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Presence of a novel form of phosphatidylinositol 4-kinase in rat liver

Henric Olsson[a,**], Wilma Martínez-Arias[a], Bjørn K. Drøbak[b], Bengt Jergil[a,*]

[a]*Biochemistry, Chemical Centre, University of Lund, PO Box 124, S-221 00 Lund, Sweden*
[b]*Department of Cell Biology, John Innes Institute for Plant Science Research, Norwich, UK*

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Abstract Rat liver microsomes contain two distinct forms of PtdIns 4-kinase which were resolved by heparin-Sepharose chromatography. One enzyme was identified as the type II PtdIns kinase previously isolated from exocytotic vesicles. The other enzyme, however, was a novel PtdIns 4-kinase isoform with properties differing from any other PtdIns kinase so far characterized. Both kinases were recognized by a monoclonal antibody specific for type II PtdIns 4-kinase, but the novel enzyme was considerably less sensitive to inhibition by adenosine and Ca^{2+} than type II enzymes, and in addition was specifically inhibited by submillimolar concentrations of dithioerythritol. The presence of a novel PtdIns 4-kinase isoform in rat liver raises the question of whether this enzyme is unique for this organ or whether it has a more widespread distribution but so far has avoided detection.

Key words: Signal transduction; Phosphoinositide; Phosphatidyl inositol 4-kinase; Rat liver

1. Introduction

PtdIns 4-kinase catalyses the first committed step in the formation of polyphosphoinositides. Increased turnover of polyphosphoinositides and second messenger production are key events in the transduction of hormonal, neuronal and growth factor signals in a wide variety of cells [1]. In addition to their roles as second messenger precursors there are now several lines of evidence which suggest that the polyphosphoinositides may have other functions, for instance in regulation of enzyme activity [2] and control of cytoskeletal dynamics [3,4].

Two forms of PtdIns 4-kinase have been identified in various tissues [5,6]. One of these, the type II PtdIns kinase [7], has been purified and characterized from several tissues [8–13], whereas the second isoform, the type III kinase, remains less well defined [14]. In rat liver a large fraction of the total PtdIns 4-kinase activity resides in low-density membranes [15,16], now identified as exocytotic vesicles [17]. We have previously isolated and partly characterized this enzyme and shown it to be a type II kinase [18]. We now report the existence of an additional, and novel, PtdIns 4-kinase in rat liver with properties differing from both the type II kinase and all other PtdIns 4-kinases so far characterized.

*Corresponding author. Fax: (46) (46) 104 534.

**Present address:* Department of Medical and Physiological Chemistry, Chemical Centre, University of Lund, Lund Sweden.

Abbreviations: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdCho, phos-phatidylcholine; PtdSer, phosphatidylserine; GPtdInsP, glycerophosphoryl-inositolphosphate; CMC, critical micelle concentration.

2. Experimental

2.1. Materials

PtdIns, PtdIns4P and PtdIns(4,5)P_2 were prepared from Sigma Type I brain extract [19]. [γ-^{32}P]ATP was prepared as described [20]. Heparin-Sepharose and the Mono S HR 5/5 column were from Pharmacia LKB Biotechnology (Sollentuna, Sweden), hydroxylapatite (fast flow) from Calbiochem (San Diego, USA) and reduced Triton X-100 from Aldrich (Steinheim, Germany). The monoclonal PtdIns 4-kinase antibody was a gift from Dr. G. Endemann, Scios Nova Icr. (Mountain View, CA, USA).

2.2. Purification of PtdIns kinases

Rat liver microsomes were prepared essentially as described in [21]. Liver tissue (16 g) was homogenized in 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0 (3 vols/g wet wt.) using a loose-fitting Potter-Elvehjem homogenizer. After centrifugation at $1000 \times g$ for 10 min, the pellet obtained was re-extracted twice by homogenization and centrifugation as above. The combined supernatants were centrifuged at $27,000 \times g$ for 10 min and the pellet re-extracted once. Microsomal membranes were recovered from the $27,000 \times g$ supernatant by centrifugation at $105,000 \times g$ for 90 min.

