Properties of a soluble inositol 1,3,4,5-tetrakisphosphate 3-phosphatase from porcine brain

Ariane HÖER, Dieter HÖER and Eckard OBERDISSE*

Institut für Pharmakologie der Freien Universität Berlin, Thielallee 69-73, D-1000 Berlin 33, Federal Republic of Germany

We have previously shown that $\text{Ins}(1,3,4,5)\text{P}_4$ is degraded to $\text{Ins}(1,4,5)\text{P}_3$ by a soluble $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase from pig brain [Höer, Kwiatkowski, Seib, Rosenthal, Schultz & Oberdisse (1988) *Biochem. Biophys. Res. Commun.* **154**, 668–675]. Here we present some properties of this enzyme using $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)\text{P}_4$ as substrate. The molecular mass, estimated by gel filtration chromatography on a Superose 6 column, was determined to be 36 kDa. The 3-phosphatase showed a high affinity towards the substrate $\text{Ins}(1,3,4,5)\text{P}_4$ ($K_m \sim 400$ nM); the $V_{\max}$ of the freshly prepared enzyme was 2 nmol/min per mg of protein. The influence of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$, the reaction products of $\text{Ins}(1,3,4,5)\text{P}_4$ hydrolysis by either 3- or 5-phosphatase respectively, on the 3-phosphatase was tested. Both isomers inhibited the enzyme, with K_i values of about 2 μM and 1.75 μM for $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ respectively. Enzyme activity was not influenced by Mg^{2+} up to 30 mM or Ca^{2+} up to 1 mM. Commercially available $\text{Ins}(3,4,5,6)\text{P}_4$ from turkey erythrocytes produced a marked inhibition of the 3-phosphatase ($K_i \sim 500$ nM). Significant inhibitory effects on enzyme activity were also found with GTP and the pyrimidine nucleotides UTP and CTP. The kinetic data presented here suggest that the $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase may be regulated by the intracellular concentrations of inositol tris- and tetrakis-phosphates.

INTRODUCTION

The role of $\text{Ins}(1,4,5)\text{P}_3$ as a second messenger that releases Ca^{2+} from intracellular stores is well established [1,2]. $\text{Ins}(1,4,5)\text{P}_3$ is generated from $\text{PtdIns}(4,5)\text{P}_2$ by phospholipase C [2]. For signal termination, $\text{Ins}(1,4,5)\text{P}_3$ is either dephosphorylated to $\text{Ins}(1,4)\text{P}_2$ by a 5-phosphatase [3] or phosphorylated to $\text{Ins}(1,3,4,5)\text{P}_4$ by a 3-kinase [4]. The physiological significance of $\text{Ins}(1,3,4,5)\text{P}_4$ is not yet clear. In some studies it has been shown that $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ may act synergistically in regulating cellular Ca^{2+} homeostasis, either by facilitating Ca^{2+} entry across the plasma membrane or by refilling empty intracellular Ca^{2+} stores [5,6]. $\text{Ins}(1,3,4,5)\text{P}_4$ is dephosphorylated to $\text{Ins}(1,3,4)\text{P}_3$ [4], which is a precursor of more highly phosphorylated inositol polyphosphates such as $\text{Ins}(1,3,4,5,6)\text{P}_5$, formed from $\text{Ins}(1,3,4,6)\text{P}_4$ [7,8]. Up until now, $\text{Ins}(1,3,4)\text{P}_3$ has been assumed to be the only degradation product of $\text{Ins}(1,3,4,5)\text{P}_4$, but there is growing evidence that $\text{Ins}(1,3,4,5)\text{P}_4$ is also degraded to $\text{Ins}(1,4,5)\text{P}_3$ by an $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase. This new metabolic pathway of $\text{Ins}(1,3,4,5)\text{P}_4$ degradation was recently described by Doughney *et al.* in erythrocyte membranes [9]. The 3-dephosphorylation of $\text{Ins}(1,3,4,5)\text{P}_4$ was also described by us in the soluble fraction of porcine brain [10], and in RBL-2H3 cells by Cunha-Melo *et al.* [11]. Cullen *et al.* [12] recently showed that $\text{Ins}(1,3,4,5)\text{P}_4$ causes release of Ca^{2+} by its rapid conversion to $\text{Ins}(1,4,5)\text{P}_3$ in electroporated mouse lymphoma L1210 cells via a 3-phosphatase. Oberdisse *et al.* [13] found that $\text{Ins}(1,4,5)\text{P}_3$ formation from $\text{Ins}(1,3,4,5)\text{P}_4$ is stimulated by thrombin and phorbol esters in saponin-permeabilized human platelets, indicating that in this system the 3-phosphatase may be regulated by a protein kinase C. Here we present some characteristics of the soluble $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase from porcine brain.

