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Lithium amplifies inhibitions of inositol phospholipid hydrolysis in mammalian brain slices

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1 We have examined the effects of lithium chloride (LiCl) on inhibitions of inositol phospholipid hydrolysis in guinea-pig and rat brain slices by assessing the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP), *in vitro*.

2 In guinea-pig and rat cerebral cortex slices the accumulation of total [^{3}H]-inositol phosphates due to the cholinoceptor agonist carbachol was inhibited by the excitatory amino acid L-glutamate, but only when LiCl was present.

3 The effects of LiCl were time and concentration-dependent. Significant inhibitions of the carbachol response by glutamate (in the presence of LiCl) being evident only after 20–30 min of stimulation at LiCl concentrations above 1.2 mM.

4 N-methyl-D-aspartate (NMDA), in the absence of LiCl, enhanced the response to carbachol at low concentrations of the amino acid and inhibited the response at higher concentrations. In the presence of 5 mM LiCl, only the inhibitory phase was observed.

5 In rat cerebral cortex slices, aluminium fluoride inhibited [^{3}H]-InsP accumulation in the presence of carbachol, noradrenaline and a depolarising concentration of KCl and these inhibitions were more marked when LiCl was present. The response to histamine was unaffected.

6 The data presented provide evidence that LiCl amplifies inhibitions of inositol phospholipid hydrolysis due to receptor and non-receptor mediated stimuli, although the mechanism underlying the effect is, as yet, obscure.

Introduction

Lithium is uniquely effective in the therapy and prophylaxis of manic-depression but its mechanism of action is unclear (Wood & Goodwin, 1987). It is now evident that lithium has effects on two important second messenger generating systems. It inhibits certain receptor-linked adenylate cyclases (Ebstein *et al.*, 1980; Newman & Belmaker, 1987) and acts at various steps in the phosphoinositide cycle, inhibiting inositol phosphatases (Hallcher & Sherman, 1980; Berridge *et al.*, 1982; Storey *et al.*, 1984) and the generation of inositol polyphosphates (Batty & Nahorski, 1987; Whitworth & Kendall, 1988; Kennedy *et al.*, 1989). Repeated lithium treatment also inhibits *ex vivo* agonist-stimulated phosphoinositide hydrolysis (Kendall & Nahorski, 1987; Godfrey *et al.*, 1989).

We have recently demonstrated that lithium, *in vitro*, enhances the inhibitory effects of muscarinic receptor stimulation on dopamine D_1-receptor-mediated adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in rat striatal slices (Whitworth & Kendall, 1989a).

Since there is a growing list of receptors whose activation inhibits inositol phospholipid hydrolysis (Hill & Kendall, 1989), we have examined the effects of lithium on a number of receptor- and non-receptor-mediated stimuli known to reduce inositol phosphate formation and we show here that lithium appears to enhance these inhibitory responses.

Some of the data have been communicated, in preliminary form to the British Pharmacological Society (Whitworth & Kendall, 1989b,c).

