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

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The influence of highly phosphorylated inositol phosphates on the Ins(1,3,4,5)P₄ 3-phosphatase enriched from the soluble fraction of pig brain was tested, using [5-³²P]Ins(1,3,4,5)P₄ as substrate. Both Ins(1,3,4,5,6)P₆ and InsP₆ are very potent inhibitors of the Ins(1,3,4,5)P₄ 3-phosphatase. The Kᵢ values were ~60 nM and ~3 nM for Ins(1,3,4,5,6)P₆ and InsP₆ respectively. Ins(1,3,4,5,6)P₄ and InsP₆ also inhibited the Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ 5-phosphatase. Using Ins(1,3,4,5,6)P₆ as substrate, the Kᵢ values were about 35 μM and 15 μM for Ins(1,3,4,5,6)P₆ and InsP₆ respectively. The concentrations which led to a 50% inhibition of Ins(4,5)P₂ (0.5 μM) degradation by the 5-phosphatase were about 20 and 10 μM for the pentakis- and hexakis-phosphate respectively. As the intracellular concentrations of Ins(1,3,4,5,6)P₆ and InsP₆ are high (up to 60 μM) 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 Ca²⁺-mobilizing inositol phosphates.

INTRODUCTION

The mobilization of Ca²⁺ from intracellular stores by Ins(1,4,5)P₃ is induced by a variety of agonists [1,2]. Ins(1,4,5)P₃ is generated from PtdIns(4,5)P₂ by the action of a phospholipase C. For inactivation, Ins(1,4,5)P₃ is either dephosphorylated to Ins(1,4)P₂ by a 5-phosphatase [3] or phosphorylated to Ins(1,3,4,5,6)P₆ by a 3-kinase [4]. The function of Ins(1,3,4,5,6)P₆ is as yet unclear. In concert with Ins(1,4,5)P₃, it may be involved in the regulation of intracellular Ca²⁺ concentrations by controlling Ca²⁺ fluxes into the cell and/or into certain compartments of the intracellular Ca²⁺ stores [5-7]. Ins(1,3,4,5,6)P₆ is dephosphorylated to Ins(1,3,4,6)P₄ [8], which is a precursor of Ins(1,3,4,5,6,7)P₇ formation via Ins(1,3,4,5,6,7)P₇ [9]. It has become evident that the metabolism of Ins(1,3,4,5,6)P₆ is more complex, because it is dephosphorylated not only to Ins(1,3,4,5,6)P₄ but also to Ins(1,4,5)P₃ by a high-affinity 3-phosphatase [10-12]. Recently we showed that Ins(1,4,5)P₃ and Ins(1,3,4,5,6)P₄ are strong inhibitors of the Ins(1,3,4,5,6)P₄ 3-phosphatase from pig brain [13].

The highly phosphorylated inositol polyphosphates, Ins(1,3,4,5,6)P₆ and InsP₆, 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 as a neurotransmitter when injected into certain regions of the central nervous system of rats [16], and extracellularly applied InsP₆ was able to enhance Ca²⁺ influx in cultured cerebellar neurons [17]. Additionally, Ins(1,3,4,5,6)P₄ and InsP₄ 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)P₄ and InsP₆ are very potent inhibitors of the Ins(1,3,4,5,6)P₄ 3-phosphatase. At higher concentrations they also inhibit the inositol polyphosphate 5-phosphatase, thereby decreasing the dephosphorylation of Ins(1,3,4,5,6)P₄ and Ins(1,4,5)P₃.

MATERIALS AND METHODS

Materials

Ins(1,3,4,5,6)P₆ was obtained from Boehringer Mannheim (Mannheim, Germany). Ins(1,4,5)P₃, Ins(1,3,4,5,6)P₄ and InsP₆ were from Calbiochem (Frankfurt am Main, Germany) and o-myoo-[¹²⁵I]inositol 1,3,4,5-tetrakisphosphate was from NEN/Du Pont de Nemours (Dréeich, Germany). PtdIns₄P and phosphatidylserine (PtdSer) (dipalmityl) were purchased from Sigma (Deisenhofen, Germany). Partisol 10 SAX and silica gel t.l.c. plates (LK6D; 20 cm x 20 cm) were from Whatman (Maidstone, Kent, U.K.), and a Bio-Gel TSK DEAE-5-PW column (75 mm x 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 ³²P-labelled substances

[5-³²P]Ins(1,3,4,5)P₄ and [5-³²P]Ins(1,4,5)P₃ were synthesized enzymically. [5-³²P]Ins(1,3,4,5)P₄ was prepared from [5-³²P]-Ins(1,4,5)P₃ using a crude Ins(1,4,5)P₃ 3-kinase from rat brain; [5-³²P]Ins(1,4,5)P₃ was generated from [5-³²P]PtdIns(4,5)P₂ with a purified phospholipase C.

