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Characterization of the effects of lithium on phosphatidylinositol (PI) cycle activity in human muscarinic m1 receptor-transfected CHO cells

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1 The effects of lithium on [^{3}H]-inositol and [^{3}H]-cytidine incorporation into [^{3}H]-inositol monophosphates ([^{3}H]-$InsP_1$) and [^{3}H]-cytidine monophosphorylphosphatidate ([^{3}H]-CMP-PA), respectively, and inositol 1,4,5-trisphosphate ($InsP_3$) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) mass were studied in carbachol-stimulated human m1 muscarinic receptor-transfected Chinese hamster ovary cells (m1 CHO cells).

2 Lithium alone (10 mM) had no appreciable effects on any of the four parameters measured; it was only in carbachol-stimulated cells that the effects of lithium became apparent.

3 In the presence of carbachol (1 mM), lithium (10 mM) caused a relatively rapid (within 5 min) accumulation of [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA which continued up to about 20–30 min, after which accumulation slowed down. On the other hand, the elevation in $InsP_3$ and $InsP_4$ levels produced by carbachol was not altered by lithium in the short-term and only at later times (>20–30 min) was the response attenuated, with $InsP_3$ and $InsP_4$ levels approaching basal.

4 The effects of lithium on carbachol-stimulated [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA accumulation and the attenuation of the carbachol-induced elevation of $InsP_3$ and $InsP_4$ were all dose-dependent, with EC_{50}s in the region of 1 mM.

5 The lithium-induced effects on [^{3}H]-CMP-PA and $InsP_3$ and $InsP_4$ in carbachol-stimulated cells could be reversed, in a dose-dependent manner, by preincubation with exogenous *myo*-inositol (EC_{50} = 2–3 mM) but not by the inactive analogue *scyllo*-inositol, indicating that these effects occur as a consequence of depletion of inositol.

6 The temporal effects of lithium are consistent with lithium inhibiting inositol monophosphatase, causing accumulation of $InsP_1$, resulting in lower free inositol levels. This leads to accumulation of CMP-PA and reduced PI synthesis which, once agonist-linked membrane inositol phospholipids are depleted, produces attenuated $InsP_3$ and $InsP_4$ responses.

7 These results in m1 CHO cells support the hypothesis that lithium affects the PI cycle cell signalling pathway by depletion of inositol due to inhibition of inositol monophosphatase.

Keywords: Lithium; phosphatidylinositol; inositol monophosphate; inositol monophosphatase; cytidine monophosphorylphosphatidate; inositol 1,4,5-trisphosphate; inositol 1,3,4,5-tetrakisphosphate; m1 CHO cells; carbachol

Introduction

It is over 40 years since the original description of the use of lithium in the treatment of manic depression (Cade, 1949), yet the mechanism by which lithium exerts its therapeutic effects remains uncertain. It has been proposed, however, that manic depression may be associated with hyperactivity of certain transmitter systems linked to the phosphatidylinositol (PI) signal transduction pathway and that lithium may act by inhibiting the PI cycle as a consequence of depletion of intracellular inositol, thereby reducing the hyperactivity in these transmitter systems (Berridge *et al.*, 1982; 1989; Nahorski *et al.*, 1991).

According to this hypothesis, lithium inhibits inositol monophosphatase (IMPase), which is a key enzyme in the PI cycle in that it dephosphorylates inositol monophosphates ($InsP_1$) producing inositol that can combine with cytidine monophosphorylphosphatidate (CMP-PA) to produce PI in a reaction catalysed by the enzyme PI synthase (Berridge & Irvine, 1989; Ragan, 1990). Intracellular inositol originates either from transport into the cell via a low- or high-affinity mechanism or from dephosphorylation of $InsP_1$, which are derived from the inositol 1,4,5-trisphosphate ($InsP_3$) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) second messengers or as an intermediate in the *de novo* synthesis of inositol from glucose 6-phosphate. In cells where inositol is derived primarily from the dephosphorylation of $InsP_1$ by IMPase rather than by transport mechanisms, inhibition of IMPase effectively depletes intracellular inositol and results in reduced membrane inositol phospholipids available for signal transduction, ultimately resulting in reduced levels of $InsP_3$ and $InsP_4$ following stimulation.

Evidence in support of this hypothesis originates primarily from work in rat or mouse cortical slice preparations in which, under stimulated conditions, lithium results in an accumulation of $InsP_1$ (Batty & Nahorski, 1985; Kennedy *et al.*, 1989). There is a concomitant accumulation of CMP-PA, presumably as a result of decreased inositol concentrations (Godfrey, 1989; Kennedy *et al.*, 1989; Nahorski *et al.*, 1991) and an attenuation of the stimulus-dependent increase in $InsP_3$ and $InsP_4$ (Kennedy *et al.*, 1989; 1990; Varney *et al.*, 1992).

