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

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1. We have studied the metabolism of Ins(1,3,4,5)P_4 (inositol 1,3,4,5-tetrakisphosphate) by rat liver homogenates incubated in a medium resembling intracellular ionic strength and pH. 2. Ins(1,3,4,5)P_4 was dephosphorylated to a single inositol trisphosphate product, Ins(1,3,4)P_3 (inositol 1,3,4-trisphosphate), the identity of which was confirmed by periodate degradation, followed by reduction and dephosphorylation to yield altritol. 3. The major InsP_2 (inositol bisphosphate) product was inositol 3,4-bisphosphate [Shears, Storey, Morris, Cubitt, Parry, Michell & Kirk (1987) Biochem. J. **242**, 393–402]. Small quantities of a second InsP_2 product was also detected in some experiments, but its isomeric configuration was not identified. 4. The Ins(1,3,4,5)P_4 5-phosphatase activity was primarily associated with plasma membranes. 5. ATP (5 mM) decreased the membrane-associated Ins(1,4,5)P_3 5-phosphatase and Ins(1,3,4,5)P_4 5-phosphatase activities by 40–50%. This inhibition was imitated by AMP, adenosine 5′-[$\beta\gamma$-imido]triphosphate, adenosine 5′-[γ-thio]triphosphate or PP_i, but not by adenosine or P_i. A decrease in [ATP] from 7 to 3 mM halved the inhibition of Ins(1,3,4,5)P_4 5-phosphatase activity, but the extent of inhibition was not further decreased unless [ATP] < 0.1 mM. 6. Ins(1,3,4,5)P_4 5-phosphatase was insensitive to 50 mM-Li^+, but was inhibited by 5 mM-2,3-bisphosphoglycerate. 7. The Ins(1,3,4,5)P_4 5-phosphatase activity was unchanged by cyclic AMP, GTP, guanosine 5′-[$\beta\gamma$-imido]triphosphate or guanosine 5′-[γ-thio]triphosphate, or by increasing [Ca^{2+}] from 0.1 to 1 μM. 8. Ins(1,3,4)P_3 was phosphorylated in an ATP-dependent manner to an isomer of InsP_4 that was partially separable on h.p.l.c. from Ins(1,3,4,5)P_4. The novel InsP_4 appears to be Ins(1,3,4,6)P_4. Its metabolic fate and function are not known.

INTRODUCTION

In a variety of cells, receptor-mediated hydrolysis of PtdIns(4,5)P_2 leads to the production of Ins(1,4,5)P_3 (reviewed by Berridge, 1984; Downes & Michell, 1985), an intracellular signal that releases Ca^{2+} from endoplasmic reticulum (reviewed by Berridge & Irvine, 1984). Ins(1,4,5)P_3 is phosphorylated by an ATP-dependent kinase to produce Ins(1,3,4,5)P_4 (Irvine *et al.*, 1986*a*; Hansen *et al.*, 1986; Hawkins *et al.*, 1986). Ins(1,3,4,5)P_4 can contribute to the activation of sea-urchin eggs, probably by facilitating Ca^{2+} influx across the plasma membrane (Irvine & Moor, 1986).

Several tissues contain enzyme activities which remove the 5-phosphate from both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, 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.*, 1986*a*,*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)P_4 and Ins(1,4,5)P_3 by liver homogenates incubated in media resembling cytosolic conditions. We have also studied the subcellular location of the liver Ins(1,3,4,5)P_4 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)P_3 that accumulates after Ins(1,3,4,5)P_4 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-Tes, 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-Tes, pH 7.2. The 100000 *g* pellets and supernatants (diluted to 30% wt. of original

