Metabolism of D-myoinositol 1,3,4,5-tetrakisphosphate by rat liver, including the synthesis of a novel isomer of myo-inositol tetrakisphosphate

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INTRODUCTION

In a variety of cells, receptor-mediated hydrolysis of PtdIns(4,5)P₂ leads to the production of Ins(1,4,5)P₃, which is released from the endoplasmic reticulum (Berridge, 1984; Downes & Michell, 1985), an intracellular signal that releases Ca²⁺ from the sarcoplasmic reticulum (Berridge & Irvine, 1984). Ins(1,4,5)P₃ is phosphorylated by a Ca²⁺-dependent kinase to produce Ins(1,4,5)P₄ (Irvine et al., 1986; Hansen et al., 1986; Hawkins et al., 1986). Ins(1,4,5)P₄ can contribute to the activation of sea-urchin eggs, probably by facilitating Ca²⁺ influx across the plasma membrane (Irvine & Moor, 1986).

Several tissues contain enzyme activities which remove the 5-phosphate from both Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, which largely inactivates their intracellular messenger actions (Downes et al., 1982; Berridge & Irvine, 1984; Storey et al., 1984; Batty et al., 1985; Connolly et al., 1985; Hansen et al., 1986; Hawkins et al., 1986; Irvine et al., 1986a,b; Shears et al., 1987). In platelet cytosol, both 5-phosphatase activities may be performed by the same enzyme (Connolly et al., 1987).

We have compared the metabolism of Ins(1,3,4,5)P₄ and Ins(1,4,5)P₄ by liver homogenates incubated in media resembling cytosolic conditions. We have also studied the subcellular location of the liver Ins(1,3,4,5)P₄ 5-phosphatase. Finally, we have added to our previous studies (Shears et al., 1987) by further investigating the metabolic fate of the Ins(1,3,4)P₄ that accumulates after Ins(1,3,4,5)P₄ hydrolysis.

MATERIALS AND METHODS

Preparation of liver homogenates and subfractions

All procedures were performed at 0-4 °C. Livers from male rats (220 g) were perfused under diethyl ether anaesthesia for 2 min with ice-cold 0.25 m-sucrose/5 mM-Tris, pH 7.2, to remove the blood. The livers were chopped and then homogenized at 500 rev./min (five up-and-down strokes) with a Teflon pestle. The homogenate was diluted to 30% (w/v) with more ice-cold 0.25 m-sucrose/5 mM-Tris, pH 7.2. The 100000 g pellets and supernatants (diluted to 30% wt. of original...
liver/vol.) were prepared as previously described (Shears et al., 1987). The supernatant fractions always contained > 90% of the lactate dehydrogenase activity of the original homogenate, indicating that cell breakage was almost complete. In some experiments, the 100000 g pellet was resuspended and centrifuged again for 1 h at 100000 g in order to obtain a washed pellet and its corresponding supernatant. These preparations were diluted with 0.25 M sucrose/5 mM-Tes, pH 7.2, to the concentration of the other liver fractions.

Density-gradient centrifugation of homogenized hepatocytes

This technique is a modification of our earlier method (Storey et al., 1984), which was in turn slightly different from that originally devised by Heyworth et al. (1983). Four 6 ml samples of hepatocytes (approx. 20 mg dry wt./ml) were each added to 20 ml of ice-cold 0.25 M sucrose/3 mM-Tes (pH 7.0)/1 mM-dithiothreitol, as remaining procedures were performed at 0-4°C. The cells were centrifuged at 100 g for 2 min, and each pellet was washed twice by resuspension in 10 ml of the sucrose-based medium, followed by re-centrifugation (100 g for 2 min). The combined pellets (made up to 12 ml) were pressurized with N₂ for 10 min to 200 kPa (30 lbf/in²). After rapid decompression, the suspension was homogenized by hand with a Teflon pestle (three up-and-down strokes). The homogenate was centrifuged (1000 g for 5 min), and 4 ml of supernatant was removed. The remainder was made up to 12 ml and the pressurization, homogenization and centrifugation procedures were repeated. A further 4 ml portion of supernatant was removed. The two supernatants were combined and diluted 1:1 with more sucrose-based medium. The final protein concentration was 1-2 mg/ml.

A 6 ml portion of the homogenate was carefully layered on a discontinuous Percoll density gradient (15 ml of density 1.06 g/ml, above 20 ml of density 1.075 g/ml). The Percoll was prepared in 0.25 M sucrose/3 mM-Tes (pH 7.0)/1 mM-dithiothreitol. Gradients (in 50 ml tubes) were centrifuged for 20 min at 65000 g in a MSE 75 ultracentrifuge with a MSE 30 rotor. Fractions (0.976 ml) were removed from the bottom of the tube with a peristaltic pump, and divided into batches and stored at -20°C until required.

Enzyme assays

Glutamate dehydrogenase (EC 1.4.1.2) and lactate dehydrogenase (EC 1.1.1.27) were assayed as described by Shears & Kirk (1984). Arylesterase (EC 3.1.1.2) was assayed as described by Shears & Hubsch (1969). Galactosyl transferase (EC 2.4.1.38) was assayed as described by Shears et al. (1987), except that incubations were quenched by placing them on ice (Brew et al., 1968) before the addition of EDTA. Alkaline phosphodiesterase 1 (EC 3.1.4.1) was assayed as described by Razzel (1963). β-Galactosidase (EC 3.2.1.23) was assayed as described by Heyworth et al. (1981).