Preparation of [5-³²P]PtdIns(4,5)P₂. [5-³²P]PtdIns(4,5)P₂ was prepared from PtdIns₄P and [γ-³²P]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 x 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-³²P]ATP prepared according to Johnson & Walshe [21] as the [³²P]P,-donor. In our experience, the reaction mixture for the preparation of [γ-³²P]ATP contains compounds which in-
terfere with PtdIns4P 5-kinase activity. Therefore, when assaying PtdIns4P 5-kinase activity, [γ-32P]ATP was diluted in a buffer containing all substances (except the enzymes) present in the reaction mixture for [γ-32P]ATP synthesis and supplemented with 3 μM unlabelled ATP. [γ-32P]ATP was applied in a volume of 25 μl; all other conditions were as described for the preparative synthesis of [5-32P]PtdIns(4,5)P2 (see below). The fraction with the highest activity of PtdIns4P 5-kinase was used for the preparation of [5-32P]PtdIns(4,5)P2. In a 2 ml polypropylene tube with screw cap (Sarstedt), 18.75 μl of PtdSer (10 mg/ml) and 50 μl of PtdIns4P (1 mg/ml), both dissolved in chloroform/methanol (2:1, v/v) were dried under a stream of nitrogen and 21 μl 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 μl of ADP (2 mM) and 22.75 μl of water were added. After sonication for 5 min at room temperature, 25 μl of [γ-32P]ATP (1.5–2 mCi) and 50 μl 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 μl of chloroform, 100 μl of HCl (100 mM), 50 μl of methanol and 25 μl 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 μl of water/methanol (4:1, v/v). The purity of [5-32P]PtdIns(4,5)P2 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-32P]ATP was incorporated in [5-32P]PtdIns(4,5)P2, which contained less than 5% radioactive PtdIns4P. This method of synthesizing [5-32P]PtdIns(4,5)P2 has been optimized for the use of radioactive PtdIns(4,5)P2, [5-32P]Ins(1,4,5)P3, 25 μl of PtdSer (10 mg/ml) and 50 μl 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 μl of chloroform, 100 μl of HCl (100 mM), 50 μl of methanol and 25 μl of water. After vortex-mixing and brief centrifugation for phase separation, the organic phase was removed and the reaction was terminated by heating. If purified [γ-32P]ATP is used, the incubation buffer should be supplemented with final concentrations of 25 mM-MgCl2, 5 mM-EGTA and 360 μM-2-phenylethylamine [23].

Preparation of [5-32P]Ins(1,4,5)P3. [5-32P]PtdIns(4,5)P2 was mixed with 15 μl of cholic acid (200 μM) dissolved in chloroform/methanol (2:1, v/v) and dried under nitrogen. The dried lipid was sonicated for 5 min in 300 μl of a buffer consisting of 80 mM-KCl, 30 mM-LiCl, 1 mM-CaCl2, 100 mM-Mg-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 μl (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. [5-32P]Ins(1,4,5)P3 was checked and purified by h.p.l.c. (see below). Usually about 90% of the available [5-32P]PtdIns(4,5)P2 was hydrolysed and recovered as [5-32P]Ins(1,4,5)P3.

Preparation of [5-32P]Ins(1,3,4,5)P4. When preparing [5-32P]Ins(1,3,4,5)P4, the reaction for the [5-32P]Ins(1,4,5)P3 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 μl, and the following reaction was started by the addition of the 100000 g supernatant from a rat brain homogenate (30–60 μg of protein/ml, final concentration) containing an Ins(1,3,4,5)P3-kinase [4]. Usually about 80–90% of the applied [5-32P]Ins(1,4,5)P3 was phosphorylated to [5-32P]Ins(1,3,4,5)P4 in 10–15 min. The reaction was stopped by the addition of 500 μl of methanol, and after centrifugation the reaction product [5-32P]Ins(1,3,4,5)P4 was purified by h.p.l.c. (see below).