Abbreviations used: Ins, Ins$P$, InsP_2, Ins(1,4)P_2, Ins(3,4)P_2, InsP_3, Ins(1,3,4)P_3, Ins(1,4,5)P_3, InsP_4, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and Ins(1,2,3,4)P_4 are *myo*-inositol and its mono-, bis-, tris- and tetrakisphosphate derivative (locants designated where appropriate, and known or assumed to be D-enantiomers); PtdIns(4,5)P_4, phosphatidylinositol 4,5-bisphosphate; p[NH]ppA, adenosine [$\beta\gamma$-imido]triphosphate; p[NH]ppG, guanosine [$\beta\gamma$-imido]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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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. The 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 N_2 for 10 min to 200 kPa (30 lbf/in^2). 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 Shephard & Hubscher (1969). Galactosyltransferase (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)P_4 5-phosphatase and Ins(1,4,5)P_3 5-phosphatase were assayed by measuring the rate of disappearance of substrate as follows: 2000 d.p.m. (approx. 3 nM) of either [^{3}H]Ins(1,3,4,5)P_4 (prepared as described by Shears *et al.*, 1987) or [^{3}H]Ins(1,4,5)P_3 (Amersham International) was added to 0.5 ml of a medium containing 120 mM-KCl, 10 mM-NaCl, 1 mM-EGTA, 4.18 mM-$MgSO_4$ (4 mM free Mg^{2+}), 0.33 mM-$CaCl_2$ (0.1 μM free Ca^{2+}), 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)P_3 and Ins(1,3,4,5)P_4 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-$HClO_4$. Samples were neutralized and loaded on to 2 cm × 0.6 cm Bio-Rad AG1-X8 (200–400 mesh) ion-exchange columns as previously described (Shears *et al.*, 1987). Greater than 98% of Ins$P$ and InsP_2 were eluted with 10 ml of 0.4 M-ammonium formate/0.1 M-formic acid [for the Ins(1,4,5)P_3 5-phosphatase assays], or > 98% of Ins$P$, InsP_2 and InsP_3 were eluted with 12 ml of 0.8 M-ammonium formate/0.1 M-formic acid [for the Ins(1,3,4,5)P_4 5-phosphatase assays]. Usually, > 95% of either Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 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)P_3 and Ins(1,3,4,5P_4. 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)P_4 hydrolysis was modified, either to change free Ca^{2+} concentration or to ensure that certain additives did not alter the Ca^{2+} and Mg^{2+} concentrations. The modified and unmodified incubation media were prepared, as described by Shears *et al.* (1987), by using the following apparent association constants: Ca^{2+}–EGTA = 6.898×10^6; Mg^{2+}–EGTA = 93.7; Ca^{2+}–ATP = 4.964×10^3; Mg^2–ATP = 1.648×10^4 (Burgess *et al.*, 1983); Ca^{2+}–AMP = 67.6; Mg^{2+}–AMP = 123 (Campbell, 1983). The association constants of p[NH]ppA and ATP[S] for Mg^{2+} and Ca^{2+} were assumed not to be substantially different from those for ATP with Mg^{2+} and Ca^{2+} (see Flodgaard & Torp-Pederson, 1978).

Preparation of inositol phosphates

[^{3}H]Ins(1,3,4,5)P_4, [4,5-^{32}P]Ins(1,3,4,5)P_4, [4,5-^{32}P]-Ins(1,4,5)P_3 and [4-^{32}P]Ins(1,4)P_2 were prepared as described by Shears *et al.* (1987). When the [^{3}H]InsP_4 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)P_3 and Ins(1,3,4)P_3. Both isomers of InsP_3 were routinely removed by re-chromatographing the preparation (Batty *et al.*, 1985), and again freeze-drying the InsP_4 fraction (see Shears *et al.*, 1987). [^{3}H]Ins(1,3,4)P_3 was prepared as follows: A 30 μl portion of a 100000 *g* washed liver pellet (see above) was added to 0.04 μCi of [^{3}H]Ins(1,3,4,5)P_4 in 0.5 ml of the medium used to assay Ins(1,3,4,5)P_4 5-phosphatase activity (see above). The washed pellet contained nearly all the hepatic Ins(1,3,4,5)P_4 5-phosphatase activity (see the Results

Table 1. First-order rate constants for hydrolysis of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 by rat liver homogenates and 100000 *g* supernatants and pellets

Liver fractions were prepared by centrifugation of liver homogenates at 100000 *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.

