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Li^+ increases accumulation of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate in cholinergically stimulated brain cortex slices in guinea pig, mouse and rat

The increases require inositol supplementation in mouse and rat but not in guinea pig

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Li^+, beginning at a concentration as low as 1 mM, produced a time- and dose-dependent increase in accumulation of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 in acetylcholine (ACh)-stimulated guinea-pig brain cortex slices prelabelled with [^{3}H]inositol and containing 1 mM-inositol in the final incubation period. Similar results were obtained by mass measurement of samples incubated with 10 mM-Li^+ by using a receptor-binding assay, although the percentage stimulation of Ins(1,4,5)P_3 accumulation by Li^+ was somewhat less by this assay. The increase in accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 by Li^+ was absolutely dependent on the presence of ACh. In the absence of added inositol, 1–5 mM-Li^+ produced smaller increases in Ins(1,4,5)P_3, but the Li^+-dependent increase in Ins(1,3,4,5)P_4 was not as affected by inositol omission. In previous studies with cholinergically stimulated rat and mouse brain cortex slices, Li^+ inhibited accumulation of Ins(1,4,5)P_3 in rat and inhibited Ins(1,3,4,5)P_4 accumulation in rat and mouse [Batty & Nahorski (1987) Biochem. J. **247**, 797–800; Whitworth & Kendall (1988) J. Neurochem. **51**, 258–265]. We found that Li^+ inhibited both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation in these species, but we could reverse this inhibition by adding 10–30 mM-inositol; we then observed a Li^+-induced increase in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. The species differences observed in the absence of supplemented inositol were explained by the fact that a much higher concentration of inositol was required to bring the Li^+-elevated levels of CDP-diacylglycerol (CDPDG) down to baseline in the rat and mouse. These data suggest that inositol is more rate-limiting for phosphatidylinositol synthesis in the presence of Li^+ in rat and mouse, which can account for the previous reports of inhibition of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation by this ion in these species. Thus, in all species examined, Li^+ could be shown to increase accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in cholinergically stimulated brain cortex slices if the slices were supplemented with sufficient inositol to bring the Li^+-elevated level of CDPDG down to near baseline, as seen in the absence of Li^+. In guinea-pig brain cortex slices, increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation could then be seen at Li^+ concentrations as low as 1 mM, which falls within the therapeutic range of plasma concentrations in the treatment of manic-depressive disorders. These observations may have therapeutic implications.

INTRODUCTION

Receptor activation of phosphodiesteratic cleavage of phosphoinositides generates intracellular second-messenger molecules, such as Ins(1,4,5)P_3, which mobilizes intracellular Ca^{2+}, and diacylglycerol, which activates protein kinase C [1–5]. The Ins(1,4,5)P_3 formed on agonist stimulation is rapidly metabolized by a kinase, which is specific for the 3-position of inositol, or by a phosphatase, which is specific for the 5-position [6–10]. The 3-kinase generates Ins(1,3,4,5)P_4, which has been suggested to act as another second messenger involving Ca^{2+} movements [11–17].

Li^+ is effective in the treatment of manic-depressive (bipolar) disorders. The mechanism of its therapeutic action is not clear, although several hypotheses have been advanced. The most well-known hypothesis is that put forward by Berridge *et al.* [18]. This is based on the fact that, in agonist-stimulated brain preparations, therapeutic concentrations of Li^+ cause accumulation of certain inositol phosphates [19–25], primarily inositol monophosphates, Ins(1,3,4)P_3 and Ins(1,4)P_2. This is brought about by inhibition of inositol monophosphatases and inositol polyphosphate 1-phosphatase(s) acting on the last two compounds. This has been suggested ultimately to decrease the supply of phosphoinositides and thus attenuate agonist-induced Ins(1,4,5)P_3 formation and intracellular Ca^{2+} mobilization [18].

Increases in the accumulation of second messenger Ins(1,4,5)P_3 or putative second messenger Ins(1,3,4,5)P_4 in cholinergically stimulated brain cortex slices have not been observed in the presence of Li^+. In slices of rat [26] and mouse cerebral cortex [27], Li^+ was reported to inhibit muscarinic-receptor-stimulated formation of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 accumulation in the rat. We find here that Li^+ increases Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in guinea-pig brain cortex slices. We confirm that Li^+ decreases Ins(1,3,4,5)P_4 in mouse and rat brain cortex slices, and we find that it has similar effects on Ins(1,4,5)P_3 levels, but if inositol is added in sufficient amounts to bring the Li^+-induced elevations in CDP-diacylglycerol (CDPDG) back to baseline, Li^+-elicited

Abbreviations used: CDPDG, CDP-diacylglycerol; KHBS, Krebs–Henseleit bicarbonate saline; ACh, acetylcholine.

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increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation can then be observed in these latter two species.