Ins(1,3,4,5)P₄ 5-phosphatase and Ins(1,4,5)P₃ 5-phosphatase were assayed by measuring the rate of disappearance of substrate as follows: 2000 d.p.m. (approx. 3 nm) of either [³H]Ins(1,3,4,5)P₄ (prepared as described by Shears et al., 1987) or [³H]Ins(1,4,5)P₃ (Amersham International) was added to 0.5 ml of a medium containing 120 mM-KCl, 10 mM-NaCl, 1 mM-EGTA, 4.18 mM-MgSO₄ (4 mM free Mg²⁺), 0.33 mM-CaCl₂ (0.1 μM free Ca²⁺), 20 mM-Hepes (pH 7.2) and 0.2 mg of saponin/ml. Incubations (at 37°C) were initiated by adding 30 μl of tissue samples (prepared as described above). The 30% (w/v) homogenates, 100000 g pellets and washed pellets were diluted 5-fold immediately before use and were then found to hydrolyse Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ with first-order kinetics (i.e. at a final tissue concentration equivalent to 0.36 wt. of original liver/vol.). The first-order rate constants obtained from incubations containing 30 μl of the 100000 g supernatants (final concn. equivalent to 1.8% wt. of original liver/vol.) were divided by 5 to be comparable with the rate constants obtained from those incubations containing homogenates and washed pellets. In further experiments, 30 μl portions of the fractions from Percoll density gradients (see above) were assayed. At appropriate times, incubations were quenched with 0.2 ml of 1.7 M-HClO₄. Samples were neutralized and loaded on to 2 cm x 0.6 cm Bio-Rad AG1-XS (200-400 mesh) ion-exchange columns as previously described (Shears et al., 1987). Greater than 98% of InsP₃ and InsP₂ were eluted with 10 ml of 0.4 M-ammonium formate/0.1 M-formic acid [for the Ins(1,4,5)P₃ 5-phosphatase assays], or > 98% of InsP₃, InsP₂ and InsP₁ were eluted with 12 ml of 0.8 M-ammonium formate/0.1 M-formic acid [for the Ins(1,3,4,5)P₄ 5-phosphatase assays]. Usually, > 95% of either Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ was next eluted in 2.5 ml of 2.8 M-ammonium formate/0.1 M-formic acid, and radioactivity in this fraction was determined (Shears et al., 1987). However, one batch of resin required 4 ml of 2.8 M-ammonium formate/0.1 M-formic acid to elute > 95% of the Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄. Then 2 ml of this eluate was added to 0.5 ml of water, and radioactivity was measured as previously described (Shears et al., 1987).

Where indicated, the incubation medium for Ins(1,3,4,5)P₄ hydrolysis was modified, either to change free Ca²⁺ concentration or to ensure that certain additives did not alter the Ca²⁺ and Mg²⁺ concentrations. The modified and unmodified incubation media were prepared, as described by Shears et al. (1987), by using the following apparent association constants:

- Ca²⁺-EGTA = 6.898 x 10⁴;
- Mg²⁺-EGTA = 93.7;
- Ca²⁺-ATP = 4.964 x 10⁵;
- Mg²⁺-ATP = 1.648 x 10⁶ (Burgess et al., 1983);
- Ca²⁺-AMP = 67.6;
- Mg²⁺-AMP = 123 (Campbell, 1983).

The association constants of p[NH]ppA and ATP[S] for Mg²⁺ and Ca²⁺ were assumed not to be substantially different from those for ATP with Mg²⁺ and Ca²⁺ (see Flodgaard & Torp-Pederson, 1978).

Preparation of inositol phosphates

[³H]Ins(1,3,4,5)P₄, [4,5-³²P]Ins(1,3,4,5)P₄, [4,5-³²P]Ins(1,4,5)P₃, [4-³²P]Ins(1,4,5)P₃ and [4-³²P]Ins(1,4,5)P₃ were prepared as described by Shears et al. (1987). When the [³H]InsP₃ was analysed by h.p.l.c. (see below), it was noted that some preparations contained a small amount (< 2%) of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄. Both isomers of InsP₃ were routinely removed by re-chromatographing the preparation (Batty et al., 1985), and again freeze-drying the InsP₃ fraction (see Shears et al., 1987). [³H]Ins(1,4,5)P₃ was prepared as follows: A 30 μl portion of a 100000 g washed liver pellet (see below) was added to 0.04 μCi of [³H]Ins(1,3,4,5)P₄ in 0.5 ml of the medium used to assay Ins(1,3,4,5)P₄ 5-phosphatase activity (see above). The washed pellet contained nearly all the hepatic Ins(1,3,4,5)P₄ 5-phosphatase activity (see the Results...
Table 1. First-order rate constants for hydrolysis of \( \text{Ins}(1,3,4,5)P_4 \) and \( \text{Ins}(1,4,5)P_3 \) by rat liver homogenates and 100 000 g supernatants and pellets

