Biochem. J. (1987) 244, 639–645 (Printed in Great Britain)

Resolution of the phosphoinositide-specific phospholipase C isolated from porcine lymphocytes into multiple species

Partial purification of two isoenzymes

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Phospholipase C isolated from porcine mesenteric lymph node lymphocytes was distributed between the soluble and particulate fractions. Enzyme activity was found predominantly in the soluble fraction with optimal activity at pH 5.5. Gel filtration chromatography of the soluble phospholipase C revealed that it was composed of multiple species of enzyme activity. The activity associated with the particulate fraction had optimal activity at pH 7.0, as also did one of the species of soluble phospholipase C. Cellulose phosphate chromatography resolved the major soluble form into two species designated PLC-A and PLC-B. Both phenyl-Sepharose chromatography and hydroxyapatite chromatography purified these species still further. PLC-A and PLC-B demonstrated similar activities against phosphatidylinositol with a pH optimum near 5.5. The phospholipase C activities were abolished against this substrate by the addition of 1 mM-EDTA. When assayed in the presence of Ca^{2+}–EDTA buffers providing a range of Ca^{2+} free concentrations, both enzymes exhibited optimal activity near 10^{-3} M free Ca^{2+}, but PLC-B was inhibited above this concentration more than PLC-A. PLC-B exhibited markedly lower activity against phosphatidylinositol 4,5-bisphosphate, suspended as liposomes of the pure phospholipid, than did PLC-A.

INTRODUCTION

Stimulation of many tissues with a number of agonists has been shown to result in a rapid turnover of phosphoinositides (Michell, 1975; Michell *et al.*, 1981; Berridge, 1984; Hawthorne, 1985). This enhanced metabolism occurs not only in response to hormone stimulation, but also in cell lines stimulated with growth factors such as platelet derived growth factor (Berridge *et al.*, 1984; Hasegawa-Sasaki, 1985), in T-lymphocytes stimulated with mitogenic lectins (Fisher & Mueller, 1968; Hui & Harmony, 1980; Hasegawa-Sasaki & Sasaki, 1983; Taylor *et al.*, 1984) and in B-lymphocytes stimulated with anti-Ig antibodies (Maino *et al.*, 1975; Guy *et al.*, 1985; Coggeshall & Cambier, 1984; Bijsterbosch *et al.*, 1985).

Since its initial discovery (Kemp *et al.*, 1959) phospholipase C (EC 3.1.4.10) activity has been found predominantly in the soluble fraction of all tissues studied to date. In some cases, where assayed with exogenous PtdIns, membrane-associated activity may also be detected (Friedel *et al.*, 1969; Keough & Thompson, 1972; Abdel-Latif *et al.*, 1980). In addition, membrane-bound phospholipase C activity can also be demonstrated by stimulating the enzyme present in membrane preparations to act upon endogenous phosphoinositides by adding deoxycholate (Lapetina & Michell, 1973) or Ca^{2+} (Downes & Michell, 1981). In other cases, evidence has been presented that membrane-bound phospholipase C activity is modulated by a GTP-binding protein, the nature of which remains to be elucidated (Cockcroft & Gomperts, 1985; Wallace & Fain, 1985; Uhing *et al.*, 1985).

Purification studies have been largely confined to the soluble activity and homogeneity has been achieved for a single form of phospholipase C isolated from rat liver (Takenawa & Nagai, 1981) and one of the two forms isolated from sheep seminal vesicles (Hofmann & Majerus, 1982). In many tissues, multiple forms of phospholipase C appear to exist, as demonstrated in rat liver, kidney and brain (Hirasawa *et al.*, 1982; Nakanishi *et al.*, 1985), human platelets (Chau & Tai, 1982; Banno *et al.*, 1986) and bovine heart (Low & Weglicki, 1983). Hofmann & Majerus (1982) demonstrated that the two forms isolated from seminal vesicular glands were immunologically distinct.

Studies of the lymphocyte phospholipase C previously reported include those by Allan & Michell (1974*a*,*b*) on an unpurified soluble preparation. In addition, we have shown that two forms separable on cellulose phosphate produce different proportions of cyclic and non-cyclic monophosphate products (Carter *et al.*, 1986). In view of the important role of stimulated metabolism of the phosphoinositides it was of interest to investigate further the nature of this phospholipase C activity, especially from a tissue in which its activity ultimately results in growth and cell division. In the present study we report the resolution and partial purification of several species of phospholipase C from porcine lymph node lymphocytes. There are two major soluble forms of the enzyme and a third species which may represent a form that is associated with, or derived from, the membrane.

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethanesulphonyl fluoride; DTE, dithioerythritol.

