Biochem. J. (1987) 242, 517–524 (Printed in Great Britain)

Evidence that inositol 1-phosphate in brain of lithium-treated rats results mainly from phosphatidylinositol metabolism

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1. In cerebral cortex of rats treated with increasing doses of LiCl, the relative concentrations of Ins(1)*P*, Ins(4)*P* and Ins(5)*P* (when Ins*P* is a *myo*-inositol phosphate) are approx. 10:1:0.2 at all doses. In rats treated with LiCl followed by increasing doses of pilocarpine a similar relationship occurs. 2. *myo*-Inositol-1-phosphatase (InsP_1ase) from bovine brain hydrolyses Ins(1)*P*, Ins(4)*P* and Ins(5)*P* at comparable rates, and these substrates have similar K_m values. 3. The hydrolysis of Ins(4)*P* is inhibited by Li^+ to a greater degree than is hydrolysis of Ins(1)*P* and Ins(5)*P*. 4. D-Ins(1,4,5)P_3 and D-Ins(1,4)P_2 are neither substrates nor inhibitors of InsP_1ase. 5. A dialysed high-speed supernatant of rat brain showed a greater rate of hydrolysis of Ins(1)*P* than of D-Ins(1,4)P_2 and a lower sensitivity of the bisphosphate hydrolysis to LiCl, as compared with the monophosphate. That enzyme preparation produced Ins(4)*P* at a greater rate than Ins(1)*P* when D-Ins(1,4)P_2 was the substrate. 6. The amount of D-Ins(3)*P* [i.e. L-Ins(1)*P*, possibly from D-Ins(1,3,4)P_3] is only 11% of that of D-Ins(1)*P* on stimulation with pilocarpine in the presence of Li^+. 7. DL-Ins(1,4)P_2 was hydrolysed by InsP_1ase to the extent of about 50%; both Ins(4)*P* and Ins(1)*P* are products, the former being produced more rapidly than the latter; apparently L-Ins(1,4)P_2 is a substrate for InsP_1ase. Li^+, but not Ins(2)*P*, inhibited the hydrolysis of L-Ins(1,4)P_2. 8. The following were neither substrates nor inhibitors of InsP_1ase; Ins(1,6)P_2, Ins(1,2)P_2, Ins(1,2,5,6)P_4, Ins(1,2,4,5,6)P_5, Ins(1,3,4,5,6)P_5 and phytic acid. 9. *myo*-Inositol 1,2-cyclic phosphate was neither substrate nor inhibitor of InsP_1ase. 10. We conclude that the 10-fold greater tissue contents of Ins(1)*P* relative to Ins(4)*P* in both stimulated and non-stimulated rat brain *in vivo* are the consequence of a much larger amount of PtdIns metabolism than polyphosphoinositide metabolism under these conditions.

INTRODUCTION

The increase in concentration of Ins(1)*P* in cerebral cortex of rats treated with Li^+, and the accompanying decrease in *myo*-inositol in the same tissue, are well-described events that have their origins in phosphoinositide metabolism (Allison & Stewart, 1971; Allison *et al.*, 1976; Sherman *et al.*, 1981) and in the inhibition by Li^+ of *myo*-inositol-1-phosphatase (InsP_1ase, EC 3.1.3.25; Hallcher & Sherman, 1980). [There are good reasons to consider changing the name of this important enzyme from *myo*-inositol-1-phosphatase to *myo*-inositol-monophosphate phosphohydrolase, or, more simply, *myo*-inositol monophosphatase. The enzyme hydrolyses both enantiomers of Ins(1)*P* (Eisenberg, 1967; Hallcher & Sherman, 1980) and of Ins(4)*P* (the present paper), as well as the *meso* Ins(5)*P* (this paper). The enzyme does not, however, hydrolyse Ins(2)*P* (Eisenberg, 1967), which is, in fact, an inhibitor (Naccarato *et al.*, 1974). The enzyme also has activity against (−)*chiro*-inositol(3)*P* and several non-inositol substrates (Eisenberg, 1967; Takimoto *et al.*, 1985).]

The metabolism responsible for the formation of Ins(1)*P* in the Li^+-treated unstimulated rat is endogenous neural activity. That metabolism can be enhanced by agents that are known to act on receptors, utilizing intracellular Ca^{2+} mobilization as part of the coupling of stimulus and response. Such stimulation is accompanied by an increase in Ins(1)*P* that is amplified by the presence of Li^+ in the tissue (Sherman *et al.*, 1985*a*,*b*, 1986*b*).

Ins(1,4,5)P_3, which is released from PtdIns(4,5)P_2 after stimulation in a number of tissues, is a potent agent for the release of Ca^{2+} from intracellular stores in a variety of cells *in vitro* (for reviews see Berridge, 1984; Berridge & Irvine, 1984). Because of the well-known intense metabolic activity of the polyphosphoinositides in neural tissues, we expected that the Ins(1)*P* produced in our studies *in vivo* would be the product of sequential dephosphorylation of Ins(1,4,5)P_3 and Ins(1,4)P_2, or of the degradation of one or more of the recently discovered inositol polyphosphates, e.g. Ins(1,3,4)P_3 or Ins(1,3,4,5)P_4 (Irvine *et al.*, 1984; Batty *et al.*, 1985). However, if Ins(1)*P* is mainly derived from polyphosphoinositide metabolism, then the larger amount of Ins(1)*P* that accumulates, relative to Ins(4)*P*, in Li^+-treated rats (Sherman *et al.*, 1985*b*) must be the result of the more rapid hydrolysis of a 4-phosphate moiety than of a 1-phosphate in the degradation of one of the inositol polyphosphates. In order to clarify the role of InsP_1ase in the degradation of these substances, we have examined

Abbreviations used: Ins, *myo*-inositol; Ins(1)*P*, *myo*-inositol 1-phosphate [similarly Ins(4)*P*, Ins(5)*P*]; Ins(1,4)P_2, *myo*-inositol 1,4-bisphosphate [similarly Ins(1,4,5)P_3, Ins(1,3,4)P_3, Ins(1,3,4,5)P_4]; InsP_1ase, *myo*-inositol-monophosphate phosphohydrolase; InsP_2ase, *myo*-inositol-1,4-bisphosphate phosphohydrolase; PtdIns, phosphatidylinositol; PtdIns(4)*P*, phosphatidylinositol 4-phosphate [similarly PtdIns(4,5)P_2]; TMS, trimethylsilyl.