The microsomes were solubilized for 60 min at 4°C in 50 mM HEPES-NaOH, pH 7.2, 0.15 M KCl, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 2% (w/v) reduced Triton X-100 at a final protein concentration of 5 mg/ml and then centrifuged at $100,000 \times g$ for 60 min. The extract obtained, containing ca 300 mg of protein and more than 90% of the microsomal PtdIns kinase activity, was diluted with an equal volume of 20 mM HEPES-NaOH, pH 7.2, 20% (v/v) glycerol, 1 mM EGTA, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 0.2% (w/v) reduced Triton X-100 and applied to a heparin-Sepharose column (2.6×10 cm) equilibrated with this buffer mixture. After washing with 0.1 M NaCl in the buffer, adsorbed proteins were eluted with a 600-ml linear 0.1–1.0 M NaCl gradient. Fractions containing the second of two peaks of PtdIns kinase activity, eluting at 0.5 M NaCl, were combined and applied to a hydroxylapatite column (1×10 cm) equilibrated with 20 mM potassium phosphate, pH 7.2, 20% (v/v) glycerol and 0.1% (w/v) reduced Triton X-100. After washing with this buffer, the enzyme was desorbed with a 60-ml linear 20–500 mM potassium phosphate gradient in the buffer. Fractions with kinase activity, eluting as a single peak at 0.26 M potassium phosphate, were combined and dialysed against 20 mM potassium phosphate, pH 6.8, 20% (v/v) glycerol, 1 mM EGTA and 0.1% (w/v) reduced Triton X-100, and applied to a Mono S H/R 5/5 column equilibrated with this buffer. After washing with 10 ml of buffer the enzyme was desorbed with a 25-ml linear 0–1.0 M NaCl gradient in the buffer. Fractions containing kinase activity, eluting as a single peak at 0.5 M NaCl, were combined and stored at −70°C (PtdIns kinase B).

For comparative studies rat liver Type II PtdIns kinase was purified as described previously [18]. This enzyme is called PtdIns kinase A. Preliminary experiments showed the first peak of microsomal PtdIns kinase activity, eluting from heparin-Sepharose at 0.25 M NaCl, to have the same properties as the type II enzyme. Type III PtdIns kinase was isolated from a cholate extract of rat brain membranes by sucrose density gradient centrifugation [27].

2.3. Assay of PtdIns kinase activity

The activity of PtdIns kinase was measured as described earlier [18], except that dithioerythritol was omitted when measuring PtdIns kinase B. The substrate was presented as PtdIns:Triton X-100 mixed micelles. Lipids in chloroform were evaporated to dryness under a stream of nitrogen, Triton X-100 was added, the solution mixed vigorously and

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sonicated for 10 min in a sonication bath (Branson 2200; Bransonic). All assays were performed in duplicate and were in the linear range with regard to protein concentration and incubation time. The difference between individual incubations in a duplicate was typically less than 2%.

2.4. Isomeric configuration of PtdInsP formed by PtdIns kinases A and B

Bulk preparations of ^{32}P-labelled reaction products, obtained by the standard assay using prolonged incubation times (10–50 min), were spiked with authentic [^{3}H]PtdIns4P (Amersham, UK) and deacylated [22]. The resulting glycerophosphoinositides were separated by isocratic HPLC using a Partisil 10-SAX column (Whatman, UK) and 100 mM NaH_2PO_4, pH 3.8, as eluant at a flow rate of 1.5 ml/min. Reproducibility between runs was ensured by inclusion of AMP and ADP as internal standards, monitored by their absorbance at 254 nm. Radioactivity was determined by liquid scintillation spectrometry after addition of Hionic scintillation fluid (Canberra-Packard, UK). Background radiation has been subtracted from all data presented.

2.5. Other methods

Protein concentrations were determined [23] using bovine serum albumin as a standard. Concentrations of phospholipids in chloroform stock solutions were determined by phosphate contents [24]. The 4C5G IgG fraction was purified from ascites fluid by binding to protein G-Agarose at pH 7.2 and eluting at pH 2.7 [25].

