The pathway of *myo*-inositol 1,3,4-trisphosphate dephosphorylation in liver

Stephen B. SHEARS, Christopher J. KIRK and Robert H. MICHELL

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

We studied the dephosphorylation pathway for $\text{Ins}(1,3,4)P_3$ (inositol 1,3,4-trisphosphate) by liver homogenates and soluble and particulate subfractions incubated in media resembling physiological ionic strength and pH. $\text{Ins}(1,3,4)P_3$ was dephosphorylated to two $\text{Ins}P_2$ (inositol bisphosphate) isomers, one of which is $\text{Ins}(3,4)P_2$ [Shears, Parry, Tang, Irvine, Michell & Kirk (1987) *Biochem. J.* **246**, 139–147]. The second $\text{Ins}P_2$ is the 1,3 isomer. $\text{Ins}(3,4)P_2$ is dephosphorylated to inositol 3-phosphate by an enzyme activity located in both soluble and particulate fractions. The phosphatase(s) that attacks $\text{Ins}(1,3)P_2$ was largely soluble, but we have not determined which phosphate(s) is removed. When the initial substrate concentration was 1 nM, the rate of dephosphorylation of $\text{Ins}(1,4)P_2 > \text{Ins}(1,3)P_2 > \text{Ins}(3,4)P_2$. None of these bisphosphates was phosphorylated when incubated with liver homogenates and 5 mM-ATP, but their rates of dephosphorylation were then decreased.

INTRODUCTION

In a variety of cells, receptor-mediated hydrolysis of $\text{PtdIns}(4,5)P_2$ leads to the production of $\text{Ins}(1,4,5)P_3$, which releases Ca^{2+} from intracellular stores (see [1,2] for reviews). $\text{Ins}(1,4,5)P_3$ can be converted to inositol by a series of phosphatases (see, e.g., [3–6]). Alternatively, $\text{Ins}(1,4,5)P_3$ can be phosphorylated to $\text{Ins}(1,3,4,5)P_4$ (e.g. [7,8]), which apparently potentiates Ca^{2+} influx across the plasma membranes of sea-urchin eggs [9]. $\text{Ins}(1,3,4,5)P_4$ is dephosphorylated to $\text{Ins}(1,3,4)P_3$ (e.g. [8,10–12]), which can then be phosphorylated to $\text{Ins}(1,3,4,6)P_4$ [12,13]. The metabolic fate of $\text{Ins}(1,3,4,6)P_4$ is unknown. $\text{Ins}(1,3,4)P_3$ is also dephosphorylated, and in tissue homogenates a major $\text{Ins}P_2$ product was identified as $\text{Ins}(3,4)P_2$ [11,13,14], which is dephosphorylated to $\text{Ins}3P$ in brain [14,15].

In some experiments, we have observed a minor unidentified $\text{Ins}P_2$ product when $\text{Ins}(1,3,4,5)P_4$ was incubated with liver homogenates [12], but not in similar experiments with $\text{Ins}(1,3,4)P_3$ [11]. We have now re-investigated the production of $\text{Ins}P_2$ from $\text{Ins}(1,3,4)P_3$, and have confirmed that two $\text{Ins}P_2$ products accumulate. We have identified the previously unknown $\text{Ins}P_2$, and studied the metabolism of both $\text{Ins}P_2$ products in liver.

MATERIALS AND METHODS

Preparation of inositol phosphates

$[^3\text{H}]\text{Ins}(1,3,4,5)P_4$, $[^3\text{H}]\text{Ins}(1,4)P_2$ and $[^{14}\text{C}]\text{Ins}3P$ (= L- $\text{Ins}1P$) were purchased from Amersham International. $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$ and $[4\text{-}^{32}\text{P}]\text{Ins}(1,4)P_2$ were prepared as described in [3], except that the erythrocytes were washed and labelled with ^{32}P in buffers without added Cl^- [16]. $[^{32}\text{P}]\text{Ins}4P$ was prepared by alkaline hydrolysis of ^{32}P -labelled $\text{PtdIns}4P$ [17].

