Subcellular localization and some properties of the enzymes hydrolysing inositol polyphosphates in rat liver

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The hydrolysis of inositol [^{32}P]trisphosphate (IP_3) and inositol [^{32}P]bisphosphate (IP_2) has been examined in subcellular fractions of rat liver. IP_3 was degraded by an enzyme located in the plasma membrane which did not degrade IP_2. Cytosolic fractions were found to degrade both IP_2 and IP_3. The IP_3 phosphatase activity of liver plasma membranes displayed a neutral pH optimum, was Mg^{2+} dependent and was not inhibited by high concentrations of Li^+. Half-maximal activity of the enzymes hydrolysing IP_3 and IP_2 was obtained with substrate concentrations in the range 1–2 μM. The significance of these observations to the proposed Ca^{2+}-mobilizing role of IP_3 is discussed.

Inositol trisphosphate *Inositol lipid* *Phosphatase* *Magnesium* *Calcium*

1. INTRODUCTION

The addition of *myo*-inositol 1,4,5-trisphosphate (IP_3) to a variety of different permeabilized cell types has been found to induce the rapid release of Ca^{2+} from a non-mitochondrial, intracellular store (review, [1]). These observations, taken together with the finding that this compound accumulates in cells stimulated with Ca^{2+}-mobilizing agonists, strongly suggest that IP_3 may act as an intracellular messenger mediating the effects of a large number of hormones and neurotransmitters whose mode of action involves an increase in the cytoplasmic free Ca^{2+} concentration [2,3]. The accumulation of IP_3 observed upon receptor occupation is thought to reflect the increased activity of a phosphodiesterase (phospholipase C) acting upon phosphatidylinositol 4,5-bisphosphate (PIP_2). Termination of the response, i.e., by displacement of the hormone from its receptor, promotes the return of IP_3 levels to control values [4,5]. It has been inferred that enzymes are present in these cells which can degrade IP_3 but the subcellar site of this degradation or the regulatory properties of these enzymes have not been studied. Further support for the presence of an enzyme catalysing IP_3 hydrolysis stems from the finding that the slow reuptake of Ca^{2+} released by IP_3 from the permeabilized hepatocyte correlates with the disappearance of this compound from the incubation medium [6]. Erythrocytes have been shown to possess an enzyme that can specifically degrade inositol 1,4,5-trisphosphate to produce inositol 1,4-bisphosphate (IP_2) [7]. A similar activity has also been observed in homogenates of insect salivary gland [8] and iris smooth muscle [9]. Here, we have utilized ^{32}P-labeled IP_3 and IP_2 to examine the subcellular distribution of the phosphatases acting on these substrates in rat liver. Our findings indicate that distinct enzymes are present in the plasma membrane and soluble fractions of the cell which can hydrolyze IP_3 and IP_2, respectively. Some properties of these enzymes are described.

2. MATERIALS AND METHODS

^{32}P-labeled IP_3 and IP_2 were prepared from their respective inositol-lipid precursors by phospholipase C-mediated hydrolysis of human erythrocyte membranes as described by Downes et al. [7]. The phospholipids were prelabeled by incubating the washed erythrocytes with ^{32}P for 12 h at 30°C as

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described by Downes and Michell [10]. It should be pointed out that in the erythrocyte this procedure labels only the monoester phosphates of the polyphosphoinositides (4 and 5 positions) without labeling the diester phosphate [7,10]. The concentrations of inositol polyphosphates were determined by measurement of phosphate content after perchloric acid digestion [11]. The basic incubation medium (pH 7.2, 30°C) used to incubate the various subcellular fractions contained, in addition to the labeled substrates, the following components (mM): KCl (110), NaCl (10), KH_2PO_4 (1), K^+-Hepes (20) and $MgCl_2$ (3). Aliquots (0.25 ml) were removed at appropriate times and deproteinized with 0.25 ml of ice-cold trichloracetic acid (20%, w/v). After removal of precipitated protein by centrifugation, the supernatant was diluted with 1 ml of water, neutralized and loaded onto small (approx. 0.6 ml) columns of Dowex-1 (formate form). Inorganic phosphate, IP_2 and the IP_3 were sequentially eluted from the column as described previously [4].