3. Results

3.1. Presence of two forms of PtdIns kinase in rat liver

Exocytotic vesicles from rat liver contain a PtdIns 4-kinase activity eluting as a single peak from heparin-Sepharose [18]. However, when rat liver microsomes, the crude source of exocytotic vesicles, were solubilized in Triton X-100 and chromatographed on heparin-Sepharose the PtdIns kinase activity was resolved into two distinct peaks (Fig. 1). The first peak (PtdIns kinase A), representing approximately 70% of the total PtdIns kinase activity recovered, eluted at exactly the same salt concentration as PtdIns 4-kinase from exocytotic vesicles, while the second peak (PtdIns kinase B) eluted at a higher salt concentration (0.5 M vs. 0.25 M NaCl). The PtdIns kinase activity of the first peak was stimulated by PtdCho and had several other properties in common with the enzyme isolated from exocytotic vesicles [18], whereas PtdIns kinase B had quite different properties. As each enzyme form retained its specific elution behaviour on re-chromatography on heparin-Sepharose, we conclude

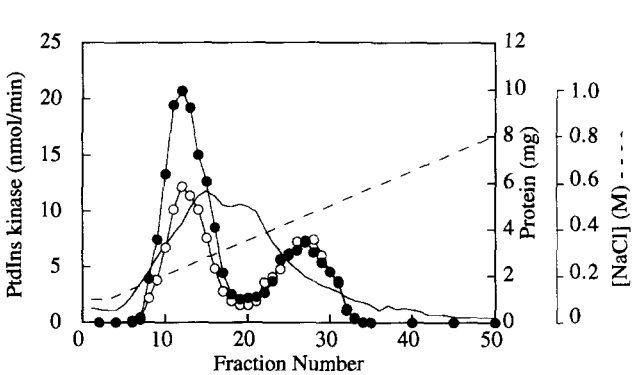

Fig. 1. Separation of two forms of PtdIns kinase on heparin-Sepharose. Rat liver microsomes (300 mg of protein) were solubilised and chromatographed on heparin-Sepharose as described in section 2. Ten-ml fractions were collected. PtdIns kinase activity was determined in the presence (●) or absence (○) of 1 mM PtdCho. Recovered activity was approximately 70%. Solid line, protein; dashed line, NaCl gradient.

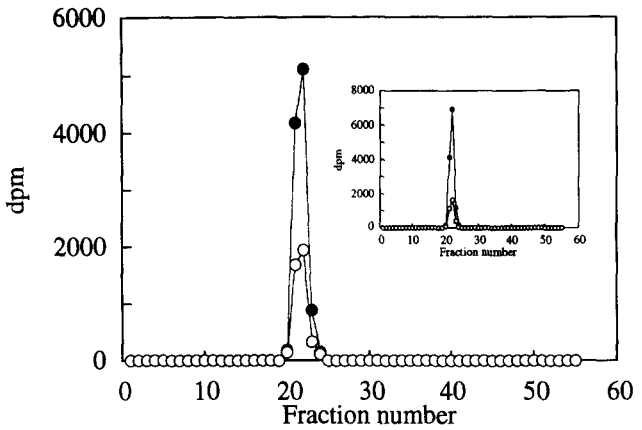

Fig. 2. Identification of reaction products. The ^{32}P-labelled reaction products produced by PtdIns kinases B and A (inset) were fortified with authentic [^{3}H]PtdInsP before deacylation and separation by isocratic HPLC as described in section 2. ●, ^{32}P label; ○, ^{3}H-label of the standard.

that rat liver microsomes indeed contain two distinct PtdIns kinases.

