The purification and properties of *myo*-inositol monophosphatase from bovine brain

Nicolas S. GEE,*† C. Ian RAGAN,* Keith J. WATLING,* S. ASPLEY,* Rosamond G. JACKSON,* Gillian G. REID,* David GANI† and Janis K. SHUTE†

*Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, U.K., and †Department of Chemistry, University of Southampton, Southampton SO9 5NH, U.K.

1. An inositol monophosphatase was purified to homogeneity from bovine brain. 2. The enzyme is a dimer of subunit M_r 29000. 3. The enzyme hydrolyses both enantiomers of *myo*-inositol 1-phosphate and both enantiomers of *myo*-inositol 4-phosphate, but has no activity towards inositol bisphosphates, inositol trisphosphates or inositol 1,3,4,5-tetrakisphosphate. 4. Several non-inositol-containing monophosphates are also substrates. 5. The enzyme requires Mg^{2+} for activity, and Zn^{2+} supports activity to a small extent. 6. Other bivalent cations (including Zn^{2+}) are inhibitors, competitive with Mg^{2+} . 7. Phosphate, but not inositol, is an inhibitor competitive with substrate. 8. Li^+ inhibits hydrolysis of inositol 1-phosphate and inositol 4-phosphate uncompetitively with different apparent K_i values (1.0 mM and 0.26 mM respectively).

INTRODUCTION

The receptor-mediated action of phospholipase C on phosphoinositides gives rise to inositol mono- and polyphosphates, which are sequentially dephosphorylated to inositol (e.g. [1–3]). The immediate precursors of inositol from this pathway are D-inositol 1-phosphate [D-Ins(1)P] and D-inositol 4-phosphate [D-Ins(4)P], which were both thought to arise from D-inositol 1,4-bisphosphate [D-Ins(1,4)P₂] [4,5]. More recent work, however, strongly suggests that D-Ins(1)P arises primarily from direct hydrolysis of phosphatidylinositol [6–10]. Inositol can also be produced from D-glucose 6-phosphate, which is converted into L-Ins(1)P by the enzyme L-Ins(1)P synthase [11]. Thus, in tissues such as brain, which lack an uptake system for inositol, the resynthesis of phosphatidylinositol and polyphosphoinositides is dependent on the dephosphorylation of both enantiomers of Ins(1)P and its isomer D-Ins(4)P.

A soluble enzyme catalysing Li^+ -sensitive hydrolysis of Ins(1)P has been partially purified from bovine brain [12] and completely purified from rat brain [13]. It was claimed that the bovine enzyme hydrolysed both D- and L-Ins(1)P [7,13] as well as D-Ins(4)P [7], but the conclusions were based on preparations that were extremely impure. On the other hand, the activity of the rat brain enzyme towards inositol monophosphates other than L-Ins(1)P was not reported.

In the present paper we describe the purification and properties of an inositol monophosphatase from bovine brain that is very similar to the rat brain enzyme. We show that it catalyses the Li^+ -sensitive hydrolysis of both enantiomers of Ins(1)P and both enantiomers of Ins(4)P at rates that are sufficient to account for monophosphatase activity in brain homogenates. A preliminary report of some of these findings has been presented elsewhere [14].

MATERIALS AND METHODS

Materials

Frozen bovine brains were obtained from Imperial Laboratories, Salisbury, Wilts., U.K. Inositol monophosphates were prepared by the method of Billington *et al.* [15]. DL-Ins(1,4)P₂ and DL-Ins(3,4)P₂ were made by the method of Angyal & Tate [16]. DL-Ins(1,3)P₂ and DL-Ins(1,3,4,5)P₄ were made as described by Billington & Baker [17]. D-Ins(1,4,5)P₃ and DL-Ins(1,3,4)P₃ were gifts from Dr. J. Huff, Department of Medicinal Chemistry, Merck Sharp and Dohme Research Laboratories, West Point, PA, U.S.A. L-[U-¹⁴C]Ins(1)P (55 mCi/mmol) was from Amersham International (Amersham, Bucks., U.K.).

Assay of inositol monophosphatase

During purification, monophosphatase activity was monitored by either of two assays. The first relied on colorimetric determination of released P_i [18] from hydrolysis of DL-Ins(1)P [6]. The second relied on the determination of [¹⁴C]inositol produced from DL-Ins(1)P containing [¹⁴C]Ins(1)P as label [6]. Since D- and L-Ins(1)P are hydrolysed at very similar rates (see below), these assays gave very similar activities.

