Rat liver contains a potent endogenous inhibitor of inositol 1,3,4,5-tetrakisphosphate 3-phosphatase

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When Ins(1,3,4,5) P_4 was incubated with a rat liver 100 000 *g* supernatant, about 93 % of the substrate was metabolized by a 5-phosphatase, and only 7 % by a 3-phosphatase. Ion-exchange chromatography of the supernatant specifically increased its 3-phosphatase activity 72 ± 3 -fold. This activated enzyme was inhibited by a heat-stable factor present in both the soluble and particulate portions of the cell.

INTRODUCTION

It is accepted that receptor-activated hydrolysis of PtdIns-(4,5) P_2 yields Ins(1,4,5) P_3 , which mobilizes intracellular Ca^{2+} stores (reviewed in [1]). More recent evidence indicates that, at least in some cell types, the product of 3-kinase-mediated phosphorylation of Ins(1,4,5) P_3 , i.e. Ins(1,3,4,5) P_4 , co-operates in the overall regulation of cellular Ca^{2+} fluxes (reviewed in [2]). The enzymic removal of the 5-phosphate group from Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 terminates the signalling functions of these compounds (reviewed in [3]). The hydrolysis of Ins(1,3,4,5) P_4 is more complex, since it can also be attacked by a 3-phosphatase [4–10]. Thus Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 are interconverted by a futile cycle that has the potential to be important in the regulation of Ca^{2+} signalling.

There is much confusion concerning the importance of Ins(1,3,4,5) P_4 3-phosphatase *in vivo*. Estimates of its activity, relative to that of the 5-phosphatase, range from 2 to 75 % in different cell types [4,5,8–10]. Furthermore, in both liver [9] and RBL-2H3 cells [4], the Ins(1,3,4,5) P_4 3-phosphatase activity in a 100 000 *g* particulate fraction was severalfold greater than that of parent homogenates; this discrepancy has yet to be resolved. A particularly puzzling observation was made with mouse lymphoma L1210 cells [7]. When these cells were permeabilized with digitonin, no 3-phosphatase activity was observed, whereas permeabilization by electroporation exposed substantial amounts of this enzyme.

In this report we provide evidence that liver contains an endogenous inhibitor of Ins(1,3,4,5) P_4 3-phosphatase, and this result may explain why this enzyme activity has proved to be so enigmatic.

MATERIALS AND METHODS

Preparation of tissue samples

Hepatic 100 000 *g* supernatants and washed particulate fractions (diluted to 30 % wt. of original liver/vol.) were prepared as described previously [11,12]. A 10 ml of portion of the supernatant was fractionated on an H/R 10/10 Mono Q column by f.p.l.c. with a NaCl gradient as previously described [11]. Some samples of either liver supernatant or washed particulate fractions were incubated at 90 °C for 10 min; precipitated protein was

removed by centrifugation (10 000 *g*, 5 min), and the remaining soluble material was designated 'heat-treated'. Some samples (2–4 ml) of supernatant were dialysed overnight (molecular-mass cut-off at 14 000 Da) against 1 litre of buffer containing 0.25 M-sucrose, 10 mM-sucrose, 10 mM-BisTris (pH 7.2) and 5 mM- $NaNO_3$.

Assay of inositol phosphatase activities

Assays were performed at 37 °C in 0.5 ml of medium containing 120 mM-KCl, 20 mM-Hepes (pH 7.2), 5 mM-MgSO₄, 1 mM-EDTA, 0.2 mg of saponin/ml and 10 000–40 000 d.p.m. of either [³H]Ins(1,4,5) P_3 or [³H]Ins(1,3,4,5) P_4 . Incubations were initiated by addition of tissue, and were quenched with 0.2 ml of 2 M-HClO₄. Samples were neutralized and analysed by gravity-fed ion-exchange columns [11], except that Ins P_3 was eluted with 5×2 ml of 0.75 M-ammonium formate/0.1 M-formic acid. Some acid-quenched incubations were neutralized with Freon/octylamine [12] and analysed by h.p.l.c., with an Absorbosphere SAX guard column (Alltech Associates, Deerfield, IL, U.S.A.) connected to a Partisil 10 SAX column (Krackler Scientific, Durham, NC, U.S.A.). The elution protocol was a modification of the method of Batty *et al.* [13], using a gradient of water and 2.2 M-ammonium formate (pH 3.7 with H₃PO₄) (solvent B): 0–5 min, B = 0 %; 5–10 min, B increased linearly to 44 %; 10–25 min, B = 44 %; 25–30 min, B increased linearly to 60 %; 30–45 min, B = 60 %; 45–46 min, B increased linearly to 100 %; 46–64 min, B = 100 %. In some experiments, 1 ml fractions of the h.p.l.c. eluate were collected; 20 μ l samples of each were mixed with scintillant to locate the Ins P_3 peaks, which were desalted [14] for structural analysis (see below). In other experiments, radioactivity in the h.p.l.c. eluate was measured [9] with a Flo-one detector (Radiomatic Instruments, Tampa, FL, U.S.A.). Elution times were: [³H]Ins(1,4) P_2 , 22.7 min; [³H]Ins(3,4) P_2 , 23.2 min; [³H]Ins(1,3,4) P_3 , 31 min; [³H]Ins(1,4,5) P_3 , 33.5 min; [³H]Ins(1,3,4,5) P_4 , 56 min.