3.2. Purification of PtdIns kinase B

The second peak of PtdIns kinase activity from heparin-Sepharose (Fig. 1) was further purified by chromatography on hydroxylapatite and a Mono S column (see section 2), both steps yielding a single peak of PtdIns kinase activity. The final preparation had a specific activity of 7 nmol/min/mg protein, 17-fold higher than the microsomal membranes and, in turn, 50-fold higher than the liver homogenate. The yield was 8% of the total PtdIns kinase activity of the membranes (or 28% after subtracting the contribution of PtdIns kinase A, assuming the same recovery of the two enzymes on heparin-Sepharose chromatography).

3.3. Identification of reaction product

The isomeric configuration of the PtdInsP produced by the PtdIns kinase B was investigated by isocratic HPLC analysis of [^{32}P]GPtdInsP obtained by O→N transacylation. The [^{32}P]GPtdInsP derived from the product of PtdIns kinase B co-chromatographed exactly with authentic [^{3}H]GPtdIns4P (Fig. 2), indicating the parent PtdInsP to be PtdIns4P. Thus, we conclude that rat liver PtdIns kinase B is a PtdIns 4-kinase. The same analysis, applied to the reaction product of the A-form (Fig. 2, inset), confirmed the previous identification of this enzyme as a type II PtdIns 4-kinase [18]. Both enzyme forms phosphorylated PtdIns specifically, as PtdIns4P and PtdIns(4,5)P_2 were phosphorylated to less than 1% of that of PtdIns under equivalent incubation conditions.

3.4. Effect of pH and divalent cations

The pH curves for the two forms of PtdIns kinase were similar, with maximum activity between pH 7 and 8 (not shown). The dependence on divalent cations differed, however. PtdIns kinase B displayed maximum activity at 4 mM Mg^{2+} (and above), and 1–1.5 mM Mn^{2+} could only partly (20% of maximum) replace Mg^{2+} (Fig. 3a). The A-form required 2 mM Mg^{2+} for maximum activity, but, in this case 0.5 mM Mn^{2+}, efficiently (80%) substituted for Mg^{2+}.

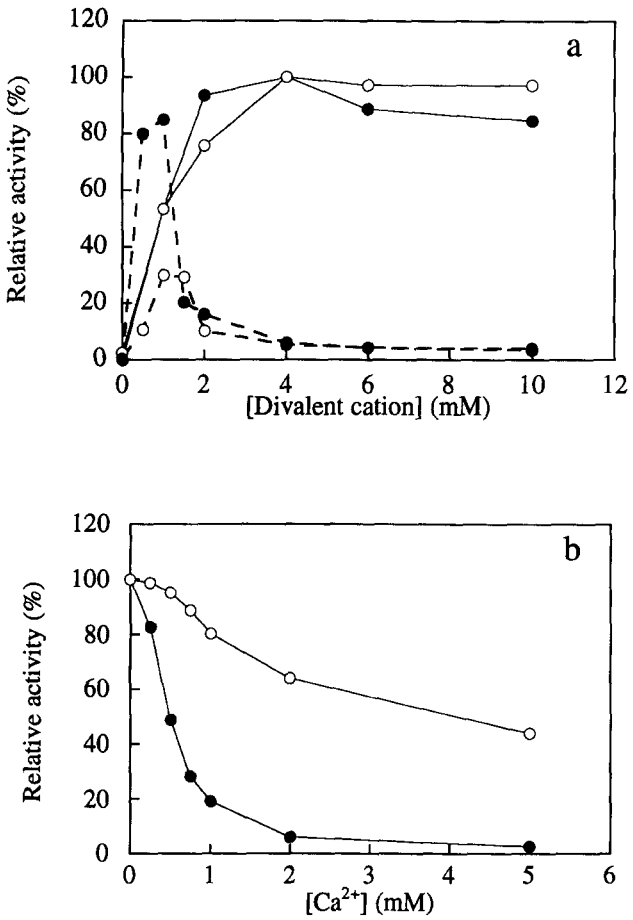

Fig. 3. Effect of divalent cations. The activities of PtdIns 4-kinase A (●) and B (○) were measured at the indicated concentrations of (a) Mg^{2+} (solid line) and Mn^{2+} (dashed line) ions and (b) Ca^{2+} ions. Data are means of duplicate incubations with deviations less than 2% from the mean.