Methods

Cross-chopped slices (350 μm × 350 μm) of cerebral cortex from male Sprague-Dawley rats (250 g), and male or female Hartley guinea-pigs (> 300 g) were prepared and preincubated for 60 min in Krebs-Henseleit buffer as described by Brown *et al.* (1984). Aliquots (50 μl) of gravity packed slices were then incubated in vials containing myo-[2-^{3}H]-inositol ([^{3}H]-MI) and Krebs-Henseleit solution for a further 60 min. In experiments in which total [^{3}H]-inositol phosphates ([^{3}H]-InsPs) were measured, 24 kBq of [^{3}H]-MI was added to each vial when LiCl was present or 48 kBq when it was absent, whereas in those involving the separation of the individual [^{3}H]-InsPs 240 kBq [^{3}H]-MI was added. Additions of lithium chloride (LiCl) (5 mM) were made 5 min before the end, or at the onset of this prelabelling period. Addition of AlF_4^- (NaF 20 mM/$AlCl_3$ 10^{-5} M) was made at the end of the prelabelling period and 5 min before the addition of stimulating agents. In experiments in which the accumulation of [^{3}H]-InsPs due to agonist stimulation was inhibited or enhanced by the simultaneous stimulation of a different receptor, both agonists were added in combination at the end of the prelabelling period. In order to study the reversibility of the glutamate inhibitions, slices were incubated for 30 min with 3 mM glutamate in the absence of LiCl followed by a 30 min washout period during which the buffer was changed 3 times. [^{3}H]-MI was added and 45 min later carbachol (1 mM) was added together with LiCl (5 mM) and glutamate when appropriate. The incubation was stopped 45 min later. Incubations were terminated after appropriate times by the addition of 100 μl of perchloric acid (10% wt/vol) and allowed to stand on ice for 20 min. For total [^{3}H]-InsPs determinations samples were neutralised with 0.9 ml KOH (0.15 M) and centrifuged at 2000 *g* for 8 min. One ml of supernatant was removed and added to 2.25 ml of 50 mM tris buffer pH 7.0, vortexed and applied to Dowex Cl− anion exchange chromatography columns. Columns were washed with 20 ml H_2O before the [^{3}H]-InsPs were eluted with 3 ml HCl (1 M).

Determinations of individual phosphates were made by anion exchange high-performance liquid chromatography (h.p.l.c.) as previously described by Whitworth & Kendall (1988).

All [^{3}H]-InsPs were quantified by liquid scintillation counting.

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Materials

Myo-[2-^{3}H]-inositol was obtained from New England Nuclear. MK801 ((±)-5-methyl-10,11-dihydro-5H-dibenzo(a, d) cyclohepten-5,10-imine maleate) was the kind gift of MSD Neuroscience Research Center, Harlow. All other drugs and chemicals were purchased from Sigma Chemical, Fisons plc or Tocris Neuramin.

Results

The effect of glutamate on carbachol-stimulated [^{3}H]-InsP accumulation in guinea-pig cerebral cortex slices is shown in Figure 1. In the absence of LiCl (Figure 1a) there was no alteration in the carbachol response due to glutamate, but in the presence of 5 mM LiCl (Figure 1b) there were significant reductions ($P < 0.05$, ANOVA) at all concentrations of carbachol examined in excess of 10^{-5} M when glutamate was also present, although glutamate alone caused small increases in [^{3}H]-InsP accumulation (Figures 4 and 5). The results obtained in rat cerebral cortex slices were very similar (Whitworth & Kendall, 1989c).

The effect of lithium on the response to carbachol in the presence of N-methyl-D-aspartate (NMDA) is shown in Figure 2). In the absence of LiCl (Figure 2a) the effect of NMDA was markedly biphasic. However, in the presence of 5 mM LiCl, (Figure 2b), the enhancement of the carbachol response due to lower concentrations of NMDA was absent. The inhibitory phase was unaltered in terms of either maximum effect or IC_{50}. NMDA alone was without effect in either the absence or presence of LiCl (data not shown).