Liver fractions were prepared by centrifugation of liver homogenates at 100 000 g (see the Materials and methods section). The first-order rate constants for inositol phosphate hydrolysis are derived from a 0.36% (wt. of original liver/vol.) final concentration of the appropriate fraction (see the Materials and methods section). Data are means ± S.E.M. from five preparations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>( 10^9 \times \text{First-order rate constant (s}^{-1}) ) for inositol phosphate hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.10 ± 0.1</td>
</tr>
<tr>
<td>Pellet</td>
<td>1.06 ± 0.12</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Washed pellet</td>
<td>0.94 ± 0.08</td>
</tr>
<tr>
<td>Supernatant from</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>washed pellet</td>
<td></td>
</tr>
</tbody>
</table>

section), but was separated from most of the \( \text{Ins}(1,3,4)P_3 \) phosphatase activity (Shears et al., 1987). After 20 min the reaction was quenched with 0.25 ml of 1.7 M-HClO₄, and the \( \text{Ins}(1,3,4)P_3 \) was purified by anion-exchange chromatography and freeze-dried (Shears et al., 1987). About 30% of the \( \text{Ins}(1,3,4,5)P_4 \) was recovered as \( \text{Ins}P_3 \), the structure of which was confirmed to be \( \text{Ins}(1,3,4)P_3 \) (see below).

Structural analysis of inositol phosphates

The \( \text{Ins}P_3 \) product of \( \text{Ins}(1,3,4,5)P_4 \) dephosphorylation (prepared as described above) was incubated with 0.1 M-periodate at neutral pH for 5 days in the dark at room temperature (Irvine et al., 1984). This treatment splits the inositol ring between two carbon atoms bearing vicinal hydroxyl groups. The samples were then reduced and dephosphorylated (Irvine et al., 1984), and the degradation products were separated by ionophoresis in 0.1 M-NaOH (Frahm & Mills, 1959) or paper chromatography (Grado & Ballou, 1961). Identification of the resulting polyol identified the isomeric configuration of the original \( \text{Ins}P_3 \).

Separation of inositol phosphates by h.p.l.c.

Samples, prepared as previously described (Shears et al., 1987), were loaded on a 25 cm x 0.46 cm main column plus a 5 cm x 0.46 cm guard column, both containing Whatman Partisil 10-SAX. Elution began with water for 5 min, and then followed the protocol of Batty et al. (1985), which utilizes gradients generated by mixing water and various proportions of 1.7 M-ammonium formate (pH 3.7 with \( \text{H}_3\text{PO}_4 \)).

Other methods

The densities of the fractions from Percoll gradients were determined as described by Heyworth et al. (1983).

Materials

\( ^{3}H\text{Ins}(1,4,5)P_3 \) and \( [U-^{14}C]\text{sucrose} \) were supplied by Amersham International, AMP, \text{pNHppA}, \text{ATP},

Fig. 1. H.p.l.c. analysis of \( ^{3}H\text{Ins}(1,3,4,5)P_4 \) metabolism by liver homogenate

Liver homogenates were incubated as described in the Materials and methods section, where details of the h.p.l.c. analyses are also given. Samples were taken at 0 (○) and 20 min (●). Immediately before h.p.l.c., the 20 min sample was ‘spiked’ with \( [4-^{32}P]\text{Ins}(1,4)P_3 \) and \( [4,5-^{32}P]\text{Ins}(1,4,5)P_3 \) (△), and these are shown on a different scale for clarity. Apart from some variation in the size of the \( ^{3}H \) peak that co-eluted with \( [4-^{32}P]\text{Ins}(1,4)P_3 \) (see the text), similar results were obtained in five additional experiments. ———, Gradient of 1.7 M-ammonium formate (adjusted to pH 3.7 with \( \text{H}_3\text{PO}_4 \)).
RESULTS

Kinetics of Ins\((1,3,4,5)P_4\) and Ins\((1,4,5)P_3\) metabolism by liver homogenate and its subfractions

Samples of liver homogenates were incubated with approx. 3 nm of either Ins\((1,3,4,5)P_4\) or Ins\((1,4,5)P_3\) in a medium with an ionic strength and pH similar to that of cell cytosol (see the Materials and methods section). The first-order rate constant for Ins\((1,3,4,5)P_4\) hydrolysis was one-third lower than that for Ins\((1,4,5)P_3\) hydrolysis. When assayed under first-order conditions, 9\% of total Ins\((1,3,4,5)P_4\) 5-phosphatase activity was located in 100000 g supernatants. The same supernatants retained a minor proportion (12\%) of Ins\((1,4,5)P_3\) 5-phosphatase activity, somewhat less than the value of 24\%, obtained in a previous report from this laboratory (Storey et al., 1984). Almost all of the Ins\((1,3,4,5)P_4\) and Ins\((1,4,5)P_3\) phosphatase activities in the initial 100000 g pellet remained membrane-bound when this fraction was resuspended and re-centrifuged (Table 1).