$[4\text{-}^{32}\text{P}]\text{Ins}(1,3,4)P_3$ was prepared from 0.05 μCi of $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)P_3$. This was converted into $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ (70% recovery) by incubation for 60 min in 5 ml of medium containing 100 mM-KCl, 20 mM-Hepes (pH 8.0 with KOH), 20 mM-phosphocreatine, 10 mM-ATP, 10 mM- MgCl_2 , 5 mM- $\text{Na}_4\text{P}_2\text{O}_7$, 1 mg of creatine kinase/ml, and 300 μl of liver supernatant (prepared as described below). The reaction was quenched with 2 ml of 5% (w/v) trichloroacetic acid. After removal of the protein by centrifugation at 1000 g for 10 min, the supernatants were neutralized by repeated washing with diethyl ether. The $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ was separated from ATP [18], desalted and freeze-dried [11]. The $[4,5\text{-}^{32}\text{P}]\text{Ins}(1,3,4,5)P_4$ was dephosphorylated to $[4\text{-}^{32}\text{P}]\text{Ins}(1,3,4)P_3$ (86% recovery) by incubation for 30 min in 15 ml of medium containing 20 mM-Tris (pH 7.5 with HCl), 4 mM-magnesium acetate, 0.2 mg of saponin/ml and 5 ml of packed human erythrocyte ghosts (cf. [18]). The membranes were removed by centrifugation at 100000 g for 1 h, and the supernatants were applied to an ion-exchange column (2 cm $\times$ 0.6 cm; Bio-Rad AG1-X8, 200–400 mesh), from which $[4\text{-}^{32}\text{P}]\text{Ins}(1,3,4)P_3$ was recovered, desalted and freeze-dried [11].

$[^3\text{H}]\text{Ins}(1,3,4)P_3$ was prepared as in [12]. $[^3\text{H}]\text{Ins}(3,4)P_2$ and $[^3\text{H}]\text{Ins}(1,3)P_2$ were prepared by incubating 0.35 μCi of $[^3\text{H}]\text{Ins}(1,3,4,5)P_4$ for 30 min with 60 μl of a liver 100000 g particulate fraction (see below) in 1 ml of the medium used to study inositol phosphate metabolism (see below). The reaction was quenched with 0.4 ml of 1.7 M- HClO_4 , neutralized and eluted by h.p.l.c. as described in [12], except that 0.5 min (0.625 ml) fractions were collected. Samples (5 μl) of each fraction were added to 0.4 ml of water and 4 ml of Triton/xylene scintillant [12] to locate the two $\text{Ins}P_2$ peaks. The later-eluted of these is $\text{Ins}(3,4)P_2$ (see the Results and discussion section, and [12]). The other peak is $\text{Ins}(1,3)P_2$ (see the Results

Abbreviations used: $\text{Ins}P$, $\text{Ins}P_2$, $\text{Ins}P_3$ and $\text{Ins}P_4$ are inositol mono-, bis-, tris- and tetrakis-phosphates, with locants designated where appropriate (enantiomeric phosphates are numbered by reference to D-inositol 1-phosphate); $\text{PtdIns}4P$, phosphatidylinositol 4-phosphate; $\text{PtdIns}(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate.

and discussion section). The fractions that constituted each peak were combined, and neutralized with a solution containing 60 mM-EDTA, 1.2 M-KOH and 75 mM-Hepes. Each sample was diluted 10-fold with water, and applied to a Bio-Rad ion-exchange column (see above). P_i was eluted with 10 ml of 180 mM-ammonium formate, and then 10 ml of 0.4 M-ammonium formate/0.1 M-formic acid was used to elute the $InsP_2$ preparations, which were desalted and freeze-dried [12]. Typical yields of $Ins(3,4)P_2$ and $Ins(1,3)P_2$ were 0.07 and 0.005 μCi respectively.

