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Purification and partial characterization of the phosphatidylinositol 4-phosphate kinase from rat brain

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Phosphatidylinositol 4-phosphate (PtdIns4*P*) kinase was purified from cytosolic and particulate material of rat brain. The purification procedure of the enzyme from cytosol consisted of $(NH_4)_2SO_4$ precipitation. DEAE-cellulose column chromatography and preparative isoelectric focusing. Other methods after DEAE-cellulose column chromatography failed to achieve further purification of the PtdIns4*P* kinase, probably caused by the tendency of the enzyme to aggregate with contaminating proteins. The final purification was 67-fold, and the recovery was 0.6%. After isoelectric focusing the fraction containing the highest PtdIns4*P* kinase activity showed only one protein as visualized by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and silver staining. The apparent M_r of this protein was 45 kDa and the isoelectric point about 5.8. The activity of PtdIns4*P* kinase was dependent on the concentration of divalent cations in the incubation medium. PtdIns4*P* kinase activity was found to be optimal at 10–30 mM-Mg^{2+}. In an attempt to compare the cytosolic with the membrane-derived kinase activity, a Triton/KCl extract from synaptic membranes was subjected to the same purification procedure as the cytosolic enzyme. A difference in isoelectric focusing was observed, possibly due to a higher tendency to form aggregates. However, we tend to conclude that also in the membranes the PtdIns4*P* kinase activity is present as a 45 kDa protein, identical with that found in the cytosol.

Although polyphosphoinositides have been found to be minor constituents of all animal tissues examined to date, increased attention is directed to their metabolism and function in a variety of membranes (Downes & Michell, 1982; Abdel-Latif, 1983). Compared with other tissues, the nervous system is rich in phosphoinositides (Hawthorne & Kai, 1970; Michell, 1975) and it has been postulated that this class of lipids is involved in the modulation of membrane-mediated processes such as chemical neurotransmission. Indeed, a variety of stimuli, including neurotransmitters, hormones, neuropeptides and electrical pulses, enhance the turnover of the phosphate groups of phosphoinositides and phosphatidic acid (Abdel-Latif, 1983).

In addition to phospholipids, phosphoproteins also seem to be involved in the regulation of neurotransmission (Rodnight, 1982). Evidence has been obtained that lipid phosphorylation and protein phosphorylation may be linked (Jolles *et al.*, 1980, 1981; Akhtar *et al.*, 1983). In a crude synaptosomal fraction corticotropin inhibits the endogenous phosphorylation of a brain-specific protein (B-50) and stimulates the formation of PtdIns4,5P_2 (Jolles *et al.*, 1981). Jolles *et al.* (1980) demonstrated that in a purified B-50/B-50 kinase fraction the extent of prephosphorylation of B-50 protein is inversely correlated with the amount of phosphate incorporated into PtdIns4*P*, suggesting that changes in the extent of phosphorylation of B-50 protein may be a regulatory factor in the metabolism of polyphosphoinositides. Recently, we were able to support this hypothesis, using specific anti-B-50 antibodies (Oestreicher *et al.*, 1983). In synaptic plasma membrane fractions these antibodies specifically inhibited the endogenous phosphorylation of B-50 protein and this inhibited phosphorylation was accompanied by a stimulated formation of PtdIns4,5P_2.

Abbreviations used: PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; SDS, sodium dodecyl sulphate.

The present paper describes a method for preparing highly purified PtdIns4*P* kinase. Previous studies on this enzyme (Kai *et al.*, 1968; Tou *et al.*, 1970; Torda, 1972; Cooper & Hawthorne, 1976; Shaikh & Palmer, 1977; Desmukh *et al.*, 1984) have been carried out using crude fractions. We report a procedure for the isolation of PtdIns4*P* kinase and give some of the properties of the enzyme.

Materials and methods

Materials

All chemicals used were of analytical grade. [γ-^{32}P]ATP was obtained from New England Nuclear. Sephadex IEF and M_r markers were purchased from Pharmacia, and Ampholines were from LKB. PtdIns4*P* was from Sigma.

Preparation of subcellular fractions

Male albino rats (150–180g body wt.) of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). Animals were killed by decapitation and synaptic plasma membranes were prepared from forebrain tissue as described previously (Zwiers *et al.*, 1980). The whole isolation procedure was performed at 0–4°C. In short, the tissue was homogenized in 0.32M-sucrose (1:10, w/v) and centrifuged for 10min at 1000*g* to remove nuclei and tissue debris. The supernatant was spun for 20min at 10000*g*. The resulting supernatant (S_2) was used for the purification of the cytosolic PtdIns4*P* kinase; the pellet, containing synaptosomes and mitochondria, was subjected to osmotic shock and again centrifuged for 20min at 10000*g*. The supernatant, containing light synaptic plasma membrane fragments was centrifuged for 30min at 100000*g*. The pellet was used for the purification of the membrane-associated PtdIns4*P* kinase.