Clearly, the effects of lithium on [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA, which accumulate as a direct effect of the inhibition of IMPase, should occur prior to any effects on the second messengers $InsP_3$ and $InsP_4$, which should occur not in parallel with the effects on [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA, but only after a lag interval that corresponds to the finite time required to deplete existing agonist-linked membrane inositol phospholipids. To examine the temporal effects of lithium on

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the PI cycle, we have measured, in Chinese hamster ovary cells transfected with the PI-linked (Fisher *et al.*, 1992) human muscarinic m1 cholinoceptor (m1 CHO cells), the incorporation of [^{3}H]-inositol into [^{3}H]-$InsP_1$ and [^{3}H]-cytidine into [^{3}H]-CMP-PA and, using radioreceptor binding assays, the mass of $InsP_3$ and $InsP_4$ second messengers. The results show that in carbachol-stimulated cells, lithium produced a relatively rapid (i.e. within 5 min) accumulation of [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA but that the attenuation of the carbachol-induced increase in $InsP_3$ and $InsP_4$ occurred only after 20–30 min. These effects were all dose-dependent and their reversal by *myo-* but not *scyllo*-inositol is consistent with them being due to a depletion of inositol secondary to inhibition of IMPase.

Methods

[^{3}H]-inositol incorporation into [^{3}H]-$InsP_1$

The method for the incorporation of [^{3}H]-inositol into [^{3}H]-$InsP_1$ was a modification of methods used in the rat cortical slice preparation (Batty & Nahorski, 1985) as described in more detail elsewhere (Atack *et al.*, 1993). Briefly, cells were grown to confluence for 2 days in Eagles minimal essential medium (inositol concentration = 11 μM) containing 10% foetal bovine serum and 0.5 μCi ml^{-1} [^{3}H]-inositol (80 Ci mmol^{-1}). Cells were harvested (2×10^6 cells ml^{-1}) into Krebs Henseleit buffer plus 0.5 μCi ml^{-1} [^{3}H]-inositol. Cells (280 μl) were then incubated at 37°C in the presence of LiCl for 30 min followed by, as required, carbachol (assay concentration = 1 mM), to give a final assay volume of 300 μl. Incubations were stopped by addition of 1 M trichloroacetic acid and samples were centrifuged, washed with diethyl ether and neutralised. Finally, [^{3}H]-$InsP_1$ were separated from [^{3}H]-inositol by Dowex-formate anion exchange chromatography (Berridge *et al.*, 1983).

[^{3}H]-cytidine incorporation into [^{3}H]-CMP-PA

The method employed was modified from that of Downes & Stone (1986). Cells were grown to confluence in 24-well plates (2×10^5 cells/well) and the medium replaced with 200 μl/well of fresh Dulbecco's minimal essential medium (inositol concentration = 40 μM) containing 3 μCi [^{3}H]-cytidine, lithium, *myo-* or *scyllo*-inositol as appropriate and were incubated at 37°C for 30 min followed by stimulation with carbachol (1 mM). Incubations were stopped with 1 ml chloroform:methanol (1:2 v/v) and a further 500 μl chloroform and 500 μl water added to each sample. Samples were aspirated from the wells and centrifuged at 1000 r.p.m. for 10 min and the upper phase removed. The lower phase was washed with 1 ml methanol:1 M HCl (1:1 v/v). Finally, 200 μl of lower phase was transferred to scintillation vials for counting.

Measurement of $InsP_3$ and $InsP_4$

Cells were grown, harvested, incubated and extracted as for the incorporation of [^{3}H]-inositol into [^{3}H]-$InsP_1$ except that no [^{3}H]-inositol was included. The concentrations of $InsP_3$ and $InsP_4$ were measured in separate triplicate 30 μl aliquots (total assay volume of 120 μl) of the same sample using previously published radioreceptor binding assays employing bovine adrenal and rat cerebellar membranes for $InsP_3$ and $InsP_4$ measurements, respectively (Challiss *et al.*, 1988; 1990; Challiss & Nahorski, 1990). Protein concentrations were determined on aliquots of harvested cells by the method of Lowry *et al.* (1951).

Materials

[^{3}H]-inositol and unlabelled $InsP_3$ and $InsP_4$ were obtained from Amersham International plc, [^{3}H]-$InsP_3$ and [^{32}P]-$InsP_4$ were from Du Pont (UK) Ltd, Dowex formate anion exchange resin from Bio-Rad, cell culture media from Gibco and organic solvents from Fisons plc. [^{3}H]-cytidine, carbachol, lithium chloride, *myo*-inositol, *scyllo*-inositol and all other reagents were purchased from Sigma.

Results

Time course of effects of lithium on [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA accumulation

[^{3}H]-$InsP_1$ In the presence of lithium alone (10 mM) or carbachol alone (1 mM) there was a slight elevation in $InsP_1$ levels, by about 20%, even at the earliest time points studied. This degree of increase in $InsP_1$ was maintained throughout the 60 min duration of the experiment with lithium alone whereas with carbachol alone $InsP_1$ levels increased to about 40% above basal at times greater than 20 min (Figure 1a).