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MATERIALS AND METHODS

Materials

Phosphatidylinositol, isolated from wheatgerm, was obtained from Lipid Products, Nutley Ridge, Surrey, U.K. L-3-Phosphatidyl[2-^{3}H]inositol (specific radioactivity 15.6 Ci/mmol) and [^{32}P]P_i (carrier-free) were purchased from Amersham International. Phosphatidyl-[2-^{3}H]inositol 4,5-bisphosphate (specific radioactivity 3.6 Ci/mmol) was obtained from Du Pont (U.K.), New England Nuclear Products Division. Unlabelled PtdIns(4,5)P_2 was prepared from Folch fraction I by the ion-exchange method of Hendrickson & Ballou (1964). Schleicher and Schüll concentrating thimbles (cut-off M_r 10000) were from Anderman and Co., East Molesey, Surrey, U.K. Sephacryl S-200, phenyl-Sepharose, PD-10 Sephadex G-25 prepacked columns and M_r markers were obtained from Pharmacia. Cellex-P and Bio-Gel HT were from Bio-Rad Laboratories and anion-exchange cellulose DE-52 was purchased from Whatman Biochemicals. All other chemicals were supplied by Sigma Chemical Co. or BDH Chemicals.

Isolation of phospholipase C

Pig mesenteric lymph nodes, obtained immediately after slaughter, were processed within 2 h. The isolation procedure was based on that originally described by Allan & Michell (1974*a*). Briefly, the nodes were dissected from surrounding tissue, cut into small pieces (5 mm), and placed in homogenization buffer (50 mM-Tris/HCl/1 mM-EGTA/0.1 M-NaCl/100 μM-PMSF/200 μM-DTE, pH 7.2) which was kept at 4 °C on ice. Thereafter all procedures were carried out at 4 °C. The tissue was homogenized in a Waring blender at full speed for two periods of 15 s. This method of tissue disruption was a compromise between speed, convenience and yield of viable cells; cell viability was 70–80 % as judged by the Trypan Blue exclusion test. The resulting dispersion was filtered through a coarse mesh sieve and centrifuged at 500 *g* for 10 min. The sedimented cells were washed twice with the homogenization buffer, resuspended and pelleted in 10 ml plastic tubes, resulting in 3 ml of packed cells in each tube. By light microscopy 85% of the cells were lymphocytes, the remainder being erythrocytes and non-lymphocytic leucocytes. The cells were disrupted by three cycles of freezing and thawing in liquid N_2. Pellets were stored frozen at −20 °C, showing no appreciable loss of activity for up to 6 weeks. The pellets were thawed as required, centrifuged at 4 °C for 1 h and 150000 *g* and the resulting supernatant used immediately as the crude enzyme preparation.

Assays for phospholipase C

Routinely, non-radioactive PtdIns (0.6 μmol) and [^{3}H]PtdIns (15000 d.p.m.) were dried and dispersed by vortex-mixing into 50 mM-Tris/maleate/NaOH/1 mM-$CaCl_2$, pH 5.5. The assay tubes were equilibrated to 37 °C and 10 μl of crude enzyme preparation or 50 μl of column fractions were added, resulting in a final assay volume of 0.5 ml. The incubation continued for 10 min and was stopped by the addition of 1.8 ml of chloroform/methanol/conc. HCl (100:200:0.3, by vol.) followed by 0.6 ml of chloroform and 0.6 ml of water. After separation of the phases, 1 ml of the aqueous phase was taken for scintillation counting. Phospholipase C assays utilizing PtdIns(4,5)P_2 were similar, with the exception that the assays were stopped by extraction with 2.5 ml of chloroform/methanol/conc. HCl (50:50:0.3, by vol.) followed by 0.75 ml of 1 mM-EDTA in 0.1 M-HCl.

When the Ca^{2+} requirement of the enzyme was investigated, the Ca^{2+} free concentration was regulated by means of Ca^{2+}–EDTA buffers made in 50 mM-Tris/maleate/NaOH adjusted to pH 5.5 or pH 7.0 (Raaflaub, 1960).

Preparation of ^{32}P-labelled phospholipids

Isolated rat hepatocytes were prepared by a collagenase perfusion technique (Elliot *et al.*, 1976). The cells were incubated in a Krebs buffer, pH 7.4, containing 5 mM-glucose, 5 mM-glutamine, 4.5 mM-lactate, 0.5 mM-pyruvate and 20 μCi of [^{32}P]P_i/ml for 2 h in an atmosphere of O_2/CO_2 (19:1). At the end of the labelling period the hepatocytes were washed and the phospholipids extracted (Bligh & Dyer, 1959) and separated by t.l.c. on silica gel 60 (Merck) using the solvent system chloroform/propan-1-ol/methyl acetate/methanol/0.25% (w/v) KCl (25:25:25:10:9, by vol.).

