Inositol 1,3,4,5,6-pentakisphosphate and inositol hexakisphosphate are inhibitors of the soluble inositol 1,3,4,5-tetrakisphosphate 3-phosphatase and the inositol 1,4,5-trisphosphate/1,3,4,5-tetrakisphosphate 5-phosphatase from pig brain

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The influence of highly phosphorylated inositol phosphates on the Ins(1,3,4,5) P_4 3-phosphatase enriched from the soluble fraction of pig brain was tested, using [5- 32 P]Ins(1,3,4,5) P_4 as substrate. Both Ins(1,3,4,5,6) P_5 and Ins P_6 were very potent inhibitors of the Ins(1,3,4,5) P_4 3-phosphatase. The K_i values were ~ 60 nM and ~ 3 nM for Ins(1,3,4,5,6) P_5 and Ins P_6 respectively. Ins(1,3,4,5,6) P_5 and Ins P_6 also inhibited the Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase. Using Ins(1,3,4,5) P_4 as substrate, the K_i values were about 35 μ M and 15 μ M for Ins(1,3,4,5,6) P_5 and Ins P_6 respectively. The concentrations which led to a 50 % inhibition of Ins(1,4,5) P_3 (0.5 μ M) degradation by the 5-phosphatase were about 20 and 10 μ M for the pentakis- and hexakis-phosphate respectively. As the intracellular concentrations of Ins(1,3,4,5,6) P_5 and Ins P_6 are high (up to 60 μ M) compared with those of the inositol trisphosphates and tetrakisphosphates, it is possible that the highly phosphorylated inositol phosphates act as regulators in the metabolism of Ca^{2+} -mobilizing inositol phosphates.

INTRODUCTION

The mobilization of Ca^{2+} from intracellular stores by Ins(1,4,5) P_3 is induced by a variety of agonists [1,2]. Ins(1,4,5) P_3 is generated from PtdIns(4,5) P_2 by the action of a phospholipase C. For inactivation, Ins(1,4,5) P_3 is either dephosphorylated to Ins(1,4) P_2 by a 5-phosphatase [3] or phosphorylated to Ins(1,3,4,5) P_4 by a 3-kinase [4]. The function of Ins(1,3,4,5) P_4 is as yet unclear. In concert with Ins(1,4,5) P_3 , it may be involved in the regulation of intracellular Ca^{2+} concentrations by controlling Ca^{2+} fluxes into the cell and/or into certain compartments of the intracellular Ca^{2+} stores [5–7]. Ins(1,3,4,5) P_4 is dephosphorylated to Ins(1,3,4) P_3 [8], which is a precursor of Ins(1,3,4,5,6) P_5 formation via Ins(1,3,4,6) P_4 [9]. It has become evident that the metabolism of Ins(1,3,4,5) P_4 is more complex, because it is dephosphorylated not only to Ins(1,3,4) P_3 , but also to Ins(1,4,5) P_3 by a high-affinity 3-phosphatase [10–12]. Recently we showed that Ins(1,4,5) P_3 , Ins(1,3,4) P_3 and Ins(3,4,5,6) P_4 are strong inhibitors of the Ins(1,3,4,5) P_4 3-phosphatase from pig brain [13].

The highly phosphorylated inositol polyphosphates, Ins(1,3,4,5,6) P_5 and Ins P_6 , are found at comparatively high concentrations in the cells of plants, birds and mammals [14,15]. Their function is still unclear. They have been shown to act like a neurotransmitter when injected into certain regions of the central nervous system of rats [16], and extracellularly applied Ins P_6 was able to enhance Ca^{2+} influx in cultured cerebellar neurons [17]. Additionally, Ins(1,3,4,5,6) P_5 and Ins P_6 have been shown to induce transmembrane ion currents when injected into neurons of *Aplysia* [18,19].

Here we demonstrate that Ins(1,3,4,5,6) P_5 and Ins P_6 are very potent inhibitors of the Ins(1,3,4,5) P_4 3-phosphatase. At higher concentrations they also inhibit the inositol polyphosphate 5-phosphatase, thereby decreasing the dephosphorylation of Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 .

