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Characterization of phosphatidylinositol-4-phosphate 5-kinase activities from bovine brain membranes

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Phosphatidylinositol-4-phosphate (PtdIns(4)P) kinase activity associated with bovine brain membranes, was released by NaCl treatment and partially purified by chromatography on phosphocellulose, phenylsepharose, Ultrogel AcA44, DEAE-cellulose and ATP-agarose. The final preparation contained a 6333-fold purified protein fraction with a specific activity of 171 $nmol \cdot min^{-1} \cdot mg^{-1}$. Under conditions where this PtdIns(4)P kinase activity (PtdIns(4)P kinase activity *b*) did not bind to DEAE-cellulose, a PtdIns(4)P kinase activity purified earlier (Moritz, A., De Graan, P.N.E., Ekhart, P.F., Gispen, W.H. and Wirtz, K.W.A. (1990) J. Neurochem. 54, 351–354) does bind (PtdIns(4)P kinase activity *a*). Both enzyme activities specifically used PtdIns(4)P as substrate and phosphorylated the inositol moiety at the 5′-position. PtdIns(4) kinase activity *a* has an apparent K_m of 18 μM for PtdIns(4)P whereas PtdIns(4)P kinase activity *b* has a K_m of 4 μM. All other measured kinetic parameters (i.e., K_m for ATP, Mg^{2+}-dependence, pH optimum, activation by phosphatidylserine and inhibition by phosphatidylinositol 4,5-bisphosphate) were similar for both enzyme activities.

Introduction

PtdIns(4,5)P_2 is a key compound in the agonist-mediated production of the second messenger molecules inositol 1,4,5-trisphosphate and diacylglycerol [1–4]. Cellular levels of PtdIns(4,5)P_2 are maintained by the phosphorylation of PtdIns(4)P by PtdIns(4)P kinase [1,4]. In order to establish the regulatory link between PtdIns(4,5)P_2 breakdown and synthesis, it is of great importance to identify and characterize the PtdIns(4)P kinase(s) involved.

PtdIns(4)P kinase activity is found both in membranes and in the cytosol [5,6,10,12–15]. In several studies the purification of the membrane bound and soluble form of PtdIns(4)P kinase has been reported [5–12,15]. In rat brain, both forms of PtdIns(4)P kinase appear to be similar [5,6]. In human red blood cells the cytosolic PtdIns(4)P kinase was found to be indistinguishable from a membrane-bound PtdIns(4)P kinase. However, PtdIns(4)P kinase activity associated with membranes could be separated into two structurally and functionally distinct enzymes showing major differences in their K_m for ATP and PtdIns(4)P, and in their activity towards PtdIns(4)P in liposomes and natural membranes [12]. From human platelet membranes two forms of Ptd(Ins)P kinase were purified which differed in apparent molecular mass and in the effects of Mn^{2+} and phosphatidylethanolamine on enzyme activities [15].

It has also been reported for PtdIns and diacylglycerol kinase that different forms occur in the same tissue. Different PtdIns 4-kinases with distinct enzymatic properties have been identified in rabbit reticulocytes, bovine brain, human platelets and human erythrocytes [16–19]. Two forms of PtdIns 4-kinase from bovine brain were shown to be immunologically different, suggesting that the two kinases are products of distinct genes [20]. Different forms of PtdIns 3-kinase

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Abbreviations: HPLC, high-performance liquid chromatography; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns kinase, phosphatidylinositol kinase (EC 2.7.1.67); PtdIns(4)P kinase, phosphatidylinositol-4-phosphate kinase (EC 2.7.1.68); PtdSer, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; v, volume; w, weight.

have been identified in 3T3 cells and bovine brain [21,22]. Similarly, diacylglycerol kinases with distinct enzymatic properties have been identified in various tissues and cell types [23–29], and also immunological differences between diacylglycerol kinases were observed [23,30]. Two cytosolic diacylglycerol kinases purified from human platelets [28], as well as a membrane-bound and a cytosolic diacylglycerol kinase purified from rat brain [29] display structural differences when analysed by peptide mapping [28,29].

In a purification published in a previous paper [10], two major PtdIns(4)P kinase activities were resolved by anion-exchange chromatography on DEAE-cellulose. The PtdIns(4)P kinase activity eluting at the highest salt concentration (PtdIns(4)P kinase activity *a*) was purified to apparent homogeneity and had an apparent molecular mass of 110 kDa [10]. Here we report on the partial purification of the second major PtdIns(4)P kinase activity (PtdIns(4)P kinase activity *b*). Kinetic parameters of both PtdIns(4)P kinase activities were determined. Both enzyme activities specifically use PtdIns(4)P as substrate and phosphorylate the inositol moiety at the 5′-position.