MATERIALS AND METHODS

Materials

All nucleotides and $\text{Ins}(3,4,5,6)\text{P}_4$ were obtained from Boehringer Mannheim (Mannheim, Germany). $\text{Ins}(1,4,5)\text{P}_3$,

$\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were from Calbiochem (Frankfurt, Germany) and *D-myo*-[2- ^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ was from Amersham Buchler (Braunschweig, Germany). Partisil 10 SAX and Partisphere WAX were purchased from Whatman (Clifton, NJ, U.S.A.). DEAE-Sephacel and a Superose 6 column (1 cm $\times$ 30 cm) were from Pharmacia (Freiburg, Germany).

Preparation of ^{32}P -labelled $\text{Ins}(1,3,4,5)\text{P}_4$

$[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)\text{P}_4$ was prepared as described previously [10] and was checked for isomeric purity using a Partisphere WAX column as described [8], which enables the separation of InsP_4 isomers. Analysis of radiolabelled InsP_4 showed that the prepared $[5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)\text{P}_4$ consisted of one radiolabelled isomer co-eluting with the $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ standard.

Phosphatase preparation

A 100 000 g supernatant was prepared from pig brain. Brains were collected after slaughtering, kept on ice and homogenized at 4 °C in 7.5 vol. of buffer consisting of 4 mM-EDTA, 20 mM-2-mercaptoethanol, 0.25 M-sucrose and 20 mM-Hepes/NaOH, pH 8.0. The following procedures were all performed at 4 °C. Large particles were removed by centrifugation (300 g) for 30 min. The resulting supernatant was diluted 2-fold with buffer (1 mM-EDTA, 20 mM-2-mercaptoethanol, 20 mM-Hepes/NaOH, pH 8.0), and membranes were sedimented by centrifugation (14 000 g) for 40 min. The supernatant was centrifuged for 30 min at 100 000 g, and the obtained supernatant (referred to as cytosol) was diluted with ethylene glycol (7:3, v/v) and loaded on DEAE-Sephacel. Proteins were eluted with a linear gradient of NaCl (0–1 M) in buffer containing 1 mM-EDTA, 20 mM-2-mercaptoethanol, 30 % ethylene glycol and 20 mM-Hepes/NaOH, pH 8.0. The 3-phosphatase activity was determined with $\text{Ins}(1,3,4,5)\text{P}_4$ as substrate (see below). 3-Phosphatase activity eluted as a single peak at 200 mM-NaCl which contained considerable amounts of 5-phosphatase activity. The peak fractions were pooled and stored at -80 °C until used.

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

* To whom correspondence should be sent.

Enzyme assays

The activities of the 3- and 5-phosphatases were determined in buffer containing 140 mM-KCl, 100 μ M-MgCl₂, 30 μ M-EDTA, 1 mM-dithiothreitol (DTT), 500 nM-Ins(1,3,4,5)P₄ with [5-³²P]-Ins(1,3,4,5)P₄ (10000–20000 c.p.m.) and 30 mM-Hepes/NaOH, pH 7.4. Samples were incubated in a volume of 100 μ l for 10–30 min at 37 °C. Variations are specified in the Figure legends. The incubation was terminated with 400 μ l of chloroform/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 analysed by h.p.l.c.

