The dephosphorylation pathway of D-myo-inositol 1,3,4,5-tetrakisphosphate in rat brain

Christophe ERNEUX, Anne DELVAUX, Colette MOREAU and Jacques E. DUMONT

Institute of Interdisciplinary Research (IRIBHN), School of Medicine, Free University of Brussels (U.L.B.), Campus Erasme (Bât. C), Route de Lennik 808, B-1070 Brussels, Belgium

Dephosphorylation of inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5) P_4] was measured in both the soluble and the particulate fractions of rat brain homogenates. Analysis of the hydrolysis of [4,5- ^{32}P]Ins(1,3,4,5) P_4 showed that for both fractions the 5-phosphate of Ins(1,3,4,5) P_4 was removed and inositol 1,3,4-trisphosphate [Ins(1,3,4) P_3] was specifically produced. In the soluble fraction, Ins(1,3,4) P_3 was further hydrolysed at the 1-phosphate position to inositol 3,4-bisphosphate [Ins(3,4) P_2]. DEAE-cellulose chromatography of the soluble fraction separated the phosphatase activities into three peaks. The first hydrolysed both Ins(1,3,4,5) P_4 and inositol 1,4,5-trisphosphate, the second inositol 1-phosphate and the third Ins(1,3,4) P_3 and inositol 1,4-bisphosphate, [Ins(1,4) P_2]. Further purification of the third peak on either Sephacryl S-200 or Blue Sepharose could not dissociate these two activities [i.e. with Ins(1,4) P_2 and Ins(1,3,4) P_3 as substrates]. The dephosphorylation of Ins(1,3,4) P_3 could be inhibited by the addition of Li^+ .

INTRODUCTION

In a variety of tissues, agonist-provoked hydrolysis of phosphatidylinositol 4,5-bisphosphate yields two intracellular messengers, Ins(1,4,5) P_3 and 1,2-diacylglycerol (Michell, 1986; Irvine, 1986). Ins(1,4,5) P_3 is responsible for mobilizing intracellular Ca^{2+} from a pool associated with the endoplasmic reticulum, whereas 1,2-diacylglycerol activates protein kinase C. Ins(1,4,5) P_3 is dephosphorylated to form Ins(1,4) P_2 (and is thus inactivated) by direct hydrolysis of the 5-phosphate by human erythrocyte membranes (Downes *et al.*, 1982). More recently, soluble and particulate phosphatases in other mammalian tissues have shown the same catalytic property (Downes *et al.*, 1982; Storey *et al.*, 1984; Joseph & Williams, 1985; Sasaguri *et al.*, 1985; Connolly *et al.*, 1985; Rana *et al.*, 1986; Erneux *et al.*, 1986). Ins(1,4,5) P_3 can also be phosphorylated by a soluble Ins(1,4,5) P_3 3-kinase to form Ins(1,3,4,5) P_4 as evidenced in rat brain, liver, pancreas and parotid gland (Irvine *et al.*, 1986; Hawkins *et al.*, 1986; Hansen *et al.*, 1986; Biden & Wollheim, 1986). Batty *et al.* (1985) suggested that the same phosphatase(s) present in erythrocyte membranes remove(s) the 5-phosphate from both Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 . The product of Ins(1,3,4,5) P_4 dephosphorylation is thus Ins(1,3,4) P_3 .

We have reported previously that in rat brain (a) Ins(1,4,5) P_3 is dephosphorylated by both soluble and particulate phosphatases to Ins(1,4) P_2 (Erneux *et al.*, 1986) and (b) Ins(1,4) P_2 is degraded by a soluble phosphatase acting on the 1-phosphate to Ins4P, and Li^+ , an antidepressant, specifically inhibits Ins(1,4) P_2 and Ins1P hydrolysis (Delvaux *et al.*, 1987a,b). As Ins(1,4,5) P_3 phosphorylation seems to play a major role in the metabolism of inositol phosphates, the enzymes responsible for the dephosphorylation of Ins(1,3,4,5) P_4

and Ins(1,3,4) P_3 have been characterized in the present study.

