Purification of a Phosphoinositide-specific Phospholipase C from Bovine Brain*

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A soluble phosphoinositide-specific phospholipase C (PLC) was purified 58,000-fold from bovine brain. The enzyme, one of six distinct PLC activities detected in brain, accounted for approximately 15% of the soluble phosphatidylinositol-4,5-bisphosphate-phospholipase C (PIP$_2$-PLC) activity in this tissue. The purification scheme included hydrophobic chromatography and phenyl-Sepharose and affinity chromatography on phosphatidylinositol-Sepharose (PI-Sepharose). The enzyme was specifically eluted from the PI-Sepharose with PI, calcium, and detergent. The purified PLC had an estimated molecular weight of 88,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and behaved as a monomeric protein during sedimentation on glycerol gradients. The enzyme required calcium for activity, exhibited a pH optimum of 6.5, and cleaved only phosphoinositides. The rates of PIP$_2$ and phosphatidyl-4-monophosphate hydrolysis exceeded the rate of PI hydrolysis under all conditions tested. These properties are consistent with a potential role for this PLC in the early events involved in cellular calcium mobilization.

The phospholipase C (PLC)$^1$-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) is a crucial step in the cellular response to calcium-mobilizing hormones and neurotransmitters (1,2). The products of this reaction serve as intracellular mediators which amplify the initial signaling event leading to cellular calcium mobilization and protein kinase C activation (1–3). Recent reports (see Refs. 4–7) suggest that activation of a phosphoinositide-specific PLC is coupled directly to cell surface receptors by a GTP-binding protein in a manner analogous to the regulation of adenylate cyclase. So far the enzyme activated by this putative GTP-binding protein has not been isolated.

Studies aimed at isolation of the PLC under receptor control have been complicated by the apparent heterogeneity of phosphoinositide-PLC. Numerous soluble phosphoinositide-specific PLC activities have been resolved chromatographically from a variety of tissues and shown to differ in molecular weight (8,9), isoelectric point and pH optima (10), and calcium dependence (11). Thus far two similar enzymes which specifically cleave the phosphoinositides have been purified to homogeneity. Takenawa and Nagai (12) isolated a phospholipase C from rat liver cytosol. The 70-kDa protein specifically hydrolyzed phosphatidylinositol (PI) and required calcium for activity. Subsequently, Hofmann and Majerus (13) purified a soluble 65-kDa phosphoinositide-specific PLC from sheep seminal vesicles. Although the enzyme isolated from seminal vesicles possessed similar properties to the liver PLC, it was purified to a much higher specific activity. In the latter study a second immunologically distinct PLC was partially purified (2600-fold) from the same source. This enzyme was specific for the phosphoinositides, was activated by calcium, and had an estimated molecular weight on gel filtration of 85 kDa. Wilson et al. (14) have demonstrated that both seminal vesicle enzymes hydrolyze polyphosphoinositides as well as PI. A PLC that demonstrates specificity for a single species of phosphoinositide has yet to be identified.

Isolation of a receptor-regulated PLC is further complicated by the heterogenous distribution of phosphoinositide-specific PLC activities in subcellular fractions. In brain (15), kidney (16), and muscle (17), a significant proportion of the total cellular phosphoinositide-specific PLC activity is associated with cellular membranes. The relationship between the membrane-bound and soluble PLC activities is unclear. It has become evident, however, that a membrane-bound PLC can be activated by calcium-mobilizing hormones in membranes isolated from various cell types (4–7). We have recently reported that thrombin and GTP or nonhydrolyzable GTP analogs stimulate a polyphosphoinositide-specific PLC that is tightly associated with fibroblast membranes (18). A polyphosphoinositide-specific PLC can be extracted from these membranes which exhibits the same chromatographic properties on ion exchange and hydrophobic resins as one of the PLC activities found in fibroblast and brain cytosol. Moreover, the PLC extracted from fibroblast membranes behaves similarly during sedimentation on glycerol gradients. Baldassare and Fisher (19) and Deckmyn et al. (20) have recently described a soluble phosphoinositide-specific PLC which is activated by nonhydrolyzable GTP analogs. These results support the idea that following cell disruption, receptor or G-protein-regulated lipases may be found in both soluble and membrane-bound states. For this reason we decided to purify a soluble PLC from an enriched source, the brain, that resembles the principal PLC solubilized from fibroblast membranes. We have designated this enzyme PLC-III to distinguish it from the two seminal vesicle PLCs previously described (13).