Plasma membranes were prepared using isotonic Percoll gradients as described by Prpic et al. [12]. Other fractions were prepared by differential centrifugation of a 10% (w/v) liver homogenate prepared in 0.25 M sucrose and 20 mM Tris-Hepes (pH 7.2). Mitochondria were isolated from this homogenate as described previously [13]. The post-mitochondrial supernatant was centrifuged at 65 000 × *g* and the resulting pellet and supernatant was used as the microsomal and cytosolic fractions, respectively. The following assay methods were used for marker enzymes: glutamate dehydrogenase [14], NADPH-cytochrome *c* reductase [15] and 5′-nucleotidase [16].

3. RESULTS AND DISCUSSION

Subcellular fractions prepared from rat liver were tested for their ability to hydrolyze added ^{32}P-IP_3 and ^{32}P-IP_2 (table 1). Plasma membranes contained the highest specific activity of an enzyme degrading IP_3. A 10-fold enrichment of this activity relative to the homogenate was obtained. The cytosolic fraction also contained IP_3-phosphatase but at a specific activity that was 20–30% of that found in the plasma membrane (table 1, fig. 4). Marker enzyme analysis indicates only a 3% contamination of the cytosolic fraction by plasma membranes and this suggests that the cytosolic IP_3-phosphatase may reflect a genuine, soluble

Table 1

Subcellular localization of the phosphatases degrading inositol trisphosphate and inositol bisphosphate in rat liver

Subcellular fraction	Rate of inositol phosphate degradation (nmol/mg protein per min)		Plasma membranes, 5′-nucleotidase		Endoplasmic reticulum NADPH-Cyt *c* reductase		Mitochondria, Glutamate dehydrogenase	
	IP_3	IP_2	– fold enrichment	% contamination	– fold enrichment	% contamination	– fold enrichment	% contamination
Homogenate	0.17	0.27						
Plasma membranes	1.86	0	28.1	–	0.7	27.4	0.03	0.7
Cytosol	0.35	0.40	0.9	3.0	0.3	11.8	0.1	1.4
Endoplasmic reticulum	0.56	0	2.2	7.8	2.5	–	0.3	7.5
Mitochondria	0.10	0	2.1	7.3	0.3	11.5	4.6	–

The degradation of ^{32}P-labeled IP_3 (3 μM) and IP_2 (6.5 μM) was measured in various subcellular fractions of rat liver. The purity of these fractions was assessed by the measurement of marker enzymes. The 'fold enrichment' was calculated as the ratio of the specific activity of the marker enzyme in a given fraction to the specific activity measured in the homogenate. The % cross-contamination was calculated with the assumption that the specific activity of the marker enzymes for plasma membranes, ER and mitochondrial fractions represent 100% purity. The results shown are the mean of 2 experiments in which the individual values differed by < 15% of the mean. The specific activity of inositol polyphosphate hydrolysis was calculated without correction for any impurity of the added substrates

form of the enzyme. Similar considerations suggest that the IP_3-phosphatase activity found in the endoplasmic reticulum and mitochondrial fractions can be entirely accounted for by plasma membrane contamination.

The time copurse of the degradation of 1.45 μM ^{32}P-IP_3 by liver plasma membranes and the accumulation of products is shown in fig. 1. Aliquots of the incubation removed at 'zero-time' and analyzed by anion exchange chromatography indicated that almost 90% of the total ^{32}P counts eluted as Ip_3. This assessment of the radiochemical purity of the IP_3 was in agreement with results obtained after thin layer chromatography of Ip_3 on polyethylenimine cellulose [17] (not shown). With increasing incubation time, the plasma membranes decreased the proportion of counts as IP_3 and this was accompanied by an increase in the counts present as inorganic phosphate and IP_2. Under these conditions linear changes were observed over a 2.5 min interval. It was consistently found that the production of P_i exceeded that of IP_2. In 5 separate experiments the relative ratio of counts in P_i and IP_2 measured after 2 min incubation was 2.1 ± 0.2 (mean ± SE). Such a result could arise if the IP_2 formed from the hydrolysis of IP_3 was further dephosphorylated by the plasma membranes. An alternative explanation is that the IP_3 derived from the erythrocyte may not be equally labeled with ^{32}P in the 4 and 5 positions of the inositol ring, and the plasma membrane enzyme may selectively cleave only the more highly labeled phosphate. Experimental support for the latter explanation comes from the finding that liver plasma membranes were unable to hydrolyse added ^{32}P-IP_2 (fig. 1B). Table 1 shows that a phosphatase acting on this compound is located exclusively in the soluble fraction of the cell. Both erythrocyte membranes [7] and salivary gland homogenates [8] also release more P_i than IP_2 when given erythrocyte ^{32}P-IP_3 as substrate. Recently, Hawkins et al. [18] have established that 60–70% of the ^{32}P-label in this molecule is present on the phosphate attached at the 5 position. Hence, our experimental results are compatible with the presence in the liver plasma membranes of an IP_3-phosphatase that selectively removes phosphate from the 5-position to produce IP_2 which can then be further dephosphorylated by a separate cytosolic enzyme.