The \(^3\)H-labelled products of Ins\((1,3,4,5)P_4\) hydrolysis were assayed by h.p.l.c. A single \(^3\)H-labelled peak was eluted 2 min before an Ins\((1,4,5)P_3\) standard, and with the expected mobility of Ins\((1,3,4)P_4\) (Fig. 1). These data confirm those reported by Hansen et al. (1986). However, they do not unequivocally identify Ins\((1,3,4)P_3\) as the only product of Ins\((1,3,4,5)P_4\) metabolism, since Ins\((3,4,5)P_3\), another potential product, would probably be eluted close to Ins\((1,3,4)P_3\) in our h.p.l.c. system (Irvine et al., 1986b). The Ins\(P_3\) product of Ins\((1,3,4,5)P_4\) hydrolysis was analysed by periodate oxidation, followed by reduction and dephosphorylation (see the Materials and methods section). Only one radioactive polyol was detected (92\% recovery), and this was identified as altitol. These experiments confirm, by a different technique, our previous determination of the Ins\(P_3\) structure as Ins\((1,3,4)P_3\) (see Shears et al., 1987). Another \(^3\)H-labelled peak that eluted 1.2 min after an Ins\((1,4)P_3\) standard (Fig. 1) was presumably Ins\((3,4)P_3\), which we previously identified as the major product of Ins\((1,3,4)P_3\) dephosphorylation (Kirk et al., 1987; Shears et al., 1987). A second minor Ins\(P_3\) peak was sometimes observed; this was presumably Ins\((1,4)P_3\) and/or Ins\((1,3)P_3\) (see Irvine et al., 1987). However, the small size of this peak precluded its further identification. In one experiment (Fig. 1) this peak accounted for 7.5\% of total Ins\(P_3\) but in other experiments the proportion varied from 0 to 6\% (n = 6) (see also Hansen et al., 1986; Shears et al., 1987).

![Fig. 2. Distribution of Ins\((1,3,4,5)P_4\) 5-phosphatase activity and various marker enzymes after density-gradient fractionation of hepatocytes](image-url)

Hepatocytes were fractionated on Percoll density gradients as described in the Materials and methods section. The following marker enzymes were assayed, and their recoveries from the original homogenate (%) are shown in parentheses: △, β-galactosidase (marker for lysosomes; 96\%); □, glutamate dehydrogenase (mitochondria; 96\%); ■, arylersterase (endoplasmic reticulum; 97\%); ●, galactosyltransferase (Golgi; 85\%); ○, alkaline phosphodiesterase 1 (plasma membranes; 92\%); △, [U-\(^14\)C]sucrose (0.2 μCi) added in a separate tube to the homogenate immediately before centrifugation, used to monitor the distribution of cytosol (98\%). ●, Ins\((1,3,4,5)P_4\) 5-phosphatase activity (99\%). Results are expressed as percentage of recovered activity/fraction and are from a single experiment, typical of two. Where percentage recovery/fraction was less than 1\%, results are omitted for clarity, except for the Ins\((1,3,4,5)P_4\) 5-phosphatase activity.
Table 2. Effects of [Ca\textsuperscript{2+}], cyclic AMP, GTP and its analogues on Ins(1,3,4,5)P\textsubscript{4} hydrolysis by liver homogenates

<table>
<thead>
<tr>
<th>Condition</th>
<th>First-order rate constant (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.1 \mu M-Ca\textsuperscript{2+})</td>
<td>1.76 ± 0.12 (8)</td>
</tr>
<tr>
<td>1 \mu M-Ca\textsuperscript{2+}</td>
<td>1.64 ± 0.16 (4)</td>
</tr>
<tr>
<td>0.1 mM-GTP</td>
<td>1.64 ± 0.16 (4)</td>
</tr>
<tr>
<td>0.1 mM-GTP[S]</td>
<td>1.88 ± 0.16 (5)</td>
</tr>
<tr>
<td>0.1 mM-p[NH]ppG</td>
<td>2.08 ± 0.32 (3)</td>
</tr>
<tr>
<td>0.1 mM-cyclic AMP + 5 mM-ATP</td>
<td>0.81 ± 0.11 (6)</td>
</tr>
<tr>
<td>5.0 mM-ATP</td>
<td>0.83 ± 0.18 (6)</td>
</tr>
</tbody>
</table>

Subcellular distribution of Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase

When liver homogenates were fractionated by centrifugation at 100000 g, approx. 90% of total Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity sedimented with the mixed particulate fraction (Table 1). The subcellular distribution of the membrane-associated Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity was studied by fractionating nuclei-free homogenates on Percoll density gradients (Heyworth et al., 1983; Storey et al., 1984). Results not shown indicated that less than 5% of total liver Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity was present in nuclei, prepared as described by Widnell & Tata (1964). Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity equilibrated as a single peak in the Percoll gradients, at a density of 1.035 g/ml, together with a marker enzyme for plasma membranes. The distribution of Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity was quite different from the distribution of markers for cytosol, Golgi apparatus, endoplasmic reticulum, lysosomes and mitochondria (Fig. 2).

Influence of potential regulators of Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity

In liver homogenates, both Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity (Table 2) and the nature of the ensuing products (results not shown) were unchanged when [Ca\textsuperscript{2+}] was increased from 0.1 to 1 \mu M. It is therefore unlikely that hormones regulate hepatic Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity in liver by changing cytosol [Ca\textsuperscript{2+}]. GTP and two GTP analogues (p[NH]ppG and GTP[S]), when added at a concentration of 0.1 mM, did not affect the rate of Ins(1,3,4,5)P\textsubscript{4} metabolism (Table 2). Thus the 5-phosphatase is unlikely to be regulated by GTP-binding proteins. The addition of 0.1 mM-cyclic AMP, in the presence of ATP, did not change Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity compared with incubations to which ATP alone was added (Table 2).