Activities with pure monophosphatase were determined by a continuous spectrophotometric assay adapted from the method of De Groot *et al.* [19]. In this, free P_i reacts with inosine to give hypoxanthine and ribose 1-phosphate (catalysed by nucleoside phosphorylase). Xanthine oxidase then catalyses reduction of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium by the hypoxanthine to give a red formazan, which is detected spectrophotometrically. Nucleoside phosphorylase (EC 2.4.2.1) and xanthine oxidase (EC 1.2.3.2) were obtained from BCL, Lewes, East Sussex, U.K. To decrease the

Abbreviations used: Ins(1)P etc., inositol 1-phosphate etc.; Ins(1,3)P₂ etc., inositol 1,3-bisphosphate etc.; Ins(1,3,4)P₃ etc., inositol 1,3,4-trisphosphate etc.; Ins(1,3,4,5)P₄, inositol 1,3,4,5-tetrakisphosphate.

† To whom correspondence should be addressed.

content of free P_i , the enzymes were sedimented in a bench centrifuge and resuspended in the original volume of 50 mM-Tris/HCl buffer, pH 7.5, saturated with $(NH_4)_2SO_4$. The washing procedure was repeated three times. Each assay contained, in 1 ml final volume, 250 mM-KCl, 50 mM-Tris/HCl buffer, pH 8.0, 3 mM-MgCl₂, 1 mM-inosine, 1 mM-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium (from a 100 mM stock solution in dimethyl sulphoxide), 0.5 unit of nucleoside phosphorylase, 0.05 unit of xanthine oxidase, 0.2–1 mM-inositol monophosphate and an amount of enzyme giving a rate at the highest substrate concentrations of approx. 2.7 nmol/min at 546 nm and 37 °C. After the addition of substrate, consumption of any free P_i was allowed to proceed to near completion before addition of enzyme. The steady-state rate was established within 2 min, as predicted from the analysis by Takagahara *et al.* [20], and followed by the increase in absorbance at 546 nm ($\epsilon = 1.84 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$), with a 1 cm light-path. The highest rates were similar to those observed with 10–20 μM free P_i in place of enzyme and substrate. This concentration of P_i would cause less than 0.5% inhibition of the enzyme in the presence of 1 mM substrate (see the Results section). In this and other assays, 1 unit of activity is defined as that amount of enzyme converting 1 μmol of substrate (0.1 mM) into product in 1 min.

Assay of inositol bisphosphatase

Activity with inositol bisphosphates was determined by the discontinuous colorimetric assay of released P_i [6,18]. The same method was used for assay of activity towards other potential substrates. During purification, activity was monitored radiochemically by using D-[³H]-Ins(1,4) P_2 as label [6].

Purification of inositol monophosphatase

Unless otherwise indicated, all operations were carried out at 0–4 °C. Four frozen bovine brains were thawed overnight and chopped with scissors. The tissue was homogenized in a Waring commercial blender operating at full speed for 1 min. The buffer (2 ml/g of tissue) was 0.1 mM-EGTA/0.5 mM-EDTA/150 mM-KCl/50 mM-Tris/HCl buffer, pH 8.5 [12]. The homogenate was centrifuged at 17000 *g* for 20 min and the supernatant was fractionated with solid $(NH_4)_2SO_4$. Protein precipitated at 40% of saturation was removed by centrifugation as above and discarded. Protein precipitated between 40 and 65% of saturation was sedimented as above, and dissolved in approx. 250 ml of 50 mM-Tris/HCl buffer, pH 8.0. The solution was dialysed overnight against 2.5 litres of the same buffer and centrifuged to remove any insoluble material.

Q-Sepharose chromatography. One half of the dialysed protein was pumped at 5 ml/min on to a Q-Sepharose column (Pharmacia, Milton Keynes, Bucks., U.K.) (2.5 cm internal diam. $\times$ 37 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 8.0. The column was washed with 100 ml of the Tris buffer and the enzyme was eluted with a linear gradient of NaCl (0–0.6 M) in a total volume of 1 litre of this buffer. Fractions (10 ml) were collected and assayed for both inositol 1-monophosphatase and inositol 1,4-bisphosphatase activities. After re-equilibration of the column, the second half of the sample was treated in the same way.