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several inositol mono- and poly-phosphates as potential substrates of this enzyme. We have also investigated the products formed by the action on Ins(1,4)P_2 of a brain extract containing InsP_2ase [Ins(1,4)P_2 phosphatase]. These experiments and others suggest that most of the Ins(1)P that we find in brain of rats treated with Li^+ alone or with Li^+ and pilocarpine is principally due to direct metabolism of PtdIns by phospholipase C rather than arising from polyphosphoinositide metabolism.

EXPERIMENTAL

Bovine brain InsP_1ase, purified up to and including the Bio-Gel 300 stage as described by Hallcher & Sherman (1980), had a specific activity of 8–11 μmol of P_i/h per mg of protein with 0.7 mM-DL-Ins(1)P as substrate. One unit of enzyme produces 1 nmol of P_i/h at 37 °C.

A crude preparation containing both InsP_1ase and InsP_2ase was prepared from fresh rat brain by homogenization with a Polytron (Brinkmann) homogenizer with 0.25 M-sucrose in 5 mM-Tris/HCl, pH 8.0 (tissue: buffer 1:2, w/v). The homogenate was centrifuged at 180000 *g* for 30 min and then pressure-dialysed against the same buffer, by using an Amicon PM 10 membrane, halving its volume. Portions of this preparation were used without further purification.

D-Ins(1,4,5)P_3 and D-Ins(1,4)P_2 were prepared from polycythaemic human red blood cells by the method of Downes & Michell (1981), with modifications to scale up the procedure as described by Wolf *et al.* (1985). Synthetic DL-Ins(1,4)P_2, DL-Ins(4)P and Ins(5)P were given by Professor Stephen Angyal, University of New South Wales, Australia. Ins(1,2)P_2, Ins(1,6)P_2, Ins(1,2,5,6)P_4, Ins(1,2,4,5,6)P_5 and Ins(1,3,4,5,6)P_5 were given by D. J. Cosgrove and G. C. J. Irvine, C.S.I.R.O., Canberra City, Australia. Phytic acid and Ins(2)P were from Sigma. The concentrations and purities of the inositol phosphates were determined after alkaline phosphatase digestion by measurement of inositol by g.l.c. and of P_i by colorimetry. The composition of each substrate (i.e., the inositol/phosphate content) was determined by fast-atom-bombardment mass spectrometry (Sherman *et al.*, 1986*a*) as well as by high-voltage paper electrophoresis (Seiffert & Agranoff, 1965). L-Ins(1)P was obtained from Elastin Products Co., St. Louis, MO, U.S.A. DL-Ins(1)P was synthesized (Kiely *et al.*, 1974).

Incubations with InsP_1ase contained 50 mM-Tris/HCl, pH 7.8, and 3 mM-Mg^{2+}, with changes or additions as noted. Incubations for the assessment of InsP_2ase activity were carried out either in the latter buffer made 1 mM in EGTA or in a 'physiological' buffer, containing NaCl (10 mM), potassium glutamate (70 mM), KCl (30 mM), $MgSO_4$ (4 mM) and EGTA (1 mM) in 10 mM-Hepes adjusted to pH 7.2, as used by Storey *et al.* (1984). Incubations in all cases were for 30 min.

Ins(1)P, Ins(4)P, Ins(5)P and *myo*-inositol were measured by g.l.c./m.s. of their trimethylsilyl (TMS) derivatives as described by Sherman *et al.* (1986*a*). Separation of the enantiomers of Ins(1)P was carried out on pentakis(trimethylsilyl)-*myo*-inositol dimethylphosphate as described by Leavitt & Sherman (1982*b*), but with a fused-silica column coated with Chirasil-Val (Alltech Associates, Waukegan, IL, U.S.A.).

Male Sprague–Dawley rats reared in our own animal facilities were used. Brain fixation, dissection and tissue analysis for inositol and inositol monophosphates were as described by Sherman *et al.* (1985*b*, 1986*a*). Drugs were administered subcutaneously.