Inhibitors of Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase compared with those of Ins(1,4,5)P\textsubscript{3} 5-phosphatase

ATP (0.1 mM) inhibited Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity by about 12%, provided that an ATP-regenerating system was present (Fig. 3). The Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity was not further inhibited unless [ATP] > 3 mM; at 5 mM-ATP (the approximate physiological concentration in rat liver cytosol; Soboll et al., 1978), the Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity was decreased by 37% (Fig. 3). The addition of 5 mM-p[NH]ppA also inhibited Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity (Table 3). Since p[NH]ppA is an ATP analogue that cannot act as a phosphate donor for kinase-mediated enzyme regulation, this suggests that the inhibition of Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity by ATP is not due to phosphorylation/dephosphorylation of the enzyme. ATP[S] inhibited the Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity more efficiently than did either ATP or p[NH]ppA (Table 3).

In these experiments the proportion of Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity inhibited by 5 mM-ATP was similar to that inhibited by 5 mM-AMP; PP\textsubscript{i} inhibited the enzyme more potently (Table 3). Adenosine and P\textsubscript{i} did not significantly affect Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity (Table 3). The effects of ATP, AMP, ATP[S] and p[NH]ppA were not due to changes in the free Mg\textsuperscript{2+} or Ca\textsuperscript{2+} concentrations in the medium, which were held constant (see the Materials and methods section). 2,3-Bisphosphoglycerate, which inhibited Ins(1,3,4,5)P\textsubscript{4} 5-phosphatase activity in brain (Irvine et al., 1986a), also inhibited the enzyme in liver homogenates (Table 3);
Table 3. Effect of ATP, ATP analogues and other compounds on Ins(1,3,4,5)P$_4$ and Ins(1,4,5)P$_3$ 5-phosphatase activities

Liver homogenates [for measuring Ins(1,3,4,5)P$_4$ hydrolysis] and 100000 g washed pellets [Ins(1,4,5)P$_3$ hydrolysis] were prepared as described in the Materials and methods section. The 5-phosphatase activity was measured in 5 min incubations. Additions to the incubation medium are as described in the Table: n.d., not determined. Where 50 mM-Li$^+$ (as LiCl) was added, 50 mM-KCl was removed to maintain a constant ionic strength. Data are means ± s.e.m., with the numbers of experiments in parentheses: *P < 0.02 compared with controls.

<table>
<thead>
<tr>
<th>Substrate metabolized (%)</th>
<th>Ins(1,3,4,5)P$_4$</th>
<th>Ins(1,4,5)P$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42 ± 2 (12)</td>
<td>54 ± 3 (9)</td>
</tr>
<tr>
<td>+ 5 mM-ATP</td>
<td>22 ± 1 (12)*</td>
<td>37 ± 5 (3)*</td>
</tr>
<tr>
<td>+ 5 mM-p[NH]ppA</td>
<td>27 ± 3 (6)*</td>
<td>36 ± 5 (5)*</td>
</tr>
<tr>
<td>+ 5 mM-ATP[S]</td>
<td>12 ± 2 (3)*</td>
<td>23 ± 7 (3)*</td>
</tr>
<tr>
<td>+ 5 mM-AMP</td>
<td>24 ± 2 (5)*</td>
<td>38 ± 5 (5)*</td>
</tr>
<tr>
<td>+ 5 mM-PP$_1$</td>
<td>9 ± 3 (5)*</td>
<td>18 ± 4 (5)*</td>
</tr>
<tr>
<td>+ 5 mM-P$_1$</td>
<td>41 ± 3 (6)</td>
<td>54 ± 4 (4)</td>
</tr>
<tr>
<td>+ 5 mM-adenosine</td>
<td>40 ± 3 (5)</td>
<td>51 ± 7 (6)</td>
</tr>
<tr>
<td>+ 5 mM-2,3-bis-phosphoglycerate</td>
<td>9 ± 2 (4)*</td>
<td>n.d.</td>
</tr>
<tr>
<td>+ 50 mM-Li$^+$</td>
<td>42 ± 2 (4)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 4. Effect of ATP on Ins(1,3,4)P$_3$ metabolism

Liver homogenates were incubated at a final concentration of 1.8% (wt. of original liver/vol.) in 0.5 ml of the incubation medium described in the Materials and methods section. ATP (5 mM) was added as indicated in the Table. The incubations also contained [3H]Ins(1,3,4)P$_3$, which was prepared as described in the Materials and methods section. At the designated incubation time, samples were quenched with 0.2 ml of 1.7 m-HClO$_4$, and inositol phosphates were separated on Bio-Rad anion-exchange columns as previously described (Shears et al., 1987). After 10 min, the radioactivity in the InsP$_3$ and InsP$_4$ fractions, plus that in the Ins, InsP and InsP$_2$ fractions, equalled the radioactivity added at zero time (results not shown). Data are means ± s.e.m. from triplicate determinations in one experiment representative of four.

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>[ATP]</th>
<th>$^3$H (d.p.m./fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>InsP$_3$</td>
</tr>
<tr>
<td>0</td>
<td>5 mM</td>
<td>952 ± 81</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>330 ± 20</td>
</tr>
<tr>
<td>10</td>
<td>5 mM</td>
<td>418 ± 36</td>
</tr>
</tbody>
</table>

50 mM-Li$^+$ did not alter Ins(1,3,4,5)P$_4$ 5-phosphatase activity (Table 3, and Hansen et al., 1986).