MATERIALS AND METHODS

Materials

Ins(1,3,4,5,6) P_5 was obtained from Boehringer Mannheim (Mannheim, Germany). Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 and Ins P_6 were from Calbiochem (Frankfurt am Main, Germany) and D-myo-[1- 3 H]inositol 1,3,4,5-tetrakisphosphate was from NEN/Du Pont de Nemours (Dreieich, Germany). PtdIns4P and phosphatidylserine (PtdSer) (dipalmitoyl) were purchased from Sigma (Deisenhofen, Germany). Partisil 10 SAX and silica gel t.l.c. plates (LK6D; 20 cm $\times$ 20 cm) were from Whatman (Maidstone, Kent, U.K.), and a Bio-Gel TSK DEAE-5-PW column (75 mm $\times$ 7.5 mm) was from Bio-Rad (München, Germany). Flow-scint IV was obtained from Canberra-Packard (Frankfurt am Main, Germany). All other chemicals were of analytical grade.

Preparation of 32 P-labelled substances

[5- 32 P]Ins(1,3,4,5) P_4 and [5- 32 P]Ins(1,4,5) P_3 were synthesized enzymically. [5- 32 P]Ins(1,3,4,5) P_4 was prepared from [5- 32 P]-Ins(1,4,5) P_3 using a crude Ins(1,4,5) P_4 3-kinase from rat brain; [5- 32 P]Ins(1,4,5) P_3 was generated from [5- 32 P]PtdIns(4,5) P_2 with a purified phospholipase C.

Preparation of [5- 32 P]PtdIns(4,5) P_2 . [5- 32 P]PtdIns(4,5) P_2 was prepared from PtdIns4P and [γ - 32 P]ATP using an enriched PtdIns4P 5-kinase according to Wreggett *et al.* [20] with some modifications. One rat brain was homogenized and centrifuged at 100 000 g, and the supernatant was applied to an h.p.l.c. ion-exchange column (Bio-Gel TSK DEAE-5-PW; 75 mm $\times$ 7.5 mm) and eluted exactly as described [20]. The eluent was collected in 1 ml fractions, which were assayed for PtdIns4P 5-kinase activity with [γ - 32 P]ATP prepared according to Johnson & Walseth [21] as the [32 P] P_i -donor. In our experience, the reaction mixture for the preparation of [γ - 32 P]ATP contains compounds which in-

Abbreviations used: DTT, dithiothreitol; fMet-Leu-Phe, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; PtdSer, phosphatidylserine.

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terfere with PtdIns4P 5-kinase activity. Therefore, when assaying PtdIns4P 5-kinase activity, [γ - 32 P]ATP was diluted in a buffer containing all substances (except the enzymes) present in the reaction mixture for [γ - 32 P]ATP synthesis and supplemented with 3 μ M unlabelled ATP. [γ - 32 P]ATP was applied in a volume of 25 μ l; all other conditions were as described for the preparative synthesis of [5- 32 P]PtdIns(4,5) P_2 (see below). The fraction with the highest activity of PtdIns4P 5-kinase was used for the preparation of [5- 32 P]PtdIns(4,5) P_2 . In a 2 ml polypropylene tube with screw cap (Sarstedt), 18.75 μ l of PtdSer (10 mg/ml) and 50 μ l of PtdIns4P (1 mg/ml), both dissolved in chloroform/methanol (2:1, v/v) were dried under a stream of nitrogen and 21 μ l of a 6-fold concentrated buffer solution [135.6 mM-MgCl₂, 22.8 mM-dithiothreitol (DTT), 3 mM-EGTA, 120 mM-Tris/HCl, pH 7.0], 6.25 μ l of ADP (2 mM) and 22.75 μ l of water were added. After sonication for 5 min at room temperature, 25 μ l of [γ - 32 P]ATP (1.5–2 mCi) and 50 μ l of the most active fraction assayed for PtdIns4P 5-kinase activity were added and incubated at 30 °C for 2.5 h. The reaction was terminated by addition of 300 μ l of chloroform, 100 μ l of HCl (100 mM), 50 μ l of methanol and 25 μ l of water. After vortex-mixing and brief centrifugation for phase separation, the upper phase was removed and the organic phase was washed twice with 50 μ l of water/methanol (4:1, v/v). The purity of [5- 32 P]PtdIns(4,5) P_2 was checked by h.p.l.c. on hydroxyapatite as described [22] or by t.l.c. on silica gel plates, which were developed with chloroform/methanol/4 M-NH₄OH (42:38:14, by vol.) Normally about 10–20% of the radioactivity from [γ - 32 P]ATP was incorporated in [5- 32 P]PtdIns(4,5) P_2 , which contained less than 5% radioactive PtdIns4P. {This method of synthesizing [5- 32 P]PtdIns(4,5) P_2 has been optimized for the use of [γ - 32 P]ATP prepared according to Johnson & Walseth [21]. In our laboratory, [γ - 32 P]ATP is prepared in a volume of about 100–150 μ l (10 mCi) and the reaction is terminated by heating. If purified [γ - 32 P]ATP is used, the incubation buffer should be supplemented with final concentrations of 25 mM-MgCl₂, 5 mM-DTT and 360 μ M-spermine [23].}

