Phosphatidylcholine enhances the activity of rat liver type II phosphatidylinositol-kinase

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Received 16 May 1993; revised version received 13 June 1993

A PtdIns 4-kinase was purified extensively from rat liver exocytotic vesicles. The enzyme had a low K_m for ATP, was inhibited by adenosine, and had an apparent molecular mass of 54 kDa, indicating it to be a type II PtdIns-kinase. The activity of the purified enzyme was enhanced several-fold by PtdCho, and to some extent by other phospholipids with basic polar head groups, and was inhibited by PtdSer. Kinetic analyses, presenting the substrate in mixed micelles of Triton X-100, PtdIns and PtdCho, showed that the effect of PtdCho was both to increase V_{max} and to decrease the apparent K_m for micellar PtdIns.

Phosphatidylinositol kinase; Phosphatidylcholine dependence; Rat liver

1. INTRODUCTION

A major signal transduction mechanism operates through agonist-stimulated hydrolysis of PtdIns(4,5)P₂ (for reviews see [1,2]). The limited supply in the plasma membrane of PtdIns(4,5)P₂ is replenished from PtdIns through two successive phosphorylation steps, in turn catalysed by PtdIns 4-kinase and PtdIns4P 5-kinase. These two enzymes differ in their subcellular distribution in rat liver [3,4] and other types of cells [5], with PtdIns 4-kinase preferentially located in intracellular membranes [6,7] and PtdIns4P 5-kinase in plasma membranes [3,8]. PtdIns 4-kinase enriched intracellular membranes were isolated from rat liver [7] and shown to contain mature export proteins [9], suggesting a close connection between the exocytotic and PtdIns(4,5)P₂ regeneration pathways in this tissue.

As part of more detailed studies of the PtdIns $(4,5)P_2$ regeneration pathway in rat liver, we are currently purifying PtdIns 4-kinase from isolated exocytotic membranes. Initially we encountered inexplicably large losses of kinase activity after various chromatographic steps. These losses were counteracted by a phospholipid extract obtained from the membranes, the activating

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Abbreviations: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanol; PtdOH, phosphatidic acid; CMC, critical micelle concentration; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid.

component turning out to be PtdCho. As activation of PtdIns 4-kinase by phospholipids has not been reported earlier, we have now more closely examined the lipid dependency of this enzyme, highly purified from rat liver exocytotic membranes.

2. EXPERIMENTAL

2.1. Materials

PtdIns, PtdIns4P and PtdIns(4,5)P₂ were purified [10] from Sigma Type I brain extract. Lyso-PtdCho and PtdGro were purchased from Sigma and other lipids from Serdary (London, Ontario, Canada). $[\gamma^{-32}P]$ ATP was prepared as described [11]. Heparin-Sepharose and the Mono S Hr 5/5 column were from Pharmacia LKB Biotechnology, hydroxylapatite (fast flow) from Calbiochem, and Silica gel 60 plates from Merck.

2.2. Assay of PtdIns 4-kinase activity

Lipids dissolved in chloroform were evaporated to dryness under a stream of nitrogen, suspended in Triton X-100 and sonicated in a sonication bath for 10 min. The standard PtdIns-kinase assay mixture contained in a final volume of 50 µl: 50 mM HEPES-NaOH, pH 7.2, 10 mM MgCl₂, 1 mM DTE, 0.5 mM PtdIns, 1 mM PtdCho, 4 mM Triton X-100, 1 mM [γ -32P]ATP (500-600 Bq/nmol) and enzyme (0.1-1 μg). The concentrations of Triton X-100 and phospholipids given as bulk ones (mM) should be regarded as formal concentrations only. After preincubation for 2 min at 25°C the reaction was started by adding ATP and terminated after 2-10 min with 1 ml CHCl₂/methanol (1:1, v/v). After addition of 0.5 ml 1.2 M HCl phosphoinositides were extracted into the lower chloroform phase, which was washed with 1 ml methanol/1 M HCl (1:1, v/v) followed by 1 ml methanol/0.1 M HCl (1:1, v/v). Routinely, [32P]PtdIns4P was isolated from the washed bottom phase by TLC on oxalate treated silica plates [3] and measured by scintillation spectrometry. Alternatively the washed bottom phase was spotted on filter paper discs (Whatman 3 MM, 2.4 cm in diameter) without prior separation of phosphoinositides. The filters were dried in air and counted. This simplified procedure could be used after partial purification of the enzyme (after the heparin-Sepharose step), yielding the same results as the more elaborate TLC protocol.