EXPERIMENTAL

Preparation of brain slices, labelling and drug treatment

Guinea-pig cerebral-cortex slices (0.5 mm × 0.35 mm × 0.35 mm) were prepared by a modification [28] of the method of Brown *et al.* [29]. Slices from the brains of two 350–400 g guinea pigs (or, where indicated, four 250 g rats or nine 25 g mice) were placed in a 50 ml Erlenmeyer flask containing 5 ml of oxygenated Krebs–Henseleit bicarbonate saline (KHBS) containing (in mM) 113 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 0.6 $MgSO_4$, 25 $NaHCO_3$, and 11.5 glucose, pH 7.4, and preincubated with vigorous shaking for 15 min at 37 °C. Before all incubations, the flasks were oxygenated for 30 s and tightly stoppered. Preincubation was repeated three times (for 15 min, 15 min and 30 min). The slices were then prelabelled with 0.25 mCi of *myo*-[2-^{3}H]inositol (sp. radioactivity 15–20 Ci/mmol) (or, where indicated, 5 μCi of [2-^{14}C]cytidine, sp. radioactivity 59 mCi/mmol) in fresh incubation medium for 1 h. At the end of this period, the slices were rinsed with 4 × 30 ml of fresh KHBS to remove free labelling compound. The slices were suspended in approx. 15 ml of incubation medium, and 1 ml samples were pipetted into Beckman Biovials. In the samples containing LiCl, ionic strength was adjusted by lowering the concentration of NaCl. The slices were then incubated with and without Li^+ for 20 min. Finally, they were incubated with or without acetylcholine (ACh) (supplemented with 100 μM-eserine) for various times at 37 °C. At the end of the incubation, the samples were transferred to tubes containing 2 ml of ice-cold 4.5% (w/v) $HClO_4$ and 20 μg of sodium phytate to terminate the reaction. Phytate was found to increase recoveries of Ins(1,3,4,5)P_4 by up to 20%, but it had no significant effect on the recovery of Ins(1,4,5)P_3 (results not shown). Because phytate interferes in the binding assays for both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, it was excluded from the quenching medium in those experiments in which the binding assay was performed.

Extraction of [^{3}H]inositol polyphosphates

The quenched samples were homogenized with a Polytron for 20 s, followed by centrifugation at 12000 *g* for 20 min. The supernatant was neutralized with KOH in the presence of 5 mM-Na_2EDTA, 10 mM-$NaHCO_3$, and 5 μl of 'pHydrion' indicator solution (range pH 1–11). The neutralized supernatant was centrifuged at 12000 *g* for 20 min to remove $KClO_4$ and subsequently stored at −20 °C. Before application to the h.p.l.c. column, a further accumulation of $KClO_4$ was removed by centrifugation. The pellets containing the particulate matter from the $HClO_4$-treated homogenate were homogenized with 3.8 ml of methanol/chloroform/water (10:5:3, by vol.), and 20 μl samples were taken to determine the radioactivity in total phosphoinositides.

Quenching and extraction of [^{14}C]CDPDG

The method of Godfrey [30] was modified as follows. Samples which had been labelled with [^{14}C]cytidine were quenched by the addition of 3.75 ml of ice-cold chloroform/methanol (1:2, v/v). After homogenization in a glass homogenizer and sequential addition, with mixing, of 1 ml of chloroform and 1 ml of water, the phases were separated by centrifugation (5000 *g*, 10 min). The lower phase was washed with 4 ml of 1 M-HCl/methanol (1:1, v/v), evaporated to dryness in a 20 ml scintillation vial, resuspended in scintillation fluid, and counted for radioactivity in a scintillation counter [30].

H.p.l.c. analysis of inositol polyphosphates

Neutralized extracts (pH 6–7) were applied to a 25 cm analytical Whatman Partisil SAX column and eluted with a linear gradient of $NH_4H_2PO_4$, pH 3.8, as follows: 0 min, water; then (in $NH_4H_2PO_4$), 5 min, 0.5 M; 35 min, 0.7 M; 40 min, 1.7 M; 80 min, 1.7 M; and then, 85 min, water; and 120 min, water. This elution program is based on the method of Dean & Moyer [31]. An automated h.p.l.c. system and peak detection system, previously described [32], were used. Continuous-flow radioactivity counting was adequate for peak detection, but quantitative measurement required collection of peaks for re-counting in a liquid-scintillation counter.

Binding assays for measurement of mass of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3

Ins(1,4,5)P_3 was assayed by the method of Challiss *et al.* [33] with the following modifications. A final incubation volume of 0.4 ml was used. The pH of the 25 mM-Tris/HCl buffer was increased to 9.0. The binding protein (from bovine adrenal cortex) was diluted in buffer, and 0.2 ml was added to the reaction mixture. The larger volume facilitated equal sampling of the particulate suspension. At the end of the binding period, the protein was separated from the reaction mixture by centrifugation (14000 *g*, 3 min) through a 0.3 ml cushion of 5% (w/v) sucrose in 25 mM-sodium acetate, pH 5.0. The lower pH of the cushion was necessary for good pelleting of the binding protein. Ins(1,3,4,5)P_4 was assayed by the method of Donié & Reiser [34]. At the conclusion of both assays, the binding protein (pellet) was dispersed in 0.35 ml of water and counted for radioactivity in 8 ml glass vials in 5 ml of Polyfluor, a biodegradable scintillation mixture.

Identification of the InsP_4 isomer as Ins(1,3,4,5)P_4

Three InsP_4 isomers have been identified as products of enzymes found in rat brain cytosol: Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and Ins(1,4,5,6)P_4 [25]. The ability of putative Ins(1,3,4,5)P_4 to displace [^{3}H]Ins(1,3,4,5)P_4 from a highly specific binding site was measured to show that Ins(1,3,4,5)P_4 levels are increased in the presence of Li^+. Because the affinity of the binding protein for Ins(1,3,4,6)P_4 and Ins(1,4,5,6)P_4 is two orders of magnitude lower than the affinity for Ins(1,3,4,5)P_4 [34,35], unrealistically large amounts of these isomers would be required to produce the results seen with the binding assay. The possibility that the other isomers are present in small amounts is not precluded, but changes in the mass of Ins(1,3,4,5)P_4 were sufficient to account for the observed changes in [^{3}H]InsP_4 in prelabelled tissue.