Preparation of [5- 32 P]Ins(1,4,5) P_3 . [5- 32 P]PtdIns(4,5) P_2 was mixed with 15 μ l of cholic acid (200 μ M) dissolved in chloroform/methanol (2:1, v/v) and dried under nitrogen. The dried lipid was sonicated for 5 min in 300 μ l of a buffer consisting of 80 mM-KCl, 30 mM-LiCl, 1 mM-CaCl₂, 100 μ M-EDTA, 1 mM-ATP, 0.1% 2-mercaptoethanol and 30 mM-Hepes, pH 7.0. The reaction was started by the addition of 1–3 μ l (100–300 ng of protein, depending on the specific activity of the enzyme) of a purified cytosolic phospholipase C from pig brain [24]. The incubation was performed overnight at 37 °C, terminated by the addition of methanol (half of the reaction volume) and centrifuged at 15000 g for 10 min. [5- 32 P]Ins(1,4,5) P_3 was checked and purified by h.p.l.c. (see below). Usually about 90% of the available [5- 32 P]PtdIns(4,5) P_2 was hydrolysed and recovered as [5- 32 P]Ins(1,4,5) P_3 .

Preparation of [5- 32 P]Ins(1,3,4,5) P_4 . When preparing [5- 32 P]Ins(1,3,4,5) P_4 , the reaction for the [5- 32 P]Ins(1,4,5) P_3 synthesis was not terminated after incubation but the medium was supplemented with 20 mM-ATP, 10 mM-MgCl₂, 3 mM-2,3-diphosphoglycerate and 1 mM-EGTA (final concentrations) in a final volume of 500 μ l, and the following reaction was started by the addition of the 100000 g supernatant from a rat brain homogenate (30–60 μ g of protein/ml, final concentration) containing an Ins(1,3,4,5) P_4 3-kinase [4]. Usually about 80–90% of the applied [5- 32 P]Ins(1,4,5) P_3 was phosphorylated to [5- 32 P]Ins(1,3,4,5) P_4 in 10–15 min. The reaction was stopped by the addition of 500 μ l of methanol, and after centrifugation the reaction product [5- 32 P]Ins(1,3,4,5) P_4 was purified by h.p.l.c. (see below).

Enrichment of Ins(1,3,4,5) P_4 3-phosphatase from pig brain

For enrichment of the 3-phosphatase, a 100000 g supernatant from pig brain was prepared, loaded on DEAE-Sephacel and eluted with a gradient from 0 to 1 M-NaCl as described [13]. 3-Phosphatase-activity-containing fractions, which also contained 5-phosphatase activity, were pooled, divided into portions and stored at –80 °C until used.

Enzyme assays

The activity of the 3-phosphatase was determined using [5- 32 P]Ins(1,3,4,5) P_4 , from which [5- 32 P]Ins(1,4,5) P_3 was generated by the 3-phosphatase. The incubation (final volume 100 μ l) was performed in buffer containing 140 mM-KCl, 100 μ M-MgCl₂, 30 μ M-EDTA, 1 mM-DTT, [5- 32 P]Ins(1,3,4,5) P_4 (10000–20000 c.p.m.) at the concentrations indicated, and 30 mM-Hepes/NaOH, pH 7.4, for 30 min at 37 °C.

The dephosphorylation of Ins(1,3,4,5) P_4 by the 5-phosphatase was determined with [5- 32 P]Ins(1,3,4,5) P_4 as substrate and by the release of [32 P]P_i as indicator of 5-phosphatase activity. Some determinations of 5-phosphatase activity were performed with [1- 3 H]Ins(1,3,4,5) P_4 (200000–400000 d.p.m.) and the accumulation of [1- 3 H]Ins(1,3,4) P_3 was measured. The incubation was carried out in the same buffer as for the 3-phosphatase assay, with the exception that 1 mM-MgCl₂ was used.