Preparation	10^3 × First-order rate constant (s^{-1}) for inositol phosphate hydrolysis	
	Ins(1,3,4,5)P_4	Ins(1,4,5)P_3
Homogenate	1.10±0.1	1.66±0.14
Pellet	1.06±0.12	1.32±0.12
Supernatant	0.10±0.02	0.20±0.02
Washed pellet	0.94±0.08	1.30±0.08
Supernatant from washed pellet	0.04±0.02	0.04±0.02

section), but was separated from most of the 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-$HClO_4$, and the Ins(1,3,4)P_3 was purified by anion-exchange chromatography and freeze-dried (Shears *et al.*, 1987). About 30% of the Ins(1,3,4,5)P_4 was recovered as InsP_3 the structure of which was confirmed to be Ins(1,3,4)P_3 (see below).

Structural analysis of inositol phosphates

The InsP_3 product of 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 (Frahn & Mills, 1959) or paper chromatography (Grado & Ballou, 1961). Identification of the resulting polyol identified the isomeric configuration of the original InsP_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 × 0.46 cm main column plus a 5 cm × 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 H_3PO_4).

Other methods

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

Materials

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

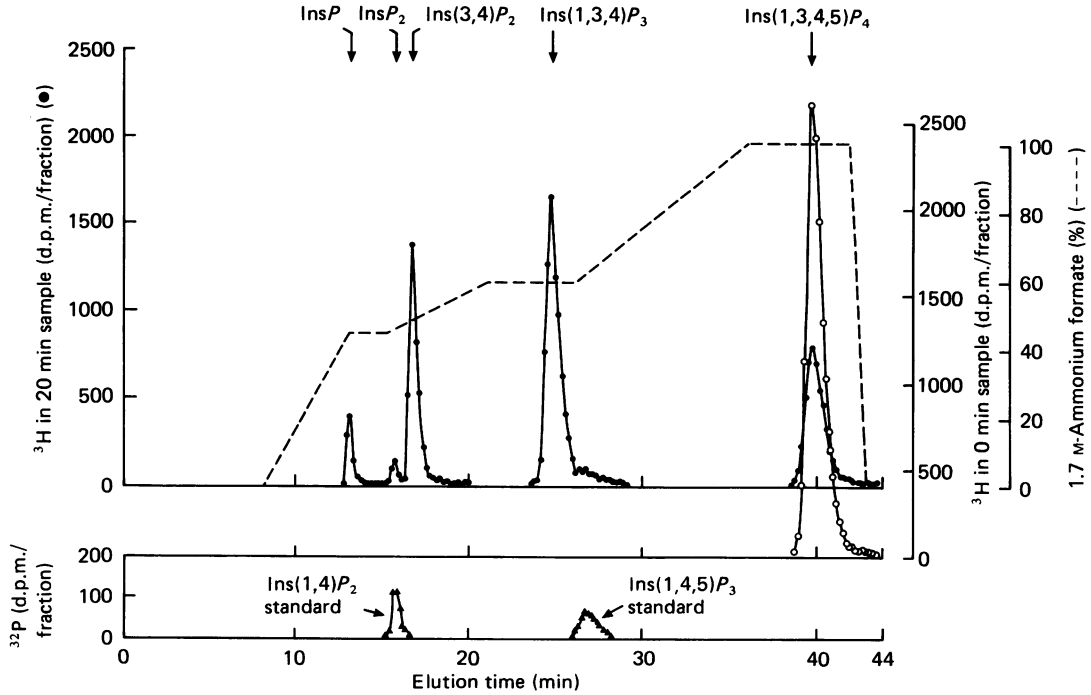

Fig. 1. H.p.l.c. analysis of [^{3}H]Ins(1,3,4,5)P_4 metabolism by liver homogenate

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]Ins(1,4)P_2 and [4,5-^{32}P]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]Ins(1,4)P_2 (see the text), similar results were obtained in five additional experiments. ----, Gradient of 1.7 M-ammonium formate (adjusted to pH 3.7 with H_3PO_4).

p[NH]ppG, GTP and Percoll were purchased from Sigma. ATP[S] was from Boehringer. Sources of other materials were as described by Shears *et al.* (1987).

