Evidence for lithium-sensitive inositol 4,5-bisphosphate accumulation in muscarinic cholinergic-stimulated cerebral-cortex slices

Stephen JENKINSON, R. A. John CHALLISS and Stefan R. NAHORSKI*

Department of Pharmacology and Therapeutics, University of Leicester, University Road, Leicester LE1 9HN, U.K.

Stimulation of [^3H]inositol-prelabelled rat cerebral-cortex slices with carbachol results in the accumulation of four [^3H]inositol bisphosphate isomeric species, $\text{Ins}(1,3)\text{P}_2$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(3,4)\text{P}_2$ and $\text{Ins}(4,5)\text{P}_2$. Although the last isomer ran as a minor peak on h.p.l.c., its accumulation was dramatically enhanced in the presence of Li^+ (1 mM), such that at 30 min it represented almost 35% of the total bisphosphate fraction. The accumulation of $\text{Ins}(4,5)\text{P}_2$ appeared to be very sensitive to Li^+ ($\text{EC}_{50} = 94 \pm 3 \mu\text{M}$), strongly implicating a Li^+ -sensitive metabolism. Evidence for this is provided from the rapid but Li^+ -sensitive decay of $\text{Ins}(4,5)\text{P}_2$ when muscarinic-receptor stimulation is antagonized by atropine at a time when accumulations have reached a new steady state. Manipulation of phospholipase D by activators and inhibitors of protein kinase C did not suggest a role for phospholipase D hydrolysis of PtdInsP_2 in the formation of $\text{Ins}(4,5)\text{P}_2$. Attempts to reveal $\text{Ins}(4,5)\text{P}_2$ metabolism, or indeed its synthesis from $\text{Ins}(1,4,5)\text{P}_3$, were not successful with broken cell preparations and strongly suggest discrete compartmentation of inositol phosphate metabolism in the intact cell.

INTRODUCTION

Agonist stimulation of a large variety of receptors in the central nervous system promotes hydrolysis of inositol phospholipids through activation of a phosphoinositide-specific phospholipase C [1,2]. Phosphodiesteratic cleavage of PtdInsP_2 by phospholipase C produces $\text{Ins}(1,4,5)\text{P}_3$ and *sn*-1,2-diacylglycerol, which release Ca^{2+} from intracellular stores and activate protein kinase(s) C (PKC) respectively [1–4]. Metabolism of $\text{Ins}(1,4,5)\text{P}_3$ is rapid and can follow two possible pathways (for review see [4]). The simple dephosphorylation route proceeds by a 5-phosphatase, to $\text{Ins}(1,4)\text{P}_2$, and $\text{Ins}(1,3,4)\text{P}_3/\text{Ins}(1,4)\text{P}_2$ 1-phosphatase, to $\text{Ins}4\text{P}$. Alternatively, phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ by 3-kinase generates the putative second messenger $\text{Ins}(1,3,4,5)\text{P}_4$, which then undergoes sequential dephosphorylation via a 5-phosphatase, to $\text{Ins}(1,3,4)\text{P}_3$, and either a 4-phosphatase or an $\text{Ins}(1,3,4)\text{P}_3/\text{Ins}(1,4)\text{P}_2$ 1-phosphatase route to produce $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}(3,4)\text{P}_2$ respectively. Subsequent dephosphorylation produces $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$, which along with $\text{Ins}4\text{P}$ are metabolized by the inositol monophosphatase to release *myo*-inositol [4]. This complex pathway of $\text{Ins}(1,4,5)\text{P}_3$ metabolism serves to terminate second-messenger action and to conserve efficiently the cellular *myo*-inositol pool for resynthesis of inositol phospholipids.

There is now substantial evidence that the anti-manic agent Li^+ can disrupt the phosphoinositide cycle in brain and that this may be of importance in its therapeutic action [5,6]. Li^+ is a potent and uncompetitive inhibitor of the inositol monophosphatase enzyme ($\text{IC}_{50} \sim 0.5 \text{ mM}$) [7,8], and to a lesser extent the $\text{Ins}(1,3,4)\text{P}_3/\text{Ins}(1,4)\text{P}_2$ 1-phosphatase ($\text{IC}_{50} \sim 5 \text{ mM}$) [9]. Therapeutically relevant concentrations of Li^+ (0.3–1.0 mM) have been shown to produce marked increases in the levels of inositol monophosphates and, by depriving phosphoinositide synthesis of a source of inositol, decreases in agonist-stimulated accumulations of $\text{Ins}(1,4,5)\text{P}_3$ [10,11] and $\text{Ins}(1,3,4,5)\text{P}_4$ [10–13] has been reported in rat and mouse cerebral-cortex slices.