For the 5-phosphatase assay with Ins(1,4,5) P_3 as substrate, the buffer contained 0.5 μ M-[5- 32 P]Ins(1,4,5) P_3 (10000–20000 c.p.m.) and 1 mM-MgCl₂. Variations are specified in the Figure legends.

All incubations were terminated with 400 μ l of methanol/water (1:1, v/v) supplemented with 10 mM-H₃PO₄, 1 mM-EDTA and 1 mM-*myo*-inositol. Samples were centrifuged for 10 min at 15000 g, and the supernatants were analysed by h.p.l.c. The differing specific activities of the enzymes in comparison with those in our previous work [13] are probably due to longer storage of the preparation used in the present study, as the 3-phosphatase appears to be more stable than the 5-phosphatase.

Purification and analysis of inositol phosphates by h.p.l.c.

For purification, [5- 32 P]Ins(1,4,5) P_3 or [5- 32 P]Ins(1,3,4,5) P_4 was loaded in a volume of 1 ml onto a Partisil 10 SAX column (200 mm $\times$ 4.6 mm) and eluted by running a gradient from 0 to 100% of eluent B (3.4 M-NH₄COOH adjusted to pH 3.7 with H₃PO₄) at a flow rate of 1.25 ml/min: 0–6 min, 0% B; 6–31 min, 0–30% B; 31–41 min, 30–50% B; 41–55 min, 50% B; 55–56 min, 50–100% B; 56–65 min, 100% B. At around the expected retention times for Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , the eluate was collected every 20 s, i.e. from 30 to 40 min and from 40 to 50 min respectively. The Čerenkov radiation of each fraction was determined in a liquid scintillation counter. Peak fractions were pooled in portions of 1.25 ml and the salt was exchanged (see below).

For analysis, samples containing 32 P-labelled reaction products were loaded on a Partisil 10 SAX column in a volume of 500 μ l and eluted in the same manner as for purification, using a gradient as previously described [13]. For analysis of 3 H-labelled inositol polyphosphates the gradient was slightly modified: 0–4 min, 0–25% B, flow 1.25 ml/min; 4–27 min, 25–33% B; 27–28 min, 33–100% B; 28–35 min, 100% B, flow 1.25–2 ml/min; 35–38 min, 100% B. The eluate was mixed with 6.25 ml of scintillation liquid (Flow-scint IV) and radioactivity was detected using an on-line h.p.l.c. radioactivity monitor (Berthold LB 506 C-1, Wildbad, Germany). Typical retention times using a new packed column were: Ins(1,4) P_2 , 12 min; Ins(1,3,4) P_3 , 22 min; Ins(1,4,5) P_3 , 25 min; Ins(1,3,4,5) P_4 , 34 min.

Exchange of salt solution for storage of radioactively labelled inositol polyphosphates

After purification of $[5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$ or $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ by h.p.l.c., the high concentrations of NH_4COOH and H_3PO_4 were exchanged for KCl. The eluted substance (volume 1.25 ml) was diluted with water to a volume of 10 ml. To this, 50 μl of Partisil 10 SAX (100 mg/ml) was added and the suspension was shaken overnight at 4 °C to bind the inositol polyphosphate. After centrifugation at 3000 g for 10 min, the supernatant was carefully removed and the inositol polyphosphate was batch-eluted with $5 \times 100 \mu\text{l}$ of HCl (1 M). The pH of the eluate was adjusted to 7.4 with 10 M-KOH, buffered with 50 μl of 1 M-Hepes, pH 7.4, and stored in the obtained 900 mM-KCl solution at -20 °C until used. $[1\text{-}^3\text{H}]\text{Ins}(1,3,4,5)P_4$ was delivered in 50 mM-ammonium phosphate buffer, which influenced the 3- and 5-phosphatase activities (see the Results and discussion section). Therefore the buffer solution was also exchanged for 900 mM-KCl and handled as above, but stored at 4 °C.

Others

Protein was determined according to Lowry *et al.* [25], with modifications [26]. Bovine serum albumin was the standard protein. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was prepared as described [21].