Assay of inositol phosphate metabolism

The radiolabelled inositol phosphates were incubated for various times at 37 °C in 0.5 ml of 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 with KOH) and 0.2 mg of saponin/ml [12]. Incubations were initiated by adding appropriate amounts of a 30 % (w/v) suspension of liver homogenate, or of 100 000 g supernatant or particulate fractions (prepared as described in [12]). Reactions were quenched with 0.2 ml of 1.7 M-HClO₄, and the protein was removed by centrifugation at 10 000 g for 3 min. The samples were then either neutralized and loaded on to Bio-Rad ion-exchange columns [12] or analysed by h.p.l.c. (see below). For those $InsP_2$ phosphatase assays which were analysed on Bio-Rad columns, > 98 % of $InsP + P_i$ were first eluted with 10 ml of 180 mM-ammonium formate. Next, > 99 % of $InsP_2$ was eluted with 4 ml of 2.8 M-ammonium formate/0.1 M-formic acid, 2 ml of which was counted for radioactivity [12].

Details of h.p.l.c. analyses

The acid-quenched samples were neutralized [12] and injected, in two 0.4 ml portions, on to a 25 cm $\times$ 0.46 cm main column plus a 5 cm $\times$ 0.46 cm guard column (both containing Partisil 10-SAX). For some experiments (Fig. 1, Table 1) the h.p.l.c. eluent was ammonium formate (pH 3.7 with H₃PO₄) [12]. Alternatively (Fig. 2), the separation of $InsP$ isomers was improved by eluting them with ammonium phosphate [19], pH 4.6 with H₃PO₄ (L. R. Stephens, personal communication), at 1 ml/min. The elution began with water until 5.5 min. Between 5.5 and 6.5 min, the elution buffer was changed to 9 % (v/v) 300 mM-ammonium phosphate, pH 4.6 with H₃PO₄. The concentration of buffer was increased from 9 to 99 % (v/v) ammonium phosphate between 23.5 and 26.5 min, and then changed to water after 36.5 min. To

each (0.5 ml) fraction, 0.5 ml of 50 % aq. (v/v) methanol and 5 ml of scintillant (see above) were added, and the radioactivity was counted.

RESULTS AND DISCUSSION

Products of $Ins(1,3,4)P_3$ dephosphorylation

Liver homogenates were incubated with [4-³²P] $Ins(1,3,4)P_3$ plus [³H] $Ins(1,3,4)P_3$. The incubation times were varied such that between 30 and 90 % of the substrate was metabolized. H.p.l.c. analysis of the products revealed two $InsP_2$ peaks, one of which was at least 98 % of total $InsP_2$. The ³²P:³H ratio of the major $InsP_2$ was identical with that of the $Ins(1,3,4)P_3$ (Table 1). Thus this product retained the radioactive 4-phosphate, is not $Ins(1,4)P_2$ [11], and must therefore be $Ins(3,4)P_2$. This conclusion confirms our earlier work [11,12]. A minor $InsP_2$ was also detected (Fig. 1), and its ³²P:³H ratio was close to zero (Table 1). This product has therefore lost the radioactive 4-phosphate and must be $Ins(1,3)P_2$. The proportion of $Ins(1,3)P_2$ never exceeded 2 % of total $InsP_2$ in incubations of up to 30 min, during which > 90 % of $Ins(1,3,4)P_3$ was metabolized (Fig. 1). The small size of the $Ins(1,3)P_2$ peak presumably explains why we failed to detect it in our earlier experiments (see [11]). Hansen *et al.* [20] had suggested that $Ins(1,3)P_2$ was the major product of $Ins(1,3,4)P_3$ dephosphorylation, but they have since acknowledged it to be $Ins(3,4)P_2$ [21].