Materials and Methods

Materials

Phosphocellulose (P 11) and DEAE-cellulose (DE 52) were purchased from Whatman (Maidstone, UK). Phenylsepharose, Ultrogel AcA44, and ATP-agarose (AGATP type 3) were from Pharmacia LKB (Uppsala, Sweden). Silica-gel plates were from Merck (Darmstadt, Germany). ATP was purchased from Boehringer (Mannheim, Germany), and bovine serum albumin from Calbiochem (USA). [γ-^{32}P]ATP (spec. act., 3000 Ci/mmol) and [^{3}H]inositol-labelled PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 were obtained from Amersham (UK). PtdIns(4)P, carrier lipid (Folch fraction I from bovine brain), phenylmethylsulfonylfluoride, leupeptin, and trypsin inhibitors from soybean and lima bean were from Sigma. PtdSer was purified from bovine brain [31].

Assay for PtdIns(4)P kinase

The assay procedure used in the purification is a slight modification of the method described previously [10]. A one-to-one mixture of PtdIns(4)P and PtdSer was dried under N_2 from a chloroform solution and subsequently exposed to the vacuum of a lyophilizer for 2 h in order to remove the last traces of chloroform. PtdSer was added to the assay to enhance PtdIns(4,5)P_2 formation [6,9]. The substrate (0.16 μmol total phospholipid) was suspended in 0.6 ml buffer consisting of 50 mM Tris-HCl (pH 7.4), 0.133% polyethylene glycol 20000, 0.333 M sucrose, 150 mM NaCl, and bovine serum albumin (0.67 mg/ml) and sonicated with a Branson probe sonifier (output 50 W) under N_2 atmosphere for 3 min (5 s on, 10 s off) on ice [32]. Phosphorylation of PtdIns(4)P was carried out in glass tubes in an incubation medium (final volume of 50 μl) containing final concentrations of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 15 mM $MgCl_2$, 1 mM EGTA, 80 μM PtdIns(4)P, 80 μM PtdSer, bovine serum albumin (0.4 mg/ml), 0.25 M sucrose, 0.1% polyethylene glycol 20000, 0.04% Triton X-100 and 50 μM [γ-^{32}P]ATP (0.5–1.5 μCi/assay). After preincubation for 5 min at 30°C, the reaction was started by addition of the enzyme solution (10 μl), and continued for 8 min at 30°C. The reaction was stopped by addition of 3 ml of chloroform/methanol/concentrated HCl (200:100:0.75 by volume). A mixture of carrier lipids consisting of PtdIns, PtdIns(4)P, PtdIns(4,5)P_2 and PtdSer (40–200 nmol each) was added, and 2-phase separation was induced by addition of 0.6 M HCl (0.6 ml) followed by vortexing. After centrifugation, the upper phase was discarded and the lower phase was washed twice with 1.5 ml of chloroform/methanol/0.6 M HCl (3:48:47 by volume). The lower phase was dried by a stream of N_2 at 50° and the lipid residue was redissolved in 60 μl of ice-cold chloroform/ methanol/water (75:25:2 by volume) by vortexing. An aliquot of 30 μl was applied on an oxalate-impregnated TLC-plate [33] and the lipids were separated by developing the plate in chloroform/ acetone/ methanol/acetic acid/water (40:15:15:12:7.5 by volume) for 60 min. Lipids were visualized by iodine staining. After sublimation of the iodine, the area containing [^{32}P]PtdIns(4,5)P_2 was scraped from the plate and counted by liquid scintillation spectrometry after addition of 3 ml of Xylofluor.

In studies where the purified PtdIns(4)P kinase was characterized, PtdSer was omitted from the assay and the Triton X-100 concentration in the assay was lowered to 0.004%. In order to achieve this, the enzyme solution containing 0.1% Triton X-100 was diluted 5 times with buffer C without Triton X-100, and 10 μl of the ensuing solution was applied to the assay medium (final volume of 50 μl). The final Triton X-100 concentration of 0.004% is far below the critical micelle concentration of 0.02% which makes the assay conditions comparable to the ones used by others [6,7,9,11,12]. Under all assay conditions, PtdIns(4,5)P_2 formation was linear with protein concentration and with time for at least 10 min. Each kinetic parameter was determined for both kinases within the same experiment.