EXPERIMENTAL PROCEDURES

Materials—Fast S-Sepharose, Fast Q-Sepharose, and phenyl-Sepharose resins were purchased from Pharmacia. Hydroxyapatite crystals were from Clarkson Chemical Co. (PA). PI-Sepharose and $^{[3]P}$H

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$^1$ The abbreviations used are: PLC, phospholipase C; PIP$_2$, phosphatidylinositol-4,5-bisphosphate; PIP$_3$, phosphatidylinositol-4-monophosphate; Ins$_4$P, inositol-1,4,5-trisphosphate; PC, phosphatidylcholine; PR, phosphatidylethanolamine; FS, phosphatidyserine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, diithiothreitol; EGTA, [ethylenebis(oxyethylenemimnitrilo)]tetracetic acid; Tris, Tris (hydroxymethyl)aminomethane hydrochloride.

2 M. J. Rebecchi and O. M. Rosen, unpublished observation.
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PI-glycan-variable surface glycoprotein protein labeled with [3H]myristic acid (8 × 10^14 dpm/mmol) were generously provided by Dr. Judith Fox, Rockefeller University, New York. [3H]PIP, [3H]PI, [3H]PI-P (3.5 Ci/mmol), [3H]PIP (3.5 Ci/mmol), [3H]PI (10 Ci/mmol) were purchased from Du Pont-New England Nuclear. [3H]InsP(1 C/mmol) was obtained from Amersham Corp. PIP, PIP, PE, PC, and PS were from Sigma. Bovine serum albumin, and aldolase and the BCA protein assay kit were obtained from Pierce Chemical Co. Dialysis tubing was from Spectrum Laboratories. Concentrating cells were from Amicon. Buffers—Buffer A consisted of 1 mM EGTA, 0.1 mM DTT, and 10 mM sodium phosphate, pH 6.7. Buffer B contained 1 mM EGTA, 0.1 mM DTT, 0.6% (w/v) octyl glucoside, and 10 mM sodium phosphate, pH 6.7. Buffer C contained 1 mM EGTA, 0.1 mM DTT, 0.6% (w/v) octyl glucoside, and 10 mM bis-Tris, pH 6.8. Buffers used to determine the effect of pH on enzyme activity were prepared with 50 mM sodium phosphate titrated with HCl or NaOH to a pH between 3.8 and 8.0 in buffer E. Solutions employed to determine the effect of calcium concentration on enzyme activity were titrated with CaCl₂ to a free calcium concentration of 0.01–1.00 mM in buffer B containing 2 mM MgCl₂. Free calcium concentrations in this range were determined using Quin-2 (free acid) as described by Tsien et al. (21). Concentrations of free calcium above 1 mM were achieved by exceeding the buffering capacity of the EGTA with a known amount of CaCl₂.

Preparation of P3-labeled Phospholipids—Hela cells (22) were incubated in 100 ml of minimal essential medium containing 10 mlCi of 200 μCi [33P]thiophosphate for 24 h at 37°C. The cells were isolated by sedimentation and extracted with 10 ml of chloroform/methanol (2:1, v/v) and 3 ml of 0.5 M KCl. The lipid content in the lower phase was dried under nitrogen and applied to Silica G plates and developed in two dimensions as described previously (23). The radioactive lipids, identified by comigration with authentic standards, were scraped from the plates, and the silica was extracted twice with chloroform/methanol (2:1, v/v). A known quantity of unlabeled phospholipid was added to the extracts which were then dried under nitrogen and resuspended in buffer as described below.