PtdIns kinase B was less sensitive to inhibition by Ca^{2+}than the A-form, with IC_{50} values of 4.1 and 0.5 mM, respectively (Fig. 3b). Free Ca^{2+} concentrations in the micromolar range (up to 200 μM; Ca^{2+}-EGTA buffers) did not affect the enzyme activity.

3.5. Effect of dithioerythritol and the monoclonal antibody 4C5G

PtdIns kinase B was inhibited to 70–80% by dithioerythritol, with an IC_{50} value as low as 0.6 mM, whereas the A-form remained unaffected (Fig. 4). Reduced glutathione also inhibited the B-form with an IC_{50} of 10 mM. *p*-Chloromercuribenzoic acid at 10 μM inhibited both enzymes completely (not shown).

The monclonal antibody 4C5G, raised to the type II PtdIns 4-kinase from bovine brain [27] at 100 μg/ml, inhibited both kinases by 70%, although the A-form was more sensitive at lower antibody concentrations (Fig. 5). Rat brain type III PtdIns kinase was not recognized by the antibody, however (Fig. 5). This antibody was earlier shown to inhibit type II kinase from bovine brain [27] and other sources [8,28], but not type III kinase from bovine or rat brain, or type I PtdIns 3-kinase [27].

3.6. Lipid dependency

PtdCho enhances the activity of PtdIns kinase A several-fold, whereas PtdSer is inhibitory [18]. These phospholipids, at the same concentration as in [18], did not affect the activity of PtdIns kinase B.

3.7. Dependence on ATP and PtdIns in Triton X-100: PtdIns mixed micelles

Kinetic analyses were performed under defined conditions, where the substrate was presented as Triton X-100:PtdIns mixed micelles (cf. [10,29,30]). At a saturating bulk PtdIns concentration of 0.2 mM, the enzyme activity was dependent only on the micellar surface concentration of PtdIns, which was varied by changing the Triton X-100 concentration. Under these conditions the two PtdIns kinases displayed saturation kinetics both for ATP and PtdIns. The K_m value for ATP was 78 μM for the B-form compared to 40 μM for the A-form. Corresponding K_m values for PtdIns were 3.4 mol% and 0.99 mol% (3.3 mol% in the presence of PtdCho), respectively.

3.8. Effect of adenosine

Adenosine inhibits PtdIns 4-kinases from various sources to different degrees [7]. The rat liver B-form was rather insensitive to increasing concentrations of adenosine, being inhibited by only 15% by 0.5 mM of the nucleoside compared to 55% for the A-form, under the analysis conditions chosen (not shown).

To obtain apparent K_i values for adenosine the PtdIns surface concentration was kept constant at a saturating level (0.5 mM bulk concentration, corresponding to a surface concentration of 9 mol%) while the ATP concentration was varied at different set adenosine concentrations. As expected for competitive inhibition, adenosine did not affect the apparent V_{max} values but caused an increase in the apparent K_m values for ATP (Fig. 6). Replots of these apparent K_m values ($K_{m'}$) vs. the adenosine concentration (Fig. 6, insets) were linear and gave K_i values for adenosine of 14 and 410 μM for kinases A and B, respectively.

4. Discussion

We have isolated a new form of PtdIns kinase from rat liver having properties distinct from an earlier isolated form. Both

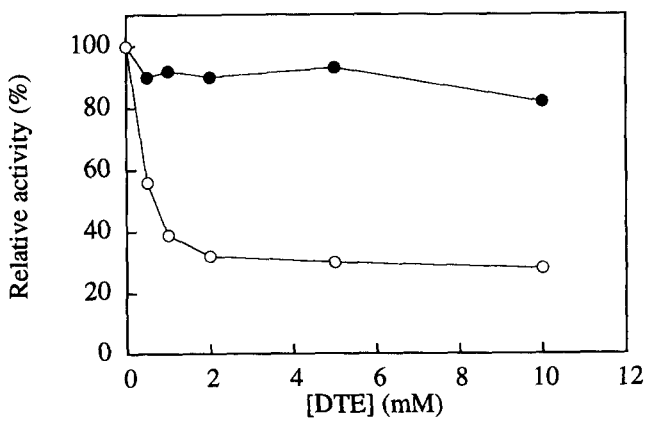

Fig. 4. Effect of dithioerythritol on the activities of PtdIns 4-kinase A and B. The activities of PtdIns 4-kinase A (●) and B (○) were measured as described in section 2 at the indicated concentrations of dithioerythritol. Data are means of duplicate incubations with deviations less than 2% from the mean.