Sephacryl S-200 chromatography. Active fractions from Q-Sepharose were pooled and concentrated to 10 ml or less by ultrafiltration on an Amicon PM 10 membrane (Amicon, Stonehouse, Glos., U.K.). The concentrated sample was chromatographed on a Sephacryl S-200 column (2.5 cm internal diam $\times$ 80 cm) equilibrated with 0.1 mM-EGTA/0.1 mM-EDTA/0.2 M-NaCl/50 mM-Tris/HCl buffer, pH 8.0, at a flow rate of 1.5 ml/min. Active fractions (3 ml each) were pooled and concentrated as above to 4.5 ml.

Chromatofocusing on Mono P. The sample was dialysed overnight against 1 litre of 25 mM-Bis-Tris/HCl buffer, pH 6.3, containing 0.2 M-betaine. Precipitated protein was removed by centrifugation at 30000 *g* for 10 min. The supernatant was divided into three equal portions. Each was successively subjected to chromatofocusing at room temperature on an HR5/20 Mono P column (Pharmacia) equilibrated with 0.2 M-betaine/25 mM-Bis-Tris/HCl buffer, pH 6.3, and developed with Polybuffer 74 (diluted 10-fold) adjusted to pH 4.0 with HCl and containing 0.2 M-betaine. The flow rate was 1.0 ml/min, and 0.5 ml fractions were collected into 0.5 ml of 50 mM-Tris/HCl buffer, pH 8.0, to raise the pH of the eluted material. Active fractions were pooled and concentrated to 5 ml. This was diluted 10-fold with the Tris buffer and reconcentrated.

Mono Q chromatography. The protein was applied to an HR5/5 Mono Q column (Pharmacia) equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, at room temperature and eluted with a linear gradient of NaCl (0–0.3 M) in the same Tris buffer (total volume 30 ml, flow rate 1 ml/min). Active fractions (0.5 ml each) were pooled and adjusted to 33% of saturation with $(NH_4)_2SO_4$ by addition of a saturated solution in 20 mM-Tris/HCl buffer, pH 7.0.

Phenyl-Superose chromatography. The protein was applied to an HR5/5 phenyl-Superose column (Pharmacia) equilibrated at room temperature with a 33%-saturated solution of $(NH_4)_2SO_4$ in 20 mM-Tris/HCl buffer, pH 7.0 (buffer A). Buffer B was 20 mM-Tris/HCl buffer, pH 8.0. The column was washed with 5 ml of buffer A/buffer B (70:30, v/v) and developed with a linear gradient of buffer A/buffer B from 70:30 (v/v) to 45:55 (v/v) (total volume 30 ml, flow rate 1 ml/min). Active fractions (0.5 ml) were pooled and stored frozen in small volumes at –70 °C.

Miscellaneous methods

SDS/polyacrylamide-gel electrophoresis was performed in 12.5% polyacrylamide gels (0.75 mm $\times$ 8 cm $\times$ 7 cm) with the Laemmli [21] buffer system. M_r markers were from BDH Chemicals, Poole, Dorset, U.K. The M_r of the native enzyme was determined from chromatography on Sephacryl S-200, with M_r markers from Pharmacia. For experiments on the monomer–dimer transformation, an HR 10/30 Superose 12 column (Pharmacia) equilibrated with the same buffer was used.

Isoelectric focusing was carried out on LKB Immobiline Dry Plates (pH range 4.0–7.0; LKB, South Croydon, Surrey, U.K.). Plates were swollen in water, then pre-run for 30 min at 2.5 kV, and enzyme samples were applied at the anodic side. Focusing was at 400 V

Fig. 1. Chromatography on Q-Sepharose

Dialysed material from $(\text{NH}_4)_2\text{SO}_4$ fractionation was applied to Q-Sepharose as described in the Materials and methods section. Unbound protein was eluted with 50 mM-Tris/HCl buffer, pH 8.0, and 10 ml fractions were collected from the start of the gradient (0–0.6 M-NaCl in a total volume of 1 litre of the Tris buffer). A_{280} (—) was measured with a 0.3 cm light-path. Fractions were assayed for activity by the radiochemical method with either DL-Ins(1)P ($\blacktriangle$) or DL-Ins(1,4)P₂ ($\bullet$) as substrate.

for 2 h and then at 1 kV for 15 h. The gel was either stained with Coomassie Blue R250 or sliced into 1 mm slices that were then incubated in buffer containing 1 mM-DL-Ins(1)P for determination of activity by the colorimetric assay [18].

Protein concentration was measured by the method of Bradford [22], with bovine serum albumin as standard.