Purification of phospholipase C

All purification procedures were carried out at 4 °C.

***(a)* Gel filtration.** The crude enzyme preparation isolated following high speed centrifugation of the disrupted lymph node lymphocytes was passed through a Millipore 8 μm filter. The sample was loaded onto a Sephacryl S-200 column (2.5 cm × 90 cm) in 50 mM-Tris/HCl/1 mM-EGTA/100 μM-PMSF/200 μM-DTE, pH 7.2, then eluted with this buffer at a flow rate of 18 ml/h. Fractions from peak 2 (see Fig. 1) were pooled (sacrificing samples to the left of this peak to avoid contamination with peak 1), concentrated and equilibrated with 50 mM-citric acid/trisodium citrate/1 mM-EGTA/100 μM-PMSF/200 μM-DTE, pH 5.8, by using a Schleicher and Schüll apparatus with a thimble retaining compounds of $M_r > 10000$. Complete equilibration with the citric acid/trisodium citrate buffer was achieved by passage through a PD-10 Sephadex G-25 column.

***(b)* Cellulose phosphate chromatography.** The concentrated fractions from peak 2 were loaded onto a cellulose phosphate column (2.5 cm × 10 cm) prepared in 50 mM-citric acid/trisodium citrate/1 mM-EGTA/100 μM-PMSF/200 μM-DTE, pH 5.8, and eluted with 50 ml of this buffer followed by a linear 0–0.6 M-NaCl gradient in the same buffer. Following initial characterization of the elution profile, it was possible to use a smaller column (1.5 cm × 5 cm), eluting stepwise with 40 ml of 0.3 M-NaCl in the column buffer. This alteration in elution conditions did not reduce the degree of purification achieved for the second peak of activity that had been retained. The two peaks of phospholipase C activity eluting from this column were pooled separately and equilibrated with the column buffer appropriate for the next step in the purification protocol, either phenyl-Sepharose or hydroxyapatite chromatography.

***(c)* Phenyl-Sepharose hydrophobic interaction chromatography.** Samples were loaded onto a phenyl-Sepharose column (1.5 cm × 4 cm), prepared in 50 mM-Tris/HCl/0.5 M-NaCl/1 mM-EGTA/100 μM-PMSF/200 μM-DTE, pH 7.2, and eluted initially with 30 ml of this

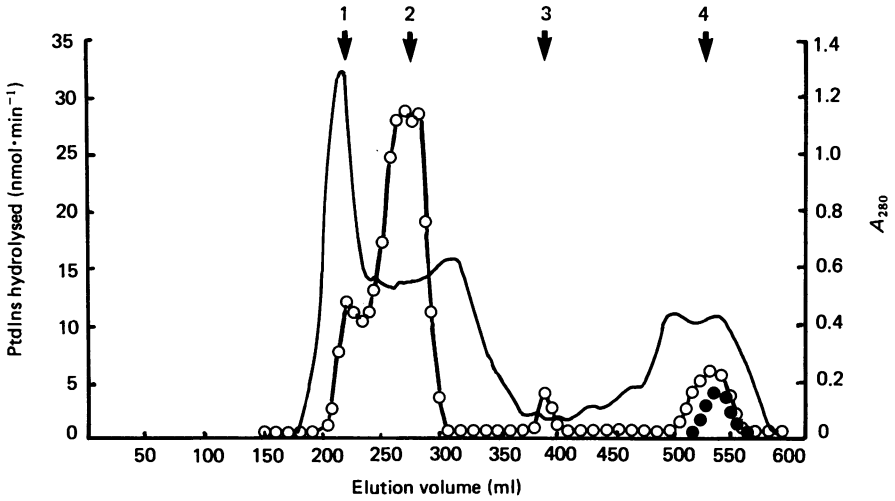

Fig. 1. Gel filtration chromatography of lymphocyte phospholipase C on Sephacryl S-200

A 15 ml portion of the soluble fraction was loaded and eluted as described in the text, and 50 μl aliquots from each fraction were assayed for phospholipase C activity using PtdIns as the substrate at pH 5.5 (○) and pH 7.0 (●). Protein concentration was monitored by A_{280} (——).

buffer. The column was then eluted in a stepwise fashion with 25% (v/v) and 50% (v/v) ethylene glycol in the column buffer.