Any Triton X-100 present in the enzyme sample and the CMC of the detergent (0.24 mM; excluded from the micelles) were accounted for when the micellar concentrations of Triton X-100 were calculated. In several cases the concentrations of Triton X-100 and phospholipids were varied as indicated. Assays were performed in duplicate, and were linear with protein concentration and incubation time.

2.3. Purification of PtdIns 4-kinase

Exocytotic membranes, 30-fold enriched in PtdIns 4-kinase, were prepared from rat liver [7] and solubilised in 50 mM HEPES-NaOH, pH 7.2, 0.15 M KCl, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithioerythritol and 2% (w/v) Triton X-100 to a final protein concentration of about 5 mg/ml. The suspension was agitated at 4°C for 30 min, and then centrifuged at 100,000 × g for 60 min. The clear supernatant (about 40 mg of protein), containing more than 90% of the PtdIns 4-kinase activity originally present in the membranes, was diluted with an equal volume of 25 mM HEPES-NaOH, pH 7.2, 20% (v/v) glycerol, 1 mM EGTA, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithioerythritol and 0.2% (w/v) Triton X-100, and applied to a heparin-Sepharose column $(1.6 \times 10 \text{ cm})$ equilibrated with this buffer mixture. After washing with 0.1 M NaCl in the buffer, the kinase was eluted with a 200-ml linear 0.1-0.6 M NaCl gradient. Fractions containing activity were combined and applied to a hydroxylapatite column (1.0 × 10 cm) equilibrated with 10 mM potassium phosphate, pH 7.2, 20% (v/v) glycerol, 1 mM dithioerythritol and 0.1% (w/v) Triton X-100. Approximately 75% of the PtdIns 4-kinase activity eluted in the flow through. This was dialysed against 20 mM potassium phosphate, pH 6.8, 20% (v/v) glycerol, 1 mM EGTA, 1 mM dithioerythritol and 0.1% (w/v) Triton X-100, and applied to a Mono S column equilibrated in the same buffer mixture. The column was washed with 10 ml of the buffer followed by elution of the enzyme with a 25-ml linear 0-1.0 M NaCl gradient in the buffer. Fractions containing kinase activity were combined and stored at -20°C. The enzyme was stable for several months under these conditions.

2.4. Renaturation of PtdIns kinase activity after SDS-PAGE

SDS-PAGE was performed as described [12] using 10% separation gels. Polypeptides were localised by silver staining [13]. One-mm-slices were excised from an unstained lane run in parallel and homogenised in 100 μ l renaturation buffer (50 mM HEPES-NaOH, pH 7.2, 2% (w/v) Triton X-100, 20 mM MgCl₂, 10 mM dithioerythritol, 0.5 mM

PtdIns, 1 mM PtdCho and 1 mM [γ -32P]ATP, 600 Bq/nmol) and incubated overnight at room temperature. Fifty- μ l-aliquots were examined for [32 P]PtdIns4P formed as for the enzyme TLC assay procedure above.

2.5. Identification of the reaction product

The identity of ³²P-labeled PtdInsP generated by the enzyme was tested by TLC on CDTA-treated silica gel 60 plates in the presence of borate [14]. The positions of labeled products were determined by autoradiography and compared to standard phospholipids.

2.6. Other methods

Protein concentrations were determined [15] using bovine serum albumin as a standard. Concentrations of phospholipids in chloroform stock solutions were confirmed by phosphate determination [16].