H.p.l.c. analysis of inositol phosphates

Samples were loaded on a Partisil 10 SAX column in a volume of 500 μ l. Elution was achieved by running a gradient from 0 to 100 % of eluent B (3.4 M-NH₄COOH adjusted to pH 3.7 with H₃PO₄) in 25 min with a flow rate of 1.75 ml/min: 0–2 min, 0 % B; 2–2.5 min, 0–5 % B; 2.5–12 min, 5–30 % B; 12–17 min, 30–60 % B; 17–20 min, 60–100 % B. This gradient was sufficient to separate P_i, Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, which were the only ³²P-labelled reaction products expected. Radioactivity was detected by an h.p.l.c. radioactivity monitor. Typical retention times were 8 min 30 s, 18 min 10 s and 21 min 10 s for P_i, Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ respectively.

Estimation of molecular mass

The molecular mass of the 3-phosphatase was determined by gel filtration at 4 °C on a Superose 6 column (1 cm $\times$ 30 cm) equilibrated with buffer consisting of 100 mM-NaCl, 1 mM-EDTA, 1 mM-DTT and 20 mM-Hepes/NaOH, pH 7.0. A part of the phosphatase preparation was concentrated to a volume of 200 μ l using an Amicon YM 10 microconcentrator and applied to the column. The flow rate was 0.1 ml/min. To each collected fraction (500 μ l), 200 μ l of ethylene glycol were added. Protein standards were detected by their absorbance at 280 nm.

Others

Protein was determined according to Lowry *et al.* [14] with bovine serum albumin as the standard protein.

RESULTS AND DISCUSSION

We investigated some characteristics *in vitro* of a soluble Ins(1,3,4,5)P₄ 3-phosphatase from pig brain. All incubations were performed in the presence of 500 nM unlabelled Ins(1,3,4,5)P₄. This is probably within the range of the physiological intracellular concentrations of Ins(1,3,4,5)P₄, which is assumed to be < 3 μ M [15]. Recently Pittet *et al.* [16] reported on the Ins(1,3,4,5)P₄ concentrations in differentiated HL-60 cells, which increased from 0.51 μ M to 2.75 μ M after stimulation with fMet-Leu-Phe.

The formation of Ins(1,4,5)P₃ from Ins(1,3,4,5)P₄ was proportional to the protein concentration of the 3-phosphatase from 10 to 60 μ g protein/ml and linear with incubation time up to 60 min (results not shown).

The phosphatase preparation also contained inositol polyphosphate 5-phosphatase. This enzyme is known to require magnesium [15]. As both 5-phosphatase and 3-phosphatase hydrolyse Ins(1,3,4,5)P₄, the influence of bivalent cations on the degradation of Ins(1,3,4,5)P₄ was investigated. To get a defined concentration of EDTA in the incubation medium, the tested samples were dialysed for 16 h against a 1000-fold volume of buffer containing 100 μ M-EDTA, 1 mM-DTT and 30 mM-Hepes/NaOH, pH 7.4. The 3-phosphatase activity was independent of magnesium up to a concentration of 30 mM (Fig. 1).

Fig. 1. Effect of Mg²⁺ on dephosphorylation of Ins(1,3,4,5)P₄ by 3- and 5-phosphatases

Incubation conditions were as described in the Materials and methods section. The free Mg²⁺ concentration was calculated using the metal chelator stability constant of EDTA. Samples (protein concentration 30 μ g/ml) were incubated for 30 min, and reaction products were analysed by h.p.l.c. ³²P_i (▲) indicates 5-phosphatase activity and Ins(1,4,5)P₃ (●) indicates 3-phosphatase activity. Shown are the means from one of five experiments, performed in triplicate, which revealed similar results.

On the other hand, the accumulation of [³²P]P_i, which was released from [5-³²P]Ins(1,3,4,5)P₄ by the 5-phosphatase, was influenced by increasing concentrations of free magnesium (MgCl₂): the enzyme was stimulated maximally at 3 mM-Mg²⁺, whereas concentrations higher than 10 mM-Mg²⁺ inhibited it. Varying the concentration of free Ca²⁺ (1 nM–1 mM) did not affect either the 3- or 5-phosphatase activities (results not shown). Thus the 3-phosphatase activity may be independent of the concentrations of intracellular Ca²⁺ and Mg²⁺. This finding does not necessarily contradict the fact that under certain conditions the 3-phosphatase appears to be affected by Mg²⁺, because Mg²⁺ may result in a very high 5-phosphatase activity, making it difficult to detect 3-phosphatase activity. This may be one reason for the observations of Doughney *et al.* [9], who observed Ins(1,4,5)P₃ release from Ins(1,3,4,5)P₄ only in the absence of Mg²⁺.