$(NH_4)_2SO_4$ precipitation of cytosolic proteins

The supernatant (S_2) was centrifuged for 1h at 105000*g* to remove all particulate material (see also Kai *et al.*, 1968). The supernatant was subjected to $(NH_4)_2SO_4$ precipitation. The protein fraction precipitating between 20 and 40% saturation was collected by centrifugation for 20min at 105000*g* and dissolved in 450ml of buffer containing 10mM-Tris/HCl (pH7.4)/0.1mM-dithiothreitol (buffer A).

Solubilization of membrane-associated PtdIns4P kinase

Analogous to the solubilization of B-50 protein and its kinase (see Zwiers *et al.*, 1980), PtdIns4*P* kinase was solubilized in a native form by treatment of the membranes with a combination of a non-ionic detergent and high salt. To achieve this, the membrane pellet was resuspended in 100ml of 6mM-Tris/HCl, pH8.1, containing 0.1mM-dithiothreitol. An equal volume of 1% Triton X-100, also containing 150mM-KCl in the same buffer, was added and the suspension was stirred for 15min. Residual particulate material was removed by centrifuging for 30min at 100000*g*. The extract was diluted to 450ml with buffer A and the pH was adjusted to 7.4 with 0.1M-HCl.

DEAE-cellulose column chromatography

The 20–40% $(NH_4)_2SO_4$ protein fraction of the cytosolic proteins, as well as the Triton/KCl extract of the membrane proteins, was subjected to DEAE-cellulose chromatography (see also Zwiers *et al.*, 1980). The proteins were bound by stirring for 15min with 20ml of (settled) DEAE-cellulose. Unbound material was removed by filtration on a sintered-glass funnel followed by four washes with 200ml of buffer A. The washed DEAE-cellulose was poured into a column (20cm × 1cm) and proteins were eluted by stepwise increases of the NaCl concentration (22.5ml of 50mM-, 22.5ml of 100mM- and 36ml of 150mM-NaCl) followed by a linear gradient (300ml total volume) of 150–250mM-NaCl. Fractions (4.5ml) were collected, dialysed overnight against buffer A and assayed for protein and PtdIns4*P* kinase activity. Active fractions were stored at −20°C for at least 4 weeks without loss of activity.

Flat bed isoelectric focusing

The final step for the purification of PtdIns4*P* kinase consisted of a separation by preparative isoelectric focusing on a flat bed of Sephadex IEF containing Ampholines (Radola, 1973; for details see Oestreicher *et al.*, 1983). After a pre-run of the flat bed for 1h at 600V, 4ml of DEAE-cellulose pooled fractions were applied as a band at 4–5cm from the cathode. The gel was focused overnight at 900V. Afterwards bands of 1cm of the gel suspension were scraped from the glass tray, 1.5ml of water was added to each and the pH was measured. The solution with the proteins was separated from the gel suspension by centrifuging for 3min at approx. 10000*g* in a Janetzki microfuge. After dialysing overnight against buffer A the fractions were assayed for protein and PtdIns4*P* kinase activity.

Determination of PtdIns4P kinase activity

PtdIns4*P* kinase activity was assayed as described previously (Jolles *et al.*, 1980). Briefly, the incubations were carried out under the following conditions unless indicated otherwise: 7.5μM-ATP, 1μCi of [γ-^{32}P]ATP (sp. radioactivity approx. 3000Ci/mmol), 10μl of the dialysed fraction

(ideally containing 2 μg of protein), 10 mM-sodium acetate, 10 mM-magnesium acetate, 0.1 mM-calcium acetate and 48 μM-PtdIns4*P* in a final volume of 25 μl at 30°C and pH 6.5. The samples were preincubated for 5 min at 30°C and the phosphorylation reaction was started by the addition of radioactive ATP with the buffer solution. The reaction was terminated 5 min later by addition of 2 ml of ice-cold chloroform/methanol/12 M-HCl (200:100:0.75, by vol.). The incubation conditions allowed linear incorporation of [^{32}P]phosphate into PtdIns4*P* for at least 5 min.

Lipid extraction and t.l.c.