Enrichment of Ins(1,3,4,5)P4 3-phosphatase from pig brain

For enrichment of the 3-phosphatase, a 100000 g supernatant from pig brain was prepared, loaded on DEAE-Sephadex 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 [5-32P]Ins(1,3,4,5)P4, from which [5-32P]Ins(1,4,5)P3 was generated by the 3-phosphatase. The incubation (final volume 100 μl) was performed in buffer containing 140 mM-KCl, 100 mM-MgCl2, 30 mM-EDTA, 1 mM-EGTA, [5-32P]Ins(1,3,4,5)P4 (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)P4 by the 5-phosphatase was determined with [5-32P]Ins(1,3,4,5)P4 as substrate and by the release of [32P]PP, as indicator of 5-phosphatase activity. Some determinations of 5-phosphatase activity were performed with [1-3H]Ins(1,3,4,5)P4 (20000–400000 d.p.m.) and the accumulation of [1-3H]Ins(1,3,4,5)P3 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)P3 as substrate, the buffer contained 0.5 μM-[5-32P]Ins(1,4,5)P3 (10000–20000 c.p.m.) and 1 mM-MgCl2. Variations are specified in the Figure legends.

All incubations were terminated with 400 μl 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 analyzed 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, [5-32P]Ins(1,4,5)P3 or [5-32P]Ins(1,3,4,5)P4 was loaded in a volume of 1 ml onto a Partisil 10 SAX column (200 mm × 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)P3 and Ins(1,3,4,5)P4, the eluate was collected every 20 s, i.e. from 30 to 40 min and from 40 to 50 min respectively. The Čerenkov radiations 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 3H-labelled reaction products were loaded on a Partisil 10 SAX column in a volume of 500 μl and eluted in the same manner as for purification, using a gradient as previously described [13]. For analysis of H3-labelled inositol polypolyphosphates the gradient was slightly modified: 0–4 min, 0–25% B; 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)P3, 12 min; Ins(1,3,4)P4, 22 min; Ins(1,4,5)P3, 25 min; Ins(1,3,4,5)P4, 34 min.
Exchange of salt solution for storage of radioactively labelled inositol polyphosphates

After elution were modifications [26]. Bovine serum albumin pH 7.4, and carefully removed the buffer solution activities and the inositol polyphosphate was batch-exchanged with 5 × 100 µl 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. [1-32P]Ins(1,3,4,5,6)P5 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. [γ-32P]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.

Ins(1,3,4,5,6)P2 and InsP6 inhibit inositol polyphosphate phosphatases

Ins(1,3,4,5,6)P2 and InsP6, a potent inhibitor of the 3-phosphatase with Ins(1,3,4,5)P2 as substrate. The Ki 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)P2 as an inhibitor of the 3-phosphatase [13].

The other inositol polyphosphate tested, InsP6, was more potent in inhibiting the 3-phosphatase than was Ins(1,3,4,5,6)P2. A Dixon plot (Fig. 2a) showed that the Ki 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 [17].

We also investigated the influence of Ins(1,3,4,5,6)P2 and InsP6 on the Ins(1,4,5)P2/Ins(1,3,4,5)P2 5-phosphatase. The dephosphorylation of Ins(1,3,4,5)P2 was inhibited by both Ins(1,3,4,5,6)P2 and InsP6. The Dixon plot revealed a Ki 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, InsP6 was also more potent in inhibiting the 5-phosphatase than was Ins(1,3,4,5,6)P2; the Ki value for InsP6 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 InsP6 (Fig. 4b).
The 5-phosphatase activity was quantified by measuring the release of $[^{32}P]P_i$ from $[^{32}P]P_i$Ins(1,3,4,5,6)$P_i$ by our enzyme preparation, since both a Mg$^{2+}$-dependent 5-phosphatase and a Mg$^{2+}$-independent 3-phosphatase, there is a possibility that $[^{32}P]P_i$Ins(1,3,4,5)$P_i$ is released from $[^{32}P]P_i$Ins(1,3,4,5)$P_i$ by the action of the 3-phosphatase when 5-phosphatase activity is measured. On the other hand, Ins(1,4,5)$P_i$ was also a substrate for the 5-phosphatase, so that the $[^{32}P]P_i$ measured could be the sum of the 5-dephosphorylation of both $[^{32}P]P_i$Ins(1,3,4,5)$P_i$ and $[^{32}P]P_i$Ins(1,4,5)$P_i$. In this case, the kinetic data would not be correct. There are, however, several reasons why our data with $[^{32}P]P_i$Ins(1,3,4,5)$P_i$ could be used to characterize the kinetic properties of the 5-phosphatase: (i) in most of the reported cases the $K_m$ value of Ins(1,4,5)$P_i$ is about 4–10-fold higher than the $K_m$ value of Ins(1,3,4,5)$P_i$ for the 5-phosphatase (for a review see [15]), and (ii) the 3-phosphatase is inhibited by very low concentrations of Ins(1,3,4,5,6)$P_i$ and Ins$P_i$ ($K_i$ ~ 60 nM and ~ 3 nM respectively), so 3-phosphatase activity is only detectable with low concentrations of inhibitor (< 10$\mu$M) added in the presence of > 0.5$\mu$M Ins(1,3,4,5)$P_i$.