Preparation of membrane supernatant

All manipulations were carried out at 0–4°C. Fifteen fresh bovine brains were collected on ice at the local slaughterhouse. The brains (5 kg wet weight) were freed of blood vessels and connective tissues. A 33% (w/v) homogenate was prepared in 50 mM Tris-HCl

(pH 7.4), 1 mM EDTA, 0.32 M sucrose, and 0.3% (v/v) 2-mercaptoethanol (buffer A) containing the protease inhibitors leupeptin (0.5 mg/l), soybean and lima bean trypsin inhibitors (1 mg/l each), and phenylmethylsulfonylfluoride (1 mM). Homogenization was carried out with an Ystral homogenizer (type 40/34, Dottingen, Germany) for 1 min at moderate speed, with the clearance adjusted to its maximum. The homogenate was centrifuged at $14000 \times g$ for 1 h. The membrane pellet was washed once by rehomogenization (33% homogenate) in buffer A containing 1 mM phenylmethylsulfonylfluoride and sedimented by centrifugation at $14000 \times g$ for 1 h. In order to release peripheral proteins the membrane pellet was subjected to a salt wash by rehomogenization (33% homogenate) in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 200 mM NaCl, 1 mM phenylmethylsulfonylfluoride and 0.3% (v/v) 2-mercaptoethanol. After stirring for 30 min, the membrane suspension was centrifuged at $14000 \times g$ for 1 h. The resulting supernatant was stabilized by addition of 0.2 vol. of buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1.5 M sucrose, 0.3% (v/v) 2-mercaptoethanol, 6% Triton X-100, 0.6% polyethylene glycol 20000, 300 μM ATP, and 200 mM NaCl.

Purification of PtdIns(4)P kinase

Table I summarizes the various steps of the purification, all manipulations were performed at 4°C.

In step 1, phosphocellulose (300 g wet weight) was equilibrated in 50 mM Tris-HCl (pH 8.0), and 200 mM NaCl, and added to the membrane supernatant (volume of 11 litres). The slurry was stirred overnight, and after settling for 90 min, the supernatant was decanted. The phosphocellulose was washed twice with 6 litres of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25 M sucrose, 0.3% (v/v) 2-mercaptoethanol, 1% Triton X-100, 0.1% polyethylene glycol 20000, 50 μM ATP, and 0.1 mM phenylmethylsulfonyl-fluoride (buffer C) containing 200 mM NaCl. The washed slurry was poured into a column (18×4.8 cm) and PtdIns(4)P kinase activity was eluted with buffer C containing 1 M NaCl, at a flow rate of 30 ml/h.

In step 2, the active fractions (310 ml) were diluted with buffer C until the ionic strength was 150 mM NaCl, and then applied to a phosphocellulose column (15×1.8 cm) equilibrated with buffer C containing 150 mM NaCl. The enzyme was eluted with a linear gradient from 150 to 1500 mM NaCl in buffer C (460 ml) at a flow rate of 16 ml/h. The activity eluted at about 800 mM NaCl.

In step 3, the active fractions (78 ml) were adjusted to 1.5 M NaCl with solid NaCl, and diluted 5 times with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25 M sucrose, 0.3% (v/v) 2-mercaptoethanol, 0.1% polyethylene glycol 20000, 50 μM ATP, 0.1 mM phenylmethylsulfonylfluoride and 1.5 M NaCl (buffer D), to lower the Triton X-100 concentration to 0.2%. The enzyme solution (390 ml) was applied to a phenylsepharose column (18×1.8 cm) which was equilibrated and eluted in buffer D containing 0.1% Triton X-100, at a flow rate of 16 ml/h. PtdIns(4)P kinase activity appeared in the flow-through of the column and was concentrated for gel filtration as follows: The flow through was dialyzed overnight against 10 vol. of buffer C, and loaded on a phosphocellulose column (14×1.4 cm) equilibrated with buffer C containing 150 mM NaCl. A concentrated solution (52 ml) of PtdIns(4)P kinase activity was eluted from the column with 1 M NaCl in buffer C at a flow rate of 8 ml/h.

In step 4, this solution was applied in two runs (2×26 ml) to an Ultrogel AcA44 column (172×2.1 cm), which was equilibrated and eluted with buffer C containing 150 mM NaCl, at a flow rate of 22 ml/h.