Structural identification of Ins P_3 product of Ins(1,3,4,5) P_4 hydrolysis

The desalted Ins P_3 product of Ins(1,3,4,5) P_4 dephosphorylation (see above) was incubated with periodate, reduced and dephosphorylated, and the resultant polyol was identified by h.p.l.c. with a Polybore-Pb column [11,15]. There were four possible Ins P_3 products, i.e. Ins(1,4,5) P_3 , Ins(1,3,4) P_3 , Ins(1,3,5) P_3 and Ins(3,4,5) P_3 . These would respectively yield the following polyols: iditol, altritol, inositol and xylitol. Since these polyols were

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resolved on the Polybore-Pb column [11,15], the structure of the parent $\text{Ins}P_3$ was determined.

Materials

Sources of all other materials are as listed elsewhere [11].

RESULTS AND DISCUSSION

$\text{Ins}(1,3,4,5)P_4$ 3-phosphatase activity in rat liver cytosol

Rat liver supernatants were incubated with $[\text{H}]\text{Ins}(1,3,4,5)P_4$ and the reaction products were analysed by h.p.l.c. (Fig. 1). In experiments where up to 50% of the substrate was metabolized under first-order conditions, the major products were identified as $[\text{H}]\text{Ins}(1,3,4)P_3$ and $[\text{H}]\text{Ins}(3,4)P_2$ (Fig. 1). Thus, in agreement with earlier studies (listed in [3]), $\text{Ins}(1,3,4,5)P_4$ was sequentially hydrolysed by a $\text{Ins}(1,3,4,5)P_4$ 5-phosphatase and $\text{Ins}(1,3,4)P_3$ 1-phosphatase. In addition, a relatively small amount of an $[\text{H}]\text{Ins}P_3$ was detected that co-eluted, upon h.p.l.c., with $\text{Ins}(1,4,5)P_3$ (Fig. 1). However, this minor $\text{Ins}P_3$ accumulated at about 7% of the rate of the $\text{Ins}(1,3,4,5)P_4$ 5-phosphatase activity (Fig. 1); no $\text{Ins}(1,4)P_2$ was detected, indicating that any $\text{Ins}(1,4,5)P_3$ that was formed was not substantially dephosphorylated. The conclusion that might be drawn from Fig. 1 is that rat liver cytosol contains very little $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase activity. However, the experiments described below complicate this interpretation.

Activation of $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase by ion-exchange chromatography

Liver supernatant was fractionated by f.p.l.c. on a Mono Q ion-exchange column, and the resultant fractions were assayed for phosphatase activity against $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ (Fig. 2). There were two peaks of $\text{Ins}(1,4,5)P_3$ phosphatase activity, possibly owing to isoenzymes [16]. There were also two peaks of $\text{Ins}(1,3,4,5)P_4$ phosphatase; the minor and earlier-eluted peak co-eluted with the earlier-eluted $\text{Ins}(1,4,5)P_3$ phosphatase peak. The $\text{Ins}(1,3,4,5)P_4$ phosphatase in this peak produced an $\text{Ins}P_3$ that, on h.p.l.c., co-eluted with $\text{Ins}(1,3,4)P_3$ (results not shown). These data are consistent with other demonstrations that soluble $\text{Ins}(1,3,4,5)P_4$ 5-phosphatase also attacks $\text{Ins}(1,4,5)P_3$ [16,17]. In three experiments the recovery of $\text{Ins}(1,3,4,5)P_4$ 5-phosphatase from the column was $67 \pm 7\%$ of that applied in the supernatant.