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

Because commercial [1-$^{3}$H]Ins(1,3,4,5)$P_i$ was delivered in...
Ins(1,3,4,5,6)P$_5$ and InsP$_6$ inhibit inositol polyphosphate phosphatases

Fig. 6. Effect of Ins(1,3,4,5,6)P$_5$ and InsP$_6$ 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 μM[5-32P]Ins(1,4,5)P$_3$ as described in the Materials and methods section. 5-Phosphatase activity was determined by the release of [32P]Pi, from [5-32P]- Ins(1,4,5)P$_3$. The concentrations of Ins(1,3,4,5,6)P$_5$ (●) and InsP$_6$ (▲) were varied as indicated. Shown are the means ± 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 μM-Ins(1,3,4,5)P$_5$ by the 3- and 5-phosphatases (Fig. 5). The Ins(1,4,5)P$_3$/Ins(1,3,4,5)P$_5$ 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 ofIns(1,4,5)P$_3$, which was generated from Ins(1,3,4,5)P$_5$ 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 [3H]Ins(1,3,4,5)P$_5$ 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$_5$ than would be expected from a decreased hydrolysis of Ins(1,4,5)P$_3$ via 5-phosphatase. When using [1-3H]Ins(1,3,4,5)P$_5$ (2–4 μl; 20 000–40 000 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 InsP$_6$ on the dephosphorylation of this substrate. The concentration of unlabelled Ins(1,4,5)P$_3$ in the incubation buffer was 0.5 μM, 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 μM and 10 μM for Ins(1,3,4,5,6)P$_5$ and InsP$_6$, respectively (Fig. 6).

Determinations of the intracellular concentrations of Ins(1,3,4,5,6)P$_5$ and InsP$_6$ range from 5 μM up to 60 μM. With n.m.r. detection, the intracellular concentrations of both the pentakisphosphate and the hexakisphosphate were found to be 5–15 μM in various tissues of mammals [28]. In fMet-Leu-Phe-stimulated HL-60 cells, Pittet et al. [29] reported concentrations of about 35–50 μM and 55–60 μM for Ins(1,3,4,5,6)P$_5$ and InsP$_6$, 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 InsP$_6$ 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$_5$ 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$_5$ 5-phosphatase, yielding Ins(1,3,4,5,6)P$_5$ and Ins(1,4,5)P$_3$ 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 InsP$_6$ on the 5-phosphatase are also interesting. The hydrolysis of Ins(1,3,4,5)P$_5$ and InsP$_6$ was affected by comparable concentrations of InsP$_1$ which was more effective than than Ins(1,3,4,5,6)P$_5$, as also seen for the 3-phosphatase. The concentrations of Ins(1,4,5,6,7)P$_5$ and InsP$_6$ necessary for inhibition of the Ins(1,4,5)P$_3$/Ins(1,3,4,5)P$_5$ 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$_5$ 5-phosphatase by InsP$_6$ in vivo would decrease the dephosphorylation and inactivation of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_5$. 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$_5$, 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 InsP$_6$ on the dephosphorylation of Ins(1,3,4,5)P$_5$ by a 3-phosphatase from rat parotid glands, with half-maximally inhibitory concentrations of 1 and 0.5 μM for Ins(1,3,4,5,6)P$_5$ and InsP$_6$, 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 InsP$_6$ 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$_5$ 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 InsP$_6$ [34]. In addition, there may exist different types of 3-phosphatase enzymes. The Ins(1,3,4,5)P$_5$ 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 InsP$_6$ on the dephosphorylation of Ins(1,3,4,5)P$_3$ 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 Ins(1,3,4,5,6)P5 and InsP6 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 Ca2+-mobilizing inositol phosphates, is speculative at the present time. If one accepts that the Ins(1,4,5)P3/Ins(1,3,4,5,6)P5-phosphatase is regulated by Ins(1,3,4,5,6)P6 and InsP6, 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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