts subjected to either h.p.l.c. or incubated with active or inactivated rat cerebral corical homogenate. Data, expressed as DPM / 2ml aliquot, represents the mean ± S.E.M. from 3 separate determinations

accumulates only Ins(1,4,5)$P_3$ and Ins(1,3,4)$P_3$ in a ratio of approximately 3:2 (Batty et al. 1985; 1989; Hawkins et al. 1986). Fig. 2.6 illustrates the region of the h.p.l.c. trace which shows the inositol trisphosphate fraction from rat cerebral cortical extracts incubated with either heat-inactivated (A) or active (B) supernatant preparations. In this particular sample, representative of several, 64% of the total d.p.m. isolated as inositol trisphosphate was present as a peak co-eluting with authentic Ins(1,4,5)$P_3$ standard and the remaining 36% as a peak co-eluting with authentic Ins(1,3,4)$P_3$ standard. Following treatment with active enzyme, however, the latter peak was completely removed with no concomitant decrease in the levels of Ins(1,4,5)$P_3$. In Table 2.3 these samples - both extracts from rat cerebral cortical slices stimulated with millimolar carbachol for 30 mins - were incubated with either heat inactivated or active cerebral homogenate supernatant. The loss of total InsP$_3$ was approximately 35% of the total, a value in close agreement with the percentage of the total InsP$_3$ believed to be present as the Ins(1,3,4)$P_3$ isomer, as calculated by h.p.l.c. In this particular sample only 70% of the drop is the [$^3$H]InsP$_3$ fraction could be accounted for by an increase in [$^3$H]InsP$_1$. However, it is important to note that, although the anion exchange chromatography described here using formate form Dowex is extremely useful, one of its main disadvantages is that elution of labelled inositol phosphates from such columns is liable to slight error and, as such, a certain amount of d.p.m. will be "lost" during the elution process.

Nevertheless, this procedure appears to provide a relatively accurate and certainly efficient and simple method of separating the Ins(1,3,4)$P_3$ and Ins(1,4,5)$P_3$ isomers in labelled extracts of rat cerebral cortical slices, rat parotid gland slices and bovine tracheal smooth muscle slice preparations. To ensure that this enzymic method did not affect [$^3$H]InsP$_4$, [$^3$H]InsP$_5$ or [$^3$H]InsP$_6$ hydrolysis, further experiments were carried out. In all the experiments discussed [$^3$H]InsP$_4$ was eluted and under no conditions
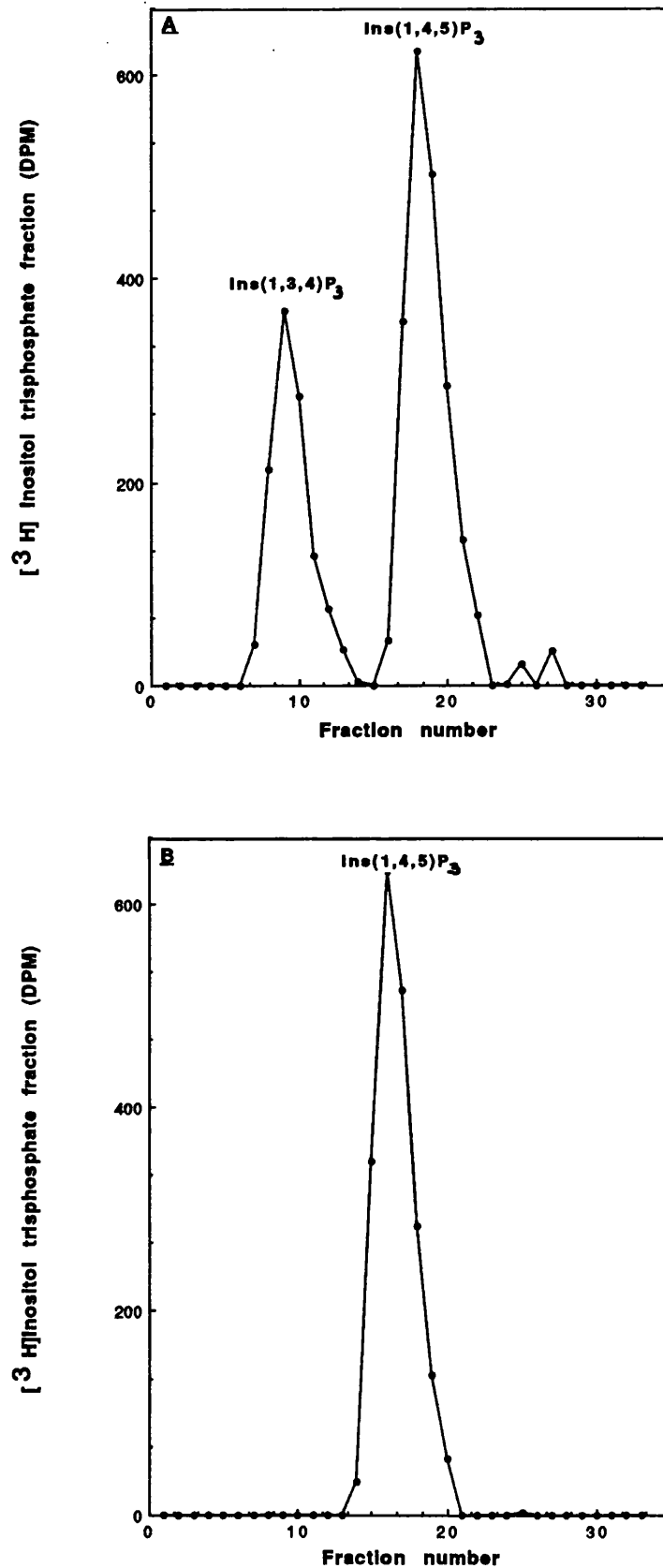
Fig. 2.6 (A and B) - Rat cerebral cortical extracts were treated with
either inactived (A) or active (B) supernatants from rat cerebral cortical
homogenate. H.P.L.C. analysis of the trisphosphate region revealed that
in this experiment, representative of several conducted, 64% was present
as Ins(1,4,5)P$_3$ and 36% as Ins(1,3,4)P$_3$ . After 30 min. Incubation at
37°C with active supernatant, complete removal of the latter was
achieved.

| | $[^3H]InsP_1$ | $[^3H]InsP_2$ | $[^3H]InsP_3$ | $[^3H]InsP_4$ |
|---|---|---|---|---|
| 30 min. (inactive enzyme) | $12415 \pm 510$ | $3070 \pm 115$ | $1150 \pm 55$ | $2100 \pm 15$ |
| 30 min. (active enzyme) | $16500 \pm 520$ | $3030 \pm 120$ | $750 \pm 40$ | $1960 \pm 100$ |
| % significance | 25% increase | no significant difference | 35% decrease | no significant difference |

Table 2.3 – Enzymic analysis of $[^3H]$inositol (poly)phosphate metabolism in the absence of $Mg^{++}$ in rat cerebral cortex.
Enzymic analysis of the samples involved treatment with active enzyme for 30 min. at 37 • C and separation using Dowex anion exchange chromatography. Data, expressed in DPM, represent the mean ± S.E.M. of at least 3 separate determinations.

did it appear to vary after treatment with the supernatant, either active or heat-inactivated, from the cerebral homogenate. Furthermore, an unlabelled extract prepared from rat cerebral cortical tissue and spiked with approximately 20,000 d.p.m. $[^3H]Ins(1,3,4,5)P_4$ was not significantly affected by the incubation. In addition, NG108-15 neuroblastoma-glioma cells incubated with $[^3H]$inositol for 48 hrs were investigated for effects on $[^3H]InsP_5$ and $[^3H]InsP_6$. In two experiments using NG108-15 cell extracts obtained from Dr. John Baird, the measurable $[^3H]InsP_5$ and $[^3H]InsP_6$ eluted from the Dowex chromatography columns with $2.0M$ $NH_4COOH/0.1M$ HCOOH, were unaffected by a 30 min incubation period with cerebral homogenate supernatant. Corresponding h.p.l.c. analysis of NG108-15 cell extracts confirmed this finding (data not shown).

Under the conditions presented here, all the tissue extracts involved in this study appeared to exhibit a $Mg^{++}$ independent hydrolysis of the $Ins(1,3,4)P_3$ isomer thus proving this enzyme method as a viable alternative to h.p.l.c. However, different tissue and cell extracts would naturally have to be tested thoroughly and compared to h.p.l.c. analysis to ensure that this method was applicable to that particular system and that other labelled $InsP_3$ isomers present were not confounding the results. Moreover, results may be complicated further by the use of a different cerebral homogenate supernatant e.g. bovine.

Some recent studies have implicated a 3-phosphatase activity which may be responsible for the back conversion of $Ins(1,3,4,5)P_4$ to $Ins(1,4,5)P_3$ (Cunha-Melo et al. 1988; Doughney et al. 1988; Dean and Moyer, 1988; Hoer et al. 1988). The presence of such an activity could be important in regulating the levels of $Ins(1,4,5)P_3$ in the cell. However, the degree of its contribution is unknown as are the optimal physiological conditions for its functioning. Doughney et al. (1988) reported that in human erythrocyte membranes in the presence of $Mg^{++}$, the conversion of $Ins(1,3,4,5)P_4$ to $Ins(1,4,5)P_3$ is rapid. As the $Mg^{++}$ concentration was lowered by omission

from the buffer or by the addition of EDTA, 80% of the product from hydrolysis of $Ins(1,3,4,5)P_4$ appeared not as the $Ins(1,3,4)P_3$ but rather as the $Ins(1,4,5)P_3$. This may lead to an important source of error in the assay described above which is performed in the absence of $Mg^{++}$. Hoer et al. (1988) demonstrated that in porcine cerebral cytosol, 3-phosphatase activity could only be observed in the presence of very high $Mg^{++}$ concentrations. As discussed previously, in routine elutions of $[^3H]InsP_4$ no significant difference was ever observed in the levels of this fraction following incubation with the cerebral supernatant.

It would appear, therefore, that, at least in the system described here, the $[^3H]InsP_3$ fraction can be separated into the component labelled $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ moieties by incubation of the tissue extracted with supernatant from a rat cerebral homogenate in the absence of $Mg^{++}$. This preferentially degrades the $Ins(1,3,4)P_3$ isomer. The method seems to be unaffected by the presence of the higher inositol (poly)phosphates whose metabolism does not complicate the results obtained. Therefore, this method has been utilised throughout the following experiments to investigate the effects of $Li^+$ on the levels of all the inositol phosphate fractions including $Ins(1,4,5)P_3$.

C H A P T E R  3.1

Lithium and Its Effects on Inositol (Poly)Phosphate Metabolism In Rat
Cerebral Cortical Slices

CHAPTER 3.1

# LITHIUM AND ITS EFFECTS ON INOSITOL (POLY)PHOSPHATE METABOLISM IN RAT CEREBRAL CORTICAL SLICES.

## Introduction

The ability of $Li^+$ to perturb inositol (poly)phosphate metabolism was first reported by Allison and Stewart(1971) who demonstrated that in rats injected intraperitoneally with $Li^+$ (10meq/kg), the levels of free inositol in the brain, in particular the cerebral cortex, fell by up to 30% within six hours of the treatment. Whilst this decrease continued in the cortex, the levels of inositol in the serum were rising over the same period. However, it was not until 1976 that Allison et al. indicated that the reason for this decrease in the levels of inositol was due to a $Li^+$-induced inhibition of the inositol monophosphatase - the enzyme responsible for removal of the remaining phosphate on the inositol ring to yield free inositol. This effect was considerable with the levels of inositol monophosphate increasing 20-fold over control values. In 1982, Berridge et al. proposed the original conjecture that $Li^+$ was influential in unbalancing normal inositol (poly)phosphate metabolism simply by acting as a block in the process of (poly)phosphoinositide lipid resynthesis. This theory and the seminal experiments which led to the discovery of the (poly) phosphoinositide metabolic pathway are discussed in Chapter 1. Experiments investigating $Li^+$ inhibition of the monophosphatase and a discussion on the inositol monophosphatase are to be found in Chapter 5.

In order to investigate the influence of $Li^+$ not only on the inositol monophosphate fraction but also on the inositol bis-, tris- and

tetrakisphosphate fractions, several experiments to consider both the

concentration and temporal dependence of their accumulation in the presence

of $Li^+$ were conducted. The experiments discussed here were all carried out

in rat cerebral cortical slices. The materials and methods used in the

course of this study are explained in Chapter 2.


## Results and Discussion


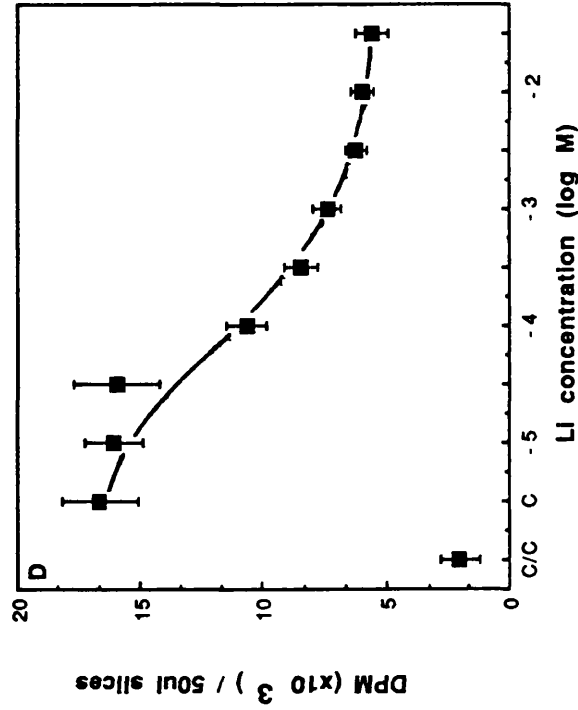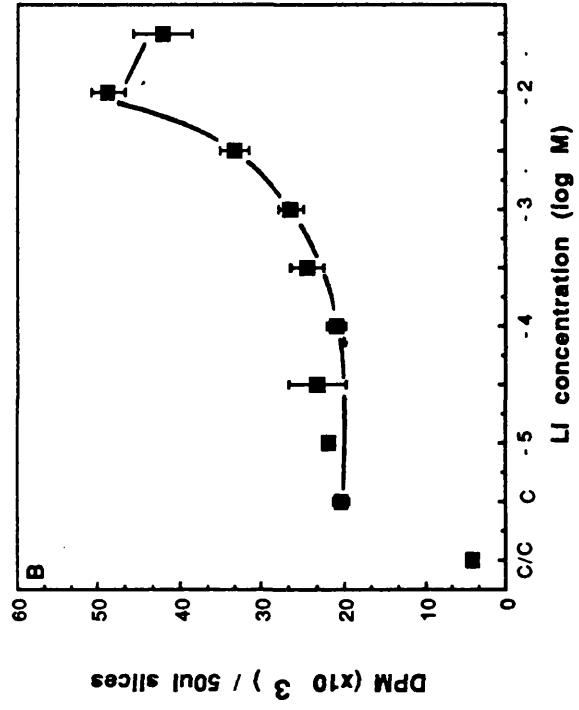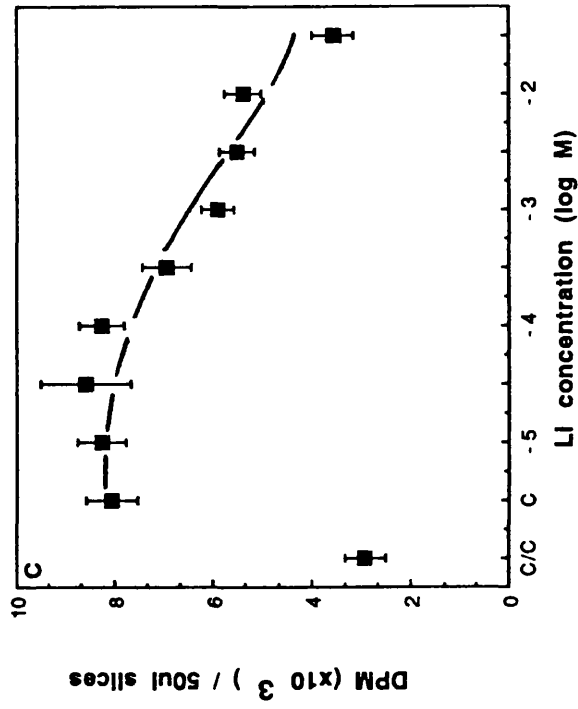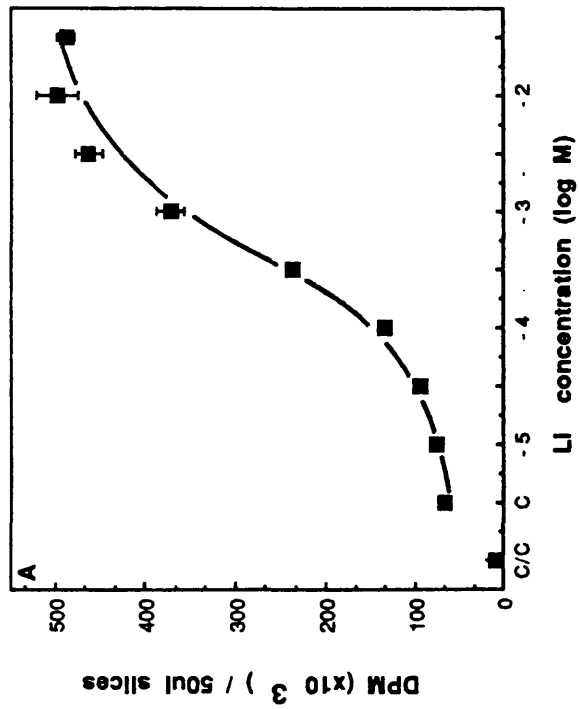## Concentration dependent effects of $Li^+$


Fig. 3.1 (A,B,C and D) shows the accumulation of inositol mono-, bis-,

(1,4,5)tris- and tetrakisphosphate fractions respectively in response to

increasing concentrations of $Li^+$ in rat cerebral cortical slices.

Fig.3.1A demonstrates that carbachol, present at a final concentration of

1mM for 30 min. causes an initial 5-6 fold increase in the levels of

detectable $InsP_1$. With increasing concentrations of $Li^+$, $InsP_1$ accumulates

in a concentration dependent manner with an $EC_{50}$ value calculated over the 30

min. experimental time period of 0.45 ± 0.03mM. This value, indicating the

concentration at which the accumulation is half that of the maximal value,

agrees with the $K_i$ value which is obtained from the purified enzyme. The

monophosphatase activity, purified to apparent homogeneity in both rat brain

(Takimoto et al. 1985) and bovine brain (Gee et al. 1988), indicates $K_i$

values of approximately 0.3mM and between 0.26mM and 1.0mM, depending upon

the predominant $InsP_1$ isomer, respectively. A similar value has also been

obtained from cloned bovine brain inositol monophosphatase (Diehl et al.

1990).

Fig. 3.1B shows the accumulation of the inositol bisphosphate fraction in

response to increasing concentrations of $Li^+$. Again, the presence of

carbachol alone is sufficient to cause a 4-5 fold stimulation in the $InsP_2$

Fig. 3.1 - Concentration dependent effects of lithium on the accumulation of individual inositol (poly)phosphates in rat cerebral cortical slices stimulated with carbachol (1mM).
A - $InsP_1$; B - $InsP_2$; C - $Ins(1,4,5)P_3$; D - $InsP_4$; C/C - control ($-$carbachol,$-Li^+$); C - control ($+$carbachol,$-Li^+$). The levels of $Ins(1,3,4)P_3$ did not change significantly during the experiment and have been omitted for clarity. Data represents the mean $\pm$ S.E.M. for at least 6 separate determinations.

**A**

DPM (x10 $^3$) / 50ul slices

500 — 400 — 300 — 200 — 100 — 0

LI concentration (log M)

C/C  C  -5  -4  -3  -2

**B**

DPM (x10 $^3$) / 50ul slices

60 — 50 — 40 — 30 — 20 — 10 — 0

LI concentration (log M)

C/C  C  -5  -4  -3  -2

**C**

DPM (x10 $^3$) / 50ul slices

10 — 8 — 6 — 4 — 2 — 0

LI concentration (log M)

C/C  C  -5  -4  -3  -2

**D**

DPM (x10 $^3$) / 50ul slices

20 — 15 — 10 — 5 — 0

LI concentration (log M)

C/C  C  -5  -4  -3  -2

levels. A further degree of accumulation does occur, albeit at higher $Li^+$ concentrations. The $EC_{50}$ value obtained here is 3.0 ± 0.39mM. Reports from several groups have indicated from partially purified preparations that $Li^+$ is much less potent in inhibiting the bisphosphatase. Delvaux et al. (1987, 1988) quote the apparent $K_i$ at 3-5mM $Li^+$ when measured in rat brain homogenates whilst Inhorn and Majerus (1987) report a slightly higher $K_i$ value of 6mM in calf brain.

Fig. 3.1C shows the effects that $Li^+$, in increasing concentrations, has upon the levels of the $[^3H]Ins(1,4,5)P_3$ fraction. Carbachol is responsible for causing a 4-fold increase in the $[^3H]Ins(1,4,5)P_3$ measured. At $Li^+$ concentrations of $10^{-5}-10^{-4}M$ this level of increase is maintained. However, at concentrations which are still submillimolar, the amount of this isomer decreases. The $IC_{50}$ value here, which represents the nominal figure ascribed to the concentration at which the accumulation has fallen to half that which it was when maximal, is 0.33 ± 0.06mM. In the presence of carbachol alone, the original levels of $[^3H]Ins(1,4,5)P_3$ were maintained. The control values, however, in several determinations, were seen to rise over the 30min. stimulation period routinely used in these experiments. As a result, as the levels of $[^3H]Ins(1,4,5)P_3$ are falling with increasing concentrations of $Li^+$, the control values of $Ins(1,4,5)P_3$ in non-carbachol, non-$Li^+$ treated samples increased slightly such that, at concentrations of $Li^+$ as high as 10mM-30mM, the difference between $Li^+$-treated samples and control samples is not significant.

The enzymic method used to separate the labelled $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ isomers from radiolabelled extracts gives sufficiently accurate estimations of not only the $[^3H]Ins(1,4,5)P_3$ isomer but also of the $[^3H]Ins(1,3,4)P_3$ isomer. The inositol phosphatase which acts on the $Ins(1,3,4)P_3$ is also sensitive to $Li^+$ and thus the levels of this isomer would be expected to increase in the presence of increasing concentrations of $Li^+$. Indeed such results have been obtained by Burgess et al. (1985). They

reported that $Li^+$ potentiated an increase in the $Ins(1,3,4)P_3$ produced in $[^3H]$inositol-labelled rat pancreatic acinar cells stimulated with 0.1 M caerulin in the presence of 20mM $Li^+$. This tissue choice was made because previously Rubin (1984) had demonstrated that in such cells, $Li^+$ caused a substantial increase in the $[^3H]InsP_3$ accumulation in response to caerulin. Several other laboratories have also indicated that $Ins(1,3,4)P_3$ accumulates in response to $Li^+$. Hanson et al. (1986) reported that 10mM $Li^+$ caused a 3-fold increase in the $[^3H]Ins(1,3,4)P3$ levels in vasopressin-stimulated rat hepatocytes whilst having no effect upon the $[^3H]Ins(1,4,5)P_3$ accumulation and Turk et al. (1986) showed a similar phenomenon in isolated pancreatic islets stimulated with glucose. In the studies reported here and in several other comparable determinations, the levels of $[^3H]Ins(1,3,4)P_3$ obtained by the enzymic method indicated that, in rat cerebral cortical slices labelled acutely and stimulated with 1mM carbachol, the production of this isomer was erratic and certainly did not accumulate to levels greater than those obtained by $Ins(1,4,5)P_3$. Carbachol alone routinely did display an ability to increase the $[^3H]Ins(1,3,4)P_3$ levels intracellularly but, again, this ability was variable between experiments and thus it was impossible to obtain any definitive data.

Lastly, Fig. 3.1D illustrates the $Li^+$ concentration dependence effects upon the levels of the tetrakisphosphate fraction. In rat cerebral cortical slices stimulated with carbachol for 30min. the major isomer present in this fraction is the $Ins(1,3,4,5)P_4$ (Batty et al. 1985, 1989). Carbachol alone causes an 8-10 fold increase in the levels of $[^3H]InsP_4$ which, in unstimulated slices in the absence of $Li^+$, appear to have resting levels much lower than those apparent in the $InsP_1$, $InsP_2$ and $Ins(1,4,5)P_3$ fractions. At very low $Li^+$ concentrations, the levels of $InsP_4$ appear to fall to levels which, in the presence of 30mM $Li^+$, are only about 2-fold over control samples. In turn, these samples, after 30 min. incubation, have not accumulated any basal $InsP_4$ in the way that the resting levels of

$Ins(1,4,5)P_3$ drift. This decline in the labelled inositol tetrakisphosphate component of the total inositol phosphate fraction has, again, a submillimolar $IC_{50}$ value ($IC_{50}$=0.1 ± 0.02mM).

The result illustrated in Fig. 3.1C showing a $Li^+$ concentration dependent decline in the levels of tritiated $Ins(1,4,5)P_3$ in carbachol-stimulated rat cerebral cortical slices was verified by examining the mass measurement of the $Ins(1,4,5)P_3$ produced under identical conditions. By so doing, the possibility that what was observed was due to changes in specific radioactivity could be effectively eliminated. Fig. 3.2 demonstrates that, in the absence of $Li^+$, the basal values of $Ins(1,4,5)P_3$ remain essentially constant. However, in the presence of millimolar carbachol plus $Li^+$ the pmol $Ins(1,4,5)P_3$/mg protein measured falls dramatically from a position well above basal to a situation where, at concentrations of 3mM and above, $Li^+$ virtually completely inhibits any carbachol-mediated production of $Ins(1,4,5)P_3$. In keeping with the labelled experiments, the $IC_{50}$ value of this activity was submillimolar ($IC_{50}$=0.42 ± 0.07mM).

Although in the labelled experiments the $Ins(1,4,5)P_3$ level did not fall to control levels, those calculated by mass measurement did and it is thus suggested that this small difference is due to changes in the specific radioactivity which are masking a decline in $Ins(1,4,5)P_3$ to control values. These results would, nevertheless, indicate one of two eventualities. As the intracellular $Li^+$ concentration rises either $Ins(1,4,5)P_3$ synthesis is blocked or its metabolism is enhanced. These possibilities will be discussed below.

## Temporal dependence of $Li^+$ effects

Further to these effects of $Li^+$ concentration on the inositol mono-, bis-, (1,4,5)tris and tetrakisphosphate fractions, experiments to determine individual inositol phosphate fraction accumulation with time in rat cerebral
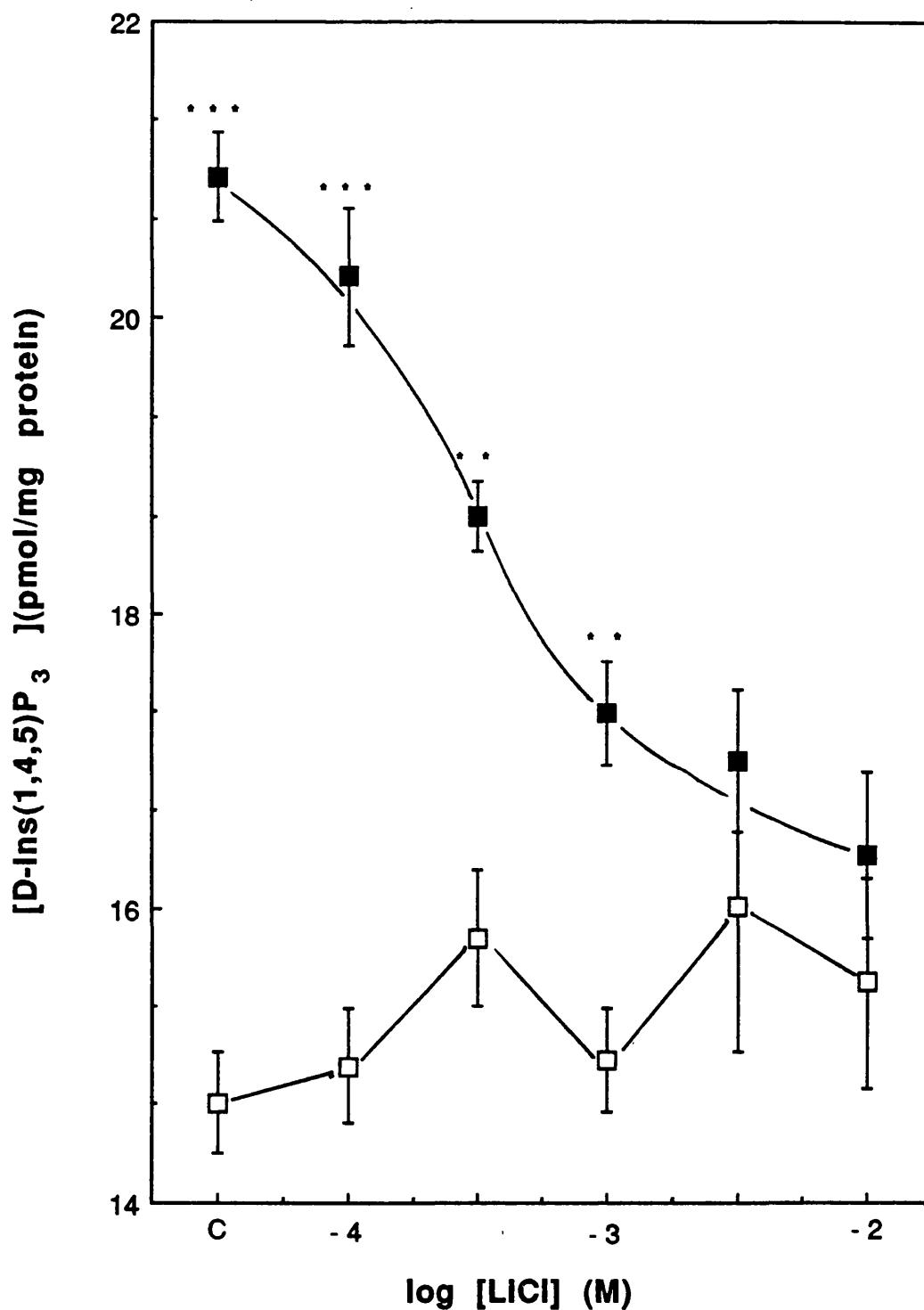
Fig. 3.2 - Concentration dependence of the effect of lithium on the concentration of Ins(1,4,5)$P_3$ in rat cerebral cortical slices.

Effect of carbachol ( ■ ) on Ins(1,4,5)$P_3$ concentration in the presence of 0 -10mM lithium. All incubations were terminated after 30 min.. Statistically significant increases over basal levels ( □ ) are indicated as **P< 0.002; ***P< 0.005. Data represents the mean ± S.E.M. of at least 3 separate determinations.

cortical slices stimulated with 1mM carbachol in the presence of 1mM $Li^+$ were undertaken. Fig. 3.3 (A,B,C and D) shows the accumulation of these fractions with time respectively.

Fig. 3.3A shows that, in the presence of carbachol, $[^3H]InsP_1$ accumulates rapidly over the 30 min. experimental time period with a 9-10 fold increase in level at 30 min. This accumulation appears to be linear throughout this interval. However, in the presence of both carbachol and $Li^+$ (1mM), the increase in intracellular $InsP_1$ is remarkable. Within the first 5 min. the accumulation is such that it is statistically significantly different from the aforementioned control values obtained in the absence of either carbachol or $Li^+$. Secondly, the linearity observed in samples treated with carbachol alone is retained in samples which are stimulated in the presence of $Li^+$. This accumulation appears to remain linear for up to 30 min. although the rate of accumulation of $[^3H]InsP_1$ is almost eight times greater than the rate of accumulation in the absence of $Li^+$.