3. RESULTS

3.1. Purification and characterisation of PtdIns 4-kinase

PtdIns 4-kinase was purified from extracts of rat liver exocytotic membranes highly enriched in the enzyme by successive chromatographies on heparin-Sepharose, hydroxylapatite and a Mono S column (see section 2 for details). The kinase bound to heparin-Sepharose in the presence of 0.1 M NaCl, and eluted as a single peak at 0.22 M NaCl in a salt gradient (Fig. 1). The enzyme also eluted as a single active peak, at 0.25 M NaCl, from the Mono S column, indicating enzyme homogeneity. The preparation procedure led to a 120-fold purification over the extract with 50% recovery of the enzyme activity (analysed in the presence of PtdCho, see below) and a specific activity of approximately 450 nmol/min · mg protein. As the enzyme in isolated exocytotic membranes was already 30-fold enriched compared to the liver homogenate [7], the overall purification was around 3,500 times.

In spite of the high purification factor, SDS-PAGE

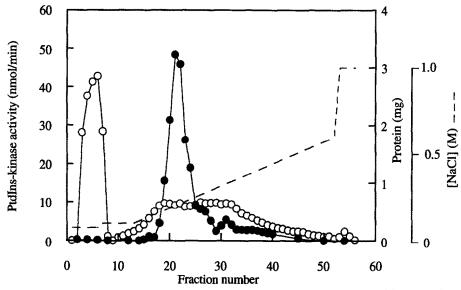


Fig. 1. Heparin-Sepharose chromatography of PtdIns 4-kinase. Rat liver exocytotic membranes were isolated from 60 g liver, solubilised in Triton X-100 (40 mg protein) and chromatographed on a heparin-Sepharose column as described in section 2. Ten ml fractions were collected during sample application and washing, and 5 ml fractions during elution. PtdIns 4-kinase activity (•) and protein concentration (o) were determined as described in section 2. Dashed line, NaCl gradient.

of the purified material followed by silver staining revealed several bands. After slicing the gel and renaturation, PtdIns-kinase activity was detected in a band with the apparent molecular mass of 54 kDa. The reaction product of the purified enzyme comigrated with a PtdIns4P standard in a TLC system able to separate PtdIns4P from PtdIns3P [14], identifying the enzyme as a PtdIns 4-kinase. These facts, together with an apparent $K_{\rm m}$ for ATP of 35 μ M and a $K_{\rm l}$ for adenosine of 10 μ M (not shown), indicated the enzyme to be a type II PtdIns-kinase [17].

3.2. Stimulation of PtdIns 4-kinase activity by PtdCho

The activity of the purified enzyme was enhanced by PtdCho (Fig. 2). Thus, PtdIns-kinase activity increased when the surface concentration of PtdCho in mixed micelles of Triton X-100:PtdIns:PtdCho was increased at constant surface and set concentrations of PtdIns (10 mol%, respectively 0.5 mM, substrate saturation). A maximum 4-fold stimulation was reached at approx. 20 mol% PtdCho. Addition of PtdCho to solubilised exocytotic membranes did not affect the endogenous enzyme activity significantly (Fig. 2), suggesting that sufficient PtdCho (or other potentially stimulating lipids) to fully stimulate the enzyme was present in the extract. The enzyme became PtdCho-dependent, however, after the heparin-Sepharose step.

3.3. Effect of micellar composition on PtdIns 4-kinase activity

The composition and the total concentration of micelles were varied in order to examine how this would affect the enzyme activity. Thus, the concentration of Triton X-100 was varied (Fig. 3) at three set levels of PtdIns (0.02, 0.1 and 0.5 mM) in the absence or presence

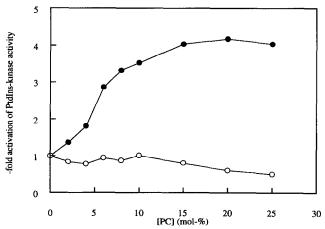
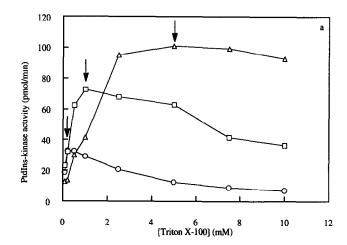


Fig. 2. Stimulation of PtdIns-kinase by PtdCho. The effect of PtdCho on the activity of purified PtdIns-kinase (•) and enzyme in solubilised exocytotic membranes (•) was determined using Triton X-100 mixed micelles containing 10 mol% PtdIns and various amounts of PtdCho. The PtdIns-kinase activity was assayed as described in section 2. Data are means of duplicate incubations. The specific activity of the enzyme in the absence of PtdCho was 138 nmol/min/mg protein.



