Purification and characterization of phosphatidylinositol 4-phosphate 5-kinases

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We detail the purification and characterization of three distinct isoforms of PtdIns4P 5-kinase present in bovine brain. One of these, PtdIns4P 5-kinase C, was purified to apparent homogeneity, and SDS/PAGE analysis demonstrated a single polypeptide and molecular mass 53 KDa. These three isoforms were shown to differ in their kinetic properties, and immunological characterization with an antibody raised to PtdIns4P 5-kinase C demonstrated that this isoform was unrelated to the other two. Furthermore, PtdIns4P 5-kinase C was shown to be the bovine brain homologue of the Type II PtdIns4P 5-kinase previously purified from human erythrocytes [Bazenet, Ruano, Brockman & Anderson (1990) *J. Biol. Chem.* 265, 18012–18022].

INTRODUCTION

Agonist-induced phosphoinositidase C activation generates three second messengers, Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , which appear to play a role in Ca^{2+} homeostasis (Streb *et al.*, 1983; Irvine, 1991), and diacylglycerol, a physiological activator of protein kinase C (Nishizuka, 1984, 1989; Berridge & Irvine, 1989).

For the sustained production of these second messengers, the mass of the precursor lipid, PtdIns(4,5) P_2 , must be maintained. Mass measurements of this lipid during agonist stimulation have demonstrated a rapid decrease, followed by a return to basal, or in some cases to above basal levels (Creba *et al.*, 1983; Turner *et al.*, 1984; Taylor *et al.*, 1984; Cockcroft *et al.*, 1985; Divecha *et al.*, 1991). Moreover, the recent demonstration of acutely stimulated PtdIns(4,5) P_2 3-phosphorylation in neutrophils (Stephens *et al.*, 1991) and in Swiss 3T3 cells (Hawkins *et al.*, 1992) suggests that the PtdIns(4,5) P_2 may also be the essential substrate for a second signalling system. Thus PtdIns(4,5) P_2 formation by PtdIns4P 5-kinase can be seen as a key reaction in the control of cell function.

Possible mechanisms for the control of this enzyme include: (1) the removal of feedback inhibition by PtdIns(4,5) P_2 ; purified PtdIns4P 5-kinase has been shown to be inhibited in this manner (Van Rooijen *et al.*, 1985; Pike & Arndt, 1988; Smith & Chang, 1989; Ling *et al.*, 1989; Pike *et al.*, 1990); (2) inhibition of the phosphomonoesterase; (3) direct activation of the enzyme during receptor stimulation. Recently Lassing & Lindberg (1990) demonstrated that stimulation of platelets with low doses of thrombin led to a very rapid (within 10 s) increase in the levels of PtdIns(4,5) P_2 . Their interpretation of these data was that receptor stimulation leads to an activation of PtdIns4P 5-kinase before activation of the phosphoinositidase C. There is other evidence which points to the regulation of this enzyme by the phosphorylated form of an activating protein, B50 (Van Dongen *et al.*, 1985), by a small and as yet uncharacterized G-protein (Urumow & Weiland, 1990) and by tyrosine phosphorylation during stimulation with epidermal growth factor in A431 cells (Cochet *et al.*, 1991) and during stimulation of neutrophils with *N*-formylmethionyl-leucyl-phenylalanine (Pike *et al.*, 1990).

Studies on the regulation of this enzyme have been hampered by the existence of poorly defined multiple isoforms and the lack

of suitable immunological tools. Thus far a 110 kDa enzyme has been purified from bovine brain membranes, although no analysis was carried out to demonstrate the site of phosphorylation on the inositol ring (Moritz *et al.*, 1990). A 53 kDa protein termed Type II PtdIns4P 5-kinase has been purified to homogeneity from human erythrocytes (Ling *et al.*, 1989; Bazenet *et al.*, 1990). Bazenet *et al.* (1990) also demonstrated the existence of a second isoform, Type I, although this was not purified to homogeneity. Interestingly, the Type II was not very active towards native erythrocyte membranes, whereas the Type I enzyme was. As there are no known agonists of human erythrocytes that stimulate turnover of these lipids, it is difficult to assess if these enzymes are involved in maintaining PtdIns(4,5) P_2 levels in a housekeeping fashion, or whether they are enzymes previously involved in the stimulated phosphoinositide cycle during differentiation to an erythrocyte, and are now redundant.