That $[^3H]InsP_1$ displays this accumulation within the first 5 min. indicates that $Li^+$ enters the cell quickly and immediately inhibits the monophosphatase enzyme present. (Poly)phosphoinositide metabolism is thought to cycle slowly in the absence of external stimuli and, indeed, in rat cerebral cortical slices, resting levels, as measured by anion exchange chromatography, are between 2 and 10 times greater than for any of the other inositol phosphate fractions (see Fig. 3.3). If this is the case, then the intracellular monophosphatase level must be such that, even at early time points, inhibition of the action of $Li^+$ is detectable. It is salient to note that this may only be applicable in brain tissue. Similar experiments looking at $Li^+$ inhibition of the monophosphatase in bovine tracheal smooth muscle indicated that a distinct lag phase before $Li^+$ inhibition could be detected in the $[^3H]InsP_1$ fraction (Chilvers et al. 1990). Thus, in peripheral tissue, this cycling of (poly)phosphoinositide lipid metabolism in the absence of agonist may be much slower than in the central nervous system.
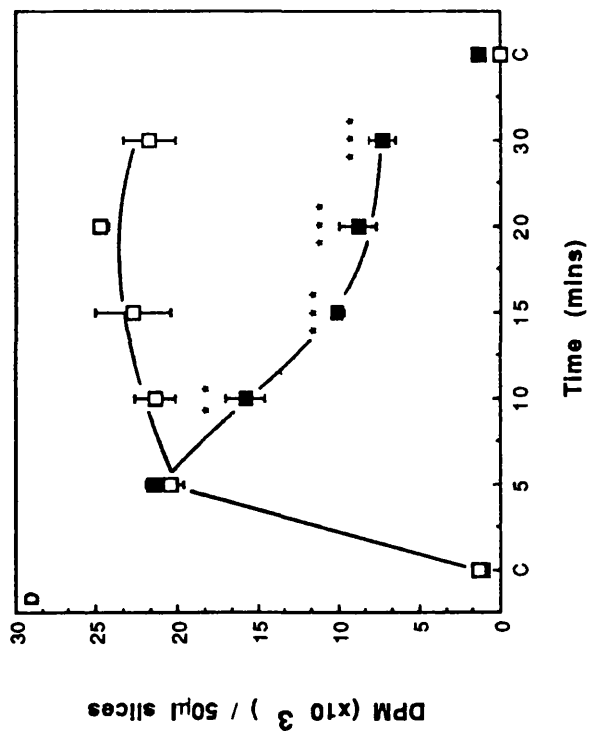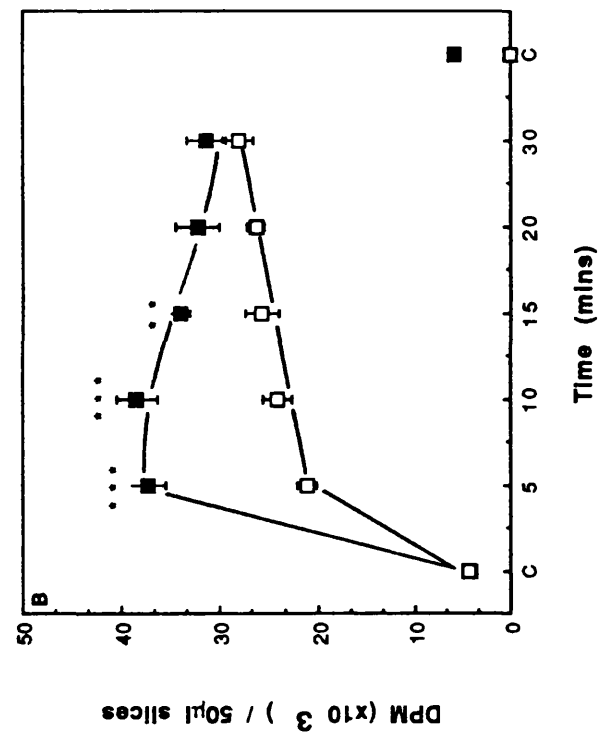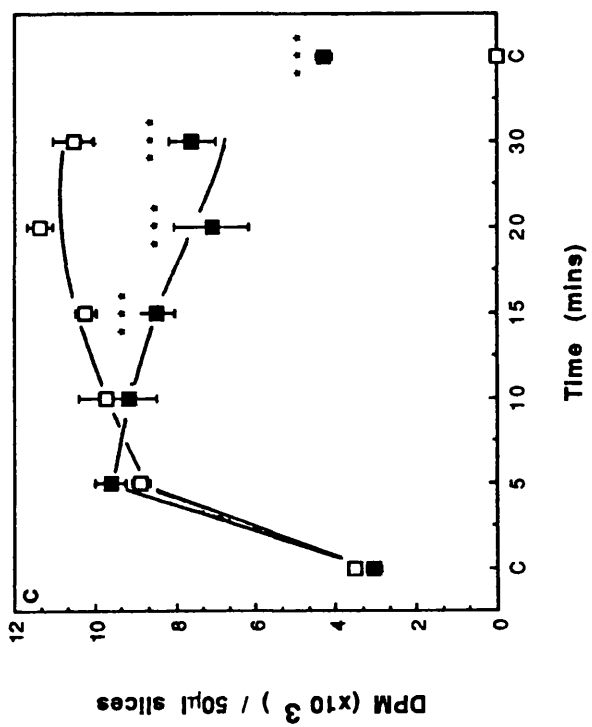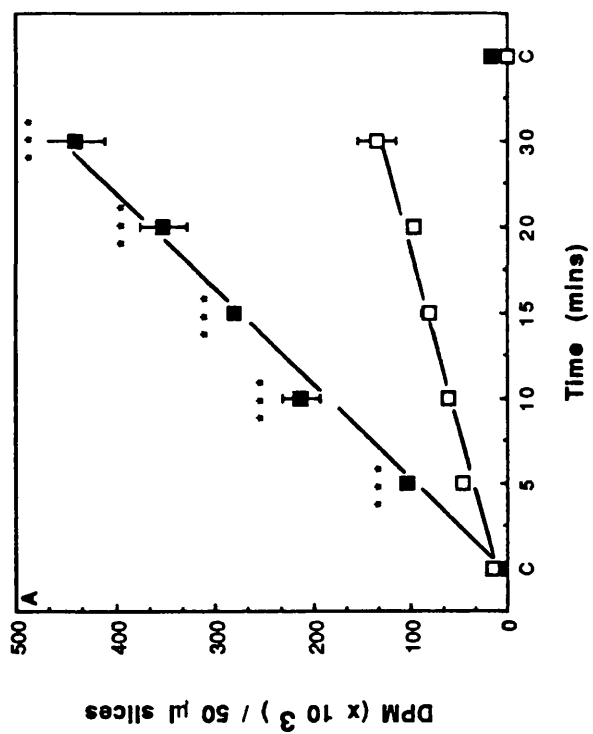
However, perhaps more interesting, is the continued linearity displayed in $[^3H]InsP_1$ accumulation for at least 30 min. after the initial agonist and $Li^+$ additions. The reasons for this are, at present, unknown but there are several possible explanations which should be considered.

In the first instance, $InsP_1$ may _not_ be accumulating linearly. It is conceivable that the effect that is seen is anomalous because changes in specific radioactivity are occurring. In cerebral cortical brain slice work, slices are labelled only acutely in comparison to cultured cells which are routinely labelled to a state approaching equilibrium (see Chapter 4). Thus, upon agonist stimulation the flux through the inositol (poly)phosphate metabolism will be increased, and, in the presence of $[^3H]$inositol, changes in specific radioactivity may be sufficient to confer linearity on $[^3H]InsP_1$ accumulation. As the levels of resynthesized (poly)phosphoinositide lipids fall in continued presence of $Li^+$ following agonist stimulation, it would be expected that the $[^3H]InsP_1$ fraction would plateau and then fall as the intracellular enzyme substrate levels become depleted. However, without a specific method for measuring the levels of mass $InsP_1$ like those for $Ins(1,4,5)P_3$ (Challiss _et al_. 1988) and $Ins(1,3,4,5)P_4$ (Donié and Reiser, 1989; Challiss and Nahorski, 1990), other possible sources of $InsP_1$ must also be considered.

Whilst current opinion believes that only $PtdIns(4,5)P_2$ is hydrolyzed upon receptor activation, theoretically at least, hydrolysis of both PtdIns PtdIns(4)P could occur. For PtdIns to split into its component parts - $InsP_1$ and DAG - PLC must catalyze esteric cleavage of the parent lipid. This has already been demonstrated, with all three (poly)phosphoinositide lipids considered here acting as substrates for the same PLC enzymes (Wilson _et al_. 1984). As such, PtdIns hydrolysis would result not only in continued DAG production but also in increased levels of $InsP_1$. Simultaneous production of $Ins(1,4,5)P_3$ would not occur under these cicumstances. Therefore, increased $Ca^{2+}$ mobilization would cease but PKC activation would continue (for review

Fig. 3.3 - Time dependent effects on the accumulation of the individual inositol (poly)phosphates in the presence ( ■ ) and absence ( □ ) of millimolar $Li^+$.
A - $InsP_1$;  B - $InsP_2$;  C - $Ins(1,4,5)P_3$;  D - $InsP_4$;  C - control (-carbachol,-$Li^+$).  Data represents the mean ± S.E.M. for at least 6 separate determinations.  Statistical significance is indicated as **$P< 0.02$;  ***$P< 0.005$ for the effect of carbachol in the presence compared to the absence of $Li^+$.

A

DPM ( x 10³ ) / 50 µl slices

500
400
300
200
100
0

C    5    10    15    20    30    C

Time (mins)

B

DPM (x10³ ) / 50 µl slices

50
40
30
20
10
0

C    5    10    15    20    30    C

Time (mins)

C

DPM (x10³ ) / 50 µl slices

12
10
8
6
4
2
0

C    5    10    15    20    30    C

Time (mins)

D

DPM (x10³ ) / 50 µl slices

30
25
20
15
10
5
0

C    5    10    15    20    30    C

Time (mins)

see Majerus et al. 1985). In vivo experiments in the presence of $Li^+$ have

also suggested that PtdIns metabolism may be as important, if not more so,

than $PtdIns(4,5)P_2$ metabolism in relation to $InsP_1$ accumulation

intracellularly (Ackermann et al. 1987; Sherman et al. 1987). This group's

evidence considered that, because in both stimulated and unstimulated rat

brain in vivo , there is 10-fold more $Ins(1)P$ than $Ins(4)P$, the only source

of the $Ins(1)P$ must be from increased hydrolysis of PtdIns. However, kinetic

analysis has now suggested that hydrolysis of $PtdIns(4,5)P_2$ alone can account

for all the $Ins(1)P$ produced. Batty et al. (1989) argue that, whilst PtdIns

hydrolysis may still be a viable proposition in in vivo studies, $Ins(1)P$

accumulation, which represents more than 90% of the total $InsP_1$ fraction in

in vitro slice preparations, can arise simply from the metabolism of the

$Ins(1,3)P_2$ isomer. This, in turn, may be sufficient to explain the increased

$InsP_1$ levels observed in Fig. 3.3A.

Thirdly, the presence of $Li^+$ over prolonged time-course experiments may

preferentially shunt $Ins(1,4,5)P_3$ metabolism through the $Ins(1,4)P_2$ pathway

rather than via the $Ins(1,3,4,5)P_4$ route. The results of this would be

two-fold. The production of $InsP_1$ would increase because the more indirect

pathway of $InsP_1$ production via the tetrakis- (1,3,4) tris- and bisphosphate

route would be attenuated whilst the more direct production of $InsP_1$ could be

achieved from $Ins(1,4,5)P_3$ by two simple dephosphorylation steps.

Furthermore, if $Li^+$ did achieve this biasing, $Ins(1,4,5)P_3$ would, with time,

decrease as the bulk of it was metabolized by the $Ins(1,4,5)P_3$

5-phosphomonoesterase and the $Ins(1,3,4,5)P_4$ levels would decline rapidly as

the 3-kinase activity responsible for its production was inhibited by some

$Li^+$-related mechanism. Such effects on the $Ins(1,4,5)P_3$ and $InsP_4$ fractions

in carbachol-stimulated rat cerebral cortical slices in the presence of $Li^+$

have been obtained and the possibilty of enhanced metabolism of both these

fractions will be discussed below.

Finally, there is the potential contribution to the inositol

monophosphate fraction by the hydrolysis of the cyclic inositol

polyphosphates. Ins(cycl:2)P, can be formed by either the sequential

dephosphorylation of its precursor molecule at both the 5- and 4-positions

(Connolly et al. 1986) or by direct PLC-catalyzed hydrolysis of PtdIns (Dixon

and Hokin, 1985). This molecule can then be hydrolyzed by an Ins(cycl:2)P

phosphodiesterase to yield Ins(1)P. Under the acid conditions of the

extraction procedure used here, cyclic inositol phosphates will not be

present. However, their contribution to the $[^3H]InsP_1$ fraction should be

considered. Whilst these inositol phosphates may be metabolized much more

slowly under the neutral conditions of in vivo studies than their non-cyclic

counterparts, their presence in stimulated cells and their subsequent

breakdown to yield free inositol via $InsP_1$ must be considered as another

putative source of $InsP_1$.

Fig. 3.3B illustrates the time-dependent accumulation of labelled $InsP_2$.

Carbachol, again, exerts its ability to cause an increase in the levels of

this molecule intracellularly. It should be noted, however, that, after the

initial large increase over the first 5 min. of stimulation, $[^3H]InsP_2$

appears to plateau. Similarly total $[^3H]InsP_2$ increases rapidly at early

time points in the presence of millimolar $Li^+$. After 5 min. the accumulation

of $[^3H]InsP_2$ is significantly different when $Li^+$-treated samples are compared

to the control samples. Interestingly, however, after this initial steady

state, the levels of $[^3H]InsP_2$ decline until, between 20 and 30 min. after

the addition of carbachol and $Li^+$, they are no longer statistically

significantly different to those achieved in control samples. Although with

simple anion exchange chromatography, as is used here, it is impossible to

detect changes within the levels of individual bisphosphate isomers, it is

proposed that the rapid accumulation $[^3H]InsP_2$ accumulation over this initial

period in the presence of $Li^+$ may be due primarily to $[^3H]Ins(1,4)P_2$. Upon

mAChR activation, the most direct metabolic route for $Ins(1,4,5)P_3$ is via the

$Ins(1,4)P_2$ isomer. With time, the $Ins(1,3)P_2$ and $Ins(3,4)P_2$ isomers will

accumulate too but it must be recognized that, with time also, the levels of Ins(1,4,5)P$_3$ and particularly InsP$_4$ decline rapidly in the presence of Li$^+$ (see below). Batty et al. (1989) have previously described increased concentrations of [$^3$H]Ins(1,4)P$_2$ following carbachol treatment as determined by h.p.l.c. The contributions made by other possible bisphosphate isomers - namely [$^3$H]Ins(1,3,)P$_2$, [$^3$H]Ins(3,4)P$_2$ and [$^3$H]Ins(4,5)P$_2$ - which these authors found were separable in rat cerebral cortical slices following mAChR stimulation - is unknown. The effect of Li$^+$ on their accumulation, although not undertaken here, has been considered by Batty and Nahorski(1989).

It should also be noted that, in the absence of Li$^+$, [$^3$H]InsP$_2$ accumulation is very rapid, again over the first 5 min. although not attaining such levels as were present in the presence of Li$^+$. Fig. 3.3B illustrates a gradual plateau indicating a new steady state of [$^3$H]InsP$_2$ metabolism has been reached. Moreover, there is a slight increase in accumulation observed throughout the remaining experimental period. This may be due to InsP$_2$ being produced from PtdIns(4)P in a manner similar to that discussed above from PtdIns under conditions of prolonged agonist exposure. However, it is possible that this increase is due to the delayed formation of Ins(4,5)P$_2$ in response to carbachol (Batty et al. 1989). This isomer was described in TRH-stimulated GH$_3$ cells (Hughes and Drummond, 1987) and it now appears that there may be two possible sources of it. Firstly, an inositol 1-phosphatase enzymic reaction on the Ins(1,4,5)P$_3$ molecule may result in Ins(4,5)P$_2$ production as suggested previously by Hughes and Drummond (1987) and van Lookern Campagne et al. (1988). Alternatively, a direct phospholipase D hydrolysis of PtdIns(4,5)P$_2$ may occur intracellularly although there is little evidence to support this mechanism. It is not known whether longer time course experiments would indicate that the levels of [$^3$H]InsP$_2$ continue to fall in the presence of Li$^+$ and if, in fact, accumulation in samples treated with carbachol alone would overtake them. H.P.L.C. analysis of the individual InsP$_2$ composition obtained in these

experiments will be required before this issue can be resolved.

When the levels of $[^3H]Ins(1,4,5)P_3$ in response to carbachol and $Li^+$ in rat cerebral cortical slices were studied, carbachol was observed to induce a rapid 3-4 fold increase in the levels of this isomer irrespective of the presence of $Li^+$. Fig. 3.3C shows, in fact, that with regard to $Li^+$, significant differences between the carbachol-treated and the carbachol- and $Li^+$-treated samples did not become apparent until 15 min. after carbachol addition. Over the remaining 15 min. of the experiment the levels of $[^3H]Ins(1,4,5)P_3$ fall steadily and, although not returned to basal values by 30 min. the amount of measurable $[^3H]Ins(1,4,5)P_3$ had fallen by almost 30% compared to the levels observed at 5 min. As seen in the $Li^+$ concentration dependence data, in the absence of carbachol and $Li^+$, the base line values creep up gradually so that, at 30 min. the levels of $[^3H]Ins(1,4,5)P_3$ in the presence of $Li^+$ are less than two-fold above control.

This figure shows clearly that $[^3H]Ins(1,4,5)P_3$ levels remain at their new steady state level for between 5 and 10 min. It is only after this apparent lag phase that any decrease in the accumulation occurs. It is possible that this decrease is due simply to the $Li^+$-induced block on the inositol monophosphatase. By preventing PtdIns resynthesis, the amount of inositol lipid available for PLC-mediated hydrolysis is effectively diminished and so $Ins(1,4,5)P_3$ and its phosphorylated derivative, $InsP_4$, may be expected to fall because of the reduction in their synthesis which will result as a consequence of this. However, as shown in Fig. 3.3A and, to a certain extent, 3.3B, the levels of $[^3H]InsP_1$ and $[^3H]InsP_2$ do not appear to be decreasing with increasing time. It would seem difficult, therefore, to reconcile these data. Without definitive data to suggest that $[^3H]InsP_1$ and $[^3H]InsP_2$ are decreasing and that it is changes in specific radioactivity which are accounting for this anomaly, it is important to consider several other lines of speculation which are open to discussion.

The reduced accumulation of $[^3H]Ins(1,4,5)P_3$ may be secondary to the

accumulation of another molecule rather than through the simple reduction in the levels of intracellular inositol which occurs following $Li^+$ treatment. This is caused by the inhibition of the monophosphatase enzyme which is responsible for the continued production of inositol following polyphosphoinositide hydrolysis. Without this activity, there will be a pronounced decrease in the levels of inositol and the cycle of lipid resynthesis and inositol (poly)phosphate metabolism will be affected (see Berridge et al. 1982,1989).

In the presence of $Li^+$ it is not only $InsP_1$ which accumulates as a precursor to lipid resynthesis. Importantly, DAG is converted to PA and thence to CMP-PA which also accumulates in the presence of $Li^+$ (see below). Without free inositol, the levels of CMP-PA could be expected to increase significantly. It is possible that CMP-PA can, under such adverse circumstances, monitor its own accumulation perhaps by a simple product inhibition or by a negative feedback mechanism. By preventing CMP-PA accumulation from reaching a level which may prove to be toxic to the cell, it might be expected that the intracellular concentration of DAG would also increase in the presence of $Li^+$ although there is little evidence to support this theory (see Hughes and Drummond, 1987). As a metabolite of PtdIns lipid signalling, DAG can have profound effects on PKC. If it accumulates under these conditions it is possible that PKC activation is subsequently enhanced in the presence of $Li^+$. PKC is known to phosphorylate muscarinic receptors (Haga et al. 1990), G-proteins (for review see Sagi-Eisenberg, 1989) and PLC, although in these instances no change in catalytic activity was observed (Rhee et al. 1986; Bennett et al. 1988). If the enhanced activity of PKC in $Li^+$-treated rat cerebral cortical slices is limited to one or all of these sites then, with time, it would be expected that, if the phosphorylation was to have negative effects on (poly)phosphoinositide lipid hydrolysis, all labelled inositol (poly)phosphate fractions would eventually be affected. As such, this may be the case but, as explained previously, the mass levels of

$InsP_1$ and $InsP_2$ have not been determined in these studies and it must be assumed, therefore, that, because only $Ins(1,4,5)P_3$ and $InsP_4$ are affected under the conditions used here, this possibility may be ruled out.

There have been several reports, however, which have indicated that, whilst PKC may not alter inositol phosphate synthesis, it may enhance the rate at which they are metabolized. The 5-phosphomonoesterase is known to play a pivotal role in terminating the signal transduced by the receptor. By removing the phosphate at the 5-position it converts not only $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ which is inactive with regard to $Ca^{2+}$ mobilization (Streb et al. 1983) but also $Ins(1,3,4,5)P_4$ to yield $Ins(1,3,4)P_3$ which is also inactive at releasing $Ca^{2+}$ (Strupish et al. 1988). Connolly et al. (1986) reported that the 5-phosphomonoesterase from platelets was phosphorylated by PKC in the presence of phosphatidylserine, $Ca^{2+}$ and DAG, thus enhancing its phosphatase activity by approximately 4-fold. They explained that this enhanced activity was due to an increase in the maximal velocity of $Ins(1,4,5)P_3$ dephosphorylation and not due to an increase in the affinity of the enzyme for $Ins(1,4,5)P_3$. Similarly, Molina y Vedia and Lapetina (1986) also investigating platelets, reported that when intact platelets were stimulated with PdBu (100-200nM) or OAG (5-20μM) for 30 sec. before treatment with Triton X-100, the $[^3H]InsP_3$ fraction was rapidly converted into $[^3H]InsP_2$. Having utilized concentrations of PdBu and OAG sufficient to stimulate PKC maximally their results again indicated that PKC was playing a potential role in inositol (poly)phosphate metabolism.

This theory of enhanced metabolism of the inositol (poly)phosphates in the presence of $Li^+$ would explain the reduced accumulation of $[^3H]InsP_4$ also. With time Fig. 3.3D indicates a dramatic, in this case, 20-fold increase from very low background values in the level of $[^3H]InsP_4$ over the first 5 min. of the experiment. Again, after a lag period, although less-pronounced in this particular figure, of 5-10 min. the $[^3H]InsP_4$ fraction decreases rapidly in the presence of $Li^+$. By 30 min. the levels have fallen to just 5-fold above

basal. The base-line values obtained in the absence of carbachol and $Li^+$, in comparison to those of the $[^3H]Ins(1,4,5)P_3$ shown in Fig. 3.3C, do not rise through some non-carbachol, non-$Li^+$ related action. That enhanced activity of the 5-phosphomonoesterase enzyme has been suggested would be a possible explanation as to why both the $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ fractions display reduced accumulation in the presence of $Li^+$.

However, these results have now proved to be controversial with other groups finding that 5-phosphomonoesterase activity, be it particulate or soluble, is unaffected by phorbol esters. For example, Orellana et al. (1985) found no effect on $[^3H]InsP_3$ metabolism in PMA-treated 1321N1 cell membrane preparations whilst Biden et al. (1988a.,1988b.) indicated that PMA was not causing enhanced $[^3H]Ins(1,4,5)P_3$ dephosphorylation in either insulin-secreting RINm5F cells or rat hepatocytes. Furthermore, use of the,albeit rather non-specific, PKC inhibitor, staurosporine, did not alter $InsP_3$ metabolism in platelets even after complete inhibition of the P47 protein purported to be the $Ins(1,4,5)P_3$ 5-phosphomonoesterase (Watson et al. 1988). Undoubtedly the most convincing data to preclude PKC involvement in enhancing $Ins(1,4,5)P_3$ metabolism via the 5-phosphomonoesterase came from Tyers et al. (1988) who searched current data bases with the coding sequence of the P47 protein (contradictorily coding for a protein of Mr 40kDa). There were no similarities between the P47 protein and the $Ins(1,4,5)P_3$ 5-phosphomonoesterase. Although platelets may prove to be an unusual system in which PKC affects the 5-phosphomonoesterase activity, it does seem unlikely from the evidence above that this is a widespread phenomenon and, therefore, does not explain the reduced accumulation of both $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ in carbachol stimulated rat cerebral cortical slices in the presence of $Li^+$.

The proposal that PKC enhances $Ins(1,4,5)P_3$ and $InsP_4$ metabolism has been further complicated by two other studies investigating PKC involvement in (poly)phosphoinositide lipid mediated cell signalling. Firstly, $Li^+$ can, at

least in <u>in vivo</u> studies of serotonin release, inhibit PKC action. PKC activation by phorbol esters causes neurotransmitter release. However, pretreatment of rats with $Li^+$ appeared to reduce the ability of phorbol esters to induce PKC translocation (Wang <u>et al</u>. 1989). This did not appear to be a direct effect. The implication was that $Li^+$ mediated its effects through one of the cofactors responsible for eliciting PKC translocation eg. cytoskeletal or vesicular proteins. Secondly, Ambrosini and Meldolesi (1989) have demonstrated that phorbol ester treatment of striatal and hippocampal neuronal primary cultures results in a complete inhibition of (poly)phosphoinositide lipid metabolism when mediated by quisqualate-preferring glutamate receptors whilst only inhibiting the carbachol response via mAChR stimulation by approximately 50%. This indicates different mechanisms of activation which, in the case of carbachol and mAChRs, may not be as sensitive to the actions of PKC as others. These data present convincing evidence that PKC may not be involved in the termination of (poly)phosphoinositide lipid hydrolysis - an activity which may be lessened still further in the presence of $Li^+$.

Two other studies have indicated that it may, in fact, be intracellular $Ca^{2+}$, and not PKC, which is the mediator of enhanced 5-phosphomonoesterase activity, however. Concentrations of $10^{-7}$ - $10^{-6}$M $Ca^{2+}$ were reported to enhance $[^3H]InsP_3$ metabolism by the cytosolic 5-phosphomonoesterase fraction in both macrophages (Kukita <u>et al</u>. 1986) and smooth muscle isolated from porcine coronary artery (Sasaguri <u>et al</u>. 1985). The proposal that $Ca^{2+}$ mobilized by mAChR-stimulated $Ins(1,4,5)P_3$ production would lead to activation of the enzyme responsible for degrading the second messenger thus preventing further $Ca^{2+}$ mobilization has been disputed. A plethora of studies have since reported that $Ca^{2+}$ can only activate 5-phosphomonoesterase activity at supraphysiological concentrations (see for example Connolly <u>et al</u>. 1985; Shears <u>et al</u>. 1987).

Thus far, it appears that the intracellular mechanisms which lead to the

decreased levels of $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ may be complex and may vary dramatically between tissues. The possibilities discussed here do not exclude the original proposal that all labelled inositol phosphate fractions are affected in the presence of $Li^+$ because of the block that this drug causes in (poly)phosphoinositide lipid resynthesis. However, to observe if PKC was having an effect on the aforementioned metabolism, a further series of experiments was undertaken.

In radioisotopically labelled rat cerebral cortical slices, various doses of the active phorbol ester, PdBu and the PKC inhibitor, staurosporine, were examined to monitor if (a)the active phorbol ester could mimic the action of $Li^+$ by direct intervention via the metabolic enzymes on which PKC has a putative stimulatory effect and (b)staurosporine would oppose this action by inhibiting PKC and thus prevent any reduced accumulation of $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$. The results obtained varied enormously from experiment to experiment and were, therefore, very difficult to interpret. Such preliminary data, however, do not preclude a PKC-mediated effect on inositol (poly)phosphate metabolism. Phorbol esters, being highly lipophilic, will partition readily into cell membranes. In tissue as heterogeneous as rat cerebral cortical slices, the phorbol ester concentration actually gaining access to neuronal cells may be much less than that which enters the more abundant glial cell population and, as the assay involves separating $[^3H]$inositol phosphates from whole samples, the effects of PdBu in neuronal cells may be obscured by its effects on the glial cells. Furthermore, only a small sub-population of the neuronal cells express muscarinic receptors thus rendering results even more difficult to interpret. Results obtained using the inactive phorbol ester, 4α-PDD, used as a control, did not differ significantly from the PdBu-treated samples. Similarly, staurosporine had no effect on the $[^3H]$inositol (poly)phosphate levels assayed in rat cerebral cortical slices in the presence of $Li^+$. Again, this does not infer a lack of PKC involvement in inositol phosphate metabolism. Staurosporine, although an

inhibitor of PKC, is non-specific and is, therefore, liable to inhibit a wide range of kinases in the cell. If "cross-talk" between the different signal transduction mechanisms is involved in inositol(poly)phosphate metabolism then this inhibition may be important and make further interpretation of results very difficult.

The difficulties surrounding any investigation into the reduced accumulation of both [$^3$H]Ins(1,4,5)P$_3$ and [$^3$H]InsP$_4$ fractions do not preclude a PKC action. It remains possible that this action is indirect rather than direct ie. PKC may phosphorylate an intermediate protein which then itself alters the 5-phosphomonoesterase activity. Alternatively, enhanced metabolism may rely upon the activation of only one of the PKC isoenzymes. The lack of specific pharmacological tools, however, for studying PKC in vitro, let alone in vivo, means that further studies into this mechanism are hindered by their non-specificity.

One further possibility which warrants discussion here, is the proposal that it is not the 5-phosphomonoesterase activity which is altered but the 3-kinase enzyme. Whilst enhancement of this activity, perhaps again through some PKC-mediated action, would not directly cause InsP$_4$ levels to fall, it would increase the rate at which [$^3$H]Ins(1,4,5)P$_3$ is metabolized.

Many aspects of the 3-kinase enzyme in the cell have been investigated. The data, however, are conflicting and indicate that the 3-kinase may exhibit species and tissue specific differences in terms of its molecular weight (Morris et al. 1988; Yamaguchi et al. 1988; Johansen et al. 1988), Ca$^{2+}$ dependency (Biden et al. 1988; Ryu et al. 1987; Biden and Wollheim, 1986; Imboden and Pattison, 1987) and PKC susceptibility (Biden et al. 1988; King and Rittenhouse, 1989). For example, in rat brain, Ca$^{2+}$ was found to inhibit the enzyme (Irvine et al. 1986; Morris et al. 1988), an effect not duplicated in bovine brain (Ryu et al. 1987). Batty and Nahorski (1987) found that in rat cerebral cortical slices stimulated with carbachol in the presence of Li$^+$, [$^3$H]InsP$_4$ levels fall but those of [$^3$H]Ins(1,4,5)P$_3$ do not. The authors

argue in their discussion that, because of the delayed onset of the inhibitory effect on $[^3H]InsP_4$ accumulation, a direct action of $Li^+$ on the 3-kinase seemed unlikely. Whilst $Li^+$ may not have a direct effect on any of the enzymes responsible for the inositol (poly)phosphate metabolism discussed here, it is still possible that the 5-10 min. lag period observed in Figs.3.3C and D is due to a $Li^+$-induced accumulation of a secondary metabolite which must first achieve a certain intracellular level before any effect is seen. That changes were not apparent in the $[^3H]Ins(1,4,5)P_3$ accumulation in the presence of, albeit higher concentrations of, $Li^+$ in the same tissue as was used here is perplexing and, as such, it is difficult to reconcile the differences between the data of Batty and Nahorski (1987) and those reported here. Again, however, the true effects of $Li^+$ on the $Ins(1,4,5)P_3$ isomer may have been masked by changes in specific radioactivity (Challiss et al. 1988; Batty and Nahorski, 1989).


Other studies


Interestingly, other studies on $[^3H]$inositol (poly)phosphate accumulation in both rat and mouse cerebral cortical slices have shown very similar results to those presented here (Batty and Nahorski, 1987; Whitworth and Kendall, 1988; Rooney and Nahorski, 1989). These groups all indicate that, in the presence of $Li^+$, $[^3H]InsP_1$ and $[^3H]InsP_2$ accumulate. Batty and Nahorski (1987) show also that $[^3H]InsP_1$ in carbachol-stimulated rat cerebral cortical slices continues to accumulate linearly for up to 45 min. but that, in the absence of $Li^+$, carbachol induces a rapid accumulation of $[^3H]InsP_1$ which is linear for only 20 min. before achieving a new steady state. Whilst this former result is in complete agreement with the data presented in Fig. 3.3A, the latter is not. The reason for this disparity is not clear. Furthermore, the results which have been obtained concerning the effect of $Li^+$ upon the $[^3H]InsP_3$ and $[^3H]InsP_4$ by various workers do not

appear to correspond.

In 1985, Batty and Nahorski reported that whilst $[^3H]InsP_1$ and $[^3H]InsP_2$ accumulated with $EC_{50}$ values of approximately 0.5mM and 4mM respectively in the presence of $Li^+$, the "$[^3H]InsP_3$" fraction decreased significantly with a half-maximal value of 1mM. The "$[^3H]InsP_3$" fraction was, however, later found to comprise not only $[^3H]InsP_3$ but also $[^3H]InsP_4$. The effect of $Li^+$, both in increasing concentrations and with time, on $[^3H]InsP_4$ is now well documented. Batty and Nahorski (1987) reported a 10 min. lag phase before $Li^+$ caused the tetrakisphosphate fraction to decrease in carbachol stimulated rat cerebral cortical slices. This effect was verified in mouse cerebral cortical slices (Whitworth and Kendall, 1988) again with the inhibition by $Li^+$ not becoming apparent until between 5 and 10 min. However, these authors demonstrated that this effect was only noticed following mAChR stimulation. Other agonists like histamine and noradrenaline did not induce a $Li^+$-mediated reduced accumulation of this fraction. They, too, pointed out that this effect could not, therefore, be due to a direct inhibition of the 3-kinase by $Li^+$ because, although carbachol caused a statistically significant decrease in the levels of $[^3H]InsP_4$, noradrenaline, histamine and KCl stimulation produced a minor enhancement in the accumulation of this fraction. $[^3H]InsP_4$ was also found to decline in the presence of increasing $Li^+$ concentrations in an investigation into the developmental aspects of mAChR-induced inositol (poly)phosphate accumulation in rat cerebral cortical slices (Rooney and Nahorski, 1989). These workers reported an $IC_{50}$ value of 0.3-0.4mM for $Li^+$ on $[^3H]InsP_4$ accumulation in 7 day- and 40 day-old rats. This accumulation was significantly greater in both young rats and also at low $Li^+$ concentrations such that measurable $[^3H]InsP_4$ is approximately 4-fold greater at 0.1mM $Li^+$ in 7 day old rats compared to 40 day old rats. The accumulation at concentrations as high as 30mM, however, only exhibited about a 2-fold difference.

The effects of $Li^+$ on the $[^3H]InsP_3$ fraction vary more between groups

than on any of the aforementioned fractions. Whitworth and Kendall (1988) reported that, in the presence of $Li^+$, all the agonists studied induced an accumulation of the total $[^3H]InsP_3$ fraction. The $Ins(1,4,5)P_3$ and the $Ins(1,3,4)P_3$ isomers were separated by h.p.l.c. and their relative accumulations in the presence of 5mM $Li^+$ measured. However, this time course was extended only to 10 min. - a time which precedes any significant reduction in accumulation in the experiments discussed above. With increasing $Li^+$ concentrations a very slight reduction in total $[^3H]InsP_3$ was observed but this decrease appeared to recover to a level that was higher than that recorded before any decrease was recorded. In contrast to this, Rooney and Nahorski (1989) observed that this reduction in accumulation in both 7 day- and 40 day-old rats was continuous throughout the $Li^+$ concentration response experiments. Moreover, Batty and Nahorski (1987) reported that $Li^+$ enhanced $[^3H]Ins(1,3,4)P_3$ accumulation in rat cerebral cortical slices in agreement with the results of Burgess et al. (1985) in pancreatic acinar cells but had no effect in the accumulation of $[^3H]Ins(1,4,5)P_3$. That such apparently different results are obtained to describe the effects of $Li^+$ on the inositol trisphosphate fraction is puzzling. It is impotant to note, however, that the techniques used in these studies revolved around the separation of the inositol trisphosphate fraction using h.p.l.c. This can be laborious and means that often only limited data are presented. This is in direct contrast to the enzymic separation which allows multiple assays to be carried out. It is possible, therefore, that because of this and also because the reduced accumulation only amounts to a fall of around 30%, that the true effects of $Li^+$ upon the $Ins(1,4,5)P_3$ were not apparent in the studies discussed above.

To verify that the effect seen in Fig. 3.3C was real, mass measurements of cold $Ins(1,4,5)P_3$ were undertaken. This technique, which had not been available for previous workers in the field, showed that, in the absence of both carbachol and $Li^+$, the basal values of $Ins(1,4,5)P_3$ stay essentially the

same highlighting the possibility that small changes in the specific radioactivity may account for the upward drift in these values observed in the radiolabelled experiments. Fig. 3.4 also shows that, in the presence of carbachol alone, the accumulation of $Ins(1,4,5)P_3$ is maintained at a level significantly above basal for up to 30 min. In the presence of 1mM $Li^+$, however, the reduced accumulation of the $Ins(1,4,5)P_3$ fraction is again apparent. After a marked 5-10 min. lag, the difference in the levels of accumulation caused by the presence of $Li^+$ becomes significant until, by 30 min., there is no apparent difference in the $Ins(1,4,5)P_3$ content between the control samples and those which contain carbachol and $Li^+$. Whilst this agrees with the data in Fig. 3.3C, there is another difference between the results presented here and those reported by other groups from the same laboratory - predominantly Batty and Nahorski (1987) but also Whitworth and Kendall (1988). With time, in either in the presence or absence of $Li^+$, both of these groups reported immediate accumulation of $Ins(1,4,5)P_3$ over basal. This accumulation would appear to have attained a maximal value within a few minutes.