Phospholipase C Assay—The substrates for the PLC assay were [3H]PIP (9.5 × 10⁶ cpm/μmol), [3H]PIP (1.3 × 10⁶ cpm/μmol), [3H]PI (3.0 × 10⁶ cpm/μmol), [3H]PI (3.0 × 10⁶ cpm/μmol), [3H]PE (4.3 × 10⁶ cpm/μmol), [3H]PE (2.6 × 10⁶ cpm/μmol), and [3H]PS (3.0 × 10⁶ cpm/μmol). Individual phospholipids were dried under nitrogen and resuspended in buffer as described below.

Purification of PLC-III—Step 1. Homogenization and Preparation of the High Speed Supernatant Fluid from Calf Brain—Ten whole fresh calf brains (wet weight, 3.5 kg) were minced, rinsed once with 0.9% (w/v) NaCl, and then with homogenization buffer consisting of 230 mM sucrose, 2 mM EGTA, 0.5 mM DTT, 10 μg/ml soybean trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM sodium phosphate, pH 6.8 at 4°C. Homogenization buffer (1.5 liters) was added, and 600 ml portions were homogenized on ice with three 10-s pulses at the maximum power of a Brinkman Polytron. A few drops of isomyl alcohol were added to decrease foaming during the homogenization. The homogenate was centrifuged at 13,000 × g for 2 h at 4°C. The supernatants were collected by filtering through sterile cotton gauze, and the pellets were rehomogenized in 2 liters of homogenization buffer on ice with three 10-s pulses at the maximum power of a Brinkman Polytron. The second homogenate was centrifuged at 13,000 × g for 1 h at 4°C. The supernatants were filtered, pooled with the first set of supernatants, and centrifuged at 13,000 × g overnight at 4°C. Approximately 2.5 liters of supernatant, containing 32 g of soluble protein, were decanted and frozen in 500-ml portions at −70°C. Approximately 90% of this material was used in the purification procedure described.

Step 2. Ammonium Sulfate Precipitation—Ammonium sulfate was added to the brain supernatant fluid to a concentration of 30% (w/v). The precipitated protein was removed, and the supernatant was centrifuged at 100,000 × g for 1 h at 4°C. Thus, the total PIP₂-PLC activity in the bovine brain supernatant was precipitated by 60% (w/v) ammonium sulfate. Greater than 90% of the total PLC activity in the bovine brain supernatant was precipitated by 60% (w/v) ammonium sulfate. The activity was recovered following resuspension of the precipitate in 40 ml of homogenization buffer and dialysis for 10 h against 7.5 liters of this same buffer using 50 kDa molecular mass cutoff dialysis tubing.

The data presented in Table I represent only the PIP₂-PLC activity associated with the enzyme we term PLC-III. PLC-III was one of six chromatographically distinct PLC activities which were resolved. It was defined as that activity which eluted from phenyl-Sepharose between 0.75 and 2.5% (w/v) octyl glucoside (see below). The content of PLC-III activity in steps 1–3 was calculated by estimating the fraction of total PLC activity recovered in each step that was definable as PLC-III in step 4. Thus, the total PIP₂-PLC activity in the bovine brain preparation described in Table I was 78,000 units (a unit is defined as the amount of enzyme needed to hydrolyze 1 nmol of PIP₂/min, using 100 μCi PIP₂ in octyl glucoside). In this preparation, PLC-III accounted for approximately 9% (6,989 units) of the total PIP₂-PLC activity in the supernatant fluid (Table 1, Step 1). In other preparations the relative content of PLC-III activity was as high as 15% of the total.