RESULTS

Purification of inositol monophosphatase

The initial stages of purification were based on those of Takimoto *et al.* [13] for the rat brain enzyme, except that we used the extraction medium of Hallcher & Sherman [12]. The elution profile from anion-exchange chromatography on Q-Sepharose is shown in Fig. 1. Preceding the main inositol monophosphatase peak there is frequently a small peak of activity that is largely insensitive to LiCl. In tissue homogenates there is a component of inositol monophosphatase activity that is not inhibited by Li⁺ [6] and might be accounted for by this other enzyme. Its properties were not further investigated. Also shown in Fig. 1 is the partial separation of inositol 1,4-bisphosphatase from the monophosphatase. Indeed, complete separation of these activities proved quite difficult and was a major factor in the development of the purification procedure.

Gel filtration on Sephacryl S-200 (Fig. 2) provided further separation of inositol monophosphatase and bisphosphatase and gave M_r values of 54000 and 46000 respectively (cf. [13]), clearly showing that these enzymes are structurally different.

Further purification by chromatofocusing on Mono P and hydrophobic chromatography on phenyl-Superose gave essentially pure enzyme and removal of all but traces of the bisphosphatase. The inclusion of an anion-exchange step on Mono Q either before or after phenyl-Superose ensured complete removal of any remaining impurities. The progress of a purification from four bovine brains is given in Table 1. From the supernatant

Fig. 2. Chromatography on Sephacryl S-200

Concentrated fractions from the Q-Sepharose step were applied to Sephacryl S-200 as described in the Materials and methods section. The first 100 ml of the void volume was discarded, and 3 ml fractions were collected thereafter. A_{280} and enzyme activities were measured as indicated in the legend to Fig. 1.

fraction of brain homogenate, the degree of purification was 940-fold with 8% yield of activity.

Molecular properties

SDS/polyacrylamide-gel electrophoresis of inositol monophosphatase gave a single polypeptide species of M_r 29000 (Fig. 3), in agreement with the finding by Takimoto *et al.* [13]. Since the native M_r was 54000 (Fig. 2), this suggests that the enzyme is a homodimer. Further evidence for this was provided by experiments in which the enzyme was denatured by urea. As shown in Table 2, assay in the presence of increasing concentrations of urea leads to progressive loss of activity. However, if the enzyme was pretreated with urea and then either dialysed (results not shown) or diluted (Table 2), a substantial return of activity was found. This suggests that denaturation by urea is readily reversible. Gel filtration of the enzyme on Superose-12 in 8 M-urea gave an M_r of 25000, showing that the enzyme had monomerized (Fig. 4). Since the enzyme was located in the column fractions by its activity after dilution, then the monomeric form can renature to give the active enzyme. As shown in Fig 4, the renatured form is dimeric, since rechromatography in the absence of urea shows a return of the M_r to 50000.

The enzyme was also homogeneous by isoelectric focusing. A single protein-staining band of pI 4.86 was found, which coincided with the inositol monophosphatase activity.

Substrate specificity

Table 3 gives kinetic data for the hydrolysis of a number of inositol mono- and bis-phosphates. In agreement with the conclusion of Hallcher & Sherman [12] for their crude enzyme, we find that $V_{\max}$ for L-Ins(1)P is somewhat higher than that for D-Ins(1)P. The K_m values are very similar. In contrast with the results reported by Ackermann *et al.* [7], though, we find that the two enantiomers of Ins(4)P are much better substrates for the enzyme, having very similar $V_{\max}$ values approx. 3 times greater than for Ins(1)P. This has been confirmed with three separate preparations of the homogeneous enzyme, in which the $V_{\max}$ values for ($\pm$)-Ins(1)P ranged from 13300 to 15600 munits/mg of protein and $V_{\max}$ values for ($\pm$)-Ins(4)P ranged from 39800 to 51000

Table 1. Purification of inositol monophosphatase

Purification was as described in the Materials and methods section. Activity was assayed by the radiochemical method. One unit of activity represents 1 μ mol of substrate hydrolysed/min at 37 °C with 0.1 mM-DL-Ins(1)*P*.