Fig. 3.5, as well as Fig. 3.3C, demonstrates that in this situation the $[^3H]Ins(1,4,5)P_3$ increases dramatically but that this increase occurs over at least a 5 min. time span. This figure shows that, in the presence of carbachol, the levels of $[^3H]Ins(1,4,5)P_3$ increase and then plateau gradually throughout the remaining 25 min. of the experiment. Different concentrations of $Li^+$, however, appear to have concentration related effects on the levels of measurable $Ins(1,4,5)P_3$. $Li^+$, at a final concentration of 10mM causes a large initial $[^3H]Ins(1,4,5)P_3$ accumulation which is not significantly greater than in the presence of carbachol alone. With time, 10mM $Li^+$ causes reduced accumulation of this isomer which is continued for up to 30 min. $Li^+$, at a final concentration of 1mM, again elicits an increase in the initial $[^3H]Ins(1,4,5)P_3$ accumulation over the first 5 min. and, whilst reduced accumulation does result, levels do not appear to continue to fall
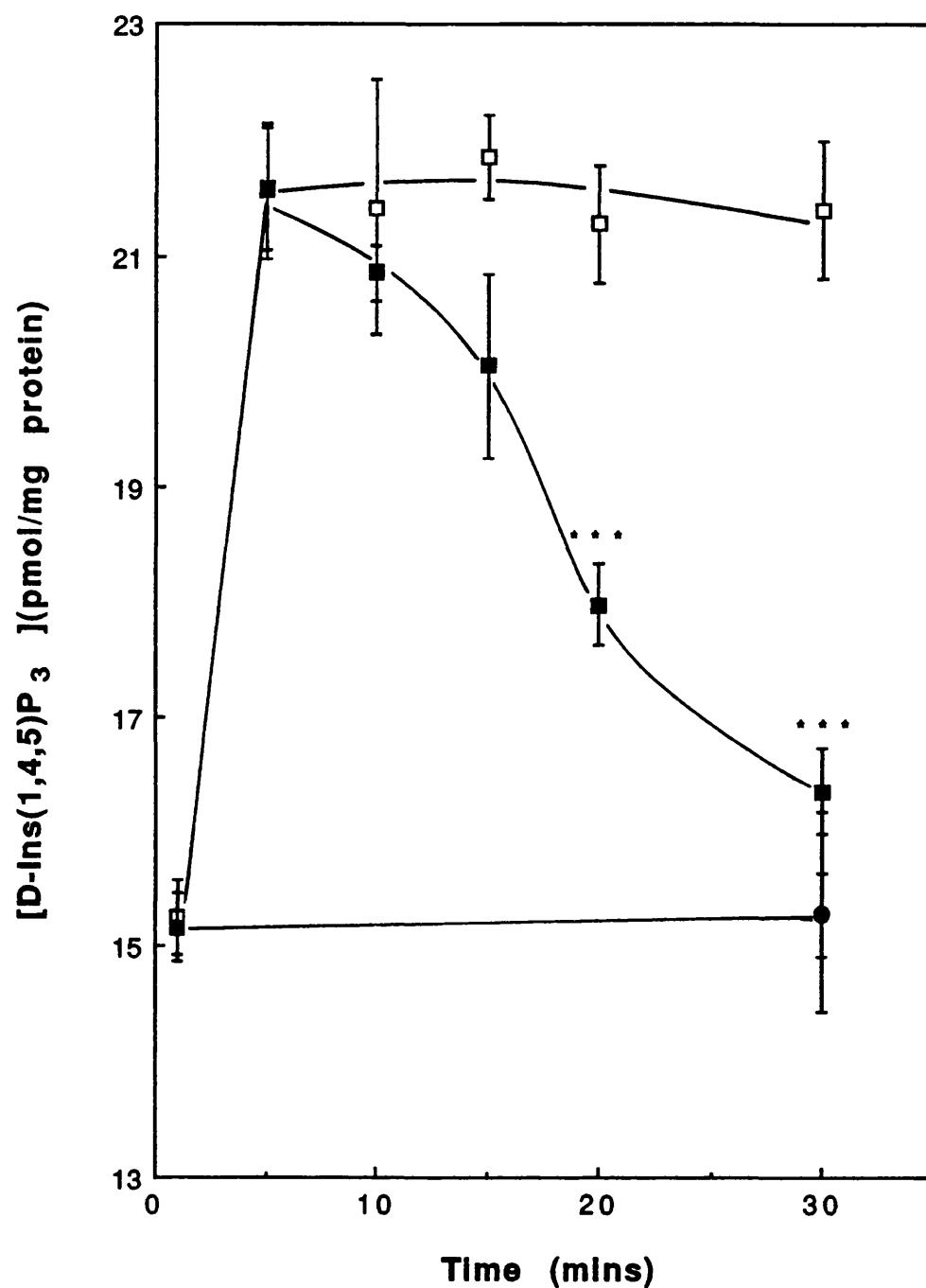
**Fig. 3.4** - Time course of changes In Ins(1,4,5)$P_3$ In the presence ($\square$,$\blacksquare$) or absence ($\bullet$) of 1mM carbachol and the presence ($\blacksquare$) of 1mM lithium. Significant differences In the effect of lithium on the agonist stimulated groups are shown as ***$P < 0.005$. Data represents the mean ± S.E.M. of at least 6 separate determinations.
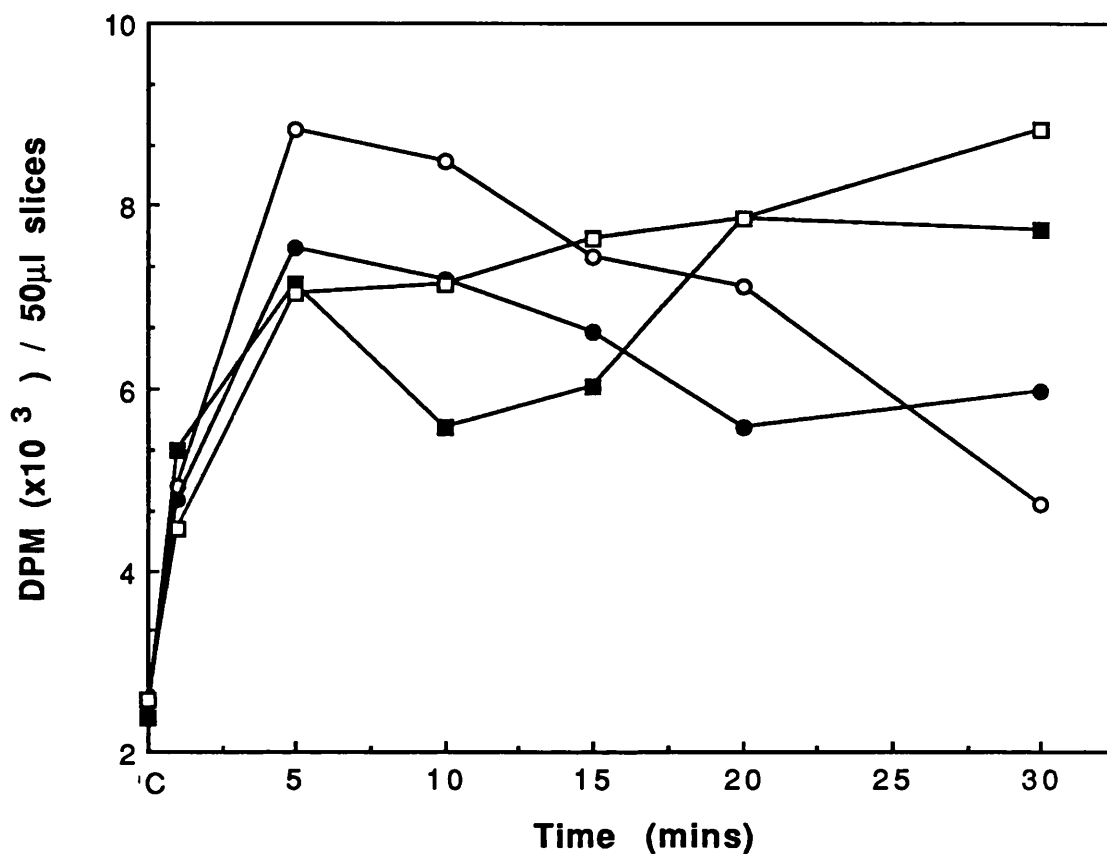
Fig. 3.5 - Time dependent effects on the accumulation of labelled Ins(1,4,5)P in rat cerebral cortex stimulated with carbachol (1mM) in the presence (o,●,■) or absence (□) of lithium. Data represents the mean for at least 3 separate determinations. S.E.M. values, which were all within 10% of the mean value, have been omitted for clarity.

o = 10mM Li ; ● = 1mM Li ; ■ = 0.3mM Li

after 20 min. Finally, in the presence of 0.3mM $Li^+$, there appears to be a marked initial accumulation which is not significantly different from the values obtained in the absence of $Li^+$. The $[^3H]Ins(1,4,5)P_3$ does not then appear to diminish but merely plateaus for up to 15 min. After this period, the accumulation appears to increase but this is not significant. As a result, the situation which prevailed at 5 min. with 10mM $Li^+$ causing more $[^3H]Ins(1,4,5)P_3$ accumulation than 1mM $Li^+$ and 1mM $Li^+$, in turn ,causing more $[^3H]Ins(1,4,5)P_3$ accumulation than 0.3mM $Li^+$ is reversed such that at 30 min., the accumulation of $[^3H]Ins(1,4,5)P_3$ is greater with 0.3mM $Li^+$ present than with 1mM $Li^+$ which is, again in turn, greater than with 10mM $Li^+$ present.

These results signify that, whilst $Li^+$ does have a definite effect on reducing the accumulation of the $[^3H]Ins(1,4,5)P_3$ in carbachol stimulated rat cerebral cortical slices - a result validated by parallel investigations on the mass $Ins(1,4,5)P_3$ accumulation in the same tissue - the effect relies upon the concentration of $Li^+$ present. This emphasizes the theory that, at higher concentrations of $Li^+$, the uncompetitive mode of its inhibition results in a complete inhibition of receptor-mediated $Ins(1,4,5)P_3$ production. At lower $Li^+$ concentrations, the possibility that more free inositol is produced via the inositol monophosphatase enzyme exists ie. the block caused by $Li^+$ may not be complete. Thus, at submillimolar $Li^+$ concentrations, whilst still causing reduced accumulation - albeit not as severe as is apparent at higher $Li^+$ concentrations - the buildup of labelled inositol phosphates, as well as that of CMP-PA, PA and DAG will also be less extreme. Indeed, the lag phase discussed above appears to lengthen at lower $Li^+$ concentrations.

## Inositol reversal studies

Further to these experiments, the ability of myo-inositol to reverse the $Li^+$-induced reduced accumulation of the $Ins(1,4,5)P_3$ was investigated. Table 3.1 indicates that, in the upper panel which involves addition of 10mM myo-inositol prior to the carbachol and $Li^+$ additions, the effects of carbachol at a final concentration of 1mM alone and also in the presence of 1mM $Li^+$, are similar to those discussed earlier (see Fig. 3.4). In the presence of 10mM myo-inositol there is no effect upon accumulation. However, in samples where $Li^+$ was also present, after 20 min., it could be seen that the presence of myo-inositol could prevent the $Li^+$-enhanced decrease in measurable $Ins(1,4,5)P_3$ levels. However, in successive experiments, 10mM myo-inositol was added to samples 20 min. after carbachol and $Li^+$ additions had been made. From former studies, it had been shown that during this initial period $Ins(1,4,5)P_3$ accumulated and plateau'ed at a new steady state before beginning to decline (see Fig. 3.4). In the lower panel of Table 3.1, it can be seen that subsequent addition of myo-inositol is without effect ie. once the effects of $Li^+$ upon $Ins(1,4,5)P_3$ accumulation have manifested, they cannot be reversed by the presence of myo-inositol even in such high concentrations as were used here. By 40 min. after carbachol and $Li^+$ additions, the $Ins(1,4,5)P_3$ measured by radioreceptor ligand binding was essentially identical in samples regardless of the myo-inositol additions.

The most obvious rationale for both $[^3H]Ins(1,4,5)P_3$ and cold $Ins(1,4,5)P_3$ diminishment in the presence of $Li^+$ lies in the $Li^+$ inhibition of the inositol monophosphatase enzyme. Thus, prior addition of myo-inositol can substantially reverse any $Li^+$-induced reduced accumulation. However, that such additions do not reverse this effect when added subsequently to the agonist and $Li^+$ indicates that other intracellular factors play important roles in inositol polyphosphate metabolism. Indeed, in in vivo situations,

<u>Time after carbachol addition (min)</u>

| Inositol<br>(10mM) | Lithium<br>(1mM) | | | |
|---|---|---|---|---|

**(A) prior addition**

| Inositol (10mM) | Lithium (1mM) | 0 | 5 | 20 |
|---|---|---|---|---|
| - | - | 14.9 ± 0.5 | 21.1 ± 0.5 | 20.7 ± 0.3 |
| + | - | 14.5 ± 0.4 | 20.3 ± 0.3 | 20.1 ± 0.3 |
| - | + | 14.9 ± 0.6 | 20.7 ± 0.5 | 16.9 ± 0.6*** |
| + | + | 13.9 ± 0.2 | 20.8 ± 0.6 | 19.1 ± 0.4 ++ |

**(B) subsequent addition**

| Inositol (10mM) | Lithium (1mM) | 0 | 5 | 20 |
|---|---|---|---|---|
| - | - | 22.0 ± 0.6 | 22.1 ± 0.7 | 21.9 ± 0.6 |
| - | + | 22.7 ± 0. 6 | 19.3 ± 0.6** | 17.9 ± 0.5*** |
| + | - | ----- | ----- | 21.2 ± 0.6 |
| - | + | ----- | ----- | 17.8 ± 0.8** |

Table 3.1 - Effect of myo-inositol addition on the action of lithium in carbachol stimulated rat cerebral cortex.
Myo-inositol was added (to a final concentration of 10mM) to incubations 60 min prior to (A) or subsequent to (B) carbachol +/- lithium addition. Ins(1,4,5)P$_3$ concentrations are expressed as mean ± S.E. (pmol/mg prot.) for 6 separate determinations. Statistical analysis was performed (by Students t test) and significant differences are shown as **P< 0.02, ***P< 0.005 for the effect of lithium in carbachol stimulated slices and ++P< 0.002 for the effect of myo-inositol addition.
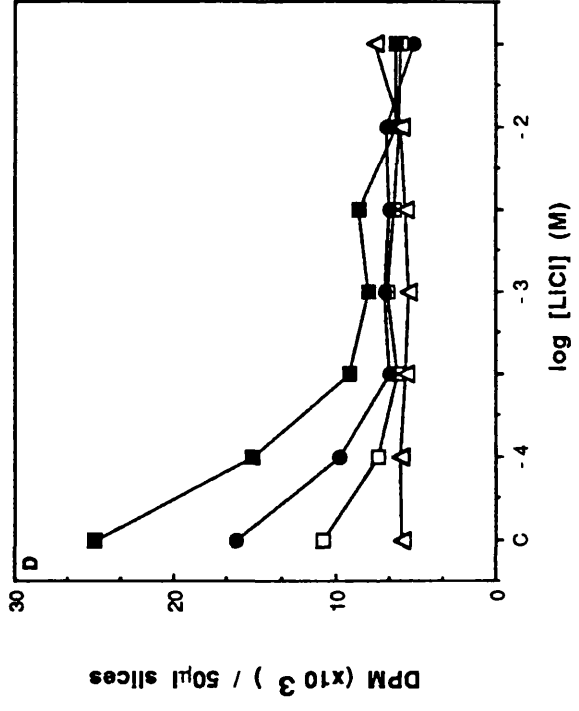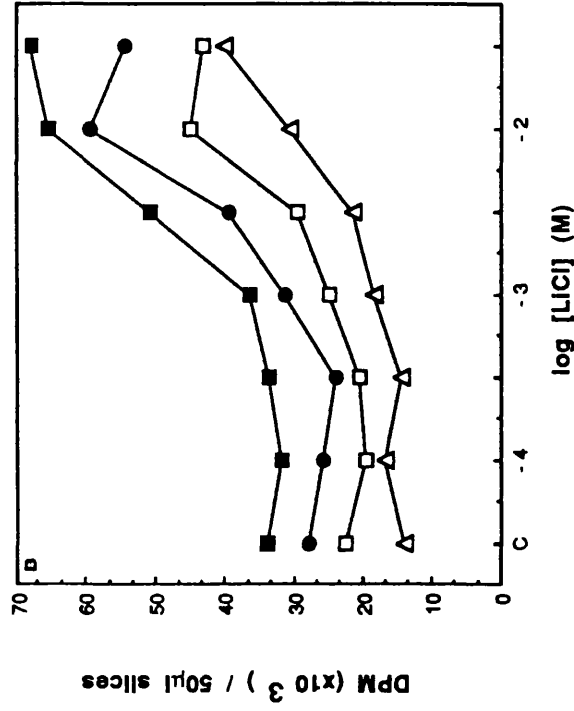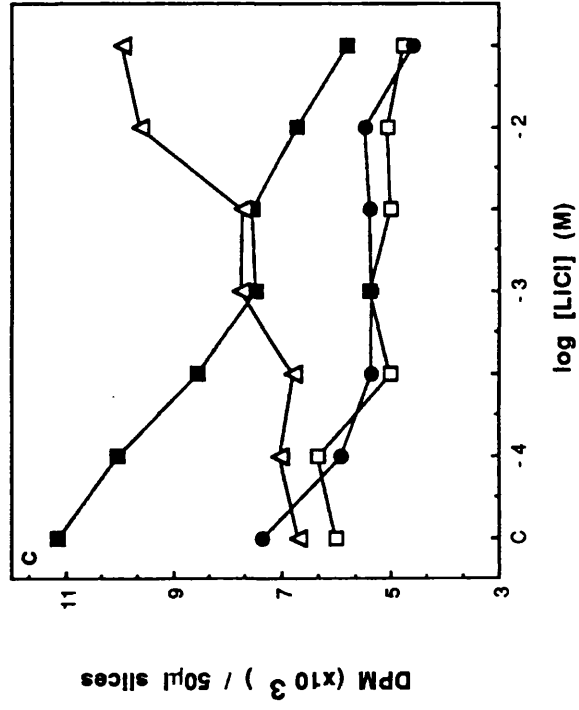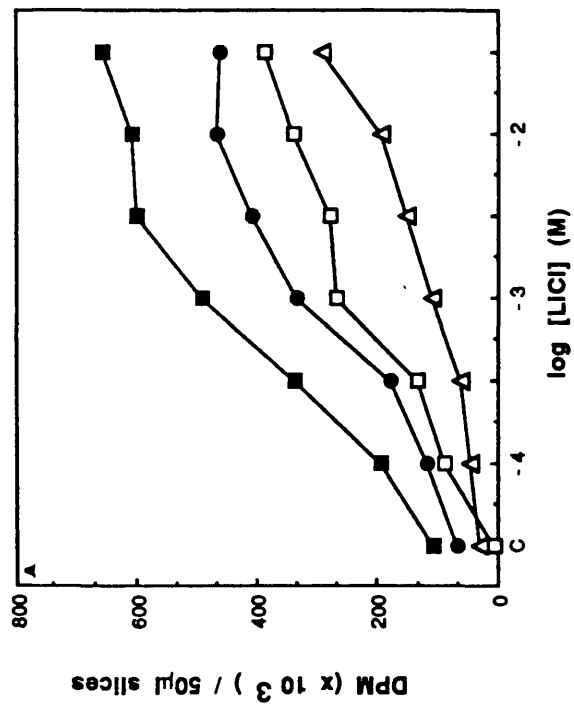
interplay between these two possibilities may occur such that $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$, the important $Ca^{2+}$-mobilizing second messengers, are degraded rapidly due to <u>both</u> decreased synthesis <u>and</u> enhanced metabolism.

The results thus far signify that rat cerebral cortical slices stimulated with millimolar carbachol accumulate labelled $InsP_1$, $InsP_2$, $Ins(1,4,5)P_3$ and $InsP_4$. The effect on $Ins(1,4,5)P_3$ has been verified using mass measurement techniques. $Li^+$ enhances the accumulation of both $[^3H]InsP_1$ and $[^3H]InsP_2$ but causes reduced accumulation of both the $InsP_3$ and $[^3H]InsP_4$ fractions. The potential causes of such phenomena have been discussed, as have the effects of <u>myo</u>-inositol addition to a system depleted of free intracellular inositol because of the presence of $Li^+$. However, it is important to note that all the aforementioned experiments have been performed in the presence of millimolar $Li^+$. The uncompetitive nature of the inhibition exerted by $Li^+$ on the inositol monophosphatase enzyme provides a strong stimulus dependency on this agent. Cells which undergo marked agonist stimulation, therefore, will be selectively deprived of this crucial source of inositol as $Li^+$ becomes more and more  effective as the amount of the enzyme.substrate (ES) complex increases. In order to illustrate this better, experiments were performed to investigate the accumulation of the labelled inositol mono-, bis-, tris- and tetrakisphosphate fractions in the presence of submillimolar carbachol concentrations as well as millimolar concentrations of arecoline, a partial mAChR agonist.

<u>Concentration dependent and temporal effects of $Li^+$ after submaximal agonist and partial agonist stimulation</u>

Fig. 3.6(A,B,C and D) indicates the effects of submillimolar carbachol concentrations and maximal partial agonist concentrations on $[^3H]InsP_1$, $[^3H]InsP_2$, $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ in the presence of increasing $Li^+$

Fig. 3.6 - Concentration dependent effects of $Li^+$ on the accumulation of the labelled inositol (poly)phosphate fractions in rat cerebral cortical slices stimulated with carbachol (1mM - ■ ; 0.3mM - ● ; 0.1mM - □ ) or arecoline (1mM - △ ). A - $InsP_1$; B - $InsP_2$; C - $Ins(1,4,5)P_3$; D - $InsP_4$; C - control [+(partial) agonist,-$Li^+$].
Data represents the mean values obtained from 3 separate experiments. S.E.M. have been omitted for clarity.

**Panel A (bottom left):**

DPM ( x 10³ ) / 50 µl slices

800
600
400
200
0

log [LiCl] (M)

-2    -3    -4    C

**Panel B (top left):**

DPM (x10³ ) / 50 µl slices

70
60
50
40
30
20
10
0

log [LiCl] (M)

-2    -3    -4    C

**Panel C (bottom right):**

DPM (x10³ ) / 50 µl slices

11
9
7
5
3

log [LiCl] (M)

-2    -3    -4    C

**Panel D (top right):**

DPM (x10³ ) / 50 µl slices

30
20
10
0

log [LiCl] (M)

-2    -3    -4    C

concentrations. The approximate $EC_{50}$ values for each of the agonist and partial agonist concentrations for [$^3$H]InsP1 accumulation are as follows:-

1.0mM carbachol = 0.59mm, 0.3mM carbachol= 1.14mM, 0.1mM carbachol= 1.33mM. Because the arecoline-stimulated accumulation of [$^3$H]InsP$_1$ is not maximal after 30 min. an $EC_{50}$ cannot be determined. It would appear, therefore, that the $EC_{50}$ values for [$^3$H]InsP$_1$ accumulation are not greatly affected in the presence of decreasing carbachol concentrations. These experiments, which were all carried out on rat cerebral cortical slice preparations that had been stimulated for 30 min., gave virtually identical results to experiments in which the stimulation period was reduced to 5 min. (data not shown).

Similarly, Fig. 3.6B indicating the apparent accumulation of [$^3$H]InsP$_2$ shows that the $EC_{50}$ values do not alter in the presence of different agonist or partial agonist concentrations. In this case, the values ranged from 4mM to 7mM in the presence of decreasing carbachol concentrations. Again, however, no value can be calculated for the accumulation in the presence of 1.0mM arecoline.

Fig. 3.6C illustrates that Li$^+$ in increasing concentrations causes a decrease in the levels of [$^3$H]Ins(1,4,5)P$_3$ in carbachol stimulated rat cerebral cortical slices. The accumulation of [$^3$H]Ins(1,4,5)P$_3$ in the presence of submillimolar carbachol concentrations is, however, not significant. Roughly speaking, both 0.1mM and 0.3mM carbachol would appear to have no major effect on [$^3$H]Ins(1,4,5)P$_3$ accumulation in the presence of Li$^+$ with 0.3mM carbachol being sufficient to cause slight reduced accumulation compared to control values. 0.1mM carbachol, on the other hand, was not. Similarly, at higher concentrations of Li$^+$, [$^3$H]Ins(1,4,5)P$_3$, in the presence of 0.3mM carbachol may be declining compared to the accumulation in the presence of 0.1mM carbachol but, because these experiments are only representative of n=2, these results can only serve as an indication of what may be occurring. If this was the case, the stimulus dependence of Li$^+$ would be evident with 0.1mM carbachol being much less efficient at eliciting

reduced accumulation of $[^3H]Ins(1,4,5)P_3$ than 0.3mM carbachol and, similarly, both these agonist concentrations would be much less efficient than 1.0mM carbachol, which causes statistically significant reductions in the levels of $[^3H]Ins(1,4,5)P_3$. Arecoline (1.0mM) appears to cause more accumulation of $[^3H]Ins(1,4,5)P_3$ at submillimolar $Li^+$ concentrations than 0.1mM and 0.3mM carbachol whilst at higher concentrations appears to <u>enhance</u> the $[^3H]Ins(1,4,5)P_3$ accumulation.

This increase at very high $Li^+$ concentrations is evident also in Fig. 3.6D which illustrates the effects of carbachol and arecoline on $[^3H]InsP_4$ accumulation in the presence of increasing concentrations of $Li^+$. The effects of carbachol exhibit a strong reliance on the prevailing agonist concentration as displayed in the $[^3H]InsP_1$, $[^3H]InsP_2$ and $[^3H]Ins(1,4,5)P_3$ fractions also. It would appear that the lower the concentration of agonist, the less effect $Li^+$ has upon labelled inositol (poly)phosphate accumulation. This would reinforce the theory that $Li^+$ acts as an uncompetitive inhibitor of (poly)phosphoinositide metabolism at the level of the inositol monophosphatase. However, the effects of arecoline are confusing. The lower levels of $[^3H]InsP_1$ and $[^3H]InsP_2$ that accumulated in the presence of increasing concentrations of $Li^+$ are in keeping with arecoline's partial mAChR agonist status. Its apparent inability to affect either $[^3H]Ins(1,4,5)P_3$ or $[^3H]InsP_4$ at concentrations of $Li^+$ which are therapeutically relevant indicates that the synthesis of these two fractions are affected in the presence of $Li^+$.

These results are reiterated in Fig. 3.7 (A,B,C and D). Both the $[^3H]InsP_1$ and $[^3H]InsP_2$ levels follow the expected pattern of accumulation. $[^3H]InsP_1$ levels are effectively linear with accumulation decreasing correspondingly with decreasing agonist concentration, The accumulation is lowest in the presence of 1.0mM arecoline. Similarly, $[^3H]InsP_2$ accumulates rapidly over the first 5 min. following (partial) agonist and $Li^+$ additions, before reaching a plateau at a new steady state of production and metabolism.

Fig. 3.7 - Time dependent effects of $Li^+$ on the accumulation of the labelled inositol (poly)phosphate fractions in rat cerebral cortical slices stimulated with carbachol
(1mM - ■ ; 0.3mM - ● ; 0.1mM - □ ) or arecoline (1mM - △ ) in the presence of 10mM $Li^+$.
A - $InsP_1$; B - $InsP_2$; C - $Ins(1,4,5)P_3$; D - $InsP_4$; C - control [+(partial) agonist,-$Li^+$].
Data represents the mean values obtained from 3 separate experiments. S.E.M. have been omitted for clarity.
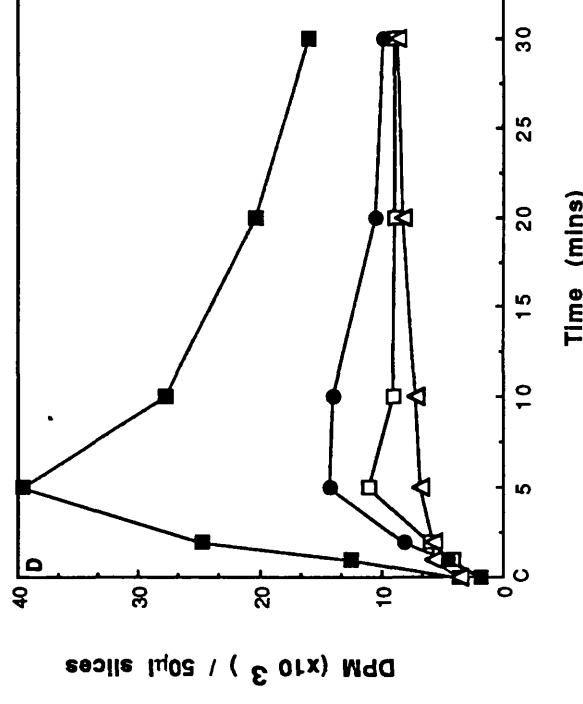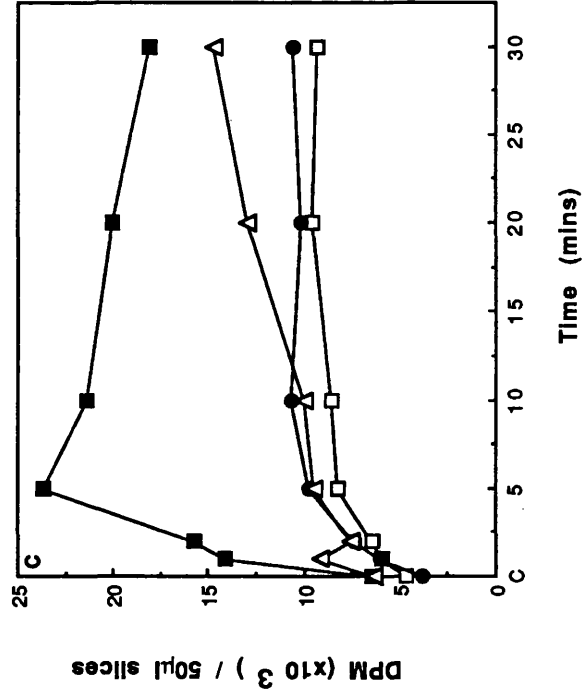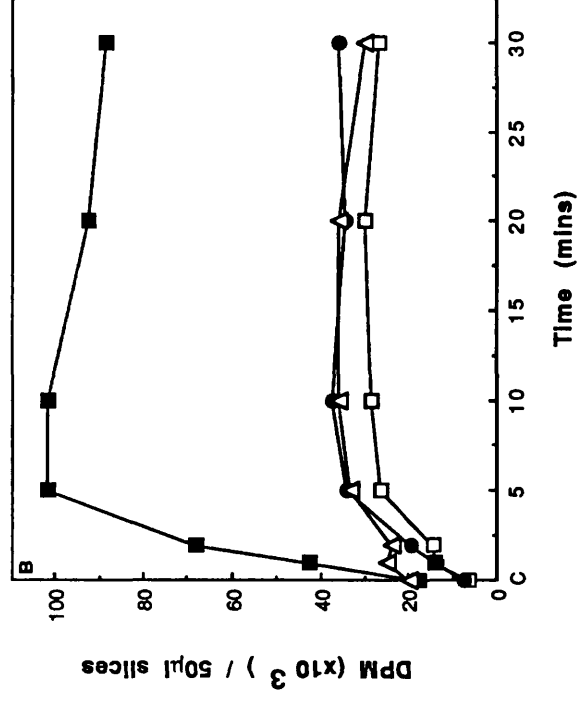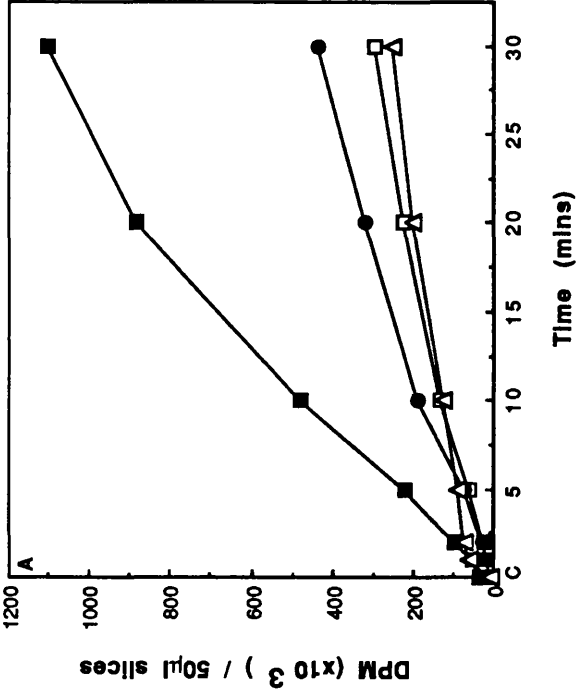
Again, the degree of accumulation matches closely the prevailing agonist or partial agonist concentration. Whilst Li$^+$ causes reduced accumulation of [$^3$H]Ins(1,4,5)P$_3$ and [$^3$H]InsP$_4$ in the presence of millimolar carbachol as before, the effect of arecoline on both of these fractions is unclear. With time, arecoline effect upon [$^3$H]InsP$_4$ accumulation. Comparison of this with the Li$^+$ concentration dependence data observed in Fig. 3.6D may indicate that arecoline, unlike carbachol, does not utilize the 3-kinase pathway and, therefore, causes no [$^3$H]InsP$_4$ accumulation in the presence of Li$^+$. The exception to this is at very high Li$^+$ concentrations which, instead of causing the expected reduced accumulation, causes enhanced accumulation. This is in agreement with the data obtained from the accumulation of [$^3$H]Ins(1,4,5)P$_3$. This also does not exhibit reduced accumulation with time which, again, is in contrast to the effects reported in the presence of carbachol.