The second peak of $\text{Ins}(1,3,4,5)P_4$ phosphatase to be eluted from the Mono Q column (Fig. 2) produced a single $[\text{H}]\text{Ins}P_3$ product, which was identified as $\text{Ins}(1,4,5)P_3$ by two experiments. First, the $[\text{H}]\text{Ins}P_3$ co-eluted, on h.p.l.c., with $\text{Ins}(1,4,5)P_3$ standards (results not shown). Second, the $[\text{H}]\text{Ins}P_3$ yielded iditol (95% of total ^3H ; $n = 2$) on incubation with periodate, followed by reduction and dephosphorylation (see the Materials and methods section). Thus the later-eluted and major $\text{Ins}(1,3,4,5)P_4$ phosphatase peak (Fig. 2) was a 3-phosphatase. No $\text{Ins}(1,3,4,5)P_4$ 5-phosphatase was detected in this peak, despite it containing some $\text{Ins}(1,4,5)P_3$ phosphatase (Fig. 1). Although we have not identified the $\text{Ins}P_2$ product of this second-eluted $\text{Ins}(1,4,5)P_3$ phosphatase peak, it seems likely to be $\text{Ins}(1,4)P_2$, produced by a species of soluble $\text{Ins}(1,4,5)P_3$ 5-phosphatase that has poor affinity towards $\text{Ins}(1,3,4,5)P_4$ [16]. The eluate from the Mono Q column contained much more 3-phosphatase activity than 5-phosphatase activity (Fig. 2). In contrast, the unfractionated supernatant expressed little 3-phosphatase activity (Fig. 1). Indeed, after ion-exchange chromatography, the 3-phosphatase activity amounted to 72 ± 3 ($n = 3$) times that applied to the column in the original supernatant (Fig. 2).

Fig. 1. Relative rates of $\text{Ins}(1,3,4,5)P_4$ 3- and 5-phosphatase activities in liver cytosol

$\text{Ins}(1,3,4,5)P_4$ hydrolysis ($\circ$) by 20 μl samples of liver supernatant was analysed as described in the Materials and methods section. Reaction products were assayed by h.p.l.c. The 5-phosphatase activity was determined from the accumulation of $\text{Ins}(1,3,4)P_3$ ($\bullet$), plus its own catabolite, $\text{Ins}(3,4)P_2$ (see [3]). The 3-phosphatase activity was evaluated from the additional $\text{Ins}P_3$ that was formed ($\blacktriangle$), which co-eluted with $\text{Ins}(1,4,5)P_3$. In this experiment, the first-order rate constants for the 5- and 3-phosphatases were 0.03 s^{-1} and 0.002 s^{-1} respectively (per ml of supernatant). Data are means of duplicate determinations in a representative experiment typical of three.

Liver contains an endogenous inhibitor of $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase

Further experiments were conducted to investigate the mechanism by which ion-exchange chromatography increased $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase activity. Incubations were performed as described in the Materials and methods section; these contained 10 μl of native supernatant [which hydrolysed $\text{Ins}(1,3,4,5)P_4$ predominantly by a 5-phosphatase; Fig. 1] plus 5 μl samples of fractions eluted from the Mono Q column that contained $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase. In these incubations, total $\text{Ins}(1,3,4,5)P_4$ phosphatase was $49 \pm 8\%$ ($n = 3$) of that expected from the sum of the individual 3- and 5-phosphatase activities. These data indicate that a factor in one tissue preparation inhibited an enzyme in the other. Further information was obtained by heat-treating the supernatant, which destroyed all of its $\text{Ins}(1,3,4,5)P_4$ phosphatase activity (Fig. 3). The $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase activity in the Mono Q fractions was inhibited by the addition of heat-treated supernatant, although there was considerable variation between experiments, particularly when less than 20 μl of heat-treated supernatant was added (Fig. 3). Thus we were unable to obtain a precise dose/response relationship. However, it was possible to demonstrate that the addition of a sufficient volume of heat-treated supernatant could completely inhibit the 3-phosphatase activity (Fig. 3). Thus liver cytosol contains an endogenous heat-stable inhibitor(s) of 3-phosphatase. It seems that the separation of the inhibitor from the 3-phosphatase by the Mono Q column causes the enzyme activation described in Fig. 2. Dialysis of the supernatant (see the Materials and methods section) did not significantly affect its ability to inhibit the 3-phosphatase (results not shown). Although this may indicate that the molecular mass of the inhibitor was above 14000 Da, it is also possible that much of the inhibitor resisted dialysis because it was tightly bound to cellular protein. The particulate fraction of the liver cell also contained a heat-stable inhibitor(s) of soluble 3-phosphatase (Fig. 3).