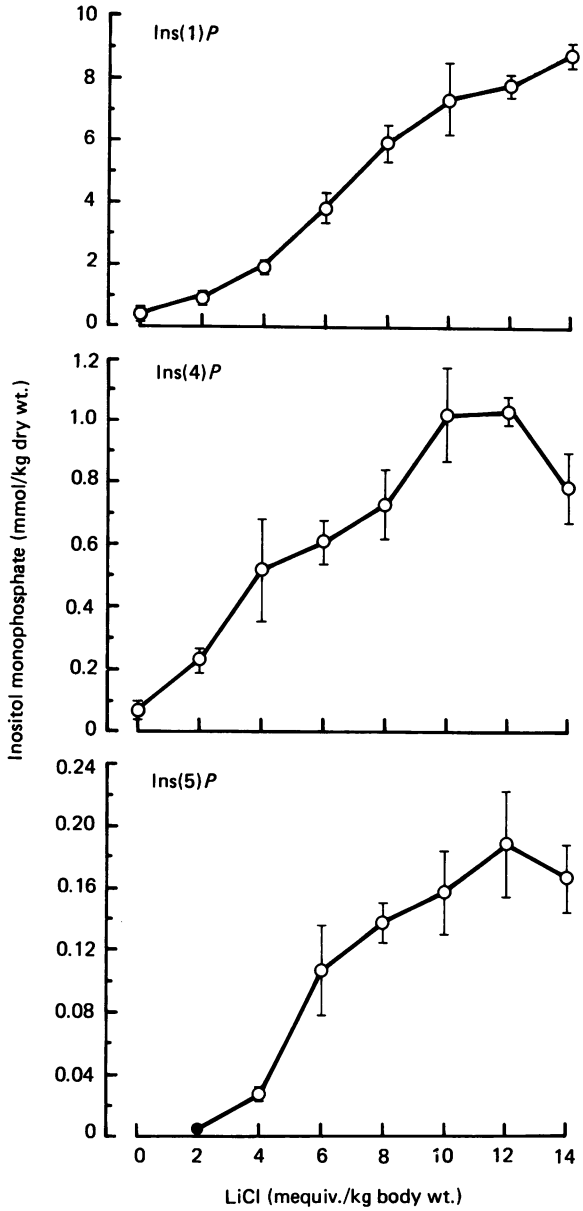

Fig. 1. LiCl dose–response of rat cerebral-cortex Ins(1)P, Ins(4)P and Ins(5)P

Rats were given LiCl subcutaneously 24 h before being killed, and the inositol monophosphates were measured by g.l.c./m.s. The results are means (±S.E.M.) for three animals, except the black circle (average of two). Note the ordinate scale changes; the values are Ins(1)P > Ins(4)P > Ins(5)P.

RESULTS

Effects of LiCl and of LiCl with pilocarpine on rat cerebral-cortex inositol monophosphates *in vivo*

These experiments were carried out by g.l.c./m.s., which allowed us to measure Ins(5)P, which we were unable to do in previous studies. In one experiment rats were given increasing doses of LiCl and killed 24 h later. The results, shown in Fig. 1, show that Ins(4)P and Ins(5)P

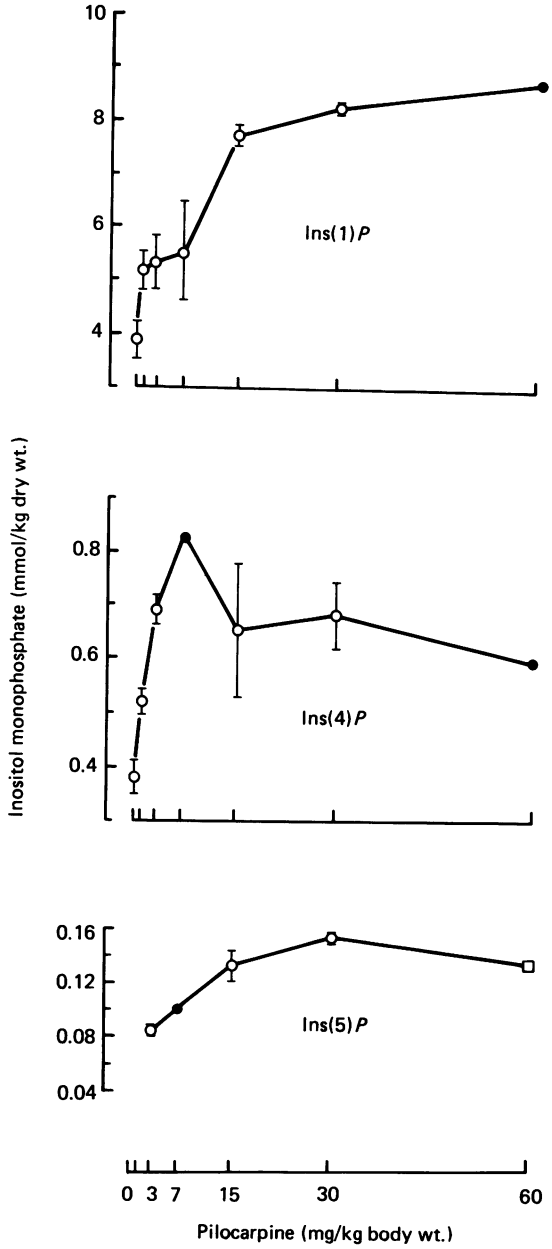

Fig. 2. Dose–response of pilocarpine in cerebral cortex of Li^+-treated rats

Rats received LiCl (5 mequiv/kg) and, 24 h later, various doses of the direct-acting cholinomimetic pilocarpine. After 30 min the animals were killed and the inositol monophosphates were measured by g.l.c./m.s. The results are means (±S.E.M.) for three animals, except the black circles, which are the average of two results, and the open square (single sample). Note the ordinate scale changes; the values of Ins(1)*P* > Ins(4)*P* > Ins(5)*P*. The apparent differences in the dose of pilocarpine giving a half-maximal response between Ins*P* isomers are not consistently observed.

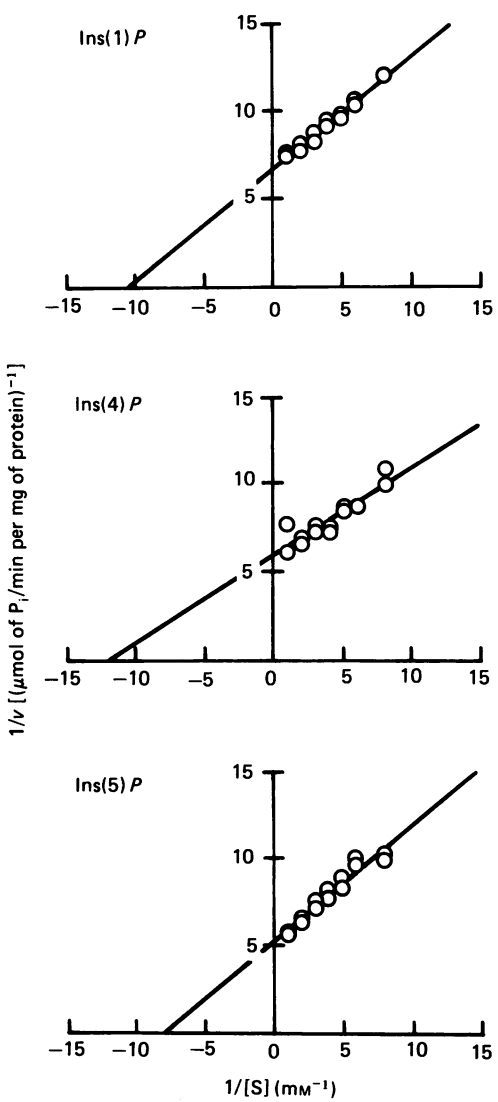

Fig. 3. Lineweaver–Burk plots of the hydrolysis of Ins(1)*P*, Ins(4)*P* and Ins(5)*P* by InsP_1ase from bovine brain