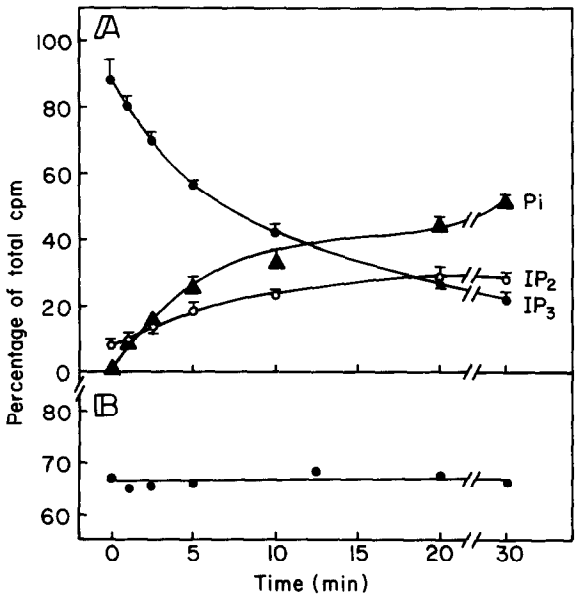

Fig. 1. Time course of degradation of ^{32}P-IP_3 (A) and ^{32}P—IP_2 (B) by rat liver plasma membranes. Liver plasma membranes (0.7 mg/ml) were incubated with ^{32}P-IP_3 (1.45 μM; cpm/nmol) and ^{32}P-IP_2 (3.5 μM; 720 cpm/nmol). Samples were removed at the indicated times, quenched with trichloracetic acid and analyzed for reaction products as described in section 2. The results shown in (A) are the mean ± SD of 4 determinations and those in (B) are the means of duplicate determinations.

The pH dependence of IP_3 hydrolysis by plasma membranes was measured over the range pH 6–9 (fig. 2). Optimal activity was obtained at pH 7.0,

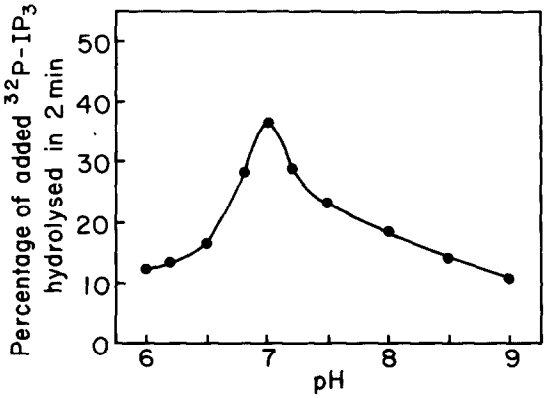

Fig. 2. pH Dependence of IP_3 hydrolysis by liver plasma membranes. The pH of the normal incubation buffer (c.f. section 2) was adjusted to the indicated pH using NaOH or HCl. The concentration of IP_3 used in the experiment was 3.6 μM.

suggesting that alkaline or acid phosphatases are unlikely to be responsible for the IP_3 dephosphorylation catalyzed by plasma membranes. Further support for the view that dephosphorylation of IP_3 is not the result of a non-specific phosphatase activity comes from the finding that the hydrolysis of 1.5 μM. ^{32}P-IP_3 is unaffected by the presence of 5 mM of either P-nitrophenylphosphate or ATP (not shown).