It was recently shown that liver particulate fractions hydrolyse

Fig. 2. Elution of phosphatase activities towards $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ during anion-exchange chromatography of liver supernatant

A 10 ml portion of liver supernatant was fractionated on a Mono Q ion-exchange column as described in the Materials and methods section. Phosphatase activities against $\text{Ins}(1,4,5)\text{P}_3$ (○) and $\text{Ins}(1,3,4,5)\text{P}_4$ (●) were assayed on 4–20 μl samples of each fraction as described in the Materials and methods section, by using gravity-fed columns. The activities in each 1 ml fraction are calculated as first-order rate constants. Two further experiments gave similar results. For details of enzyme recoveries from the column, see the text.

$\text{Ins}(1,3,4,5)\text{P}_4$ with both 5- and 3-phosphatases, in a ratio of about 5:1 when the incubation buffer (see the Materials and methods section) was supplemented with 9 μM - $\text{Ins}(1,3,4,5)\text{P}_4$, plus 5 mM-MgATP to inhibit specifically the 5-phosphatase [9]. We obtained similar results in the present study, in 15 min incubations containing 8 μl of washed particulate fraction (results not shown). The further addition of heat-treated samples (33 μl) of either supernatant or washed particulate fractions decreased

3-phosphatase activity by $78 \pm 6\%$ and $80 \pm 6\%$ respectively. The 5-phosphatase activity in the same incubations was not inhibited by the heat-treated soluble and particulate preparations (the activities were respectively $106 \pm 5\%$ and $107 \pm 1\%$ of control) ($n = 4$, one experiment, typical of two).

General discussion

It has previously been puzzling that hepatic particulate fractions contain significant amounts of $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase, an enzyme never previously observed in liver homogenates (see above and [9]). However, we have now shown that, in liver, $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase is inhibited by a heat-stable endogenous factor(s) in both the soluble and particulate regions of the cell. Presumably the particulate 3-phosphatase activity increases upon its separation from the inhibitor in the soluble fraction. Despite this, the particulate inhibitor may continue to prevent the full expression of particulate 3-phosphatase. The inhibitor, if widespread, would explain why fractionation of RBL-2H3 cell homogenates into soluble and particulate fractions also increased $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase activity [4]. In any case, our data indicate a necessity to reconsider previous determinations [4–10] of the amount of 3-phosphatase activity in both the particulate and the soluble fractions of the cell. We may need to understand the interaction between the inhibitor and the 3-phosphatase *in vivo*, before any studies *in vitro* with cell-free systems are going to help to elucidate the physiological activity of this enzyme.

Our results may also be particularly relevant to an earlier observation that brain contains considerable amounts of $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase [6]. In this study, the source of the enzyme was fractions of cytosol that had been chromatographed on DEAE-Sephacel. The authors of that study did not quote recoveries obtained during their chromatographic procedures, but, in view of our data, we suggest that they may have assayed an activated 3-phosphatase. Certainly the possibility of considerable enzyme activation should be carefully considered during purification. It should also be noted that $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase may be activated by electroporation of cells [7].

Three major questions remain to be addressed: (i) what is the

Fig. 3. Inhibition of soluble $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase by heat-treated supernatant and particulate fractions

The 3-phosphatase preparation was obtained by combining the eight f.p.l.c. fractions containing the most activity (see Fig. 2); these fractions were diluted 5-fold, and samples (20 μl) were assayed as described in the Materials and methods section, by using gravity-fed columns. Incubations also contained 0–80 μl of heat-treated cytosol (○) or particulate fractions (▲). The inhibition of 3-phosphatase activity is plotted as a percentage relative to the activity in incubations with 0 μl of the heat-treated tissue. Heat-treated preparations did not hydrolyse $\text{Ins}(1,3,4,5)\text{P}_4$. Five experiments were performed, each of which generated between one and four data points, which in turn represent the means ($\pm$ S.E.M. where these exceed the size of the symbol) from 3–4 determinations. Data from different experiments were not combined.

nature of the 3-phosphatase inhibitor(s)?; (ii) what is the extent of Ins(1,3,4,5) P_4 3-phosphatase activity *in vivo*?; (iii) is the inhibitory factor part of some regulatory process that contributes to the control of cellular levels of Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , and hence their second-messenger activities?

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Received 2 February 1990/23 February 1990; accepted 28 February 1990