After termination of the phosphorylation reaction, carrier polyphosphoinositide (10 μg) was added, isolated as described by Dawson & Eichberg (1965). The extraction procedure and t.l.c. were carried out as described in detail by Jolles *et al.* (1981). The lipids were visualized with iodine vapour and ^{32}P-labelled spots were detected by autoradiography on Kodak Royal X-Omat film (10–20 h). After scraping the spots from the plate the amount of radioactivity in PtdIns4,5P_2 was estimated by liquid-scintillation counting (Jolles *et al.*, 1981).

Separation of proteins by polyacrylamide gel electrophoresis

SDS/polyacrylamide-gel electrophoresis (11% acrylamide) was performed on slab gels (see Zwiers *et al.*, 1976). Proteins were stained with Fast Green and occasionally with the more sensitive silver-staining method described by Merril *et al.* (1981).

Other analyses

The apparent M_r of proteins was determined by comparison with M_r marker proteins. The method of Lowry *et al.* (1951) was used to determine the amount of total protein. The amount of pure PtdIns4*P* kinase was estimated by densitometric scanning of the stained gel patterns with known amounts of bovine serum albumin as standard. Scanning was performed at 650 nm with a Zeiss PM-QII spectrophotometer with the KM3 chromatography attachment.

Results

PtdIns4*P* kinase was purified from cytosolic as well as from membrane-associated fractions of rat brain. In Table 1 the quantitative aspects of the isolation procedures are summarized.

Since a reliable measurement of the activity of PtdIns4*P* kinase in membranes is very difficult due to high endogenous ATPase activity and possible exhaustion of endogenous PtdIns4*P* pools, activity values of the homogenate and membrane fraction were not determined.

Table 1 shows the different steps of purification of cytosolic PtdIns4*P* kinase. $(NH_4)_2SO_4$ precipitation between 20 and 40% saturation yielded some enrichment in terms of specific activity. Further fractionation of proteins was achieved by DEAE-cellulose column chromatography and flat bed isoelectric focusing. The final purification was 67-fold compared with the 105000*g* supernatant protein fraction. The recovery of activity was only 0.6%. The amount of protein recovered was very

Table 1. *Purification of PtdIns4P kinase from cytosol and synaptic plasma membranes*

Cytosolic and membrane-derived fractions of rat brain were assayed for PtdIns4*P* kinase under standard incubation conditions allowing linear incorporation of radioactivity into PtdIns4*P* with time. The data presented in this Table are from one representative experiment that was repeated with similar results at least four times. One unit is defined as the amount of PtdIns4*P* kinase activity transferring 1 pmol of phosphate to PtdIns4*P* to produce PtdIns4,5P_2 in 1 min at 30°C. Abbreviation used: n.d., not determined.

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/μg)	Purification (-fold)	Recovery (%)
Cytosol					
Homogenate	4000	n.d.	–	–	–
105000*g* supernatant	420	46000	0.11	1	100
20–40% $(NH_4)_2SO_4$ precipitate	210	32000	0.15	1.4	70
DEAE-cellulose pooled fractions 30–60 (Fig. 1)	16	9900	0.62	5.6	22
Isoelectric focusing pooled fractions 14–17 (Fig. 3)	0.038	280	7.4	67	0.61
Membranes					
Synaptic plasma membranes	220	n.d.	–	–	–
Triton/KCl extract	165	16500	0.10	1	100
DEAE-cellulose pooled fractions 35–55 (Fig. 2)	10	2500	0.25	2.5	15

low after flat bed isoelectric focusing; this was probably caused by the fact that most proteins, including part of the kinase, precipitated at the origin.

The specific activity of the Triton/KCl extract from synaptic plasma membranes (0.10 units/μg of protein) was comparable with the specific activity found in the 105000*g* supernatant protein fraction (Table 1). DEAE-cellulose chromatography of the extract yielded a 2.5-fold enrichment.

After flat bed isoelectric focusing of the DEAE-cellulose pool obtained from membrane-derived material, no activity was found apart from that at the origin. Pilot studies with DEAE-cellulose chromatography indicated that PtdIns4*P* kinase tended to elute with the majority of proteins if a linear salt gradient from 0–400mM-NaCl was applied. Therefore, an elution system was developed consisting of a stepwise enhancement of the ionic strength (22.5ml of 50mM- and 100mM-NaCl followed by 36ml of 150mM-NaCl). Under these conditions the bulk of the cytosolic (Fig. 1) and of the membrane-derived (Fig. 2) proteins were eluted. The PtdIns4*P* kinase activity, however, was eluted after application of a shallow salt gradient. In both instances the peak of PtdIns4*P* kinase activity was eluted at about 180–190mM-NaCl in the gradient (Figs. 1 and 2). If the active fractions were incubated with [^{32}P]ATP without added PtdIns4*P*, no ^{32}P-labelled phospholipids were detected, suggesting the absence of phospholipid precursors in these column eluate fractions.