Step 3. Fast S Sepharose Chromatography—The dialyzed preparation of enzyme (118 ml) was mixed with Fast S Sepharose which had been equilibrated with buffer A at 4°C (see
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PLC-III was separated from five other soluble PIP2-PLC activities present in bovine brain in steps 1–4. A unit of activity is defined as the amount of enzyme necessary to hydrolyze 1 nmol of PIP2/min using 100 μM PIP2 in buffer containing 1% octyl glucoside and 1 mM free calcium.

| Step | Total units | Total protein | Specific activity | Yield |
|------|-------------|---------------|-------------------|-------|
| 1. Supernatant fluid | 6,989 a | 7,031 | 1.0 | 93 |
| 2. Ammonium sulfate | 6,500 a | 2,471 | 2.6 | 93 |
| 3. Fast S Sepharose-1 | 6,132 a | 370.9 | 16.5 | 88 |
| 4. Phenyl-Sepharose | 6,500 | 34.85 | 186 | 93 |
| 5. Fast Q Sepharose | 3,208 | 3,370 | 961.9 | 46 |
| 6. Fast S Sepharose-2 | 2,665 | 2,664 | 1,000 | 38 |
| 7. Hydroxylapatite | 1,527 | 0.760 | 2,010 | 22 |
| 8. PI-Sepharose | 804 a | 0.031 d | 25,900 | 11 |
| 9. Concentration | 498 a | 0.007 d | 7 |
| 10. Glycerol gradient | 402 | 0.007 d | 57,400 | 6 |

a The content of PLC-III activity in steps 1–3 was calculated from the amount of PIP2-PLC activity recovered as PLC-III in step 4.

* Percent recovery of the total PIP2-PLC activity in step 1 (78,000 units).

Two thirds of this material was further purified.

* Protein content was estimated by densitometry of silver-stained proteins.

* Seventy percent of the concentrated enzyme was further purified.

* Not determined.

"Experimental Procedures" for buffer compositions). Resin was added to the dialysate until no further binding of PIP2-PLC activity was detected (24 ml). The mixture was centrifuged at 800 g for 1 min, and the supernatant fluid which contained approximately 50% of the PLC activity (36,000 units) was removed. The resin was washed three times at 4 °C with buffer A, and adsorbed PLC activity was eluted with two 25-ml volumes of buffer A containing 0.4 M NaCl. If the adsorbed PLC activity is eluted from the Fast S resin using a linear gradient of NaCl of 0–400 mM, at least three distinct peaks of PIP2-PLC activity are observed. Two major PLC activities elute at: 0.4–4 mM NaCl and 4–10 mM NaCl; a third minor activity elutes at 10–13 mM NaCl (not shown).

Step 4. Phenyl-Sepharose Chromatography—The material eluted from Fast S Sepharose with 0.4 M NaCl was adjusted to 0.6 M NaCl and shaken for 2 h at 4 °C with phenyl-Sepharose that had been equilibrated with buffer A containing 0.6 M NaCl. Resin was added until no further adsorption of PIP2-PLC activity was detected (12 ml of resin). The resin was allowed to settle, and the supernatant fluid which contained about 50% of the total PLC activity (18,000 units) was removed. The phenyl-Sepharose was then poured into a column (1.5 × 7 cm) and eluted with 8 bed volumes of buffer A without NaCl followed by a 100-ml linear octyl glucoside gradient of 0–3% (w/v) in buffer A at 5 ml/4 min/fraction. Five PIP2-PLC activities were resolved in this step. One of them did not bind to phenyl-Sepharose, two eluted from the resin as the NaCl concentration fell toward zero, and a minor activity eluted between 0.40 and 0.75% octyl glucoside. A major peak of activity was eluted from the resin between 0.75 and 2.5% octyl glucoside. This latter activity was designated PLC-III and further purified. The chromatographic properties of PLC-III on phenyl-Sepharose were the same as the major PIP2-PLC activity extracted from fibroblast membranes. The chromatogram in Fig. 1 (which is from a purification other than the one described in Table I) illustrates the resolution of the four PIP2-PLC activities that adsorb to phenyl-Sepharose. Thus, the first three steps of the purification scheme resolved six PIP2-PLC activities. One of them failed to bind to Fast S Sepharose, another bound to Fast S Sepharose but not to phenyl-Sepharose, and four eluted from phenyl-Sepharose at low ionic strength or with detergent. The relationship of these activities to each other was not determined.