$Ins(1,3)P_2$ and $Ins(3,4)P_2$ were also formed when $Ins(1,3,4)P_3$ was incubated with 100 000 g supernatants and pellets (Fig. 1, Table 1). The proportion of total $InsP_2$ that was $Ins(1,3)P_2$ was greatest (5–20 %) in the experiments with the particulate fractions, which may reflect their low $Ins(1,3)P_2$ phosphatase activity (see below). $Ins(1,3)P_2$ was detected in only two of four experiments with supernatants, and amounted to < 5 % of total $InsP_2$ (Fig. 1). Others [15] have reported that $Ins(1,3,4)P_3$ was dephosphorylated to equal amounts of $Ins(1,3)P_2$ and $Ins(3,4)P_2$ in incubations with a calf brain supernatant [15]. However, our incubation medium was different from that used in [15], and the initial $Ins(1,3,4)P_3$ concentration may have been different. Moreover, the proportions of bisphosphates accumulating during $Ins(1,3,4)P_3$ metabolism depend on the kinetic parameters of the phosphatases, which could be subject to species and tissue differences.

Table 1. Determination of the $InsP_2$ products of $Ins(1,3,4)P_3$ metabolism

Approx. 6000 d.p.m. of [³H] $Ins(1,3,4)P_3$ and 3000 d.p.m. of [4-³²P] $Ins(1,3,4)P_3$ were incubated as described in the Materials and methods section with either 1.8 % (w/v) liver homogenates or equivalently diluted supernatant or particulate fractions. Incubations were performed for 10, 20 or 30 min, and then quenched and analysed by h.p.l.c. using an ammonium formate/H₃PO₄ gradient (see the Materials and methods section). Results are means $\pm$ S.E.M., with the numbers of determinations in parentheses. The ³²P/³H ratio for the $Ins(1,3,4)P_3$ substrate was 0.45 ± 0.04 ($n = 13$).

	³² P/ ³ H ratio of $InsP_2$ eluted at 16–16.8 min	Identification	³² P/ ³ H ratio of $InsP_2$ eluted at 17.2–18 min	Identification
Homogenate	0.012 ± 0.01 (5)	$Ins(1,3)P_2$	0.45 ± 0.06 (5)	$Ins(3,4)P_2$
Supernatant	0.01 (2)		0.45 ± 0.08 (4)	
Particulate	0.012 ± 0.01 (4)		0.50 ± 0.06 (4)	

Metabolism of $\text{Ins}(1,3)P_2$ and $\text{Ins}(3,4)P_2$

For these experiments, $\text{Ins}(1,3)P_2$ and $\text{Ins}(3,4)P_2$ were both prepared with $\text{Ins}(1,3,4,5)P_4$ as a precursor (see the Materials and methods section). Their purity was checked by h.p.l.c., whereupon neither $\text{Ins}P_2$ preparation co-eluted with standards of $\text{Ins}(1,5)P_2$ or $\text{Ins}(4,5)P_2$ (results not shown). An $\text{Ins}(1,4)P_2$ standard was eluted only

Fig. 1. Analysis, by h.p.l.c., of the $\text{Ins}P_2$ products of $\text{Ins}(1,3,4)P_3$ metabolism by liver homogenates and 100000 g supernatant and particulate fractions

Approx. 6000 d.p.m. of $[^3\text{H}]\text{Ins}(1,3,4)P_3$ and 3000 d.p.m. of $[4\text{-}^{32}\text{P}]\text{Ins}(1,3,4)P_3$ were incubated as described in the Materials and methods section with either 1.8% (w/v) liver homogenates (●, ○), or equivalent amounts of 100000 g supernatant (▲, △) or particulate (■, □) fractions. Incubations were performed for 10, 20 or 30 min, and then quenched and analysed by h.p.l.c. using an ammonium formate/ H_3PO_4 gradient (see the Materials and methods section). Two $\text{Ins}P_2$ peaks were detected (see [12]). The first of these (○, △, □) was eluted after 16–16.8 min, and the other (●, ▲, ■) after 17.2–18 min. Each data point shows the conversion of $\text{Ins}(1,3,4)P_3$ into the $\text{Ins}P_2$ products in a single incubation. The remainder of the radioactivity was recovered in the Ins , $\text{Ins}P$ and P_i fractions. Results were obtained from four homogenates and corresponding fractions.