In the presence of both lithium and carbachol, there was a pronounced increase in [^{3}H]-$InsP_1$ levels over and above levels in control (i.e. lithium-alone or carbachol-alone) by 5 min after which [^{3}H]-$InsP_1$ continued to accumulate linearly

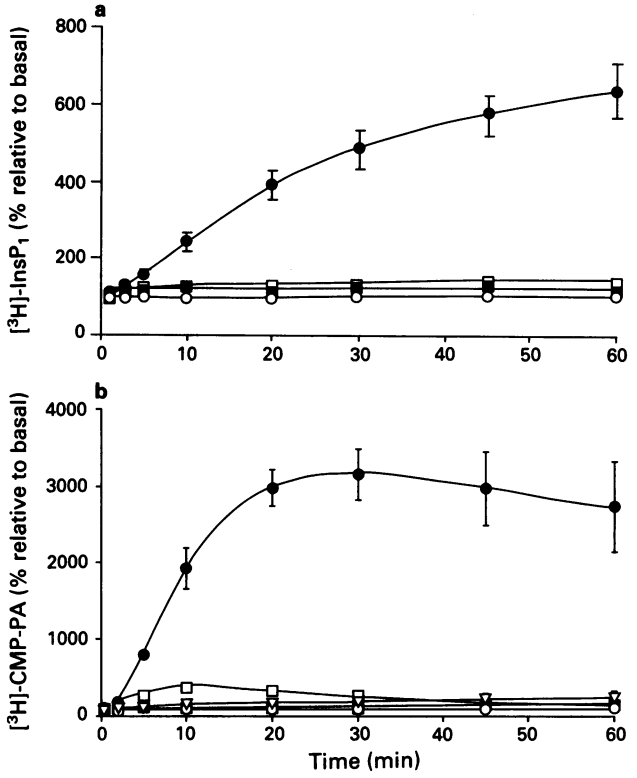

Figure 1 Levels of [^{3}H]-inositol monophosphates ([^{3}H]-$InsP_1$) (a) and [^{3}H]-cytidine monophosphorylphosphatidate ([^{3}H]-CMP-PA) (b) in m1 CHO cells as a function of time after addition of carbachol or buffer under a variety of experimental conditions: (○) basal (no lithium and no carbachol); □ plus carbachol (no lithium plus stimulation with 1 mM carbachol for varying times); (■) plus lithium (preincubation for 30 min with 10 mM lithium followed by addition of buffer); (●) plus lithium and carbachol (preincubation for 30 min with 10 mM lithium and stimulation with 1 mM carbachol); (▽) plus lithium, carbachol and inositol (preincubation for 30 min with 10 mM lithium and 10 mM *myo*-inositol and stimulation with 1 mM carbachol). Values are expressed as a percentage of basal and are presented as mean ± s.e.mean ($n = 7$ and $n = 4$ for [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA, respectively). Note that in carbachol-stimulated cells, accumulation of both [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA occurred within 5 min and that 10 mM *myo*-inositol reversed the lithium-induced accumulation of [^{3}H]-CMP-PA.

until after 20–30 min of stimulation. After 30 min the rate of accumulation slowed down considerably. Maximum levels of [^{3}H]-InsP$_1$ levels reached about 600% of basal levels (Figure 1a).

[^{3}H]-CMP-PA Generally, the effects of lithium on the accumulation of [^{3}H]-CMP-PA (Figure 1b) were similar to those seen with [^{3}H]-InsP$_1$. Thus, lithium alone (10 mM) elevated [^{3}H]-CMP-PA levels by about 20% and carbachol alone (1 mM) also stimulated accumulation but to a greater extent than seen for [^{3}H]-InsP$_1$, with [^{3}H]-CMP-PA achieving levels after 10 min of cholinoceptor stimulation about 4 fold of basal. With lithium plus carbachol there was a 70% increase in levels at 2 min which became pronounced after 5 min. As with [^{3}H]-InsP$_1$, there was a linear accumulation of [^{3}H]-CMP-PA up to about 20 min after which levels plateaued, with maximum [^{3}H]-CMP-PA levels reaching about 30 fold higher than basal.

When cells were preincubated with *myo*-inositol (10 mM) in addition to lithium and then stimulated with carbachol, the effects were dramatic with the large increase in [^{3}H]-CMP-PA seen with lithium and carbachol in the absence of *myo*-inositol being abolished. However, although [^{3}H]-CMP-PA levels were initially (up to about 2 min) around 40% lower than basal, at later time points there was a slight, progressive increase in levels to about 250% of basal by 60 min, suggesting that the reversal of the effects of lithium was about 95%.