forms of the enzyme are particulate and phosphorylate PtdIns in the 4-position. The new B-form was associated with so far unidentified material present in the microsomal fraction, whereas the A-form was present in exocytotic vesicles derived from these microsomes [18].

PtdIns kinase B differed in properties from the A-form in several respects. It was inhibited by dithioerythritol, was comparatively insensitive to inhibition by adenosine and calcium ions, and was affected only slightly by various phospholipids. Furthermore, it differed from the A-form in K_m values for ATP and PtdIns and dose–response curves to Mg^{2+}/Mn^{2+} and the monoclonal antibody 4C5G. These facts, together with the different subcellular distribution of the two enzyme forms and the inability of PtdIns 4-kinase B to renature after SDS-gel electrophoresis, indicate that they are distinct enzymes and that one form is not derived from the other form by proteolysis.

The A-form was identified as a type II PtdIns kinase by its sensitivity to inhibition by adenosine and Ca^{2+}, its apparent molecular mass of 56 kDa upon SDS-PAGE and renaturation, and its inhibition by the antibody 4C5G [7,27].

The B-form also differed from all the type III kinases identified to date. It had a K_m for ATP of 78 μM and a K_i for adenosine of 410 μM, both constants intermediate between reported values for the type II and the type III enzymes [14]. Furthermore, it was inhibited up to 70% by the 4C5G antibody which did not affect the activity of the type III enzyme from rat brain. The enzyme was excluded from a Superdex 200 gel filtration column, indicating a molecular mass above 600 kDa under conditions when the type III enzyme from bovine uterus [5] and rat brain [26] elute at 220 and 80 kDa, respectively. This behaviour of PtdIns kinase B indicates that it is in an aggregated form, probably also reflected by its refractiveness to exhaustive purification. In contrast to the A-form, PtdIns kinase B activity could not be recovered after SDS-gel electrophoresis and, therefore, the minimum molecular mass of PtdIns kinase B could not be determined by this method.

Another novel aspect of PtdIns 4-kinase B, and also a property which clearly disinguished it from PtdIns kinase A, was its sensitivity to inhibition by dithioerythritol. The IC_{50} value of 0.6 mM suggests that the enzyme may be regulated by the cellular redox state, also indicated by the inhibitory effect of physiological concentration of reduced glutathione.

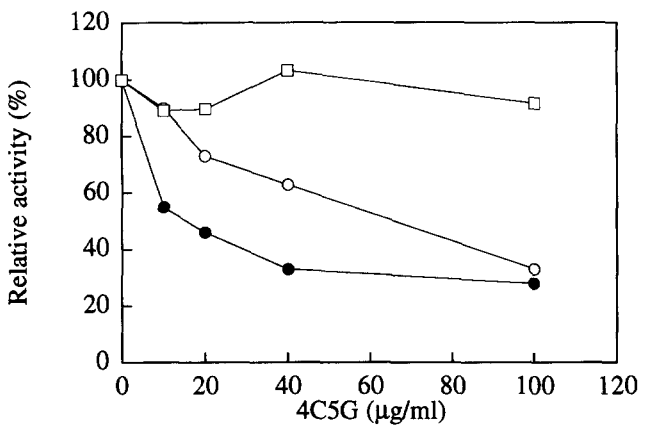

Fig. 5. Effect of the monoclonal antibody 4C5G on PtdIns 4-kinase activity. PtdIns 4-kinase A (○) and B (●), and the type III enzyme (□) isolated from rat brain, were incubated for 30 min at 4°C with the indicated concentrations of purified 4C5G before PtdIns 4-kinase activity was assayed as described in section 2. Data are means of duplicate incubations with deviations less than 2% from the mean.