RESULTS

Effect of increasing concentrations of ACh on the levels of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in guinea-pig brain cortex slices in the presence and absence of Li^+

Li^+ (10 mM) increased the level of all three inositol polyphosphates above that of samples incubated with ACh alone (Fig. 1). Unlike Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3, Ins(1,4,5)P_3 was present at 0.1 μM-ACh, an ineffective agonist concentration. The presence of Ins(1,4,5)P_3 in the absence of agonist has been previously observed [36]. The Li^+ stimulation was dependent on the presence of ACh. The stimulation of Ins(1,3,4)P_3 accumulation by Li^+ has been well documented [37]. However, the present paper is the first study showing that messenger Ins(1,4,5)P_3 and putative messenger Ins(1,3,4,5)P_4 accumulate in response to

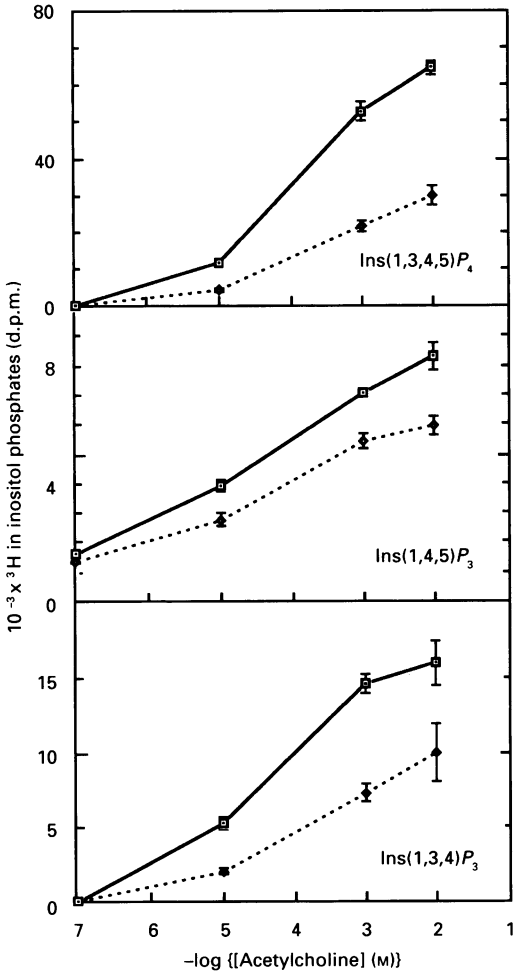

Fig. 1. Effects of increasing concentrations of ACh, with and without Li^+, on accumulations of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in guinea-pig brain cortex slices

[^{3}H]Inositol-prelabelled brain cortex slices were preincubated for 20 min with and without 10 mM-LiCl, followed by incubation for 10 min with various concentrations of ACh with (⊡) and without (◇, ◆) Li^+. An inositol phosphate extract was analysed by h.p.l.c. Labelling, quench-extraction and h.p.l.c. separation are described in the Experimental section.

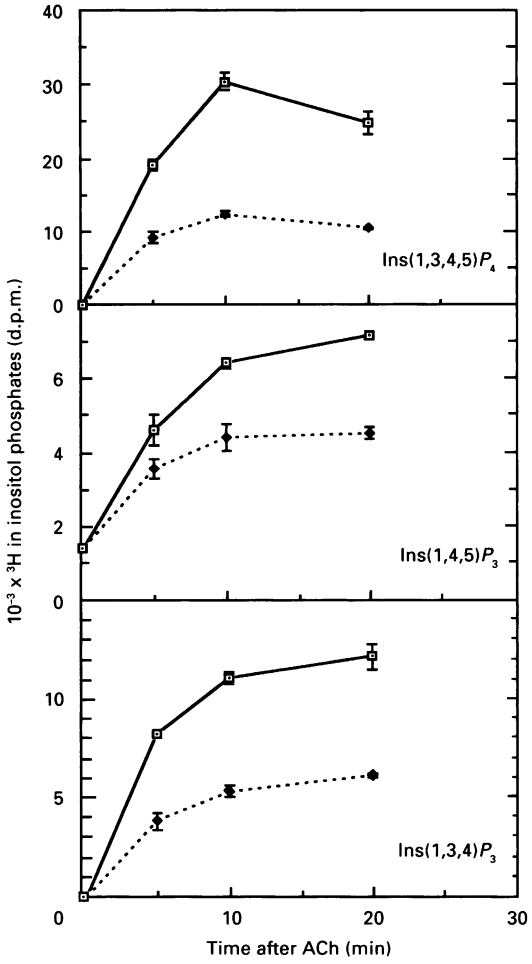

Fig. 2. Time-dependent formation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in guinea-pig brain cortex slices incubated with ACh in the presence and absence of Li^+

[^{3}H]Inositol-prelabelled brain cortex slices were preincubated for 20 min with and without 10 mM-Li^+. They were then incubated for various times with 0.1 mM-ACh with (⊡) and without (◆) 10 mM-Li^+. An inositol phosphate extract was analysed by h.p.l.c. Labelling, quench-extraction and h.p.l.c. separation are described in the Experimental section.

Li^+ in cholinergically stimulated brain cortex slices. The failure to reach saturation at the highest experimentally feasible concentration of agonist is a characteristic of muscarinic stimulation of inositol phosphate formation in brain slices, previously noted by Downes [38].

Note that at the high ACh concentration, the absolute level of Ins(1,3,4,5)P_4 was about 4 times that of Ins(1,3,4)P_3 and about 8 times that of Ins(1,4,5)P_3. The relatively low levels of Ins(1,3,4)P_3 in brain, as compared with many other tissues, could be due to an active 4-phosphatase pathway for breakdown of Ins(1,3,4)P_3, which is present in brain [39]. This pathway is unaffected by Li^+ [40]. The functional significance of the unusually high levels of Ins(1,3,4,5)P_4 in brain cortex is unclear.

Time-dependent accumulation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 with and without Li^+ in the presence of ACh

Fig. 2 shows the time-dependent accumulation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 with and without 10 mM-Li^+ in the presence of ACh. There was a time-dependent increase in the formation of all three inositol phosphates above ACh controls in the presence of Li^+. The Li^+-induced increments essentially reached a maximum at 10 min and then remained more or less constant up to 20 min.