0.2 min after the peak of the putative $[^3\text{H}]\text{Ins}(1,3)P_2$, so the latter's purity was studied by incubating 2000 d.p.m. with 5000 d.p.m. of $[^{32}\text{P}]\text{Ins}(1,4)P_2$ in 0.5 ml of 0.2 M- NaIO_4 /0.1 M-HCl for 5 days in the dark at room temperature. This treatment oxidizes inositol phosphates with vicinal hydroxyl groups, but only *p*-isomers of $\text{Ins}P_2$ are totally destroyed, yielding P_i [3,22]. The incubations were quenched with 400 μl of ethylene glycol, diluted 10-fold with water and applied to ion-exchange columns. The columns were eluted as described in the Materials and methods section: 84% of the $[^{32}\text{P}]\text{Ins}(1,4)P_2$ was converted into $[^{32}\text{P}]P_i$, whereas 100% of the ^3H -labelled product had the polarity of $\text{Ins}P_2$, and was presumably a pentitol bisphosphate (see [22]). Thus the original $\text{Ins}P_2$ preparation contained no $[^3\text{H}]\text{Ins}(1,4)P_2$.

The rate of dephosphorylation of 1 nM- $\text{Ins}(1,3)P_2$ by liver homogenates was about twice that of 1 nM- $\text{Ins}(3,4)P_2$ (Table 2). Both substrates were metabolized much more slowly than $\text{Ins}(1,4)P_2$. $\text{Ins}(3,4)P_2$ 4-phosphatase activity was equally distributed between 100000 g supernatant and particulate fractions, whereas $\text{Ins}(1,3)P_2$ phosphatase activity was largely soluble (Table 2). When 5 mM-ATP was added to these incubations, none of the bisphosphates were phosphorylated (results not shown); these are conditions where around 20% of $\text{Ins}(1,3,4)P_2$ is converted into $\text{Ins}(1,3,4,6)P_4$ [12]. Thus the $\text{Ins}(1,3,4)P_3$ 6-kinase in rat liver displays a specificity for the trisphosphate substrate. In those incubations with ATP, the rates of dephosphorylation of $\text{Ins}(1,4)P_2$, $\text{Ins}(1,3)P_2$ and $\text{Ins}(3,4)P_2$ were respectively inhibited by 30%, 56% and 80% as compared with incubations performed without ATP (Table 2). ATP also inhibits the dephosphorylation of $\text{Ins}(1,4,5)P_3$, $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,3,4)P_3$ [12].

The $\text{Ins}P$ products of $[^3\text{H}]\text{Ins}(3,4)P_2$ dephosphorylation were analysed by h.p.l.c. After incubations with liver homogenates for 20 min (Fig. 2), or for 10 and 30 min (results not shown) the only $[^3\text{H}]\text{Ins}P$ isomer detected co-eluted with an $\text{Ins}3P$ standard, and not with $\text{Ins}4P$. Thus liver dephosphorylates $\text{Ins}(3,4)P_2$ by a 4-phosphatase. We did not determine the pathway of $\text{Ins}(1,3)P_2$ metabolism, since the two potential enantiomeric products ($\text{Ins}1P$ and $\text{Ins}3P$) are not separable by h.p.l.c. However, when $[^{32}\text{P}]\text{Ins}(1,3)P_2$ was dephosphorylated by brain homogenates, the $\text{Ins}P$ product was shown to be $\text{Ins}1P$ [15]. The $\text{Ins}P$ product(s) formed by dephosphorylation of $\text{Ins}(3,4)P_2$ and $\text{Ins}(1,3)P_2$ are presumably

Table 2. Rates of metabolism of inositol bisphosphates by liver homogenates and 100000 g supernatant and particulate fractions