The data presented in Figs. 3.6 and 3.7 is inconclusive. It remains possible that Li$^+$ causes enhanced metabolism of the [$^3$H]Ins(1,4,5)P$_3$ and [$^3$H]InsP$_4$ fractions only in situations in which there is prolonged chronic stimulation and by a mechanism which has yet to be determined. Lower carbachol concentrations cause similar effects though not as pronounced. Arecoline may shift inositol (poly)phosphate metabolism via the 5'-phosphomonoesterase in the presence of Li$^+$. This would account for the accumulation of [$^3$H]Ins(1,4,5)P$_3$ as it only has one metabolic outlet in this hypothesis and the lack of detectable [$^3$H]InsP$_4$. Theoretically, therefore, because carbachol stimulation in the presence of Li$^+$ encourages [$^3$H]Ins(1,4,5)P$_3$ metabolism through the 3-kinase and 5-phosphomonoesterase -mediated routes, effects on both the [$^3$H]Ins(1,4,5)P$_3$ and [$^3$H]InsP$_4$ fractions should be detectable. This, indeed, is the case. However, the reason why arecoline should cause this modification in inositol (poly)phosphate metabolism is unclear. It is possible that, by some undefined agonist-induced mechanism, PtdIns and PtdIns(4)P are preferentially

hydrolyzed perhaps via stimulation of a different PLC isoenzyme or that intracellular $Ca^{2+}$ mobilization may be sufficient to overcome the requirements of the partial mAChR agonist without subsequent extracellular $Ca^{2+}$ influx through $Ins(1,4,5)P_3$ synergy with $InsP_4$. These effects would account for the observation that little $[^3H]Ins(1,4,5)P_3$ and virtually no $[^3H]InsP_4$ are detected under the conditions described above. The effects of $Li^+$ within this situation remain uncertain and, because these experiments represent data points obtained from only one or two experiments ,little emphasis should be placed on them.

This section has established, however, that $Li^+$ has extensive effects on the metabolism of carbachol stimulated inositol (poly)phosphate metabolism in rat cerebral cortical slices. Differences between the data presented here and that of other workers (Batty and Nahorski, 1987; Whitworth and Kendall, 1988; Rooney and Nahorski, 1989) may be due to technical reasons such as changes in specific radioactivity, the preparation and handling of the tissue and the enzymic $[^3H]InsP_3$ separation technique for example. However, what has been confirmed is that $Li^+$ is a potent inhibitor of the production of free inositol. In an attempt to investigate the block exerted by $Li^+$ on the inositol monophosphatase enzyme, work was carried out to study the sensitivity of CMP-PA accumulation to the presence of $Li^+$ because this precursor molecule combines with inositol to allow PtdIns lipid resynthesis. This will be discussed in detail in the second part of this chapter.

# C H A P T E R  3.2

Lithium and its Effects on CMP-PA Accumulation in Carbachol Stimulated Rat
Cerebral Cortical Slices

CHAPTER 3.2

LITHIUM AND ITS EFFECTS ON CMP-PA ACCUMULATION IN CARBACHOL STIMULATED RAT
CEREBRAL CORTICAL SLICES.

Introduction

In Chapter 3.1, it was established that $Li^+$ in both increasing

concentrations and with increasing time has extensive and profound effects

upon the accumulation of $[^3H]$inositol mono-, bis-, (1,4,5)tris- and tetrakis-

phosphate fractions in rat cerebral cortical slices stimulated with

carbachol. In the absence of $Li^+$, inositol combines with CMP-phosphatidic

acid (CMP-PA) to effect PtdIns resynthesis. Theoretically, therefore, in the

presence of $Li^+$, as the cell becomes depleted of myo-inositol, CMP-PA levels

should increase. However, quite how much DAG undergoes conversion back into

PtdIns via the CMP-PA intermediary is unknown. 1,2-DAG can be incorporated

into the pathway of synthesis of other lipids or diglycerides for example.

Nevertheless, the $K_m$ for inositol that the CMP-PA: myo-inositol transferase

has, at least in liver, is relatively high ($K_m$ 2.5mM, Takenawa and Egawa,

1977) and, as a result, the accumulation of CMP-PA in the presence of $Li^+$

should be a sensitive indicator of inositol depletion.

CMP-PA levels have been shown previously to accumulate in the absence of

$Li^+$ in pancreatic islets stimulated with glucose (Frienkel et al. 1975). In

the presence of $Li^+$, however, the resulting rise in the levels of

intracellular CMP-PA is dramatic, as shown in both rat parotid gland (Downes

and Stone, 1986) and rat cerebral cortex (Godfrey, 1989).

In this section measurements of inositol CMP-PA in stimulated rat

cerebral cortical slices in the presence and absence of $Li^+$ are made in
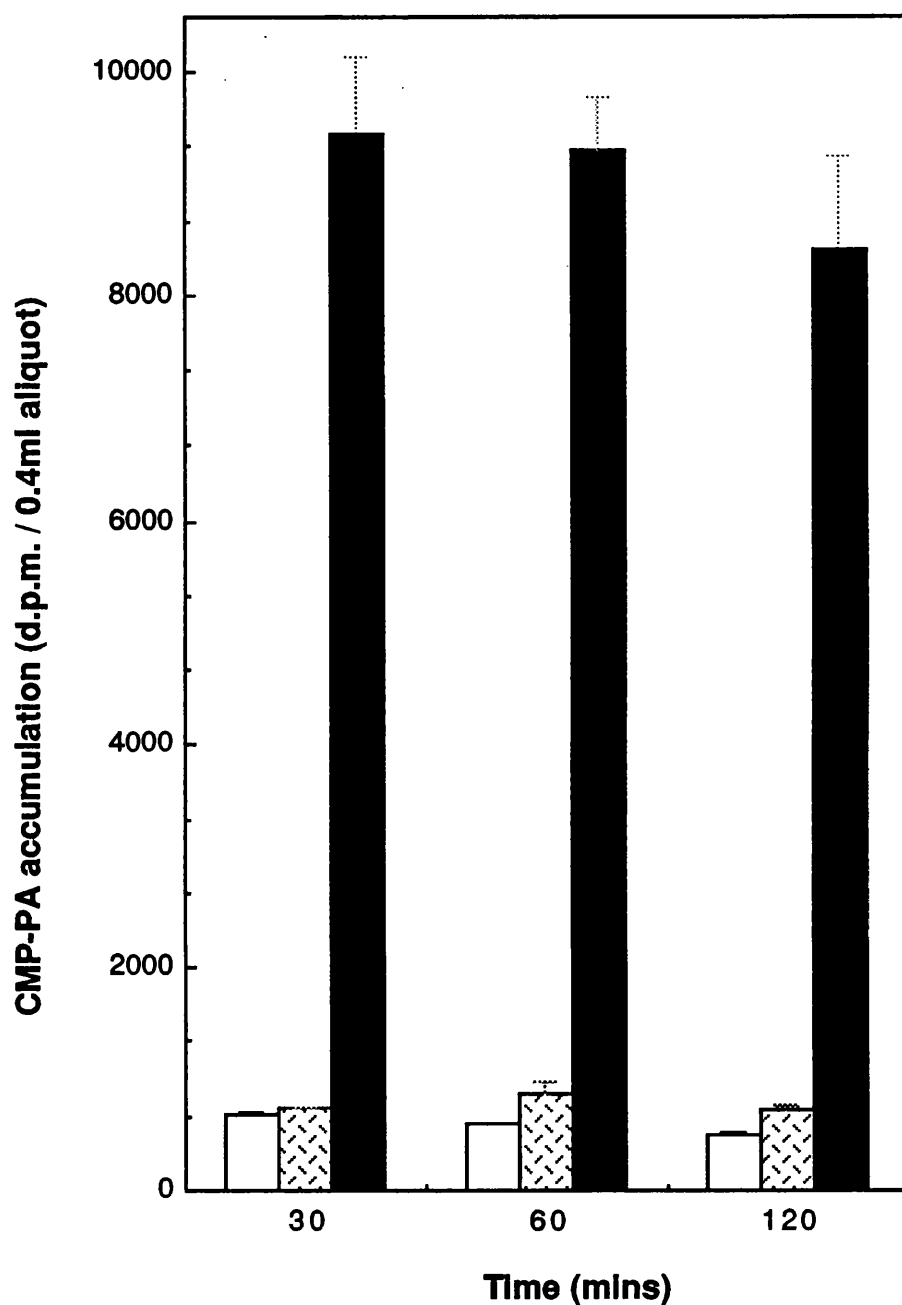
Fig. 3.8 - Effect of the length of the labelling period upon the accumulation of [$^{14}$C] CMP-PA.

Rat cerebral cortical slices were preincubated and subsequently labelled for either 30, 60 or 120 min. with $^{14}$C-cytidine (0.2μCi/vial). 1mM carbachol ± 5mM lithium were then added for a further 30 min. before reactions were terminated and the [$^{14}$C] CMP-PA extracted. Data represents the mean ± S.E.M. for 2 separate experiments, each performed in triplicate.

☐ = -CCH; -Li $^+$    ▨ = +CCH; -Li $^+$    ■ = +CCH; +Li $^+$
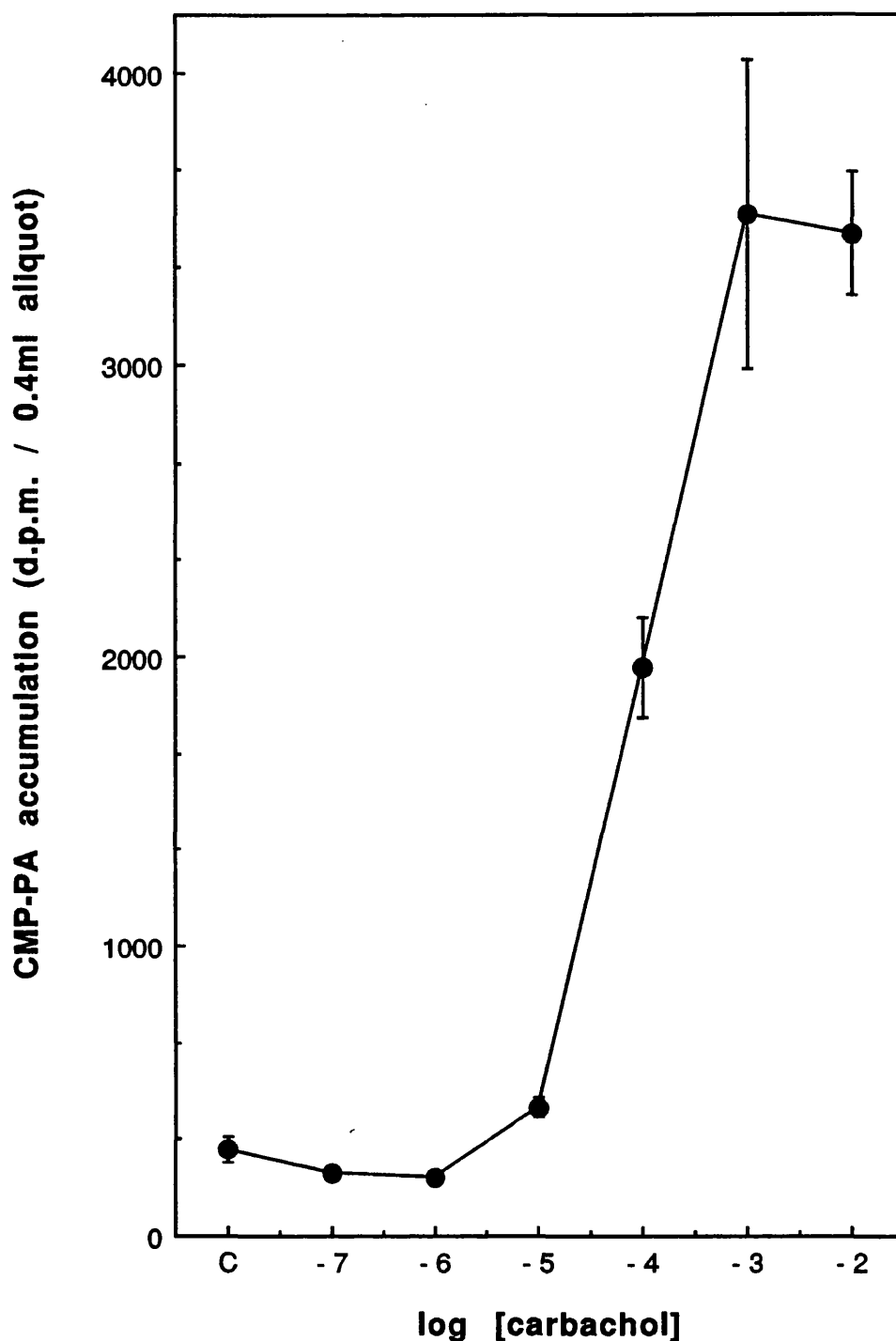
Fig. 3.9 - Concentration dependent effect of carbachol on the accumulation of [$^{14}$C] CMP-PA in rat cerebral cortical slices.

50μl aliquots of tissue labelled with 0.1μCi/vial [$^{14}$C]-cytidine were stimulated with various concentrations of carbachol in the presence of lithium (1mM) for 30 min. before reactions were terminated and the [$^{14}$C] CMP-PA extracted. Data represents the mean ± S.E.M. for 2 separate determinations, each performed in triplicate.
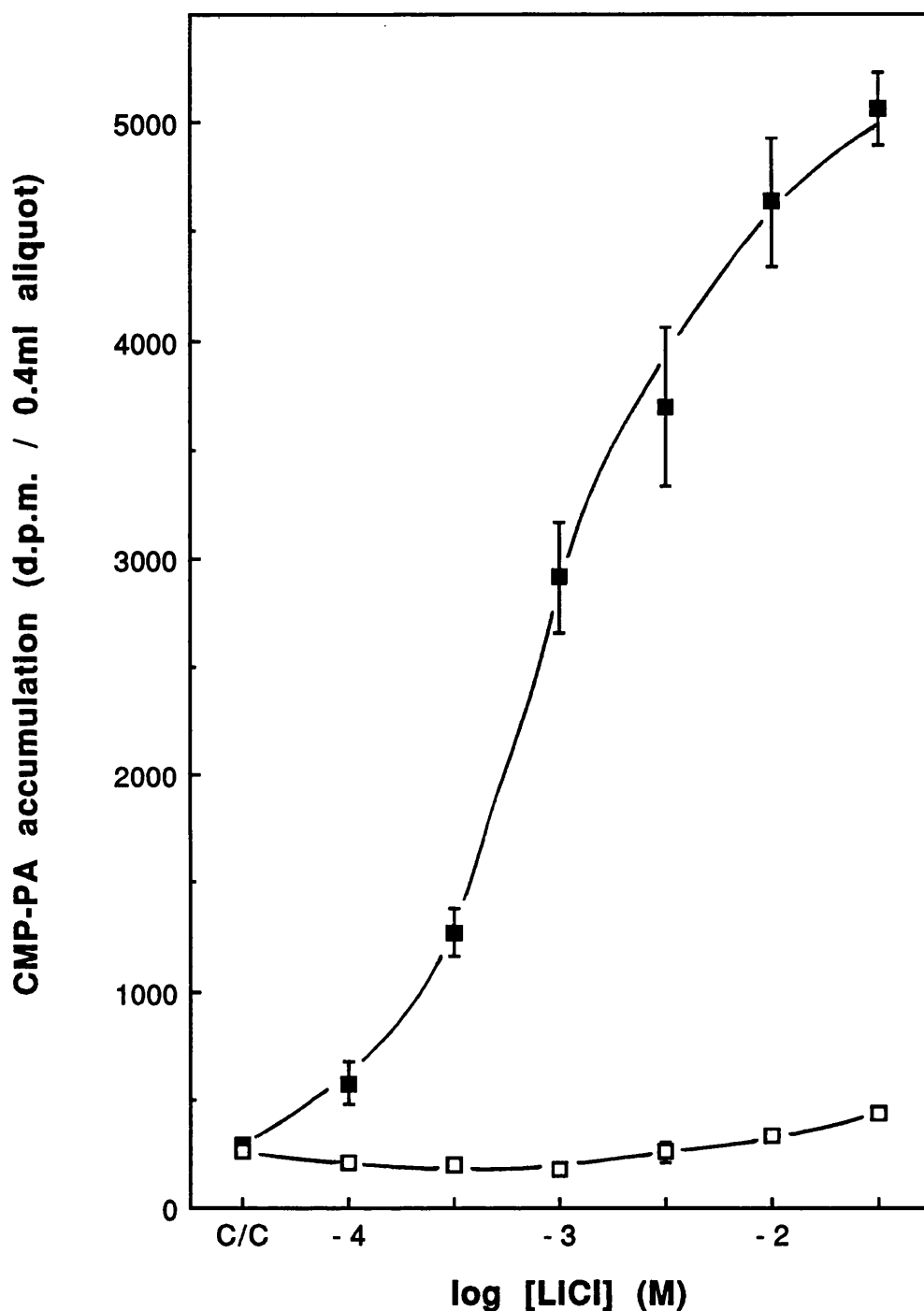
Fig. 3.10 - Concentration dependent effect of lithium on the accumulation of [$^{14}$C] CMP-PA In rat cerebral cortical slices.

Slices were incubated in the presence of lithium ( □ ) or lithium plus carbachol ( ■ ) for 30 min. Data represents the mean ± S.E.M. for at least 6 separate determinations, each performed in triplicate. Addition of 1mM carbachol in the absence of lithium for 30 min. increased CMP-PA accumulation by 58% (basal = 229 ± 11; 1mM CCH = 362 ± 16 dpm / 0.4ml aliquot.
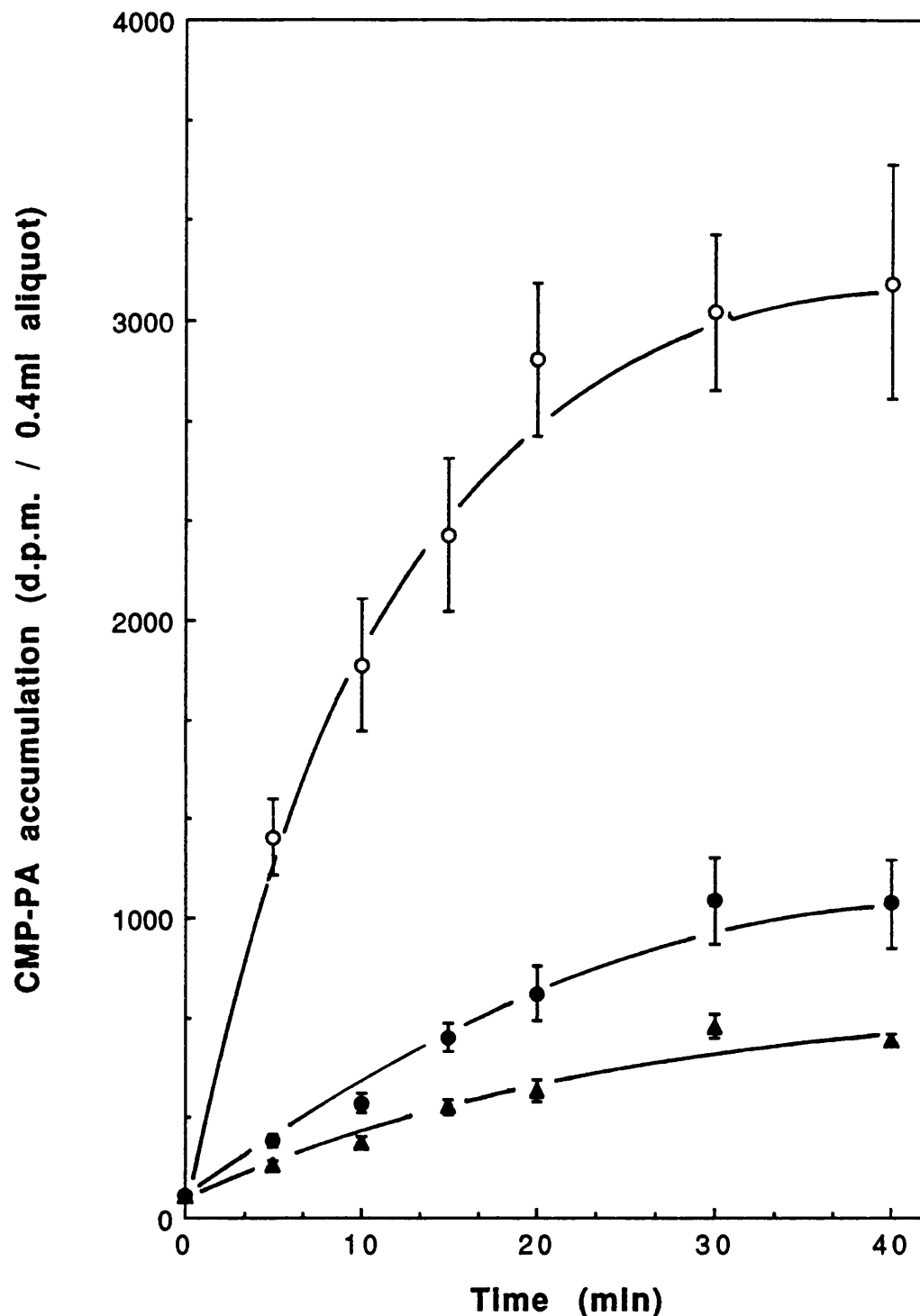
Fig. 3.11 - Effect of myo-inositol on the time courses of accumulation of [$^{14}$C] CMP-PA in the presence of lithium and carbachol in cerebral cortical slices. Lithium (1mM) and carbachol (1mM) were added at t=0 after preincubation of the slices for 60 min. in the absence (O) or presence of 10mM (●) or 30mM (▲) myo inositol. Data represents the mean ± S.E.M. for at least 3 separate determinations each performed in triplicate. The presence of myo-inositol (10 or 30mM) has no effect on basal CMP-PA accumulation at t=0 but significantly attenuated the increase in accumulation subsequent to lithium and carbachol additions (P < 0.01 for all data points, Students t test)

parallel with mass measurements of both the Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$ fractions. The ability of exogenously added myo and scyllo-inositol to reverse these observed effects are also examined. The materials and methods used in the following experiments are detailed in Chapter 2.1.

## Results and Discussion

These investigations involve [$^{14}$C]CMP-PA determinations from rat cerebral cortical slices which had been labelled for 60 min. in the presence of [$^{14}$C]cytidine. Fig. 3.8 displays data from experiments carried out to observe the optimal labelling period. There appears to be very little difference between [$^{14}$C]CMP-PA accumulation in slices labelled for 30, 60 or 120 min. in the presence of both carbachol and Li$^+$. Likewise this figure also shows the dramatic effect of accumulation of this labelled lipid precursor in the presence of Li$^+$ compared to the accumulation which occurs in the presence of carbachol alone. In view of the proposed kinetic studies, a 60 min. labelling period was adopted. This was the period of preincubation with label during other experiments involving [$^3$H]inositol phosphate extraction from rat cerebral cortical slices so differences in the handling of the slice preparations were minimalized. Furthermore, a carbachol concentration dependence curve illustrated that [$^{14}$C]CMP-PA accumulation was maximal at concentrations of 1mM and above with half maximal accumulation occurring at 0.27 ± 0.01 mM. For this reason 1mM carbachol was routinely used in the following experiments (see Fig. 3.9).

Fig. 3.10 illustrates that [$^{14}$C]CMP-PA accumulation in a dose-dependent manner in the presence of 1mM carbachol and increasing concentrations of Li$^+$. This process has an EC$_{50}$ value of 0.61 ± 0.19mM. In the absence of carbachol, Li$^+$ is seen to have no direct effect on [$^{14}$C]CMP-PA accumulation

except at supraphysiological concentrations where the levels drift very slightly upwards. In the absence of $Li^+$, carbachol is seen to effect less than a 2-fold increase in the levels of $[^{14}C]CMP-PA$. The $EC_{50}$ value observed here is similar to that quoted earlier for $Li^+$ induced increases in $[^3H]InsP_1$. The concomitant increases in these two inositol lipid precursors indicates their intracellular relationship and highlights the accumulation of CMP-PA as a sensitive indicator of inositol depletion by $Li^+$.

When the time-course of this accumulation was observed, in the presence of millimolar $Li^+$, $[^{14}C]CMP-PA$ levels increase immediately with no apparent lag phase (Fig. 3.11). However, this accumulation does appear to plateau gradually after 20 min. indicating that a new steady state of production and metabolism has been reached. This accumulation is, therefore, much slower than the rate at which $[^3H]InsP_1$ accumulates, and although this may be due to differences in the rate of labelling, it may also be indicative of $[^{14}C]CMP-PA$ being re-routed into the de novo synthesis of other phosphoglycerides. For example, CMP-PA can react with the hydroxyl group of a polar alcohol like serine to effect phosphatidylserine synthesis although how likely it is that the cell would respond to situations of chronic mAChR stimulation by expending energy on the synthesis of other, non(poly)phosphoinositide lipids is unknown. Similarly, the CMP-PA precursor, DAG, can, as an alternative to accumulation, circumvent this synthetic stage and become involved with triacylglycerol synthesis for example. There is also the possibility that under conditions of chronic mAChR stimulation, the presence of $Li^+$ causes a shift in DAG metabolism away from a DAG kinase-mediated pathway towards a DAG-lipase interaction. This enzyme causes extensive deacylation of DAG providing arachidonate which is, in turn, important in the generation of bioactive metabolites like prostaglandins, thromboxanes and leukotrienes (Rittenhouse and Simmons, 1981; Irvine, 1982; Lenstra and Mauco, 1984). It is obvious, therefore, that the potential uses for both DAG and CMP-PA are greater than those known for

$InsP_1$. This latter molecule, which accumulates linearly in the presence of $Li^+$, has only one apparent metabolic outlet i.e. via the monophosphatase enzyme. However, in the presence of $Li^+$, if it is assumed that both CMP-PA and DAG, by some negative feedback mechanism which prevents further accumulation of CMP-PA, accumulate, linear accumulation may be prevented by their accommodation into other pathways. It is salient also, that if DAG accumulation does occur as a result of $Li^+$ inhibition of the inositol monophosphatase enzyme, PKC activation will be increased correspondingly and may be important in preventing further CMP-PA accumulation by enhancing its metabolism.

## Temporal effects of exogenously added myo-inositol

Fig. 3.11 also demonstrates the effects on $[^{14}C]$CMP-PA levels in the presence of exogenous, unlabelled myo-inositol. In agreement with Godfrey (1989), these results demonstrate that $[^{14}C]$CMP-PA accumulation can be severely attenuated by the prior incubation of the slices with myo-inositol. If myo-inositol was added prior to the addition of carbachol and $Li^+$ at a final concentration of 10mM, its presence could evoke a partial inhibition of $[^{14}C]$CMP-PA. This reduction was calculated from several separate determinations to be between 65% and 70%. Similarly, the presence of 30mM myo-inositol during the 60 min. labelling period with $[^{14}C]$cytidine further increased the extent of this prevention of $[^{14}C]$CMP-PA accumulation. Whilst the presence of such high concentrations of myo-inositol extracellularly could effect over 80% inhibition of accumulation, it is interesting to note that, even when present at such concentrations, there is not sufficient free inositol available in the cell to the CMP-PA to overcome completely the effects of the $Li^+$ blockade. In this case an accumulation representative of approximately 20% of that observed in the absence of a preincubation period with myo-inositol occurs. Inositol can be taken up by brain slices by a

saturable transport mechanism (Spector, 1975). However, because reversal of $[^{14}C]$CMP-PA accumulation is not complete even at external concentrations of up to 30mM myo-inositol, it could be suggested that, in this in vitro preparation, this process may not be particularly efficient. It should be pointed out, however, that although poor efficacy of inositol transport may be the cause of the lack of complete reversal of $[^{14}C]$CMP-PA accumulation by myo-inositol, there may be additional factors which must be taken into consideration. One of the most important of these is the difficulty which may exist in 're-loading' the cerebral cortical slices with inositol. During the preparation of the slices and the subsequent washing stages described in Chapter 2.1, a significant proportion of the unbound, intracellular inositol is lost. Sherman et al. (1986) have reported that brain slices which have undergone such preincubation lose up to 80% of their original inositol. Furthermore, 0.1mM myo-inositol in the medium did not appear to be sufficient to restore this deficit but these authors found that 10mM myo-inositol was enough to restore levels of inositol to those found in fresh tissue. This fact may explain the high myo-inositol requirement to overcome the $Li^+$ blockade. It may also indicate why in rat parotid gland, Downes and Stone (1986) noted a 15 min. lag period before $Li^+$ caused an elevation in detectable levels of CMP-PA. Whilst this may be indicative of larger or more accessible pools of inositol within the tissue, it may also imply that in the preparation of the cerebral cortical slices used here more of these pools were lost than in comparable preparations of the parotid gland slices (Downes and Stone, 1986) or, indeed, the rat cerebral cortical slices preparation used by Sherman et al. (1986) in which 10mM myo-inositol was sufficient to replenish the intracellular inositol pools.

Having established in Fig. 3.11 that $[^{14}C]$CMP-PA accumulation is roughly linear over the 20 min. period following agonist and $Li^+$ additions, experiments were carried out to determine the effects of subsequent addition

of 10mM and 30mM myo-inositol on $[^{14}C]CMP$-PA accumulation (Fig. 3.12) when added at 20 min. for a further 30 min. 10mM myo-inositol can virtually prohibit any further accumulation of $[^{14}C]CMP$-PA but noticeably not reverse the process. Addition of 30mM myo-inositol after 20 min. however, could reduce the $[^{14}C]CMP$-PA levels significantly towards basal accumulation values obtained in the absence of either carbachol or $Li^+$. Even so, at concentrations as high as 30mM, this reversal by myo-inositol was not quite complete after 30 min. with accumulation still almost two-fold above the levels of $[^{14}C]CMP$-PA seen in the absence of either carbachol or $Li^+$ at this time. Interestingly, however, the addition of 10mM scyllo-inositol appeared to have absolutely no effect upon accumulation of $[^{14}C]CMP$-PA. The levels of the labelled lipid continued to increase with time in the presence of carbachol and $Li^+$ with values which, in the presence of 10mM scyllo-inositol also, were not significantly different from those in which no inositol additions had been made. Control values remained constant throughout the 50 min. experimental period. The addition of myo-inositol (10mM or 30mM) to control samples which contained neither carbachol nor $Li^+$ led to slight reductions in the levels of detectable $[^{14}C]CMP$-PA present, approximately halving the very low basal figures. Control samples at 60 min. in the absence of carbachol, $Li^+$ and inositol had an average count of 168 ± 14 DPM/0.4ml aliquot which was further reduced to 84 ± 6 DPM/0.4ml aliquot in the presence of 10mM myo-inositol and 62 ± 3 DPM/0.4ml aliquot in the presence of 30mM myo-inositol. These results indicate that whilst myo-inositol can prevent or reverse accumulation of CMP-PA, the naturally occuring scyllo-isomer cannot even though it can gain access to the cell by the same transport mechanism as the myo-inositol. Therefore, unlike myo-inositol, scyllo-inositol cannot be utilised as the head-group molecule during inositol lipid synthesis. Moreover, that myo-inositol does not fully reverse $[^{14}C]CMP$-PA accumulation may infer more than one site of action of $Li^+$. If $Li^+$ only inhibited the monophosphatase enzyme then 10mM-30mM
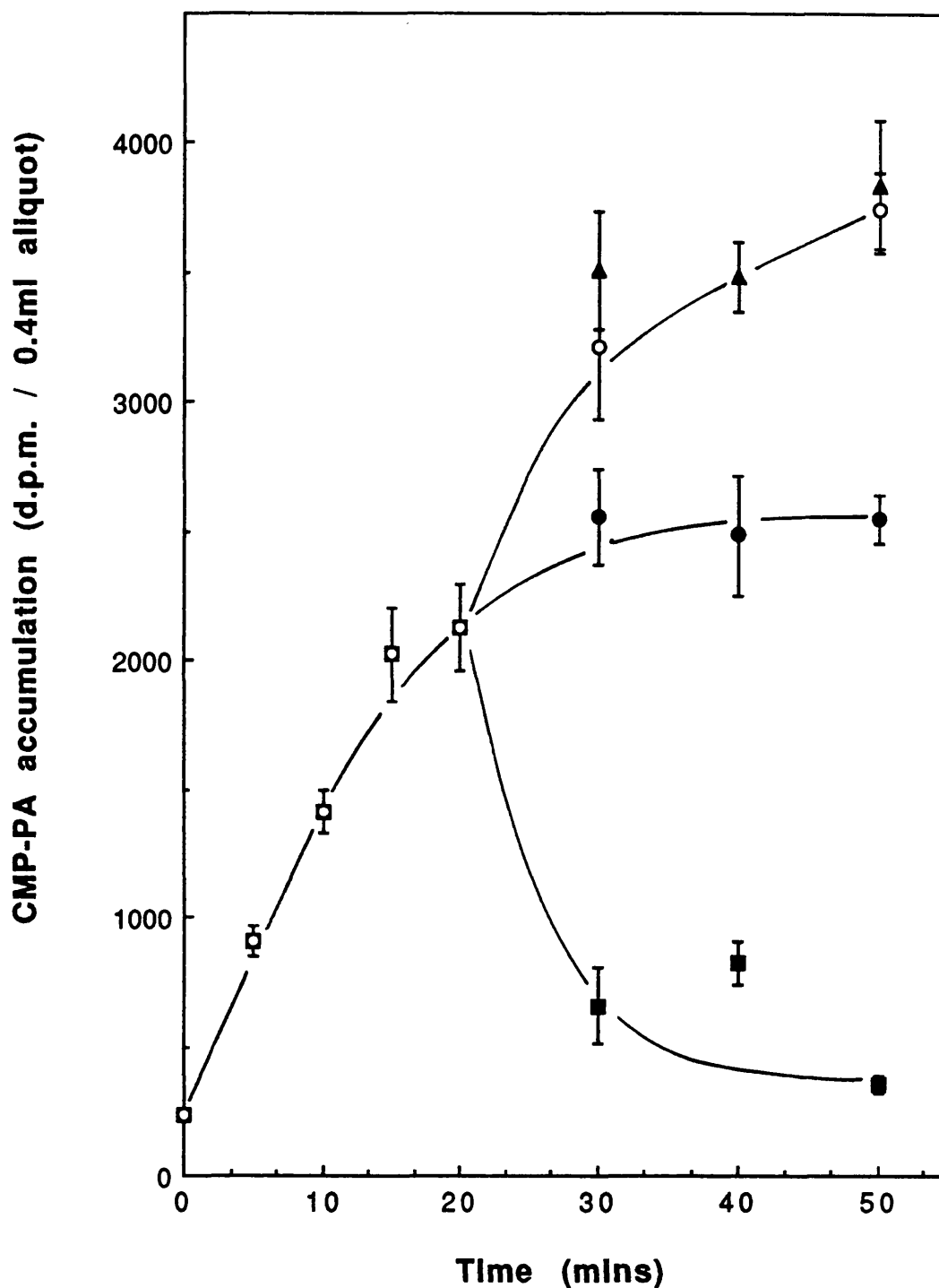
Fig. 3.12 - Effects of subsequent addition of myo- or scyllo-inositol on the accumulation of [14 C]CMP-PA in the presence of lithium and carbachol in cerebral cortical slices. Lithium (1mM) and carbachol (1mM) were added at t=0. After 20 min., final concentrations of 10mM ( ● ) or 30mM ( ■ ) myo-inositol or 10mM scyllo-inositol ( ▲ ) were added. Data represents the mean ± S.E.M. for at least 3 separate determinations, each performed in triplicate. The addition of myo-inositol (10 or 30 mM) significantly attenuated the increase in accumulation of labelled CMP-PA (P< 0.05 for data points; Student's t test); scyllo-inositol addition did not significantly affect accumulation.

myo-inositol should be sufficient to reverse completely and rapidly any $[^{14}C]CMP-PA$ accumulation upon its addition. It remains possible therefore that whilst this is a site of action of $Li^+$, there may be another site of action which is not reversible by the addition of myo-inositol e.g. covalent modification of one of the enzymes involved in inositol (poly)phosphate metabolism.