***(d)* Hydroxyapatite chromatography.** Fractions of the first peak derived from cellulose phosphate chromatography (PLC-A) were pooled and equilibrated with 0.01 M-sodium phosphate/100 μM-PMSF/200 μM-DTE, pH 6.8, and loaded onto a column (1.5 cm × 5 cm) of hydroxyapatite prepared in this buffer. The fractions containing the second peak of phospholipase C activity which eluted from the cellulose phosphate column with 0.3 M-NaCl (PLC-B) were pooled and brought to equilibrium with 0.05 M-sodium phosphate/100 μM-PMSF/200 μM-DTE, pH 6.8, and allowed to run into a column of hydroxyapatite also prepared in this buffer. Both columns were eluted with 30 ml of the respective column buffer followed by a linear concentration gradient of sodium phosphate from the starting concentration to 0.4 M with an otherwise similar buffer composition.

Other methods

SDS/polyacrylamide-gel electrophoresis was according to the method of Laemmli (1970) using a 5–15% linear gradient of polyacrylamide and a 3% stacking gel. Proteins were visualized using Coomassie Brilliant Blue R-250. Protein determination of column eluates was routinely assessed by the absorbance at 280 nm, or measurements were made using the method of Lowry *et al.* (1951) using bovine serum albumin to calibrate the standard curve.

RESULTS

Distribution of activity

Phospholipase C activity was found predominantly in the soluble fraction under all the assay conditions used. When the thawed cells, used for enzyme preparation, were centrifuged at 150000 *g* for 1 h, some phospholipase C activity remained with the particulate fraction: 14.5% of the pH 7.0 activity as opposed to 1.9% of the total pH 5.5 activity. When the phospholipase C assay was carried out in the presence of sodium deoxycholate (0.005–0.2%) the activity at pH 5.5 was severely inhibited at all concentrations, with total inhibition at the higher concentrations tested. In contrast the activity detected at pH 7.0 was stimulated 2-fold, optimally with 0.1% sodium deoxycholate, and not inhibited over the range tested. However, the pK_a of deoxycholate is near pH 6, so that these results may reflect differential effects of the ionized and un-ionized forms of the bile acid on the enzyme rather than different sensitivities to deoxycholate of the pH 5.5 and pH 7.0 activities.

Stability of phospholipase C

During enzyme isolation and purification procedures the buffers used routinely contained EGTA, PMSF and DTE in an effort to retain as much activity as possible. Enzyme activity was stable for several months (−20 °C) in the form of frozen lymphocyte pellets, but was far less stable once isolated as the soluble fraction. The activity assayed at pH 5.5 was judged to have a half-life of approx. 6 days when stored at 4 °C in the crude form of isolated cytosol. This was markedly reduced as the purification procedures progressed, making an accurate assessment of purification achieved somewhat difficult. The activity assayed at pH 7.0 in the soluble fraction was extremely labile and could not be detected after 3 days storage, possibly explaining the rather variable recovery of this enzyme species. The labile nature of this activity precluded any attempt at purification in the present study.

Purification of phospholipase C

An initial attempt to remove the bulk of contaminating proteins in the crude preparation of cytosolic phospholipase C by precipitation with $(NH_4)_2SO_4$, as often used by other groups (Takenawa & Nagai, 1981; Hakata *et al.*, 1982), resulted in an extremely poor recovery of phospholipase C activity. A poor recovery was also noted by Low & Weglicki (1983) and may possibly be

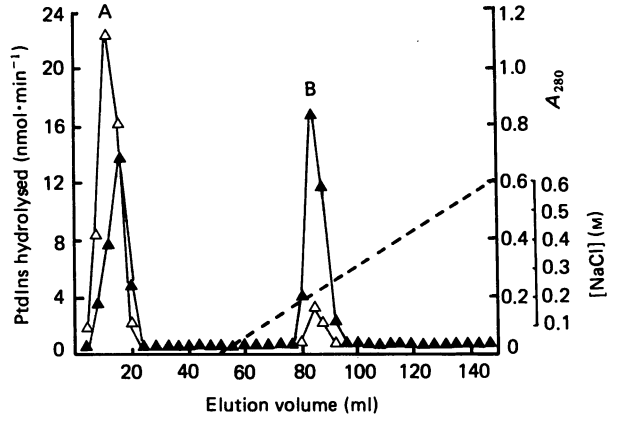

Fig. 2. Cellulose phosphate chromatography of phospholipase C peak 2 eluted from gel-filtration chromatography resolves the two species PLC-A and PLC-B

Peak 2 eluting from Sephacryl S-200 was pooled, concentrated and equilibrated with the column buffer. The sample was loaded and eluted with a 0–0.6 M-NaCl gradient (broken line) in column buffer as described in the text, and 50 μl aliquots from each fraction (4 ml) were assayed for phospholipase C activity at pH 5.5 (▲) and protein was monitored by A_{280} (△).

accounted for by loss of some species of phospholipase C activity.