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 analysed 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_3 (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.*, 1986*b*). The InsP_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 altritol. These experiments confirm, by a different technique, our previous determination of the InsP_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_2 standard (Fig. 1) was presumably Ins(3,4)P_2, 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 InsP_2 peak was sometimes observed; this was presumably Ins(1,4)P_2 and/or Ins(1,3)P_2 (see Irvine *et al.*, 1987). However, the small size of this peak precluded its further identification. In one experiment (Fig. 1) this isomer accounted for 7.5% of total InsP_2, but in other experiments the proportion varied from 0 to 6% ($n = 6$) (see also Hansen *et al.*, 1986; Shears *et al.*, 1987).

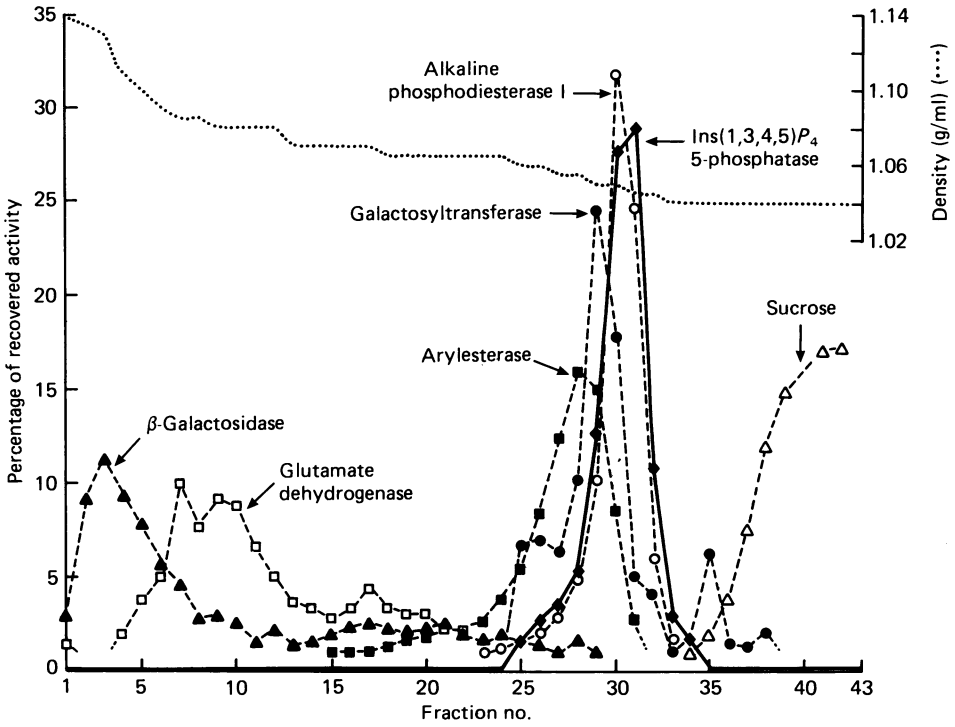

Fig. 2. Distribution of Ins(1,3,4,5)P_4 5-phosphatase activity and various marker enzymes after density-gradient fractionation of hepatocytes

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%); ■, arylesterase (endoplasmic reticulum; 97%); ●, galactosyltransferase (Golgi; 83%); ○, 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^{2+}], cyclic AMP, GTP and its analogues on Ins(1,3,4,5)P_4 hydrolysis by liver homogenates

Ins(1,3,4,5)P_4 hydrolysis during 5 min incubations of liver homogenates was measured as described in the Materials and methods section. The incubations containing 0.1 mM-cyclic AMP also included 5 mM-theophylline. Data are means ± S.E.M. from the numbers of experiments in parentheses.