RESULTS AND DISCUSSION

When the enriched 3-phosphatase was incubated over 60 min, a linear substrate decomposition was seen at a concentration of 15 μg of protein/ml (results not shown). Therefore all experiments were performed at this protein concentration.

Fig. 1. Inhibition of the 3-phosphatase by Ins(1,3,4,5,6) P_5 .

The enzyme assay was carried out for 30 min as described in the Materials and methods section. Shown are the means from one of two experiments performed in triplicate. (a) Dixon plot. The concentration of Ins(1,3,4,5,6) P_5 was varied as indicated; the concentrations of Ins(1,3,4,5) P_4 were: ●, 0.5 μM ; ■, 1 μM ; ▲, 2 μM ; ◆, 4 μM . (b) Lineweaver-Burk plot. The concentrations of Ins(1,3,4,5,6) P_5 were: ◆, 0 μM ; ▼, 0.1 μM ; ▲, 0.2 μM ; ■, 0.5 μM ; ●, 1 μM .

Fig. 2. Inhibition of the 3-phosphatase by Ins P_6 .

The 3-phosphatase was assayed as described in the Materials and methods section by the formation of $[5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$ from $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ within 30 min. Shown are the means from one of five experiments performed in triplicate revealing similar results. (a) Dixon plot. The concentration of Ins P_6 was varied as indicated; the concentration of Ins(1,3,4,5) P_4 were: ●, 0.5 μM ; ■, 1 μM ; ▲, 2 μM ; ◆, 4 μM . (b) Lineweaver-Burk plot. The concentrations of Ins P_6 were: ◆, 0 nM; ▲, 1 nM; ■, 5 nM; ●, 10 nM.

Ins(1,3,4,5,6) P_5 was a very potent inhibitor of the 3-phosphatase with Ins(1,3,4,5) P_4 as substrate. The K_i value was about 60 nM, as estimated from a Dixon plot (Fig. 1a). The Lineweaver-Burk plot indicated complex inhibition kinetics (Fig. 1b): it revealed curves instead of straight lines. This could be due to several enzymes catalysing the same reaction or to one enzyme with two substrate-binding sites, of which only one is affected by the inhibitor [27]. It is more likely that the 3-phosphatase is one enzyme with two substrate-binding sites, because it was eluted as a single peak when molecular exclusion chromatography was performed [13]. We recently described the same complex kinetics for Ins(1,4,5) P_3 as an inhibitor of the 3-phosphatase [13].

The other inositol polyphosphate tested, Ins P_6 , was more potent in inhibiting the 3-phosphatase than was Ins(1,3,4,5,6) P_5 . A Dixon plot (Fig. 2a) showed that the K_i value was about 3 nM. When the data are presented in an $1/v$ versus $1/[S]$ plot, a mixed-type inhibition is seen (Fig. 2b). Both the linear Dixon plot and the slope $1/[S]$ and $1/v$ -axis intercept replots (not shown) indicated a linear mixed-type inhibition, i.e. a mixture of partial competitive and pure non-competitive inhibition [27].

We also investigated the influence of Ins(1,3,4,5,6) P_5 and Ins P_6 on the Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase. The dephosphorylation of Ins(1,3,4,5) P_4 was inhibited by both Ins(1,3,4,5,6) P_5 and Ins P_6 . The Dixon plot revealed a K_i value of 35 μM for the pentakisphosphate as inhibitor (Fig. 3a), and the Lineweaver-Burk plot fitted best to a competitive inhibition (Fig. 3b). As shown for the 3-phosphatase, Ins P_6 was also more potent in inhibiting the 5-phosphatase than was Ins(1,3,4,5,6) P_5 ; the K_i value for Ins P_6 was estimated to be about 15 μM in a Dixon plot (Fig. 4a). The presentation in an $1/v$ versus $1/[S]$ plot indicated a competitive inhibition for Ins P_6 (Fig. 4b).