Gel filtration chromatography resolved the phospholipase C activity of the crude preparation into a number of peaks (designated 1–4 in Fig. 1). All peaks were apparent when the profile was assayed at pH 5.5, but only peak 4 could be detected at pH 7.0. When the protease inhibitors PMSF and EGTA were included in both homogenization and elution buffers, peak 3 disappeared, without detectable increase in any other peaks. The apparent M_r of each peak was assessed from calibration of the column with standard M_r marker proteins: peak 1, 300000; peak 2, 175000; peak 3, 57000; peak 4, 11000.

Cellulose phosphate chromatography of peak 2 revealed that this peak itself was composed of two species of phospholipase C activity in approximately equal proportions (Fig. 2). The first peak of activity eluting from such a column was designated PLC-A and the second peak which eluted at about 0.2 M-NaCl was designated PLC-B. Both species retained their initial elution characteristics when rechromatographed on freshly prepared cellulose phosphate columns, thereby confirming their separate identities. The separation achieved by this method was thought to be due to some degree of affinity for the matrix, as cation-exchange chromatography using carboxymethylcellulose failed to resolve these two species. The recovery of activity following this step was consistently high and the degree of purification achieved for PLC-B was particularly good. Stepwise elution with 0.3 M-NaCl was equally effective and offered a fast and efficient stage in the purification protocol.

PLC-A and PLC-B could be further purified by using either phenyl-Sepharose or hydroxyapatite chromatography. Both species of phospholipase C were retained by phenyl-Sepharose and elution with 25% (v/v) ethylene glycol was unsuccessful; both species required

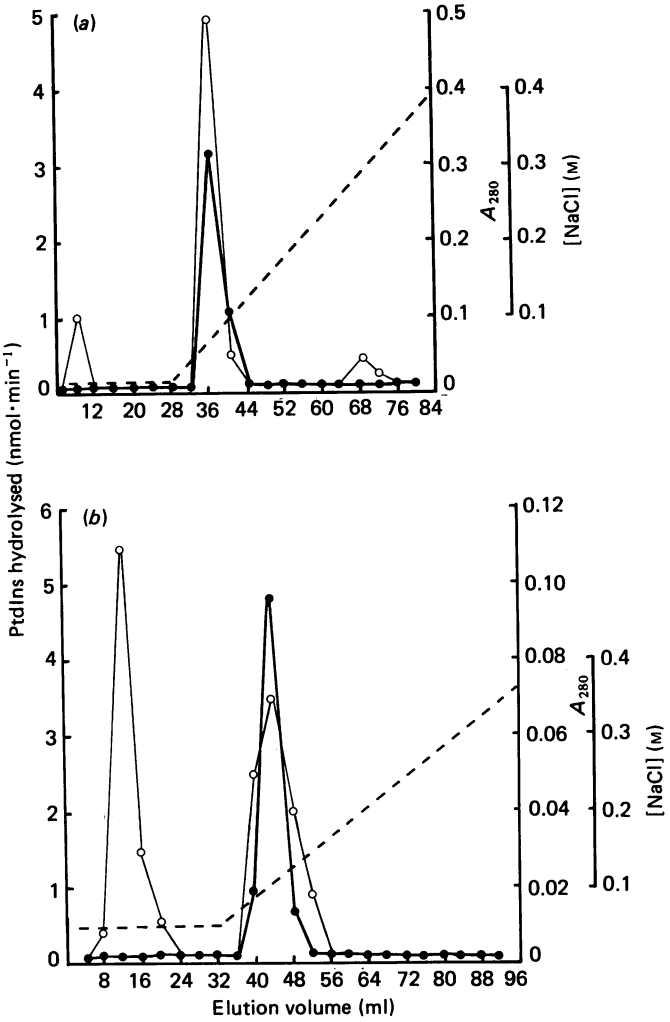

Fig. 3. Hydroxyapatite chromatography of PLC-A or PLC-B

PLC-A (*a*) and PLC-B (*b*) eluted from cellulose phosphate were pooled, concentrated and equilibrated with the new column buffer. Samples were loaded and eluted as described in the text. The elution profile was followed for protein (○) by A_{280} and phospholipase C activity (●) in 50 μl aliquots from each fraction (4 ml). NaCl concentration is indicated by the broken line.

50% (v/v) ethylene glycol for elution. These elution characteristics for both species imply strong hydrophobic interactions with the column matrix, possibly reflecting their ability to interact with the membrane *in vivo*.