Effects of increasing concentrations of Li^+ on the accumulation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in cholinergically stimulated guinea-pig brain cortex slices

Fig. 3 shows the effects of low concentrations of Li^+ on the accumulation of the two InsP_3s and Ins(1,3,4,5)P_4 in the presence and absence of 1 mM-inositol. In the absence of inositol (Fig. 3*a*), there was a small increase in the accumulation of Ins(1,4,5)P_3 as the Li^+ concentration was increased to 5 mM. Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 increased to a greater extent.

In the presence of inositol (Fig. 3*b*), the results for Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 were similar to those seen in the absence of inositol, but the Li^+-induced increase in Ins(1,4,5)P_3 was consistently greater on a percentage basis than in samples without supplemental inositol. All three inositol polyphosphates showed

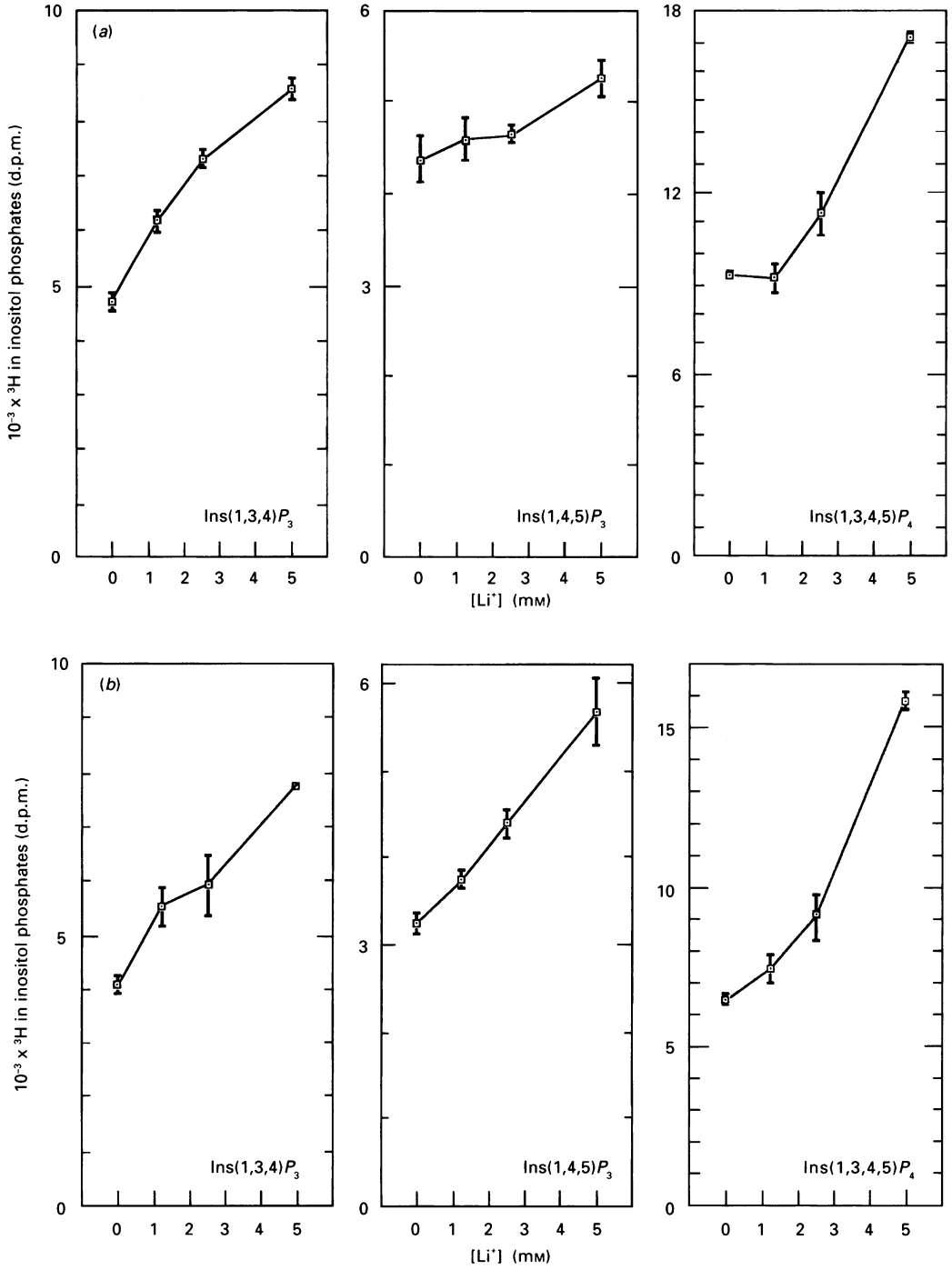

Fig. 3. Effect of low concentrations of Li^+, with and without inositol supplementation, on the accumulation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in guinea-pig brain cortex slices in the presence of ACh

Brain cortex slices, labelled with [^{3}H]inositol, were preincubated for 20 min with various concentrations of LiCl and then incubated in the same medium for 10 min with 0.1 mM-ACh. (*a*) No inositol supplementation; (*b*) 1 mM-inositol present during 20 min preincubation and during stimulation. An inositol phosphate extract was analysed by h.p.l.c. Labelling, quench-extraction and h.p.l.c. separation are described in the Experimental section. Experiments shown in (*a*) and (*b*) were done on separate days, so the absolute radioactivities cannot be directly compared.

some increase at 1 mM-Li^+, which is the midpoint of the therapeutic range of plasma concentrations for Li^+. If the target neurones could be isolated, the sensitivity and maximum stimulation would probably be greater.