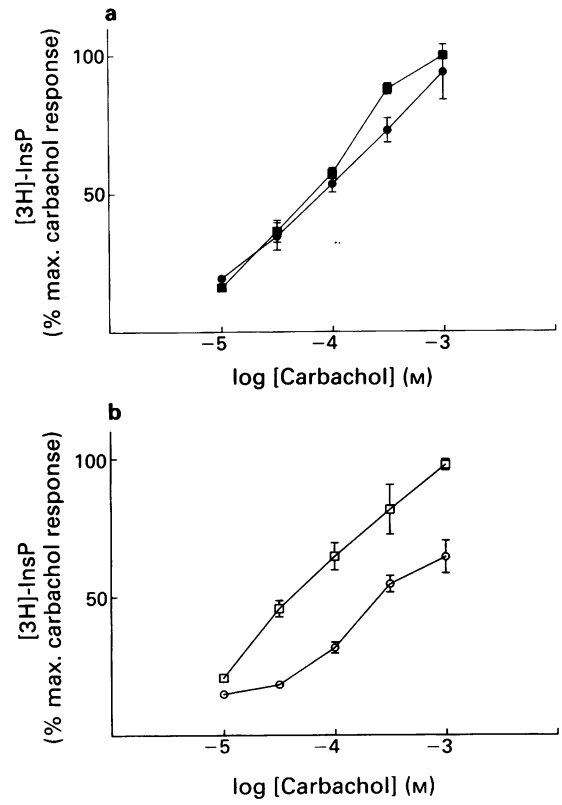

Figure 1 Effect of LiCl on the glutamate inhibition of carbachol-stimulated accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP) in guinea-pig cerebral cortex slices. Slices were prelabelled with myo-[2-^{3}H]-inositol ([^{3}H]-MI) for 60 min before addition of increasing concentrations of carbachol, in the absence (■, □) or presence (●, ○) of 3 mM L-glutamate. (a) Represents experiments conducted in the absence and (b) in the presence of 5 mM LiCl. Incubations were stopped by the addition of perchloric acid after 45 min and total [^{3}H]-InsP separated by anion exchange chromatography. Basal accumulations were 993 ± 82 d.p.m. and 612 ± 50 d.p.m. in the presence and absence of LiCl, respectively. In the presence of carbachol (1 mM) and carbachol plus LiCl there were accumulations of 2100 ± 90 and 10200 ± 525 d.p.m., respectively. The data represent the means of three experiments each with quadruplicate tissue incubations; vertical lines show s.e.mean.

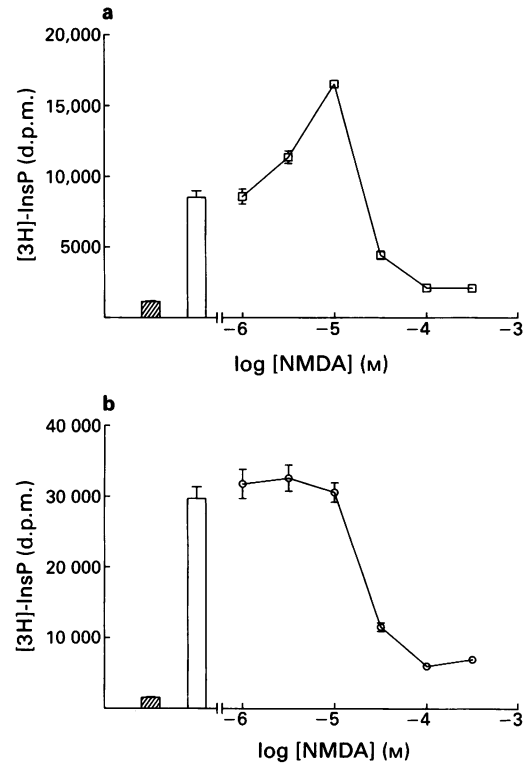

Figure 2 The effects of LiCl on N-methyl-D-aspartate (NMDA) inhibition of carbachol stimulated accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP) in guinea-pig cerebral cortex slices. The cross-hatched column represents control, the open column the presence of 1 mM carbachol. The graphs show the effects of increasing concentrations of NMDA in the presence of carbachol. (a) Represents experiments in the absence and (b) in the presence of 5 mM LiCl. The data points depict the means of quadruplicate incubations on a single occasion and vertical lines show s.e.mean. The experiment was repeated twice with essentially identical results (apart from some inter-experiment variability in absolute levels of radioactivity accumulating).

The inhibition caused by glutamate was not immediate (Figure 3). There was no effect of glutamate on the carbachol response either 5 or 15 min after addition, in the presence of LiCl, but there were significant reductions at both 30 and 45 min. In the absence of LiCl, there was no inhibition due to glutamate and at earlier time points there were small enhancements of the carbachol response.