Incubations were with 14 units of enzyme, for 30 min at 37 °C. Each point represents the average of duplicate incubations. Intercepts gave K_m (mM) and $V_{max.}$ values (μmol/min per mg of protein) respectively of: Ins(1)*P*, 0.094, 0.15; Ins(4)*P*, 0.085, 0.17; Ins(5)*P*, 0.125, 0.19.

increase with increasing doses of LiCl, as was shown previously for Ins(1)*P* (Sherman *et al.*, 1985*b*). The concentrations of Ins(4)*P* and Ins(5)*P* are respectively approx. 12% and 2% that of Ins(1)*P* at all LiCl doses.

The dose–response of cerebral cortex to pilocarpine was examined in rats that had, 24 h earlier, received 5 mequiv. of LiCl/kg subcutaneously, followed by increasing doses of pilocarpine 30 min before death. The treatment (Fig. 2) results in increases in Ins(1)*P*, Ins(4)*P* and Ins(5)*P* that approach a maximum at about 15 mg of pilocarpine/kg. As with Li^+, the concentrations of Ins(4)*P* and Ins(5)*P* are approx. 10% and 2% of the cortex Ins(1)*P* at all pilocarpine doses. As in previous experiments (Honchar *et al.*, 1983; Sherman *et al.*, 1985*a*), the combination of LiCl and pilocarpine causes the rats to undergo limbic seizures. The seizures become behaviourally apparent at pilocarpine doses above 10 mg/kg.

K_m and $V_{max.}$ determinations with inositol monophosphates and InsP_1ase

DL-Ins(1)*P*, DL-Ins(4)*P* and Ins(5)*P* were incubated separately with the same preparation of InsP_1ase (14 units) over a concentration range of 0.12–1.0 mM. The reactions were followed by P_i measurement, and the

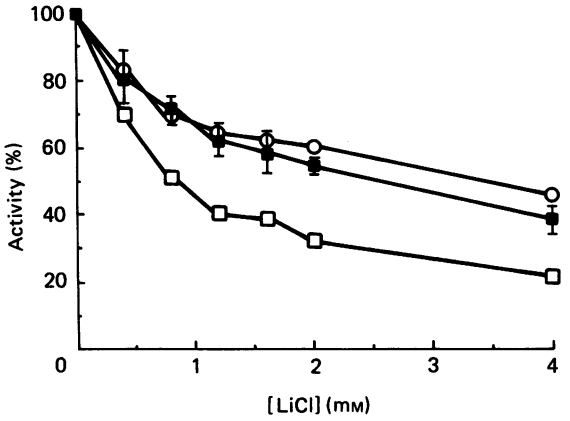

Fig. 4. Li^+ inhibition of the hydrolysis of Ins(1)*P*, Ins(5)*P* and Ins(4)*P* by InsP_1ase

Each incubation contained 0.7 mM-Ins*P*, For Ins(5)*P* (○) and Ins(4)*P* (□) each point is the average of two incubations, and for Ins(1)*P* (■) each point is the mean ± S.E.M. for three experiments carried out in duplicate.

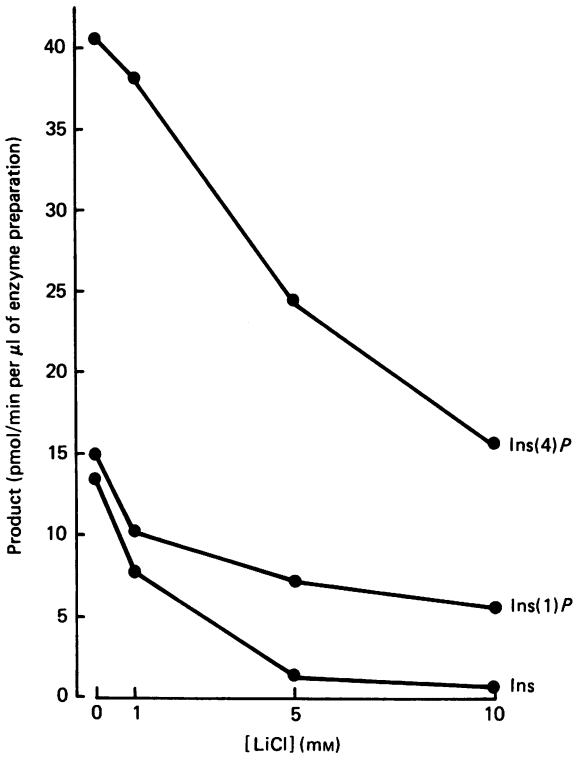

Fig. 5. Effect of LiCl concentration on the hydrolysis of D-Ins(1,4)P_2 in an incubation with a dialysed high-speed supernatant of rat brain