In this study we have characterized the existence of three forms of PtdIns4P 5-kinases and purified one of these to homogeneity from bovine brain. Antibodies raised to this purified enzyme do not cross-react with the other forms of the enzymes, but do recognize the Type II enzyme purified from human erythrocytes.

MATERIALS AND METHODS

Materials

Bovine brains were obtained from Adkins Abattoir, Church End, Cherry Hinton, Cambridge, U.K., or from Canvin International, Sandy, Bedfordshire, U.K. [^{32}P]ATP and ECL detection systems were from Amersham International. Protein assay reagent was from Bio-Rad. Q-Sepharose Fast Flow was from Pharmacia LKB. Heparin-agarose Type I, proteinase inhibitors and all conjugated antibodies were from Sigma Chemical Co. Phosphocellulose P11 was from Whatman. Silica-gel t.l.c. plates were from Merck. All other chemicals were of analytical grade.

Assay for PtdIns4P 5-kinase

PtdIns4P 5-kinase was assayed by two methods. The rapid method was used to assay column fractions. PtdIns4P (500 pmol) was adsorbed to micro-titre wells in 100 μ l of 50 mM-Tris/acetate (pH 7.4)/80 mM-KCl/10 mM-magnesium acetate/2 mM-EGTA (kinase buffer) overnight. The plate was rinsed with water, and

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; GroPIns, glycerophosphoinositol.

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the assays were carried out in 200 μ l of kinase buffer containing 1–20 μ l of enzyme and 5 μ M-ATP plus 0.5 μ Ci of [32 P]ATP/well. The plates were incubated at room temperature for 10 min, then rinsed with water. The plate was then cut into separate wells and the radioactivity incorporated into PtdIns(4,5) P_2 was determined by Čerenkov counting. Alternatively, for comparison of isoenzyme properties, assays were carried out with micelles of PtdIns4P prepared by sonication into kinase buffer. Assays in this case were quenched by addition of 750 μ l of chloroform/methanol/HCl (80:160:1, by vol.). The phases were split by addition of 250 μ l of chloroform and 250 μ l of 2.4 M-HCl. The washed lower phases were then evaporated to dryness and spotted on to t.l.c. plates previously dipped in 1% potassium oxalate/2 mM-EDTA/50% methanol, dried at room temperature and activated at 110 °C for 30 min. The plates were developed in chloroform/methanol/ammonia/water (45:35:2:8, by vol.). Spots corresponding to PtdIns(4,5) P_2 were scraped, and radioactivity incorporated was determined by scintillation counting.

Purification of PtdIns4P 5-kinases

The following procedure was carried out at 4 °C. Two fresh bovine brains were homogenized in 20 mM-Tris/HCl (pH 8.3)/0.5 mM-EDTA/0.1 mM-EGTA/0.25 M-sucrose/24 mM-2-mercaptoethanol/0.4 mM-phenylmethanesulphonyl fluoride (PMSF)/5 mM-leupeptin to obtain 3 litres of homogenate. This was cleared by centrifugation at 25000 g for 60 min in a Sorvall Europa centrifuge. The pH of the supernatant was adjusted to 8.3 by addition of ice-cold 1 M-Tris. The supernatant could be stored at –80 °C for several months without loss of PtdIns4P 5-kinase activity.

The supernatant was bound to 300 ml of Q-Sepharose Fast Flow equilibrated in 20 mM-Tris/HCl (pH 8.3)/0.5 mM-EDTA/0.1 mM-EGTA/24 mM-2-mercaptoethanol/0.4 mM-PMSF (buffer A) for 90 min, then rinsed in a sintered funnel with 5 litres of buffer A to remove unbound protein. The resin was packed into a column and was developed with a linear gradient of 1.6 litres of buffer A to buffer A + 1.0 M-NaCl.