We have also compared these effects of ATP and other phosphate esters with their effects on the 5-phosphatase activity that attacks Ins(1,4,5)P$_3$. We did not perform these experiments with liver homogenates, since they contain a cytosolic ATP-dependent Ins(1,4,5)P$_3$ kinase activity (Hansen et al., 1986; Irvine et al., 1986a; Kirk et al., 1987), which would have complicated our assays. Thus a 100000 g washed pellet was prepared from liver (see the Materials and methods section), which contained about 80% of the total Ins(1,4,5)P$_3$ 5-phosphatase activity present in liver homogenates (Table 1). These washed pellets converted < 0.2% of added Ins(1,4,5)P$_3$ to Ins(1,3,4,5)P$_4$ during our incubations and no Ins(1,3,4,5)P$_4$ was formed (results not shown). Ins(1,4,5)P$_3$ 5-phosphatase activity in the washed particulate fraction was inhibited by about one-third by 5 mM-ATP, -AMP or -p[NH]ppA; ATP[S] was again a more potent inhibitor. Neither 5 mM-adenosine nor 5 mM-LiCl, affected Ins(1,4,5)P$_3$ 5-phosphatase activity (Table 3). In the presence of 5 mM-ATP, the addition of physiological concentrations of either AMP (0.4 mM; see Soboll et al., 1978) or PP$_1$ (0.04 mM; see Guynn et al., 1974) did not further affect either Ins(1,4,5)P$_3$ or Ins(1,3,4,5)P$_4$ 5-phosphatase activities (results not shown).

Ins(1,3,4)P$_3$ kinase in liver homogenates

We considered the possibility that some of the apparent inhibition of Ins(1,3,4,5)P$_4$ 5-phosphatase by ATP (Fig. 3, Table 3) might have been caused by a phosphorylation of some of the Ins(1,3,4)P$_4$ produced in these experiments. We therefore studied the influence of ATP on Ins(1,3,4)P$_4$ metabolism. In 10 min incubations with liver homogenates, the addition of 5 mM-ATP halved the amount of Ins(1,3,4)P$_4$ that was dephosphorylated, indicating that ATP inhibits Ins(1,3,4)P$_4$ 5-phosphatase. In these experiments, ATP also promoted the conversion of 25% of the added Ins(1,3,4)P$_4$ into a more ionized compound that was eluted from Dowex columns in an "InsP$_3$" fraction (Table 4). These incubations were performed for twice the time, and contained 5 times the homogenate concentration, as compared with the incubation conditions used to study Ins(1,3,4,5)P$_4$ hydrolysis (cf. Tables 3 and 4). Thus these data do not affect the conclusion that ATP inhibits Ins(1,3,4,5)P$_4$ hydrolysis. When the product of the putative Ins(1,3,4)P$_4$ kinase was analysed by h.p.l.c., it was eluted 0.4 min after a [4,5-32P]Ins(1,3,4,5)P$_4$ internal standard (Fig. 4). It therefore appears that Ins(1,3,4)P$_4$ is phosphorylated to an InsP$_4$ that is not Ins(1,3,4,5)P$_4$. However, there was not a baseline separation of Ins(1,3,4,5)P$_4$ standard from the novel InsP$_4$ so it is not excluded the possibility that some Ins(1,3,4,5)P$_4$ was also a phosphorylation product of Ins(1,3,4)P$_4$.

The discovery of a novel isomer of InsP$_4$ led us to consider whether it might have contaminated our original preparations of [3H]Ins(1,3,4,5)P$_4$. This was unlikely, because the Ins(1,3,4,5)P$_4$ was produced under the conditions used by Shears et al. (1987), in which very little Ins(1,3,4,5)P$_4$ would have formed; Ins(1,3,4,5)P$_4$ was produced from Ins(1,4,5)P$_3$ by using a 100000 g liver supernatant that contained little Ins(1,3,4,5)P$_4$ 5-phosphatase, and 2,3-bisphosphoglycerate was included to inhibit Ins(1,3,4)P$_3$ production from Ins(1,3,4,5)P$_4$ (Table 3). Although our Ins(1,3,4,5)P$_4$ preparations were...
Fig. 4. H.p.l.c. analysis of the products of Ins(1,3,4,5)P₄ metabolism in the presence of ATP

Liver homogenates (1.8%, w/v) were incubated, for 0 (a) and 10 min (b), with [³H]Ins(1,3,4)P₃ in 0.5 ml of the medium described in the Materials and methods section, to which 5 mM-ATP was added. Immediately before h.p.l.c. the 0 min sample was ‘spiked’ with [⁴,⁵-³²P]Ins(1,4,5)P₃ and the 10 min sample was ‘spiked’ with [⁴,⁵-³²P]Ins(1,4,5)P₃. The radioactivity was assessed by liquid-scintillation spectrometry. Results are from a single experiment, typical of three.