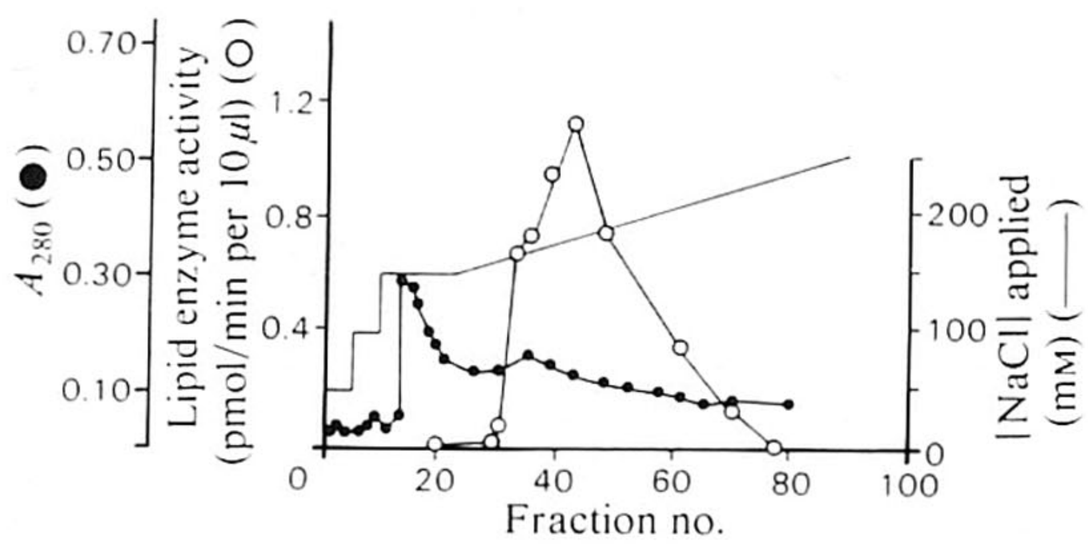

Fig. 1. *DEAE-cellulose column chromatography of cytosolic proteins*

Proteins (210mg) from the $(NH_4)_2SO_4$ 20–40% fraction obtained from rat brain cytosol were further fractionated by DEAE-cellulose chromatography as described in the Materials and methods section. The proteins were eluted by stepwise increase of the NaCl concentration followed by a linear gradient from 150 to 250mM. Fractions (4.5ml) were collected. ●, A_{280}; ○, PtdIns4*P* kinase activity assessed using 10μl per fraction.

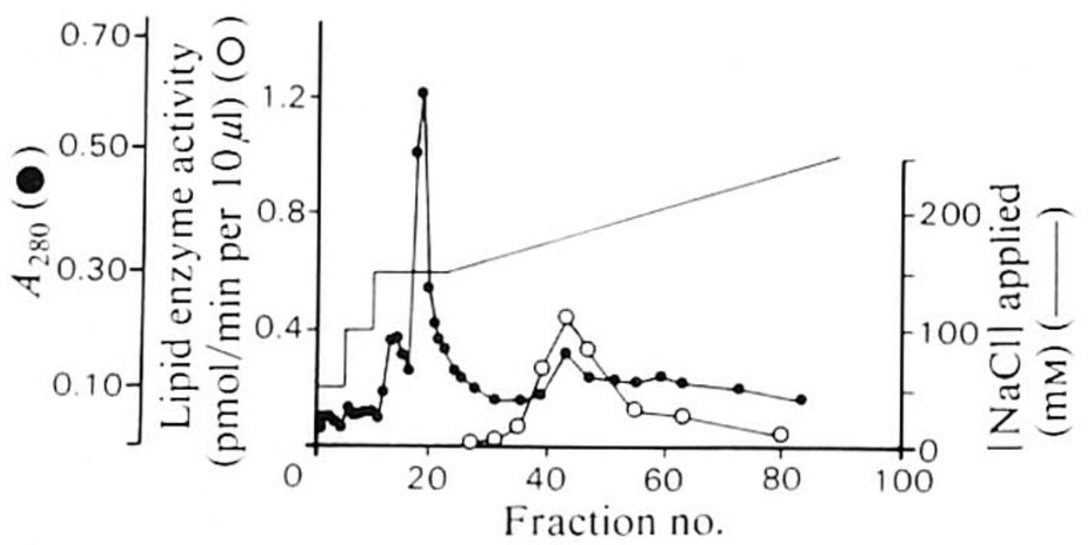

Fig. 2. *DEAE-cellulose column chromatography of proteins solubilized from synaptic plasma membranes*

Solubilized synaptic plasma membrane proteins (220mg) were subjected to DEAE-cellulose column chromatography. For details see the legend to Fig. 1.