The Q-Sepharose peak was diluted by a factor of 3 with buffer A and applied to a heparin-agarose column (5.0 cm $\times$ 2.5 cm) equilibrated in 20 mM-Tris/HCl (pH 7.5)/0.5 mM-EDTA/0.1 mM-EGTA/2 mM-MgCl₂/10% glycerol/12 mM-2-mercaptoethanol/0.4 mM-PMSF (buffer B). This was then washed with 75 ml of buffer B containing 0.3 M-NaCl, then developed with 240 ml of a linear gradient of 0.3–1.0 M-NaCl in buffer B. The peak of enzyme activity was dialysed overnight against buffer B containing 5 μ M-ATP and 0.01% Triton X-100, which was found to stabilize the enzyme (Moritz *et al.*, 1990).

The dialysed heparin-agarose peak was applied to a phosphocellulose column (3 cm $\times$ 1.5 cm) at 0.75 ml/min, washed with 60 ml of buffer B + 0.01% Triton X-100 + 300 mM-NaCl, and then developed with a linear gradient of 0.3–1 M-NaCl. This resolved two peaks of activity, which were then concentrated to 0.5 ml by using an Amicon Centriprep 10 concentrator.

Each peak from the previous column was diluted to 10 ml in buffer B + 0.01% Triton X-100 and then loaded on to a Mono Q column. The column was washed for 8 min in 10% NaCl and then developed with a 32 ml gradient to 0.5 M-NaCl in buffer B.

Preparation of antibodies

Purified PtdIns4P 5-kinase C was prepared for immunization by precipitation with trichloroacetic acid, followed by acetone washing. The pellet was then resuspended into 50 mM-Tris/HCl, pH 7.4, and emulsified in either Freund's complete or incomplete adjuvant before injection. One rabbit was injected with approx. 80 μ g of protein.

Western blotting

Proteins were separated by SDS/PAGE on 10%-acrylamide gels by the method of Laemmli (1970), and then transferred to nitrocellulose in accordance with Towbin *et al.* (1979). The nitrocellulose was blocked overnight in Tris/saline buffer (TBS) containing 3% BSA and 0.05% Tween 20. The membrane was incubated with primary antibody (serum at a dilution of 1:100 or affinity-purified antibody at 10 μ g/ml) for 2 h, followed by extensive washing in TBS/Tween (0.2%). This was then incubated with secondary antibody for 1 h, washed as above and developed with 3,3'-diaminobenzidine or with the ECL kit.

Determination of the position of phosphorylation of PtdIns4P by the purified kinases

Each of the three peaks of kinase activity was used to phosphorylate PtdIns4P micelles as described above. After quenching of the samples, [3 H]PtdIns(4,5) P_2 (10000 d.p.m.) was added. The samples were then deacylated, or deacylated and deglycerated, and then subjected to analysis by ion-exchange h.p.l.c. on a Partisphere 5-SAX column as described by Stephens *et al.* (1991).

RESULTS

Purification of cytosolic PtdIns4P 5-kinases

The supernatant from two bovine brains was applied to a Q-Sepharose column and eluted with a linear salt gradient. Two peaks of PtdIns4P 5-kinase were detected by using the novel multi-well plate assay described in the Materials and methods section. This assay is extremely rapid, and allows the characterization of 100 column fractions within 30 min. The assay was simple, suitable for multiple samples and did not require tedious phase extractions followed by t.l.c.

The two peaks of activity were pooled, to avoid duplication of the purification at this early stage, and applied to a heparin column. All the enzyme activity was found to bind to the column, and was eluted as a single peak of activity at approx. 0.7 M-NaCl (Fig. 1a). The recovery of activity from this column was extremely high (> 90%) and the purification was approx. 400-fold. This peak of activity was dialysed and applied to a phosphocellulose column. Elution from this column with a linear salt gradient yielded two peaks of activity. The first was eluted at a salt concentration of 0.3 M and the second at 0.8 M-NaCl (Fig. 1b).