The effects of LiCl were concentration-related (Figure 4) with inhibitions due to glutamate evident at LiCl concentrations of 1.2 mM and above.

The inhibitory effect of glutamate on the carbachol response was reversible. After the glutamate had been washed out the carbachol response in the glutamate-treated slices was 83 ± 9% (mean ± s.e.mean, $n = 4$) of the response to carbachol (calculated as d.p.m. over basal) observed in slices treated in an identical fashion apart from the inclusion of glutamate, compared with a reduction to 46 ± 4% ($n = 4$) of the control carbachol response in slices to which glutamate was added in combination with carbachol at the start of the 45 min agonist incubation period.

The inhibitory effects of glutamate in the guinea-pig cerebral cortical slice are at least somewhat selective for muscarinic receptor responses. In Figure 5 it can be seen that the combination of glutamate- and noradrenaline-stimulated [^{3}H]-InsP accumulations was greater than additive and that LiCl had no effect on this enhancement. In the absence of

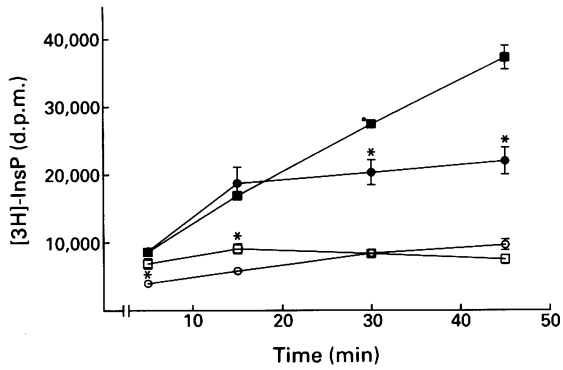

Figure 3 Time course of glutamate inhibition of carbachol stimulated accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP) in the presence and the absence of 5 mM LiCl, in guinea-pig cerebral cortex slices. The symbols represent the following conditions: (○) 1 mM carbachol, no LiCl; (□) 1 mM carbachol plus 3 mM glutamate, no LiCl; (■) 1 mM carbachol plus 5 mM LiCl; (●) 1 mM carbachol, 3 mM glutamate plus 5 mM LiCl. The data represent the results of a single experiment with triplicate tissue incubations which was repeated with essentially identical results on two further occasions. *$P < 0.05$ (unpaired t test) compared with the absence of glutamate.

LiCl the combined response to glutamate/noradrenaline was 233 ± 30% of that due to noradrenaline alone and in the presence of LiCl it was 228 ± 21% ($n = 3$).

Non-receptor-mediated inhibitions of phosphoinositide hydrolysis also appeared to be affected by LiCl. A combination of $AlCl_3$ and NaF (AlF_4^-) has previously been shown to inhibit muscarinic receptor-stimulated [^{3}H]-InsP accumulation in rat brain (Godfrey & Watson, 1988). In a manner reminiscent of glutamate, AlF_4^- alone stimulated [^{3}H]-InsP accumulation in rat cerebral cortex slices and this was enhanced by LiCl (see Figure 7), but in combination with carbachol the response was significantly less than that due to carbachol alone (Figure 6a). The addition of LiCl markedly potentiated the reductions due to AlF_4^- (Figure 6b). Responses to noradrenaline (3×10^{-4} M) and elevated KCl (31 mM) were also reduced to a greater extent by AlF_4^- in the presence compared with the absence of LiCl, but the response to a combination of histamine plus AlF_4^- was approximately additive with respect to the effects of AlF_4^- and histamine alone, regardless of the presence of LiCl (Figure 7).