Fig. 3 shows that the hydrolysis of ^{32}P-IP_3 by plasma membranes was entirely dependent on the presence of Mg^{2+} in the incubation medium. Similar results were obtained for the hydrolysis of ^{32}P-IP_2 by the cytosolic fraction (not shown). The concentration of Mg^{2+} required for half-maximal IP_3-phosphatase activity was approximately 0.25 mM (fig. 3). Lithium is known to inhibit the activity of inositol 1-phosphate phosphatase [19] and pretreatment of hepatocytes with this ion causes an increase in the accumulation of inositol phosphates upon stimulation with vasopressin. A maximal effect on IP_3 accumulation was obtained with 20 mM LiCl [4]. However, 20 mM LiCl had no effect on the hydrolysis of IP_3 catalyzed by plasma membranes when tested over a range of IP_3 and Mg^{2+} concentrations (not shown). Thus these effects of Li^+ on IP_3 accumulation observed in hormone-treated hepatocytes is unlikely to be the result of an inhibitory effect of Li^+ on IP_3-phosphatase. Alternative indirect effects of this cation on inositol lipid metabolism remain to be explored.

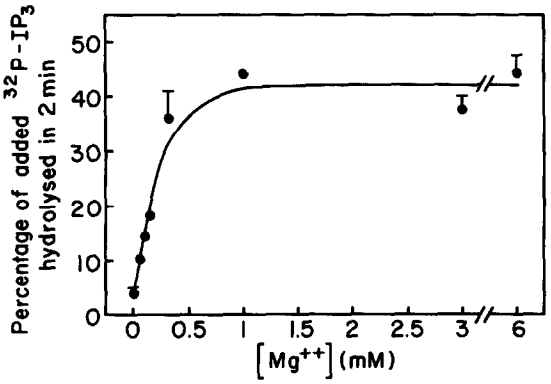

Fig. 3. Magnesium dependence of IP_3 hydrolysis by liver plasma membranes. The experimental conditions were as described for fig. 1. Each determination was made in duplicate and the results shown are the mean of 2–3 separate experiments.

The dependence of enzyme activity on the concentration of added IP_3 using plasma membranes and cytosolic fractions is shown in fig. 4A. IP_3-phosphatase activity in the cytosolic fraction

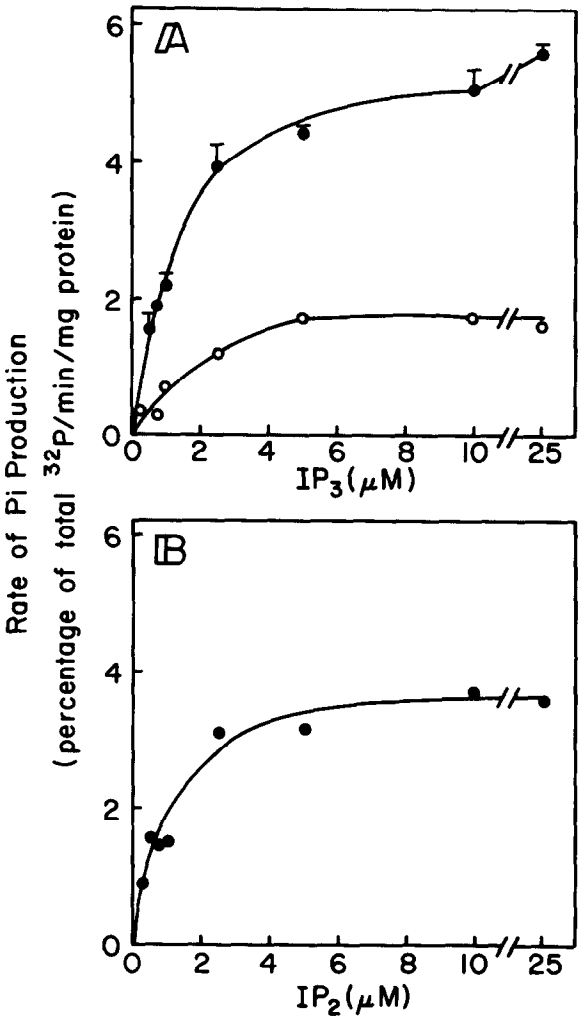

Fig. 4. Concentration dependence of IP_3 (A) and IP_2 (B) hydrolysis. (A) Liver plasma membranes (0.7 mg protein/ml; •—•) or a cytosolic fraction (1.6 mg protein/ml; ○—○) were incubated with the indicated concentrations of ^{32}P-IP_3 for 2 min. The ^{32}P-phosphate released over this interval was measured and is expressed as a percentage of the total ^{32}P added to the incubation. The results shown are the mean of 2 experiments or, where error bars are shown, the mean ± SE of 3 experiments. (B) The experimental conditions were identical except that ^{32}P-IP_2 was used as the substrate for the enzyme present in the cytosolic fraction.