A 3-phosphatase which dephosphorylates Ins(1,3)P₂ has been described in calf brain [17]. It is independent of Mg²⁺ and is not inhibited by Li⁺. We cannot exclude the possibility that the enzyme from porcine brain, which is also independent of Mg²⁺ and Li⁺ [10], is identical with this Ins(1,3)P₂ 3-phosphatase. An Ins(1,3)P₂ phosphatase in bovine brain has recently been described [18]. As the authors used unlabelled substrate, they were unable to determine the site of dephosphorylation. As Ins(1)P is the only known product of Ins(1,3)P₂ dephosphorylation, the described enzyme from bovine brain could be a 3-phosphatase. This Ins(1,3)P₂ phosphatase is independent of Mg²⁺ and inhibited by KCl (conc. causing 50 % inhibition 265 mM). The enzyme described by us is activated by KCl [10] and is therefore not identical with the Ins(1,3)P₂ phosphatase from bovine brain.

To estimate the molecular mass of the enzyme, molecular-exclusion chromatography was performed on Superose 6 (Fig. 2); the molecular mass of the 3-phosphatase was determined to be 36 kDa. This molecular mass is of the same order of magnitude as for other inositol phosphatases [18–20].

Fig. 2. Determination of the molecular mass by gel-filtration chromatography on Superose 6

The molecular mass of the 3-phosphatase was determined as described in the Materials and methods section using a Superose 6 column (1 cm $\times$ 30 cm). Marker proteins with known molecular masses were: ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa) and ribonuclease (13.7 kDa). The standard curve is indicated by the broken line (Δ). 3-Phosphatase activity was assayed as described in the Materials and methods section by the formation of Ins(1,4,5) P_3 from Ins(1,3,4,5) P_4 (●). The elution volume of the peak of the 3-phosphatase activity was 17.5 ml, corresponding to a molecular mass of 36 kDa.

To determine the substrate affinity, we investigated the dependency of the enzyme activity on the Ins(1,3,4,5) P_4 concentration. The estimated K_m of approx. 400 nM (results not shown) indicates a high substrate affinity of the 3-phosphatase for Ins(1,3,4,5) P_4 . A comparably high affinity for Ins(1,3,4,5) P_4 was found for the 3-phosphatase from human platelets ($K_m \sim 2.4 \mu\text{M}$) [13] and also for 5-phosphatases from human platelets [21] and rat brain [22] (K_m values of around $1 \mu\text{M}$), whereas the affinity of the 5-phosphatases for Ins(1,4,5) P_3 was lower ($K_m \sim 10 \mu\text{M}$). The initial $V_{\max}$ was calculated to be 2 nmol/min per mg of protein. If stored at -80°C the 3-phosphatase was stable for several months, but enzyme activity decreased within weeks when stored at 4°C .

The formation of Ins(1,4,5) P_3 from Ins(1,3,4,5) P_4 could theoretically be catalysed by the Ins(1,4,5) P_3 3-kinase, which under certain conditions acts as a phosphatase, e.g. in the absence of ATP. Therefore we tested the influence of ATP and other nucleotides on the 3-phosphatase (Table 1). ATP was a weak inhibitor of the 3-phosphatase. GTP ($100 \mu\text{M}$) produced a marked inhibition of the enzyme, and the pyrimidine nucleotides UTP and CTP were more effective inhibitors than was GTP. Both pyrimidine nucleotides inhibited the 3-phosphatase half-maximally at concentrations of $10 \mu\text{M}$. Other nucleotides tested, such as non-hydrolysable adenine and guanine nucleotides or deoxyribonucleotides, had no or at least only slight effects on the 3-phosphatase activity (results not shown). Whether this nucleotide specificity is of physiological importance or is a property occurring only *in vitro* has yet to be elucidated.