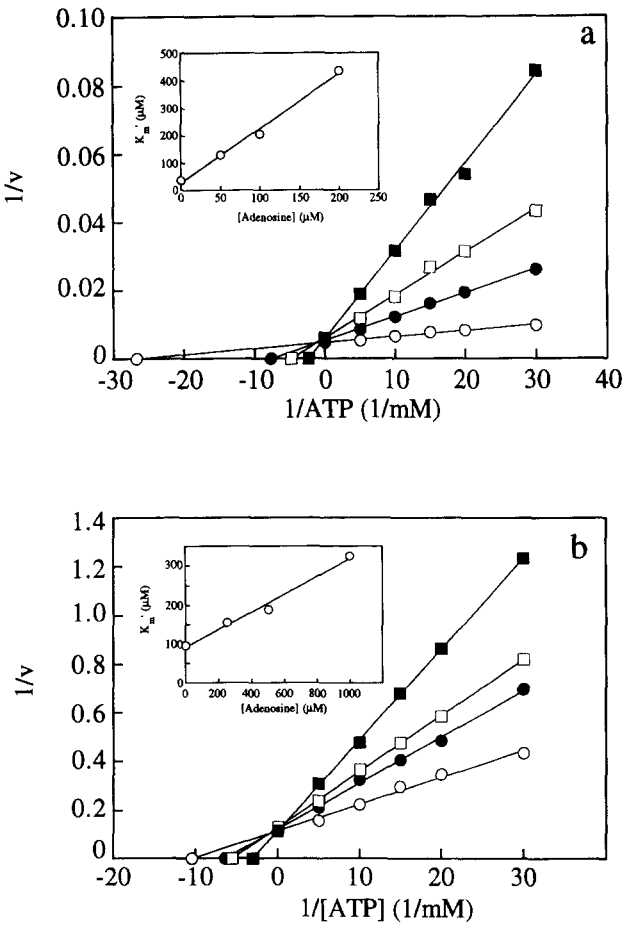

Fig. 6. Inhibition of PtdIns 4-kinase A and B by adenosine. The activity of PtdIns 4-kinase A (a) and B (b) were measured as a function of the concentration of ATP at 0 (○), 0.05 (●), 0.10 (□) and 0.20 (■) mM adenosine (a) and 0 (○), 0.25 (●), 0.50 (□) and 1.00 (■) mM adenosine (b), respectively. The concentration of PtdIns was 11 mol% (0.5 mM bulk concentration). The data are means of duplicate incubations and plotted as $1/v(nmol^{-1} \times min \times mg)$ vs. the reciprocal of each ATP concentration. The insets are replots of the apparent $K_{m'}$ values obtained in a and b, respectively, vs. the concentration of adenosine. The lines were drawn using least-squares analysis of the data. The intercepts with abscissa and ordinate are also indicated by symbols.

The presence of a second PtdIns 4-kinase (kinase B) distinct from the type II enzyme (kinase A), in rat liver cells raises several questions. One is whether this enzyme is unique to liver cells, carrying out some specific function in this organ, or whether B-kinase analogues are widespread in eukaryotes. Another question, in view of the multiplicity of PtdIns-4 kinases, is how the different forms are related structurally to each other. One possibility is that they are products of alternative splicing, although this is speculative so far due to the lack of structural data. A third question which needs addressing is that of the physiological roles of the A- and B-kinases. Some clues may lie in their different sensitivity to dithioerythritol, phospholipids and Ca^{2+}, but a final assessment must await more detailed information about their subcellular distribution and in vivo activity. It is also possible that the kinases are part of distinct phosphoinositide signalling systems operating within the cell,

particularly as isolated cytoskeletons and nuclei each seem to contain a set of enzymes involved in phosphoinositide turnover [31–33].

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