Fig. 4 shows the effects of Li^+ concentrations ranging from 5 to 100 mM on the accumulation of inositol polyphosphates in cerebral cortex slices in the absence of added inositol. Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 increased progressively up to 50 mM-Li^+, beyond which they reached a plateau or fell. At 50 mM-Li^+, Ins(1,4,5)P_3 was about 3.3 times the control without Li^+ and Ins(1,3,4,5)P_4 was about 12.5 times its control. Ins(1,3,4)P_3 showed a different pattern. It increased sharply up to 10 mM-Li^+ and then reached a plateau. Similar profiles were obtained by using trichloroacetic acid/phytate quench and extraction, though recoveries were much lower (results not shown). Inhibition at 75 mM- and 100 mM-Li^+ may be caused by insufficient Na^+

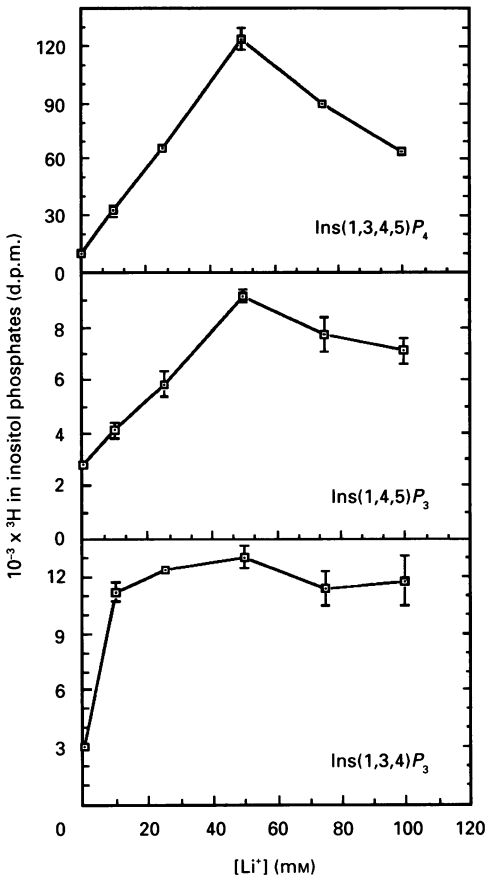

Fig. 4. Effect of high concentrations of Li^+, without inositol supplementation, on the accumulation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in guinea-pig brain cortex slices incubated in the presence of ACh

Brain cortex slices, labelled with [^{3}H]inositol, were preincubated for 20 min with various concentrations of LiCl and then incubated in the same medium for 10 min with 0.1 mM-ACh. An inositol phosphate extract was analysed by h.p.l.c. Labelling, quench-extraction and h.p.l.c. separation are described in the Experimental section.

concentration, because the concentration of Na^+ was lowered proportionately to maintain proper ionic strength in the medium. Inhibition of inositol polyphosphate formation at high Li^+ was also seen in mini-lobules from pancreas with a boiling extraction method (J. F. Dixon & L. E. Hokin, unpublished work). Addition of atropine to slices which had been stimulated as above for 10 min in the presence of 50 mM-Li^+ resulted in a time-dependent return of [^{3}H]inositol polyphosphates to control levels (results not shown).

Li^+ had no effect on $HClO_4$ extraction of inositol polyphosphates

To exclude the possibility that the Li^+-induced increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were due to effects on the extraction process, two sets of control samples were incubated with ACh but without Li^+, and a third set of samples was incubated with ACh and 50 mM-Li^+. After incubation, 50 mM-Li^+ was added to one of the two sets of control samples at the moment of quenching. This addition of Li^+ to a control set had no effect (Fig. 5) on the levels of any of the inositol polyphosphates. On the other hand, incubation with 50 mM-Li^+ showed the usual increases in all three inositol polyphosphates.

Effect of Li^+ on the accumulation of inositol polyphosphates in cholinergically stimulated rat and mouse brain cortex slices in the presence and absence of inositol

Figs. 6(*a*) and 6(*c*) show that Li^+ suppressed the levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in cholinergically stimulated rat and mouse brain cortex slices incubated without inositol supplementation. This confirms previous observations [26,27] in the rat and the mouse (in the mouse, the isomers of InsP_3 were not separated). One possible explanation for the species differences between guinea pig, on the one hand, and rat and mouse, on the other hand, is that in the presence of Li^+ inositol may be more rate-limiting for resynthesis of phosphoinositides in the rat and mouse. Figs. 6(*b*) and 6(*d*) show that this was, in fact, the case. If sufficient inositol was added so it was not rate-limiting for PtdIns synthesis (see the next section), a Li^+-induced increase in both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 levels could be seen. At later times, this increment decreased (results not shown). As previously reported [26,41,42], Li^+ increased the accumulation of Ins(1,3,4)P_3, even in the absence of added inositol, in the rat. This is probably due to the strong inhibition of Ins(1,3,4)P_3 1-phosphatase by Li^+.

Effect of Li^+ on the accumulation of CDPDG in guinea pig, rat and mouse cerebral cortex slices in the presence and absence of increasing concentrations of inositol

It has been previously reported that, in rat brain cortex slices incubated in the presence of Li^+, CDPDG accumulates because of insufficient inositol to react with it to form PtdIns, as a consequence of the inositol being trapped as inositol phosphates [30,43]. Fig. 7 shows the effects of increasing concentrations of inositol on CDPDG levels in the different species in the presence of Li^+. In rat and mouse, the concentration of inositol required to bring CDPDG levels to 50% of maximum were 6 and 19 times higher, respectively, than in guinea pig. CDPDG levels in rat and mouse remained slightly above the controls (without Li^+) at 30 mM-inositol. In guinea pig, 2 mM-inositol was sufficient to decrease CDPDG to the control level without Li^+.