The 3-phosphatase was inhibited by its reaction product Ins(1,4,5) P_3 , with a K_i of about $2 \mu\text{M}$ (Fig. 3a). The $1/v$ versus $1/[S]$ plot performed to determine the inhibition type produced curves instead of straight lines (Fig. 3b). These complex inhibition kinetics are due either to multiple enzymes which catalyse the same reaction but are not inhibited to the same extent by the

Table 1. Influence of nucleotides on the formation of Ins(1,4,5) P_3 from Ins(1,3,4,5) P_4 by a 3-phosphatase

Ins(1,3,4,5) P_4 (500 nM) was incubated with cytosolic Ins(1,4,5) P_3 3-phosphatase for 30 min in a volume of $100 \mu\text{l}$ and reaction products were analysed by h.p.l.c. as described in the Materials and methods section. The data are representative of three experiments which revealed similar results.

Nucleotide	Ins(1,4,5) P_3 (% of control)
100 μM -ATP	90.8
100 μM -GTP	59.5
10 μM -UTP	64.5
100 μM -UTP	16.3
10 μM -CTP	53.6
100 μM -CTP	14.6

Fig. 3. Influence of the reaction product Ins(1,4,5) P_3 on Ins(1,3,4,5) P_4 3-phosphatase activity

The enzyme assay was carried out for 30 min (protein concentration $30 \mu\text{g/ml}$) as described in the Materials and methods section; the concentrations of Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 were varied. (a) A Dixon plot of the data revealed a K_i of $\sim 2 \mu\text{M}$. The concentrations of Ins(1,3,4,5) P_4 were: ●, $0.5 \mu\text{M}$; ▲, $1 \mu\text{M}$; ◆, $2 \mu\text{M}$; ■, $4 \mu\text{M}$. (b) The $1/v$ versus $1/[S]$ plot shows the complex inhibition kinetics. The concentrations of Ins(1,3,4,5) P_4 were: ●, $0 \mu\text{M}$; ■, $1 \mu\text{M}$; ▲, $2 \mu\text{M}$; ◆, $5 \mu\text{M}$; *, $10 \mu\text{M}$. Shown are the means from one of three experiments performed in triplicate.

inhibitor, or to one enzyme with two substrate-binding sites, of which only one is affected by the inhibitor [23]. In this case it is more likely that the reaction is catalysed by one enzyme, because the 3-phosphatase activity was eluted as a single peak from a Superose 6 gel filtration column (see above).

We also tested the influence of $\text{Ins}(1,3,4)\text{P}_3$, the other degradation product formed from $\text{Ins}(1,3,4,5)\text{P}_4$ by the 5-phosphatase. The Dixon plot revealed a K_i of about $1.75\ \mu\text{M}$ for this inositol trisphosphate isomer (Fig. 4a). The $1/v$ versus $1/[S]$ plot indicated a mixed-type inhibition (results not shown). Since the Dixon plot is linear, this indicates a linear mixed-type inhibition, i.e. a mixture of partial competitive and pure non-competitive inhibition [23].

The K_i values of the tested trisphosphate isomers are 4–5-fold higher than the K_m value for the substrate $\text{Ins}(1,3,4,5)\text{P}_4$, indicating a higher affinity of the enzyme for the latter compound. However, the K_i values are within the concentrations measured for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ in cells. The data reported on basal intracellular concentrations of $\text{Ins}(1,4,5)\text{P}_3$ range from 0.1 to $0.4\ \mu\text{M}$ in resting cells, and up to 1.0 – $2.5\ \mu\text{M}$ after agonist stimulation [15]. The intracellular level of $\text{Ins}(1,3,4)\text{P}_3$ is assumed to range from $1.1\ \mu\text{M}$ (basal) to $3.6\ \mu\text{M}$ (stimulated) [24]. Therefore

it is likely that these compounds influence the 3-phosphatase *in vivo*.