Step 5. Fast Q Sepharose Anion Exchange Chromatography—The fractions eluted from phenyl-Sepharose between 0.75 and 2.5% octyl glucoside were pooled, mixed with 3 ml of Fast Q Sepharose that had been equilibrated in buffer C, and poured into a column (1.5 × 2 cm). Greater than 90% of the PLC activity bound to the resin. The column was eluted with a 34-ml linear gradient of NaCl from 0 to 500 mM in buffer C. PLC activity eluted as a single peak between 70 and 210 mM NaCl. Similar behavior was observed for the fibroblast membrane PIP2-PLC activity which eluted from Fast Q Sepharose between 0.1 and 0.3 M NaCl.

Step 6. Fast S Sepharose Chromatography-2—Peak fractions from the Fast Q column were pooled and the volume was reduced to 1.5 ml using an Amicon concentrator with a 30-kDa cutoff. The material was diluted to 12 ml with buffer B and mixed with 1 ml of Fast S resin equilibrated in buffer B. Ninety-five percent of the PIP2-PLC activity bound to the Fast S Sepharose. The resin was poured into a column (0.9 × 2 cm) and eluted with a 12-ml linear gradient of NaCl from 0 to 400 mM. The PLC activity eluted as a single peak between 80 and 275 mM NaCl. This step often resulted in a 2-fold purification. However, for the preparation shown in Table I this procedure provided no enrichment. The PIP2-PLC activity solubilized from fibroblast membranes also eluted from Fast S Sepharose between 0.1 and 0.3 M NaCl.

Step 7. Hydroxylapatite Chromatography—The peak fractions from the Fast S column were pooled, diluted with an equal volume of buffer B, and mixed with 0.5 ml of hydroxylapatite crystals equilibrated in buffer B. Greater than 94% of the PLC activity bound to the crystals which were then poured into a column (1.2 × 0.9 cm) and eluted stepwise in 25 mM increments from 25 to 450 mM Na2HPO4 in buffer B at pH 6.8 and 4 °C. The PLC activity eluted as a single peak.
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between 50 and 225 mM Na$_2$PO$_4$. At this point PLC-III had been purified about 2000-fold; however, SDS-PAGE revealed numerous protein bands upon silver staining. One-third of the material from the hydroxylapatite column was frozen, and the remainder was subjected to further purification.

Steps 8–9. Phosphatidylinositol-Sepharose Affinity Chromatography—Phosphatidylinositol that had been covalently linked to epoxy-activated Sepharose as described by Saltiel et al. (27) was used as a substrate affinity matrix. The peak fractions from the hydroxylapatite column were pooled, concentrated 10-fold using a centricon concentrator (30-kDa cutoff), and rediluted in buffer B. This material was then mixed with 2.7 ml of PI-Sepharose equilibrated with buffer B containing 0.4 mg/ml of cytochrome c as carrier protein at 4 °C for 30 min. The resin was poured into a column (1.5 × 1.7 cm) and washed at room temperature with four column volumes of buffer B. One column volume of buffer B containing 2 mg/ml PI, 2 mM CaCl$_2$, and 1% octyl glucoside was applied, and 1 ml was allowed to flow through. The column was closed off for 2 min and then eluted with four more volumes of the buffer. Nearly 80% of the applied PLC activity was eluted (Fig. 2B). Various elution procedures including either PI, CaCl$_2$, or octyl glucoside alone or PI plus octyl glucoside or calcium plus octyl glucoside were less effective in releasing the PLC from the resin. To obtain good recoveries of PLC activity from the PI-Sepharose, it was important to slightly exceed the binding capacity of the resin for PIP$_2$-PLC activity. It was also necessary to close off the column for several minutes following the initial application of the elution buffer.