Fig. 3. Influence of $\text{Ins}(1,3,4,5,6)P_5$ on the dephosphorylation of $\text{Ins}(1,3,4,5)P_4$ by the $\text{Ins}(1,4,5)P_3/\text{Ins}(1,3,4,5)P_4$ 5-phosphatase

Incubations were performed for 30 min as described in the Materials and methods section. Shown are the means from one of three experiments performed in triplicate revealing similar results. (a) Dixon plot. $\text{Ins}(1,3,4,5,6)P_5$ was applied at various concentrations as indicated. The concentrations of $\text{Ins}(1,3,4,5)P_4$ were: $\bullet$, 0.5 μM ; $\blacksquare$, 1 μM ; $\blacktriangle$, 2 μM ; $\blacklozenge$, 4 μM . (b) Lineweaver-Burk plot. The concentrations of $\text{Ins}(1,3,4,5,6)P_5$ were: $\blacklozenge$, 0 μM ; $\blacktriangle$, 10 μM ; $\blacksquare$, 50 μM ; $\bullet$, 100 μM .

Fig. 4. Influence of $\text{Ins}P_6$ on the dephosphorylation of $\text{Ins}(1,3,4,5)P_4$ by the $\text{Ins}(1,4,5)P_3/\text{Ins}(1,3,4,5)P_4$ 5-phosphatase

The enzyme was incubated for 30 min as described in the Materials and methods section. Shown are the means from one of three experiments, which were performed in triplicate, revealing similar results. (a) Dixon plot. $\text{Ins}P_6$ was applied at the concentrations indicated. The concentrations of $\text{Ins}(1,3,4,5)P_4$ were: $\bullet$, 0.5 μM ; $\blacksquare$, 1 μM ; $\blacktriangle$, 2 μM ; $\blacklozenge$, 4 μM . (b) Lineweaver-Burk plot. The concentrations of $\text{Ins}P_6$ were: $\blacklozenge$, 0 μM ; $\blacktriangle$, 20 μM ; $\blacksquare$, 50 μM ; $\bullet$, 100 μM .

The 5-phosphatase activity was quantified by measuring the release of $[^{32}\text{P}]P_i$ from $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$. Because our enzyme preparation contained both a Mg^{2+} -dependent 5-phosphatase and a Mg^{2+} -independent 3-phosphatase, there is a possibility that $[5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$ is released from $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ by the action of the 3-phosphatase when 5-phosphatase activity is measured. On the other hand, $\text{Ins}(1,4,5)P_3$ is also a substrate for the 5-phosphatase, so that the $[^{32}\text{P}]P_i$ measured could be the sum of the 5-dephosphorylation of both $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ and $[5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$. In this case, the kinetic data would not be correct. There are, however, several reasons why our data with $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ could be used to characterize the kinetic properties of the 5-phosphatase: (i) in most of the reported cases the K_m value of $\text{Ins}(1,4,5)P_3$ is about 4–10-fold higher than the K_m value of $\text{Ins}(1,3,4,5)P_4$ for the 5-phosphatase (for a review see [15]), and (ii) the 3-phosphatase is inhibited by very low concentrations of $\text{Ins}(1,3,4,5,6)P_5$ and $\text{Ins}P_6$ ($K_i \sim 60$ nM and ~ 3 nM respectively), so 3-phosphatase activity is only detectable with low concentrations of inhibitor (< 10 μM) added in the presence of ≥ 0.5 μM $\text{Ins}(1,3,4,5)P_4$.

To ensure that the kinetic data obtained with ^{32}P -labelled $\text{Ins}(1,3,4,5)P_4$ were correct, we also performed the kinetic investigations for the 5-phosphatase with $[1\text{-}^3\text{H}]\text{Ins}(1,3,4,5)P_4$ as substrate, which allows us to discriminate between 3- and 5-phosphatase activities. In these experiments, the K_i values for $\text{Ins}(1,3,4,5,6)P_5$ and $\text{Ins}P_6$ were determined with 55 μM and ~ 17.5 μM respectively (results not shown) and confirmed the kinetic data for the 5-phosphatase obtained with ^{32}P -labelled substances.

Because commercial $[1\text{-}^3\text{H}]\text{Ins}(1,3,4,5)P_4$ was delivered in

Fig. 5. Influence of ammonium phosphate buffer on $\text{Ins}(1,3,4,5)P_4$ dephosphorylation by the $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase and $\text{Ins}(1,4,5)P_3/\text{Ins}(1,3,4,5)P_4$ 5-phosphatase

The incubation was performed as described in the Materials and methods section using 0.5 μM $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ as the substrate in the presence of increasing concentrations of ammonium phosphate buffer, pH 8.0. 3-Phosphatase activity ($\bullet$) was determined by measuring the accumulation of $[5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$ in the presence of 100 μM MgCl_2 and 30 μM EDTA . 5-Phosphatase activity ($\blacksquare$) was quantified by the release of $[^{32}\text{P}]P_i$ from $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ in the presence of 1 mM MgCl_2 . Shown are the means $\pm$ S.D. from one of three triplicate determinations revealing similar results.