Validation by mass measurements of the [^{3}H]inositol-prelabelling technique for measuring Li^+-induced changes in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4

Table 1 shows the effects of ACh and Li^+ on Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation, as determined by mass measurements. The effects of ACh and ACh plus Li^+ on the accumulation of Ins(1,3,4,5)P_4, as measured by mass measurements, were very similar to those measured by the radioisotope technique. In the experiment shown in Table 1, no non-radioactive inositol was added, and the Li^+ concentration was 10 mM, so these results can be directly compared with those in Fig. 2. The stimulations of Ins(1,4,5)P_3 accumulation, as determined by mass measurements, were statistically significant, but the percentage increases were less than with the prelabelling technique. This was due, at least in part, to the considerably higher basal levels of Ins(1,4,5)P_3. We have found that even with rapid extraction in the cold with $HClO_4$ there may have been some hydrolysis of PtdInsP_2, as evidenced by the continuous release of considerable amounts of Ins(1,4,5)P_3 on repeated extraction. Many other methods of extraction were tried in which acid was avoided, but very little extraction of Ins(1,4,5)P_3 was achieved.

It should be pointed out that in mouse pancreatic acini, where the ACh-stimulated accumulations of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and the enhancement by Li^+ were large, and where there is much less PtdInsP_2 than in brain, the percentage stimulations were the same with either [^{3}H]inositol prelabelling

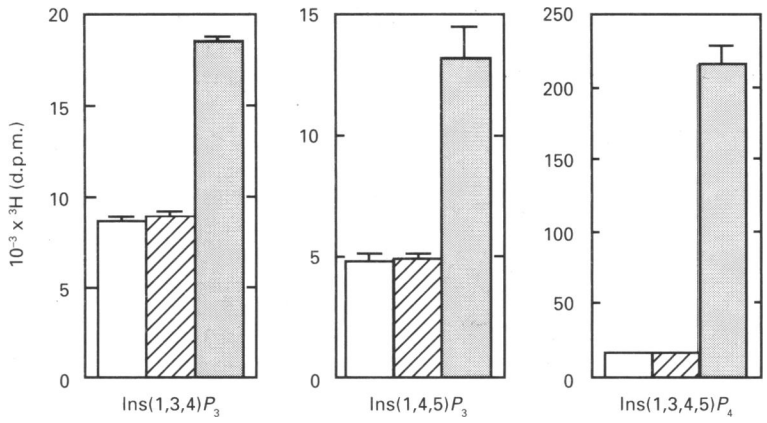

Fig. 5. Effect of Li^+ on the extraction of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in rat and mouse brain cortex slices in the presence of ACh

Brain cortex slices, labelled with [^{3}H]inositol, were preincubated for 20 min in the absence and presence of 50 mM-Li^+ and then incubated in the same medium for 10 min with 0.1 mM-ACh. Immediately before quenching, Li^+ (50 mM final concn.) was added to some of the samples which had been incubated without Li^+ as indicated above. An inositol phosphate extract was analysed by h.p.l.c. Labelling, quench-extraction and h.p.l.c. separation are described in the Experimental section. Key: □, incubation without Li^+; ▨, incubation without Li^+, extraction with 50 mM-Li^+; ▩, incubation with 50 mM-Li^+, extraction with 50 mM-Li^+.

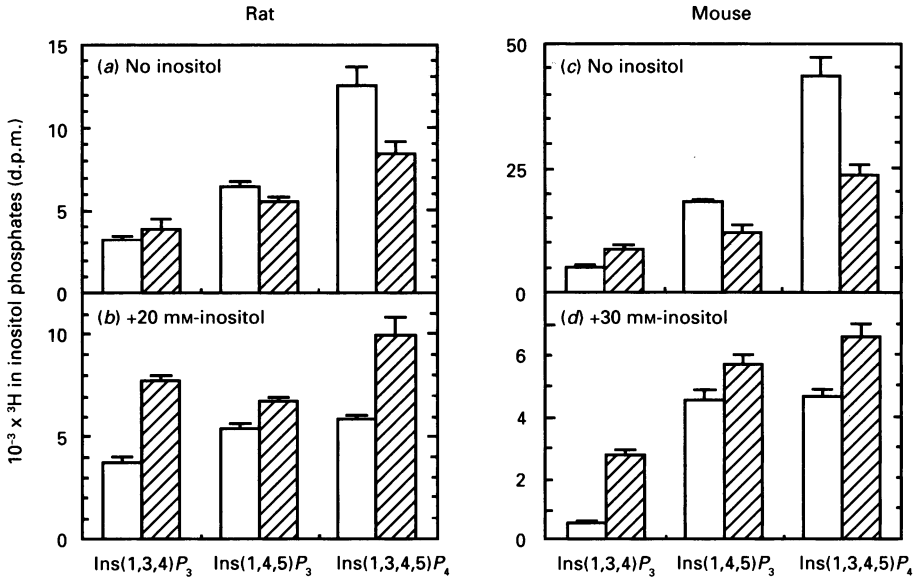

Fig. 6. Effect of Li^+ on the accumulation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 in rat and mouse brain cortex slices in the presence of ACh, with and without inositol supplementation

Brain cortex slices, labelled with [^{3}H]inositol, were preincubated for 20 min with (▨) or without (□) 10 mM-LiCl and, as indicated, with or without inositol. The slices were then incubated in the same medium with 0.1 mM-ACh for 5 min in (*b*) and for 10 min in (*a*), (*c*) and (*d*). An inositol phosphate extract was analysed by h.p.l.c. Labelling, quench-extraction and h.p.l.c. separation are described in the Experimental section.

or mass measurement (J. F. Dixon, G. Los & L. E. Hokin, unpublished work).

DISCUSSION

We show here that, in addition to its well-known stimulatory effects on Ins(1,3,4)P_3 accumulation, Li^+ increased accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in cerebral cortex slices of all species examined if sufficient inositol was made accessible to the PtdIns-synthesizing system. Thus, in cholinergically stimulated mouse and rat brain cortex slices, where Li^+ has been previously shown to inhibit Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 accumulation [26,27,43], supplementation with sufficient inositol reversed these inhibitions and led to a Li^+-induced accumulation of both compounds. The Li^+ stimulations in guinea-pig brain cortex slices were ACh-dependent, and for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 they increased with increasing Li^+ concentrations up to 50 mM, at which point the stimulations were 3.3- and 12.5-fold respectively. In addition to the guinea pig, which has been the main experimental model used here, we have found stimulations of accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in cortical slices of rabbit (C. Moummi & L. E. Hokin, unpublished work).