In step 5, the active fractions (77 ml) were diluted with buffer C to 70 mM NaCl, and then applied to a DEAE-cellulose column (10.5×1.4 cm) which was equilibrated and eluted with buffer C containing 70 mM NaCl, at a flow rate of 8 ml/h. The activity appearing in the flow-through of the column was used. The active fraction was applied to an ATP-agarose column (8×1.3 cm) equilibrated with buffer C containing 70 mM NaCl. The enzyme was eluted from the column with a linear gradient from 70 to 1000 mM NaCl in buffer C (total volume of 160 ml), at a flow rate of 8 ml/h. PtdIns(4)P kinase activity was eluted at

TABLE I

Purification of phosphatidylinositol-4-phosphate 5-kinase from bovine brain membranes

Purification step	Protein (mg)	Total act. (nmol/min)	Spec. act. (nmol min^{-1} mg^{-1})	Purification (-fold)	Yield (%)
Membrane supernatant	21900	591	0.027	1	100
Phosphocellulose (stepwise)	1302	482	0.37	13	81
Phosphocellulose (gradient)	406	313	0.77	28	54
Phenylsepharose	187	318	1.7	63	54
Ultrogel AcA44	4.9	215	43.9	1625	36
DEAE-cellulose/ATP-agarose	0.36	61.6	171	6333	10

about 500 mM NaCl. The final PtdIns(4)P kinase preparation (PtdIns(4)P kinase activity *b*) was stored in small aliquots at −20°C and was stable for several months.

Preparation of PtdIns(3)P and of PtdIns(3,4)P_2

PtdIns(3)P was prepared by enzymatic phosphorylation of PtdIns with PtdIns 3-kinase. The PtdIns 3-kinase activity used was associated with phosphotyrosyl-proteins [34] immuno-isolated from epidermal growth factor-stimulated A431 cells. The immuno-isolation of the phosphotyrosyl-proteins was carried out as described by Payrastre et al. [35]. Sonicated vesicles of PtdIns (300 μM) were incubated with this protein preparation in 50 mM Tris-HCl (pH 7.4), 70 μM [γ-^{32}P]ATP (10 μCi), 0.5 mM Na_3VO_4, and 10 mM $MgCl_2$ at 37°C during 15 min, and the lipids were immediately extracted. Lipids were analyzed by HPLC as described below. A major peak of [γ-^{32}P]PtdIns(3)P was detected, with only a minor peak of [γ-^{32}P]PtdIns(4)P. In order to prepare PtdIns(3,4)P_2, sonicated vesicles of pure PtdIns(4)P were used instead of PtdIns.

Lipid kinase assay for determination of the phosphorylation of PtdIns(3)P and PtdIns(3,4)P_2

PtdIns(4)P kinase solutions in buffer C containing 0.1% instead of 1% Triton X-100 were diluted 10 times with 50 mM Tris-HCl (pH 7.4). Aliquots of these PtdIns(4)P kinase preparations (20 μl) were assayed in a final volume of 100 μl containing 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (10 μCi), and 5–10 nM PtdIns(3)P or 5–10 nM PtdIns(3,4)P_2. The reaction was started by addition of the enzyme solution, continued for 10 min at 30°C, and then stopped by addition of 400 μl of chloroform/methanol (1 : 1, by volume). Lipids were immediately extracted as described [36].

Lipid analysis

Lipids were separated by thin-layer chromatography [37], and radioactive lipids were scraped off the TLC-plate after detection by autoradiography. Lipids were deacylated as described by Auger et al. [38], and analyzed by HPLC [39]. Authentic deacylated tritiated PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 were used as standard for identification. ^{32}P was counted using the Cerenkov effect, while tritiated samples were quantified after admixture of Liquiscint 303.

Other procedures

PtdIns(4)P kinase activity *a* was purified as described [10] and stored in small aliquots at −20°C. Protein concentrations were determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL, USA) with bovine serum albumin as the standard. Before protein determination, proteins were precipitated with trichloroacetic acid in the presence of sodium deoxycholate [40]. SDS-PAGE was performed by the method of Kristjansson et al. [41] using 11% slab gels. Proteins were visualized by silver staining according to Merril et al. [42].

Results and Discussion

Purification of PtdIns(4)P kinase activity b

PtdIns(4)P kinase activity associated with bovine brain membranes, was released by extraction with 200 mM NaCl. In a previous purification [10] the first step consisted of DEAE-cellulose chromatography of the membrane extract, yielding two major activity peaks. One peak appeared in the flow-through (PtdIns(4)P kinase activity *b*), the other peak eluted with the salt gradient used (PtdIns(4)P kinase activity *a*). The latter activity was purified to apparent homogeneity [10]. In the present study PtdIns(4)P kinase was purified by an entirely new procedure (Table I). In the first step, PtdIns(4)P kinase activity was concentrated from the membrane supernatant by binding to phosphocellulose. This concentration of enzyme activity proved necessary in order to be able to scale-up to 15 bovine brains as the starting material. As in the previous purification [10], EDTA, sucrose, NaCl, 2-mercaptoethanol, Triton X-100, polyethylene glycol 20 000, phenylmethylsulfonylfluoride and ATP were included in all buffers used to prevent a rapid loss of enzyme activity during the purification.