Time course of effects of lithium on InsP$_3$ and InsP$_4$

InsP$_3$ Lithium alone (10 mM) had essentially no effect on InsP$_3$ concentrations whereas carbachol alone (1 mM) rapidly produced, within 10 s, a large increase in InsP$_3$ levels to about 300% of basal (Figure 2a). Thereafter, InsP$_3$ concentrations were maintained at between 200–300% of basal throughout the duration of the experiment. In the presence of lithium and carbachol, the short-term (< 10 min) effects (i.e. large increase in InsP$_3$ levels) were essentially the same as in the absence of lithium. However, by 30 min lithium had clearly attenuated the increase in InsP$_3$ caused by carbachol, with InsP$_3$ concentrations reaching basal (Figure 2a). The attenuation of the carbachol-induced elevation of InsP$_3$ by lithium could be reversed by the addition of exogenous *myo*-inositol (10 mM) with the response in lithium/carbachol/*myo*-inositol-treated cells being the same as in cells stimulated by carbachol alone (i.e. *myo*-inositol cancels out the effects of lithium). Indeed, if anything, InsP$_3$ levels were enhanced in the presence of exogenous *myo*-inositol (see also Lee *et al.*, 1992).

InsP$_4$ The effects of lithium on InsP$_4$ (Figure 2b) were qualitatively the same as seen with InsP$_3$. Hence, lithium alone (10 mM) had no effect on InsP$_4$ levels whereas carbachol alone (1 mM) produced a rapid increase in InsP$_4$ levels to about 500% of basal within the first minute. The

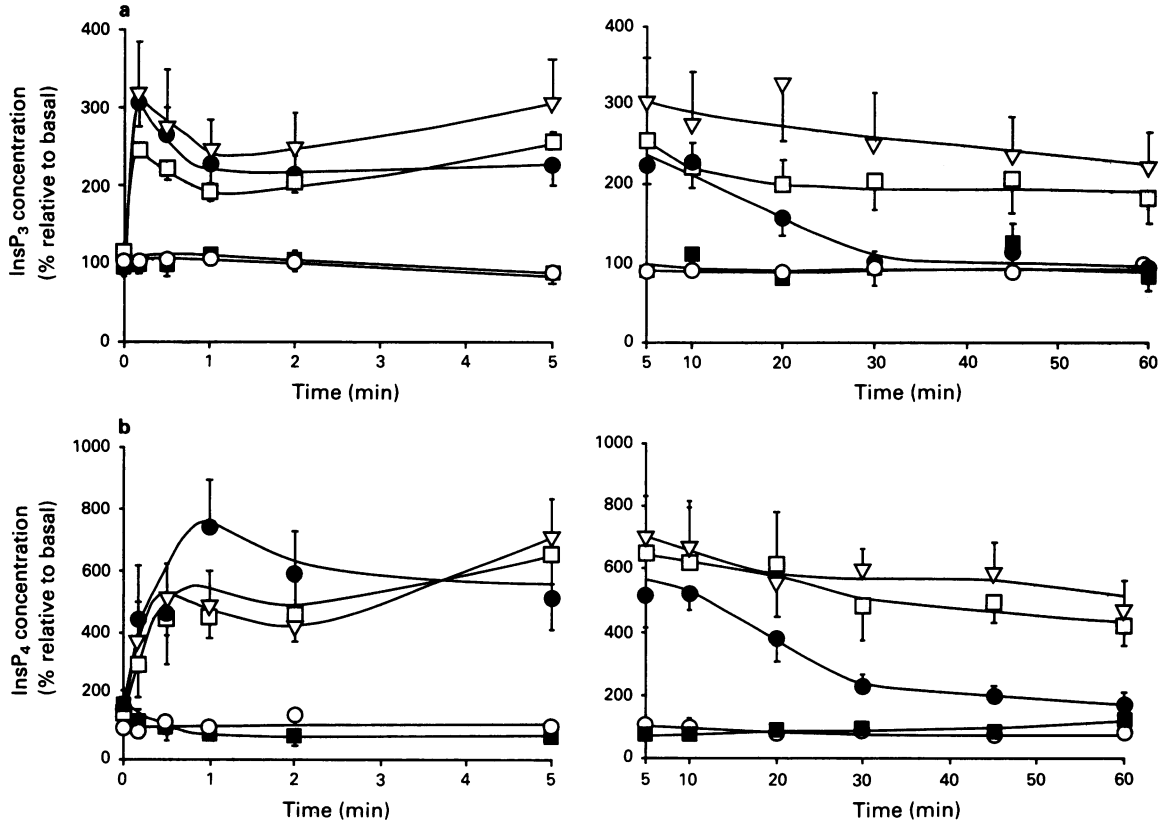

Figure 2 Levels of inositol 1,4,5-trisphosphate (InsP$_3$) (a) and inositol 1,3,4,5-tetrakisphosphate (InsP$_4$) (b) in m1 CHO cells as a function of time after addition of carbachol or buffer under a variety of experimental conditions (figures on left show 0–5 min, figures on right show 5–60 min): (○) basal (no lithium and no carbachol); (□) plus carbachol (no lithium plus stimulation with 1 mM carbachol for varying times); (■) plus lithium (preincubation for 30 min with 10 mM lithium followed by addition of buffer); (●) plus lithium and carbachol (preincubation for 30 min with 10 mM lithium and stimulation with 1 mM carbachol for varying times); (▽) plus Li, carbachol and inositol (preincubation for 30 min with 10 mM lithium and 10 mM *myo*-inositol plus stimulation with 1 mM carbachol). Values are expressed as a percentage of basal values (averaged across the various time points) and are presented as mean ± s.e.mean ($n = 7$ and $n = 4$ for InsP$_3$ and InsP$_4$, respectively). Basal InsP$_3$ and InsP$_4$ concentrations were in the region of 30 and 10 pmol mg^{-1} protein, respectively. Note that lithium only started to attenuate the carbachol-induced elevation of InsP$_3$ and InsP$_4$ after 20–30 min and that this attenuation was reversed by the addition of exogenous *myo*-inositol (10 mM).