The two peaks of activity were kept separate from this point onwards. Each was concentrated, re-diluted and applied to a Mono Q column. Peak 1 from the phosphocellulose column yielded two peaks of activity, eluted at 0.125 M-NaCl (which we have called PtdIns4P 5-kinase A) and 0.4 M-NaCl (PtdInsP 5-kinase B) (Fig. 1c). Peak 2 from the phosphocellulose column yielded a single peak of activity at 0.38 M-NaCl (Fig. 1d, which we have defined as PtdIns4P 5-kinase C). Table 1 shows the purification and recovery of the enzyme at each column stage. Purification of PtdIns4P 5-kinase C was 4500-fold, with a recovery of only 6%. However, if one assumes that only 23% of the initial activity was from PtdIns4P 5-kinase C, the rest being from PtdIns4P 5-kinases A and B (see data for the phosphocellulose column), then the purification is approx. 20000-fold with a recovery of 30%. Analysis of PtdIns4P 5-kinase C by SDS/PAGE demonstrated the presence of a single protein of molecular mass 53 kDa which co-eluted with PtdIns4P 5-kinase activity (Fig. 1e).

Characterization of the position of phosphorylation of PtdIns4P

With the recent characterization of a number of kinases that will phosphorylate PtdIns4P in the 3-position by PtdIns 3-kinase (Carpenter *et al.*, 1990), it was important to establish the position

Fig. 1. Purification of the three forms of PtdIns4P 5-kinase

The initial homogenate was fractionated on a Q-Sepharose column, which yielded two peaks of PtdIns4P kinase activity. These were then loaded on to the heparin-agarose column and eluted with a linear salt gradient of 0.3–1 M-NaCl (a). PtdIns4P kinase activity (●) was pooled and applied to a phosphocellulose column (b). Two peaks of activity were found (●). Peak 1 was then concentrated and applied to the Mono Q column (c). This yielded two peaks of activity, eluted at approx. 125 mM-NaCl (peak A) and 380 mM-NaCl (peak B). Peak 2 from the phosphocellulose column (b) was also applied to a Mono Q column (d). A single peak of activity was eluted at 380 mM-NaCl. (----, protein or A₂₈₀; —, NaCl gradient.) The gel photograph (e) shows the SDS/PAGE analysis of 20 µl of each of column fractions 15–21 (lanes 2–8). Lane 1 contains molecular-mass markers of 200, 97, 69, 46, 30 and 21 kDa from top to bottom. A single 53 kDa polypeptide co-migrated with the PtdIns4P 5-kinase activity. Gels were run under reducing conditions essentially as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R250 (0.25 %, in 40 % methanol/10 % acetic acid) overnight and destained with several washes of the above solution without the Coomassie Blue.

Table 1. Summary of the purification of the three different PtdIns4P 5-kinases

The data shown are from a single experiment typical of at least three different purifications. Assays were linear with respect to time and to the amount of protein added. Mono Q 1 was the chromatography of peak 1 from the phosphocellulose column and yielded two peaks of PtdIns4P 5-kinase activity, defined as peaks A and B. Mono Q 2 was chromatography of peak 2 from the phosphocellulose column and yielded a single peak of PtdIns4P 5-kinase activity, defined as peak C.

	Protein (mg/ml)	Total protein (mg)	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Purification (fold)	Recovery (%)
Supernatant	12 500	94000	0.0001	9.4	1.0	100
Q-Sepharose	2350	2300	0.0013	3.05	1.3	32.4
Heparin	0.175	17.5	0.464	8.12	464	86
Phosphocellulose						
Peak 1	0.440	4.4	1.5	6.6	1511	70
Peak 2	0.165	2.3	0.987	2.2	987	23
Mono Q 1						
Peak A	0.245	0.486	1.623	0.79	1500	8.3
Peak B	0.268	0.539	0.705	0.38	705	4.0
Mono Q 2						
Peak C	0.082	0.164	4.54	0.74	4500	7.8

of phosphorylation of the products of these enzymes. Therefore, after phosphorylation the lipid was either deacylated, or deacylated and deglycerated, and then subjected to analysis of the subsequent head groups by h.p.l.c. As shown in Fig. 2, all three kinases yielded products which co-migrated with

GroPIns(4,5)P₂. No other ³²P-labelled products were found during the analysis. In the profile shown, GroPIns(3,4)P₂ was found to migrate five fractions before GroPIns(4,5)P₂. After deacylation and deglyceration all the ³²P-labelled products co-migrated exactly with Ins(1,4,5)P₃ (results not shown).