Purification of PtdIns4P kinase by flat bed isoelectric focusing

Since the specific activity of PtdIns4*P* kinase in the DEAE-cellulose fractions obtained from cytosolic proteins was 3-fold higher than observed with the solubilized membraneous material, a pool of the cytosolic DEAE-cellulose proteins (fractions 30–60; Fig. 1), was used for fractionation by isoelectric focusing on a flat bed of Sephadex IEF (Table 1). After running to equilibrium overnight, scrapings of 1cm of the flat bed were tested for the presence of proteins by SDS/polyacrylamide-gel electrophoresis and for PtdIns4*P* kinase activity. Activity was recovered in fractions 19–21 (representing the place of application of the sample) and in fractions 14–17 (Fig. 3). Analysis of proteins revealed that fraction 20 contained a high number of protein bands (Fig. 4*a*) also present in the original sample, the 45kDa band being the most prominent one. In fraction 15 (Fig. 4*b*) only the 45kDa protein

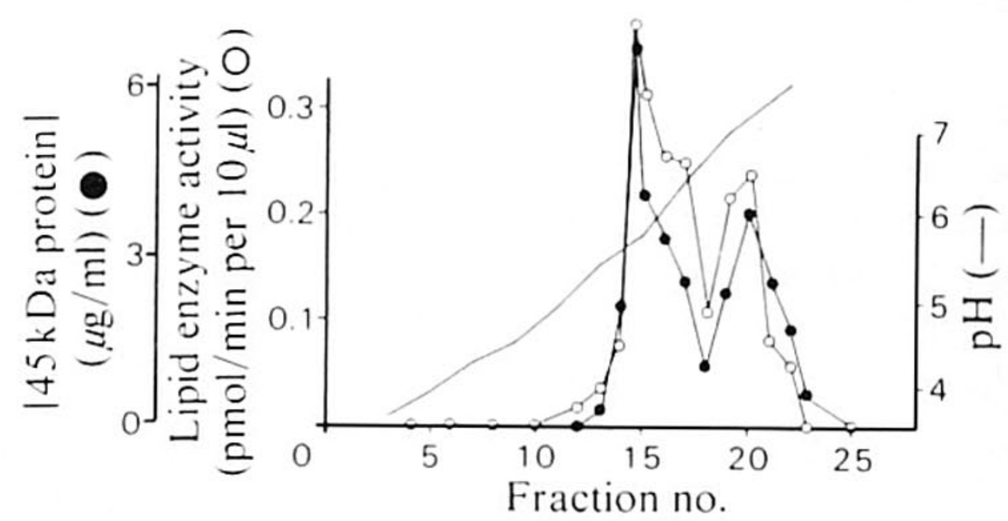

Fig. 3. *Flat bed isoelectric focusing of DEAE-cellulose pool of cytosolic proteins*

DEAE-cellulose fractions 30–60 from cytosolic proteins were pooled and concentrated to 8ml by an Amicon ultrafiltration cell equipped with a PM10 filter membrane. After dialysis, 4ml of the pool (8mg) was applied to a flat bed of Sephadex IEF. The sample was applied at fractions 19–21. After separation, bands of 1cm were scraped from the plate. The pH was measured (——) and PtdIns4*P* kinase activity was assessed using 10μl per fraction (○). The amount of 45kDa was estimated by densitometric scanning at 650nm (●).