Polyacrylamide gel electrophoresis of the enzyme eluted from PI-Sepharose indicated that numerous protein bands had been removed from the bulk of the PLC activity (Fig. 2A). Three major protein bands of 77, 88, and 101 kDa coeluted with the PLC activity (fraction 8, Fig. 2B; lane 8, Fig. 2A). A fourth band at 47 kDa proved to be a contaminant in the elution buffer (lane 12, Fig. 2A). The bands which appeared at the very bottom of the gel were from the cytochrome c used as carrier protein.

A comparison of the silver-staining intensity (as determined by densitometry) of the 77-, 88-, and 101-kDa protein bands to the PLC activity in each fraction demonstrated that the 88-kDa band (designated by the asterisk, Fig. 2A) correlated best with PLC activity. This band which constituted about 1% of the total protein in the hydroxylapatite-purified material was enriched nearly 13-fold in the peak of PLC activity specifically eluted from the PI-Sepharose. In contrast, both the 77- and 101-kDa proteins appeared to be significantly depleted by this step. In subsequent experiments both the 77- and 101-kDa bands were removed by applying the hydroxylapatite-purified material in buffer containing deoxycholate (1 mg/ml) and 10 mM bis-Tris, pH 6.7.

Step 10. Sedimentation on Glycerol Gradients—The peak fractions of PLC activity from step 9 were pooled. The PI was removed from the preparation by binding the enzyme to Fast Q Sepharose equilibrated in buffer C and washing the resin with 1% octyl glucoside in buffer C. The enzyme was then eluted with 0.5 M NaCl in buffer C containing 0.4 mg/ml cytochrome c, and the volume was reduced with an Amicon concentrator (30-kDa molecular mass cutoff). Seventy percent of this material (0.38 ml) was further purified on a 12 to 35% glycerol gradient (11 ml) in buffer B by centrifugation at 39,000 × g for 41.5 h at 4 °C. The PLC activity was detected as a single symmetric peak sedimenting between the bovine serum albumin (66 kDa, 4.3 s) and aldolase (150 kDa, 7.3 s) standards (Fig. 3B). The s value for the PLC, determined as described by Martin and Ames (28), was 5.2 ± 0.13 S.D. in

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**Fig. 2.** A, SDS-polyacrylamide gel electrophoresis of the fractions eluted from PI-Sepharose. A portion (120 μl) of fractions 1–11 from the PI-Sepharose column described below were boiled in 0.1% SDS and 20 mM DTT, and subjected to electrophoresis in a 7.5% polyacrylamide gel. The gel was stained with silver to visualize protein bands. Lanes 1–11 correspond to fractions 1–11 from the PI-Sepharose column. Lane 12 contained buffer used to elute the PLC. The 88-kDa protein band is designated by an asterisk. B, affinity chromatography on PI-Sepharose. The peak fractions from the hydroxylapatite column were pooled and chromatographed on PI-Sepharose as described under “Results.” One column volume per fraction was collected. The arrow indicates addition of the elution buffer containing PI, calcium, and octyl glucoside.