In an attempt to analyse more fully the inositol phosphate metabolite profile in Li^+ -treated cerebral cortex, we have observed

a marked accumulation of a labelled bisphosphate which we had previously detected in this tissue as a very minor product after muscarinic-receptor stimulation and had tentatively identified as $\text{Ins}(4,5)\text{P}_2$ [14]. The accumulation of this metabolite suggests an additional Li^+ -sensitive enzyme and an added complexity to inositol polyphosphate metabolism in cerebral tissue.

MATERIALS AND METHODS

Studies with [^3H]inositol-labelled brain slices

Cerebral-cortex slices were prepared from male Wistar rats (250–300 g) as described previously [10]. In brief, tissue was cross-chopped (350 $\mu\text{m} \times 350 \mu\text{m}$) with a McIlwain tissue chopper and the slices were transferred to a flat-bottomed flask containing 150 ml of Krebs–Henseleit buffer [KHB (in mM): NaCl, 118; KCl, 4.7; NaHCO_3 , 25; CaCl_2 , 1.3; MgSO_4 , 1.2; KH_2PO_4 , 1.2; glucose, 10; pH 7.4 after saturation with O_2/CO_2 (19:1)]. After extensive initial washing, the slices were incubated for 60 min at 37 °C in a shaking water-bath with KHB replacements every 15 min. Gravity-packed slices (50 μl) were transferred to vials containing 230 μl of KHB supplemented with 5 μCi of [^3H]inositol/vial and incubated for 60 min. After this labelling period, drugs were added at the concentrations and times stated in the text; the vials were gassed with O_2/CO_2 (19:1) and returned to the water-bath for the appropriate periods of time (see the Results section). In all cases, the final incubation volume was adjusted to 300 μl .

Incubations were terminated by addition of 300 μl of ice-cold HClO_4 (10%, v/v). Samples were extracted on ice for 15 min and centrifuged (3000 g, 20 min, 4 °C). A portion of the supernatant (450 μl) was sampled, EDTA (125 μl , 10 mM) was added, and the acid was extracted by addition of freon/octylamine (750 μl ; 1:1, v/v). After thorough mixing and centrifugation (3000 g, 10 min, 4 °C), a portion of the aqueous phase (350 μl) containing the various inositol (poly)phosphate isomers was analysed by h.p.l.c. as previously described [14].

Abbreviations used: PKC, protein kinase C; PLD, phospholipase D; PDBu, phorbol 12,13-dibutyrate.

* To whom correspondence should be addressed.

Preparation of an [^3H]Ins(4,5) P_2 standard

[^3H]Ins(4,5) P_2 was prepared by mild alkaline phosphatase treatment of [^3H]Ins(1,4,5) P_3 [15]. Calf intestinal alkaline phosphatase (10 units) was dissolved in a BSA solution (500 μl , 0.1 %). Then 50 μl of this stock solution was added to 1940 μl of 0.2 M-Tris/maleate (pH 9.0)/2 mM-MgCl $_2$, to which 0.05 μCi of [^3H]Ins(1,4,5) P_3 was added. After 60 min, at 37 °C, the reaction was terminated by addition of 0.2 ml of HClO $_4$ (10 %, v/v). Acid was extracted from the sample as described above.

Materials

myo-[2- ^3H]Inositol (15.4 Ci/mmol) was purchased and [^3H]Ins(1,4,5) P_3 (17 Ci/mmol) obtained as a gift, from NEN DuPont (U.K.) Ltd. (Stevenage, Herts., U.K.). Ro 318220 was obtained as a gift from Roche Products (Welwyn Garden City, Herts., U.K.). Alkaline phosphatase was purchased from Sigma. All other agents were of analytical grade.