Each point is the average of two incubations. Product was measured by g.l.c./m.s. The enzyme preparation contains both InsP_1ase and InsP_2ase, the former having greater activity with 0.7 mM-Ins(1)*P* than the latter at the same concentration of D-Ins(1,4)P_2 (considering all products formed by the mixed-enzyme preparation). Approx. 50% inhibition occurs at LiCl concentrations of 7.3 mM for the appearance of Ins(4)*P*, 4.6 mM for Ins(1)*P* and 1.8 mM for Ins. Since Fig. 3 shows the Li^+-free InsP_1ase to have comparable rates with Ins(1)*P* and Ins(4)*P* the zero-Li^+ reactions in this Figure show that the 1-phosphate is removed more rapidly than the 4-phosphate of InsP_2. Thus, if anything, in this enzyme preparation, which is close to the enzyme composition of brain cytosol, Ins(4)*P* content would give an overestimate of the amount of Ins(1,4)P_2 in the system.

results plotted by the Lineweaver–Burk method (Fig. 3). The respective K_m and $V_{max.}$ values were: for Ins(1)*P*, 94 μM and 0.15 μmol/min per mg of protein; for Ins(4)*P*, 85 μM and 0.17 μmol/min per mg; for Ins(5)*P*, 125 μM and 0.19 μmol/min per mg. In one experiment (not shown) the K_m of Ins(1)*P* was determined over the range 0.01–1.0 mM, by using g.l.c./m.s. to analyse for *myo*-inositol. The K_m was 80 μM, essentially the same as that measured previously, and the Lineweaver–Burk plot was linear over the entire range. DL-Ins(4)*P* was completely hydrolysed by InsP_1ase, i.e. the D and L enantiomers are both substrates of the enzyme. Not having access to pure D- or L-Ins(4)*P*, we do not know if there is any difference in the rate of hydrolysis of the two enantiomers, as is the case with D- and L-Ins(1)*P*, which have a $V_{max.}$ ratio of L:D = 1.3 (Hallcher & Sherman, 1980).

myo-Inositol 1:2-cyclic phosphate was not a substrate of InsP_1ase (34.5 units of enzyme, 0.77 mM substrate), nor did bovine intestine *Escherichia coli* alkaline phosphatase hydrolyse the cyclic ester.

Inhibition by Li^+ of the hydrolysis of the inositol monophosphate isomers by InsP_1ase

The effect of Li^+ on each of the inositol monophosphates was determined by incubation of 0.7 mM solutions of each with 6.9 units of InsP_1ase in the presence of 0.4–4.0 mM-LiCl. The results (Fig. 4) show the hydrolysis of Ins(4)*P* to be inhibited by Li^+ to a greater degree than is that of the 1- or 5-isomers: 50% inhibition of the hydrolysis of the Ins(1)*P*, Ins(5)*P* and Ins(4)*P* was at approx. 2.4 mM, 3.2 mM and 0.8 mM-LiCl respectively.

Action of InsP_1ase on inositol polyphosphates

In early experiments, where the reactions were followed by phosphatase analysis, less than 1% of D-Ins(1,4)P_2 or of D-Ins(1,4,5)P_3 from red blood cells was hydrolysed by InsP_1ase (0.7 mM solutions with 34 units of enzyme; 1 h; P_i measurement). Under these conditions 50% of the Ins(1)*P* would have been hydrolysed. Incubations under the same conditions, but including 0.7 mM-Ins(1)*P*, revealed no inhibition of the hydrolysis of the Ins(1)*P*; thus these inositol phosphates have little affinity for the enzyme's catalytic site. Neither is there any inhibitor present in the substrate preparation that prevents InsP_1ase from acting on Ins(1)*P*. In later experiments, where the effect of the InsP_1ase preparation on Ins(1,4)P_2 was examined by g.l.c./m.s. analysis of products, we found that a 10-fold larger amount of the enzyme preparation than used above, when incubated with 0.65 mM-Ins(1,4)P_2, produced 730 pmol of *myo*-inositol, but no detectable Ins(1)*P* or Ins(4)*P*, in 30 min. This amount of product represents about 1% conversion of the starting material, and thus would have not been measured in the phosphate-analysis experiments. The source of this inositol is unclear; approximately the same amount of product was formed at three lower InsP_2 concentrations, to as low as 28 μM. Appropriate controls showed it not to be a contaminant.

To explore further the specificity of InsP_1ase on inositol polyphosphates, and the effect of these substrates on the enzyme, Ins(1,6)P_2 (0.4 mM), Ins(1,2)P_2 (0.2 mM), Ins(1,2,5,6)P_4 (0.7 mM), Ins(1,2,4,5,6)P_5 (0.4 mM), Ins(1,3,4,5,6)P_5 (0.6 mM) and InsP_6 (phytic acid; 0.7 mM) were each incubated with 34 units of InsP_1ase, with and without 0.7 mM-Ins(1)P. No hydrolysis occurred, nor did any of the substrates affect the ability of the enzyme to hydrolyse Ins(1)P. Each of the substrates (except phytic acid; not tested) was completely hydrolysed by bovine intestine alkaline phosphatase (18 units; 3 h).

Action of a rat brain supernatant preparation on D-Ins(1,4)P_2

Fig. 5 shows the effects of a 30 min incubation of the brain supernatant on D-Ins(1,4)P_2 in the 'physiological' buffer under conditions where no more than 2% of the substrate was hydrolysed, in the absence of LiCl and at three LiCl concentrations. LiCl inhibited the appearance of Ins(4)P by 50% at a concentration of 7.3 mM. The formation of Ins(1)P was half-inhibited at 4.6 mM, and that of Ins at 1.8 mM. The enzyme preparation that was used hydrolysed 0.7 mM-Ins(1)P at a rate of 231 pmol/min per μl in the absence of Li^+ (results not shown) and acted on 0.7 mM-Ins(1,4)P_2 to form Ins(1)P, Ins(4)P and Ins at a combined rate of 69 pmol/min per μl. In 10 mM-LiCl only 5% of the hydrolytic rate of Ins(1)P remains (results not shown), whereas InsP_2 is hydrolysed at 32% of the rate in the absence of Li^+ [i.e. the sum of all products from Ins(1,4)P_2 when incubated with 10 mM-Li^+ as compared with all products after incubation without Li^+]. Although the InsP_1ase is 3.4 times more active than the Ins(1,4)P_2 hydrolase at high substrate concentrations, nevertheless both Ins(1)P and Ins(4)P accumulate, the latter at 3 times the rate of the former.