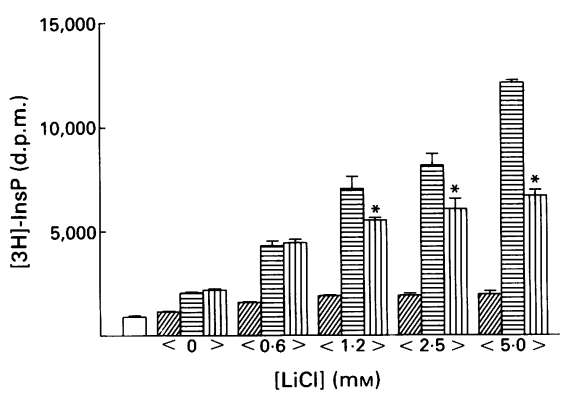

Figure 4 Concentration-response relationship of LiCl with glutamate inhibition of carbachol-stimulated accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP) in guinea-pig cerebral cortex slices. The open column represents control, the diagonally hatched columns the presence of 3 mM glutamate, the horizontally hatched columns the presence of 1 mM carbachol and the vertically hatched columns carbachol plus glutamate. LiCl was added 5 min before the agonists and incubations proceeded for 45 min.

The figure represents a single experiment with triplicate tissue incubations which was repeated with essentially identical results on two further occasions. *$P < 0.005$ with respect to carbachol alone (ANOVA).

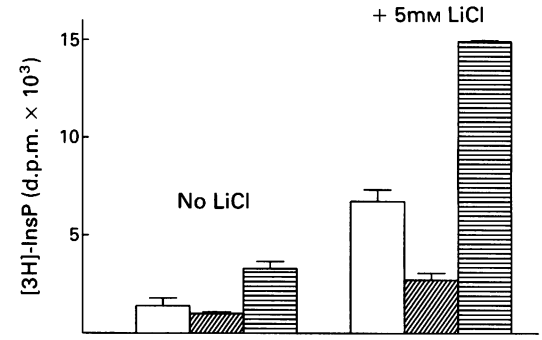

Figure 5 The effect of LiCl on the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP) due to glutamate and noradrenaline in guinea-pig cortical slices. The following conditions are represented in both the presence and absence of 5 mM LiCl: open columns, noradrenaline 3×10^{-4} M; diagonally-hatched columns, glutamate 3 mM; horizontally-hatched columns, noradrenaline plus glutamate.

The data are expressed as d.p.m. [^{3}H]-InsP in excess of control and represent a single experiment, conducted in quadruplicate, which was repeated with essentially similar results on two other occasions.

The inhibitory effects of AlF_4^- were more rapid in onset than those due to receptor activation. Table 1 shows the effects of AlF_4^- on carbachol-stimulated formation of individual [^{3}H]-inositol phosphates 5 min after agonist addition in the presence and absence of 5 mM LiCl. In contrast to its effect on the accumulation of total [^{3}H]-inositol phosphates, LiCl