In the presence of inositol, stimulations of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 formation were seen in guinea-pig brain cortex slices with concentrations of Li^+ as low as 1 mM, which is the midpoint of the therapeutic range in the treatment of bipolar disorder. It is likely that, if the target neurones could be isolated,

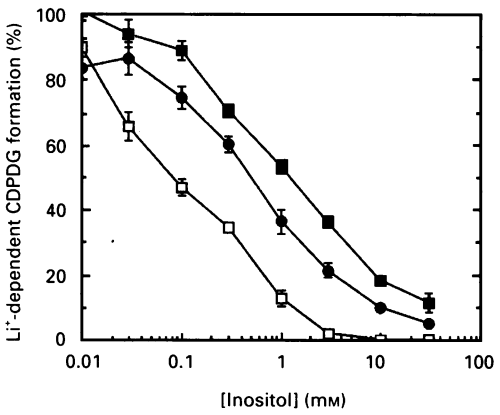

Fig. 7. Effect of increasing concentrations of inositol on the accumulation of [^{14}C]CDPDG in brain cortex slices of various species in the presence of ACh and Li^+

Brain cortex slices were preincubated with [^{14}C]cytidine for 60 min, then for 20 min with 10 mM-Li^+ and various concentrations of inositol and finally incubated for 10 min with 0.1 mM-ACh in the same medium. The reaction was terminated, and CDPDG was extracted and measured as described in the Experimental section. The radioactivity in [^{14}C]CDPDG, which accumulated in the absence of Li^+, was subtracted from radioactivities observed in the presence of Li^+, before calculation of Li^+-enhanced [^{14}C]CDPDG. Inositol had no effect on the [^{14}C]CDPDG level in the absence of Li^+. Key: ■, mouse; ●, rat; □, guinea pig.

Table 1. Effect of Li^+ on mass of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in guinea-pig cerebral cortex slices

Slices from guinea pig cerebral cortex were prepared as usual and carried through the same series of incubations as used in the [^{3}H]inositol experiments, except that [^{3}H]inositol was omitted. Subsequently, the slices were preincubated for 20 min with or without 10 mM-Li^+ and finally incubated with or without 1 mM-ACh for 10 min and quenched with $HClO_4$ in the absence of phytic acid. The neutralized extracts were analysed by the binding assays for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Incubation, quench-extraction and assay are described in the Experimental section. Values are means ± S.E.M.

	Ins(1,4,5)P_3 (pmol/mg)	Ins(1,3,4,5)P_4 (pmol/mg)
−ACh	11.4±0.8	2.71±0.1
+ACh	19.9±1.1	14.5±0.6
−ACh+Li^+	10.9±0.3	3.28±0.1
+ACh+Li^+	23.5±0.4	32.3±1.1
(ACh) Δ	8.5±1.1	11.8±0.6
(ACh+Li^+) Δ	12.6±0.3	29.1±1.1
Li^+ stimulation	48% (P = 0.036)	123% (P = 0.0015)

there would be greater stimulations and greater sensitivity of Li^+, since Li^+-insensitive neurones are likely to be present in cerebral cortex slices.

As stated above, if the slices were supplemented with sufficient inositol, stimulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation by Li^+ could be demonstrated in rat and mouse brain cortex slices. The explanation for the differences in Li^+ responses in rat and mouse without inositol supplementation versus the other species examined is likely to be that Li^+ causes a greater decrease in relative inositol levels, and therefore a greater decrease in the amount of PtdIns which can be synthesized in rat and mouse than in guinea pig and rabbit. Measurements of Li^+-dependent CDPDG formation are consistent with this hypothesis. Any decrease in the concentration of cytosolic inositol near the K_m of PtdIns synthase for inositol (4.6 mM for rat cerebral cortex [44]) will slow down the rate of PtdIns synthesis [18] and increase accumulations of CDPDG. The concentration of inositol required to reverse Li^+-dependent CDPDG formation in rat and mouse (Fig. 7) lies in the range of the K_m of the rat brain synthase for inositol, whereas reversal in guinea pig is achieved at significantly lower concentrations. One or both of the following explanations for this species difference could account for the results. It is possible that there is a difference in the requirement for inositol for normal PtdIns synthesis, based on differences in the affinity of the synthase for inositol in various species and in various tissues. Or, assuming similar K_m values, the amount of inositol present in washed mouse and rat brain slices may be much less than in guinea-pig slices.

Whitworth & Kendall [27] found that in mouse brain cortex slices the effect of Li^+ on stimulated levels of Ins(1,3,4,5)P_4 depended on the agonist used (i.e. inhibition with carbachol and enhancement with KCl, histamine and noradrenaline). Such agonist specificity could be due to differences in the rates of turnover of the phosphoinositide cascade, availability of inositol, requirement for inositol, and/or local concentrations of Li^+. Clearly, Li^+ can enhance the stimulated level of InsP_4 with a variety of agonists. We have found that 10 mM-Li^+ enhances by 100% levels of Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 in the presence of 1 mM-5-hydroxytryptamine in cerebral cortex slices from guinea-pig brain and by 20% for Ins(1,4,5)P_3 (P. S. Sastry & L. E. Hokin, unpublished work). Whitworth & Kendall [27] found no inhibition of Ins(1,3,4,5)P_4 formation after 5-hydroxytryptamine stimulation of mouse brain, but observed no statistically significant enhancement. This difference might reflect the species difference noted with muscarinic stimulation.