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**Table 5. ATP-dependent phosphorylation of [³H]Ins(1,4,5)P₃ and [⁴,⁵-³²P]Ins(1,4,5)P₃**

Samples (30 μl) of 30% (wt. of original liver/vol.) 100000 g liver supernatants were incubated in 0.5 ml of medium consisting of 0.25 mM-sucrose, 50 mM-Tris (pH 7.5, 20 mM-MgCl₂, 10 mM-ATP, 25 mM-2,3-bisphosphoglycerate (Na⁺ salt), 0.2 mg of saponin/ml plus [³H]Ins(1,4,5)P₃ and [⁴,⁵-³²P]Ins(1,4,5)P₃ as described in the Table. After 45 min at 37 °C, incubations were quenched with 0.2 ml of 1.7 M-HClO₄, and inositol phosphates were separated on Bio-Rad anion-exchange columns as previously described (Shears et al., 1987). Data are means ± S.E.M. from triplicate incubations in one experiment, representative of three.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Time (min)</th>
<th>Radioactivity (d.p.m./fraction)</th>
<th>³H</th>
<th>³²P</th>
<th>³²P/³H</th>
</tr>
</thead>
<tbody>
<tr>
<td>InsP₄</td>
<td>0</td>
<td>2837 ± 14</td>
<td>591 ± 6</td>
<td>0.21 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>InsP₄</td>
<td>0</td>
<td>16 ± 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>InsP₄</td>
<td>45</td>
<td>512 ± 18</td>
<td>96 ± 10</td>
<td>0.19 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>InsP₄</td>
<td>45</td>
<td>1232 ± 33</td>
<td>243 ± 9</td>
<td>0.20 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

eluted as a single peak during h.p.l.c. (Fig. 1), in such experiments a small proportion of the novel InsP₄ isomer could have been masked by a larger proportion of Ins(1,3,4,5)P₄. The purity of the Ins(1,3,4,5)P₄ was therefore assessed by a different method. [³H]Ins(1,4,5)P₃ and [⁴,⁵-³²P]Ins(1,4,5)P₃ were simultaneously phosphorylated under exactly these conditions normally used to prepare [³H]Ins(1,3,4,5)P₄ [see Shears et al. (1987) and Table 5]. The ³²P in the [⁴,⁵-³²P]Ins(1,4,5)P₃ precursor was distributed between the 5- and 4-phosphate groups in the ratio 63:37 (see Downes et al., 1982; Shears et al., 1987). Any accumulation of Ins(1,3,4)P₃, followed by its phosphorylation by non-radioactive ATP, would have decreased the ³²P/³H ratio of the InsP₄ and InsP₃ fractions obtained in these experiments. However, no such decrease was observed (Table 5), confirming the isotopic homogeneity of our Ins(1,3,4,5)P₄ preparations.

**Characterization of the InsP₄ produced by phosphorylation of Ins(1,3,4,5)P₃**

Aliquots (approx. 1000 d.p.m.) of three preparations of the [³H]InsP₄ produced by phosphorylation of [³H]Ins(1,3,4)P₃ were each incubated for 1 h with 0.3 ml of human erythrocyte ghosts (prepared as described by Downes et al., 1982) plus 0.5 ml of 4 mM-magnesium acetate/20 mM-Tris/HCl (pH 7.3), containing 0.2 mg of saponin/ml and 1000 d.p.m. of [⁴,⁵-³²P]Ins(1,4,5)P₄. Under these incubation conditions, 78 ± 5% of the Ins(1,3,4,5)P₃ was hydrolysed by the 5-phosphatase activity in the ghosts (see Downes et al., 1982). However, 98 ± 0.8% of the original [³H] label was recovered as InsP₄. Thus the [³H]InsP₄ contained no Ins(1,3,4,5)P₄ and, assuming that no phosphate migration occurred during the phosphorylation of Ins(1,3,4,5)P₃, the novel InsP₄ must be Ins(1,2,3,4)P₄ or Ins(1,3,4,6)P₄, or a mixture of both of these isomers. Ins(1,2,3,4)P₄ possesses vicinal hydroxyl groups and should be sensitive to periodate; Ins(1,3,4,6)P₄ should be periodate-insensitive. However, past experience suggests that periodate oxidation of InsP₄ isomers, especially those with pairs of trans-hydroxyl groups, occurs only under stronger oxidizing conditions than are needed for oxidation of periodate-
sensitive Ins$_{P4}$ isomers (Tomlinson & Ballou, 1962; Lim & Tate, 1973). Thus the novel [PH]Ins$_{P4}$ was incubated at room temperature for 4 days at pH 2.0 in the dark with 0.1 m-periodate (Tomlinson & Ballou, 1962). Reduction and dephosphorylation was then performed as described in the Materials and methods section, except that 0.4 mg of alkaline phosphatase/ml was used. On analysis of the resultant polyols (see the Materials and methods section) there was no detectable altritol, which would have been the expected product of Ins(1,2,3,4)$_{P4}$ degradation. The only labelled polyol detected was inositol (70% recovery). These results suggest that most of the Ins$_{P4}$ that is formed from Ins(1,3,4)$_{P4}$ is Ins(1,3,4,6)$_{P4}$. However, a definitive assignment of its structure must await further experiments.