Fraction	Total activity (munits)	Total protein (mg)	Sp. activity (munits/mg)	Yield (%)
Supernatant	114 500	13 900	6.1	100
(NH ₄) ₂ SO ₄ cut	111 700	4 900	22.8	98
Q-Sepharose	46 400	350	133	41
Sephacryl S-200	39 000	107	364	34
Mono P	17 500	12.8	1370	15
Mono Q	14 900	6.4	2330	13
Phenyl-Superose	8840	1.54	5740	8

Fig. 3. SDS/polyacrylamide-gel electrophoresis of inositol monophosphatase

Purified enzyme (2 μ g) was analysed by electrophoresis as described in the Materials and methods section and detected by staining with Coomassie Blue. The M_r was determined by reference to markers of M_r values 78 000, 66 000, 45 000, 29 000, 17 000 and 12 000.

munits/mg of protein. The K_m values for the Ins(4)*P* enantiomers are also somewhat greater. The pure monophosphatase shows no activity towards DL-Ins(1,4)*P*₂, DL-Ins(3,4)*P*₂, Ins(1,3)*P*₂, D-Ins(1,4,5)*P*₃, DL-Ins(1,3,4)*P*₃ and DL-Ins(1,3,4,5)*P*₄ (Table 4). Ackermann *et al.* [7] also reported no activity of their crude enzyme towards D-Ins(1,4,5)*P*₃. Takimoto *et al.* [13] found activity of their rat brain enzyme with a wide range of non-inositol-containing monophosphates, in particular 2'-AMP, 2'-GMP and β -glycerophosphate, which were almost as good substrates as L-Ins(1)*P*. Qualitatively similar results were found with the bovine brain enzyme (Table 4) except that hydrolysis rates were considerably lower. Hallcher & Sherman [12] also noted hydrolysis of 2'-AMP at lower rates but failed to find any hydrolysis of α - or β -glycerophosphate.

Effects of bivalent cations

In agreement with previous work on the rat and bovine enzymes [12,13] we found that activity was totally dependent on Mg²⁺ (K_m 2.0 mM). At high concentrations

(> 10 mM) Mg²⁺ was inhibitory [12]. Other bivalent cations inhibited Mg²⁺-stimulated activity in a competitive manner, as reported previously. In particular, we confirmed the potent inhibition by Ca²⁺ [12], but we found that Zn²⁺ is an even better inhibitor with a K_i of approx. 2 μ M. In the absence of Mg²⁺, Zn²⁺ can also support activity to a maximum extent of 20% of that obtained with an optimal Mg²⁺ concentration. However, much higher concentrations of Zn²⁺ (greater than 1 mM) are required to achieve this.

Inhibition by products

Inositol is not an inhibitor at concentrations up to 50 mM, suggesting that release of inositol is essentially irreversible. On the other hand, *P*_i inhibits competitively with substrate with a K_i value of 0.52 mM.

Inhibition by Li⁺

We have confirmed the uncompetitive nature of Li⁺ inhibition [12,13]. The apparent K_i values determined

Table 2. Denaturation of inositol monophosphatase by urea

Partially purified enzyme (sp. activity 25 munits/mg of protein) was used for these experiments. In Expt. 1, 0.6 munit of enzyme was incubated with 0.1 mM-DL-Ins(1)*P* for 15 min in the presence of the indicated urea concentrations. Production of inositol was measured radiochemically. In Expt. 2, 6 munits of enzyme were incubated with 0.1 mM-DL-Ins(1)*P* for 1.5 min in the presence of the indicated urea concentrations. In Expt. 3, 6 munits of enzyme were treated with 8.2 M-urea for 20 min at 37 °C exactly as in Expt 2 but in the absence of substrate. A portion (0.6 munit) was then diluted 10-fold into assay medium and incubated for 15 min at 37 °C in the presence of 0.1 mM substrate as in Expt. 1.

Expt. no.	Concn. of urea in preincubation (M)	Concn. of urea in assay (M)	Activity (% of original activity)
1	0	0.1	100
	0	4.1	89
	0	6.4	49
	0	7.9	27
	0	8.7	19
2	0	0	112
	0	8.4	15
3	8.2	0.82	74

Fig. 4. Dissociation of inositol monophosphatase in urea and its re-association

(a) Crude inositol monophosphatase (see Table 2) (60 munits in 0.2 ml) was made 8 M in urea (volume now 0.31 ml) and incubated at 37 °C for 20 min. A portion (100 µl) was applied to an HR 10/30 Superose 12 column equilibrated with 0.1 mM-EGTA/0.1 mM-EDTA/0.2 M-NaCl/50 mM-Tris/HCl buffer, pH 8.0, containing 8 M-urea at room temperature. Fractions (0.2 ml) were collected at a flow rate of 0.5 ml/min. Portions (20 µl) of each fraction were diluted 10-fold with assay buffer [6] and left for 20 min at room temperature before addition of radiolabelled substrate. Markers were: DB, Dextran Blue; BSA, bovine serum albumin; OV, ovalbumin; CT, chymotrypsinogen A; RN, ribonuclease. (b) Crude inositol monophosphatase (30 munits in 100 µl) was treated with 8 M-urea as in (a). The sample was diluted 10-fold with column buffer (without urea), and 200 µl was applied to the Superose 12 column equilibrated with the same buffer. Fractions (0.2 ml) were collected as before, and 50 µl portions were assayed radiochemically for inositol monophosphatase activity.