carbachol-induced elevation of $InsP_4$ was sustained throughout the experiment, with values varying between about 500–600% of basal. The short-term (< 10 min) effects of lithium on the carbachol-stimulated increase in $InsP_4$ were essentially negligible, but by 20 min the response to carbachol became attenuated. However, this attenuation was not as marked as with $InsP_3$ (which reached basal levels by 30 min) with $InsP_4$ levels approaching basal only by 60 min. Again as with $InsP_3$, the lithium attenuation of the carbachol-induced increase in $InsP_4$ could be reversed by *myo*-inositol, with the lithium/carbachol/*myo*-inositol time course being the same as seen with carbachol alone.

Lithium dose-response curves

Figure 3 shows that the effects of lithium on [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA accumulation and on the attenuation of the carbachol-induced elevation of $InsP_3$ and $InsP_4$ levels were all dose-dependent. Moreover, the mean EC_{50}s (± s.e.mean) for these various effects were all comparable: 1.2 ± 0.4 mM ([^{3}H]-$InsP_1$ response); 0.8 ± 0.2 mM ([^{3}H]-CMP-PA); 0.9 ± 0.2 mM ($InsP_3$); and 0.9 ± 0.2 mM ($InsP_4$).

Reversal of effects of lithium by myo- *but not* scyllo-*inositol*

Figure 4 shows that the effects of lithium on [^{3}H]-CMP-PA accumulation and on the attenuation of the carbachol-induced elevation of $InsP_3$ and $InsP_4$ levels could all be reversed, in a dose-dependent manner, by *myo*- but not *scyllo*-inositol. The mean EC_{50}s (± s.e.mean) for reversal by *myo*-inositol were: 1.4 ± 0.2 mM for prevention of lithium-induced [^{3}H]-CMP-PA accumulation and 2.7 ± 0.3 mM and 3.3 ± 0.4 mM for the reversal of the lithium-induced attenuation of the $InsP_3$ and $InsP_4$ responses, respectively.

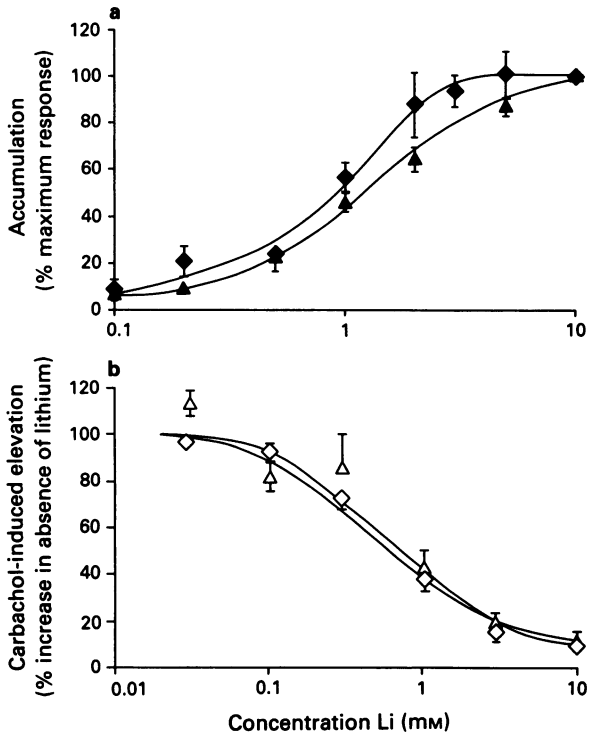

Figure 3 Effects of various concentrations of lithium (preincubation = 30 min) in carbachol-stimulated (1 mM; duration = 60 min) m1 CHO cells on accumulation of [^{3}H]-inositol monophosphates ([^{3}H]-$InsP_1$) (◆) and [^{3}H]-cytidine monophosphorylphosphatidate ([^{3}H]-CMP-PA) (▲) (a) and attenuation of carbachol-induced increase in inositol 1,4,5-trisphosphate ($InsP_3$) (◇) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) (△) (b). Mean (± s.e.mean) EC_{50} values were 1.2 ± 0.4 mM (n = 7) for [^{3}H]-$InsP_1$; 0.8 ± 0.2 mM (n = 6) for [^{3}H]-CMP-PA; 0.9 ± 0.2 mM (n = 6) for $InsP_3$; and 0.9 ± 0.2 mM (n = 5) for $InsP_4$.