**DISCUSSION**

In liver, the membrane-bound Ins(1,3,4,5)$_{P4}$ and Ins(1,4,5)$_{P4}$ 5-phosphatase activities have several similarities. The major proportion of each enzyme activity is associated with plasma membranes. A small fraction of each activity is cytosolic, but little or no activity was detected in mitochondria, lysosomes, endoplasmic reticulum, nuclei or Golgi apparatus [compare Fig. 2 with data in Storey et al. (1984) and Shears et al. (1987), and see also Seyfried et al. (1984) and Joseph & Williams (1985)]. Both substrates are hydrolysed with similar first-order rate constants by particulate fractions (Table 1). Moreover, both membrane-associated 5-phosphatase activities are insensitive to 50 mM-Li$^+$, but are substantially inhibited by 2,3-bisphosphoglycerate [compare Table 3 with results of Joseph & Williams (1985)]. Both enzyme activities are inhibited by ATP, AMP, PP$_i$, p[NH]ppA and ATP[S] (Table 3). Neither enzyme activity is affected by GTP or GTP analogues, by cyclic AMP or by increasing the free Ca$^{2+}$ concentration from 0.1 to 1 $\mu$M [compare Table 2 with results of Shears et al. (1987)]. Taken together these similarities strongly suggest that, in liver, a single Ins(1,4,5)$_{P4}$ and Ins(1,3,4,5)$_{P4}$ 5-phosphatase may catalyse both membrane-associated activities. Platelet cytosol contains an enzyme which, when purified, hydrolyses both Ins(1,4,5)$_{P4}$ and Ins(1,3,4,5)$_{P4}$; each substrate competes with the other, again suggesting that both 5-phosphatase activities may be catalysed by the same enzyme (Connolly et al., 1985, 1987). Erythrocyte plasma membranes also contain 5-phosphatase activities against both Ins(1,4,5)$_{P4}$ and Ins(1,3,4,5)$_{P4}$ (Downes et al., 1982; Batty et al., 1985; Irvine et al., 1986a,b).

An in vivo physiological role of these phosphatases remains to be established. Ins(1,4,5)$_{P4}$ and Ins(1,3,4,5)$_{P4}$ 5-phosphatase activities by 40–50% (Fig. 3 and Table 3). Thus the lifetime of Ins(1,3,4,5)$_{P4}$ and Ins(1,4,5)$_{P4}$ in cells will be extended by the presence of ATP. In certain extreme nutritional states, the cytosolic ATP of rat liver may vary within the range 3–7 mM (Soboll et al., 1978). Even under conditions this extreme, there would be little effect on the activity of Ins(1,4,5)$_{P4}$/Ins(1,3,4,5)$_{P4}$ 5-phosphatase.

Best (1986) has shown that 10 $\mu$M-Ca$^{2+}$ activates polyphosphoinositol hydrolysis in permeabilized pancreatic islets. The subsequent accumulation of inositol phosphates, including Ins(1,4,5)$_{P4}$ [and presumably also Ins(1,3,4,5)$_{P4}$, although this was not measured], was enhanced several-fold by 5 mM-ATP. Best (1986) suggested that ATP sustained the supply of PtdIns(4,5)$_{P4}$ from phosphatidylinositol, thereby promoting the production of inositol phosphates. Our data (Table 3) indicate that ATP may also have promoted the accumulation of inositol phosphate(s) in these experiments by inhibiting the dephosphorylation of Ins(1,4,5)$_{P4}$ and Ins(1,3,4,5)$_{P4}$ to free inositol.

Our discovery that Ins(1,3,4)$_{P4}$ can be phosphorylated to a novel Ins$_{P4}$, which is probably Ins(1,3,4,6)$_{P4}$, has revealed yet another aspect of inositol polyphosphate metabolism in mammalian cells. H.p.l.c. analyses of [PH]Ins$_{P4}$, isolated from several [PH]inositol-labelled and stimulated tissues, have not previously indicated that more than one isomer was present (Batty et al., 1985; Downes et al., 1986). However, in these short-term experiments, there was no evidence that dephosphorylation of Ins(1,3,4,5)$_{P4}$ to Ins(1,3,4)$_{P4}$ was therefore there may not have been sufficient Ins(1,3,4)$_{P4}$ to support substantial synthesis of the novel Ins$_{P4}$. We have yet to make a direct search for the novel Ins$_{P4}$ in stimulated hepatocytes.

The metabolic fate of the novel Ins$_{P4}$ isomer is not known. An intriguing possibility is that the Ins$_{P4}$ might be a precursor for Ins$_{P4}$ and Ins$_{P4}$ (see Heslop et al., 1985). However, there was no evidence for this in our experiments, in which radioactivity lost from Ins(1,3,4)$_{P4}$ was quantitatively recovered as either Ins$_{P4}$ or Ins$_{P4}$ (Fig. 4). Nevertheless, the ability of liver tissue to re-arrange the isomeric configuration of Ins(1,3,4,5)$_{P4}$ involves two enzymes and represents a cost to cellular energy stores. Presumably the novel Ins$_{P4}$, or some metabolite derived from it, has an intracellular function which remains to be uncovered.

**Note added in proof (received 19 June 1987)**

Since this paper was accepted for publication, we have become aware that Ins(1,3,4)$_{P4}$ can also be phosphorylated to an Ins$_{P4}$ in bovine adrenal glomerulosa cells [T. Balla, G. Guillemette, A. Baukal & K. J. Catt (1987) J. Biol. Chem., in the press]. These workers also propose that their Ins$_{P4}$ is the (1,3,4,6) isomer.

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**REFERENCES**


Metabolism of inositol tetrakisphosphate


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