RESULTS

Effect of Li $^+$ on inositol bisphosphate isomer accumulation

In agreement with our previous studies in carbachol-stimulated cerebral-cortex slices [14], four labelled bisphosphate products could be separated by h.p.l.c. (Fig. 1). Identification of three of the metabolites as Ins(1,3) P_2 , Ins(1,4) P_2 and Ins(3,4) P_2 has been previously achieved by co-elution of standards and analysis of appropriate alditols [14]. The late-running minor peak had been tentatively identified as Ins(4,5) P_2 [14], and in these experiments support for this identification came from an identical elution of the alkaline phosphatase product of authentic [^3H]Ins(1,4,5) P_3 (Fig. 1). The accumulation of each of the inositol bisphosphate products was increased upon muscarinic-receptor stimulation (47-, 4-, 12- and 23-fold respectively after 30 min), albeit from very different basal levels (Fig. 2).

Li $^+$ (10 μM –10 mM) had either no effect, or a relatively weak effect, on Ins(1,3) P_2 , Ins(1,4) P_2 and Ins(3,4) P_2 accumulations. However, this univalent ion produced an 11-fold increase in carbachol-stimulated Ins(4,5) P_2 accumulation, and this enhancement was observed at low concentrations of Li $^+$ ($\text{EC}_{50} = 94 \pm 3 \mu\text{M}$).

The results in Fig. 3 demonstrate the time course of accumulation of inositol bisphosphate isomers in carbachol-stimulated (1 mM) cerebral-cortex slices in the presence and

absence of 1 mM-Li $^+$. Carbachol-stimulated Ins(1,3) P_2 , Ins(1,4) P_2 and Ins(4,5) P_2 accumulation in the absence of Li $^+$ was rapid, reaching a new steady state by 5–10 min. Although Ins(3,4) P_2 appeared to increase throughout the 30 min incubation, its relatively low accumulation did not suggest a clear kinetic difference from the other bisphosphate isomers.

In the presence of Li $^+$ (1 mM) a significant enhancement of carbachol-stimulated Ins(1,3) P_2 at 5 and 10 min and in Ins(1,4) P_2 accumulation at 10 min was observed. However, at the 20 and 30 min time points the accumulations of these bisphosphates were not significantly different in the absence and presence of Li $^+$. Ins(3,4) P_2 accumulation was not influenced by this ion at any of the incubation times investigated. However, Li $^+$ produced

Fig. 2. Effect of Li $^+$ on carbachol-stimulated inositol bisphosphate isomer accumulation in cerebral-cortex slices

[^3H]Inositol bisphosphate isomer accumulation in carbachol-stimulated (1 mM) rat cerebral-cortex slices in the presence of increasing concentrations of Li $^+$ (10 μM –10 mM). All incubations were for 30 min. Values are means $\pm$ S.E.M. of data normalized from three separate experiments each performed in triplicate. Carbachol-stimulated values were (in d.p.m.) as follows: Ins(1,3) P_2 , 4107 $\pm$ 785; Ins(1,4) P_2 , 11047 $\pm$ 1019; Ins(3,4) P_2 , 2098 $\pm$ 392; Ins(4,5) P_2 , 558 $\pm$ 96.

Fig. 3. Time courses of carbachol-stimulated inositol bisphosphate isomer accumulation in cerebral-cortex slices

[^3H]Inositol bisphosphate accumulation in carbachol-stimulated (1 mM) rat cerebral-cortex slices in the absence (O) and presence (●) of Li $^+$ (1 mM). Values are means $\pm$ S.E.M. of data normalized from three separate experiments each performed in triplicate. Basal values of [^3H]inositol bisphosphates were (in d.p.m.) as follows: Ins(1,3) P_2 , 64 $\pm$ 11; Ins(1,4) P_2 , 2428 $\pm$ 85; Ins(3,4) P_2 , 104 $\pm$ 27; Ins(4,5) P_2 , 51 $\pm$ 8. Statistically significant differences (Student's t test) are indicated for the absence versus the presence of Li $^+$ at given time points: * P < 0.05; ** P < 0.01; *** P < 0.001.