MATERIALS AND METHODS

Materials

The sources of chemicals, materials and the preparation of [4- ^{32}P]Ins(1,4) P_2 and [4,5- ^{32}P]Ins(1,4,5) P_3 from ^{32}P -labelled erythrocyte membranes have been described previously (Delvaux *et al.*, 1987a). [2- 3H]Ins1P (sp. radioactivity 8 Ci/mmol), [2- 3H]Ins(1,4) P_2 (sp. radioactivity 2 Ci/mmol) and [2- 3H]Ins(1,4,5) P_3 (sp. radioactivity 3.6 Ci/mmol) were obtained from New England Nuclear Corp. [^{32}P]P_i and unlabelled inositol phosphates were from Amersham. DEAE-cellulose (DE-52) and the h.p.l.c. column Partisil 10 SAX (25 cm analytical column) were from Whatman. Sephacryl S-200, Blue Sepharose and Sephadex G-10 were supplied by Pharmacia.

Preparation of [3H]- and [^{32}P]-Ins(1,3,4,5) P_4 and [3H]Ins(1,3,4) P_3

[3H]Ins(1,3,4,5) P_4 was prepared by phosphorylation of [3H]Ins(1,4,5) P_3 by rat brain 180000 g supernatant fraction as described by Irvine *et al.* (1986). The incubation mixture contained (in 100 μ l) 1.5 μ M-[3H]Ins(1,4,5) P_3 (0.5 μ Ci), 10 mM-ATP, 10 mM-MgCl₂, 25 mM-2,3-bisphosphoglycerate and 0.04 mg of rat brain crude enzyme. Incubation was for 5 min at 37 °C. [3H]Ins(1,3,4,5) P_4 was purified by h.p.l.c., desalted by Sephadex G-10 chromatography, freeze-dried, and resuspended in 1 ml of water. [^{32}P]Ins(1,3,4,5) P_4 was prepared by the same method with [^{32}P]Ins(1,4,5) P_3 (0.1 μ Ci). [3H]Ins(1,3,4,5) P_4 was used as substrate for human erythrocyte membranes to prepare [3H]Ins(1,3,4) P_3 . Purification was performed on Dowex (formate)

Abbreviations used: Ins1P, inositol 1-phosphate; Ins4P, inositol 4-phosphate; Ins P_2 , inositol bisphosphate; Ins(3,4) P_2 , inositol 3,4-bisphosphate; Ins(1,3) P_2 , inositol 1,3-bisphosphate; Ins(1,4) P_2 , inositol 1,4-bisphosphate; Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; Ins(1,3,4) P_3 , inositol 1,3,4-trisphosphate; Ins(1,3,4,5) P_4 , inositol 1,3,4,5-tetrakisphosphate.

Fig. 1. H.p.l.c. analysis of the products of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ hydrolysis by rat brain soluble (a) and particulate (b) fraction

$[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (about 5000 c.p.m.) was incubated for 5 min in the presence of rat brain soluble (a) or particulate (b) fraction and 50 mM-Hepes/NaOH (pH 7.5), 2 mM- MgCl_2 , 5 mM-2-mercaptoethanol and 1 mg of bovine serum albumin/ml. The reaction was terminated by adding 0.1 ml of 20% (v/v) trichloroacetic acid. After centrifugation (3000 g for 30 min), removal of trichloroacetic acid, neutralization and filtration on a Centricon TM micro-concentrator to remove proteins, a sample, spiked with $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ and $[^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$, was loaded on a Partisil SAX anion-exchange h.p.l.c. column. Elution was performed as described in the Materials and methods section; the fraction volume was 0.6 ml. The elution of $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ and $[^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$ is indicated by arrows; $\bullet$, ^3H (ordinate).

columns by two successive runs to remove overlapping $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$, and the product was desalted as above. Final purity of each preparation was checked by h.p.l.c.

Incubation and assay of inositol phosphate phosphatases

Incubation and assay of inositol phosphate phosphatases in triplicates were as previously described (Erneux *et al.*, 1986). The dephosphorylated products were separated on 0.2 ml (wet bed vol.) Bio-Rad AG1X8 Dowex columns (Downes *et al.*, 1986). Substrates were $[^{32}\text{P}]$ - or $[^3\text{H}]$ - $\text{Ins}(1,3,4,5)\text{P}_4$, $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$, $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$, $[^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$, $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ and $[^3\text{H}]\text{Ins}1\text{P}$. When $[^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$ was used as substrate (only the 4-phosphate is ^{32}P -labelled; see Downes *et al.*, 1982; Hawkins *et al.*, 1984), it was dephosphorylated to $[^{32}\text{P}]\text{Ins}4\text{P}$ (Delvaux *et al.*, 1987b). We measured $\text{Ins}(1,4)\text{P}_2$ 1-phosphatase activity with either $[^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$ (μM) or $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ (nM) as substrate, with no difference in the profile of activity on DEAE-cellulose. H.p.l.c. analysis of the product(s) of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ or $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ hydrolysis was done with a Whatman Partisil 10 SAX column (0.46 cm $\times$ 25 cm), which was eluted as described by Hansen *et al.* (1986).