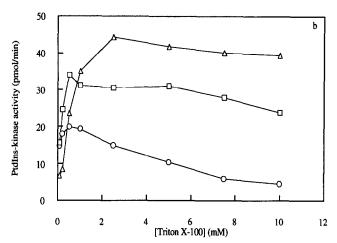


Fig. 3. Effect of Triton X-100 concentration on PtdIns 4-kinase activity. The activity of purified PtdIns-kinase was assayed as described in section 2 at various Triton X-100 concentrations at three set concentrations of PtdIns; $0.02 (\bigcirc)$, $0.1 (\square)$ and $0.5 (\triangle)$ mM, in the presence (a) or absence (b) of PtdCho in a 2:1 molar ratio to PtdIns. Data are means of duplicate incubations. Arrows indicate activity maximum at each set concentration of PtdIns.

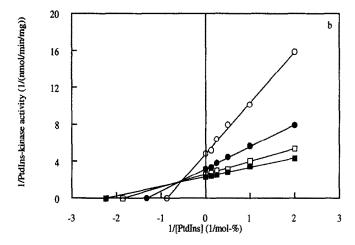
of PtdCho (2:1 ratio to PtdIns). The PtdIns kinase activity depended not only on the set level of PtdIns, but also on the relative amount of PtdIns in the micelles, i.e. on the PtdIns surface concentration (at each set level of PtdIns this decreased with increasing Triton X-100 concentration). This dependency was most pronounced in the presence of PtdCho, when activity maximum at each set concentration of PtdIns was observed at a micellar surface concentration of 7.7 mol% PtdIns (indicated by arrows). A further decrease in the micellar surface concentration of PtdIns, brought about by increasing the amount of Triton X-100 and keeping PtdIns constant, led to a decrease in the kinase activity. This decrease was more pronounced at low set PtdIns levels. Similar results were obtained in the absence of PtdCho, although higher PtdIns surface concentrations (approx.

17 mol%) were required than in the presence of PtdCho at 0.1 and 0.5 mM PtdIns (Fig. 3).

A kinetic analysis of the effect of PtdCho was also performed by examining the substrate saturation curves at different PtdCho concentrations (Fig. 4a), keeping the micellar concentration constant. Double reciprocal plots of PtdIns-kinase activity versus PtdIns surface concentration (Fig. 4b) indicated that PtdCho affected both $V_{\rm max}$ and the apparent $K_{\rm m}$ for PtdIns.

3.4. Effect of various phospholipids on PtdIns-kinase activity

We also examined how other phospholipids would affect the enzyme activity (Table I). Only those with basic polar head groups were stimulatory, although less so than PtdCho; lyso-PtdCho enhanced the activity 2.5-fold, PtdEtn and sphingomyelin less than 2-fold.



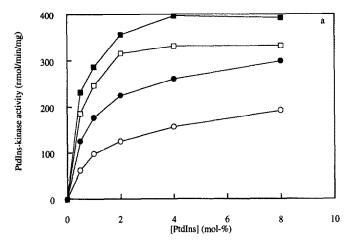


Fig. 4. Dependency of PtdIns 4-kinase activity on micellar surface concentration of PtdIns at various surface concentrations of PtdCho. (A) PtdIns-kinase activity was measured as a function of the micellar concentration of PtdIns at 0 (○), 4 (●), 6 (□) and 10 (■) mol% of PtdCho. The sum of the molar concentrations of Triton X-100, PtdIns and PtdCho was kept constant at 5 mM. (B) Lineweaver—Burke plot of the data using least-squares analysis to fit the lines. The intercepts with abscissa and ordinate are also indicated by symbols.