Fig. 2. Demonstration that all three PtdIns4P kinases phosphorylate at the 5 position

PtdIns4P was phosphorylated in the presence of [32 P]ATP with each of the different isoforms, (a), (b) or (c). The labelled lipids were then separated from ATP and deacylated in the presence of [3 H]-PtdIns(4,5) P_2 . The water-soluble products were then chromatographed on a Partisphere h.p.l.c. column (Stephens *et al.*, 1991). All the 32 P-labelled products ($\bullet$) co-migrated with [3 H]GroPIns(4,5) P_2 ($\circ$). GroPIns(3,4) P_2 migrated five fractions before [3 H]-GroPIns(4,5) P_2 .

Table 2. Summary of the kinetic data

Assays were carried out such that they were linear with respect to time and to the amount of protein added. The assays contained 1 nmol of PtdIns4P in the assay for the determination of the K_m for ATP, and contained 5 μ M-ATP for the K_m determination of PtdIns4P. All assays were repeated for two independent preparations of each isoform, and the K_m values were within 10% of those given.

PtdIns4P 5-kinase isoform	K_m for ATP (μ M)	K_m for PtdIns4P (μ M)
A	25	0.3
B	20	7.8
C	2.7	40

Fig. 3. A bovine brain PtdIns4P 5-kinase is the homologue of the Type II PtdIns4P kinase purified from human erythrocytes

Purified preparations of either bovine brain PtdIns4P 5-kinase C (lane 1) or purified human erythrocyte Type II PtdIns4P 5-kinase (lane 2) were separated by SDS/PAGE, transferred to nitrocellulose and probed with an antibody raised to the bovine enzyme. The antibody recognizes the human enzyme. Reciprocal experiments carried out with an antibody raised to the human enzyme yielded identical results.

Kinetic analysis of the three PtdIns4P 5-kinases

Although it is difficult to interpret kinetic data of lipid enzymes for their substrates in physiological terms, we carried out this analysis to establish if any of the isoforms isolated demonstrated different kinetic patterns. The data (Table 2) show that the K_m of the enzymes for ATP for PtdIns4P 5-kinase A and B were 25 and 20 μ M, compared with PtdIns4P 5-kinase C, which had a K_m of 2.7 μ M. The K_m measurements for PtdIns4P gave a value for peak C of 40 μ M, whereas that for peak B was 7 μ M. Interestingly, PtdIns4P 5-kinase A had a much lower K_m for PtdIns4P, being 0.34 μ M. This enzyme also showed potent inhibition by high levels of PtdIns4P.

Immunological characterization

Antibodies were prepared to the PtdIns4P 5-kinase C enzyme. As this appears to be the bovine homologue of the type II enzyme purified by Bazenet *et al.* (1990) from human erythrocytes, we tested our antibodies against pure human erythrocyte type II enzyme (kindly donated by R. Anderson). As shown in Fig. 3 (lane 2), antibodies raised against the bovine PtdIns4P 5-kinase C recognized the human erythrocyte enzyme. We also demonstrated that antibodies against the erythrocyte enzyme also detected our preparation from bovine brain (results not shown).

We then tested the three enzyme preparations to determine if any of the activities that we had purified could be explained by proteolytic degradation of the PtdIns4P 5-kinase C enzyme. As shown in Fig. 4, the antibodies against PtdIns4P 5-kinase C did not recognize any of the proteins present in phosphocellulose peak 1 (lane 2) or in the other preparations after Mono Q chromatography (lanes 4 and 5), even though the same amount of activity as for PtdIns4P 5-kinase C was loaded on the gel (lane 6).

Fig. 4. An antibody raised to the bovine brain isoform-C PtdIns4P 5-kinase does not recognize the other isoforms of the enzyme

Pooled peaks from the heparin column (lane 1), phosphocellulose peak 1 (lane 2) and peak 2 (lane 3), Mono Q peak A (lane 4), Mono Q peak B (lane 5) and purified PtdIns4P 5-kinase (Mono Q peak C; lane 6) were separated by SDS/PAGE, transferred to nitrocellulose and probed with the antibody against isoform C. A single band was recognized in the heparin fraction. No proteins were recognized in phosphocellulose peak 1 or in peak A or B from the chromatography of phosphocellulose peak 1 on Mono Q.