	10^3 × First-order rate constant (s^{-1})
Control (0.1 μM-Ca^{2+})	1.76 ± 0.12 (8)
1 μM-Ca^{2+}	1.64 ± 0.16 (4)
0.1 mM-GTP	1.64 ± 0.16 (4)
0.1 mM-GTP[S]	1.88 ± 0.16 (5)
0.1 mM-p[NH]ppG	2.08 ± 0.32 (3)
0.1 mM-cyclic AMP + 5 mM-ATP	0.81 ± 0.11 (6)
5.0 mM-ATP	0.83 ± 0.18 (6)

Subcellular distribution of Ins(1,3,4,5)P_4 5-phosphatase

When liver homogenates were fractionated by centrifugation at 100000 *g*, approx. 90% of total Ins(1,3,4,5)P_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_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_4 5-phosphatase activity was present in nuclei, prepared as described by Widnell & Tata (1964). Ins(1,3,4,5)P_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_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_4 5-phosphatase activity

In liver homogenates, both Ins(1,3,4,5)P_4 5-phosphatase activity (Table 2) and the nature of the ensuing products (results not shown) were unchanged when [Ca^{2+}] was increased from 0.1 to 1 μM. It is therefore unlikely that hormones regulate hepatic Ins(1,3,4,5)P_4 5-phosphatase activity in liver by changing cytosol [Ca^{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_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_4 5-phosphatase activity compared with incubations to which ATP alone was added (Table 2).

Inhibitors of Ins(1,3,4,5)P_4 5-phosphatase compared with those of Ins(1,4,5)P_3 5-phosphatase

ATP (0.1 mM) inhibited Ins(1,3,4,5)P_4 5-phosphatase activity by about 12% provided that an ATP-regenerating system was present (Fig. 3). The Ins(1,3,4,5)P_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_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_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_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_4 5-phosphatase activity more efficiently than did either ATP or p[NH]ppA (Table 3).

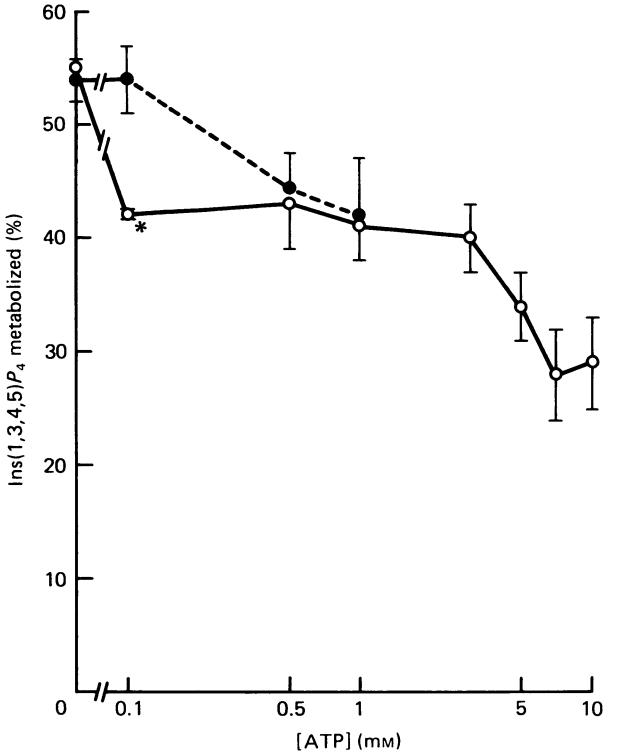

Fig. 3. Effect of ATP on Ins(1,3,4,5)P_4 5-phosphatase activity

Liver homogenates were incubated for 5 min with [^{3}H]Ins(1,3,4,5)P_4 and 10 mM-phosphocreatine, with (●) or without (○) creatine kinase. Various concentrations of ATP were added as described in the Materials and methods section. Data are means ± S.E.M. from three experiments: *$P < 0.025$ comparing incubations with and without creatine kinase. When [ATP] > 1 mM, Ins(1,3,4,5)P_4 metabolism was unaffected by creatine kinase (results not shown).

In these experiments the proportion of Ins(1,3,4,5)P_4 5-phosphatase activity inhibited by 5 mM-ATP was similar to that inhibited by 5 mM-AMP; PP_i inhibited the enzyme more potently (Table 3). Adenosine and P_i did not significantly affect Ins(1,3,4,5)P_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^{2+} or Ca^{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_4 5-phosphatase activity in brain (Irvine *et al.*, 1986*a*), 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.