Table I

Effects of various lipids on PtdIns 4-kinase activity

Lipid added to PtdIns	% of control
None	100 ± 3
Phosphatidylcholine	378 ± 15
Lyso-phosphatidylcholine	246 ± 7
Sphingomyelin	172 ± 11
Sphingosine	96 ± 16
Phosphatidylethanolamine	161 ± 16
Phosphatidylglycerol	111 ± 16
Phosphatidic acid	77 ± 10
Phosphatidylserine	55 ± 9
PtdIns4P	50 ± 9
PtdIns(4,5)P ₂	70 ± 14

PtdIns 4-kinase activity was tested as described in section 2 using mixed micelles of Triton X-100 containing 10 mol% (0.5 mM) PtdIns and 20 mol% (1.0 mM) of the lipids to be tested. Data are means ± S.E. based on four experiments performed in duplicate. The specific activity of the control (PtdIns alone) was 138 nmol/min/mg.

PtdSer, in contrast, decreased the activity to half, whereas PtdGro, PtdIns(4,5)P₂ and PtdOH only had slight effects. Sphingosine, in contrast to sphingomyelin, did not affect the activity. PtdIns4P was inhibitory, presumably due to product inhibition, similar to an earlier observation as regards PtdIns4P 5-kinase [18].

4. DISCUSSION

We have quite extensively, and with a high yield, purified PtdIns-kinase from rat liver exocytotic vesicles. Several properties of the enzyme (PtdIns4P as reaction product, a low K_m for ATP, adenosine sensitivity and an apparent molecular mass of 54 kDa) indicated it to be a type II PtdIns 4-kinase [17]. The same type of PtdIns 4-kinase has been purified extensively from other sources [19–24], but this has not been possible earlier for the rat liver enzyme (cf. [25]). The finding of the PtdCho dependency of the enzyme facilitated the design of a purification protocol. Another liver PtdIns-kinase, isolated from porcine liver microsomes [26], differed from the type II kinases by not being dependent on Triton X-100.

PtdCho stimulated the activity of the purified enzyme severalfold when included in the Triton X-100:PtdIns mixed vesicles used as substrate. Other phospholipids with basic polar head groups were less stimulatory, whereas PtdSer inhibited the enzyme activity. Such a sensitivity of PtdIns-kinases to the phospholipid composition of the micellar substrate has not been reported before, although the substrate presentation form has turned out to be important for other lipid-metabolising enzymes [27–30].

More detailed studies showed that the effect of PtdCho was to increase $V_{\rm max}$ and to decrease the apparent $K_{\rm m}$ for the lipid substrate. The micellar surface concentration of PtdIns was also critical for enzyme activ-

ity, particularly at subsaturating substrate concentrations. Thus, great care has to be taken to deliver the substrate to PtdIns kinase in an optimal form. A defined micellar composition is particularly critical in kinetic analyses as shown in studies on PtdIns-kinase from yeast [19,31], in which Triton X-100:PtdIns mixed micelles were used. It should be of interest to test the effect of additional phospholipids on these enzymes, and on other PtdIns-kinases under defined conditions. In addition to the PtdCho sensitive type II enzyme, we have isolated a second PtdIns 4-kinase from rat liver microsomes (unpublished work). This enzyme is not stimulated by PtdCho and has a high K_m for ATP, a high K_i for adenosine, and is relatively insensitive to Ca2+ inhibition, and, therefore, has other properties than the type II kinases.

One question is whether the effect of PtdCho and PtdSer also applies in the natural membrane environment. Studies on protein kinase C show that a micellar surface composition may mimic the membrane in regulating the enzyme activity [28,32]. Variations in membrane phospholipid concentrations might then be a means to modulating PtdIns-kinase activity, for instance, to assure PtdIns4P production in the proper compartment along the exocytotic pathway.

Acknowledgements: We thank Mrs Hildegun Lundberg for skilful technical assistance. This work was supported by the Swedish Natural Science Research Council.

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