Thus the 1-phosphate moiety of Ins(1,4)P_2 is removed more rapidly than the 4-phosphate, and both the Li^+-sensitivity and the activity of the bisphosphatase are less than for the monophosphatase.

Although separate experiments showed that the brain supernatant contained an EGTA-inhibitible phosphatase activity that hydrolysed Ins(1)P, all of the experiments reported here contained EGTA; thus that activity did not contribute to the products formed. Levamisole had little or no effect on this activity.

Effect of stimulation of L-Ins(1)P [D-Ins(3)P]

Whole cortex from a rat that had been administered LiCl (10 mequiv/kg) and pilocarpine (30 mg/kg) was derivatized (Allison *et al.*, 1976) and then subjected to g.l.c., and the TMS-Ins(1)P was collected and converted into the pentakis(trimethylsilyl)dimethyl phosphate (Leavitt & Sherman, 1982*b*). That derivative of Ins(1)P undergoes enantiomeric separation on g.l.c. when a chiral liquid phase is used. The L-Ins(1)P content was 0.80 mmol/kg dry wt of cortex and that of the D-form 7.2 mmol/kg. In previous experiments, wherein the same degree of increase in total Ins(1)P was obtained by using only Li^+ (Sherman *et al.*, 1981), the L-Ins(1)P and D-Ins(1)P contents were, for control rats, 0.09 ± 0.01 and 0.22 ± 0.06 mmol/kg dry wt., and for Li^+-treated rats, 0.50 ± 0.01 and 6.52 ± 0.55 mmol/kg. Thus, although both enantiomers increased on Li^+ treatment, the D-form increased to a much larger degree in each of these experiments.

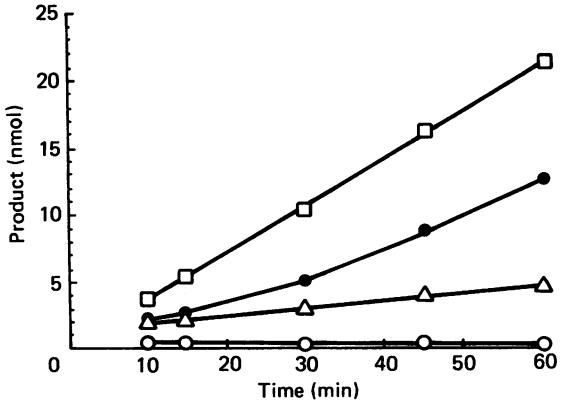

Fig. 6. Time course of the hydrolysis of L-Ins(1,4)P_2 by InsP_1ase

Each time point is for a single incubation: □, P_i; ●, *myo*-inositol; △, Ins(4)P; ○, Ins(1)P. P_i was measured colorimetrically, and inositol and inositol phosphates by g.l.c./m.s. Expansion of the ordinate shows Ins(1)P first to decrease, then to increase in amount. That, and the shape of the inositol curve, suggest that the result may be the product of more than one enzyme activity; however, a similar experiment with 0.8 mM-LiCl gave identical appearance curves for Ins(1)P and Ins(4)P, as would be expected if both steps were inhibited to the same degree. The rates of production of *myo*-inositol were both decreased by Li^+.

Action of InsP_1ase on DL-Ins(1,4)P_2

Having a sample of synthetic (i.e. racemic) Ins(1,4)P_2, we investigated its behaviour with InsP_1ase and found it to be partially hydrolysed by the enzyme. In subsequent experiments, with amounts of enzyme up to 345 units and incubation times as long as 2 h, only 40–46% was hydrolysed. The substrate was analysed by g.l.c. of the TMS ether/esters (Sherman *et al.*, 1986*a*) and found to contain no inositol monophosphates but between 6% and 16% of an uncharacterized and only partially separated bisphosphate isomer [not Ins(4,5)P_2, which we know is well separated from Ins(1,4)P_2 as the TMS derivative; Sherman *et al.*, 1971].

The hydrolysis of DL-Ins(1,4)P_2 was monitored over a 1 h period, by measuring the release of P_i, *myo*-inositol, Ins(1)P and Ins(4)P (Fig. 6). An effort was made to collect Ins(1)P from the gas chromatograph so as to examine its chirality (Leavitt & Sherman, 1982*a*); however, so little accumulates in the reaction that the determination could not be made. Unfortunately our chiral g.l.c. method does not separate D- and L-Ins(4)P.

The hydrolysis of DL-Ins(1,4)P_2 by InsP_1ase is inhibited by Li^+. When we followed the reaction over a 45 min period in the presence of Li^+, we found *myo*-inositol to be produced more slowly, but there was no difference between experiments with and without Li^+ in the rate of production of either Ins(1)P or Ins(4)P. This result is to be expected if the dephosphorylation of both the bisphosphate and the monophosphates is Li^+-sensitive.

We also examined the effect of the InsP_1ase inhibitor Ins(2)P on the hydrolysis of DL-Ins(1,4)P_2, using P_i production to monitor the reaction. When 0.7 mM-Ins(1)P and -DL-Ins(1,4)P_2 were incubated separately with and without 1 mM-Ins(2)P, and P_i was measured,

the Ins(1)*P* reaction was inhibited by 87% [Ins(4)*P* is similarly inhibited] and the Ins(1,4)P_2 reaction by 44%. Calculation of the P_i formed, considering that the inhibition of hydrolysis of the monophosphates produced in the intermediate stage of hydrolysis would be nearly complete, suggests that Ins(2)*P* did not inhibit the hydrolysis of the bisphosphate.