The specificity of the enzyme for its substrate $\text{Ins}(1,3,4,5)\text{P}_4$ was examined using commercially available $\text{Ins}(3,4,5,6)\text{P}_4$ from turkey erythrocytes. This preparation contains 12% $\text{Ins}(2,4,5,6)\text{P}_4$, but the effects are probably due to $\text{Ins}(3,4,5,6)\text{P}_4$. The enzyme was strongly inhibited by this compound; the inhibition constant was determined to be about $500\ \text{nM}$ by a Dixon plot (Fig. 4b) and approximated to the K_m for $\text{Ins}(1,3,4,5)\text{P}_4$. The $1/v$ versus $1/[S]$ plot indicated a linear mixed-type inhibition of the 3-phosphatase by $\text{Ins}(3,4,5,6)\text{P}_4$ (results not shown). $\text{Ins}(3,4,5,6)\text{P}_4$, which was first detected in chick erythrocytes [25] and other avian cells, also occurs in murine macrophages [26], bovine adrenal glomerulosa cells [27] and differentiated HL-60 cells [16]. In avian and some mammalian cells, $\text{Ins}(3,4,5,6)\text{P}_4$ is the main tetrakisphosphate isomer in unstimulated cells and increases, after a delay, after agonist stimulation. In HL-60 cells, it was found to increase from a basal concentration of about $1.4\ \mu\text{M}$ to $3.5\ \mu\text{M}$ after stimulation with fMet-Leu-Phe within 5 min [16]. In contrast, $\text{Ins}(1,3,4,5)\text{P}_4$ is formed more rapidly in stimulated cells. $\text{Ins}(3,4,5,6)\text{P}_4$ may play a role in the synthesis of more highly phosphorylated inositol phosphates [26], although its origin is still unknown. The low K_i of approx. $500\ \text{nM}$ of $\text{Ins}(3,4,5,6)\text{P}_4$ as an inhibitor indicates a high affinity of the 3-phosphatase for this inositol tetrakisphosphate isomer. It cannot be ruled out that the isomer is also a substrate for the enzyme. Additionally, the intracellular concentration of $\text{Ins}(3,4,5,6)\text{P}_4$ in resting cells is 3-fold higher than that of $\text{Ins}(1,3,4,5)\text{P}_4$ (see above). Therefore it might be that $\text{Ins}(1,3,4,5)\text{P}_4$ is hydrolysed only in very small amounts by the 3-phosphatase *in vivo*. Evidence for this comes from preliminary studies in which we tested the effects of $\text{Ins}(1,3,4,5,6)\text{P}_5$ on the 3- and 5-phosphatases. $\text{Ins}(1,3,4,5,6)\text{P}_5$ is assumed to have an intracellular concentration of between $10\ \mu\text{M}$ [24] and $50\ \mu\text{M}$ [16]. We found that this inositol pentakisphosphate is a very potent inhibitor of the 3-phosphatase, but is also an inhibitor of the 5-phosphatase. In discussing the probability of activity of the 3-phosphatase investigated in this study *in vivo*, it has to be considered that the inositol polyphosphates may be unequally distributed in the cell, as has been discussed for the receptor mechanisms generating $\text{Ins}(1,4,5)\text{P}_3$ and for the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pools [28].

All inositol phosphates tested affected the 3-phosphatase in concentrations which are found in unstimulated or stimulated cells. Therefore the activity of this enzyme *in vivo* may strongly depend on the concentrations of inositol phosphates other than the substrate, $\text{Ins}(1,3,4,5)\text{P}_4$. As the intracellular concentrations of inositol tris- and tetrakis-phosphates vary depending on agonist stimulation in various cells, the 3-phosphatase activity is possibly indirectly regulated by these changes in the concentrations of $\text{Ins}(1,4,5)\text{P}_3$ and its metabolites. These assumptions have to be confirmed in whole cell systems.

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Fig. 4. Influence of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(3,4,5,6)\text{P}_4$ on $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase (Dixon plot)

Incubations were performed as described in the Materials and methods section. The enzyme ($30\ \mu\text{g}/\text{ml}$ of protein) was incubated for 30 min with various concentrations of $\text{Ins}(1,3,4)\text{P}_3$ or $\text{Ins}(3,4,5,6)\text{P}_4$ as indicated. The concentrations of $\text{Ins}(1,3,4,5)\text{P}_4$ were: ●, $0.5\ \mu\text{M}$; ▲, $1\ \mu\text{M}$; ■, $2\ \mu\text{M}$. (a) $\text{Ins}(1,3,4)\text{P}_3$. Shown are the means from one of three experiments performed in triplicate. (b) $\text{Ins}(3,4,5,6)\text{P}_4$. Shown are the means from one of two experiments, performed in triplicate, which revealed similar results.

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