Purification of rat brain inositol phosphate phosphatase activities

Rat brain homogenate was prepared at 0–4 $^{\circ}\text{C}$ in 20 mM-Tris/HCl (pH 7.5)/0.4 mM-phenylmethanesulphonyl fluoride/5 μM -leupeptin/0.25 M-sucrose. In general, 4 g of fresh tissue was homogenized in 20 ml of buffer. Soluble and particulate fractions were prepared by centrifugation at 180000 g for 1 h. Chromatography on a DEAE-cellulose column (0.9 cm $\times$ 14 cm) of the rat brain soluble fraction was performed by overnight elution as previously described (Delvaux *et al.*, 1987a).

Peak fractions of $\text{Ins}(1,4)\text{P}_2$ phosphatase activity were concentrated with an Amicon ultrafiltration unit (PM 10 membrane). $\text{Ins}(1,4)\text{P}_2$ phosphatase was further purified either by gel filtration or by Blue Sepharose chromatography. A part of the concentrated fraction (0.5 mg in 0.5 ml) was chromatographed on a Sephacryl S-200 column (0.9 cm $\times$ 85 cm) equilibrated with 20 mM-Tris/HCl (pH 7.5)/0.1 mM-EDTA/0.2 M-NaCl/10% (v/v) glycerol/0.4 mM-phenylmethanesulphonyl fluoride/5 mM-2-mercaptoethanol. The column was eluted with the equilibration buffer at a flow rate of 10 ml/h in 1 ml fractions. Alternatively, the concentrated fraction was applied to a column (0.3 cm $\times$ 4 cm) of Blue Sepharose equilibrated with 20 mM-Pipes/NaOH buffer (pH 6.3)/2 mM- MgCl_2 /0.1 mM-EGTA. The enzyme was eluted with 30 ml of a linear gradient of NaCl (0–1 M) in the equilibration buffer. Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Specificity of $\text{Ins}(1,3,4,5)\text{P}_4$ dephosphorylation

Previous studies have shown that $\text{Ins}(1,3,4,5)\text{P}_4$ can be dephosphorylated in the 5-position to form $\text{Ins}(1,3,4)\text{P}_3$ (Batty *et al.*, 1985; Irvine, 1986; Irvine *et al.*, 1986). Our data obtained with both soluble and particulate fractions of rat brain and red cell membranes support these results. H.p.l.c. analysis of the products of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ hydrolysis showed that peak B (Fig. 1) chromatographed with the mobility of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ (Hawkins *et al.*, 1986). However, $\text{Ins}(1,3,4)\text{P}_3$ was rapidly metabolized by rat brain soluble fraction to an isomer of inositol bisphosphate distinct from $\text{Ins}(1,4)\text{P}_2$ [i.e. $\text{Ins}(1,3)\text{P}_2$ or $\text{Ins}(3,4)\text{P}_2$]. The latter metabolite (peak C in Fig. 1a) could also be detected by direct analysis of

Fig. 2. InsP_4 dephosphorylation by rat brain particulate fraction

Rat brain particulate fraction was incubated in the presence of $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$ and $[\text{^32P}]\text{Ins}(1,3,4,5)\text{P}_4$ in the assay buffer mixture for 0, 5 and 10 min. Inositol phosphates were separated on columns (2 cm $\times$ 0.6 cm) of Bio-Rad AGIX-8. Elution fractions (8 ml): InsP_1 (0.2 M-ammonium formate/0.1 M-formic acid), InsP_2 (0.4 M-ammonium formate/0.1 M-formic acid), InsP_3 (0.8 M-ammonium formate/0.1 M-formic acid), InsP_4 (1.2 M-ammonium formate/0.1 M-formic acid). Data represent means of triplicates (the S.E.M. values for these samples are smaller than the symbols and are therefore not indicated).