Fig. 6. Effect of Ins(1,3,4,5,6) P_5 and Ins P_6 concentration on the degradation of Ins(1,4,5) P_3 by the 5-phosphatase

The enzyme was incubated for 30 min with 0.5 μ M-[5- 32 P]Ins(1,4,5) P_3 as described in the Materials and methods section. 5-Phosphatase activity was determined by the release of [32 P] P_i from [5- 32 P]Ins(1,4,5) P_3 . The concentrations of Ins(1,3,4,5,6) P_5 (●) and Ins P_6 (■) were varied as indicated. Shown are the means $\pm$ S.D. from one of three experiments performed in triplicate. The curves were fitted by non-linear regression using GraphPAD (version 2.0; ISI Software, Philadelphia, PA, U.S.A.).

50 mM-ammonium phosphate buffer, pH 8.0, we tested the influence of this buffer on the dephosphorylation of 0.5 μ M-Ins(1,3,4,5) P_4 by the 3- and 5-phosphatases (Fig. 5). The Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase was half-maximally inhibited by concentrations of ammonium phosphate buffer of about 10 mM, but the 3-phosphatase seemed to be activated by ammonium phosphate buffer applied at concentrations from 1 mM to 10 mM; higher amounts of the buffer were inhibitory. This increased formation of Ins(1,4,5) P_3 , indicating 3-phosphatase activity, is partly caused by an inhibition of the 5-phosphatase by ammonium phosphate, thus decreasing the hydrolysis of Ins(1,4,5) P_3 which was generated from Ins(1,3,4,5) P_4 by the 3-phosphatase. However, it is also due to a slight activation of the 3-phosphatase by ammonium phosphate. We confirmed this by investigations with [3 H]Ins(1,3,4,5) P_4 as substrate (results not shown). In these experiments there was more Ins(1,4,5) P_3 formed from Ins(1,3,4,5) P_4 than would be expected from a decreased hydrolysis of Ins(1,4,5) P_3 via 5-phosphatase. When using [1- 3 H]Ins(1,3,4,5) P_4 (2–4 μ l; 200 000–400 000 d.p.m.) as delivered, the final concentration of ammonium phosphate would be 1–2 mM. At this concentration both enzymes were only slightly affected. However, to avoid any influence, we exchanged the ammonium phosphate buffer for KCl as described in the Materials and methods section.

Since Ins(1,4,5) P_3 is also degraded by a 5-phosphatase, we examined the effects of Ins(1,3,4,5,6) P_5 and Ins P_6 on the dephosphorylation of this substrate. The concentration of unlabelled Ins(1,4,5) P_3 in the incubation buffer was 0.5 μ M, approximating to the concentration of Ins(1,4,5) P_3 in unstimulated cells [15]. Both inositol polyphosphates tested were comparably effective, with concentrations causing 50% inhibition of around 20 μ M and 10 μ M for Ins(1,3,4,5,6) P_5 and Ins P_6 respectively (Fig. 6).

Determinations of the intracellular concentrations of Ins(1,3,4,5,6) P_5 and Ins P_6 range from 5 μ M up to 60 μ M. With n.m.r. detection, the intracellular concentrations of both the pentakisphosphate and the hexakisphosphate were found to be

5–15 μ M in various tissues of mammals [28]. In fMet-Leu-Phe-stimulated HL-60 cells, Pittet *et al.* [29] reported concentrations of about 35–50 μ M and 55–60 μ M for Ins(1,3,4,5,6) P_5 and Ins P_6 respectively, as measured by h.p.l.c. with a metal dye detection system.

Considering the intracellular concentrations of Ins(1,3,4,5,6) P_5 and Ins P_6 and their inhibitory potencies on the 3-phosphatase, it is conceivable that this enzyme has not been detected in intact cells as yet. Indeed, all studies of this enzyme have been reported with cell-free systems [10–12,30] or permeabilized cells [31,32]. In the latter system, the endogenous inositol pentakis- and hexakis-phosphate will be diluted by the incubation buffer.