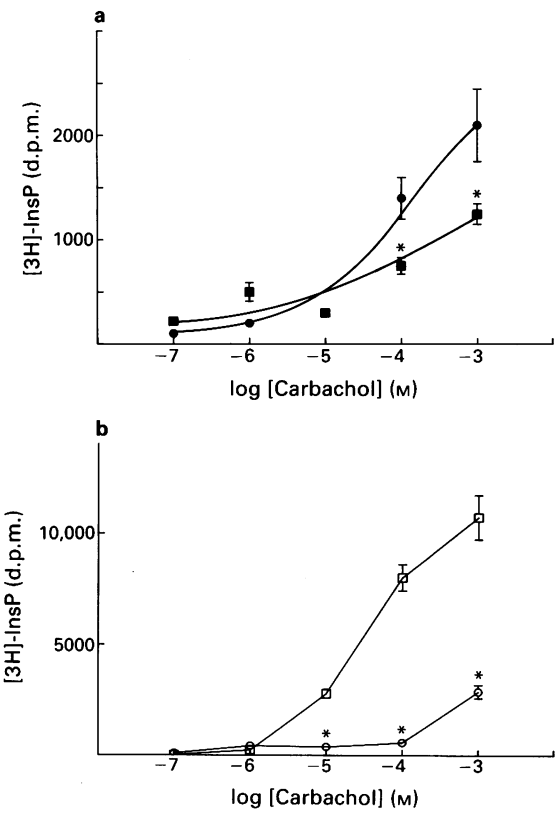

Figure 6 The effect of LiCl on AlF_4^- inhibition of carbachol-stimulated accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP) in rat cerebral cortex slices. (a) The effect of combining AlF_4^- with increasing concentrations of carbachol (■), and that of carbachol alone (●) in the absence of LiCl. (b) The effect of combining AlF_4^- with increasing concentrations of carbachol (○), and that of carbachol alone (□) in the presence of 5 mM LiCl. *Significantly less ($P < 0.05$, unpaired t test) than corresponding concentration of carbachol in the absence of AlF_4^-. The points represent the means of 3 separate experiments each conducted in triplicate; vertical lines show s.e.mean.

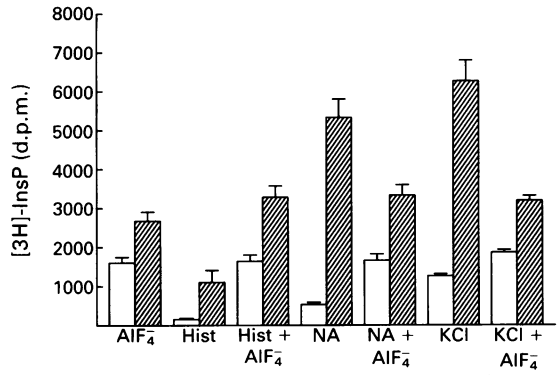

Figure 7 The effect of AlF_4^- on the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsP) in rat cerebral cortex slices. Hist = histamine (10^{-3} M), NA = noradrenaline (3×10^{-4} M), KCl = potassium chloride (50 mM). Open columns represent the absence and hatched columns the presence of 5 mM LiCl.

In the absence of LiCl the combined responses were; NA + AlF_4^- —79%; hist + AlF_4^- —92%; KCl + AlF_4^- —65%; carbachol + AlF_4^- (Figure 6) 69% of the sums of the individual responses. In the presence of LiCl the combined responses were; NA + AlF_4^- —42%; hist + AlF_4 —88%; KCl + AlF_4^- —36%; carbachol + AlF_4^- —41% of the sums of the individual responses. Each column represents the mean of 3 separate experiments each conducted in triplicate and vertical bars show s.e.mean.

did not enhance the effects of AlF_4^- on inositol trisphosphate ($InsP_3$) or inositol tetrakisphosphate ($InsP_4$) formation. In fact, LiCl significantly reduced AlF_4^--stimulated $InsP_4$ production. AlF_4^- reduced carbachol stimulation of 1,3,4-$InsP_3$, 1,4,5-$InsP_3$ and $InsP_4$ and all of these reductions were potentiated by the addition of LiCl. In the case of 1,4,5-$InsP_3$ there was no accumulation in excess of that due to LiCl alone in the presence of carbachol, AlF_4^- and LiCl.

It was not possible to investigate the effects of LiCl on amino acid-induced inhibition of muscarinic receptor-mediated inositol polyphosphate formation, since the accumulations of 1,4,5-$InsP_3$ and $InsP_4$ are much smaller in guinea-pig brain slices compared with those seen in rat or mouse brain (Whitworth & Kendall, unpublished observations).

Discussion

It is now clear that lithium has multiple effects on the phosphoinositide cycle. In addition to inhibiting inositol mono- and bisphosphate phosphatases (see Nahorski, 1988) lithium has been shown to reduce the *in vitro* formation of the primary product of phosphatidylinositol 4,5-bisphosphate (Ptd $InsP_2$) hydrolysis i.e. 1,4,5-$InsP_3$ (Kennedy *et al.*, 1989) and of $InsP_4$ (Batty & Nahorski, 1987; Whitworth & Kendall, 1988) which has also been suggested to have a second messenger function (Irvine & Moor, 1986).