PtdIns(4)P kinase activity bound to phosphocellulose was eluted from the material by first pouring a column followed by washing with 1 M NaCl. This activity could not be eluted with a salt gradient, since gradient elution consistently resulted in clogging up of the column. This problem was not observed when in the second step the active fraction was applied to a phosphocellulose column followed by elution of PtdIns(4)P kinase activity with a salt gradient. During gel filtration on Ultrogel AcA44, all PtdIns(4)P kinase activity appeared just before the salt peak, similar to what was observed during the purification of PtdIns(4)P kinase activity *a* [10]. This retarded elution, probably due to hydrophobic interactions with the column material, led to an excellent separation of PtdIns(4)P kinase from other proteins. Of all the steps used in the purification procedure, this step yielded the highest enrichment (i.e., 25-fold) of PtdIns(4)P kinase activity. Subsequent chromatography of the active fraction on DEAE-cellulose yielded PtdIns(4)P kinase activity in the flow-through. Because of this behaviour this activity was assumed to be PtdIns(4)P kinase activity *b* (see above).

This last ion-exchange chromatography step in combination with affinity chromatography on ATP-agarose resulted in a 6333-fold purified protein with a specific activity of 171 nmol · min^{-1} · mg^{-1} (see Table I). The

specific activity of the final preparation of PtdIns(4)P kinase activity *a* was reported to be 85 nmol · min^{-1} · mg^{-1} [10]. Since under the present assay conditions PtdIns(4)P kinase activity is 2-fold higher due to the activation by PtdSer, one may confer that the specific activity of PtdIns(4)P kinase activities *a* and *b* are comparable.

Despite the extensive purification, the final pool of PtdIns(4)P kinase activity *b* still contained several protein bands, as revealed by silver staining after SDS-PAGE (Fig. 1). So far, numerous attempts to obtain a homogeneous preparation have failed, and it can not be excluded that PtdIns(4)P kinase activity *b* is an oligomer of several subunits with different molecular mass. The estimated molecular mass of PtdIns(4)P kinase activity *a* is 110 kDa [10]. No 110 kDa protein is detectable in the PtdIns(4)P kinase activity *b* preparation, suggesting that we are dealing with different proteins.

Characteristics of PtdIns(4)P kinase activities a and b

The phosphorylation site on the inositol ring of PtdIns(4)P was determined after deacylation of the product of the PtdIns(4)P kinase reaction, followed by analysis of the deacylated product by HPLC. For both kinase activities, the elution profile shows a single peak comigrating with authentic deacylated PtdIns(4,5)P_2 (Fig. 2). These results indicate that both kinase activities specifically phosphorylate PtdIns(4)P on the 5′-position. Previously, PtdIns(4)P kinase from erythrocyte membranes has also been reported to specifically phosphorylate PtdIns(4)P on the 5′-position [9].

Recently, a novel phosphatidylinositol phosphate compound, PtdIns(3)P, has been identified in vitro [43]

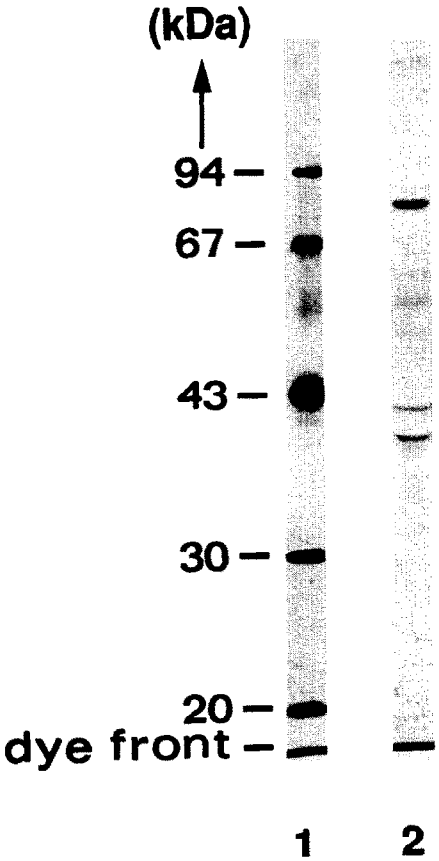

Fig. 1. Purified PtdIns(4)P kinase *b*. The final preparation obtained after chromatography on ATP-agarose was subjected to SDS-PAGE. Protein was visualized by silver staining. Lane 1: molecular mass markers; lane 2: purified enzyme.