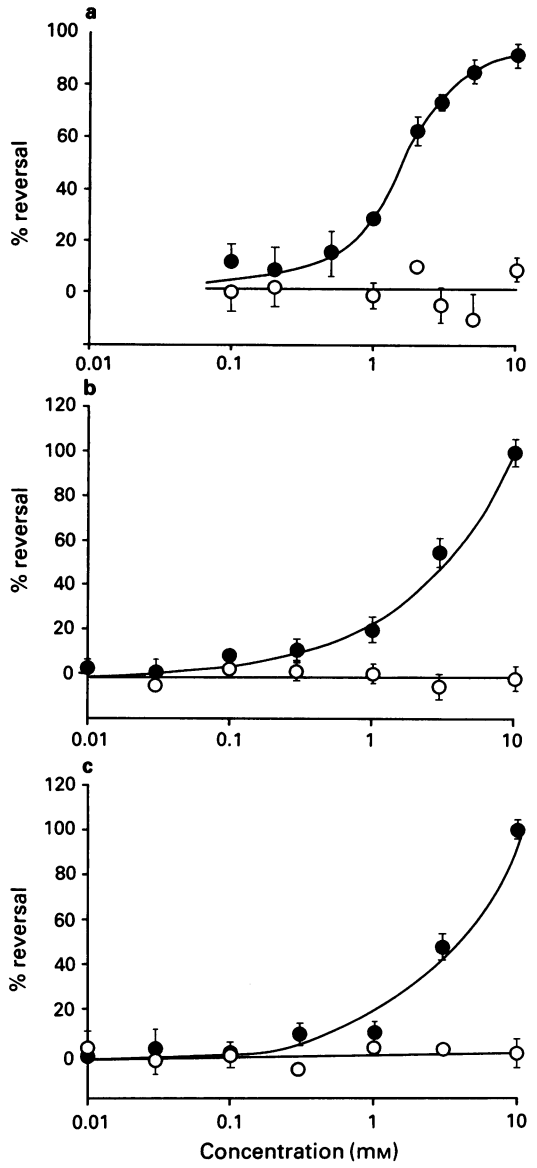

Figure 4 Reversal of the effects of lithium (10 mM, preincubation = 30 min) on carbachol-stimulated (1 mM; duration = 60 min) [^{3}H]-cytidine monophosphorylphosphatidate ([^{3}H]-CMP-PA) accumulation (a) and inositol 1,4,5-trisphosphate ($InsP_3$) (b) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) (c) levels by *myo*-inositol (●) but not *scyllo*-inositol (○) (i.e. experimental conditions plus lithium, carbachol, and [various] *myo*- or *scyllo*-inositol). Mean (± s.e.mean) EC_{50} values for *myo*-inositol reversal for [^{3}H]-CMPA-PA, $InsP_3$ and $InsP_4$ were 1.4 ± 0.2 mM (n = 6), 2.7 ± 0.3 mM (n = 4) and 3.3 ± 0.4 mM (n = 3), respectively.

Discussion

The linearity of [^{3}H]-$InsP_1$ accumulation over the first 20–30 min in carbachol-stimulated m1 CHO cells is comparable to that seen in the rat cortical slice preparation (Gonzales & Crews, 1984; Kennedy *et al.*, 1989) and the EC_{50} for this lithium-induced effect in m1 CHO cells (1.2 mM, Figure 3; in agreement with a previous report of 1 mM; Atack *et al.*, 1993) was equivalent to that in cortical slices (1–2 mM for total inositol phosphates i.e. inositol mono- plus polyphosphates [Berridge *et al.*, 1982; Brown *et al.*,

1984] and 0.5 and 0.3 mM for $InsP_1$ [Batty & Nahorski, 1985; Kennedy *et al.*, 1989]). However, the EC_{50} for lithium in mouse cortical slices was much lower (ca. 0.1 mM; Whitworth & Kendall, 1988). The reduced rate of [^{3}H]-$InsP_1$ accumulation after about 20–30 min was also very similar to that seen in rat cortical slices (Gonzales & Crews, 1984) and in thyrotropin-releasing hormone-stimulated GH_3 pituitary tumour cells (Drummond *et al.*, 1984; Hughes & Drummond, 1987) and is consistent with reduced PI cycle activity, presumably as a consequence of inositol depletion. Unfortunately, using the present method involving [^{3}H]-inositol prelabelling, it is not possible to address the issue of whether exogenous *myo*-inositol can reverse this effect (i.e. can exogenous inositol maintain stimulated PI cycle turnover resulting in a continued, linear $InsP_1$ accumulation?) since exogenous inositol would upset the established isotopic equilibrium. However, this issue could be addressed by mass measurements of $InsP_1$ isomers (Atack *et al.*, 1992).