Fig. 1. H.p.l.c. separation of [^3H]inositol bisphosphates

H.p.l.c. separation of the [^3H]inositol bisphosphates present in an extract from carbachol-stimulated (1 mM) cerebral-cortex slices in the absence (O) and presence (●) of 1 mM-Li $^+$ (30 min incubation). The dotted line demonstrates the co-elution of [^3H]Ins(4,5) P_2 prepared by alkaline phosphatase treatment of [^3H]Ins(1,4,5) P_3 (see the Materials and methods section). Positions of other bisphosphate isomer peaks are indicated.

Fig. 4. Reversibility of carbachol-stimulated inositol phosphate isomer accumulations after atropine addition in cerebral-cortex slices

[³H]Inositol phosphate isomer accumulation in carbachol-stimulated (1 mM) rat cerebral-cortex slices in the absence (△, ▲) and presence of Li⁺ (○, ●) (1 mM). The non-selective muscarinic antagonist atropine (10 μM) was added at 10 min (▲, ●), and the decay rates for each inositol phosphate isomer are shown. Values are means ± S.E.M. of data normalized from three separate experiments each performed in triplicate. Basal values of [³H]inositol phosphates were (in d.p.m.) as follows: Ins1P, 5846 ± 1442; Ins4P, 2902 ± 326; Ins(1,3)P₂, 73 ± 17; Ins(1,4)P₂, 2150 ± 324; Ins(4,5)P₂, 33 ± 8; Ins(1,3,4)P₃, 146 ± 41; Ins(1,4,5)P₃, 1840 ± 410; Ins(1,3,4,5)P₄, 429 ± 151.

Table 1. Effects of PDBu or Ro 318220 on carbachol-stimulated Ins(4,5) P_2 , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 accumulations in rat cerebral-cortex slices

Cerebral-cortex slices were preincubated and labelled with [^{3} H]inositol as described in the Materials and methods section. PDBu (5 μ M) or Ro 318220 (5 μ M) was added 10 min before LiCl (1 mM) and/or carbachol (1 mM) addition for 15 min. Values are means $\pm$ S.E.M. for three experiments performed in triplicate and are expressed as d.p.m./sample.

InsP species...	Ins(4,5) P_2		Ins(1,4,5) P_3		Ins(1,3,4,5) P_4	
	Li ⁺ ...	— +	— +	— +	— +	— +
Control		31 ± 7 42 ± 9	1513 ± 20 1320 ± 274	428 ± 16 262 ± 48		
PDBu		28 ± 5 55 ± 11	1729 ± 218 1412 ± 312	389 ± 25 253 ± 70		
Carbachol		839 ± 116 5109 ± 220	4498 ± 456 4210 ± 215	12051 ± 1646 6253 ± 645		
Carbachol + PDBu		1190 ± 51 6464 ± 599	4640 ± 321 3845 ± 568	16826 ± 2223 7116 ± 981		
Carbachol + Ro 318220		882 ± 118 4898 ± 264	4959 ± 264 3879 ± 398	12348 ± 1300 5345 ± 485		

a dramatic enhancement in Ins(4,5) P_2 accumulation at all time points studied, such that at 30 min Ins(4,5) P_2 made up 34.0 $\pm$ 3.6 % of the total inositol bisphosphate fraction. Ins(4,5) P_2 accumulation appeared to be linear with time in the presence of Li $^+$, indicative of an inhibition of its metabolism.

Kinetic analysis of inositol phosphate metabolism

Attempts to detect metabolism of Ins(4,5) P_2 by cerebral-cortical homogenates were not successful. Negligible loss of [^{3} H]Ins(4,5) P_2 (1 μ M) was observed even after long incubations in an intracellular buffer with a variety of cerebral homogenates. However, metabolism of Ins(4,5) P_2 in cortex slices was easily detected by a kinetic decay approach previously employed in this laboratory [12]. In brief, inositol phosphate accumulation is stimulated to an elevated steady state by an agonist; the muscarinic antagonist atropine is then added to suppress stimulated synthesis, and the decay in inositol phosphate isomer accumulation provides a measure of metabolism. The results in Fig. 4 demonstrate the time course of accumulation of inositol phosphate isomers in the absence and presence of Li $^+$ in carbachol-stimulated (1 mM) cerebral-cortex slices. In the absence of Li $^+$ all isomers examined reached steady-state levels by 10 min, at which time atropine (10 μ M) was added and the decay kinetics of the individual isomers were examined. As expected, Ins(1,4,5) P_3 levels fell dramatically, reaching a new steady state, slightly higher than basal, within 1 min (see below). Other accumulated inositol phosphate isomers decayed to basal more slowly, indicative of slower rates of metabolism.