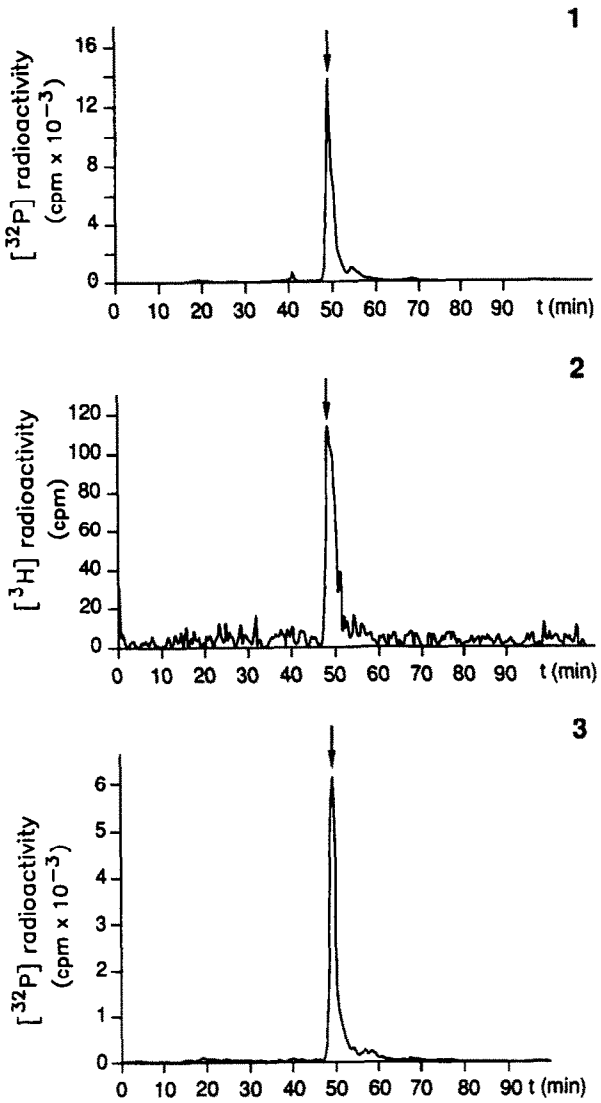

Fig. 2. Identification of the phosphorylation site on the inositol ring. PtdIns(4,5)P_2 was produced by phosphorylation of PtdIns(4)P with [γ-^{32}P]ATP using PtdIns(4)P kinase under standard conditions. The ^{32}P-labeled PtdIns(4,5)P_2 was deacylated and analyzed by HPLC. Authentic deacylated tritiated PtdIns(4,5)P_2 was mixed prior to HPLC-analysis with the deacylated product of the reaction catalyzed by PtdIns(4)P kinase *a*. 1: HPLC elution of the deacylated product of the reaction catalyzed by PtdIns(4)P kinase activity *a*. 2: HPLC elution of deacylated tritiated PtdIns(4,5)P_2 standard. Deacylated PtdIns(3,4)P_2 standard elutes at 44 min (not shown). 3: HPLC elution of the deacylated product of the reaction catalyzed by PtdIns(4)P kinase activity *b*. Arrows indicate the elution position of deacylated PtdIns(4,5)P_2.

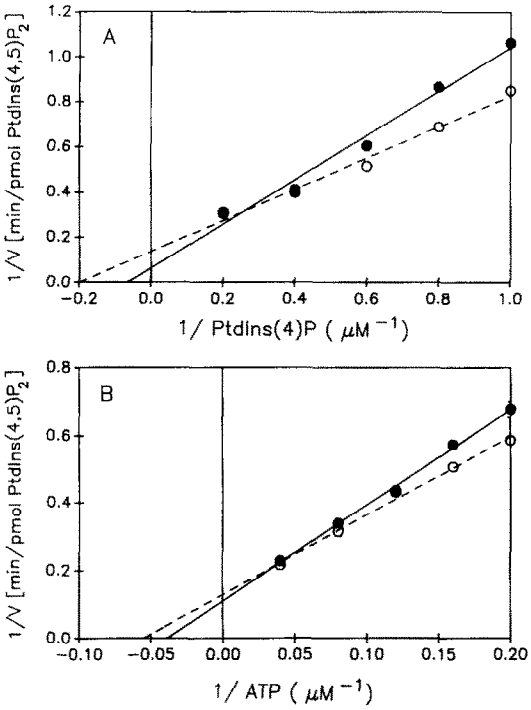

Fig. 3. Lineweaver-Burk plots for the utilization of PtdIns(4)P and ATP by PtdIns(4)P kinase. A: Variation of PtdIns(4)P concentrations in the presence of 50 μM ATP. B: Variation of ATP concentrations in the presence of 80 μM PtdIns(4)P. Data are an average of two experiments. Kinase activity *a*: ●——●; kinase activity *b*: ○——○.