	Substrate metabolized (%)	
	Ins(1,3,4,5)P_4	Ins(1,4,5)P_3
Control	42±2 (12)	54±3 (9)
+5 mM-ATP	22±1 (12)*	37±5 (5)*
+5 mM-p[NH]ppA	27±3 (6)*	36±3 (5)*
+5 mM-ATP[S]	12±2 (3)*	23±7 (3)*
+5 mM-AMP	24±2 (5)*	38±5 (5)*
+5 mM-PP_i	9±3 (5)*	18±4 (5)*
+5 mM-P_i	41±3 (6)	54±4 (4)
+5 mM-adenosine	40±3 (5)	51±7 (6)
+5 mM-2,3-bis-phosphoglycerate	9±2 (4)*	n.d.
+50 mM-Li^+	42±2 (4)	n.d.

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 [^{3}H]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, Ins$P$ 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.

Incubation time (min)	[ATP]	^{3}H (d.p.m./fraction)	
		InsP_3	InsP_4
0	5 mM	952±81	50±4
10	0	330±20	22±7
10	5 mM	418±36	227±15

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.*, 1986*a*; 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 into Ins(1,3,4,5)P_4 during our incubations and no Ins(1,3,4)P_3 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-P_i 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_i (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).

The activities of Ins(1,4,5)P_3 5-phosphatase in washed 100000 *g* pellets, and of Ins(1,3,4,5)P_4 5-phosphatase in homogenates, were inhibited to about the same extent by each of the various compounds listed in Table 3. Moreover, the degree of inhibition of Ins(1,3,4,5)P_4 5-phosphatase activity by ATP, p[NH]ppA, AMP or PP_i was identical in homogenates and in the washed pellets (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_3 produced in these experiments. We therefore studied the influence of ATP on Ins(1,3,4)P_3 metabolism. In 10 min incubations with liver homogenates, the addition of 5 mM-ATP halved the amount of Ins(1,3,4)P_3 that was dephosphorylated, indicating that ATP inhibits Ins(1,3,4)P_3 phosphatase. In these experiments, ATP also promoted the conversion of 25% of the added Ins(1,3,4)P_3 into a more ionized compound that was eluted from Dowex columns in an 'InsP_4' 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_3 kinase was analysed by h.p.l.c., it was eluted 0.4 min after a [4,5-^{32}P]Ins(1,3,4,5)P_4 internal standard (Fig. 4). It therefore appears that Ins(1,3,4)P_3 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 these data do not exclude the possibility that some Ins(1,3,4,5)P_4 was also a phosphorylation product of Ins(1,3,4)P_3.

The discovery of a novel isomer of InsP_4 led us to consider whether it might have contaminated our original preparations of [^{3}H]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)P_3 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