products of $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ from hydrolysis by rat brain soluble fraction (results not shown). The simultaneous dephosphorylation of $[\text{^32P}]\text{Ins}(1,3,4,5)\text{P}_4$ and $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$ was also studied in order to determine the $^{32}\text{P}/^{3}\text{H}$ ratio of the InsP_3 fraction (Shears *et al.*, 1987). In a typical experiment with the particulate fraction, ^{32}P , but no ^3H , was eluted in the 'InsP + P_1 ' fraction after 5–10 min incubation of the mixture of $[\text{^32P}]$ - and $[\text{^3H}]\text{InsP}_4$ (Fig. 2). At the same time, the $^{32}\text{P}/^3\text{H}$ ratio of the InsP_3 product was 0.13–0.12, or 38–41 % of the InsP_4 precursor {the ^{32}P label of our $[\text{^32P}]\text{Ins}(1,3,4,5)\text{P}_4$ was distributed between the 5- and 4-phosphate in the ratio 63:37}. This identified the product of $\text{Ins}(1,3,4,5)\text{P}_4$ degradation as $\text{Ins}(1,3,4)\text{P}_3$. Similar results were obtained for the soluble fraction, although the $\text{Ins}(1,3,4)\text{P}_3$ produced was rapidly degraded to an InsP_2 isomer (in confirmation of the h.p.l.c. data), which was ^{32}P -labelled. This isomer retained the same $^{32}\text{P}/^3\text{H}$ ratio as $\text{Ins}(1,3,4)\text{P}_3$ (i.e. 35–40 % of the InsP_4 precursor), suggesting that $\text{Ins}(1,3,4)\text{P}_3$ is degraded at the 1-phosphate to $\text{Ins}(3,4)\text{P}_2$.

DEAE-cellulose chromatography and sensitivity to LiCl inhibition

Previous results have shown that chromatography of the soluble fraction of rat brain homogenates on DEAE-cellulose resolved three phosphatase activities: an $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase, an $\text{Ins}(1,4)\text{P}_2$ 1-phosphatase and an InsP phosphatase. If LiCl (10 mM) was included in the assay mixture, it inhibited dephosphorylation of both InsP and $\text{Ins}(1,4)\text{P}_2$, suggesting the existence of two Li^+ -sensitive phosphatases (Delvaux *et al.*, 1987a,b). In the present study, we re-assayed each fraction of the DEAE-cellulose profile with $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ as substrates. A single peak of inositol phosphatase activity co-eluted with either $\text{Ins}(1,3,4,5)\text{P}_4$ or $\text{Ins}(1,4,5)\text{P}_3$ as substrate (results not shown). LiCl had no effect on $\text{Ins}(1,3,4,5)\text{P}_4$ phosphatase activity as measured with both ^3H - and ^{32}P -labelled $\text{Ins}(1,3,4,5)\text{P}_4$.

We also measured $\text{Ins}(1,3,4)\text{P}_3$ phosphatase activity with the ^3H -labelled substrate (Fig. 3a). A single peak of activity was eluted from the column, co-purifying with $\text{Ins}(1,4)\text{P}_2$ phosphatase activity. LiCl inhibited crude and DEAE-cellulose-purified $\text{Ins}(1,3,4)\text{P}_3$ phosphatase. A typical concentration/response curve for the purified enzyme is shown in Fig. 4; 50 % inhibition occurred at 3–5 mM-LiCl at 1–2 nM- $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$. The addition of 10 mM-KCl or - NH_4Cl had no effect. The concentrated enzyme obtained after DEAE-cellulose (peak III) was applied to either a Sephacryl S-200 column or a Blue Sepharose column. A single peak of phosphatase activity was co-purified, acting on both $[\text{^32P}]\text{Ins}(1,4)\text{P}_2$ and $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ (the data are shown in Fig. 3b for the Sephacryl column). Recoveries of phosphatase activities amounted to 40–70 % of that applied to the columns, with either substrate.

DISCUSSION

The two important features of this paper can be summarized as follows: (1) the dephosphorylation of $\text{Ins}(1,3,4)\text{P}_3$ in rat brain cytosol is Li^+ -sensitive; (2) the dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ involves different phosphatases, which can be separated by DEAE-cellulose.