The dramatic accumulation of [^{3}H]-CMP-PA seen in m1 CHO cells resembled that seen following cholinoceptor stimulation in parotid gland slices (Downes & Stone, 1986) and rat cerebral cortex slices (Godfrey, 1989; Kennedy *et al.*, 1990; Nahorski *et al.*, 1991). However, whereas in rat cortical slices (Godfrey, 1989; Kennedy *et al.*, 1990) accumulation was immediate, there was a 2–5 min lag period in m1 CHO cells whilst in parotid gland there was a slightly longer lag (Downes & Stone, 1986). These differences are presumably due to the different tissue preparations employed. The dose-dependency of the effect of lithium on [^{3}H]-CMP-PA accumulation in m1 CHO cells (0.8 mM; Figure 3) was similar to that seen in the rat cortical slice preparation (0.6 mM: Godfrey, 1989; Kennedy *et al.*, 1990). A striking feature of the response in both m1 CHO cells and the rat cortical slice preparation is the similarity between the [^{3}H]-CMP-PA accumulation and the [^{3}H]-$InsP_1$ accumulation i.e. 2–5 min lag in m1 CHO cells and immediate response in rat cortical slices (Figure 1 and Kennedy *et al.*, 1989; 1990). These data confirm that [^{3}H]-CMP-PA accumulation is a sensitive indicator of inositol depletion caused by accumulation of [^{3}H]-$InsP_1$ due to inhibition of IMPase (Downes & Stone, 1986; Kennedy *et al.*, 1990; Nahorski *et al.*, 1991).

With respect to $InsP_3$ and $InsP_4$, the greater increase in $InsP_4$ compared to $InsP_3$ following cholinoceptor stimulation in the absence of lithium is similar to that reported in rat, mouse and guinea-pig cortical slices (Whitworth & Kendall, 1988; Kennedy *et al.*, 1990; Nahorski *et al.*, 1991; Lee *et al.*, 1992). As regards the time course of carbachol-stimulated increases in $InsP_3$ and $InsP_4$, there was an initial spike of $InsP_3$ within the first min, whereas $InsP_4$ levels rose less dramatically with no evidence of a peak within the first min. These results are in agreement with previous observations using m1 CHO cells (Lambert *et al.*, 1991b) or the human neuroblastoma cell line SH-SY5Y, which express endogenous m3 muscarinic receptors (Lambert & Nahorski, 1990; Lambert *et al.*, 1991a). In the presence of lithium, there was a definite lag time of about 20–30 min before lithium started to attenuate the carbachol-induced elevation in $InsP_3$ and $InsP_4$; at early time points, carbachol-stimulated $InsP_3$ and $InsP_4$ concentrations were essentially the same in the presence and absence of lithium. A similar lag period of about 10–20 min has been reported for reductions in $InsP_3$ and $InsP_4$ in the rat (Kennedy *et al.*, 1989; 1990; Nahorski *et al.*, 1991; Varney *et al.*, 1992) and mouse (Whitworth & Kendall, 1988) cortical slice preparations. Furthermore, in electrophysiological studies, the ability of muscarinic stimulation to block the inhibitory actions of adenosine in a hippocampal preparation was inhibited by lithium only after a 10–15 min lag (Worley *et al.*, 1988) and the densitization of carbachol-induced hippocampal cell firing was attenuated by lithium (an effect that could be reversed with 1 mM *myo*-inositol) only after an appreciable delay (Pontzer & Crews, 1990). This lag period presumably represents the time required for depletion of agonist-linked membrane inositol phospholipids after which the response to stimulation becomes reduced. The EC_{50} of lithium's effect on $InsP_3$ and $InsP_4$ levels in m1 CHO cells were the same, 0.9 mM (Figure 3), which is higher than seen in the rat cortical slice preparation for $InsP_3$ and $InsP_4$ (0.3 and 0.1 mM, respectively: Kennedy *et al.*, 1989).

The various time course data are consistent with lithium exerting its effect on the PI cycle via inhibition of IMPase, resulting in accumulation of [^{3}H]-$InsP_1$ and reduced free inositol available for PI synthesis, thereby causing a concomitant increase in [^{3}H]-CMP-PA levels. Although [^{3}H]-CMP-PA accumulation and therefore reduced PI synthesis occurred within the initial 5 min after carbachol stimulation, PI signal transduction presumably continues as normal as judged by the linearity of $InsP_1$ accumulation (suggesting that the rate of $InsP_1$ production is constant) and the lack of effect of lithium on carbachol-stimulated increases in $InsP_3$ and $InsP_4$. However, after about 20–30 min, membrane inositol phospholipids become depleted due to continued stimulation of the cells such that carbachol-induced increases in $InsP_3$ and $InsP_4$ become attenuated. At this point flux through the PI cycle becomes reduced resulting in reduced $InsP_1$ production, and hence the decreased rate of [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA accumulation (Figure 1).