## Effects of exogenous inositol additions upon Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$

In addition to the experiments described above, further experiments were carried out in conjunction to these to observe the ability of $Li^+$ to reduce Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$ in rat cerebral cortical slices stimulated with carbachol as above, using the mass measurement techniques discussed in Chapter 2.1. In Fig. 3.13A the capacity which $Li^+$, at a concentration of just 1mM, has to reduce Ins(1,4,5)$P_3$ levels in cholinergically stimulated brain slices is seen. These results agree closely with those displayed in Table 3.1 (upper panel) which represents prior addition of 10mM myo-inositol in a variety of different situations. In all samples regardless of the additions made at t=0, large increases in the levels of detectable Ins(1,4,5)$P_3$ are evident over the first 5 min. of the experiment. These levels indicate a 40%-50% increase in the accumulation of Ins(1,4,5)$P_3$ following mAChR stimulation. In the presence of carbachol alone, the presence of 10mM myo-inositol has no apparent effect on Ins(1,4,5)$P_3$ levels with carbacahol demonstrating, as in Table 3.1, its ability to cause a sustained Ins(1,4,5)$P_3$ response for at least 20 min. The presence of $Li^+$, however, causes a dramatic reduction in Ins(1,4,5)$P_3$ accumulation effectively back to control values within 15-20 min. This effect had already been noted in Fig. 3.3C which measured $[^3H]Ins(1,4,5)P_3$ and Table 3.1A which measured unlabelled Ins(1,4,5)$P_3$ although due to changes in the specific

Fig. 3.13 - Effects of $Li^+$ and myo-inositol on the time courses of changes in Ins(1,4,5)$P_3$ (A) and Ins(1,3,4,5)$P_4$ (B) concentrations in carbachol-stimulated rat cerebral cortical slices.
Carbachol (1mM) only ( ● , ○ ), or carbachol plus 1mM $Li^+$ ( ■ , □ ) were added at t=0, after preincubation of slices in the absence ( ○ , □ ) or presence ( ● , ■ ) of 10mM myo-inositol for 60 min. Data represents the mean ± S.E.M. for at least 3 separate determinations, each performed in triplicate. Statistically significant attenuating effects of inositol on carbachol-stimulated inositol polyphosphate accumulation by $Li^+$ are indicated as *P< 0.05; **P< 0.01; ***P< 0.001.
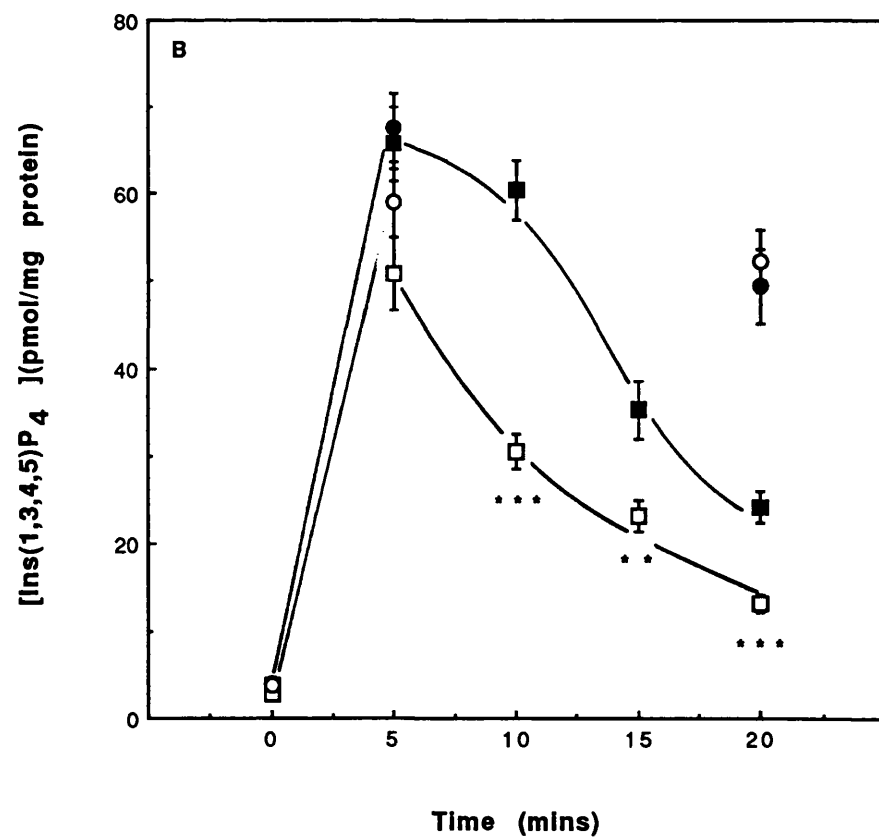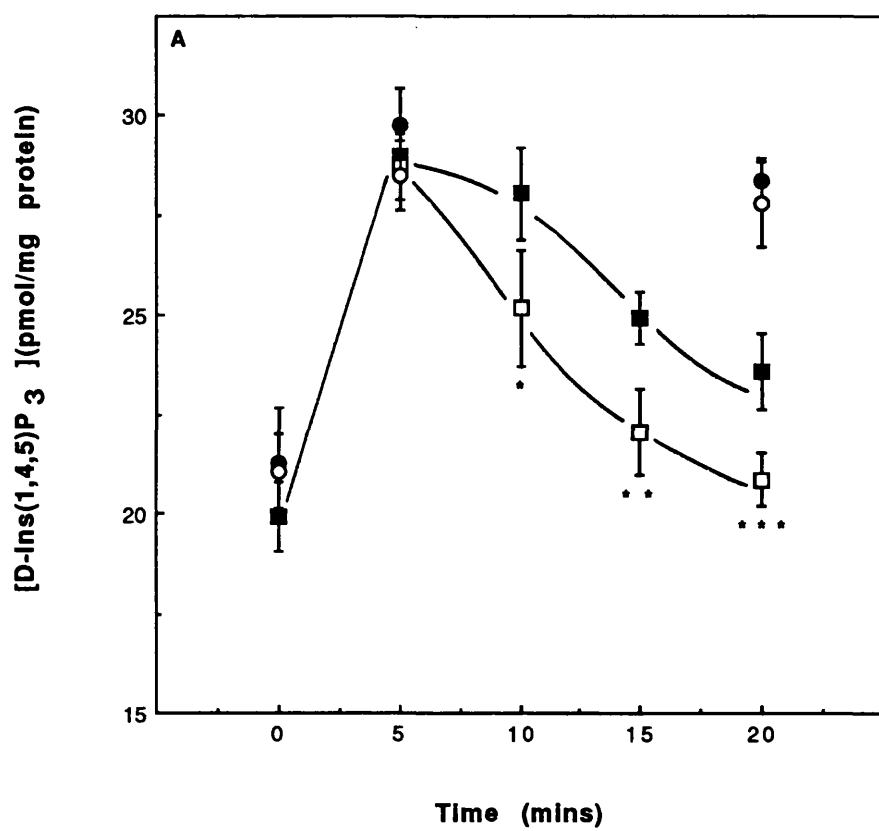
radioactivity, levels of $Ins(1,4,5)P_3$ in the former case did not regain basal values. When the slices had been preincubated with 10mM myo-inositol, however, there appeared to be a delay in the $Li^+$-induced reduced accumulation of $Ins(1,4,5)P_3$ such that by 20 min. levels were approximately 10% greater in the presence of myo-inositol compared to in its absence.

Fig. 3.13B illustrates a similar phenomenon occurring in the levels of $Ins(1,3,4,5)P_4$ as measured by radioreceptor ligand binding techniques. Carbachol, again irrespective of $Li^+$ or inositol additions, causes a 30-40 fold increase in the accumulation of this isomer from very low background levels. Although not so clearly defined as in Fig. 3.13A carbachol, regardless of myo-inositol additions, also causes a sustained stimulation of $Ins(1,3,4,5)P_4$ production. In the presence of millimolar $Li^+$, $Ins(1,3,4,5)P_4$ accumulation is reduced drastically as observed previously in radio-isotopically labelled experiments (see Fig. 3.3D). The prior incubation of the slices with 10mM myo-inositol, however, once more caused a time-dependent delay in the onset of the reduction in the accumulation seen also with the $Ins(1,4,5)P_3$. The attenuation in the $Li^+$ associated decrease in $Ins(1,3,4,5)P_4$ levels is significantly different after only 10 min. Preincubation of the slices with 10mM scyllo-inositol had no effect on preventing the inhibitory effect of $Li^+$ on inositol (poly)phosphate accumulation (data not shown). This is in agreement with Fig. 3.12 in which scyllo-inositol was found to be ineffective in the reversal of $[^{14}C]$CMP-PA accumulation but where myo-inositol could result in a significant reversal of $[^{14}C]$CMP-PA levels in the presence of $Li^+$.

In the attempt to determine whether the decreased agonist-induced accumulation of inositol (poly)phosphates is related to the ability of $Li^+$ to inhibit the inositol monophosphatase and thus remove an important source of inositol for (poly)phosphoinositide synthesis, the results obtained illustrate clearly the protective effect of 10mM myo-inositol upon $Li^+$-induced reduced accumulation of the levels of these two second

messengers. This effect was not substantially improved by preincubation of the slices in the presence of 30mM myo-inositol. The results also highlight both the inability of scyllo-inositol to combine with CMP-PA and effect PtdIns resynthesis and the central role played by myo-inositol in (poly)phosphoinositide lipid signalling.

That the effects described here are real could be open to experimental debate from several angles. For example, the radioactivity appearing in the organic phase of this extraction procedure was merely assumed to be [$^{14}$C]CMP-PA. However, this assumption was made on the basis that the only lipid known to contain cytidine is CMP-PA. Furthermore, under similar circumstances Godfrey (1989) indicated that more than 90% of the radioactivity obtained via this method co-chromatographed identically to appropriate phospholipid standards on oxalate-impregnated t.l.c. plates run with three different solvent systems. Likewise, using the method of Jolles et al. (1981), Downes and Stone (1986) identified [$^3$H]cytidine-containing phospholipids and other [$^{32}$P]labelled phospholipids by t.l.c. suggesting that, in rat parotid gland, also, the only cytidine-containing lipid was, indeed, CMP-PA. The case for assuming that what was measured was actually [$^{14}$C]CMP-PA is, then, strong.

Secondly, other experiments revealed that changes in the ionic strength of the extracellular medium caused by the addition of various concentrations of up to 30mM Li$^+$, did not influence the production of [$^{14}$C]CMP-PA per se (data not shown). These modifications involved altering the final univalent ion concentration in each sample using NaCl to ensure that anomalous results were not being observed simply due to changes in ionic strength.

Finally, the two mass assays described in Chapter 2.1 and utilised in this study, have very low levels of cross-specificity - generally less than 1%. The most likely source of error is probably due to the Ins(1,3,4,5,6)P$_5$. However, the EC$_{50}$ value for this is approximately two orders of magnitude greater than for Ins(1,3,4,5)P$_4$ in the Ins(1,3,4,5)P$_4$ binding assay (Donié

and Reiser, 1989). Therefore, although Ins(1,3,4,5,6)$P_5$ may be present in high concentrations in various tisues, the likelihood of it influencing the results obtained from acutely labelled rat cerebral cortical slices is minimal.

These results, as a whole then, indicate that, whilst there is overwhelming evidence to suggest that $Li^+$ inhibits the inositol monophosphatase enzyme, the possibility that $Li^+$ exerts subtle effects within the cell still exists. The reviews of Berridge et al. (1982; 1989) highlight that it is this former property which most probably explains the therapeutic benefits of $Li^+$. By preventing free inositol accumulation the presence of $Li^+$ slows down the process of (poly)phosphoinositide lipid resynthesis. However, obtaining data which suggests that incorporation of label into the (poly)phosphoinositide lipid fraction diminishes under such experimental parameters has proved problematical. Drummond and Raeburn (1984) demonstrated that in $GH_3$ pituitary tumour cells treated with thyrotropin-releasing hormone (TRH) in the presence of $Li^+$, there were significant reductions in the levels of both PtdIns and PtdIns(4)P - approximately 50% and 20% respectively - compared to control values but, interestingly not in the levels of PtdIns(4,5)$P_2$, the lipid to which most importance has been ascribed. In similar studies on parotid gland, both PtdIns(4)P and PtdIns(4,5)$P_2$ labelling was affected in the presence of carbachol and $Li^+$ but these reductions were found not to be significantly different when averaged over several experimental determinations. In addition the labelling of PtdIns(4,5)$P_2$ in carbachol- stimulated rat cerebral cortical slices is not affected in the presence of $Li^+$ by more than 15% compared to control samples (Batty and Nahorski, unpublished data).

One possible explanation for these apparently contradictory results may be that the lipid pool is turning over rapidly in the presence of agonist and, therefore, to observe changes in the levels of each of the (poly)phosphoinositide lipids under discussion here would be extremely

difficult. Furthermore, the actual size of the agonist-sensitive pools is not known. Pool-sizes even within single cells have not yet been determined (Monaco and Woods, 1983); therefore, in tissue as heterogeneous as cerebral cortical brain slices, such measurments are complicated still further. In addition, regardless of the pool size, if $PtdIns(4,5)P_2$ is the only inositide lipid which undergoes PLC-catalyzed hydrolysis - although even this is debatable - then, under chronic stimulation, the cell might be expected to maintain its levels of $PtdIns(4,5)P_2$ by increasing the flux through the kinases responsible for phosphorylating PtdIns at the 4- and 5- positions. Consequently, changes in the levels of PtdIns and PtdIns(4)P may be easier to detect. These problems emphasise the advantages of quantifying $[^{14}C]CMP-PA$ accumulation as this is, at least, selective in observing the agonist-sensitive impairment of lipid synthesis in the presence of $Li^+$.

If it is, as suggested, that $Li^+$ will be most effective in the most acutely stimulated cells - which may, in turn, underlie the symptoms of manic-depressive illness (Berridge et al. 1989) - then this may account for cell specificity with regards to $Li^+$ as well as explain agonist-specificity of $Li^+$ on $Ins(1,3,4,5)P_4$ accumulation. In 1988 Whitworth and Kendall demonstrated that in mouse cerebral cortical slices only mAChR stimulation led to reduced accumulation of the $InsP_4$ fraction in the presence of $Li^+$ whilst agonists such as histamine and noradrenaline did not. Curiously, the $InsP_1$ and, to a certain extent, $InsP_2$ accumulation in the presence of all the aforementioned agonists were very similar. These less effective agonists were tested against carbachol by Godfrey (1989) to observe their ability to accumulate CMP-PA in the presence of $Li^+$. All the agonists and even depolarising concentrations of $K^+$ led to increased CMP-PA levels in cerebral cortical slices and, whilst these were all significant, carbachol manifested a much larger fold increase in CMP-PA accumulation compared to the others.

These results would imply that, because the $[^3H]InsP_1$ and $[^3H]InsP_2$ accumulation elicited by the other agonists in mouse cerebral cortical slices

is similar to that evoked by carbachol, the explanation for variation in the $[^3H]InsP_4$ accumulation is not especially due to differences in receptor density i.e. there is not a greater density of muscarinic receptor compared to those for histamine, noradrenaline etc. It is possible that PLC catalyses the hydrolysis of PtdIns or PtdIns(4)P in histamine or noradrenaline-linked responses although the experimental problems facing such measurements make estimations at this possibly very difficult. If it is assumed, therefore, that only PtdIns(4,5)$P_2$ is hydrolyzed then these agonists including carbachol, must all stimulate similar rates of PLC coupling but cells expressing muscarinic receptor must preferentially undergo greater flux through the $InsP_4$ pathway particularly in the presence of $Li^+$. Hansen et al. (1986) indicated that, at least in rat hepatocytes, $Li^+$ has no demonstrable effect on Ins(1,3,4,5)$P_4$ accumulation so the results shown here in both labelled and unlabelled experiments as well as those of other workers (Whitworth and Kendall, 1988; Godfrey, 1989) may be characteristic only of PtdIns-linked receptors in brain. Again it should be reiterated that in brain slices different cell populations may complicate further the interpretation of such results. However, what has been established is that CMP-PA accumulation is critically dependent upon the supply of myo-inositol regardless of the agonist and because the $K_m$ for inositol of the CMP-PA: myo-inositol transferase enzyme, at least in the liver, is 2.5mM (Takenawa and Egawa, 1977) it seems likely that the intracellular inositol concentration is the rate-limiting step in the resynthesis of PtdIns. $Li^+$, by inhibiting the inositol monophosphatase, deprives the brain cells of its most important source of myo-inositol - namely inositol (poly)phosphate metabolism.

Nevertheless, the results involving myo- and scyllo-inositol additions to such assays presented above cannot be accommodated into such a simplistic interpretation of the action of $Li^+$. If myo-inositol addition can overcome the $Li^+$ inhibition of the inositol monophosphatase then at such high

concentrations as 10-30mM added prior to agonist and $Li^+$ additions, it would be expected that no $[^{14}C]$CMP-PA would accumulate. Data from Fig. 3.11 indicates that this clearly is not the case. Similarly subsequent addition of myo-inositol could be expected to reverse completely any carbachol and $Li^+$-induced accumulation of this PtdIns precursor molecule. Again, this was not apparent with either 10mM or 30mM myo-inositol added exogenously. Furthermore, in the experiments in which myo-inositol was added prior to or subsequent to agonist and $Li^+$ additions and the mass $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ measured, the expected reversal of the $Li^+$-induced reduced accumulation was not apparent. Although the fall in $Ins(1,4,5)P_3$ levels was substantially decreased, the values obtained were still significantly different to those achieved in control samples. This result demonstrates the inability of prior incubation with myo-inositol to prevent a decline in the mass of $Ins(1,4,5)P_3$ or $Ins(1,3,4,5)P_4$, simply delay them. Preincubation, therefore, of the cerebral cortical slices with myo-inositol does not reverse the reduced accumulation in the presence of $Li^+$, it merely prolongs the period over which the second messenger levels fall by approximately 5 min.

These results tend to indicate two fundamental phenomena. Firstly, at least in cerebral cortical tissue in vitro, $Li^+$ has profound effects on inositol (poly)phosphate metabolism, suggesting that this system is exquisitely sensitive to modest depletions in the intracellular inositol concentration. Secondly, and perhaps more importantly, is the implication that $Li^+$, although inhibiting the monophosphatase enzyme, has one or more other sites of action which could account for the reduced accumulation of both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ in the presence of $Li^+$ whilst $InsP_1$ accumulation displays continued linearity. The inability of myo-inositol either to prevent or reverse these manifestations underline this hypothesis. Various possibilities which would, in part, explain the data presented here have been considered. These included PKC involvement, increased participation of cyclic inositol (poly)phosphate metabolism and the potential

shift which $Li^+$ induces, either directly or indirectly, in the lipid source of PLC-catalyzed hydrolysis.

Since myo-inositol does have an effect on the accumulation of PtdIns(4,5)$P_2$ metabolites in the presence of $Li^+$, it would be of considerable interest to know whether it can affect other systems in which $Li^+$ has been shown to be influential. For example, several groups have demonstrated the ability of myo-inositol but not of its isomers or synthetic analogues, to reverse $Li^+$-induced effects. Busa and Gimlich (1989) have shown that the teratogenic effects elicited by $Li^+$ in Xenopus embryos can be reduced and, in some cases, abolished by the co-injection of myo but not epi-inositol. Similarly, myo-inositol can reverse $Li^+$-induced changes during mitosis in sea urchin zygotes. Scyllo-inositol, sorbitol, mannitol and glucose were all tested as a representative sample of cyclitols and sugars but none could replace myo-inositol in the (poly)phosphoinositide lipid cycle (Forer and Sillers, 1987). Furthermore, $Li^+$-induced effects on T-lymphocyte activation (Mustelin et al. 1986) and in $\alpha_1$-adrenoceptor effects in guinea pig heart (Mantelli et al. 1988) could also be overcome in the presence of myo-inositol. However, a study by Worley et al. (1988) found that in hippocampal slices, the action of carbachol, which inhibits the adenosine response, is diminished in the presence of therapeutically relevant $Li^+$ concentrations. Whether this ability is either reversed or prevented by myo-inositol is unknown.

(Poly)phosphoinositide metabolism is strongly implicated in multiple cellular functions. The potent and apparently selective actions of $Li^+$, particularly in in vitro cerebral cortical slice preparations, are of great importance in defining the psychotropic actions of this unique drug and, as such, warrant much more investigation. That $Li^+$ may have more than one site of action is still open to question. However, because of the obvious problems surrounding studies on inositol (poly)phosphate metabolism in cerebral cortical slices more emphasis is now being put on investigations

using cultured cells. The following chapter will deal with several preliminary experiments involving the effects if $Li^+$ in mAChR stimulated cells.

# C H A P T E R  4

The Effects of Lithium on Inositol (Poly)Phosphate Accumulation in Continuous

Cell Culture

CHAPTER 4


THE EFFECTS OF $Li^+$ ON INOSITOL (POLY)PHOSPHATE ACCUMULATION IN CONTINUOUS
CELL CULTURE


Introduction


Having established that $Li^+$ has profound effects upon the inositol
(poly)phosphates and CMP-PA assayed from acutely labelled rat cerebral
cortical slices it was of interest to consider the action of $Li^+$ when applied
to homogenous populations of adherent cell lines in continuous culture. This
would then allow comparisons to be drawn between the two systems. In the
studies presented here, two cell lines have been used:


$\underline{CHO}_{m1}$ - Chinese hamster ovary cells were transfected with a stable $M_1$
transfection. CHO cells are widely available and used in a variety of
different studies. What makes the transfected cell particularly attractive,
however, for the work described here is that the activation by carbachol is
due solely to the stimulation of the $M_1$ muscarinic receptor subtype. CHO
cells do not express any endogenous muscarinic receptors and the results are
therefore not complicated by the presence of other muscarinic receptor
subtypes.


SH-SY5Y - These cells are a subclone derived from the SK-N-SH cell
line. This parent line was originally identified in a bone marrow metastases
in a 4 year old girl (Biedler et al. 1973) and is known to express muscarinic
receptors which are linked to (poly)phosphoinositide metabolism (Fisher and
Snider, 1987; Fisher and Heacock (1988). The SK-N-SH cell has, however, two

morphologically distinct cell types, namely the SH-SY5Y and SH-EP1 cells. Only the former is neuroblast-like and this had been purported to express predominantly on $M_1$ receptor subtype (Serra et al. 1988). Binding studies using a variety of muscarinic receptor antagonists has contradicted this with the recent report that SH-SY5Y cells express a homogeneous population of $M_3$ muscarinic receptors (Lambert et al. 1989). Having a putative homogeneous population like the $CHO_{m1}$ cells, the SH-SY5Y cells are ideal for the work included in this chapter. Also, perhaps more importantly, the SH-SY5Y cell is of neuronal origin and, therefore, of more immediate interest in the investigation of $Li^+$, a drug which appears to affect the central nervous system profoundly.

The advantages of using clonal continuous cell culture as a model system are numerous. The population of cells are homogeneous. Results will not, therefore, be complicated by the presence of other cells, as is the case in cerebral cortical slice work. Handling of cells is also much less harsh than corresponding experiments using cerebral tissue. Cells which are scraped off the surface upon which they are growing will not lose as much free inositol as the cerebral cortical slices do during the cross-chopping procedure. As a result, for labelled inositol phosphate studies, intact cells are a useful system. The major disadvantage, however, is that these cells are immortal i.e. they are derived from cells which have undergone an intracellular transformation which has rendered them tumorogenic. This may alter the observed responses through some unknown modification. The only way to circumvent this problem is to utilize primary embryonic cultures which are prepared from the tissue under investigation. For the purposes of this study, however, it was both sufficient and simpler to use the $CHO_{m1}$ and SH-SY5Y cells as systems in which to investigate the inositol (poly)phosphate production in response to $Li^+$.

## Results and Discussion

Experiments were carried out by Dr. D. Lambert to investigate the binding of selective muscarinic antagonists to both $CHO_{m1}$ and SH-SY5Y cells. The $CHO_{m1}$ cells have a very high affinity for pirenzipine, an $M_1$ selective antagonist. This is not surprising. As stated previously, the only muscarinic receptor that these cells express is an $M_1$ subtype. The SH-SY5Y cells demonstrate a very high affinity for 4-DAMP this indicating that these cells express only $M_3$ receptors. This has already been reported by Lambert et al. (1989)

The experiments involving the concentration and temporal effects of $Li^+$ upon labelled inositol (poly)phosphate production were carried out in $CHO_{m1}$ and SH-SY5Y cells labelled for 48 hours. This ensures that the cells are labelled to a state which is approaching equilibrium and, thus, have the advantage over rat cerebral cortical slice work in which the tissue could only be labelled acutely.

Fig. 4.1 (A,B,C and D) indicates the concentration dependent effects of $Li^+$ upon labelled inositol mono-, bis-, tris- and tetrakisphosphate accumulation in response to carbachol in $CHO_{m1}$ cells. Fig 4.1A shows the accumulation of total $[^3H]InsP_1$. $Li^+$ alone has no effect upon the accumulation regardless of the prevailing concentration. However, in the presence of millimolar carbachol, $[^3H]InsP$, accumulates with an $EC_{50}$ value of $0.68 \pm 0.04mM$. This is very similar to that observed in Fig. 3.1A. Similarly $[^3H]InsP_2$ appears to accumulate at higher concentrations of $Li^+$ in the presence of carbachol (Fig. 4.1B). The $EC_{50}$ value which cannot be accurately calculated will be greater than 12mM and is significantly greater than that observed in rat cerebral cortical slice extract.

Fig. 4.1C shows the $Li^+$ concentration dependence of total $[^3H]InsP_3$ accumulation in carbchol stimulated $CHO_{m1}$ cells. At submillimolar concentrations of $Li^+$, the total $[^3H]InsP_3$ appears to decline by

Fig. 4.1 - Concentration dependent effects of lithium on the accumulation of individual inositol phosphates in $CHO_{m1}$ cells in the presence ( ■ ) or absence ( □ ) of carbachol (1mM).
A - $InsP_1$;  B - $InsP_2$;  C - $InsP_3$;  D - $InsP_4$;  C - control (-carbachol,-$Li^+$). Control values in the presence of carbachol alone after 30 min. were A - 25.05 ± 2.4;  B - 14.94 ± 0.61; C - 5.03 ± 0.27;  D - 7.32 ± 0.44 (values expressed as DPM / 50µl aliquot). Data represents the mean ± S.E.M. for 3 separate determinations.
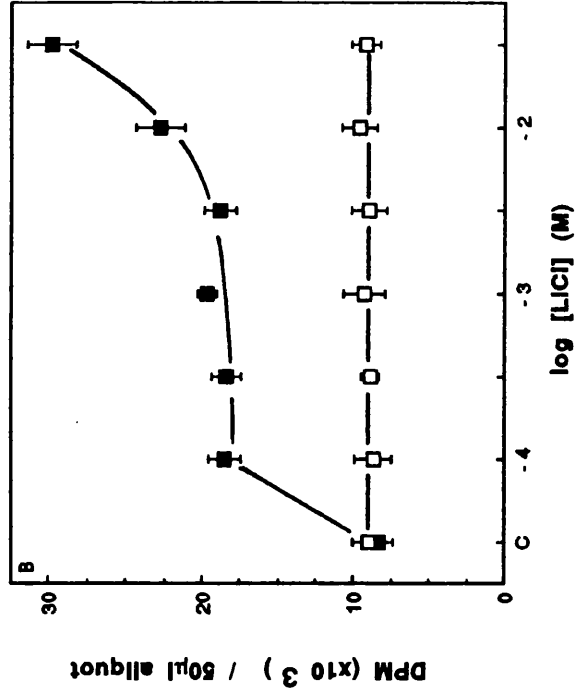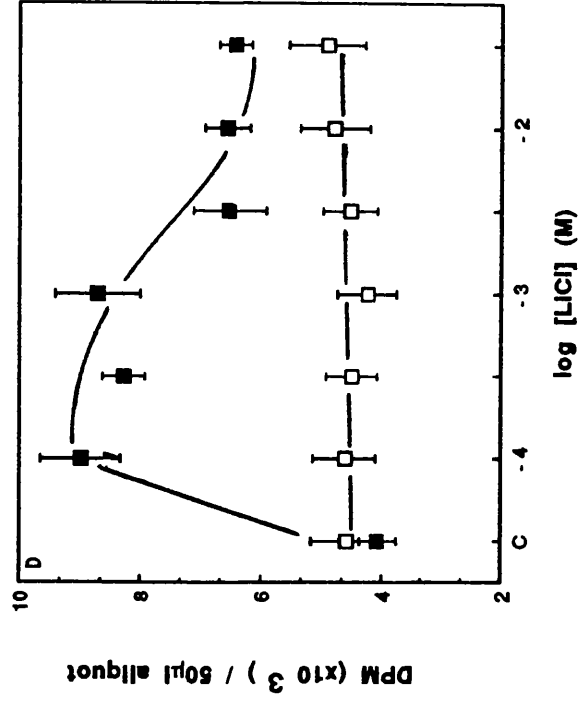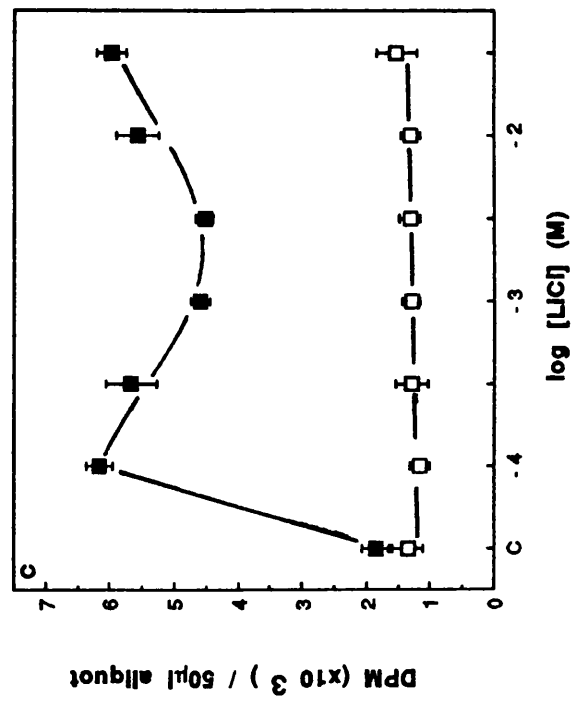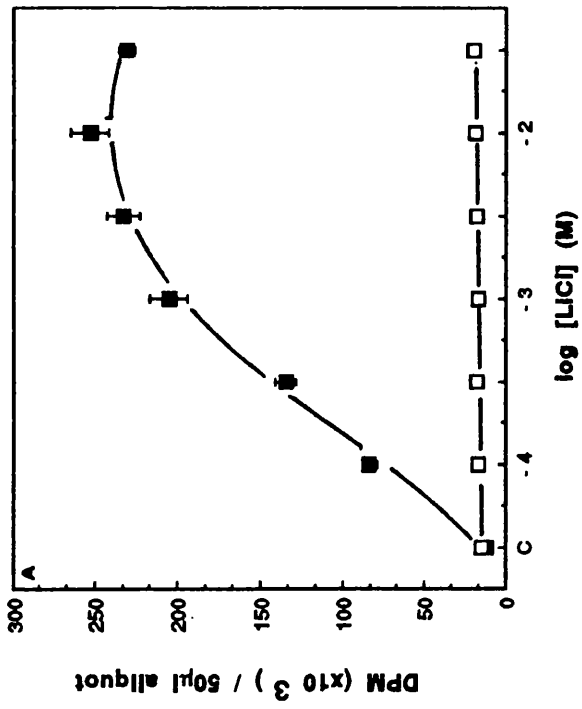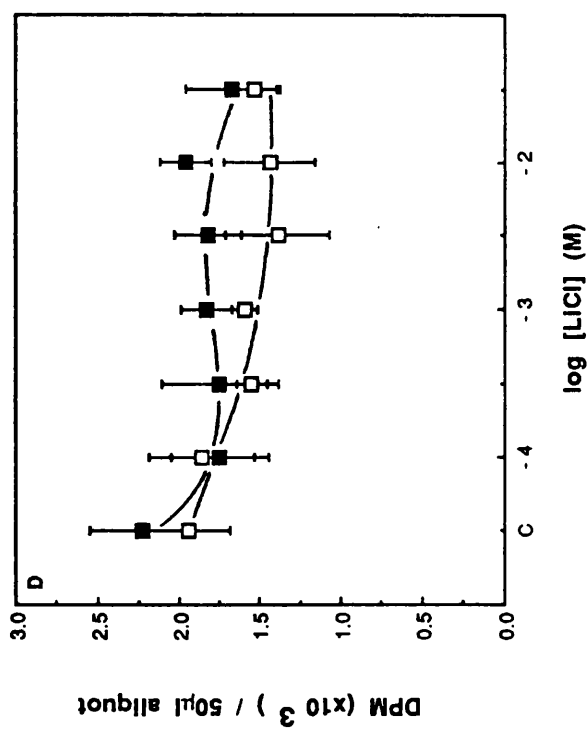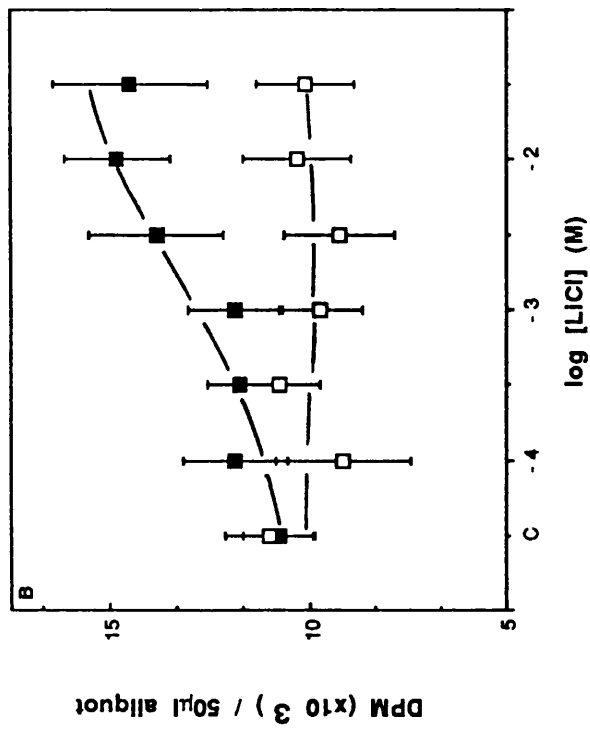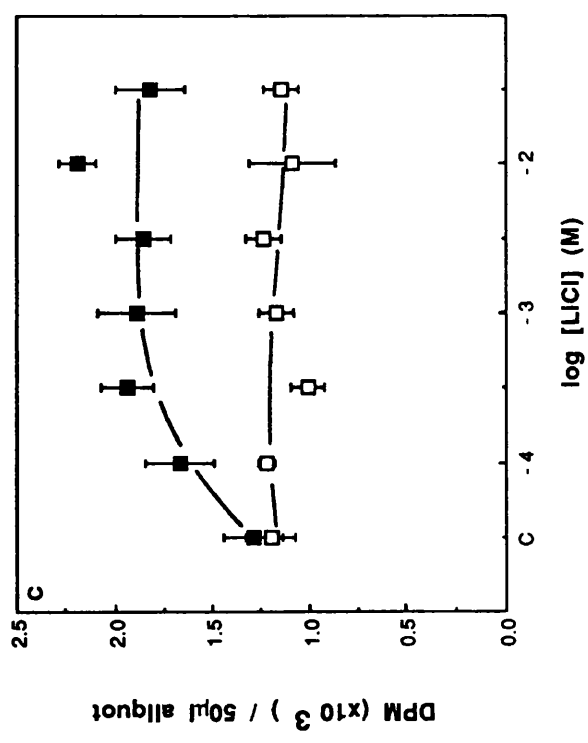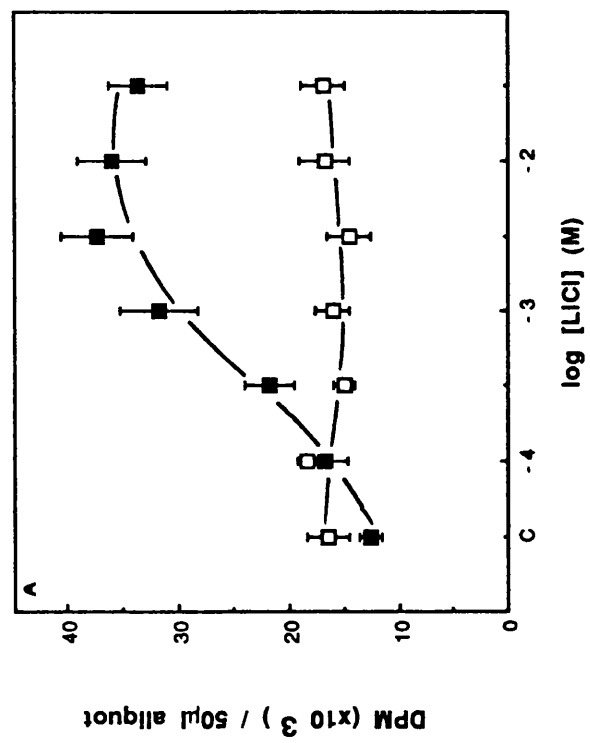
approximately 25% over a $Li^+$ concentration difference of just one log unit.