In *ex vivo* experiments LiCl treatment reduces receptor-mediated and non-receptor-mediated phosphoinositide hydrolysis (Kendall & Nahorski, 1987; Godfrey *et al.*, 1989). Since the catalogue of inhibitory influences of lithium on the phosphoinositide cycle is growing and because there is increasing evidence that there are receptors linked to phospholipase C in a negative as well as a positive manner (Linden & Delahunty, 1989), it was of interest to examine the effects of lithium on inhibitory phosphoinositide responses.

Excitatory amino acids have previously been shown to inhibit as well as to stimulate phosphoinositide hydrolysis, but the negative effects have been suggested to be a non-specific neurotoxic phenomenon (Godfrey *et al.*, 1988). We do not believe this to be so for two major reasons; firstly, the effects can be 'washed out' i.e. brain slices can be pre-incubated with glutamate followed by removal of the amino acid by washing without loss of subsequent muscarinic receptor responsiveness (see Results). Secondly, the effect of glutamate on a different receptor response i.e. that due to the α_1-adrenoceptor is to potentiate that response (Figure 5), although it should be noted that in rat brain slices noradrenaline stimulated inositol phospholipid hydrolysis has been shown to be inhibited by excitatory amino acids (Jope & Li, 1989). However, this effect seemed to be largely due to decreased incorporation of [^{3}H]-MI (which was not evident in the guinea-pig cortical slices; data not shown) and the amino acids did not produce irreversible cell damage.

In this study the very modest inhibitory effect of glutamate on the response to muscarinic receptor stimulation in the absence of lithium was markedly potentiated when lithium was present (Figure 1). This inhibitory effect appeared to be due to a reduction in the maximum response to carbachol rather than a change in EC_{50}, suggesting that a change in the affinity of the muscarinic receptor for carbachol was not caused by lithium.

NMDA had a markedly biphasic effect on the response to carbachol. We do not yet have sufficient information to identify the receptor mediating the potentiation (although we have found that it is blocked by the NMDA antagonist MK801 (Kendall, unpublished)), but it was abolished by lithium. In contrast, the inhibitory phase was unaffected by lithium, but given the greater efficacy of NMDA compared with glutamate this is perhaps not surprising. If, for example, the lithium effect is due to an increase in the coupling of negatively linked receptors, NMDA and glutamate could perhaps be considered to be full and partial agonists respectively at such a putative, negatively-linked site leaving no capacity for an increase in the effect of NMDA. Examination of the effects of combinations of glutamate and NMDA on the carbachol response would be a useful test of this hypothesis.

A direct negative linkage of excitatory amino acid receptors with phospholipase C is perhaps not fully in keeping with the time delay before the inhibition due to glutamate is evident (Figure 3), although this could represent indirectness of the

Table 1 Effect of LiCl on the inhibition of carbachol-stimulated [^{3}H]-inositol trisphosphate ([^{3}H]-$InsP_3$) and [^{3}H]-inositol tetrakisphosphate ([^{3}H]-$InsP_4$) accumulation in rat cerebral cortical slices due to AlF_4^-

	[^{3}H]-*1,3,4-InsP$_3$*	[^{3}H]-*1,4,5-InsP$_3$*	[^{3}H]-*InsP$_4$*
AlF_4^-	428 ± 491	1614 ± 923	1025 ± 281
AlF_4^- plus LiCl	201 ± 104	616 ± 329	480 ± 49*
Carbachol (10^{-3} M)	2435 ± 164	5514 ± 862	28767 ± 3008
Carbachol plus LiCl	3753 ± 401	3829 ± 717	26314 ± 1147
Carbachol plus AlF_4^-	949 ± 316†	2306 ± 265†	5121 ± 1537†
Carbachol plus AlF_4^- plus LiCl	527 ± 134§	0	1295 ± 149§