**Fig. 3.** A, SDS-polyacrylamide gel electrophoresis of the glycerol gradient fractions. A portion of fractions 14–30 from the glycerol gradient described below were subjected to SDS-polyacrylamide gel electrophoresis. The gel was stained with silver to visualize the protein bands. Lanes 14–30 correspond to fractions 14–30 from the glycerol gradient. The 88-kDa protein band is designated by an asterisk. B, PLC activity after glycerol gradient sedimentation. Seventy percent of the concentrated PI-Sepharose-purified material (0.38 ml) was layered on top of a 12–35% (11 ml) glycerol gradient in buffer B and centrifuged at 39,000 × g for 41.5 h at 4 °C. The gradient was fractionated into 0.25-ml portions and assayed for PIP$_2$-PLC activity. Standards, cytochrome c (1.8 s), ovalbumin (3.6 s), bovine serum albumin (4.3 s), and aldolase (7.3 s) were resolved in a parallel glycerol gradient.
three separate experiments. The absence of detergent in the gradient buffer did not significantly affect the sedimentation properties of the PLC activity (not shown). Individual fractions from the gradient were analyzed by SDS-polyacrylamide gel electrophoresis. The intensity of the silver-stained band migrating at 88 kDa on SDS-PAGE (designated with an asterisk, Fig. 3A) correlated best with PLC activity. In the peak fraction (fraction 21), the 88-kDa band represented approximately 35% of the total protein. This protein was enriched about 2.5-fold from the previous step. In contrast the peak of the 77-kDa protein was almost entirely displaced from the peak of enzyme activity. In three independent experiments the peak of staining intensity of the 101-kDa band was consistently shifted one fraction further toward the bottom of the gradient than the peak of PLC activity. Moreover, the 101-kDa band was removed when the peak of PLC activity from the glycerol gradient was passed over a second PI-Sepharose column (not shown). Thus, the 88-kDa band was designated PLC-III.

Substrate Specificity
The PI-Sepharose-purified enzyme demonstrated specificity for the phosphoinositides. PC, PE, and PS (1 mM) in either octyl glucoside or deoxycholate were not hydrolyzed. The outer surface glycoprotein of trypanosomes which is linked to PI (29) also did not serve as a substrate. Octyl glucoside and deoxycholate were tested for their ability to support the hydrolysis of the phosphoinositides. The optimal concentrations of these two detergents were 1% and 1 mg/ml for PIP2 and PI, respectively. The rates of hydrolysis of PIP2 and PIP were about 12- and 2-fold greater in the presence of octyl glucoside (1%, w/v) than deoxycholate (1 mg/ml) (Table II). In contrast, hydrolysis of PI was significantly inhibited by octyl glucoside; only deoxycholate supported the efficient hydrolysis of this lipid. The rates of substrate hydrolysis were compared using detergents which were optimal for each phosphoinositide. Under these conditions, the rates of hydrolysis of PIP2 and PIP, determined in buffer containing octyl glucoside, were as much as 36- and 60-fold greater, respectively, than the rate of PI hydrolysis measured in buffer containing deoxycholate (Fig. 4). When cleavage of 1 mM PIP2 and 1 mM PI were compared in the presence of deoxycholate, the rate of PIP2 hydrolysis was more than three times greater than PI (Table II). The relative substrate affinities for PIP2 and PIP, in the presence of 1% octyl glucoside and PI in the presence of 1 mg/ml deoxycholate were of the order PI > PIP2 > PIP (Fig. 4). The half-maximal concentrations of PI and PIP2 were 250 and 350 μM, respectively; at concentrations up to 1 mM PIP was unable to saturate the enzyme. Both PIP2 and PIP were actively hydrolyzed by this lipase; there were 46,000 mol of PIP2 and 72,000 mol of PIP, hydrolyzed per min per mol of enzyme. The rates of hydrolysis of the polyphosphoinositides exceeded the rate of PI hydrolysis under all conditions tested. It is possible that in intact cells, this enzyme may also exhibit specificity for PIP2 and PIP. In support of this idea we found that the ratio of the rates of hydrolysis of PIP2 to PI (assayed at 500 μM substrate) in the crude bovine brain supernatant and the PI-Sepharose-purified enzyme were 2.6 and 2.7, respectively, suggesting that other phosphoinositide-PLC activities having substrate specificities different from PLC-III were depleted during the purification of this enzyme.