When incubations were performed in the presence of Li $^+$ (1 mM) a sustained enhancement in carbachol-stimulated Ins1P, Ins4P and Ins(4,5) P_2 , and a decrease in Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , was observed, as shown in Fig. 4. Li $^+$ also altered the decay kinetics of several isomers. The decay of both inositol monophosphate isomers was decreased although not totally arrested, by Li $^+$ (1 mM). The metabolism of Ins(1,3) P_2 was essentially unaffected, whereas that of Ins(1,4) P_2 was slightly delayed, probably owing to a weak inhibitory effect of Li $^+$ on the dephosphorylation of Ins(1,4) P_2 by Ins(1,3,4) P_3 /Ins(1,4) P_2 1-phosphatase. Ins(4,5) P_2 metabolism was clearly suppressed by Li $^+$ (1 mM), and there was a delay of approx. 4 min after atropine blockade before any decrease in the accumulation of this isomer was observed. However, a clear loss at 10 min suggests that the metabolism of Ins(4,5) P_2 is not completely blocked by 1 mM-Li $^+$.

Ins(1,4,5) P_3 metabolism was rapid and unaffected by Li $^+$ (1 mM) with Ins(1,4,5) P_3 , reaching a new lower steady state within 1 min after addition of atropine. It should be noted, however, that in both the absence and the presence of Li $^+$ the addition of atropine resulted in the decay of Ins(1,4,5) P_3 to new steady-state levels slightly greater than basal. It seems probable that this reflects changes in the specific radioactivity of [^{3} H]Ins(1,4,5) P_3 after

carbachol stimulation of the cerebral cortex slices. We have previously described this phenomenon in cortex slices [12]. This specific-radioactivity change is only a significant feature with the immediate product of receptor-stimulated PtdIns P_2 hydrolysis, Ins(1,4,5) P_3 . Both Ins(1,3,4) P_3 and Ins(1,3,4,5) P_4 decayed to basal and were unaffected by Li $^+$ (1 mM).

Effect of PKC-activated phospholipase D (PLD) on Ins(4,5) P_2 accumulation

The possibility that Ins(4,5) P_2 is the product of PLD hydrolysis of PtdIns P_2 was examined indirectly. The present evidence strongly implicates a PKC activation of PLD [15] and therefore effects of the active phorbol 12,13-dibutyrate (PDBu) and the PKC inhibitor Ro 318220 [16] were examined (Table 1) on carbachol-stimulated accumulations of Ins(4,5) P_2 and other possible precursors, Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 . Basal and agonist-stimulated levels of Ins(4,5) P_2 , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , in both the absence and the presence of Li $^+$ (1 mM), were unaffected by either activation or inhibition of PKC by PDBu or Ro 318220 respectively. These results are not consistent with PKC-activated PLD involvement in the production of Ins(4,5) P_2 in cerebral-cortex slices.

DISCUSSION

There is now substantial evidence, from both the accumulation of metabolites in mammalian cells and the products resulting from homogenate incubations, that Ins(1,4,5) P_3 can be phosphorylated to Ins(1,3,4,5) P_4 or dephosphorylated by 5-phosphatase to Ins(1,4) P_2 (see [3,4]). Although the presence of substantial Ins(1,4,5) P_3 1-phosphatase activity in the slime mould *Dictyostelium discoideum* [17] generates the unusual inositol bisphosphate isomer Ins(4,5) P_2 , there is little evidence to date that this latter metabolite accumulates in agonist-stimulated mammalian cells. Hughes & Drummond [18] first reported a late-running [^{3} H]inositol bisphosphate h.p.l.c. peak in thyroliberin (TRH)-stimulated GH $_3$ cells that was somewhat enhanced by 10 mM-Li $^+$. We also previously observed accumulation of a minor late-running bisphosphate peak in carbachol-stimulated cerebral-cortex slices [14], and its tentative identification as Ins(4,5) P_2 has been supported here by the co-elution of the alkaline phosphatase product of Ins(1,4,5) P_3 . Further analysis of this product by examining the corresponding [^{3} H]alditols cannot unequivocally identify Ins(4,5) P_2 [14], but other separations are being considered. However, at present its elution profile and co-elution with standard strongly suggest its identity as Ins(4,5) P_2 . The major observation of this study is that Li $^+$ greatly enhances the accumulation of this unusual metabolite, such that at 30 min it represents almost 35 % of the total bisphosphate fraction. This should not, however, be compared quantitatively, since agonist-