The difficulty in studying the 3-phosphatase in prelabelled intact cells is due to the fact that Ins(1,4,5) P_3 is a metabolic intermediate. It is generated not only from PtdIns(4,5) P_2 by phospholipase C but also from Ins(1,3,4,5) P_4 by 3-dephosphorylation. Furthermore, Ins(1,4,5) P_3 is the substrate of both the Ins(1,4,5) P_3 3-kinase and the Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase, yielding Ins(1,3,4,5) P_4 and Ins(1,4) P_2 respectively. Additionally, nothing is known about the compartmentation of inositol phosphates within the cell, making it difficult to estimate both local concentrations and the resulting inhibitory effects of these compounds on distinct enzymes. Altogether, it is as yet almost impossible to determine how active the 3-phosphatase is *in vivo*.

The inhibitory effects of Ins(1,3,4,5,6) P_5 and Ins P_6 on the 5-phosphatase are also interesting. The hydrolysis of Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 was affected by comparable concentrations of Ins P_6 which was more effective than Ins(1,3,4,5,6) P_5 , as also seen for the 3-phosphatase. The concentrations of Ins(1,3,4,5,6) P_5 and Ins P_6 necessary for inhibition of the Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase are three orders of magnitude higher than those needed to inhibit the 3-phosphatase, but are in the reported range of the intracellular concentrations for higher-phosphorylated inositol polyphosphates. The inhibition of the Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase by Ins P_6 *in vivo* would decrease the dephosphorylation and inactivation of Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 . As Ins(1,4,5) P_3 is the intracellular second messenger releasing Ca^{2+} , and as this Ca^{2+} release is probably also controlled by Ins(1,3,4,5) P_4 , the intracellular Ca^{2+} signal may be indirectly modified by the higher-phosphorylated inositol phosphates.

Recently, Hughes & Shears [33] described inhibitory effects of Ins(1,3,4,5,6) P_5 and Ins P_6 on the dephosphorylation of Ins(1,3,4,5) P_4 by a 3-phosphatase from rat parotid glands, with half-maximally inhibitory concentrations of 1 and 0.5 μ M for Ins(1,3,4,5,6) P_5 and Ins P_6 respectively. These discrepancies compared with our findings may be due to the enzyme preparation: we used a 3-phosphatase partially purified by anion-exchange chromatography. It is likely that during this procedure endogenous Ins(1,3,4,5,6) P_5 and Ins P_6 are removed. Moreover, Hughes & Shears [33] clearly pointed out that their kinetic determinations were complicated by endogenous inhibitory factor(s) present in the soluble fraction of rat parotid gland. Hodgson & Shears [30] described an unknown endogenous inhibitor of the Ins(1,3,4,5) P_4 3-phosphatase from rat liver which was removed by ion-exchange chromatography. These endogenous inhibitors might be Ins(1,3,4,5,6) P_5 and Ins P_6 [34]. In addition, there may exist different types of 3-phosphatase enzymes. The Ins(1,3,4,5) P_4 3-phosphatase from rat parotid gland is described as a Mg^{2+} -dependent enzyme [33], whereas we showed that the 3-phosphatase of pig brain cytosol is Mg^{2+} -independent [13].

Hughes & Shears [33] also investigated the influence of Ins(1,3,4,5,6) P_5 and Ins P_6 on the dephosphorylation of Ins(1,3,4,5) P_4 by the 5-phosphatase from rat parotid gland. The

enzyme was not affected by concentrations of up to 10 μM of both inositol polyphosphates tested, which is congruent with our results.

The inhibitory effects of $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 have to be taken into consideration when investigating the metabolism of inositol phosphates, because other enzymes involved in inositol phosphate metabolism may also be affected by these compounds. To postulate a physiological role for the higher-phosphorylated inositol phosphates, by regulating the activities of enzymes involved in the metabolism of Ca^{2+} -mobilizing inositol phosphates, is speculative at the present time. If one accepts that the $\text{Ins}(1,4,5)\text{P}_3/\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphatase is regulated by $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 , it has to be concluded that the activity of the 3-phosphatase *in vivo* is negligible. This is supported by the observation that 3-phosphatase activity could not be detected in intact cells. On the other hand, the question arises as to why the 3-phosphatase is expressed in a cell without having a physiological function. Thus this enzyme may play an as yet unknown role in the metabolism of inositol phosphates, but probably it is not an important participant in signal transduction.

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