DISCUSSION

Three forms of PtdIns4P 5-kinase have been substantially purified from bovine brain. One of these, isoform C, was purified to homogeneity, and SDS/PAGE analysis demonstrated a single protein of molecular mass 53 kDa. The kinetic data for this enzyme were in very good agreement with the data obtained for the Type II human erythrocyte PtdIns4P 5-kinase (Bazenet *et al.*, 1990) and that purified from the same source by Ling *et al.* (1989). Immunological analysis using antibodies raised against either the human enzyme or the bovine enzyme cross-reacted. Indeed, two-dimensional peptide mapping of the bovine enzyme gave a very similar pattern (results not shown) to that observed by Bazenet *et al.* (1990), suggesting that they are closely related.

We have presented several lines of evidence that the isoforms A and B are different from C. Isoforms A and B were easily separated from C by the phosphocellulose column, being eluted together at approx. 350 mM-NaCl. All the three isoforms show very different kinetic characteristics with respect to their K_m for PtdIns4P, although all three were determined to be 5-kinases. Isoform A has a very low K_m for PtdIns4P, and higher concentrations of PtdIns4P, above 6 μ M, showed potent inhibition. The other isoforms did not show this property. Kinetic data for isoform B suggest that it closely resembles the Type I activity from human erythrocytes (Bazenet *et al.*, 1990), having a similar K_m for ATP and for its substrate. Bazenet *et al.* (1990) also demonstrated that their Type I enzyme was not inhibited by heparin even at concentrations of up to 250 μ g/ml. However, all of the isoforms purified in the present study were totally inhibited by heparin at a concentration of 1 μ g/ml (results not shown). They also suggested that the Type I enzyme was predominantly

associated with the erythrocyte membrane. As yet, we have not undertaken a full study on the subcellular localization of the different isoforms.

Immunological analysis using an antibody raised either to the bovine PtdIns4P 5-kinase C or to the human erythrocyte Type II enzyme (kindly provided by R. Anderson) did not recognize any proteins in the PtdIns4P 5-kinase A or B peaks. This suggests that these activities are unlikely to be due to proteolytic degradation of the PtdIns4P-kinase C enzyme, and represent true isoforms of the activity.

It has been suggested that PtdIns4P kinase may be inhibited by PtdIns(4,5) P_2 and that the removal of this inhibition during receptor stimulation may be a method for the activation of the enzyme (Ling *et al.*, 1989; Pike & Arndt, 1988). In our hands, inhibition of the enzymes was only seen at concentrations of PtdIns(4,5) P_2 which were 5–10-fold higher than the PtdIns4P concentration. The inhibition seen by other workers may be due to the way in which the substrate was presented. In other studies, up to 0.1% Triton and other lipids such as phosphatidylserine have been used in the assay. Indeed, it has been shown that the inhibition of rat brain PtdIns4P 5-kinase by PtdIns(4,5) P_2 was either competitive or non-competitive, depending on the presence of different lipid species such as diacylglycerol (Smith, 1988).

Whether all three isoforms are present in all cells or whether they show tissue-specificity has not been determined. A preliminary screening of cell lines and tissues has found that there are very different levels present. For example, 3T3 cells appear to have very little PtdIns4P 5-kinase C, whereas Jurkat cells, a T-cell line, contain significant amounts. It is possible that the different isoforms are stimulated by different second-messenger systems, such that one form is stimulated by tyrosine phosphorylation whereas another isoform is linked to a G-protein, rather reminiscent of the function of the phospholipase C enzymes (Rhee, 1991). Another possibility is that the different isoforms are linked to different pathways. For example, it has been suggested that the likely candidate for a second messenger during *N*-formylmethionyl-leucyl-phenylalanine stimulation of the PtdIns3P kinase pathway in neutrophils is the production of PtdIns(3,4,5) P_3 , which appears to come directly from the phosphorylation of PtdIns(4,5) P_2 (Stephens *et al.*, 1991). Interestingly, there is also a 10–20-fold activation of PtdIns4P 5-kinase during this stimulation (L. R. Stephens, personal communication). Thus it appears that PtdIns(4,5) P_2 is at the branch point of two fundamental second-messenger pathways, and it will therefore be important to understand the regulation of the enzymes involved in the biosynthesis of this key intermediate.

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