Approx. 1000 d.p.m. (final concn. 1 nM) of the appropriate $[^3\text{H}]\text{Ins}P_2$ was incubated as described in the Materials and methods section with 3.75 μl [for $\text{Ins}(1,4)P_2$ assays], 30 μl [for $\text{Ins}(1,3)P_2$] or 60 μl [for $\text{Ins}(3,4)P_2$] of 30% (w/v) homogenate, or equivalent amounts of 100000 g supernatant or particulate fractions. Incubations were performed for 7–10 min, during which times the reaction rates were linear. Results are means $\pm$ S.E.M. for three preparations of liver fractions, each assayed in triplicate: n.d., not determined.

Fraction	ATP	Substrate metabolized (%/min per μl of liver fraction)		
		$\text{Ins}(1,4)P_2$	$\text{Ins}(1,3)P_2$	$\text{Ins}(3,4)P_2$
Homogenate	0	2.0 ± 0.1	0.09 ± 0.02	0.04 ± 0.003
Homogenate	5 mM	1.4 ± 0.02	0.04 ± 0.02	0.008 ± 0.004
Supernatant	0	n.d.	0.08 ± 0.009	0.02 ± 0.004
Particulate	0	n.d.	0.02 ± 0.007	0.02 ± 0.003

Fig. 2. Analysis by h.p.l.c. of $\text{Ins}(3,4)\text{P}_2$ metabolism by liver homogenates

Approx. 6000 d.p.m. of $[^3\text{H}]\text{Ins}(3,4)\text{P}_2$ was incubated as described in the Materials and methods section with 3.6% (w/v) liver homogenates for 20 min. The samples were quenched and neutralized as described in the Materials and methods section, and then 'spikes' of $[^{14}\text{C}]\text{Ins}3\text{P}$ and $[^{32}\text{P}]\text{Ins}4\text{P}$ were added. The samples were analysed by h.p.l.c. using an ammonium phosphate gradient (see the Materials and methods section). The samples containing the InsP fractions were counted for radioactivity twice, first with a $^{14}\text{C}/^3\text{H}$ program, and again with a $^{32}\text{P}/^3\text{H}$ program; ●, ^3H ; ○, ^{32}P or ^{14}C . Note that two inositol peaks are present, because each sample was loaded in two portions. Similar results were obtained in two further experiments.

metabolized by the InsP phosphatase [23], which, at least in brain, has well characterized activity towards both $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$.

General conclusions

Our experiments have added two new reactions to those previously known to be involved in the dephosphorylation of $\text{Ins}(1,3,4)\text{P}_3$ by rat liver, namely the removal of the 4-phosphate groups from $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(3,4)\text{P}_2$. From these and previous experiments with broken-cell preparations (see the Introduction for references), it appears that $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}(3,4)\text{P}_2$ are probably produced as a result of $\text{Ins}(1,4,5)\text{P}_3$ metabolism in intact stimulated cells. $\text{Ins}(1,4)\text{P}_2$ might also be formed by hydrolysis of $\text{PtdIns}4\text{P}$. Other, as yet unidentified, InsP_2 isomers may accumulate during $\text{Ins}(1,3,4,6)\text{P}_4$ metabolism. Considerable further work will be necessary to establish, in intact cells, the relative contributions of these proven and possible pathways of InsP_2 production.

After stimulation of intact hepatocytes, the major InsP_2 (s) is eluted during h.p.l.c. with the characteristics

of $\text{Ins}(1,4)\text{P}_2/\text{Ins}(1,3)\text{P}_2$, whereas the isomer(s) chromatographically similar to $\text{Ins}(3,4)\text{P}_2$ is a minor product [20]. None of these h.p.l.c. peaks has yet been subjected to rigorous chemical analysis, nor have changes in their individual concentrations been analysed either as a function of the period of stimulation or in the presence of phosphatase inhibitors such as Li^+ .

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