DISCUSSION

The original goal of this study was to understand what appeared to be a discrepancy between the developing picture of the importance of Ins(1,4,5)P_3 produced in response to specific stimuli as a second messenger for the mobilization of Ca^{2+} and our observation that, in Li^+-treated animals, D-Ins(1)*P* is produced in much larger amounts than Ins(4)*P* (Sherman *et al.*, 1985*b*). If, for example, the major part of the phospholipase C-dependent metabolism of the phosphoinositides is via polyphosphoinositide metabolism, with PtdIns serving principally as precursor of PtdIns(4)*P* and PtdIns(4,5)P_2, then, to explain our observation, there must be a large difference between the rates of hydrolysis of Ins(1)*P* and Ins(4)*P* by InsP_1ase or in the rate of removal of one of the phosphate moieties of Ins(1,4)P_2 by InsP_2ase. It is also possible that Ins(1,3,4)P_3 or Ins(1,3,4,5)P_4 is metabolized by preferential removal of a 4-phosphate, although, on the basis of present knowledge, this seems unlikely for InsP_4. There might also be differential rates of hydrolysis that result from unequal Li^+-sensitivity of these reactions.

In our previous study (Sherman *et al.*, 1985*b*) we found that, after a single dose of LiCl, the brain contents of Ins(1)*P* and Ins(4)*P* changed in parallel over time. These findings are expanded in the present paper. In Fig. 1 we show that the LiCl dose–response of Ins(4)*P* and Ins(5)*P* *in vivo* parallels that of Ins(1)*P* and that the concentration of the last is about 10 and 50 times that of Ins(4)*P* and Ins(5)*P* respectively. In addition, we have found that the responses of Ins(4)*P* and Ins(5)*P* to cortical stimulation by pilocarpine in the LiCl-treated rat also parallel that of Ins(1)*P* (Fig. 2), again with the large differences in concentration of Ins(4)*P* and Ins(5)*P* relative to Ins(1)*P*.

Fig. 3 shows that, with InsP_1ase, DL-Ins(1)*P*, DL-Ins(4)*P* and Ins(5)*P* have K_m and $V_{max.}$ values that are practically the same. Fig. 4, however, shows that the greater inhibition of Ins(4)*P* hydrolysis by Li^+ would increase, rather than decrease, the tissue contents of that isomer. Thus there appears to be no basis in the kinetics of the InsP_1ase reaction that would account for the differences in the amounts of Ins(1)*P* and Ins(4)*P* that we find in rat cerebral cortex *in vivo*.

Two previous reports on InsP_2ase activity disagree as to whether there is hydrolytic specificity for the 4-phosphate group of Ins(1,4)P_2. In one study, a homogenate of blowfly salivary gland was found to release [^{32}P]P_i from [4-^{32}P]Ins(1,4)P_2. In the presence of 10 mM-LiCl no Ins(4)[^{32}P]P was found to accumulate, which was taken as evidence that the 4-phosphate was preferentially removed from the bisphosphate and that the hydrolysis was Li^+-insensitive (Berridge *et al.*, 1983). In the second study (Storey *et al.*, 1984), a high-speed supernatant of rat liver homogenate, comparable with our rat brain preparation, was found to contain Ins(1,4)P_2 phosphatase and Ins(1)*P* phosphatase activities. With the same 4-labelled Ins(1,4)P_2 substrate as in the study by Berridge *et al.* (1983), that enzyme preparation produced both [^{32}P]P_i and an inositol mono[^{32}P]phosphate; thus both phosphate groups of InsP_2 were hydrolysed by the enzyme preparation. The monophosphatase activity was less active than the bisphosphatase; both activities were inhibited by Li^+.

Our rat brain supernatant preparation contained phosphatase activity towards Ins(1)*P* that was 3.4-fold greater in the absence of Li^+ than that toward Ins(1,4)P_2 as measured by the sum of all products formed [thus including the InsP_1ase action on Ins(1)*P* and Ins(4)*P*]. Fig. 5 shows that the hydrolytic activity toward the 1-phosphate of Ins(1,4)P_2 to produce Ins(4)*P* is 2.7 times greater than that toward the 4-phosphate moiety of InsP_2 in the absence of Li^+. The Li^+-sensitivity of InsP_1ase was also found to be greater than that of the InsP_2ase activity. In addition, Li^+ differentially affects the hydrolysis of Ins(1,4)P_2, removing the 1-phosphate at about half the rate of the 4-phosphate. Thus, with this crude enzyme preparation, which should reflect the situation in intact brain, there is nothing in the kinetics or Li^+-sensitivity that would account for the 10-fold difference in concentration of Ins(1)*P* and Ins(4)*P* that we find in our experiments *in vivo*.

We considered the possibility that the Ins(1)*P* that we were measuring in the pilocarpine-stimulated experiments was, in part or in its entirety, the L-enantiomer, not from the synthesis *de novo* of L-Ins(1)*P* (Eisenberg, 1967; Leavitt & Sherman, 1982*c*), but from phosphoinositide metabolism via D-Ins(1,3,4)P_3 (Irvine *et al.*, 1984) or from D-Ins(1,3,4,5)P_4 (Batty *et al.*, 1985). Our previous finding was that D-Ins(3)*P* [i.e. L-Ins(1)P] is present in brain of the Li^+-treated rat at a concentration only 7–13% that of D-Ins(1)*P* (Sherman *et al.*, 1981). As we have reported here, the same situation prevails after pilocarpine stimulation in the presence of Li^+. Both results suggest that, if D-Ins(1,3,4)P_3 is being formed *in vivo*, either it is produced in much smaller amounts than D-Ins(1)*P*, or it is being metabolized in a way that removes the D-3-phosphate moiety much more rapidly than the D-1 ester. Other evidence suggests that, in hepatocytes, Ins(1,3,4)P_3 is metabolized to an InsP_2 that is not Ins(1,4)P_2 (Hansen *et al.*, 1986), implying that rapid 3-phosphate removal is not occurring. The other possibility, that L-Ins(1,3,4)P_3 is the enantiomer that was isolated by Irvine *et al.* (1984), seems unlikely, as it would require a different PtdIns(4,5)P_2 precursor, the unknown L-phosphatide.