had a maximum activity that was 30% of that found in the plasma membranes. However, both fractions required approximately the same concentration of IP_3 for half-maximal activity. Double reciprocal plots of the data gave values of 1.4 and 1.0 μM, respectively, for the membrane and soluble forms of the enzyme. Dose-response studies were carried out with IP_2 as a substrate for the enzyme activity present in the cytosolic fraction (fig. 4B). Maximal activity of this enzyme was 2-fold greater than the IP_3-phosphatase found in this fraction (fig. 4A). Half-maximal hydrolysis of IP_2 was obtained at a concentration of 0.8 μM.

The apparent K_m of the IP_3 phosphatase present in the erythrocyte membrane has been reported to be 25 μM [7], which is substantially higher than we find for the liver enzyme. Nevertheless, in many respects the two enzymes display identical properties. This applies to their pH optima, Mg^{2+} dependence and selective hydrolysis of the 5-phosphate on the inositol ring. Our findings are in qualitative agreement with a recent study of IP_3 hydrolysis in liver reported by Seyfred et al. [20].

At present, it cannot be excluded that the soluble form of the IP_3 phosphatase (table 1, fig. 4A) does not arise from the dissociation of the plasma membrane enzyme during subcellular fractionation. It is clear, however, that the greater portion of this activity is located in the plasma membrane. If this result can be extrapolated to the intact cell, the conclusion can be drawn that the plasma membrane is both the site of production and degradation of this messenger molecule. However, Ca^{2+} release promoted by IP_3 occurs from a specialized part of the hepatic endoplasmic reticulum [3]. Presumably, IP_3 reaches this site as a consequence of diffusion from the plasma membrane. Very little is known concerning the exact location of the IP_3-releasable Ca^{2+} store within the cell or the diffusion properties of IP_3. The rapidity with which hormones can cause the internal mobilization of Ca^{2+} prompts the speculation that the IP_3-sensitive Ca^{2+} store may also lie in close proximity to the plasma membrane.

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REFERENCES

[1] Joseph, S.K. (1984) TIBS 9, 420–421.
[2] Berridge, M.J. (1984) Biochem. J. 220, 345–360.
[3] Williamson, J.R., Cooper, R.H., Joseph, S.K. and Thomas, A.P. (1985) Am. J. Physiol., in press.
[4] Thomas, A.P., Alexander, J. and Williamson, J.R. (1984) J. Biol. Chem. 259, 5574–5584.
[5] Downes, C.P. and Wusteman, M.M. (1981) Biochem. J. 216, 633–640.
[6] Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F. and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077–3081.
[7] Downes, C.P., Mussat, M.C. and Michell, R.H. (1982) Biochem. J. 203, 169–177.
[8] Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–482.
[9] Akhtar, R.A. and Abdel-Latif, A.A. (1980) Biochem. J. 192, 783–791.
[10] Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140.
[11] Hess, H.H. and Derr, J. (1975) Anal. Biochem. 63, 607–613.
[12] Prpic, V., Green, K.C., Blackmore, P.F. and Exton, J.H. (1984) J. Biol. Chem. 259, 1382–1385.
[13] Schneider, W.C. and Hogeboom, G.H. (1950) J. Biol. Chem. 183, 123–128.
[14] Schmidt, E. (1974) in: Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.) vol. 2, pp. 650–656, Academic Press, New York.
[15] Master, B.S.S., Williams, C.H. and Kamin, H. (1962) Methods Enzymol. 10, 565–567.
[16] Ipata, P.L. (1967) Anal. Biochem. 10, 30–36.
[17] Emilsson, A. and Sundler, R. (1984) J. Biol. Chem. 259, 3111–3116.
[18] Hawkins, P.T., Michell, R.H. and Kirk, C.J. (1984) Biochem. J. 218, 785–793.
[19] Allison, J.H. (1978) in: Cyclitols and Phosphoinositides (Eisenberg, F. and Wells, W.W. eds) pp. 507–519, Academic Press, New York.
[20] Seyfred, M.A., Farrell, L.E., and Wells, W.W. (1984) J. Biol. Chem. 259, 13204–13208.