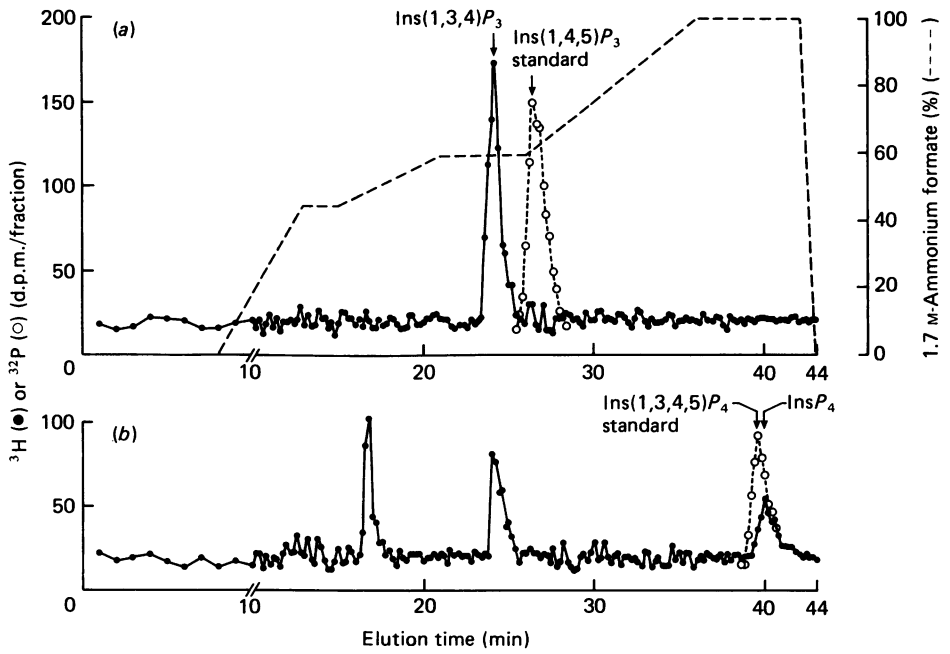

Fig. 4. H.p.l.c. analysis of the products of Ins(1,3,4,)P_3 metabolism in the presence of ATP

Liver homogenates (1.8%, w/v) were incubated, for 0 (*a*) and 10 min (*b*), with [^{3}H]Ins(1,3,4)P_3 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-^{32}P]Ins(1,4,5)P_3 and the 10 min sample was 'spiked' with [4,5-^{32}P]Ins(1,3,4,5)P_4. ●, ^{3}H; ○, ^{32}P (d.p.m./fraction). The radioactivity was assessed by liquid-scintillation spectrometry. Results are from a single experiment, typical of three. ----, Gradient of 1.7 M-ammonium formate (adjusted to pH 3.7 with H_3PO_4).

Table 5. ATP-dependent phosphorylation of [^{3}H]Ins(1,4,5)P_3 and [4,5-^{32}P]Ins(1,4,5)P_3

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 M-sucrose, 50 mM-Tes (pH 7.5), 20 mM-$MgCl_2$, 10 mM-ATP, 25 mM-2,3-bisphosphoglycerate (Na^+ salt), 0.2 mg of saponin/ml plus [^{3}H]Ins(1,4,5)P_3 and [4,5-^{32}P]Ins(1,4,5)P_3 as described in the Table. After 45 min at 37 °C, incubations 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). Data are means ± S.E.M. from triplicate incubations in one experiment, representative of three.

Fraction	Time (min)	Radioactivity (d.p.m./fraction) ^{3}H	^{32}P	^{32}P/^{3}H
InsP_3	0	2837±14	591±6	0.21±0.003
InsP_4	0	16±3	0	-
InsP_3	45	512±18	96±10	0.19±0.02
InsP_4	45	1232±33	243±9	0.20±0.01

eluted as a single peak during h.p.l.c. (Fig. 1), in such experiments a small proportion of the novel InsP_4 isomer could have been masked by a larger proportion of Ins(1,3,4,5)P_4. The purity of the Ins(1,3,4,5)P_4 was therefore assessed by a different method. [^{3}H]Ins(1,4,5)P_3 and [4,5-^{32}P]Ins(1,4,5)P_3 were simultaneously phosphorylated under exactly these conditions normally used to prepare [^{3}H]Ins(1,3,4,5)P_4 [see Shears *et al.* (1987) and Table 5]. The ^{32}P in the [4,5-^{32}P]Ins(1,4,5)P_3 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)P_3, followed by its phosphorylation by non-radioactive ATP, would have decreased the ^{32}P:^{3}H ratio of the InsP_3 and InsP_4 fractions obtained in these experiments. However, no such decrease was observed (Table 5), confirming the isotopic homogeneity of our Ins(1,3,4,5)P_4 preparations.