The metabolism and function of Ins(1,3,4,5)P_4 have recently been reviewed [45]. There is recent suggestive evidence that Ins(1,3,4,5)P_4 may be a second messenger in Ca^{2+} influx [11–17]. Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 appear to interact synergistically to activate a cholinergically stimulated Ca^{2+}-dependent K^+ current (a measure of Ca^{2+} influx across the plasma membrane) in patch-clamp studies of perfused lacrimal acinar cells [13]. In the absence of Ins(1,3,4,5)P_4, the current was not activated. Highly specific binding sites for Ins(1,3,4,5)P_4 have been characterized in crude cerebellar membranes from rat [35], and highly specific binding proteins for Ins(1,3,4,5)P_4 have been solubilized and isolated from the same tissue [46].

Li^+ has multiple sites of action in the phosphoinositide cascade, and it is likely that action at one or more of these sites is the basis for the therapeutic action of Li^+ in the treatment of manic-depressive illness. It has already been shown that inositol monophosphates and certain inositol polyphosphates [Ins(1,4)P_2 and Ins(1,3,4)P_3] accumulate on exposure of agonist-treated cells to Li^+; this appears to be due to an inhibitory action of Li^+ on inositol monophosphatases and inositol polyphosphate 1-phosphatase(s), although not all inositol polyphosphate 1-phosphatases [19–25] are affected. Berridge *et al.* [18] have proposed that the trapping of inositol as inositol phosphates (mainly as inositol monophosphates) causes inositol to become rate-limiting for the synthesis of phosphoinositides, eventually leading to less availability of second messengers, and they suggest that this may be a mechanism for the therapeutic action of Li^+. The data presented here, showing reversal of Li^+-induced inhibitions of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulations by inositol in rat and mouse cerebral cortex slices, suggest that the mechanism proposed by Berridge *et al.* [18] is operative, at least in rat and mouse brain cortex slices. However, on the basis of the data presented here, the increases in levels of second messengers

by Li^+ under conditions where inositol is not rate-limiting must also be taken into consideration.

The mechanism of the Li^+-induced accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is not known. We have shown that Li^+ retards the breakdown of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in atropine-quenched guinea-pig brain cortex slices prelabelled with [^{3}H]inositol and stimulated with ACh (C. H. Lee, J. F. Dixon & L. E. Hokin, unpublished work). This could be responsible for the accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, but other explanations are possible. The enzymic mechanism for the Li^+-induced accumulation of these compounds requires investigation.

Experiments similar to those in Figs. 1, 2 and 4 were initially done by using a trichloroacetic acid/phytate quench-extraction method. The $HClO_4$ extraction method was initially tested as a substitute for trichloroacetic acid/phytate to be used in the binding assay, since, although phytate has a dramatic effect on recoveries in the trichloroacetic acid system [47], it interferes with the binding assay. $HClO_4$ extraction resulted in increases over trichloroacetic acid/phytate extraction in recoveries of inositol polyphosphates in the presence and absence of Li^+, of 80–120% (results not shown). The recoveries with trichloroacetic acid/phytate extraction were sufficiently low that effects which were relatively modest with $HClO_4$, such as increments in Ins(1,4,5)P_3 at low Li^+ concentrations, lost statistical significance after extraction with trichloroacetic acid/phytate. To test the possibility that Li^+ might be interacting with $HClO_4$ to show falsely higher levels of the inositol polyphosphates, we incubated samples with and without Li^+, followed by addition of Li^+ after incubation to half of the samples incubated without Li^+. Addition of Li^+ to samples incubated without Li^+ had no effect on the levels of inositol polyphosphates (Fig. 5). It also appears that Li^+ has no effect on a possible hydrolysis of PtdInsP_2 by $HClO_4$, as demonstrated by the finding that Li^+ had no effect on the levels of inositol polyphosphates in the absence of agonist (results not shown). In addition, the Li^+ effect has been demonstrated in pancreas without acid, by using a boiling quench method (J. F. Dixon, G. Los & L. E. Hokin, unpublished work). Atropine decreases ACh-stimulated accumulations of all the inositol polyphosphates to control levels both with and without Li^+, showing that stimulated levels are receptor-mediated with and without Li^+. We conclude that extraction artifacts play no role in Li^+-induced enhancements of ACh-stimulated levels of inositol polyphosphates.

Almost all work on cerebral phosphoinositide metabolism in slices and the effects of Li^+ thereon has utilized rat cerebral cortex. This is probably why Li^+-induced increases in levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in cholinergically stimulated brain preparations have not been observed. In view of the work presented here, if the rat or the mouse model system is to be used, the incubation media should be supplemented with inositol.

Relative inositol deficiency owing to trapping of inositol in the form of inositol phosphates may not occur on Li^+ treatment *in vivo*. In slice experiments, owing to the need for prelabelling of phosphoinositides, there is extensive incubation and washing of slices. This may contribute to excessive inositol deficiency in those systems where no Li^+-induced increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are seen without added inositol. This interpretation is experimentally supported by studies *in vivo* in mouse which demonstrate an incremental effect of chronic Li^+ treatment on the mass of brain Ins(1,4,5)P_3 with and without pilocarpine stimulation and on levels of brain [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 after stimulation with pilocarpine [48]. However, there is a caveat. In comparable experiments, an inhibition of these levels by Li^+ is seen in the rat brain *in vivo* [49]. Obviously more experiments must be done *in vivo*, especially in higher species.

Why does the guinea pig respond differently from rat and mouse? Recently, DNA analyses suggest that the guinea pig may not be a rodent [50]. The rabbit, which also is not a rodent, also does not require supplementation with inositol to demonstrate a stimulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 levels by Li^+. Perhaps rodents, the most commonly used models, are unique in maintaining inadequate relative levels of inositol in brain slices.

The salient observation presented here is that, if supplemented with inositol, there is a consistent stimulation of Ins(1,4,5)P_3 and of Ins(1,3,4,5)P_4 accumulation by Li^+ in all species examined, and this occurs in guinea pig over a therapeutic concentration range. This may have implications in the treatment of bipolar disorders with Li^+.

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