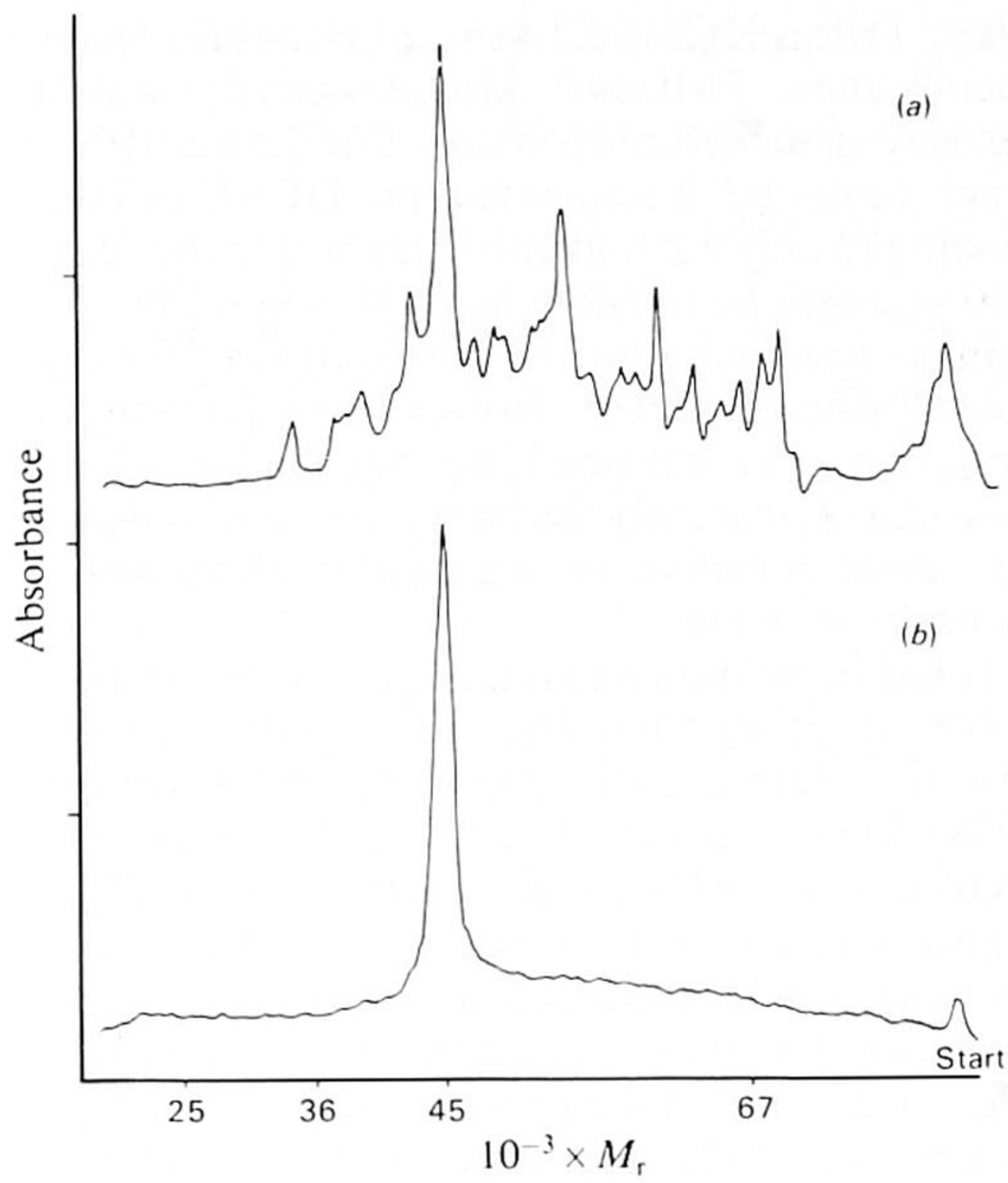

Fig. 4. *Densitometric scans of gel patterns of isoelectric focusing fractions after SDS/polyacrylamide-gel electrophoresis*

Isoelectric focusing fractions 20 (*a*) and 15 (*b*) (30 μl each) were separated by SDS/polyacrylamide-gel electrophoresis. Proteins were stained with a sensitive silver-staining method. Densitometric tracings were obtained by scanning at 650 nm. The vertical axis gives the absorbance in arbitrary units.

band was detected. The isoelectric point of this fraction was determined to be 5.8. The specific activity in fraction 15 was the highest and was 5.7 pmol/min per μg of protein.

Detection of the 45 kDa protein by densitometric scanning

The relative amount of 45 kDa protein in the flat bed fractions was determined by densitometric scanning of the gel pattern obtained after SDS/polyacrylamide-gel electrophoresis. The presence of 45 kDa protein appeared to correlate well with the level of PtdIns4*P* kinase activity (Fig. 3).

After separation of the DEAE-cellulose eluate fractions by SDS/polyacrylamide-gel electrophoresis, an intensively stained 45 kDa band was present as a major protein in fractions containing PtdIns4*P* kinase activity. This appeared to hold for the cytosolic as well as for the membrane-derived kinase activities (results not shown).