This level is then maintained but at concentrations of $Li^+$ above 3mM, the

total $[^3H]InsP_3$ accumulation is seen to increase again, until at

concentrations of $Li^+$ as high as 30mM, the level of accumulation has returned

to its original level. This figure is obviously complicated by the fact that

it represents total $[^3H]InsP_3$ and not just $[^3H]Ins(1,4,5)P_3$ as before. It

remains possible that this biphasic effect is due to the accumulation of

another trisphosphate isomer. In cells labelled to a state approaching

equilibrium inositol trisphosphate isomers other than $Ins(1,4,5)P_3$ and

$Ins(1,3,4)P_3$ are known to be labelled (see for example Stephens et al. 1989).

Therefore, whilst the reduced accumulation evident at submillimolar $Li^+$

concentrations may be due to the $Ins(1,4,5)P_3$ isomer, the second phase of

increased accumulation may be due to another isomer. The effects of $Li^+$ upon

all the enzymes involved in the metabolic pathway to free inositol have not

yet been fully determined. At high $Li^+$ concentrations, the metabolism of one

may be inhibited and thus its substrate will accumulate. Indeed, such an

action is known to inhibit the metabolism of $Ins(1,3,4)P_3$ and, at higher $Li^+$

concentrations, it may well be this isomer which is accumulating. To

discover which inositol trisphosphate isomer is responsible for this biphasic

concentration dependence response enzymic separation of the $Ins(1,4,5)P_3$ and

$Ins(1,3,4)P_3$ and h.p.l.c. analysis would be required.

Fig. 4.1D shows that $Li^+$ causes reduced accumulation of the $[^3H]InsP_4$

fraction in response to carbachol. The $IC_{50}$ value for this activity would

appear to be higher than that quoted in similar experiments in rat cerebral

cortical slices (see Chapter 3). This highlights the subtle differences in

$Li^+$ sensitivity that may occur between species and indeed, between tissues.

As a direct comparison to the experiments described above, identical

experiments were conducted using labelled SH-SY5Y cells in suspension. Fig.

4.2 (A,B,C and D) illustrates these results. Under these conditions notably

the accumulation of $[^3H]InsP_1$ appears to be much lower than in either CHO$_{m1}$

Fig. 4.2 - Concentration dependent effects of lithium on the accumulation of individual inositol phosphates in SH-SY5Y cells in the presence ( ■ ) or absence ( □ ) of carbachol (1mM). A - $InsP_1$; B - $InsP_2$; C - $InsP_3$; D - $InsP_4$; C - control (-carbachol,-Li+). Control values in the presence of carbachol alone after 30 min. were A - 11.57 ± 0.81; B - 7.35 ± 0.76; C - 1.43 ± 0.26; D - 1.21 ± 0.06 (values expressed as DPM / 50μl aliquot). Data represents the mean ± S.E.M. for 3 separate determinations.

**B**

DPM (x10³) / 50µl aliquot

15

10

5

log [LiCl] (M)

-2  -3  -4  C

**D**

DPM (x10³) / 50µl aliquot

3.0  2.5  2.0  1.5  1.0  0.5  0.0

log [LiCl] (M)

-2  -3  -4  C

**A**

DPM (x10³) / 50µl aliquot

40  30  20  10  0

log [LiCl] (M)

-2  -3  -4  C

**C**

DPM (x10³) / 50µl aliquot

2.5  2.0  1.5  1.0  0.5  0.0

log [LiCl] (M)

-2  -3  -4  C

cells or rat cerebral cortical slices with carbachol stimulation. In the presence of increasing concentrations of $Li^+$ there is only a 1.5-2 fold increase in levels of this fraction (Fig. 4.2A). The $EC_{50}$ value again is 0.69 ± 0.08mM which is not significantly different from that observed in either of the two other systems investigated.

Fig. 4.2B shows the accumulation of the $[^3H]InsP_2$ fraction in SH-SY5Y cells in response to carbachol and in the presence of $Li^+$. The $EC_{50}$ value is approximately 2.75mM. This value appears to vary from the values obtained for $[^3H]InsP_2$ accumulation in both $CHO_{ml}$ cells and rat brain cerebral cortical slices. Whilst the $EC_{50}$ values for $[^3H]InsP_1$ accumulation in the presence of increasing concentrations of $Li^+$ remain similar in all three systems, those for the accumulation of $[^3H]InsP_2$ exhibit more variation. As stated previously, the most obvious explanation for this is differences in the physical properties of the enzyme(s) responsible for the metabolism of $InsP_2$. It is possible, for example, that the degree of noncompetitive and uncompetitive inhibition exerted by $Li^+$ varies between systems.

Figs 4.2C and D indicate that, under the conditions being used in these experiments, neither the total $[^3H]InsP_3$ nor the $[^3H]InsP_4$ fractions appear to acumulate significantly above control values or, indeed, the values obtained in the presence of $Li^+$ alone.

The results obtained here indicate that increasing concentrations of $Li^+$ have very different effects on the accumulation of the individual inositol phosphate fractions isolated from $CHO_{ml}$ and SH-SY5Y cells. Of the two, the $CHO_{ml}$ cell response appears to bear the closer similarity to rat cerebral cortical slices, whilst the SH-SY5Y cells do not, certainly inasmuch as the $[^3H]InsP_3$ and $[^3H]InsP_4$ fractions are concerned. To verify these effects the time course of accumulation of the $[^3H]$inositol phosphate fractions were investigated in both $CHO_{ml}$ and SH-SY5Y cells in response to carbachol and $Li^+$.
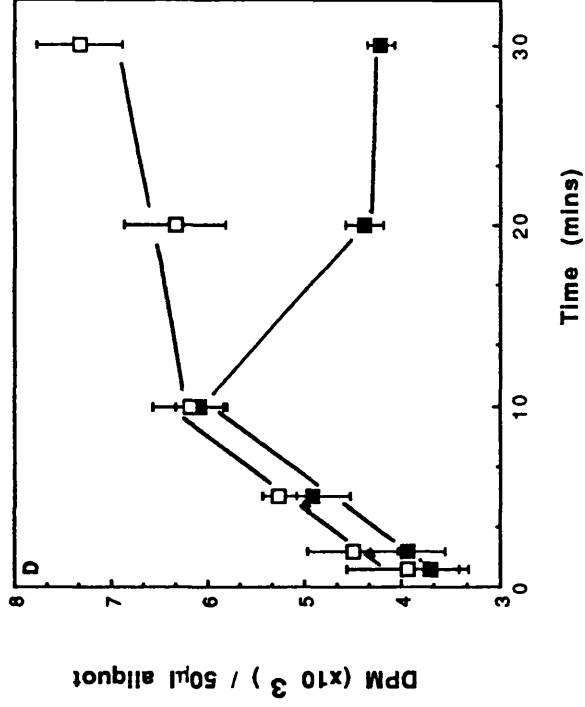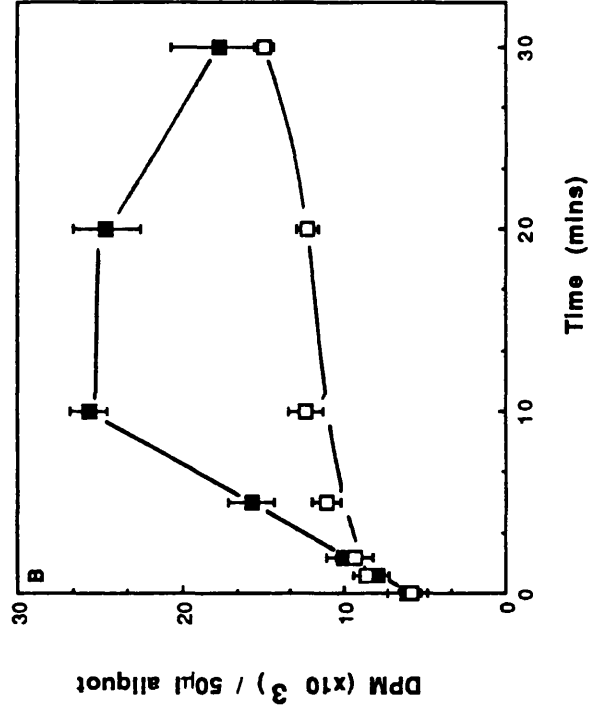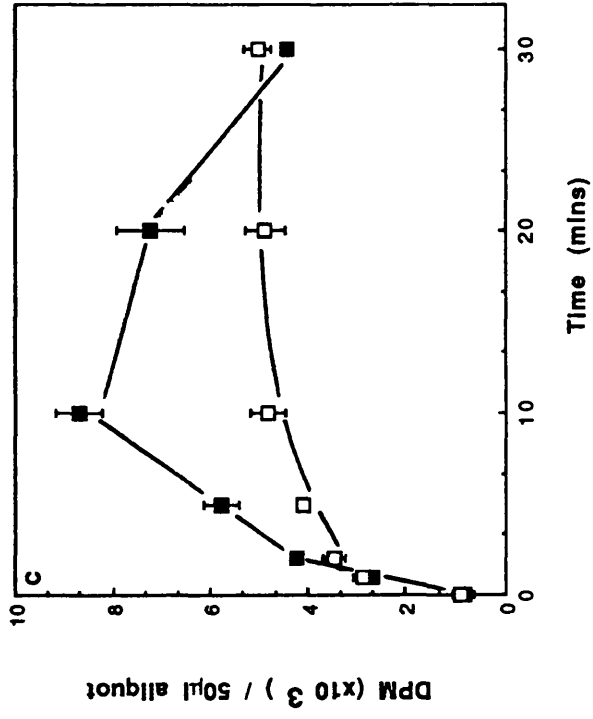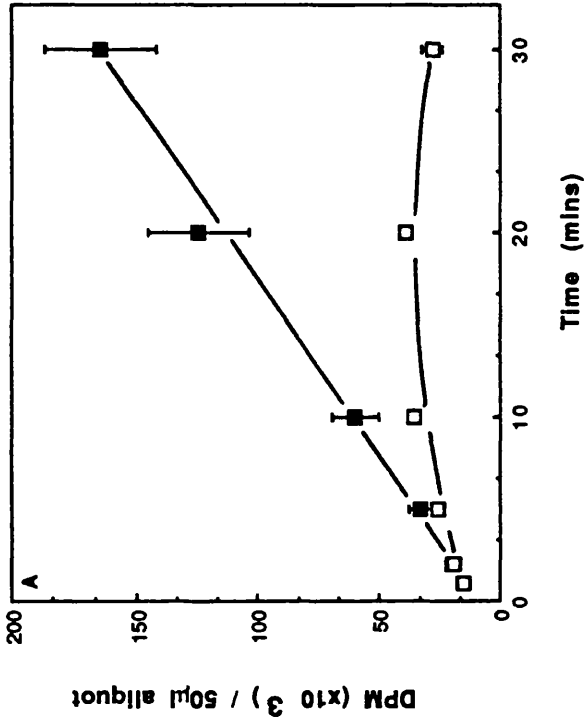
As can be seen in Fig. 4.3 (A,B,C and D) $Li^+$ exerts effects on all the inositol mono-, bis-, tris- and tetrakisphosphate fractions isolated. In

Fig. 4.3A, $[^3H]InsP_1$ accumulates in the presence of carbachol alone, although clearly this effect is not linear as was observed in the accumulation of $[^3H]InsP_1$ in rat cerebral cortical slices. Indeed, after 20 mins, the accumulation appears to decline. The reason for this is unknown. In the presence of $Li^+$, the accumulation of $[^3H]InsP_1$ appears to be approaching linearity for up to and including 30 mins after addition of the agonist. Interestingly, however, it takes 10 mins before the difference in accumulation between carbachol-treated samples and carbachol- and $Li^+$-treated samples becomes significantly different. This is in direct contrast to the accumulation of $[^3H]InsP_1$ in rat cerebral cortical slices which was rapid and exhibited significant differences between the conditions by 5 mins. The rate of accumulation thereafter, however, appears to be similar. This may indicate that in $CHO_{m1}$ cells there is a very slow cycle of (poly)phosphoinositide metabolism in the absence of any external stimuli and, unlike rat cerebral cortical slices, $CHO_{m1}$ cells therefore have a low level of resting $InsP_1$. $Li^+$ will, as a result, take longer to exert a discernible effect upon the accumulation.

This theory is borne out by Fig. 4.3B which represents the accumulation of $[^3H]InsP_2$. Again, in the absence of $Li^+$, there is a slight accumulation up to 30 mins. The $[^3H]InsP_2$, however, in the presence of $Li^+$ indicates a rapid acumulation such that by 5 mins there is a significant difference in the levels detected in carbachol- and $Li^+$-treated samples as compared to non-$Li^+$-treated samples. If, as suggested, $InsP_1$ is derived mainly from $InsP_2$ under these conditions, then $[^3H]InsP_1$ would be expected to demonstrate a delay before accumulating. In addition, Fig. 4.3B indicates that after 20 mins the levels of $[^3H]InsP_2$ decline. This appears to be more dramatic than in the corresponding figure for rat cerebral cortical slices (see Fig. 3.3B) probably because in $CHO_{m1}$ cells upward drift of accumulation in the non-$Li^+$-treated samples is not as pronounced. By 30 mins however, again there is no significant difference in the accumulation regardless of the

Fig. 4.3 - Time dependent effects on the accumulation of the individual labelled inositol (poly)phosphates in the presence ( ■ ) or absence ( □ ) of $Li^+$ (10mM) in $CHO_{m1}$ cells stimulated with carbachol (1mM).
A - $InsP_1$;  B - $InsP_2$;  C - $InsP_3$;  D - $InsP_4$;  C - control (-carbachol,-$Li^+$).  Data represents the mean ± S.E.M. for 3 separate determinations.

**A**

DPM (x10$^3$) / 50µl aliquot

200
150
100
50
0

Time (mins)

0   10   20   30

**B**

DPM (x10$^3$) / 50µl aliquot

30
20
10
0

Time (mins)

0   10   20   30

**C**

DPM (x10$^3$) / 50µl aliquot

10
8
6
4
2

Time (mins)

0   10   20   30

**D**

DPM (x10$^3$) / 50µl aliquot

8
7
6
5
4
3

Time (mins)

0   10   20   30

presence of $Li^+$.

Fig. 4.3C illustrates the effects of carbachol and $Li^+$ upon total
$[^3H]InsP_3$ accumulation. In the presence of carbachol alone, there appears to
be very little accumulation of the $[^3H]InsP_3$ fraction. This is in direct
contrast to the situation in the corresponding experiments in labelled rat
cerebral cortical slices. In this latter case, the accumulation in the
presence of carbachol alone or in the presence of carbachol and $Li^+$ was
virtually identical for up to 10 mins following administration. This only
represented the $[^3H]Ins(1,4,5)P_3$ isomer, however, whilst in the experiments
discussed here involving $CHO_{ml}$ cells, no such distinction was made.
Nevertheless, $Li^+$ appears to enhance the ability of carbachol to cause total
$[^3H]InsP_3$ accumulation to rise between 15% and 45% above control levels.
This enhancement is such that, by between 1 min and 5 mins the accumulation
of $[^3H]InsP_3$ is significantly greater in the presence of $Li^+$ compared to in
its absence. This is in keeping with the time-course of accumulation of both
the $[^3H]InsP_1$ and $[^3H]InsP_2$ fractions. The accumulation of $[^3H]InsP_3$ appears
to be the most rapid, being significantly greater in the presence of $Li^+$
before 5 mins. In agreement, the $[^3H]InsP_2$, which is derived from the
$[^3H]InsP_3$ fraction, does not display significantly different accumulation
until the 5 mins time point. Likewise, the $[^3H]InsP_1$ formed by
dephosphorylation of the $[^3H]InsP_2$ fraction, does not display significant
difference in its accumulation until 10 mins after agonist and $Li^+$ aditions.
This series of events corresponds to the dogma that, following receptor
activation, PLC-catalyzed hydrolysis of $PtdIns(4,5)P_2$ leads to the production
of $Ins(1,4,5)P_3$ which is sequentially dephosphorylated to produce $InsP_2$ and
$InsP_1$. As each of these events occurs in progression, the production of each
fraction would be expected to lag behind that of its precursor. This is
evidently occurring in $CHO_{ml}$ cells although detailed h.p.l.c. analysis would
be required to investigate the isomeric species involved. In the conditions
utilized here, the inositol (poly)phosphate fractions have very low resting

levels. This may suggest that, at early time points, the pathway involving the 5-phosphomonoesterase is particularly active.

Furthermore, the rate of accumulation of $[^3H]InsP_3$ in $CHO_{ml}$ cells is much slower than in rat cerebral cortical slices, taking 10 mins to reach a maximal value. In previous experiments in cortex the greatest accumulation was achieved by 5 mins. As a result, any lag phase which may be occurring, in a manner similar to that in cortical tissue, is not immediately evident. This does not automatically suggest that such a phase does not occur. If time points had been taken at 15 mins for example, a lag phase may have been apparent. If this were the case, this would be consistent with the delayed production of all the fractions mentioned previously. In rat cerebral cortical slices, where production of the labelled inositol mono-, bis-, (1,4,5)tris- and tetrakis- phosphate fractions are rapid in response to carbachol and $Li^+$, the lag phase before the reduced accumulation of the $[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ fractions occurs between 5 and 10 mins. In $CHO_{ml}$ cells, with their proposed lower resting levels of inositol (poly)phosphates, the inhibition of the cycle by $Li^+$ will take longer to implement and, accordingly, if this lag phase is dependent upon the build-up of some secondary metabolite, then this too would be delayed. Therefore, theoretically, if such a phase does occur, it may be apparent between 10 and 15 mins, rather than between 5 and 10 mins.

The reason why $Li^+$ should enhance the production of total $[^3H]InsP_3$ over carbachol-only treated samples is also puzzling. It should be noted that in these experiments $Li^+$ is present at a final concentration of 10mM compared to the rat cerebral cortical slice experiments which utilized only 1mM $Li^+$. This modification was made to accommodate any difference in the ability of the cell to take up $Li^+$. As explained previously, the rate which $Li^+$ takes to enter cells appears to be largely dependent on the $Na^+$-$H^+$ exchange mechanism (for review see Ehrlich and Diamond, 1980). Whilst this apparatus has been identified in neuronal tissue (see Ehrlich and Diamond, 1980 and

refs. therein) neither the presence nor the efficiency of this mechanism has been widely studied in $CHO_{ml}$ cells. It was therefore deemed necessary to increase the concentration of $Li^+$ used to safeguard against this possibility.

As shown in Fig. 3.5, 10mM $Li^+$, in the presence of millimolar carbachol, causes accumulation of $[^3H]Ins(1,4,5)P_3$ in rat cerebral cortical slices which is very similar to the accumulation in the presence of carbachol alone. In contrast, $Li^+$ appears to enhance total $[^3H]InsP_3$ production in $CHO_{ml}$ cells compared to carbachol alone. The enhancement evident in Fig. 4.3C may be due primarily to $[^3H]Ins(1,3,4)P_3$. At concentrations as high as 10mM, $Li^+$ may be affecting one or more elements of (poly)phosphoinositide cell signalling directly. For example, $Li^+$ may enhance the receptor G-protein interaction, activate GTPase activity or increase PLC activity. There are no reports to suggest that this occurs, however. Indeed the only study into the effect of $Li^+$ on G-proteins indicated that $Li^+$ inhibited the formation of the muscarinic cholinergic receptor and G-protein complex rather than stimulate it (Avissar et al. 1988). These situations would account, however, for the $Li^+$-induced increased synthesis of total $[^3H]InsP_3$ assuming that the difference between the carbachol- and the carbachol- and $Li^+$- treated samples is due solely to $Ins(1,4,5)P_3$. Such affects would appear, at least in the experiments reported here, to occur only at very high concentrations and will, therefore, not be relevant therapeutically.

In agreement with previous studies (see Fig. 3.3D) $Li^+$ has an effect upon the accummulation of $[^3H]InsP_4$. In the absence of $Li^+$, carbachol elicits an increase in the accumulation of $[^3H]InsP_4$ by approximately 2-3 fold over basal values. After 10 mins following the addition of agonist, the accumulation of this fraction appears to reach a new steady state of production and metabolism. In the presence of 10mM $Li^+$, the accumulation is identical to that in its absence. However, between 10 mins and 30 mins the reduced accumulation evident in rat cerebral cortical slices, occurs. This effectively reduces the levels of measurable $[^3H]InsP_4$ to around 1.5 fold
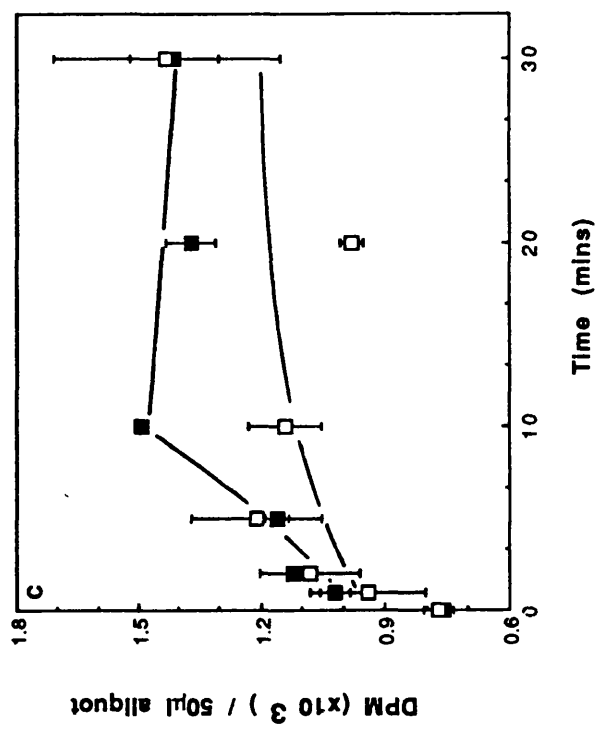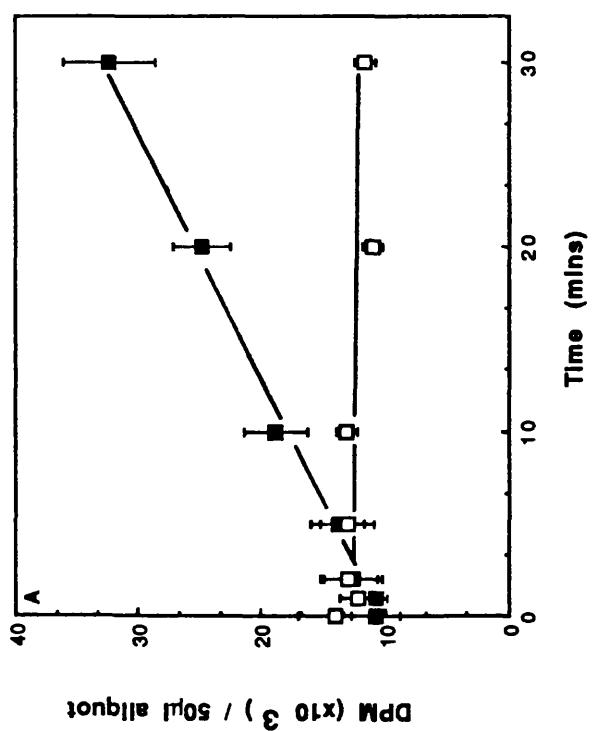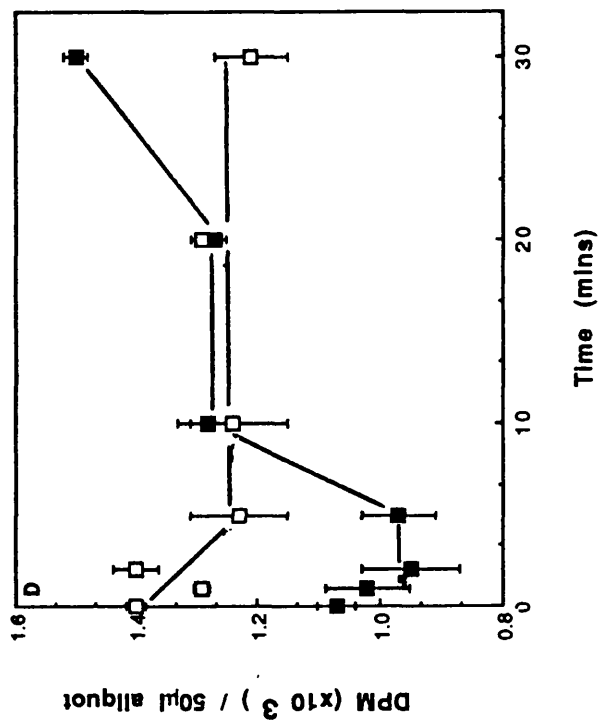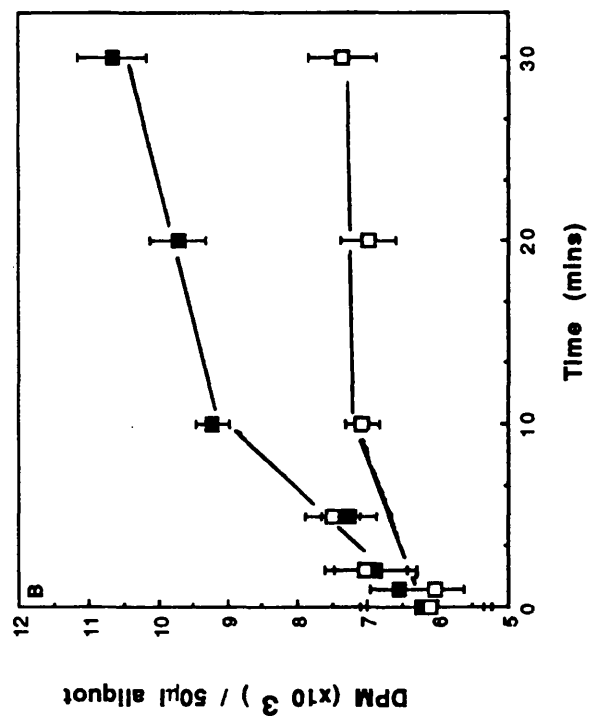
above basal.

Further to these experiments, the time course of accumulation of the individual labelled inositol phosphate fractions were investigated in SH-SY5Y cells in response to carbachol (1mM) and $Li^+$ (10mM). The results are shown in Fig. 4.4 (A,B,C and D). Fig. 4.4A indicates that, in the absence of $Li^+$, carbachol does not appear to cause any $[^3H]InsP_1$ to accumulate over a 30 mins period whilst in its presence there is approximately a 3.5 fold increase in the accumulation of this fraction. Again this accumulation is apparently linear for up to 30 mins. Like the $CHO_{m1}$ cells, but unlike the rat cerebral cortical slices, a signifiant difference in the accumulation due to the presence of $Li^+$ is not evident until the 10 mins time point. The $[^3H]InsP_2$ (Fig. 4.4B) does not display pronounced accumulation in the presence of carbachol and $Li^+$. Again, it is only after 5 mins following agonist and $Li^+$ additions that there is a marked difference in the accumulation. Unlike both $CHO_{m1}$ and rat cerebral cortical slices experiments, the $[^3H]InsP_2$ accumulation does not decline however slightly after 15-20 mins. In fact, the fraction appears to continue accumulating for periods of up to and including 30 mins. The total $[^3H]InsP_3$ and $[^3H]InsP_4$ fractions, as were evident in Fig. 4.2 (C and D) do not accumulate to a level at which it is possible to state that definite changes in their levels are occurring in response to $Li^+$.

The results thus far indiate that there are substantial differences in the accumulation of labelled inositol (poly)phosphates in cells in response to carbachol and $Li^+$. In so far as the $Li^+$ concentration dependence data is concerned, although the $[^3H]InsP_1$ and $[^3H]InsP_2$ fractions appear to follow the familiar pattern displayed in acutely labelled rat cerebral cortical slice extracts, the $[^3H]InsP_3$ and $[^3H]InsP_4$ fractions appear to differ. Essentially, SH-SY5Y cells do not accumulate either of these fractions to the degree that $CHO_{m1}$ cells and rat cerebral cortical slices do. In these latter systems both $[^3H]InsP_3$ and $[^3H]InsP_4$ accumulate significantly above basal but

Fig. 4.4 - Time dependent effects on the accumulation of the individual labelled inositol (poly)phosphates in the presence ( ■ ) or absence ( □ ) of Li+ (10mM) in SH-SY5Y cells stimulated with carbachol (1mM).

A - InsP$_1$; B - InsP$_2$; C - InsP$_3$; D - InsP$_4$; C - control (-carbachol,-Li+). Data represents the mean ± S.E.M. for 3 separate determinations.

**A**

DPM (x10 $^3$) / 50 µl aliquot

40
30
20
10
0

Time (mins)

0   10   20   30

**B**

DPM (x10 $^3$) / 50 µl aliquot

12
11
10
9
8
7
6
5

Time (mins)

0   10   20   30

**C**

DPM (x10 $^3$) / 50 µl aliquot

1.8
1.5
1.2
0.9
0.6

Time (mins)

0   10   20   30

**D**

DPM (x10 $^3$) / 50 µl aliquot

1.6
1.4
1.2
1.0
0.8

Time (mins)

0   10   20   30

then exhibit reduced accumulation at both higher $Li^+$ concentrations and longer time intervals. That this process may be reversed in $CHO_{ml}$ cells by the presence of other labelled $InsP_3$ isomers has been discussed.

There are several possible explanations for these observed differences. The two which probably exert most influence are: (a) the time of exposure to radioisotopic labelling, and, (b) the receptor subtype which is predominantly expressed. That the cells have been subjected to media containing label for 48 hrs prior to use, compared to the rat cerebral cortical slices which had only an acute labelling period of 60 mins, may account for certain differences in which changes in specific radioactivity have been implicated. However, it is of interest that the suggestion that it was changes in specific radioactivity which was the cause of the continued linearity in the $[^3H]InsP_1$ accumulation observed in cerebral tissue (Fig. 3.3A) has not been borne out by the studies presented here. If it were such changes, then in cells labelled to a state approaching equilibrium, theoretically, the $[^3H]InsP_1$ accumulation in response to carbachol and $Li^+$ should reflect a truer representation of what is occurring in an in vivo situation. Furthermore, if $Li^+$ was simply preventing (poly)phosphoinositide lipid synthesis by inhibiting the inositol monophosphatase enzyme, then the levels of $[^3H]InsP_1$, would be expected to plateau and decline as less and less lipid became available for hydrolysis following intracellular inositol depletion. Clearly, this is not the case in either labelled cells or in labelled rat cerebral cortical slices. Accumulation appears linear for periods up to and including 30 mins. This, however, does not effectively eliminate this possibility. $[^3H]InsP_1$ accumulation may be diminishing after 30 mins but, because none of the experiments herein were extended beyond 30 mins this eventuality has not been examined. If this were true, then each of the sytems under discussion here, would have to have substantial intracellular levels of $InsP_1$ to allow this linear accumulation to continue for 30 mins. Other possible sources of $InsP_1$ like direct PLC catalyzed cleavage of PtdIns

and the cyclization of glucose may play a substantial part in furthering

$InsP_1$ accumulation. The possibilities warrant further investigation.