Results represent the means ± s.e.mean (in d.p.m.) in excess of basal (i.e. no addition or 5 mM LiCl where appropriate) of 3 separate experiments, each of which was a combination of 5 tissue incubations. Statistical significance: * with respect to AlF_4^-; † with respect to carbachol; § with respect to carbachol plus AlF_4^-, all $P < 0.05$ (ANOVA).

lithium, as opposed to the glutamate effect. It should be noted that a similar delay occurs before the modulation of histamine-stimulated phosphoinositide hydrolysis due to adenosine (Hill & Kendall, 1987; Kendall & Hill, 1988).

The effects of lithium are not uniquely related to amino acid receptors. Recently it has been shown that fluoroaluminate ions (AlF_4^-) both stimulate basal inositol phospholipid hydrolysis and inhibit agonist-stimulated responses (Blackmore *et al.*, 1985; Godfrey & Watson, 1988). The analogy of this situation with that of the adenylate cyclase system is compelling, since despite the well known stimulant effects of AlF_4^- on adenylate cyclase activity in membrane preparations, only inhibition of stimulated cyclic AMP formation is evident in most intact cell systems (Blackmore *et al.*, 1985; Paris & Pouyssegur, 1987).

In this study AlF_4^- inhibited [^{3}H]-InsP accumulation due to carbachol, noradrenaline and elevated KCl and all of these inhibitions were more marked in the presence of lithium. However, the effects of AlF_4^- and histamine were simply additive regardless of the presence of lithium.

The inhibitory effects of AlF_4^- on carbachol stimulation were rapid in onset with significant reductions in the formation of the immediate product of Ptd $InsP_2$ hydrolysis i.e. 1,4,5-$InsP_3$ after 5 min. The formation of the products of 1,4,5-$InsP_3$ metabolism i.e. $InsP_4$ and 1,3,4-$InsP_3$ were also reduced, arguing against an accelerated flux of inositol phosphates through the cycle. All of these reductions were enhanced in the presence of lithium.

It must be admitted that, because of the multiple effects of LiCl on the inositol phospholipid cycle, there are difficulties in the interpretation of some of the data presented. This is particularly true when accumulations of total phosphates (mainly $InsP_1$) in the presence of LiCl are compared with those in its absence. However, the observation that LiCl enhances the inhibition of carbachol-stimulated 1,4,5-$InsP_3$ formation by AlF_4^- supports the contention that there is an additional target for lithium apart from the inositol phosphate phosphatases.

The site of action of AlF_4^- with regard to its inhibitory effects is uncertain, but it is unlikely to be a direct effect on receptor recognition sites or on phospholipase C given its effect and lack of effect on the responses to elevated KCl and histamine respectively.

It is tempting to assume that AlF_4^- effects are G-protein mediated (as an extension of the analogy with the adenylate cyclase system), with the net effect being due to the relative activities of positively and negatively coupled proteins. This being so and recalling that lithium has been shown to inhibit the coupling of both muscarinic receptors and β-adrenoceptors to pertussis toxin- and cholera toxin-sensitive G proteins respectively (Avissar *et al.*, 1988), it seems a reasonable working hypothesis to suggest that the enhancement of inhibitory effects by lithium is due to an interaction at the level of a G-protein negatively linked to phospholipase C.

Given the complexity of the brain slice preparation, it cannot be assumed that the inhibitory agents are acting directly on the same cells in which stimulation of inositol phospholipid hydrolysis is elicited. Hence, the elucidation of the mode of action of lithium will require the use of homogeneous cell populations that express inhibitory responses (e.g. GH_3 pituitary tumour cells, Delahunty *et al.*, 1988) in future experiments.

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