Ion sensitivity of PtdIns4P kinase

The sensitivity of this enzyme to variations in the incubation conditions was investigated. The activity was tested under conditions allowing

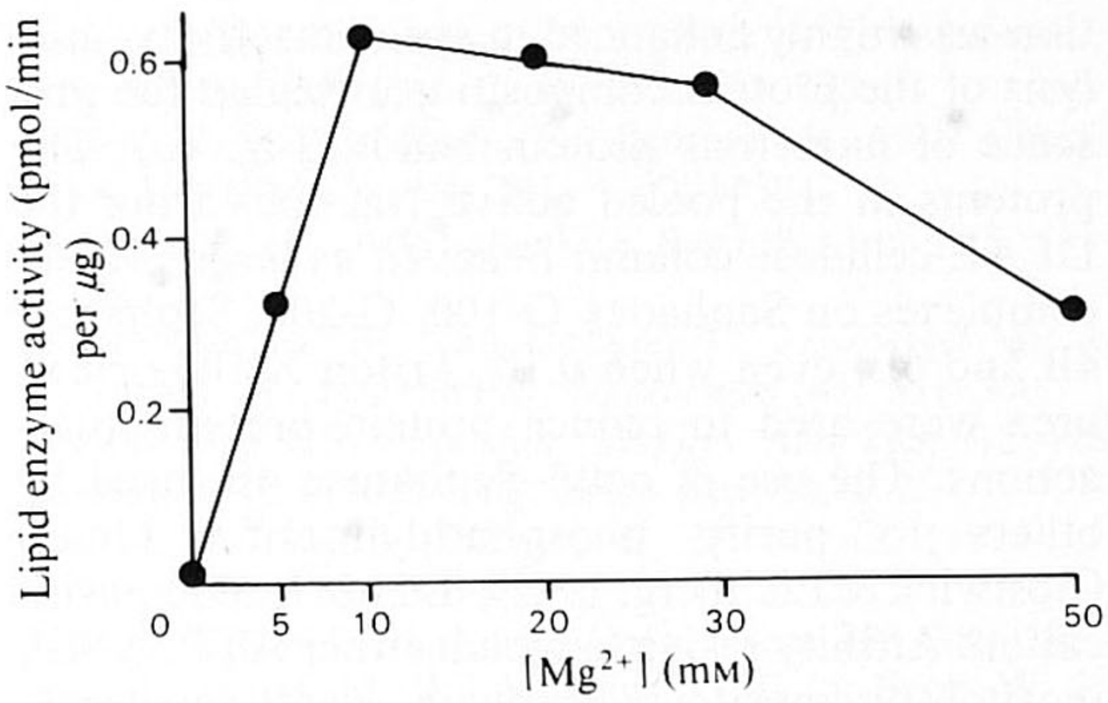

Fig. 5. *Influence of Mg^{2+} on PtdIns4P kinase*

A sample of the DEAE-cellulose pool of cytosolic proteins (2 μg total protein) was incubated for 5 min in the presence of 7.5 μM-ATP and 48 μM-PtdIns4*P*. The Mg^{2+} concentration in the medium was varied from 0 mM (no addition) to 50 mM. The Ca^{2+} concentration was 0.1 mM. The activity of PtdIns4*P* kinase was measured in duplicate at each Mg^{2+} concentration.

linear phosphorylation of PtdIns4*P* with time. In addition, it appeared that the incorporation was proportional to the amount of protein used (0.5–10 μg; results not shown).

Fig. 5 shows that under standard conditions (7.5 μM-ATP, 2 μg of protein, 48 μM-PtdIns4*P*, 0.1 mM-Ca^{2+}), Mg^{2+} is an essential cofactor for PtdIns4*P* kinase, with optimal stimulation between 10–30 mM-Mg^{2+}.

Maximal PtdIns4*P* kinase activity was observed at very low Ca^{2+} concentrations (0–0.1 mM added Ca^{2}; results not shown).

Discussion

To date there is only limited information in the literature on the purification of brain PtdIns4*P* kinase. Kai *et al.* (1968) and Torda (1972) obtained a fraction enriched in PtdIns4*P* kinase activity by precipitation of cytosolic rat brain proteins between 20% and 40% $(NH_4)_2SO_4$ saturation. This is confirmed by our findings. However, other methods of purification used by Kai *et al.* (1968) and Torda (1972) (gel filtration on Sephadex G-200 and Sepharose 4B) did not appear to be successful in our hands, since all proteins and enzyme activity were eluted in the void volume of the columns of these two types of molecular sieves (results not shown). From this behaviour in filtration procedures, we concluded that we possibly were dealing with large aggregates of proteins.