and in vivo [44]. The metabolism of PtdIns(3)P appears to be linked to oncogenic and mitogenic events, yet the physiological role of PtdIns(3)P and its metabolites are not known to date (for review, see Ref. 45). We determined whether PtdIns(3)P and its metabolic product PtdIns(3,4)P_2 were an in vitro substrate for PtdIns(4)P kinase activities *a* and *b* from bovine brain membranes. Neither lipid was phosphorylated by PtdIns(4)P kinase activities *a* and *b*. However, PtdIns(4)P under these conditions present as a minor contaminant (5% of total), was phosphorylated to PtdIns(4,5)P_2 (data not shown). This indicates that PtdIns(4)P kinase is able to discriminate between two isomers at very low substrate concentrations, showing a specificity for the PtdIns(4)P isomer. In contrast, the PtdIns(4)P kinase from human erythrocyte membranes could be shown to also phosphorylate PtdIns(3)P [45]. PtdIns was not phosphorylated by either kinase. In the presence of deoxycholate, a condition optimal for measurement of diacylglycerol kinase activity [46], diacylglycerol was phosphorylated by either kinase activity to less than one percent of the enzyme activity observed with PtdIns(4)P as a substrate.

The dependence of the PtdIns(4)P kinase reaction on the concentrations of ATP and PtdIns(4)P was analyzed by Lineweaver-Burk plots (Fig. 3). For the two enzymes the K_m values for PtdIns(4)P differed

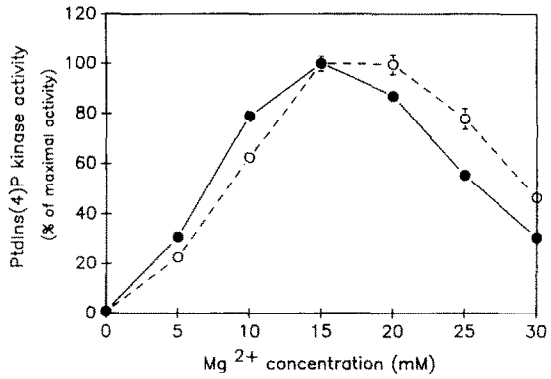

Fig. 4. Dependence of PtdIns(4)P kinase activity on Mg^{2+} concentrations. PtdIns(4)P kinase was assayed in the presence of the indicated concentrations of Mg^{2+}. Data are an average of two experiments. Kinase activity *a*: ●——●; kinase activity *b*: ○——○.

3.5-fold, being 18 μM for PtdIns(4)P kinase activity *a*, and 4 μM for PtdIns(4)P kinase activity *b*. The K_m values for ATP did not differ significantly; the K_m for ATP was 25 μM for PtdIns(4)P kinase activity *a* and 20 μM for PtdIns(4)P kinase activity *b*. All these K_m values are in the range of those reported for other purified PtdIns(4)P kinase preparations, which differ from 3–60 μM for PtdIns(4)P and from 2–130 μM for ATP [6–9,11,12].

For both PtdIns(4)P kinase activities *a* and *b*, maximal activation was found at a Mg^{2+} concentration of about 15 mM. With regard to the overall dependence on Mg^{2+}, however, a slight difference between kinase activities *a* and *b* was reproducibly found (see Fig. 4). Other PtdIns(4)P kinases have similar optimal activities around 10–30 mM Mg^{2+} [5–9,11–15]. In Fig. 5 it is shown that both forms of PtdIns(4)P kinase have a broad optimum of activity around pH 7.0. This pH optimum is similar to values reported by others [7,8,13,14].

It has been reported by several authors that PtdIns(4)P kinase is stimulated by PtdSer [6,9] and

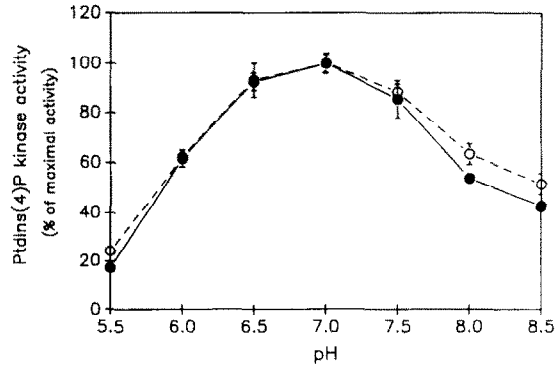

Fig. 5. Dependence of PtdIns(4)P kinase activity on different pH values. Buffers used were: 50 mM sodium acetate (pH 5.5), 50 mM Bistris-HCl (pH 6.0–7.0), 50 mM Tris-HCl (pH 7.5–8.5). Data are an average of two experiments. Kinase activity *a*: ●——●; kinase activity *b*: ○——○.