Secondly, the apparent difference in the accumulation of each of the labelled inositol phosphate fractions may be due to the muscarinic receptor subtype which is predominantly expressed on the cell surface. The $CHO_{m1}$ cells have a homogeneous population of $M_1$ receptors whilst the SH-SY5Y cell apparently has a homogeneous population of $M_3$ receptors. Rat cerebral cortical slices obviously express a large variety of receptors. The mAChR population undoubtedly contains $M_1$ and $M_3$ both of which elicit a (poly)phosphoinositide-linked response. However, pharmacological and molecular analysis of the receptors and the intracelular mRNA has indicated that $M_1$ is probably more prevalent in cerebral cortex than the $M_3$ (for discussion see Chapter 1). This may account for the similarity in response to carbchol and $Li^+$ of $CHO_{m1}$ cells and rat cerebral cortical slices. In order to substantiate this, a neuronal cell line which expresses $M_1$ and $M_3$ receptors in approximately the same proportion as the cerebral cortex would be required. Plainly, investigations into the actions of $Li^+$ in various cell lines are important but because $Li^+$ displays selectivity towards the central nervous system, primary or continuous cultures which originate from this tissue will inevitably be of the greatest interest.

It has, however, been established that $Li^+$ enters both the cell lines under consideration here and affects their (poly)phosphoinositide metabolism in response to carbachol. The site(s) of its action, has still not been determined. Obviously, $Li^+$ is inhibiting the inositol monophosphatase enzyme but whether it is also affecting the synthesis of the second messenger, $Ins(1,4,5)P_3$, affecting the rate of its metabolism or is, indeed, responsible for both, has not been proven. The theory that $Li^+$ acts at another site within the cell has not been substantiated and, apparently unopposed, its only known site of action remains the inositol monophosphatase. Therefore, the following chapter will deal exclusively with this enzyme, discuss its

properties and address some fundamental questions which have not yet been

dealt with.

# C H A P T E R   5

The Inositol Monophosphatase Enzyme

CHAPTER 5

## THE INOSITOL MONOPHOSPHATASE ENZYME

## Introduction

One of the first reports which indicated that there was an enzymic activity which corresponded to an inositol monphosphatase was in 1966. Chen and Charlampous, studying the cyclization of glucose-6-phosphate to inositol in yeast defined the enzyme activities responsible for this pathway, the latter of which was defined as inositol-1-phosphatase. The intermediate between the two stages was characterized as inositol-1-phosphate. One year later, Eisenberg (1967) published data confirming this enzymic activity in the supernatant from rat testis homogenate and noted that no rearrangement of the glucose carbon chain occurred during the process of cyclization. Since then a plethora of studies have indicated that the enzyme is apparently universal and catalyzes the hydrolysis of inositol monophosphate to inositol.

The inositol monophosphatase has now been purified from several sources including bovine brain (Gee et al. 1988; Attwood et al. 1988; Meek et al. 1988; Hallcher and Sherman, 1980), rat brain (Takimoto et al. 1985), chick erythrocytes (Roth et al. 1981) and even lily pollen (Gumber et al. 1984). In all cases where the enzyme has been purified to homogeneity it appears to be dimeric with a native molecular weight of approximately 60kDa. Cloning and expression of the bovine brain inositol monophosphate has been successful with inositol monophosphatase activity being observed which was indistinguishable from the purified enzyme (Diehl et al. 1990). The enzyme appears to be encoded by an open reading frame of 277 amino acids and, because of the similarities between the cloned and the purified enzymes, it would appear that no significant post-translational modifications occur which

alter the activity of the native enzyme.

The inositol monophosphate enzyme plays a pivotal role in the maintenance of intracellular inositol levels. The main sources of inositol come from three potential sites - the extracellular medium, de novo synthesis from glucose and metabolism of the inositol (poly)phosphates formed following agonist-induced PLC-catalyzed hydrolysis of the inositide lipids. Inositol can be transported across the membrane by a saturable uptake mechanism (Spector, 1976) which, at least under the experimental conditions discussed in Chapter 3, may not be particularly active. Both de novo synthesis of inositol from glucose and the metabolism of the inositol (poly)phosphates depend upon inositol monophosphatase activity though and latterly, this enzyme has been shown to be responsible for inositol production from Ins(1,4,5)$P_3$ via the Ins(1,4)$P_2$ and Ins(4)P (Ragan et al. 1988; Ackermann et al. 1987) and from Ins(1,3,4,5)$P_4$ via the Ins(1,3,4)$P_3$ and Ins(3,4)$P_2$ or Ins(1,3)$P_2$ (Hansen et al. 1986; Inhorn et al. 1987; Shears et al. 1987). The inositol monophosphatase hydrolyzes Ins(1)P and its enantiomer Ins(3)P (Hallcher and Sherman, 1980), Ins(4)P and Ins(5)P (Ackermann et al. 1987). This latter group showed that DL-Ins(1)P, DL-Ins(4)P and Ins(5)P were all hydrolyzed by a crude enzyme preparation with similar $K_m$ and $V_{max}$ values - 94$\mu$M and 0.15$\mu$mol/min per mg of protein for Ins(1)P; 85$\mu$M and 0.17$\mu$mol/min per mg of protein for Ins(4)P and 125$\mu$M and 0.19$\mu$mol/min per mg of protein for Ins(5)P. However, in contrast to this, Gee et al. (1988) reported that the D- and L-enantiomers of Ins(4)P are, in fact, better substrates for the enzyme quoting $V_{max}$ values much greater than those for D or L-Ins(1)P. Several other non-inositol containing molecules are also now known to act as substrates for the inositol monophosphatase enzyme including 2'-AMP, 2'GMP and $\beta$-glycerophosphate (Takimoto et al. 1985; Gee et al. 1988). The rates of hydrolysis of these non-inositol-containing monophosphates were considerably lower in the report by this latter group compared to that of the former who found that these substances were almost as good substrates as L-Ins(1)P. This

may be indicative of a species related difference between the rat and the bovine enzyme. The report by Ackermann et al. (1987) that $Ins(1,4)P_2$ was also a substrate for the inositol monophosphatase enzyme has now been refuted by Gee et al. (1988) who found that it, in fact, has a very low $V_{max}$ value when assayed with the purified bovine brain enzyme. They attribute the original finding to the use of a bovine brain enzyme preparation which was not sufficiently pure.

It now appears that none of the more highly phosphorylated inositol phosphates are metabolized by the inositol monophosphatase with no activity reported towards $DL-Ins(1,4)P_2$, $DL-Ins(3,4)P_2$, $Ins(1,3)P_2$, $D-Ins(1,4,5)P_3$, $DL-Ins(1,3,4)P_3$ or $DL-Ins(1,3,4,5)P_4$ (Gee et al. 1988) or $Ins(c1:2)P$ (Ackermann et al. 1987). Interestingly, $Ins(2)P$ does not appear to be a substrate for the enzyme (Eisenberg, 1967) and indeed, Naccarato et al. (1974) have described its ability to inhibit the monophosphatase enzyme. The exception to this appears to be chick erythrocyte. A partially purified inositol monophosphatase preparation from 7-day old chick erythrocytes hydrolyzed $Ins(2)P$ with a similar affinity to $Ins(1)P$ and $Ins(3)P$ which were also tested. Noticeably, however, none of the other inositol phosphates described above, or indeed, inositol up to a concentration of 50mM appear to inhibit enzyme activity in any system.

Several other, non-inositol containing metabolites do appear to be inhibitory though. Inorganic phosphate acts as a competitive inhibitor with a $K_i$ value of 0.52mM (Gee et al. 1988). Similarly sodium fluoride, present as $F^-$ ions shows competitive inhibition against glycerol 2-phosphate yet displays noncompetitive inhibition with $Ins(1)P$ (Ganzhorn and Chanal, 1990). $Mg^{++}$, however, appears to both essential for activation and yet inhibitory at high concentrations. Hallcher and Sherman (1980) showed that the inositol monophosphatase is completely inactive in the absence of $Mg^{++}$ but displays maximal activity at concentrations of 1mM-2mM. At concentrations greater than 10mM, $Mg^{++}$ was found to be inhibitory. In agreement with Hallcher and

Sherman (1980), Gee et al. (1988) found that other divalent cations could inhibit inositol monophosphatase activity by competing competitively with $Mg^{++}$. $Ca^{2+}$, for example has a $K_i$ value of 18-20μM whilst $Mn^{++}$ has a much lower $K_i$ of 2.3μM. Both these studies were carried out using bovine brain preparation. Interestingly both Eisenberg (1967) and Naccarato et al. (1984) found that $Mn^{++}$ was not an inhibitor and, in fact, could partially substitute for $Mg^{++}$. Both these studies however, were undertaken in rat tissues highlighting the possibility that tissue- and species-specific differences could account for these apparently anomalous results.

The discovery that $Li^+$ can inhibit the inositol monophosphatase enzyme has proved to be the most interesting, at least therapeutically. In 1971 Allison and Stewart reported that in rats treated subcutaneously with $Li^+$ at a dose of 10meq/kg of body weight, the levels of myo-inositol in the cerebral cortex of these animals decreased and remained at such depressed levels for a further 12 hours. Indeed, the measurable inositol did not return to its previous levels until 72 hours after the administration of the drug. These authors also described how the effect of $Li^+$ on the myo-inositol levels could be, under certain conditions, reversed entirely in the presence of atropine or scopolamine, two mAChR antagonists. Later, the effect of $Li^+$ on decreasing inositol levels in cortical tissue was found to be accompanied by a marked increase in the intracellular Ins(1)P levels. This, in turn, led Berridge et al. (1982) to propose that $Li^+$ could have profound effects on the (poly)phosphoinositide metabolism in cells by reducing the amount of free myo-inositol available for reincorporation into new inositol lipids. As such, the result of this would be a depletion in the levels of (poly)phosphoinositide lipid available for the PLC-catalyzed hydrolysis into the second messenger $Ins(1,4,5)P_3$ and DAG moieties. The net effect would, therefore, be that the cycle of inositol lipid hydrolysis and resynthesis would be slowed down. Furthermore, $Li^+$ would preferentially affect cells in which this particular pathway was being abnormally active. That $Li^+$ can

elicit such selectivity may be possible.  In clinical situations several days

of $Li^+$ treatment are required before any abatement or cessation of

manic-depressive behaviour is observed in bipolar patients (Bunney and

Murphy, 1976).

As discussed previously in in vitro situations acute $Li^+$ treatment leads

to the immediate accumulation of all the labelled inositol phosphate

fractions measured after 10 min. Subsequently, the accumulation of

$[^3H]Ins(1,4,5)P_3$ and $[^3H]InsP_4$ declines.  These results do not represent an

in vitro situation, however, where, for example, inositol cannot cross the

blood-brain barrier to replenish the fall in free inositol which occurs as a

result of $Li^+$ treatment (Margolis et al. 1971; Barkai, 1979) and

investigations into the effect of chronic $Li^+$ treatment have been made by

several groups.  Patients undergoing $Li^+$ treatment do not become tolerant to

the drug.  This suggests that there is no compensatory shift in any of the

enzymes responsible for inositol (poly)phosphate metabolism.  However, in

1986 Renshaw et al. reported that in cerebral cortical preparations from rats

fed with $Li^+$ in the form of $Li_2CO_3$-containing pellets, the activity

attributed to the myo-inositol monophosphatase enzyme increased.  Indeed,

after 28 days of maintenance on a low level $Li^+$ diet, this activity appeared

to double.  Honchar et al. (1989) in contrast, have found no evidence to

support this, finding that activity ascribed to inositol monophosphatase

activity remained the same both in rats which had been injected daily with

LiCl for 29 days or had been maintained on a $Li_2CO_3$-containing diet.

Furthermore, these authors also contradicted a publication concerning the

effect of injected or dietary $Li^+$ on phospholipid levels in rat cerebral

cortex.  Joseph et al. (1987) found that, following acute LiCl administration

only the levels of phosphatidylethanolamine (PE) were affected whilst in rats

which had been subjected to chronic dietary $Li^+$ administration levels of both

PE and PtdIns were significantly reduced from control values yet meaurable PC

was increased.  Honchar et al. (1989) could not detect any change in the

absolute levels of the brain (poly)phosphoinositide lipids. The effect, then, that $Li^+$ has, either on the levels of the inositol monophosphatase enzyme or on the levels of the (poly)phosphoinositide lipids in rat cerebral cortex following chronic $Li^+$ treatment, appears to be controversial. Godfrey et al. (1989) reported that $[^3H]$inositol labelling of $PtdIns(4,5)P_2$ was enhanced following three days of twice-daily LiCl treatment (3meq/kg) whilst PtdIns and PtdIns(4)P were unaffected and that there was a concomitant 25%-40% reduction in labelled inositol phosphates extracted under these same conditions. In fact, following chronic $Li^+$ treatment the levels of $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_4$ were all substantially reduced compared to the levels of these tritiated inositol phosphate fractions from saline-treated controls.

Whatever the long-term effects of $Li^+$ in vitro, at present its only known site of action remains the inositol monophosphatase. The mode of inhibition appears to be primarily uncompetitive although $Li^+$ itself can alter the binding characteristics of other ions. $Li^+$ appears to act as a noncompetitive inhibitor of $Mg^{++}$ (Hallcher and Sherman, 1980) - a situation which can be complicated by the presence of $F^-$ (Ganzhorn and Chanal, 1990). Theoretically, there are three main types of reversible inhibition - competitive, non-competive and uncompetitive - that are distinguishable by kinetic comparison with each other. Lineweaver-Burke plots that are typical for each of these types of inhibition are shown in Fig. 5.1.

## 1. Competitive Inhibition:

Competitive inhibition is relatively common. The inhibitor binds to the active site of the enzyme in a reversible manner thus competing with the normal substrate. High concentrations of substrate will, therefore, overcome the inhibition of a competitive inhibitor. As a result the apparent $K_m$ is increased but the $V_{max}$ remains unchanged (see Fig. 5.1A).

## 2. Non-competitive Inhibition:

Noncompetitive inhibitors bind reversibly to free enzyme or to the ES complex i.e. they bind to sites other than the active site. This can, in turn, lead to a deformation of the enzyme which results in the ES complex neither forming nor degrading at its normal rates. As a result of this, high substrate concentrations are not sufficient to overcome this type of inhibition and the $V_{max}$ decreases (Fig. 5.1B). In the simplest case, the $K_m$ remains unchanged. Alternatively, competitive inhibitors of second substrates often appear as noncompetitive inhibitors of the first.

## 3. Uncompetitive Inhibition:

This type of inhibition is particularly unusual in single substrate reactions and few natural examples are known to exist. In such cases, the inhibitor combines only with the ES complex and not with the free enzyme. This prevents it from undergoing further reaction to yield its normal products (Fig. 5.1C).

Uncompetitive inhibition can have enormous effects on the levels of the metabolic intermediates, indeed, much more so than competitive inhibition (Cornish-Bowden, 1986). The resulting toxic effects of uncompetitive inhibition may explain why cells have evolved in such a way as to avoid expressing too many examples of it. This is the case with $Li^+$, which, as an uncompetitive inhibitor of the inositol monophosphatase, can have toxic effects. The therapeutic concentration range over which $Li^+$ can be administered safely is, in fact, very narrow. It would be particularly beneficial, therefore, to develop a replacement for $Li^+$. This may prove to be problematical, however, because the unique, stimulus-dependent ability of
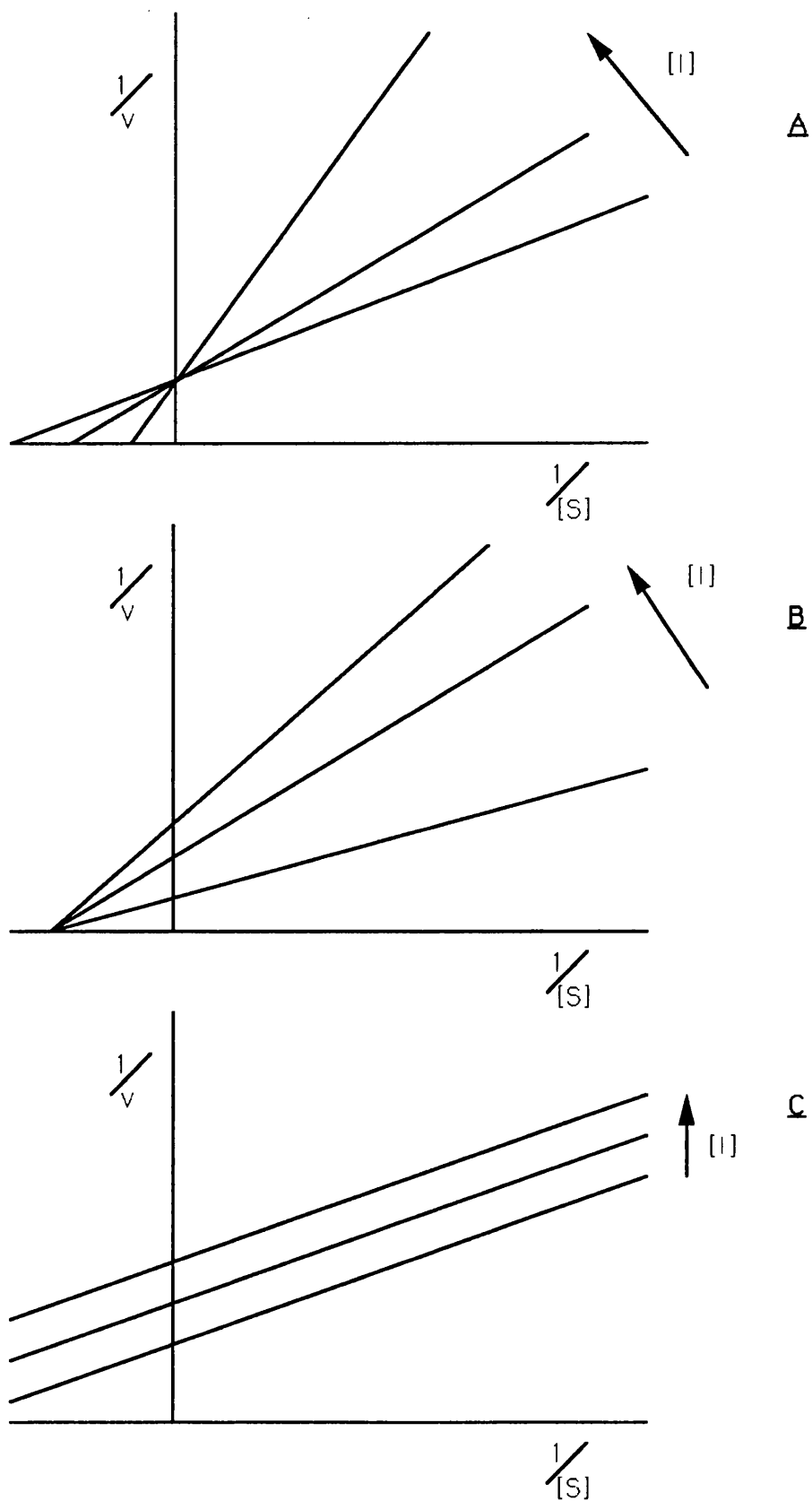
Fig. 5.1 - Comparison of Lineweaver - Burke plots for competitive (A), noncompetitive (B) and uncompetitive (C) inhibition.

(v = velocity; S = substrate; I = inhibitor )

$Li^+$ to target those cells in the central nervous system which are abnormally active will be dificult to duplicate. Cornish-Bowden (1986) has suggested that designing a competitive inhibitor to the "key enzyme", in this case the inositol monophosphatase, should result in negligible toxicity. However, this may not necessarily be the better alternative as it may be the very uncompetitive nature of $Li^+$ which provokes such serendipitous pharmacological effects.

In this chapter the effects of $Li^+$ on a crude preparation of rat cerebral homogenate supernatant will be examined. Furthermore, the ability of two polyclonal antibodies raised to purified inositol monophosphatase from bovine brain to inhibit the inositol monophosphatase from a variety of different systems will be investigated. All the Materials and Methods used throughout this study are described in Chapter 2.1.

## Results and Discussion

Fig. 5.2 illustrates the inhibition by $Li^+$ of a crude supernatant prepared from rat cerebral homogenate. This has been displayed as a direct plot (A) and as a double reciprocal, Lineweaver-Burke plot (B). Clearly both the $V_{max}$ and $K_m$ values have been altered from those apparent in the uninhibited reaction. When the data obtained was computer fitted to calculate the most appropriate mode of inhibition the following results were acquired:

## Uncompetitive Fit:

$$V_{max} = 0.186 \pm 0.019$$

$$K_m = 0.172 \pm 0.042 \text{ mM}$$
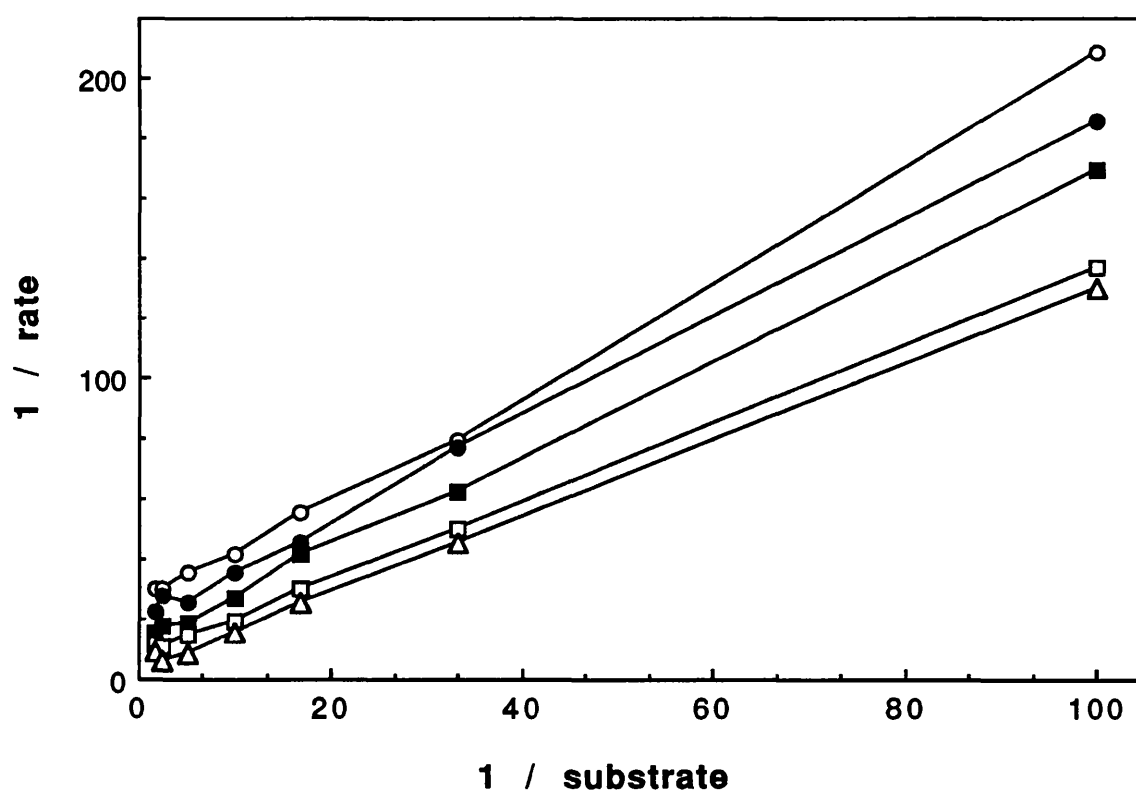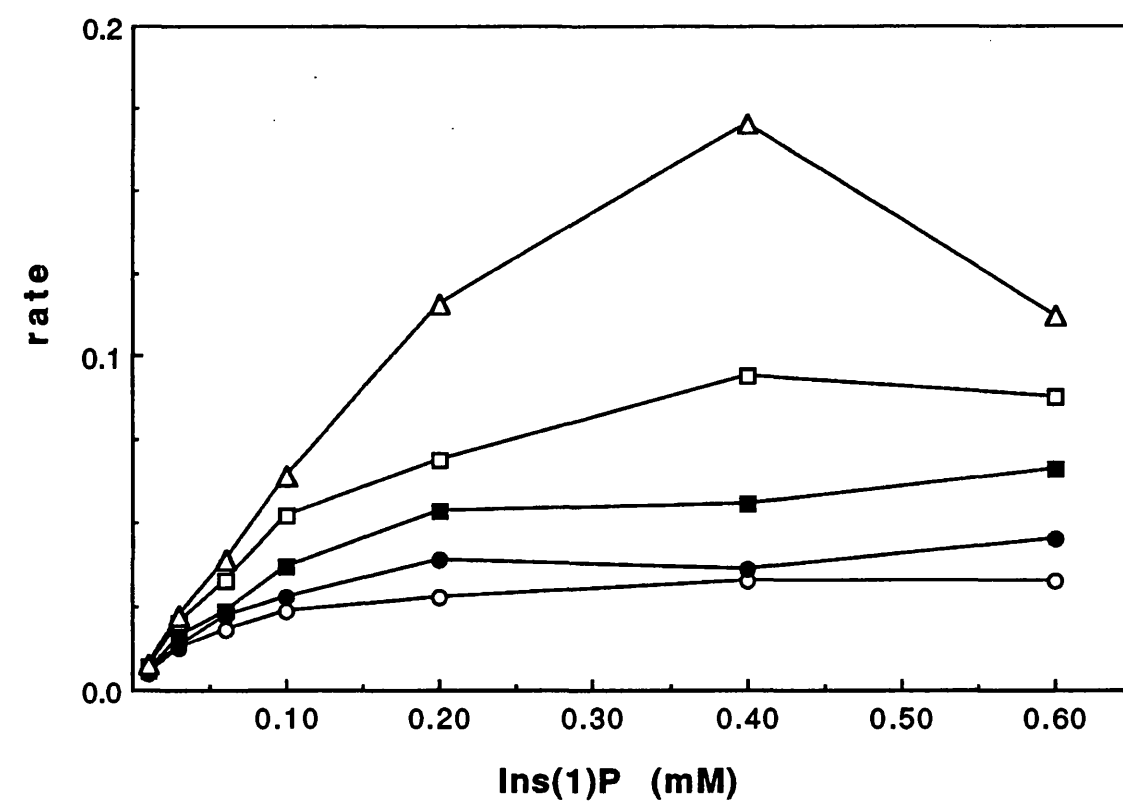
$$K_i = 0.380 \pm 0.06 \text{ mM}$$

Fig. 5.2 - Direct and double reciprocal plots of inositol monophosphatase activity in the presence of increasing concentrations of lithium.

0mM = △ ; 0.4mM = □ ; 0.8mM = ■ ; 1.4mM = ● ; 2.0mM = ○ . Data represents values obtained from one experiment.

## Noncompetitive Fit:

$$V_{max} = 0.165 \pm 0.014$$

$$K_m = 0.119 \pm 0.027 \text{ mM}$$

$$K_i = 0.631 \pm 0.091 \text{ mM}$$

Where:  $V_{max}$ = maximum rate

$K_m$ = Michaelis constant

$K_i$ = the inhibition constant

The data points did not fit to competitive inhibition at all. Because of the rather erratic data obtained from the [I]=0 samples and, perhaps less so for the [I]=0.4mM, noticeable in Fig. 5.2A, it is difficult to assume that the mode of $Li^+$ inhibition on the inositol monophosphatase is either purely uncompetitive or noncompetitive. It is possible that the inhibition is "mixed". At very low substrate concentrations the inhibition appears to be uncompetitive whilst at high substrate concentrations the inhibition tends more towards a noncompetitive mode. Under different conditions it may, therefore be possible to express preferentially either uncompetitive or noncompetitive inhibition. The implications of this are not fully understood. However, an important property of uncompetitive inhibitors of enzyme activity is that they increase in potency as the degree of substrate saturation of the enzyme increases. If (poly)phosphoinositide metabolism is enhanced in manic depressive patients as has been suggested (Berridge et al. 1982; 1989) then the amount of substrate will be increased. Although this cannot reverse either uncompetitive or noncompetitive inhibition in the same way that it can reverse competitive inhibition, it can be seen that the amount of ES complex will be increased. However, as the inhibitor concentration is raised to a higher level, it is possible that the inhibitor will be present in sufficient excess to cause it to bind noncompetitively ie.

to the free enzyme as well as to the ES complex of the inositol monophosphatase. As a result, a more noncompetitive type of inhibition may be observed at much higher $Li^+$ concentrations. Concentrations above 1mM were not investigated in this study however.

Having established that $Li^+$ is a potent inhibitor of the inositol monophosphatase enzyme in a crude cerebral homogenate, the effects of polyclonal antibodies raised to the inositol monophosphatase enzyme purified from bovine brain were tested in a variety of systems. In in vitro situations the C-19 and C-20 antibodies had been shown to inhibit the inositol monophosphatase enzyme. The antibodies were tested against an enriched bovine brain supernatant, the rat brain supernatant used for the studies of $Li^+$ inhibition and three different cells lines. The rapid freeze-thawing method exploited to obtain supernatants containing inositol monophosphatase enzyme from these cells is described in Chapter 2.1. Table 5.1 indicates the percentage inhibition that each of these antibodies displayed assuming that the values obtained with the preimmune sera were 100%. The figures represent the inhibition obtained when 50μl of the appropriate antisera was added to a final volume of 300μl containing approximately 2-7,000 dpm $[^{14}C]Ins(1)P$.

The enriched bovine brain supernatant exhibited about 80% inhibition in the presence of either the C-19 or C-20 antibodies. This is not surprising as the antibodies were originally raised to purified bovine brain monophosphatase. A high degree of recognition would, therefore, be expected. Under these conditions the presence of just 5μl of antisera was sufficient to cause between 60% and 70% inhibition within 15 min. Both the human cell lines - SH-SY5Y and HL-60 - were also inhibited by the C-19 and C-20 antibodies by between 60% and 80%. Interestingly neither displayed increasing inhibition with increasing bleed number. Normally, the titre of each bleed increases such that, in this case, more inhibtion would be expected in the 4th bleed than in the 2nd and 3rd bleeds. However, it was

| Sample | Bleed No. | C-19 Antibody | C-20 Antibody |
|--------|-----------|---------------|---------------|
| SH-SY5Y | 2 | 72.7 | 76.6 |
| | 3 | 76.8 | 76.5 |
| | 4 | 76.6 | 66.9 |
| HL-60 | 2 | 66.0 | 85.0 |
| | 3 | 59.0 | 84.9 |
| | 4 | 71.0 | 75.2 |
| CHOml | 2 | ZERO | 86.8 |
| | 3 | ZERO | 88.8 |
| | 4 | ZERO | 82.3 |
| Rat Brain Supernatant | 2 | ZERO | 23.4 |
| | 3 | ZERO | 4.0 |
| | 4 | ZERO | 7.0 |
| Bovine Brain Supernatant | 2 | 78.0 | 72.2 |
| | 3 | 88.0 | 73.0 |
| | 4 | 89.4 | 76.5 |

Table 5.1

Effects of two inhibitory polyclonal antibodies on the production of free inositol in selected cell extracts and brain supernatants.

Figures are expressed as percentage inhibition assuming the preimmune sera represents 0% i.e. no inhibition and 100% represents total inhibition. Data represents the mean of 2 separate experiments.

the effects of the antibodies on supernatants obtained from CHO cells with a stable $M_1$ receptor transfect and crude rat brain homogenate that were the most striking. Firstly, the C-19 antibody appeared to have no inhibitory effect upon the inositol monophosphatase from $CHO_{m1}$ cells whilst the C-20 antibody inhibited enzymic activity by over 80%. Perhaps most surprising is the apparent lack of effect of either the C-19 or C-20 antibodies on inositol monophosphatase from rat cerebral homogenate supernatant. Recent evidence using monoclonal antibodies raised to the inositol monophosphatase has suggested that the inhibitory epitope at which these antibodies are directed is within the 10 amino acids found at the $-NH_2$ terminal of the inositol monophosphatase protein (Ian Ragan, pers. commun.). Certainly in mutants of the bovine brain enzyme cloned by Diehl et al.(1990) where one or more of the amino acids is deleted or altered there appears to be a clear reduction in the phosphatase activity of the enzyme. This may explain the data observed in Table 5.1. The $-NH_2$ terminal sequence of the inositol monophosphatase may be the same in bovine brain, SH-SY5Y and HL-60 cells, whilst that found in rat brain may be different. This difference may be in just one amino acid or it may affect several in the sequence if this is indeed the site of action of the polyclonal antibodies used in these experiments. The potential that evolutionary differences do occur should not be overlooked. If this is the case there may be other examples of systems in which these C-19 and C-20 antibodies would not elicit an inhibition of the inositol monophosphatase activity. Having established that, at least in the majority of the conditions examined here, the C-19 and C-20 antibodies have an inhibitory action by binding to the native inositol monophosphatase, it was important to substantiate this and the theory that the antibodies were binding to a particular sequence of the protein by denaturing the enzyme.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) causes disruption of most protein-protein and protein-lipid interactions thus causing the native conformation of the protein to be lost. Comassie blue

Fig. 5.3 - SDS-PAGE analysis of the proteins in both rat and bovine brain homogenates and in extracts prepared from $CHO_{m1}$ and HL-60 cells. The protein markers run in the lane indicated are as follows:

| | | |
|---|---|---|
| Rabbit muscle phosphorylase | M.W. = | 97400 |
| Bovine serum albumin | | 66200 |
| Hen egg white ovalbumin | | 42699 |
| Bovine carbonic anhydrase | | 31000 |
| Soybean trypsin inhibitor | | 21500 |
| Hen egg white lysozyme | | 14400 |

(values expressed in daltons).