from secondary plots are 1.0 mM with DL-Ins(1)P as substrate and 0.26 mM with DL-Ins(4)P as substrate. The greater sensitivity of inositol 4-monophosphatase activity to Li^+ had previously been reported by Ackermann *et al.* [7] for their crude bovine brain preparation. Since uncompetitive inhibitors do not combine with free enzyme or with any form of the enzyme whose concentration can be decreased by increasing substrate concentration, it is not unexpected that the apparent K_i should depend on the nature of the substrate. In the simplest possible model, for example, the apparent K_i values would be equal to the dissociation constants of the enzyme-substrate- Li^+ complexes.

Table 3. Kinetic properties of inositol monophosphatase

Activities with the indicated substrate were determined as described in the Materials and methods section by use of the continuous spectrophotometric assay. Initial rates determined with several substrate concentrations were fitted to a Michaelis-Menten expression by non-linear least-squares-regression analysis. Values for K_m and V_{max} are given as means $\pm$ S.E.M. with the numbers of substrate concentrations used in parentheses. The absolute stereochemistry of the enantiomers of Ins(4)P is not yet known.

Substrate	K_m (mM)	V_{max} ($\mu\text{mol}/\text{min per mg of protein}$)
DL- or ($\pm$)-Ins(1)P	0.16 ± 0.02 (6)	13.3 ± 0.9 (6)
D- or ($\pm$)-Ins(1)P	0.16 ± 0.02 (6)	12.7 ± 0.5 (6)
L- or ($-$)-Ins(1)P	0.19 ± 0.08 (6)	17.0 ± 2.7 (6)
($\pm$)-Ins(4)P	0.23 ± 0.03 (6)	39.8 ± 2.4 (6)
(+)-Ins(4)P	0.19 ± 0.05 (5)	43.7 ± 5.8 (5)
(-)-Ins(4)P	0.23 ± 0.06 (5)	47.7 ± 6.0 (5)

Table 4. Substrate specificity of inositol monophosphatase

Activities were determined by colorimetric assay of released P_i or by the continuous assay. Except when indicated, rates were determined at concentrations 10-fold higher than the K_m to give approximate values for V_{max} . These are expressed relative to V_{max} for DL-Ins(1)P. Activity with DL-Ins(1,4)P₂, DL-Ins(3,4)P₂, Ins(1,3)P₂, D-Ins(1,4,5)P₃, DL-Ins(1,3,4)P₃ and DL-Ins(1,3,4,5)P₄ (all at 0.1 mM) was not detected.

Substrate	Maximum relative velocity
DL-Ins(1)P	100
DL-Ins(1,4)P ₂	< 0.3
DL-Ins(3,4)P ₂	< 0.3
Ins(1,3)P ₂	< 0.7
D-Ins(1,4,5)P ₃	< 0.7
DL-Ins(1,3,4)P ₃	< 0.7
DL-Ins(1,3,4,5)P ₄	< 1.5
2'-AMP	50
2'-GMP	44
β -Glycerophosphate	33
D,L- α -Glycerophosphate (2 mM)	13

DISCUSSION

The purification of bovine brain inositol monophosphatase clarifies several aspects of inositol polyphosphate metabolism in brain that were previously unclear. Firstly, we have shown that the homogeneous enzyme catalyses hydrolysis of both enantiomers of Ins(1)P and both enantiomers of Ins(4)P. Moreover, the relative rates of hydrolysis of these substrates are the same as those encountered in crude brain homogenates or high-speed supernatants, and the latter activities are substantially inhibited by Li^+ at millimolar concentrations [6]. Thus the inositol monophosphatase is

responsible for inositol production in the cell not only from breakdown of D-Ins(1,4,5) P_3 {via D-Ins(1,4) P_2 and D-Ins(4) P [6,7]} but also from D-glucose 6-phosphate {via L-Ins(1) P [11]} and from D-Ins(1,3,4,5) P_4 . The last-mentioned molecule is dephosphorylated to D-Ins(1,3,4) P_3 [22,24] and then to D-Ins(3,4) P_2 [10,25] with perhaps some minor contribution from D-Ins(1,3) P_2 [10,25,26]. The metabolite of D-Ins(3,4) P_2 is D-Ins(3) P [10], whereas D-Ins(1,3) P_2 can only be hydrolysed to either D-Ins(3) P [the same as L-Ins(1) P] or D-Ins(1) P , both of which are substrates of the monophosphatase.