The source of Ins(5)*P* in Li^+- and Li^+/pilocarpine-stimulated rat brain must be PtdIns(4,5)P_2. Perhaps this is a degradation product of Ins(1,4,5)P_3 that escapes 5-dephosphorylation either at the trisphosphate level or after conversion into Ins(1,3,4,5)P_4. The clear increase in concentration, after stimulation, is consistent with its production after stimulation of phosphoinositide metabolism.

The lack of significant activity of InsP_1ase toward D-Ins(1,4)P_2 and D-Ins(1,4,5)P_3 is not surprising in the context of regulation of phosphoinositide metabolism, but we did not expect that the substrates would have so little affinity for the catalytic site of the enzyme. Studies in the past (Eisenberg, 1967) have suggested that InsP_1ase is generally active toward equatorial inositol phosphate groups as well as some analogous substances (Takimoto *et al.*, 1985). It is now clear that the role of this enzyme can be defined as that of an inositol monophos-

phatase, with the peculiar exception of the activity toward DL-Ins(1,4)P_2.

The partial hydrolysis of DL-Ins(1,4)P_2 suggests that the L-enantiomer is a substrate for InsP_1ase. The degree to which the hydrolysis fell short of 50% seems to be explained by the presence of a phosphorylated impurity in the sample. Possibly the activity is due to another enzyme, however; in the Li^+ experiment both stages of hydrolysis seem to be equally inhibited and, in addition, the enzyme preparation has very little activity toward the pure D-enantiomer. The time-course experiments with and without Li^+ suggest the following relationships to exist:

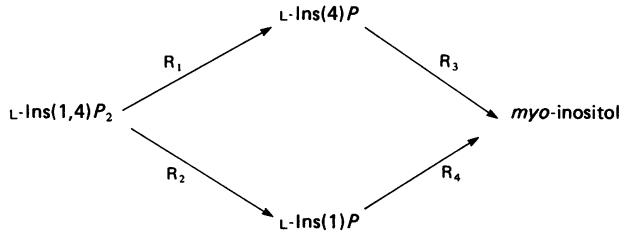

Fig. 6 shows that, in the hydrolysis of L-Ins(1,4)P_2, Ins(4)P accumulates over time, but Ins(1)P does not. Since we found that $R_3 = R_4$, with the racemic substrates in Fig. 3, then, to an approximation, $R_1 > R_3$, $R_2 \leqslant R_4$ and $R_1 > R_2$, i.e. the same situation that is found with the InsP_2ase activity in our brain supernatant preparation. The enzyme thus appears to hydrolyse the L-1 phosphate group of the diester (a natural conformation) better than an L-4 (unnatural) phosphate group. It may be significant that the L-1 phosphate is the 3-phosphate in the D-series, i.e. in D-Ins(1,3,4)P_3.

Burgess *et al.* (1985) report that Li^+ enhances the accumulation of Ins(1,3,4)P_3 in stimulated guinea-pig hepatocytes and pancreatic acinar cells. Hansen *et al.* (1986) have also found the hydrolysis of Ins(1,3,4)P_3 to be inhibited by Li^+ in a rat hepatocyte preparation, and also demonstrated Li^+ inhibition of formation of the product of hydrolysis of Ins(1,3,4)P_3. They propose that product to be Ins(1,3)P_2. Thus there seem now to be three Li^+-inhibited inositol phosphate phosphohydrolases.

The apparent lack of inhibition of the action of our InsP_1ase on Ins(1,4)P_2 by Ins(2)P remains unexplained. It is possible that there are two phosphatases in the enzyme preparation, but they would both have to be inhibited by Li^+ to about the same degree to give the result that we obtained, and the second enzyme would have to be incapable of hydrolysing D-Ins(1,4,5)P_3 and D-Ins(1,4)P_2.

We conclude that the larger amount of Ins(1)P that we find in rat cerebral cortex relative to Ins(4)P represents a larger amount of PtdIns metabolism relative to that of PtdIns(4)P and PtdIns(4,5)P_2 in both stimulated and unstimulated brain. A necessary caveat is that the Ins(1)P is produced by polyphosphoinositide metabolism that takes place by the pathway which produces inositol cyclic polyphosphates (Wilson *et al.*, 1985*b*,*c*), with *myo*-inositol 1:2-cyclic phosphate as the end-product of that metabolism and the precursor of Ins(1)P. We are by no means the only group to provide evidence that, in response to stimuli, a larger amount of PtdIns is metabolized than is PtdIns(4,5)P_2. For example, half of total platelet PtdIns is degraded by the direct action of phospholipase C after thrombin stimulation (Wilson *et al.*, 1985*a*). Others have described similar situations (for references see Majerus *et al.*, 1986).

If there is 10 times more PtdIns metabolism than that of the polyphosphoinositides in brain, the question must be asked as to what processes that metabolism serves.

A suggestion that is raised by this work is that the effect of Li^+ on brain Ins(1,4)P_2 contents in Li^+-treated animals is not very great in molar amounts. This suggestion awaits confirmation by direct measurement of InsP_2, but is supported by the close reciprocal relationship between the molar amounts of Ins(1)P and Ins in Li^+-treated rats (Sherman *et al.*, 1985*a*,*b*), i.e. most of the inositol decrease in Li^+-treated rats can be accounted for as Ins(1)P.

We gratefully acknowledge the support of U.S. Public Health Service Grants NS-05159 and AM-20579, and the Washington University Mass Spectrometry Resource RR-00954.

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Received 8 September 1986; accepted 1 November 1986