DEAE-cellulose column chromatography appeared to give a fraction enriched in PtdIns4*P* kinase activity (Table 1). Although the peak frac-

tion was highly enhanced in specific activity, analysis of the protein composition revealed the presence of numerous protein bands (Fig. 4*a*). The proteins in the pooled active fractions from the DEAE-cellulose column behaved as large protein complexes on Sephadex G-100, G-200, Sepharose 4B and 6B, even when 0.1% Triton X-100 or 6M-urea were used to reduce protein–protein interactions. The use of octyl–Sepharose 4B, used by others to purify phosphatidylinositol kinase (Bostwick & Eichberg, 1981), did not lead to purification. Affinity resins to which either ATP, AMP, inositol or inositol phosphate were covalently coupled appeared not to bind any of the proteins nor PtdIns4*P* kinase activity.

Methods previously used in this laboratory to isolate the membrane-associated B-50 protein kinase (isoelectric focusing in polyacrylamide-slab gels; Zwiers *et al.*, 1980) were also not helpful. The only procedure that resulted in a partial separation of cytosolic PtdIns4*P* kinase from the aggregates of DEAE-cellulose pool proteins was isoelectric focusing on a flat bed of Sephadex IEF containing Ampholines (pH range 3.5–10). By this method fractions were obtained containing only one protein as visualized by SDS/polyacrylamide-gel electrophoresis. Although the kinase activity might be associated with a protein present at concentrations lower than the detection limit of the protein staining method, we tend to conclude that the 45kDa protein is indeed the lipid kinase. This is further supported by the fact that the 45kDa protein was always co-eluted with the kinase activity (observed in DEAE-cellulose fractions and in isoelectric focusing fractions; see also Fig. 3). Additional evidence was obtained from recent experiments in which we could show that immunoglobulins directed against the 45kDa band inhibited the PtdIns4*P* kinase activity in the DEAE-cellulose pool (C. J. Van Dongen, unpublished work).

Flat bed isoelectric focusing resulted in two areas containing enzyme activity. From the protein patterns in these two areas we conclude that the activity at the origin might be contained in an aggregate of proteins. The pH of the fractions that did migrate into the gel and contained the peak activity was 5.6–6.0. The final PtdIns4*P* kinase preparation (pool of fractions 14–17 after isoelectric focusing) showed a high specific activity (7.4pmol/min per μg of protein). However, the overall yield was very low (0.6%), probably due to the partial removal of essential cofactors such as lipids and proteins (Kai *et al.*, 1968; Desmukh *et al.*, 1984).

In the present paper also an attempt was made to characterize the membrane-associated PtdIns4*P* kinase activity. Jolles *et al.* (1980) observed that after Triton/KCl treatment of synaptic plasma membranes, PtdIns4*P* kinase activity was recovered in the soluble fraction. The Triton/KCl extract could be fractionated on DEAE-cellulose, leading to only a 2-fold enrichment. Further purification might be possible by $(NH_4)_2SO_4$ precipitation, as was found for the purification of the cytosolic PtdIns4*P* kinase. However, no further fractionation was obtained by flat bed isoelectric focusing. Apparently the membrane-derived entity was more sensitive to aggregation than was the cytosolic enzyme.

It has been shown that a number of enzymes involved in phosphoinositide metabolism are sensitive to divalent cations (Griffin & Hawthorne, 1978; Abdel-Latif *et al.*, 1979; Jolles *et al.*, 1981; Akhtar *et al.*, 1983). In the present study, PtdIns4*P* kinase was shown to be sensitive to Mg^{2+} in the presence of 0.1mM added Ca^{2+}. The optimal Mg^{2+} concentration was 10–30mM. In the absence of added Ca^{2+} and in the presence of 1–10mM-Mg^{2+}, Jolles *et al.* (1981) found the highest labelling of PtdIns4,5P_2 in a crude synaptosomal fraction of rat brain. Other investigators, using higher ATP concentrations (1–5mM) found an optimum for Mg^{2+} between 15 and 30mM (Kai *et al.*, 1968; Tou *et al.*, 1970; Cooper & Hawthorne, 1976) and an inhibition by Ca^{2+} ions in the presence of Mg^{2+} (Kai *et al.*, 1968; Akhtar *et al.*, 1983).

In view of recent data obtained in this laboratory, which suggest that a correlation exists between the extent of phosphorylation of the brain synaptic protein B-50 and the level of PtdIns4*P* kinase activity in synaptic plasma membranes (Jolles *et al.*, 1980; Oestreicher *et al.*, 1983; Jork *et al.*, 1984), we are presently testing the modulatory effects of the protein B-50 on the highly purified PtdIns4*P* kinase described in this study.

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