stimulated accumulations of the other major bisphosphate isomers, Ins(1,3)P₂ and Ins(1,4)P₂, are relatively Li⁺-insensitive and their rate of turnover at steady state could greatly exceed that for Ins(4,5)P₂ (see below).

The enhanced and linear accumulation of Ins(4,5)P₂ with time in the presence of low concentrations of Li⁺ is reminiscent of the effects of this ion on inositol monophosphate accumulation [10,12] (Fig. 4), which results from the potent uncompetitive inhibition of inositol monophosphatase [7,8]. Thus it is tempting to suggest that Ins(4,5)P₂ is generated in cerebral tissue after receptor activation and that it is metabolized by an enzyme that is potently inhibited by Li⁺. Indeed, the EC₅₀ for Li⁺ enhancing its accumulation by carbachol (94 ± 3 μM) could suggest a more potent inhibition than on the monophosphatase (however, see below). Alternatively, Li⁺ may enhance Ins(4,5)P₂ formation from putative precursors, assuming that the metabolism of Ins(4,5)P₂ is rate-limiting. This should be considered in light of the unexpected agonist-stimulated accumulations of Ins(1,4)P₂ and Ins(1,3)P₂ observed at 5–10 min in the presence of 1 mM-Li⁺. From our knowledge of the specificity and Li⁺-sensitivity of the Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase [4], this concentration of Li⁺ should only minimally affect the accumulation of the former and be totally ineffective against the latter isomer. Thus the possibility remains that Li⁺ enhances dephosphorylation of Ins(1,4,5)P₃ and/or Ins(1,3,4,5)P₄, resulting in the depletion of these inositol polyphosphate second messengers and enhancement of the three bisphosphate isomers. However, there is no support for this hypothesis from the atropine reversal experiments, since the rate of metabolism of either Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ was not influenced by Li⁺. This hypothesis would also be inconsistent with the clear effects of Li⁺ on inositol depletion and suppression of phosphoinositide synthesis [5,6] being the major determinants of reduced Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ accumulation.

After receptor blockade, increased Ins(4,5)P₂ in the absence of Li⁺ quickly decays, suggesting that in the intact cell metabolism occurs rapidly. Our failure to observe such metabolism with homogenates may suggest strict control of hydrolysis in the intact cell. Further homogenate experiments using a variety of incubation conditions are needed. The linear accumulation of this bisphosphate with Li⁺ is more likely a consequence of inhibition of its metabolism. Indeed, receptor blockade in the presence of Li⁺, which produces a rapid and dramatic decrease in Ins(1,3)P₂ and Ins(1,4)P₂, results in no decay of Ins(4,5)P₂ within the first 4 min after atropine addition. However, by 10 min a significant decrease in Ins(4,5)P₂ accumulation is observed, indicating that the enzyme(s) involved in metabolizing Ins(4,5)P₂ are not fully inhibited. This contrasts somewhat with the results mentioned above, in which half-maximal enhancement of Ins(4,5)P₂ accumulation occurred at 94 ± 3 μM and appears maximal at 1 mM-Li⁺. This anomaly would be consistent if Ins(4,5)P₂ was being produced from Ins(1,4,5)P₃ via a Li⁺-sensitive 1-phosphatase similar to that found in *Dictyostelium discoideum* [17], though with a somewhat higher K_i for Li⁺. This would be consistent with the bell-shaped Li⁺-concentration/response curve obtained for carbachol-stimulated Ins(4,5)P₂ accumulation and the inability of 1 mM-Li⁺ to block Ins(4,5)P₂ decay kinetics completely. Finally, in the presence of Li⁺ (1 mM) the metabolism of Ins1P and Ins4P is very slow and less than that for Ins(4,5)P₂, suggesting that the blockade of Ins1P and Ins4P metabolism by Li⁺ (IC₅₀ ~ 0.5 mM) is more potent than that for Ins(4,5)P₂. This suggests an IC₅₀ for Li⁺ inhibition of the enzyme(s) metabolizing Ins(4,5)P₂ of greater than 0.5 mM, rather than the 90 μM that results from a truncated concentration-response curve incurred by inhibitory effects of higher Li⁺ concentrations on Ins(4,5)P₂ synthesis. These data would also