Secondly, we have shown that the monophosphatase has no activity towards inositol bis- or poly-phosphates, which are therefore dephosphorylated by one or more different enzymes, as previous workers had suggested [5,7,8]. We disagree with the conclusion by Ackerman *et al.* [7] that L-Ins(1,4) P_2 is a substrate for inositol monophosphatase and attribute their finding to the use of an impure enzyme preparation.

Lastly, our results support recent ideas that Ins(1) P arises primarily from direct hydrolysis of phosphatidylinositol and not from hydrolysis of Ins(1,4) P_2 [6–10]. Although the V_{max} of the monophosphatase is higher for Ins(4) P than for Ins(1) P , this cannot account for the much greater concentration of the latter isomer in rat cerebral cortex either before or after systemically administered Li^+ [27], particularly when we consider that Ins(1,4) P_2 is predominantly hydrolysed to Ins(4) P [6,7]. Clearly, the Ins(1) P must arise from another source, of which phosphatidylinositol is the obvious candidate [7–10].

The differential sensitivity of Ins(4) P and Ins(1) P hydrolysis to Li^+ can be explained by the uncompetitive nature of the inhibition. Although this property of Li^+ has been known for some time [12], the effects to be expected of an uncompetitive inhibitor on inositol polyphosphate metabolism have not been considered. Cornish-Bowden [28] has recently highlighted the enormous effects that such inhibitors can have on the concentrations of metabolic intermediates in a pathway. Increasing the substrate concentration to compensate for the presence of an inhibitor is particularly difficult for uncompetitive inhibitors, since the substrate potentiates the inhibition. In contrast, the effect of competitive inhibitors is relatively easily overcome. It is unrealistic to expect metabolite concentrations to adjust so as to completely negate the effect of the inhibitor, but, as shown by Cornish-Bowden [28], the effect of an uncompetitive inhibitor on metabolite concentrations is always greater than that of a competitive inhibitor, particularly if the affected enzyme has a low flux control coefficient. For inositol monophosphatase in rat cerebral cortex we can calculate the effects of systemic Li^+ on metabolic flux if we assume that the rat enzyme has the same kinetic parameters as the bovine enzyme. Ackermann *et al.* [7] and Sherman *et al.* [27] give data for the Li^+ , Ins(1) P and Ins(4) P concentrations in rat cortex following systemic Li^+ treatment. In the absence of Li^+ , the Ins(1) P concentration is 0.22 mmol/kg dry wt. or approx. 0.044 mM. This would support monophosphatase activity at 22% of its maximum rate with this substrate. At a high dose of Li^+ , e.g. 10 mmol/kg body wt. the Ins(1) P concentration rises 33-fold to 7.3 mmol/kg dry wt. or approx. 1.5 mM. This Li^+ dose provides a cortical concentration of 21 mmol/kg dry wt. or approx. 4.2 mM. Under these conditions monophosphatase activity is

19% of its maximum rate. Thus Li^+ has very little effect on flux through the monophosphatase, and clearly the cell attempts to compensate for the presence of Li^+ by raising the Ins(1) P concentration dramatically. A very similar conclusion can be reached for Ins(4) P , which in the absence of Li^+ supports activity at 5.7% of the maximum rate and in the presence of 4.2 mM- Li^+ supports activity at 5.5% of the maximum rate. Similar calculations for lower doses of Li^+ suggest that monophosphatase flux actually increases somewhat in the presence of Li^+ . A competitive inhibitor of the same K_i would have much less effect on the Ins(1) P concentration. Thus 19% of the maximum rate would be obtained in the presence of 4.2 mM of a competitive inhibitor (K_i 1.0 mM) by an Ins(1) P concentration of only 0.20 mM.

Clearly, the cell attempts to maintain constant flux through inositol monophosphatase by elevating the substrate concentrations. Since this is true for Ins(1) P and Ins(4) P , then all pathways producing monophosphates must maintain a constant rate. Therefore the effect of Li^+ can be largely accounted for by its inhibitory action on inositol monophosphatase, and the increased Ins(1) P and Ins(4) P pools do not reflect any stimulation by Li^+ of the synthesis of these compounds. This idea is strongly supported by the minimal effects of systemic Li^+ on the cortical concentrations of Ins(1,4) P_2 , inositol trisphosphates and phosphoinositides [27,29]. It is likely therefore that the pharmacology of Li^+ is linked to the drastic decrease in cellular inositol that is needed to raise the Ins(1) P concentration. Depletion of inositol leads to a rise in the concentrations of CMP-phosphatidate (the precursor of phosphatidylinositol) and phosphatidic acid, which may then result in increased diacylglycerol concentrations [30,31]. Since the magnitude of the decrease in inositol concentration is a function of the uncompetitive nature of inhibition by Li^+ , it may be that the mode of action is as important as the site of action in the pharmacology of Li^+ .