In confirmation of the report of Hawkins *et al.* (1986) in rat parotid and Shears *et al.* (1987) in rat liver, we have observed that, in rat brain, $\text{Ins}(1,3,4,5)\text{P}_4$ is degraded to $\text{Ins}(1,3,4)\text{P}_3$, which is further hydrolysed to $\text{Ins}(3,4)\text{P}_2$. The effect of Li^+ on $\text{Ins}(1,3,4)\text{P}_3$ degradation is unclear. Data provided by Hansen *et al.* (1986) in liver homogenate showed an effect of Li^+ on $\text{Ins}(1,3,4)\text{P}_3$ hydrolysis. More recent studies by Shears *et al.* (1987), in the same tissue did not repeat these observations. These discrepancies could be due to the fact that phosphatase assays were carried out on crude homogenate, or to assay conditions etc. We therefore decided to purify the inositol phosphate phosphatases on a DEAE-cellulose column. Each fraction could then be tested with various inositol phosphates as substrate. As $\text{Ins}(1,3,4)\text{P}_3$ phosphatase co-purified with the Li^+ -sensitive $\text{Ins}(1,4)\text{P}_2$ 1-phosphatase, we investigated the effect of Li^+ on the hydrolysis of $\text{Ins}(1,3,4)\text{P}_3$. We could estimate enzyme activity from both the substrate hydrolysed and the reaction product [i.e. the radioactivity eluted in the $\text{Ins}(3,4)\text{P}_2$ fraction]. $\text{Ins}(1,3,4)\text{P}_3$ phosphatase was shown to be inhibited by Li^+ (Fig. 4). These data may explain the potentiation by Li^+ of $\text{Ins}(1,3,4)\text{P}_3$ formation in stimulated rat cerebral-

Fig. 3. Separation of inositol phosphatases by DEAE-cellulose chromatography (a) and Sephacryl S-200 gel filtration (b)

(a) DEAE-cellulose chromatography was performed as described in the Materials and methods section. Fractions (8 ml) were assayed for activity with $30 \mu\text{M}$ - $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ ($\bullet$), $0.03 \mu\text{M}$ - $[^3\text{H}]\text{Ins}1\text{P}$ ($\blacktriangle$), $0.01 \mu\text{M}$ - $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ ($\square$) and 2 nM - $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ ($\blacksquare$). (b) A sample (0.5 ml) of the DEAE-cellulose-purified $\text{Ins}(1,3,4)\text{P}_3$ phosphatase or peak III was applied to a column ($0.9 \text{ cm} \times 85 \text{ cm}$) of Sephacryl S-200. Fractions (1 ml) were assayed for activity with 2 nM - $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ ($\blacksquare$) and $2.7 \mu\text{M}$ - $[^{32}\text{P}]\text{Ins}(1,4)\text{P}_2$ ($\square$) respectively. The column was calibrated with Blue Dextran 2000 (V_0), bovine serum albumin (BSA; M_r 67000) and chymotrypsinogen A (ChA; M_r 25000).

Fig. 4. Concentration/response curve of the LiCl inhibition of DEAE-cellulose-purified $\text{Ins}(1,3,4)\text{P}_3$ phosphatase activity

$[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ was used at 2 nM and the incubation time was 6 min. Data are expressed as percentages of the control value (means of triplicates $\pm$ s.d.). Control activity was measured in the absence of LiCl .

cortical slices and other cells (Batty *et al.*, 1985; Burgess *et al.*, 1985; Hansen *et al.*, 1986; Balla *et al.*, 1986).

Although the dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ involves different phosphatases, as evidenced

by DEAE-cellulose chromatography, the failure to separate $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ phosphatase activities by this method (or other techniques) does not necessarily imply that these two inositol phosphates are hydrolysed by the same enzyme. We believe that the same comment applies for the results of Inhorn *et al.* (1987), who isolated an inositol 1-phosphatase from calf brain with the same specificity as that described in our study. Our data show co-purification of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ phosphatases by DEAE-cellulose followed by gel filtration or Blue Sepharose chromatography. It is possible that co-purification is coincidental, although two additional arguments for the existence of a single enzyme are (a) the specificity of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ hydrolysis in the 1-phosphate position by a cytosolic enzyme, and (b) the sensitivity of both phosphatase activities to Li^+ inhibition. Further purification of these enzyme activities should clarify the situation.

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