The profiles resulting from chromatography of PLC-A (Fig. 3*a*) and PLC-B (Fig. 3*b*) on hydroxyapatite show the different elution conditions required for these two species, again indicating some physical differences between them. This technique proved to be somewhat more effective as a purification step for PLC-B than for PLC-A. Nevertheless, the profiles of the gels that were run after these procedures remain complicated, and neither protein is obtained as a homogeneous preparation by this sequence of techniques.

Properties of phospholipase C

The activity recovered in the soluble fraction was optimal at pH 5.5, as indicated in Fig. 4. There was a shoulder of activity at pH 7.0 which is thought to

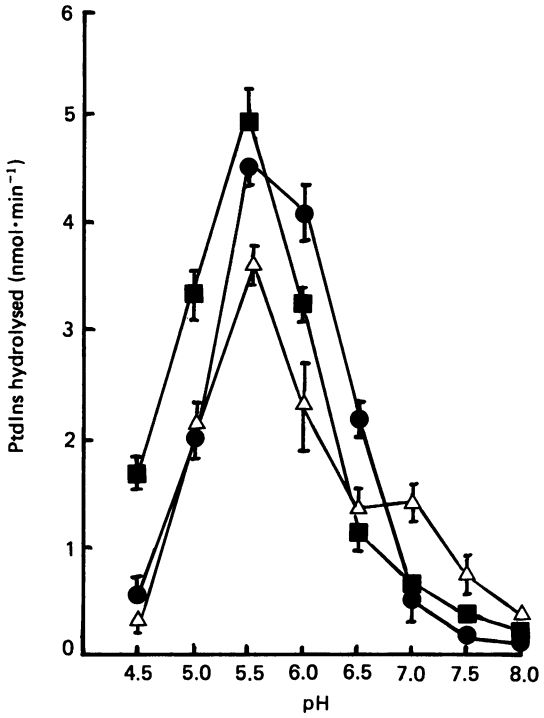

Fig. 4. pH profile of the soluble lymphocyte phospholipase C activity against PtdIns

Phospholipase C activity upon PtdIns was assayed over a range of pH values using the buffers 50 mM-Tris/maleate/NaOH (pH 5.0–8.0) and 50 mM-citric acid/tri-sodium citrate (pH 4.5), containing 1 mM-$CaCl_2$. Assays were made on crude phospholipase C (△), PLC-A (■) and PLC-B (●), under standard conditions.

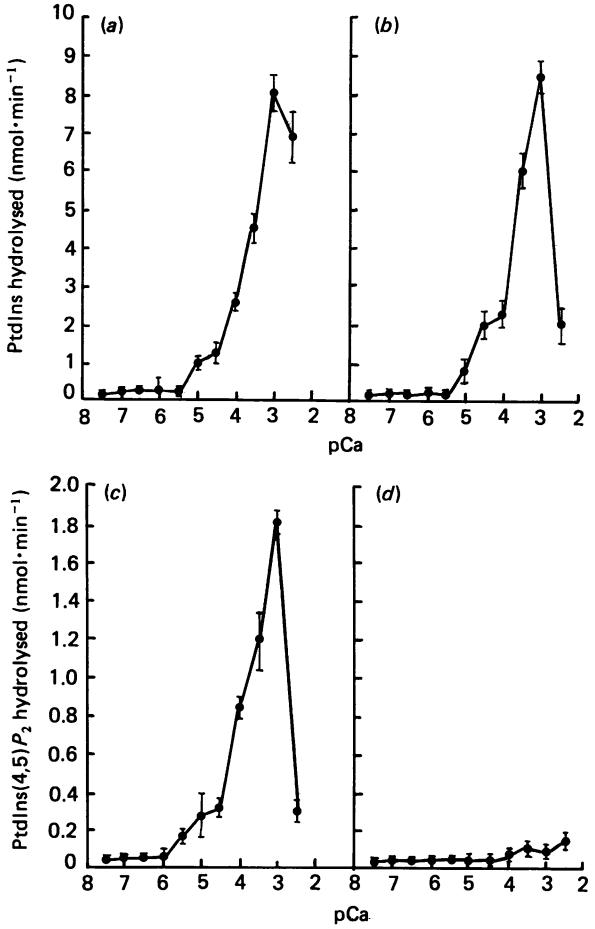

Fig. 5. Effect of Ca^{2+}–EDTA buffers on the two species of phospholipase C, PLC-A and PLC-B, using PtdIns or PtdIns(4,5)P_2 as substrate

PLC-A activity against PtdIns (*a*) or PtdIns(4,5)P_2 (*c*) was assayed over a range of Ca^{2+} free concentrations regulated by Ca^{2+}–EDTA solutions buffered to pH 5.5. PLC-B activity against PtdIns (*b*) or PtdIns(4,5)P_2 (*d*) was also investigated in a similar way.

represent a further species of phospholipase C activity, as it may be resolved from the majority of pH 5.5 activity by gel filtration and is eluted along with the activity designated peak 4.