REFERENCES

1. Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
2. Downes, C. P. & Michell, R. H. (1985) in *Molecular Mechanisms of Transmembrane Signalling* (Cohen, P. & Houslay, M. D., eds.), pp. 3–56, Elsevier, Amsterdam
3. Abdel-Latif, A. A. (1986) *Pharmacol. Rev.* **38**, 227–272
4. Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) *Nature (London)* **312**, 374–376
5. Michell, R. H., Cubitt, A. B., Downes, C. P., Hawkins, P. T., King, C. E., Kirk, C. J., Morris, A. J., Mussatt, M. C., Shears, S. B. & Storey, D. J. (1986) *Horm. Cell Regul.* **139**, 71–79
6. Ragan, C. I., Watling, K. J., Gee, N. S., Aspley, S., Jackson, R. G., Reid, G. G., Baker, R., Billington, D. C., Barnaby, R. J. & Leeson, P. D. (1988) *Biochem. J.* **249**, 143–148
7. Ackermann, K. E., Gish, B. G., Honchar, M. P. & Sherman, W. R. (1987) *Biochem. J.* **242**, 517–524
8. Balla, T., Baukal, A. J., Guillemette, G., Morgan, R. O. & Catt, K. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9323–9327
9. Wilson, D. B., Newfield, E. J. & Majerus, P. W. (1985) *J. Biol. Chem.* **260**, 1046–1051
10. Inhorn, R. D., Bansal, V. S. & Majerus, P. W. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2170–2174

11. Eisenberg, F. (1967) *J. Biol. Chem.* **242**, 1375–1382
12. Hallcher, L. M. & Sherman, W. R. (1980) *J. Biol. Chem.* **255**, 10896–10901
13. Takimoto, K., Okada, M., Matsuda, Y. & Nakagawa, H. (1985) *J. Biochem. (Tokyo)* **98**, 363–370
14. Aspley, S., Baker, R., Billington, D. C., Gee, N. S., Jackson, R. G., Kulagowski, J. J., Mawer, I. M., Ragan, C. I., Reid, G. G. & Watling, K. J. (1987) *Br. J. Pharmacol.* **91**, 303P
15. Billington, D. C., Baker, R., Kulagowski, J. J. & Mawer, I. M. (1987) *J. Chem. Soc. Chem. Commun.* 314–316
16. Angyal, S. J. & Tate, M. E. (1961) *J. Chem. Soc.* 4122–4128
17. Billington, D. C. & Baker, R. (1987) *J. Chem. Soc. Chem. Commun.* 1011–1013
18. Itaya, K. & Ui, M. (1966) *Clin. Chim. Acta* **14**, 361–366
19. De Groot, H., De Groot, H. & Noll, T. (1985) *Biochem. J.* **230**, 255–260
20. Takagahara, I., Yamauti, J.-I., Fujii, K., Yamashita, J. & Horio, T. (1983) *J. Biochem. (Tokyo)* **93**, 1145–1157
21. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
22. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–252
23. Hawkins, P. T., Stephens, L. & Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
24. Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
25. Shears, S. B., Storey, D. J., Morris, A. J., Cubitt, A. B., Parry, J. B., Michell, R. H. & Kirk, L. J. (1987) *Biochem. J.* **242**, 303–402
26. Hansen, C. A., Mah, S. & Williamson, J. R. (1986) *J. Biol. Chem.* **261**, 8100–8103
27. Sherman, W. R., Munsell, L. Y., Gish, B. G. & Honchar, M. P. (1985) *J. Neurochem.* **44**, 798–807
28. Cornish-Bowden, A. (1986) *FEBS Lett.* **203**, 3–6
29. Meek, J. L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4162–4166
30. Drummond, A. H. & Raeburn, C. A. (1984) *Biochem. J.* **224**, 129–136
31. Downes, C. P. & Stone, M. A. (1986) *Biochem. J.* **234**, 199–204

Received 22 June 1987/17 September 1987; accepted 5 October 1987