suggest that there could be a dramatic flux through Ins(4,5)P₂ in cerebral cortex under agonist-stimulated conditions, and that this is underestimated because of the inability to dissect pharmacologically the suppression component of its metabolism with Li⁺.

The route of dephosphorylation of Ins(4,5)P₂ is unknown. Ins(4,5)P₂ has been shown to be a substrate, albeit a poor one, for the Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ 5-phosphatase [19]. Whether Ins(4,5)P₂ is dephosphorylated to Ins4P or Ins5P by a 5- or a 4-phosphatase activity respectively is still to be answered. However, significant Ins5P accumulation has been reported in cerebral cortex of rats treated with Li⁺ and the muscarinic agonist pilocarpine *in vivo* [20].

Two possible routes should be considered as the source of Ins(4,5)P₂. Firstly, PLD could hydrolyse PtdInsP₂ to form Ins(4,5)P₂ (and phosphatidic acid) directly, by-passing the production of the second messenger Ins(1,4,5)P₃. PLD has been shown to be stimulated by activators of PKC, such as phorbol esters (see [15] for review), suggesting that agonists may use such a route of activation. However, recent studies do not indicate a carbachol-stimulated PLD activity in rat cerebral-cortex slices [21]. In the present study PKC-stimulation of PLD produced by PDBu had no effect on either basal or carbachol-stimulated levels of Ins(4,5)P₂ in either the presence or the absence of Li⁺. Similarly the selective PKC inhibitor Ro 318220, which has been shown to inhibit PKC-stimulated PLD activity in Swiss 3T3 cells [16], did not produce a significant decrease in carbachol-stimulated Ins(4,5)P₂ accumulation. These results therefore suggest that at least a PKC-activated PLD is probably not involved in the formation of Ins(4,5)P₂. Alternatively, either the metabolism of Ins(1,4,5)P₃ by a 1-phosphatase, or indeed the sequential dephosphorylation of Ins(1,3,4,5)P₄ by a 1-phosphatase and a 3-phosphatase, could produce Ins(4,5)P₂. At first sight this route does not appear likely to account for the relatively large amount of Ins(4,5)P₂ accumulating, since this bisphosphate is not produced when Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ is metabolized by crude brain homogenates ([14]; S. Jenkinson, R. A. J. Challiss & S. R. Nahorski, unpublished work). Compartmentation of 1-, 3- and the overwhelming 5-phosphatase activities within the cell, however, could account for the production of Ins(4,5)P₂ from either Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄. As discussed above, a less sensitive effect of Li⁺ on such enzyme(s) would be totally consistent with all our results previously reported.

The significance of agonist-stimulated Ins(4,5)P₂ accumulation is not known. Ins(4,5)P₂ has been shown to release Ca²⁺ from intracellular stores in a similar manner to Ins(1,4,5)P₃, albeit with a potency which is ~ 100-fold less than that for Ins(1,4,5)P₃ ([22]; S. Jenkinson, S. Safrany, R. A. J. Challiss & S. R. Nahorski, unpublished work). Any calculation of the accumulated mass of Ins(4,5)P₂ is complicated by (i) the change in specific radioactivity of labelled Ins(1,4,5)P₃ and (ii) the inability to assess the true accumulation in a heterogeneous cell population. However, Ins(4,5)P₂ would seem unlikely to play a major role in Ca²⁺ mobilization, in view of its rate of accumulation and the sustained increase in Ins(1,4,5)P₃ seen in cerebral-cortex slices. Other roles for this alternative route of metabolism for Ins(1,4,5)P₃ and Ins(4,5)P₂ accumulation await further investigation. However, the present results reveal that, in the presence of Li⁺ and agonist, Ins(4,5)P₂ accumulates linearly with time, and this at the very least suggests further complexity to our present appreciation of inositol polyphosphate metabolism in brain.

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