Using the isolation procedure described here phospholipase C activity was found to be dependent upon Ca^{2+}, 1 mM-Ca^{2+} resulting in maximum stimulation of enzyme activity. No activity was detected in the presence of EDTA or EGTA, indicating negligible contamination with lysosomal phospholipase C (Irvine *et al.*, 1978; Matsuzawa & Hostetler, 1980). Phospholipase C activity declined rapidly when warmed to temperatures exceeding 40 °C for 10 min, and the enzyme was totally inactive when heated to temperatures in excess of 55 °C.

The crude enzyme preparation was specific for the phosphoinositides; there was no detectable hydrolysis of ^{32}P-labelled phosphatidylcholine, phosphatidylethanolamine or phosphatidic acid. Limited activity was observed when phosphatidylserine was the substrate, but this represented less than 2% of the activity found using PtdIns as the substrate. This was considerably less than that reported to occur when the phospholipase C isolated from human platelet cytosol was assayed for activity against phosphatidylserine (Chau & Tai, 1982).

It was possible that the unlabelled PtdIns, which was derived from wheatgerm and used routinely in the assays, might not be as effective a substrate for phospholipase C activity as that derived from mammalian sources and used here as the radiolabelled [^3H]PtdIns. When phospholipase C activity was compared using the purchased PtdIns and that purified in the laboratory from rat tissue, the soluble lymphocyte phospholipase C hydrolysed both substrates at equal rates, confirming previous reports (Chau & Tai, 1982; Holub & Celi, 1984).

As a function of time, the hydrolysis of PtdIns by the crude preparation of lymphocyte phospholipase C and the two partially purified species, PLC-A and PLC-B, slowed markedly at a point at which approx. 65% of the substrate was hydrolysed. The arrest of activity at this point may be due to the effect of accumulation of one of the end products, diacylglycerol, on the physical state of the substrate liposomes. Inclusion of diacylglycerol in mixed vesicles with PtdIns strongly inhibited phospholipase C activity with virtual cessation of activity when present in a 1:1 ratio (H. R. Carter & A. D. Smith, unpublished work). Phospholipase C activity was also found to be directly proportional to protein concentration, again diverging from a linear relationship at the point when approx. 65% of the substrate had been utilized. Identification of the water-soluble end products

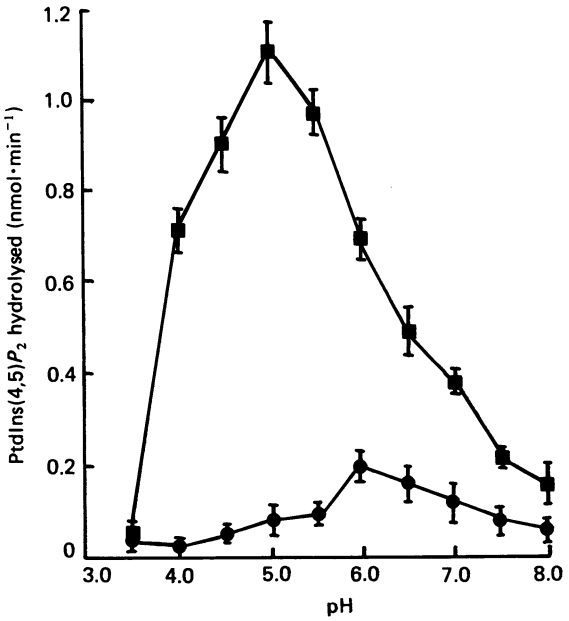

Fig. 6. pH profile of PLC-A and PLC-B activity against PtdIns(4,5)P_2

PLC-A (■) and PLC-B (●) were assayed over a range of pH values in the presence of 1 mM-$CaCl_2$. Buffers were 50 mM-Tris/maleate/NaOH in the range pH 5.0–8.0, and 50 mM-citric acid/trisodium citrate in the range pH 3.5–4.5.

of phospholipase C action upon PtdIns has been reported (Carter *et al.*, 1986).