The key aspect of this sequence of events is the reduction in free inositol available for PI synthesis as a consequence of inhibition of IMPase. If this is correct then replenishment by exogenous *myo*-inositol should reverse all these features (i.e. accumulation of [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA and attenuation of the carbachol-induced increases in $InsP_3$ and $InsP_4$). Although it was not possible to study the effects of *myo*-inositol on reversal of $InsP_1$ accumulation (see above), *myo*-inositol was able to abolish the effect of lithium on [^{3}H]-CMP-PA and $InsP_3$ and $InsP_4$. Hence, lithium-induced accumulation of [^{3}H]-CMP-PA following cholinoceptor stimulation in m1 CHO cells was almost completely reversed by addition of 10 mM *myo*- but not *scyllo*-inositol with an EC_{50} of 1.4 mM and the attenuation of $InsP_3$ and $InsP_4$ responses by lithium were reversed by *myo*-inositol with respective EC_{50}s of 2.7 and 3.3 mM (Figure 4). These results are similar to those seen in parotid gland where [^{3}H]-CMP-PA accumulation could be restored to levels seen in the absence of lithium by 10–30 mM *myo*-inositol (Downes & Stones, 1986). In cholinoceptor-stimulated rat brain slices, Godfrey (1989) observed complete reversal of lithium-induced [^{3}H]-CMP-PA accumulation by 10 mM *myo*-inositol, with an EC_{50} of 0.8 mM and in an autoradiographic study of [^{3}H]-cytidine incorporation into membrane [^{3}H]-CMP-PA, Hwang *et al.* (1990) reported a reversal of the effects of lithium by *myo*-inositol at a concentration of 20 mM. However, also in rat brain slices, Nahorski and colleagues (Kennedy *et al.*, 1990; Nahorski *et al.*, 1991) reported only partial reversal of lithium-induced [^{3}H]-CMP-PA accumulation and attenuation of $InsP_3$ and $InsP_4$ responses by 10 and 30 mM *myo*-inositol (but no effect of *scyllo*-inositol), presumably due in these particular preparations to relatively poor transport of exogenous *myo*-inositol into inositol-depleted cells. The differences in responses of different tissues may, as suggested by Nahorski *et al.* (1991), reflect differences in inositol reserves, inositol transport systems, resting inositol monophosphates and differences in the kinetic properties of PI synthase between tissues (e.g. different EC_{50} values for *myo*-inositol for the reversal of [^{3}H]-CMP-PA accumulation in rat, mouse and guinea-pig cortical slice preparations: Lee *et al.*, 1992).

It should be emphasised that in the present study, the effects of lithium were readily observed in cells grown in low inositol concentration medium. Indeed, the validity of the hypothesis that lithium may exert its effects *in vivo* via its actions on PI cycle activity due to inositol depletion has recently been challenged by observations that although lithium does indeed attenuate agonist-induced increases in $InsP_3$ and $InsP_4$ in rat and mouse cortical slices, there is no comparable effect in guinea-pig or monkey brain cortical slices (Dixon *et al.*, 1992; Lee *et al.*, 1992). These species

differences were ascribed to differences in endogenous inositol levels in the different cortical slice preparations.

Clearly, in tissues in which inositol depletion can be easily achieved (e.g. rat or mouse cortical slices or m1 CHO cells), the effects of lithium on PI cycle activity are readily observed. However, the key issue, highlighted by the studies of Hokin and colleagues (Dixon *et al.*, 1992; Lee *et al.*, 1992), is whether sufficient inositol depletion to impair PI cycle functioning occurs *in vivo*. Thus, it is possible that in the brain *in vivo*, although free inositol levels fall after lithium administration, the decrement of about 35% (Allison & Stewart, 1971; Sherman *et al.*, 1986) may not be sufficient to be rate-limiting in the synthesis of PI and consequently the levels of brain $InsP_3$ and $InsP_4$ may not be altered *in vivo* by lithium-treatment. However, it has recently been reported that lithium can attenuate pilocarpine-induced elevations in rat cortical $InsP_3$ levels (Jope *et al.*, 1992). Moreover, this effect was observed at 60 min but not 20 min, a time course consistent with the effects seen in m1 CHO cells (Figure 2). It therefore seems that the reduced levels of inositol in the rat cortex *in vivo* are indeed sufficient to impair PI-linked intracellular signalling (or at least $InsP_3$), presumably as a consequence of reduced PI synthesis caused by inositol depletion.

In summary, we have characterized the effects of lithium on [^{3}H]-$InsP_1$ and [^{3}H]-CMP-PA accumulation and $InsP_3$ and $InsP_4$ concentrations in cholinoceptor-stimulated m1 CHO cells. The effects of lithium were all dose-dependent with EC_{50}s in the region of 1 mM, which is approximately equivalent to therapeutic plasma levels of lithium (Drummond, 1987) and also comparable to the K_i for lithium vs inositol monophosphatase (Hallcher & Sherman, 1980; Gee *et al.*, 1988; McAllister *et al.*, 1992). The effects of lithium on [^{3}H]-CMP-PA and $InsP_3$ and $InsP_4$ were all consistent with being due to inositol depletion since exogenous *myo-* but not *scyllo*-inositol were able to reverse these effects. These data support the hypothesis that lithium can modulate PI cycle activity due to inositol depletion as a consequence of inhibition of IMPase.

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