Characterization of the InsP_4 produced by phosphorylation of Ins(1,3,4)P_3

Aliquots (approx. 1000 d.p.m.) of three preparations of the [^{3}H]InsP_4 produced by phosphorylation of [^{3}H]Ins(1,3,4)P_3 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-^{32}P]Ins(1,3,4,5)P_4. Under these incubation conditions, 78±5% of the Ins(1,3,4,5)P_4 was hydrolysed by the 5-phosphatase activity in the ghosts (see Downes *et al.*, 1982). However, 98±0.8% of the original ^{3}H label was recovered as InsP_4. Thus the [^{3}H]InsP_4 contained no Ins(1,3,4,5)P_4 and, assuming that no phosphate migration occurred during the phosphorylation of Ins(1,3,4)P_3, the novel InsP_4 must be Ins(1,2,3,4)P_4 or Ins(1,3,4,6)P_4, or a mixture of both of these isomers. Ins(1,2,3,4)P_4 possesses vicinal hydroxyl groups and should be sensitive to periodate; Ins(1,3,4,6)P_4 should be periodate-insensitive. However, past experience suggests that periodate oxidation of InsP_4 isomers, especially those with pairs of *trans*-hydroxyl groups, occurs only under stronger oxidizing conditions than are needed for oxidation of periodate-

sensitive InsP_3 isomers (Tomlinson & Ballou, 1962; Lim & Tate, 1973). Thus the novel [^{3}H]InsP_4 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)P_4 degradation. The only labelled polyol detected was inositol (70% recovery). These results suggest that most of the InsP_4 that is formed from Ins(1,3,4)P_3 is Ins(1,3,4,6)P_4. However, a definitive assignment of its structure must await further experiments.

DISCUSSION

In liver, the membrane-bound Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 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 Seyfred *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 μM [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)P_3 and Ins(1,3,4,5)P_4 5-phosphatase may catalyse both membrane-associated activities. Platelet cytosol contains an enzyme which, when purified, hydrolyses both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4; 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)P_3 and Ins(1,3,4,5)P_4 (Downes *et al.*, 1982; Batty *et al.*, 1985; Irvine *et al.*, 1986*a*,*b*).

A near-physiological concentration of ATP (5 mM; see Soboll *et al.*, 1978) inhibited the membrane-associated Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 5-phosphatase activities by 40–50% (Fig. 3 and Table 3). Thus the lifetime of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 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)P_3/Ins(1,3,4,5)P_4 5-phosphatase.

Best (1986) has shown that 10 μM-Ca^{2+} activates polyphosphoinositide hydrolysis in permeabilized pancreatic islets. The subsequent accumulation of inositol phosphates, including Ins(1,4,5)P_3 [and presumably also Ins(1,3,4,5)P_4, 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)P_2 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)P_3 and Ins(1,3,4,5)P_4 to free inositol.

Our discovery that Ins(1,3,4)P_3 can be phosphorylated to a novel InsP_4, which is probably Ins(1,3,4,6)P_4, has revealed yet another aspect of inositol polyphosphate metabolism in mammalian cells. H.p.l.c. analyses of [^{3}H]InsP_4, isolated from several [^{3}H]inositol-labelled and stimulated tissues, have not previously indicated that more than one isomer was present (Batty *et al.*, 1985; Downes *et al.*, 1986; Hansen *et al.*, 1986; Palmer *et al.*, 1986; Turk *et al.*, 1986). Structural analysis of the InsP_4 from tissue samples stimulated for less than 1 min identified only Ins(1,3,4,5)P_4 (Batty *et al.*, 1985; Downes *et al.*, 1986). However, in these short-term experiments, there was relatively little dephosphorylation of Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3. Therefore there may not have been sufficient Ins(1,3,4)P_3 to support substantial synthesis of the novel InsP_4. We have yet to make a direct search for the novel InsP_4 in stimulated hepatocytes.

The metabolic fate of the novel InsP_4 isomer is not known. An intriguing possibility is that the InsP_4 might be a precursor for InsP_5 and InsP_6 (see Heslop *et al.*, 1985). However, there was no evidence for this in our experiments, in which radioactivity lost from Ins(1,3,4)P_3 was quantitatively recovered as either InsP_2 or InsP_4 (Fig. 4). Nevertheless, the ability of liver tissue to re-arrange the isomeric configuration of Ins(1,3,4,5)P_4 involves two enzymes and represents a cost to cellular energy stores. Presumably the novel InsP_4, 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)P_3 can also be phosphorylated to an InsP_4 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 InsP_4 is the (1,3,4,6) isomer.

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