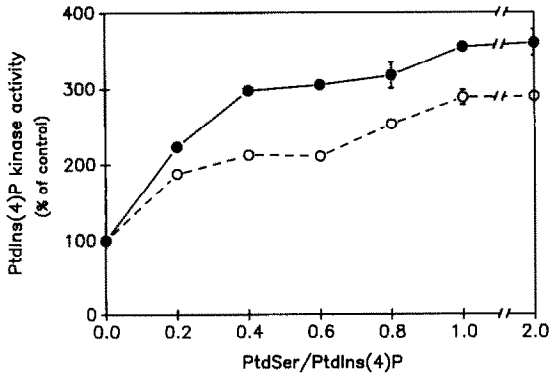

Fig. 6. Stimulation of PtdIns(4)P kinase by PtdSer. Various amounts of PtdSer were added to the assay containing 80 μM PtdIns(4)P. The molar ratio of PtdSer/PtdIns(4)P was varied from 0.2 to 2. Data are an average of two experiments. Kinase activity *a*: ●——●; kinase activity *b*: ○——○.

inhibited by its product PtdIns(4,5)P_2 [7,9,47]. Such a stimulation by PtdSer and inhibition by PtdIns(4,5)P_2 is also found for both PtdIns(4)P kinase activities *a* and *b* in this study. Fig. 6 shows that for both activities the activation by PtdSer reaches a maximum at a ratio of PtdIns(4)P/PtdSer of 1:1, yet there is a difference between type kinase activities *a* and *b* in the extent of the activation. For PtdIns(4)P kinase activity *a*, maximal activation by PtdSer is 3.5-fold, whereas PtdIns(4)P kinase *b* activity is increased to a maximum of 2.5-fold. At a molar ratio of PtdIns(4)P/PtdIns(4,5)P_2 of 1:1, both PtdIns(4)P kinase activities are inhibited by about 40%, and at a molar ratio of PtdIns(4)P/PtdIns(4,5)P_2 of 3:1 both enzyme activities are inhibited by as much as 70% (Fig. 7). The extent of activation by PtdSer and of inhibition by PtdIns(4,5)P_2 are comparable with observations for other PtdIns(4)P kinase preparations [6,7,9,47].

Recently we have found that phosphatidic acid activates PtdIns(4)P kinase to an even higher extent than PtdSer, an effect which could play a direct role in an

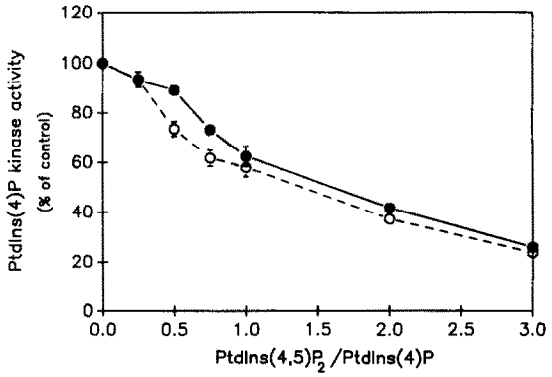

Fig. 7. Inhibition of PtdIns(4)P kinase activity by PtdIns(4,5)P_2. Various amounts of PtdIns(4,5)P_2 were added to the assay containing 80 μM PtdIns(4)P. The molar ratio of PtdIns(4,5)P_2 /PtdIns(4)P was varied from 0.25 to 3. Data are an average of two experiments. Kinase activity *a*: ●——●; kinase activity *b*: ○——○.

enhanced resynthesis of PtdIns(4,5)P_2 after ligand-receptor stimulated breakdown [48]. Both PtdIns(4)P kinase activities *a* and *b* showed a similar susceptibility to activation by phosphatidic acid, their activity being enhanced up to 20-fold upon addition of the lipid [48].

Concluding Remarks

Although PtdIns(4)P kinase activities *a* and *b* could be separated completely by chromatography on DEAE-cellulose, their kinetic parameters were rather similar, except for the K_m for PtdIns(4)P which was significantly different. Moreover, except during chromatography on DEAE-cellulose, both PtdIns(4)P kinase activities behaved as a single activity during purification. These data show that PtdIns(4)P kinase activities *a* and *b* have many properties in common.

Further research will be required to establish whether a structural relationship exists between PtdIns(4)P kinase activities *a* and *b* and to elucidate whether they have different functions in the cell.

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