# SDS-Page of Cell Extracts and Brain Homogenates



Rat    Bovine    HL-60    CHO$_{m1}$    Markers

RM37-02
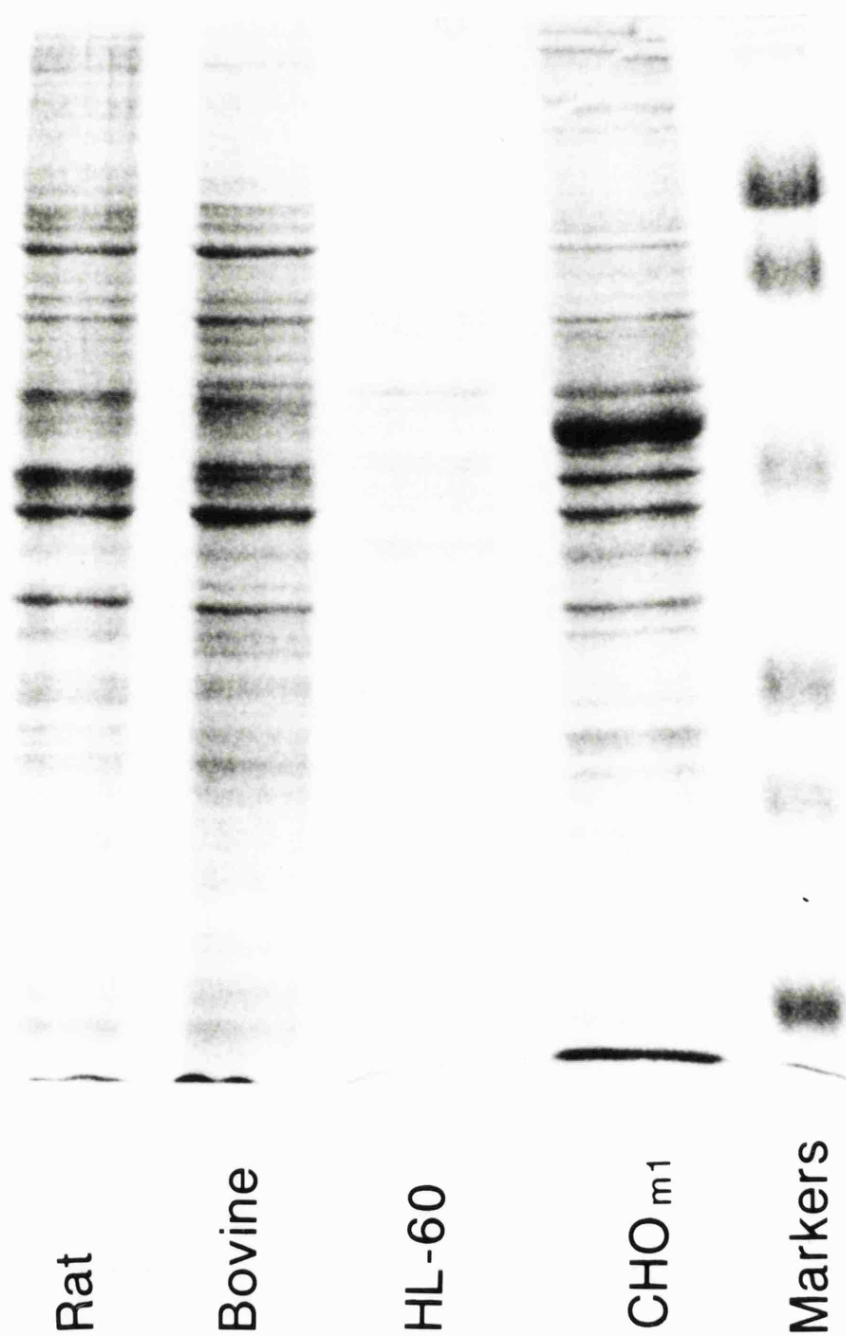
staining will highlight the protein bands obtained after electrophoresis. Fig. 5.3 shows the gel obtained by this process. Approximately 20μg protein was loaded into each track and, as can be seen, proteins from $CHO_{m1}$ cell extract, enriched bovine brain homogenate and crude rat brain homogenate supernatants are stained heavily. Unfortunately, because of difficulties in maintaining HL-60 cells in culture, the quantities obtained from the freeze-thawing process were much less and, correspondingly, less protein was loaded onto the gel. As a result, the protein in the HL-60 track is much more faintly stained compared to the other three.
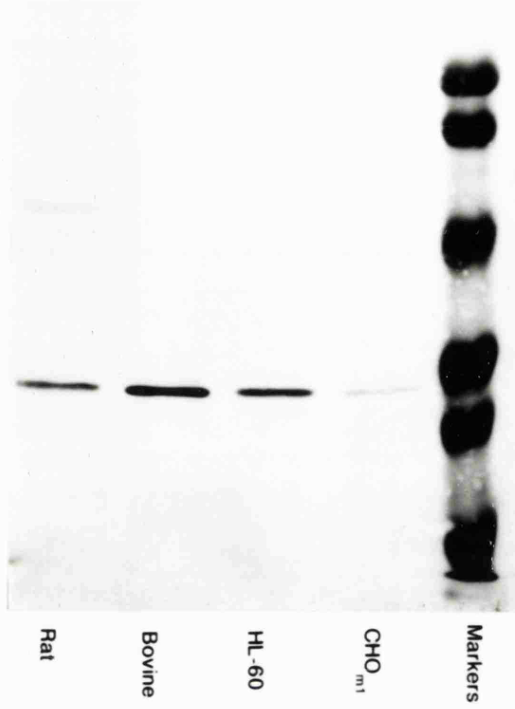
Two other gels which had been run were loaded onto nitrocellulose paper in order to obtain western blots. Fig. 5.4 displays the western blots obtained. The blots were washed in a TBS/Tween/milk powder medium containing the appropriate dilution of the antibody - either C-19 or C-20 for 2 hrs. Upon termination of the reaction, both the C-19 and C-20 antibodies could be seen to have detected a band assumed to be the inositol monophosphatase enzyme. This assumption was made on the basis of the apparent molecular weight. This was approximately 30kDa which corresponds to the subunit molecular weight of the enzyme in question. Detection of this same band was present in $CHO_{m1}$ and HL-60 cell extracts as well as in the bovine brain and rat brain supernatants. The specificity of both these antibodies is apparent with a dilution of 1:1000 being sufficient. In all four western blots in Fig. 5.4, the strongest bands appears in the track which contained enriched bovine brain homogeate supernatant. Again, this is to be expected as the antibodies were raised to the purified bovine brain enzyme. Rat brain, $CHO_{m1}$ and HL-60 cell extracts are all detected although the $CHO_{m1}$ cell extract is consistently less strongly stained than the others. Interestingly, in the presence of either antibody at either dilution rat brain inositol monophosphatase is detected.

These results indicate that both the C-19 and C20 antibodies appear to be recognizing a sequence within the polypeptide chain and not a conformation

Fig. 5.4 - Western analysis of crude extracts prepared from rat and bovine brain homogenates and $CHO_{m1}$ and HL-60 cells. The two polyclonal antibodies, C-19 and C-20, are present in two different dilutions. The molecular weight markers used were the same as those detailed in Fig. 5.3. This would indicate that the protein the antibodies specifically recognize has a molecular weight of between 21500 and 31000 daltons. The molecular weight of the inositol monophosphatase is approximately 30kDa (Takimoto et al. 1985; Gee et al. 1988).

# C-19 Antibody

## 1:200 dilution



Rat

Bovine

HL-60

CHO$_{m1}$

Markers

## 1:1000 dilution

Rat

Bovine

HL-60

CHO$_{m1}$

Markers

# C-20 Antibody

## 1:200 dilution

Markers

CHO$_{m1}$

HL-60

Bovine

Rat

## 1:1000 dilution

Markers

CHO$_{m1}$

HL-60

Bovine

Rat
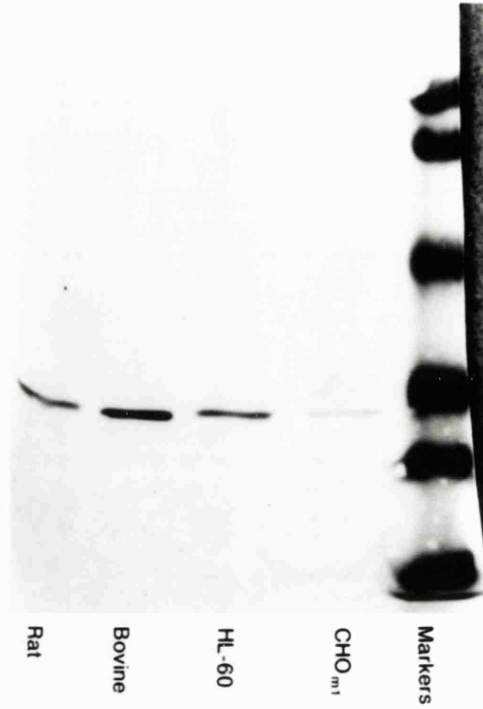
which would have been lost under this type of denaturing condition. When compared to the data in Table 5.1, it is clear that both C-19 and C-20 antibodies, even at high dilutions, recognize the rat brain inositol monophosphatase and that the C-19 recognizes 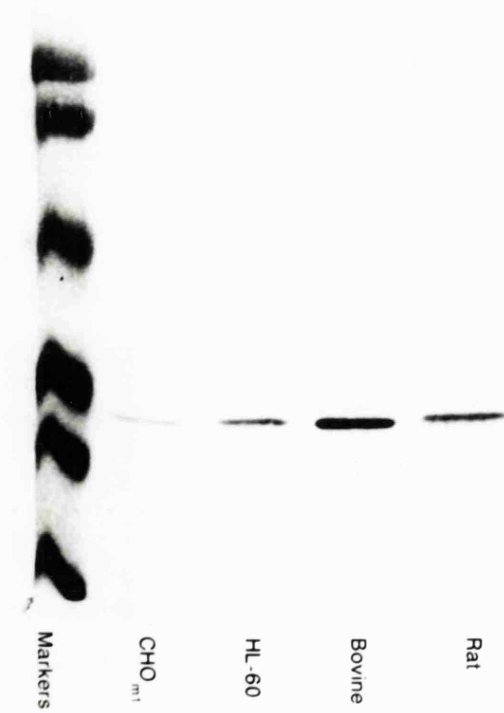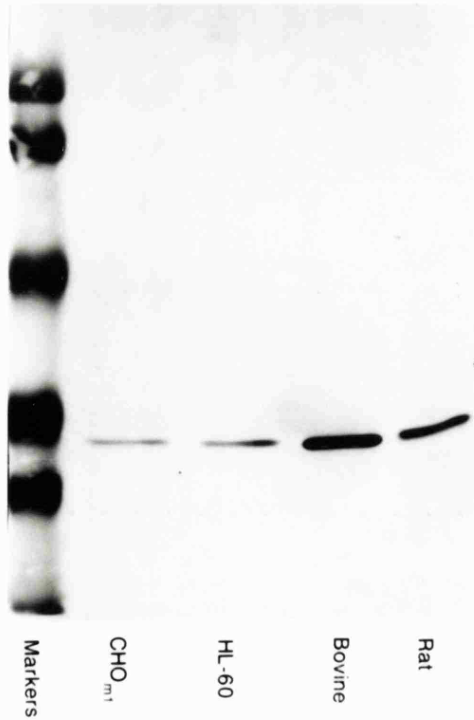the same enzyme from $CHO_{m1}$ cell extracts yet in none of these examples were the antibodies inhibitory towards the activity of native inositol monophosphatase. In these instances at least, there must be epitopes to which antibodies bind without causing inhibition.

Having established that in most systems the C-19 and C-20 antibodies potently inhibit the inositol monophosphatase activity and with the knowledge that $Li^+$ also inhibits the inositol monophosphatase enzyme it was important to try to compare them. From the studies described above and in previous chapters, it was confirmed that $Li^+$ could inhibit inositol monophosphatase activity in tissue and cell preparations as well as in cell-free extracts. However, the experiments involving the polyclonal antibodies had only been conducted in cell-free supernatants. As a result, several experiments were attempted to introduce antibodies into cells. The aim was to compare and contrast the effects of $Li^+$ in the cell with the effects of the antibody in the cell. Previous data and discussion has suggested that $Li^+$ actions within a cell involve more than a simple mixed inhibition of the inositol monophosphatase. The only action of the antibody, in comparison, is the inhibition of this enzyme. Therefore, if the antibody could elicit the same effects as $Li^+$ within a cell then a good case could be made for $Li^+$ only having one site of action, namely the inositol monophosphatase enzyme. Theoretically, however, if the antibody could only increase the intracellular $[^3H]InsP_1$, for example, in carbachol-stimulated cells, whilst having no effect on the accumulation of $[^3H]Ins(1,4,5)P_3$ or $[^3H]InsP_4$, then this might be indicative of another site of $Li^+$-induced activity within the cell.

In 1984 McNeil et al. described a novel technique for incorporating macromolecules into adherent cells grown in culture. This method, termed

"scrape-loading" was found to incorporate dextran molecules of up to $Mr=70,000$ very efficiently and, indeed, dextran of up to $Mr=2,000,000$ was loaded although this was much less effective. As described in Chapter 2, cells were "scrape-loaded" and allowed to replate for about 24 hrs. Fig. 5.5 shows the effects of this method on $CHO_{ml}$ cells grown in culture. Photograph A is of confluent $CHO_{ml}$ cells. Photographs B and C are taken approximately 8 hrs and 24 hrs after loading. McNeil et al. (1984) quote that, after "scrape-loading" about 40% of the cells will be lost but that of the remaining cells, 90% will replate. Fig. 5.5 shows the data obtained from "scrape-loaded" $CHO_{ml}$ cells with the C-20 antibody (4th bleed). There appears to be no significant difference between the results obtained from cells scraped in the presence of antibody and those scraped in the absence i.e. the antibody did not enter the cells sufficiently to cause detectable inhibition of the inositol monophosphatase.

Most of the work which has utilized the "scrape-loading" technique has been involved in loading molecules of a much lower molecule weight than an antibody. Assuming most of the immunoglobulin present is IgG then the molecular weight is approximately 150kDa. McNeil et al. (1984) have calculated that the pore opened by scraping will have a diameter of around 50nM, which at least in theory, should be large enough to accommodate a IgG molecule. However, because of other factors including steric hindrance and the transiency of the openings in the membrane, it must be assumed that little, if any, antibody entered the cell. If antibody did enter the cell, it is also possible that during the 24 hrs between "scrape-loading" and experimentation, the antibody was proteolytically digested or in some other way modified such that it could no longer function as an inhibitor of the inositol monophosphatase.

A commercially available kit which permeabilises cell membranes transiently was also tested. The main advantage of this was that the antibody was added to cells already in suspension and the reaction terminated

A

Confluent $CHO_{m1}$ Cells
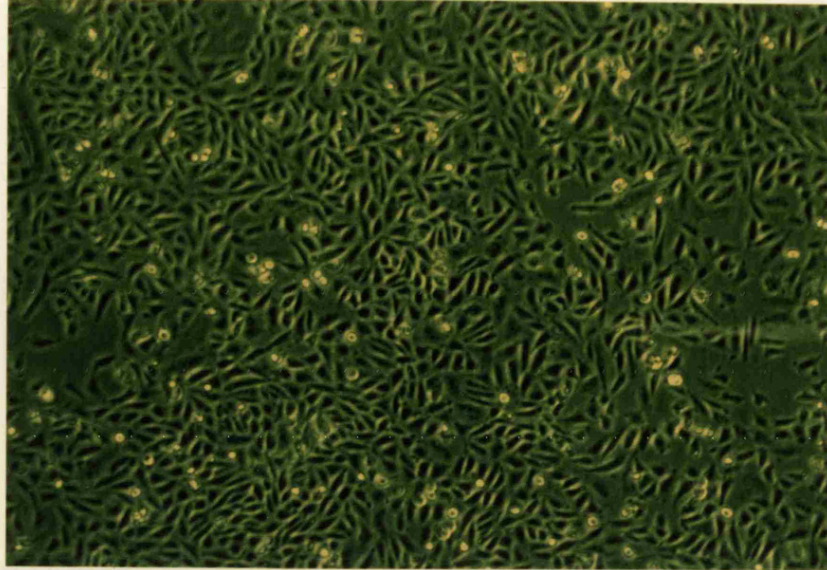


B CHO$_{m1}$ Cells 6 Hours After Scrape Loading
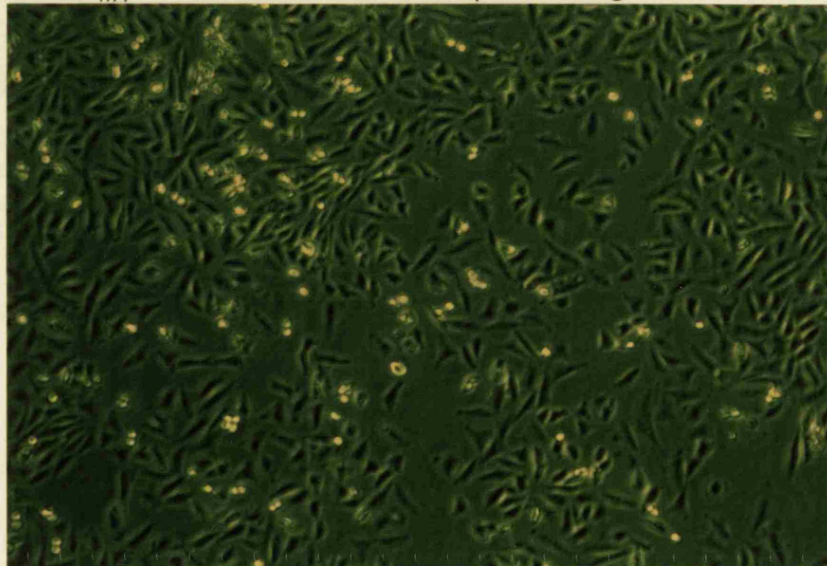


C CHO$_{m1}$ Cells 24 Hours After Scrape Loading

Fig. 5.6 - Effect of "scrape-loading" C-20 polyclonal antibody on inositol monophosphatase activity in CHOm1 cells grown in continuous culture. The methods used are described fully in Chapter 2. The data here is from a single experiment representative of several carried out.
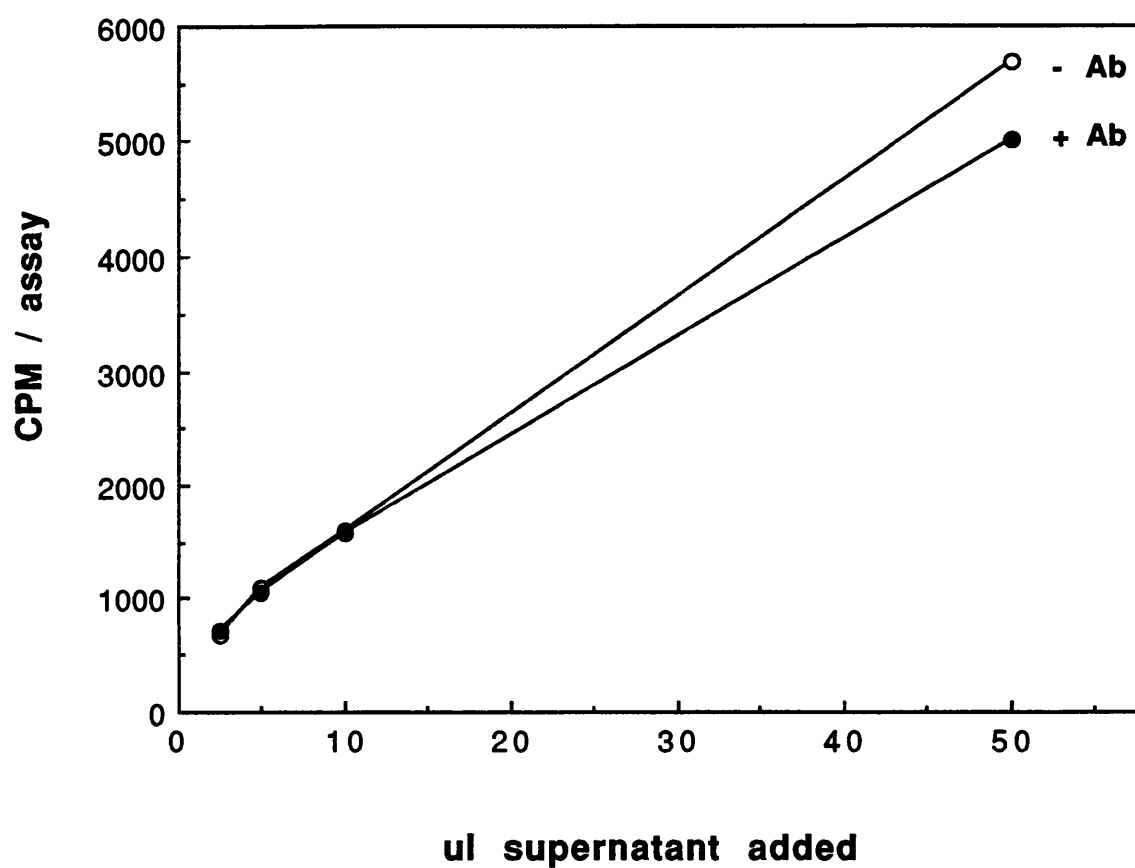
within 15 min. The antibody should therefore be able to inhibit the enzyme without suffering any long term effects. The main disadvantage, however, is that the exclusion limit for this technique is approximately 6500 Da. Further to these experiments it was hoped to use fluorescent tagging molecules to give an indication of how successful the "scrape-loading" of antibodies was at introducing macromolecules into both $CHO_{ml}$ and SH-SY5Y cells but time did not permit these experiments to be carried out. Experiments such as these hold the key to discovering if the reported actions of $Li^+$ are all due to its inhibition of the inositol monophosphatase or if $Li^+$, either directly or indirectly, causes perturbation of inositol (poly)phosphate metabolism via another site of action. However, with these antibodies probably being too large to introduce into a cell, the antibodies would, firstly have to be purified and then cleaved proteolytically into the "heavy" and "light" chain moieties. The Fab fragments would then have to be tested in cell free extracts to ensure inhibition of the inositol monophosphatase was still occurring. With a molecular weight of 25-30kDa, "scrape-loading" of these fragments may be more successful in elucidating the intracellular actions of $Li^+$. The opportunities that this type of experiment present to the furtherance of understanding the intricacies of inositol (poly)phosphate metabolism as well as to pinpointing the site(s) of action of $Li^+$ will be discussed in the next chapter which deals with the conclusions drawn from this study and the possibilites available to future research in this field.

# CHAPTER 6

Conclusions and Further Perspectives

CHAPTER 6

CONCLUSIONS AND FURTHER PERSPECTIVES

The work presented in this thesis fulfils some of the aims of the original research proposal. In this final chapter, a brief summary of the conclusions drawn from these studies will be presented followed by an overview of future studies which could be considered.

The theory that $Li^+$ perturbs (poly)phosphoinositide metabolism has been proven by studies investigating the concentration and temporal dependent effects of this ion on the accumulation of the individual inositol phosphate fractions. The inositol monophosphate fraction has been shown to be potently affected in the presence of $Li^+$ in all three of the systems which were studied here, namely rat cerebral cortical slices, $CHO_{m1}$ cells and SH-SY5Y cells. The $EC_{50}$ values obtained for the accumulation of both the inositol mono- and bisphosphate fractions agreed effectively with the $K_i$ values which have been obtained from studies on purified preparations of the corresponding enzymes. This is particularly true of the rat cerebral cortex.

It has also been shown that, whilst the effects of $Li^+$ on these two fractions may be easy to predict, the effects on the tris- and tetrakisphosphate fractions are not. During the course of this research an enzymic method for separating labelled $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ has been developed. This has proved particularly efficient in studies of rat cerebral cortical slices which, under the labelling conditions used here, only accumulate these two trisphosphate isomers. As a result of this method, coupled to studies of the mass amount of $Ins(1,4,5)P_3$ as measured by a radioreceptor assay it has been shown for the first time that $Li^+$ causes reduced accumulation of $Ins(1,4,5)P_3$ in carbachol stimulated rat cerebral cortical slices. Previous to this study in work from this laboratory, Batty et al. (1987) had indicated that $Li^+$ potently inhibited the accumulation of

$InsP_4$ either by reducing its synthesis or by enhancing its metabolism. This, too, has been confirmed in experiments here using rat cerebral cortical slices. The results obtained from these two fractions in the corresponding experiments in cells have been more difficult to explain however. Such results are complicated by the fact that the cells are labelled for 48 hours compared to the 60 mins labelling period for the rat cerebral cortical slices. This, in turn, can lead to the labelling of trisphosphate isomers, which, although may be present in this latter system, are not labelled under the conditions used here. Furthermore, in the experiments discussed in Chapter 4, the trisphosphate fractions are not enzymically split to isolate the $Ins(1,4,5)P_3$ fraction. Therefore, the results are further confused by the presence of all the labelled trisphosphate isomers.

In Chapter 3 work was presented which suggested that, in the presence of $Li^+$, $[^{14}C]CMP-PA$ accumulates dramatically in rat cerebral cortical slices. As a precursor to PtdIns resynthesis, these results demonstrate the potent effects of $Li^+$ on the production of free inositol via inositol (poly)phosphate metabolism. Indeed, the inhibitory effect of $Li^+$ on the inositol monophosphatase enzyme in a rat cerebral cortical preparation was also established. Latterly, also, the ability of polyclonal antibodies raised to the inositol monophosphatase enzyme was investigated.

This aspect of the work is undoubtedly the most promising in the attempts currently being made to decipher the intracellular actions of $Li^+$. It is widely accepted that, as the major drug commonly prescribed for the treatment of manic depression, $Li^+$ selectively targets the central nervous system. It is highly effective in stabilizing both unipolar and bipolar manic depression and yet its mechanism of action has remained elusive ever since Cade (1949) first used $Li^+$ salts in the treatment of mania over 40 years ago. With the discovery that $Li^+$ inhibits the inositol monophosphatase enzyme, a good case for this being the intracellular target for $Li^+$ was made (Allison et al. 1976). However, the profusion of reports which have followed since, indicate

that $Li^+$ has perhaps more than one side of action (see for example Avissar et al. 1988; Newman and Belmaker, 1987). Indeed, the work presented here also implies that this may be true.

Several studies involving the additions of myo-inositol before and after $Li^+$ has exerted its effect(s), have indicated that, even at very high concentrations, myo-inositol cannot fully reverse the $Li^+$-induced actions on the inositol polyphosphate fractions. The addition of myo-inositol prior to stimulation caused significant reductions in the levels of measurable $[^{14}C]$CMP-PA. Subsequent addition of 10mM myo-inositol could prevent, but, noticeably, not reverse the accumulation of $[^{14}C]$CMP-PA. Indeed, concentrations as high as 30mM were required before any such reversal was apparent. Similarly, myo-inositol addition in experiments in which the mass amounts of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ were made indicated that, although its presence could delay the onset of the observed response by around 5 mins, it could not prevent fully the reduced accumulation. Whilst this may be indicative of a less efficient inositol transport mechanism, the fact that total reversal of the effects described above is not apparent may suggest that not all the actions of $Li^+$ are accountable for solely in its inhibition of the inositol monophosphatase enzyme. This is clearly an important aspect if the block that it causes on inositol (poly)phosphate metabolism has other, more wide-ranging implications on the cell. During the stimulatory period, another action which is not reversible by the presence of exogenous myo-inositol may be occurring. Covalent modification of one or more of the components of the pathway would result in a modification which would be irreversible by myo-inositol. The degree to which these possibilities are expressed in the presence of $Li^+$ compared to the effects caused simply by the inhibition of the inositol monophosphatase is not known. With the advent of antibodies which are selective for the inositol monophosphatase, this confusion may soon be resolved.

If these antibodies can be introduced into a cell type in which all

preliminary investigations involving $Li^+$ have been well characterized, then they will render the enzyme inactive. The effects of a non-functional inositol monophosphatase enzyme can be compared with the original results. If the observed effects are identical then it will be clear that $Li^+$, by the simple inhibition of the monophosphatase, can result in its repetoire of known actions. If, however, the two sets of data do not correspond, then a clearer indication that $Li^+$ can act at a site distal to the inositol monophosphatase will be evident.

Unfortunately, gaining access to the cell in question may prove difficult for the antibody. With its enormous molecular weight, the only feasible alternatives are to cleave the antibody into its constituent heavy and light chains or to inject the antibody into the cell. Both these solutions have major drawbacks, however. By separating the immunoglobulin into its Fab Fc parts, the intrinsic activity of the antibody may be destroyed. Only the Fab fragments have antigenic binding capacity but the Fc fragment may play a pivotal role in aligning the Fab "arms" with the epitope on the enzyme i.e. the so-called "segmental flexibility" would be destroyed. If the antibody is to be injected intact into the cell, the cell type will obviously be limited. Xenopus oocytes are often used for studies of this nature, for example, because of their large size. Furthermore, the long-term stability of antibodies inside cells is not fully understood. It may be possible that proteases will immediately attack the antibody and start to digest it.

Another possibility of deleting the endogenous activity of the inositol monophosphatase enzyme is through the use of antisense RNA. When present in a cell, antisense RNA will cause the translation of a protein which is different from its native state. This is usually done by altering the nucleic acid code at the active site of the enzyme. In so doing, the antisense RNA present will cause the inositol monophosphatase enzyme made by the intracellular protein synthesis apparatus to be non-functional. This, in effect, is the same as using agents to inhibit the native enzyme. The

antisense RNA could be introduced into the cell by injection or by the attachment of a lipophilic headgroup molecule which would facilitate its transport across the cell membrane.

None of the examples suggested are likely to be therapeutically beneficial however. $Li^+$, with its very narrow safe therapeutic range and unpleasant side effects, is an obvious target for todays pharmaceutical industries. Therefore, to discover a replacement drug which would alleviate psychological disorders as effectively as $Li^+$, would obviously be advantageous. If a relevant uncompetitive inhibitor of the inositol monophosphatase can be found then this may be effective. However, as discussed in Chapter 5, uncomptetitive inhibition is unusual and such inhibitors can be toxic. Competitive inhibitors, although common, may not be particularly effective as they would lead to very high resting levels of the inositol (poly)phosphates in all the cells in the central nervous system and would not selectively target those cells which were subject to chronic stimulation . This would occur because the competitive inhibitor would not be stimulus dependent.

Importantly, the raison d'etre of (poly)phosphoinositide lipid hydrolysis must be expounded. The role of inositol (poly)phosphates in intracellular neuronal $Ca^{2+}$ homeostasis has recently been reviewed (Nahorski, 1988). In it, it is suggested that they are fundamental in controlling ion channel excitability in neuronal tissue e.g. $Ca^{2+}$ activated $K^+$ channels. Indeed, work has suggested that PKC activation by DAG can block such activity in hippocampal pyramidal cells (Baraban et al. 1985) indicating that perhaps both the moieties derived from (poly)phosphoinositide lipid hydrolysis play essential roles in the acute effects of receptor stimulation upon ion channel conductance. Furthermore, if $Li^+$ does have profound effects both upon the metabolism and/or synthesis of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ and the accumulation of DAG in vivo, then it might be expected that this ion will alter the intracellular $Ca^{2+}$ concentration in cells undergoing cholinergic

stimulation. This would lead to an alteration in the activity of the neuron and, indeed, may radically affect the enzymes involved in (poly)phosphoinositide signalling. For example, the substrate specificity of the PLC enzymes may change as might the translocation of PKC.

The effects on the long-term action(s) of (poly)phosphoinositide metabolism are clearly also of importance. There is early evidence linking increases in intracellular $Ca^{2+}$ to increased expression of the early response genes like c-fos, c-jun and c-myc (see for example Morgan and Curran, 1988). If gene expression is enhanced, what genes are transcribed  This so-called "signal transcription" pathway could evolve quickly as interest in the potential links between (poly)phosphoinositide metabolism and cell growth increases. (Poly)phosphoinositide metabolism may hold the key to understanding the factors which cause quiescent cells to transform and proliferate.

It can, therefore, be seen that the (poly)phosphoinositide cell signalling pathway has a long way to go before its complexities are fully understood and obviously extensive further research is required. $Li^+$ will undoubtedly remain one of the most useful tools in these investigations and, although its actions may soon be unravelled, the long-term effects of $Li^+$ treatment may provide insight into the long-term effects of (poly)phosphoinositide metabolism within the cell.

A P P E N D I C E S

## ABBREVIATIONS

Unless otherwise stated, "inositol" refers to myo-inositol. $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$ and $InsP_6$ refer to myo-inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis- phosphates respectively. Where appropriate the positions of the phosphate locants around the inositol ring are given to denote a specific isomer e.g. $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$. Enantiomeric definitions of D- and L- are given only where it is essential. Similarly, the (poly)phosphoinositide lipid abbreviations are as follows:

PtdIns = 1-(3-sn-phosphatidyl)-D-myo-inositol

PtdIns(4)P = 1-(3-sn-phosphatidyl)-D-myo-inositol-4-monophosphate

$PtdIns(4,5)P_2$ = 1-(3-sn-phosphatidyl)-D-myo-inositol-4,5-bisphosphate


AEC = 3-amino-9-ethylcarbazole

APS = ammonium persulphate

CMP-PA = CMP phosphatidic acid

DAG = diacylglycerol

DTT = dithiothreitol

EDTA = ethylenediaminotetraacetic acid

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

ER = endoplasmic reticulum

GroPtdIns = glycerophosphorylinositol

$H_2O_2$ = hydrogen peroxide

HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

$K_m$ = Michaelis-Menten constant

mAChR = Muscarinic acetylcholine receptor

OPH = 1,10-phenanthroline monohydrate

PA = phosphatidic acid

SDS = sodium dodecyl sulphate

TEMED = N,N,N',N' tetramethylethylenediamine

TBS = Tris-buffered saline

TCA = trichloroacetic acid

$V_{max}$ = maximum velocity of an enzyme catalyzed reaction

2'-AMP = 2'-adenosine monophosphate

2'-GMP = 2'-guanine monophosphate

4-DAMP = 4-diphenylacetoxy-N-methyl piperidine methiodide


## MATERIALS


All radiochemicals were purchased from either New England Nuclear or Amersham International. Other chemicals were obtained from Fisons, Aldrich, BDH or Sigma.

Dowex anion exchange resin was from Sigma as were all drugs.

Liquid scintillation cocktails were from Fisons or May and Baker

All media and supplements used for cell culture were obtained from Gibco.

Scyllo-inositol was a gift from Merck, Sharp and Dohme Research Laboratories, Harlow, Essex.

## BUFFERS

| Krebs-Henseleit: | | (g/l) | (mM) |
|---|---|---|---|
| | NaCl | 6.94 | 118.6 |
| | KCl | 0.35 | 4.7 |
| | $MgSO_4.7H_2O$ | 0.29 | 1.2 |
| | $CaCl_2.2H_2O$ | 0.19 | 1.3 |
| | $KH_2PO_4$ | 0.16 | 1.2 |
| | $NaHCO_3$ | 2.10 | 25.0 |
| | Glucose | 2.10 | 11.7 |

Running Gel Buffer: 1.5M TRIS pH 8.8

Running Gel: 3ml 30% acrylamide, 2.5ml running gel buffer, 32$\mu$l 10% APS, 100$\mu$l 10% SDS, 10$\mu$l TEMED, 4.35ml $H_2O$

Stacking Gel Buffer: 1.0M TRIS pH 6.8

Stacking Gel: 400$\mu$l 30% acrylamide, 1ml stacking gel buffer, 30$\mu$l 10% APS, 40$\mu$l 10% SDS, 10$\mu$l TEMED, 2.5ml $H_2O$

Gel Loading Buffer: 50mM TRIS pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue

Running Buffer: 25mM TRIS, 250mM glycine, 0.1% SDS, pH 8.3

Destain: 10% acetic acid, 40% methanol

RIPEA (x2.5): 50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 1% deoxycholate, 1% w/v Triton X-100, 1% SDS

R E F E R E N C E S

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