Both PLC-A and PLC-B had optimal activity at pH 5.5 when assayed with PtdIns, with no significant activity at pH 7.0 (Fig. 4). This was expected as these species were derived from peak 2 (gel filtration, Fig. 1) which demonstrated activity only at pH 5.5 when assayed under both conditions of pH. When the concentration of Ca^{2+} was controlled with Ca^{2+}–EDTA buffers, both PLC-A and PLC-B had optimal activity near 10^{-3} M-Ca^{2+} (Figs. 5*a* and 5*b*), but PLC-B was more markedly inhibited above this concentration than was PLC-A.

When assayed using PtdIns(4,5)P_2, there was a marked difference in the activities of PLC-A and PLC-B. Figs. 5(*c*) and 5(*d*) show the activity found with PtdIns(4,5)P_2 using Ca^{2+}–EDTA buffers to give a range of Ca^{2+} free concentrations. This is a useful distinction between the two forms of the enzyme assayed using suspensions of pure lipid, but may not relate to the activities in the cell, in which the substrate is presented in more complex mixtures. Fig. 6 indicates the pH profile of activity using PtdIns(4,5)P_2 as substrate.

DISCUSSION

We have shown that the soluble phospholipase C activity from porcine lymphocytes, as initially investigated by Allan & Michell (1974*a*,*b*), is composed of a number of different activities. Such multiple species have also been found in other tissues with a variety of different purification techniques (Hirasawa *et al.*, 1982; Hofmann & Majerus, 1982; Chau & Tai, 1982; Low *et al.*, 1984; Nakanishi *et al.*, 1985; Banno *et al.*, 1986). Occasionally, earlier studies on some of these tissues have revealed a single form of phospholipase C, for example in rat liver (Takenawa & Nagai, 1981) and also from tissues which have since been shown to possess multiple species. It is possible that the initial stages of purification in these cases may have employed a step, for example precipitation with $(NH_4)_2SO_4$, that may inactivate one (or some) of the species of phospholipase C, thereby resulting in the identification of only one form. Indeed precipitation with $(NH_4)_2SO_4$ resulted in a very poor recovery of activity when used by us with the lymphocyte soluble enzyme.

Our purification techniques resolved four species of activity. Peak 3 recovered from the gel filtration step is thought to arise as a result of proteinase activity, as the inclusion in the elution buffers of EGTA and PMSF, agents which would inhibit Ca^{2+}-dependent proteinase and serine proteinase activities respectively, results in loss of this species. The M_r found for the remaining species ($\sim$ 300000 for peak 1 and $\sim$ 175000 for peak 2) are comparable with those reported by Low *et al.* (1984) for the various species isolated from tissues such as brain, liver and heart with M_r values in the region of 420000, 290000, 150000 and 75000–110000. Although the SDS/polyacrylamide gels of the purified preparations remain complicated, a prominent band is present corresponding to M_r 78000 when PLC-B is purified using either phenyl-sepharose or hydroxyapatite. This M_r is close to that found using SDS/polyacrylamide gels after purification of phospholipase C from rat liver (Takenawa & Nagai, 1981) or sheep vesicular gland (Hofmann & Majerus, 1982). Peak 4 was eluted from the gel filtration column with an apparent M_r of 11000. This was considerably smaller than expected in view of previously published M_r values for this type of enzyme activity. It is possible that there is some degree of association with the column matrix, causing retardation of this species. However chromatography on Sephadex G-200 resulted in a similar profile to that found for Sephacryl S-200 (Carter & Smith, 1985). Alternatively, this peak may represent a fragment originating from proteolysis, despite the precautions taken. Another possibility is that this small protein could represent a dissociated catalytic unit from a complex multisubunit enzyme.

One of the more important questions remaining to be answered concerns the relationship of the soluble and particulate phospholipase C activities in the lymphocyte. Very recently, it has been shown (Kamisaka *et al.*, 1986) that the small amount of particulate activity can be solubilized using 0.4% deoxycholate and subjected to several stages of purification. There is agreement between the results of these workers and ourselves that the membrane-associated activity exhibits more activity at pH 7 than does the soluble form, especially in the presence of deoxycholate. The observation of two pH optima for the lymphocyte confirms the earlier report of Allan & Michell (1974*a*), but the two pH optima may be due to different isoenzymes rather than, as suggested previously, to an effect of pH on the physical nature of the substrate, since if the latter were the case it would be expected that a similar effect would be observed with the purified enzymes, which is not so. It cannot, however, be excluded that interaction between the different forms occurs in the crude supernatant, particularly between the M_r 11000 protein and the other enzymes, and that under these conditions both factors may play a role. We have

yet to investigate the possibility that these different forms of the enzyme in fact represent subunits of a complex enzyme that is dissociated by the separation procedures we have employed.

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Received 3 October 1986/11 February 1987; accepted 5 March 1987