| Substrate | Rate of hydrolysis | μmol/min/mg protein |
|-----------|--------------------|---------------------|
| PIP2      | 28.5               | 2.3                 |
| PIP       | 20.2               | 0.6                 |
| PI        | 0.2                | 0.7                 |

Calcium and pH Requirements
The effect of calcium concentration on PIP2-PLC activity was assessed using buffers which simulated cytosolic ionic conditions (2 mM MgCl2, 100 mM KCl, pH 7.0). Calcium was required for PLC activity; MgCl2 (up to 5 mM) would not substitute for calcium. Calcium activated the enzyme over a wide range of concentrations (nearly four log orders) and the activation curve consistently appeared somewhat biphase (Fig. 5) with one phase of activation occurring between 3 × 10⁻⁷ and 1 × 10⁻⁵ M and the other between 3 × 10⁻⁴ and 1 × 10⁻³ M.
The enzyme hydrolyzed PIP₂ over a broad pH range from 4.0 to 7.5, with optimal activity at pH 6.5. Activity was significantly diminished above pH 7.5.

**Stability**

The PI-Sepharose-purified enzyme required carrier protein (cytochrome c, 0.4 mg/ml) and detergent (octyl glucoside, 0.6%) for stability. It retained about 75% of its activity when stored frozen, between pH 6.0 and 7.0, at -70 °C, for 1 month.

**Discussion**

The enzyme designated PLC-III was resolved and purified from a mixture of six chromatographically distinguishable PIP₂-PLC activities in calf brain cytosol. The apparent heterogeneity of these soluble brain phospholipases is consistent with the results reported by others for a variety of tissues including brain (8, 10, 11), liver (11), platelets (9, 30), seminal vesicles (13), heart, lung, and kidney (8). It is presently unclear what the relationship is between PLC-III and these other PIP₂-PLC activities.

The enzyme we have called PLC-III was purified 58,000-fold. Since it constituted about 35% of the protein in the final preparation we estimate that purification to homogeneity would require an enrichment of greater than 100,000-fold. Thus, the calculated abundance of PLC-III is less than 0.001% of brain cytosolic protein. The enzyme is sufficiently active, however, to account for as much as 15% of the total soluble brain PIP₂-PLC activity.

One of the crucial steps in the isolation of PLC-III was hydrophobic chromatography on phenyl-Sepharose. Five PLC activities were resolved by this resin including PLC-III. This chromatographic method was also used to separate several PIP₂-PLC activities from fibroblast cytosol and to isolate a PIP₂-PLC associated with fibroblast membranes.² Chau and Tai (30) and Banno et al. (9) have previously found that hydrophobic chromatography on phenyl-Sepharose and elution with either ethylene glycol or detergent was useful in the partial purification of several phosphoinositide-PLC activities present in platelet cytosol.

Another key step in the purification of PLC-III was the use of PI-Sepharose as an affinity matrix. Elution of the enzyme from this resin was dependent upon the presence of PI, calcium, and detergent in the eluting buffer. These components, in other combinations, were less effective in releasing the enzyme from the column. The requirement for calcium suggested that the enzyme bound to the PI-Sepharose is released when calcium activates the enzymatic hydrolysis of PI.

Three major proteins of 77, 88, and 101 kDa coeluted with the PLC activity from the PI-Sepharose. However, both the 77-kDa and the 101-kDa proteins were more prevalent in the flow-through fractions which contained much less PLC activity than the peak of activity that was eluted with PI, calcium, and detergent. In contrast the 88-kDa protein was almost completely adsorbed to the resin and was recovered in high yield by the specific elution step. Thus, of the three proteins detected during PI-Sepharose chromatography only the 88-kDa protein correlated with PLC activity. Identification of the 88-kDa PLC-III was supported by its cosementation with PLC activity on glycerol gradients. Sedimentation analysis demonstrated that the 88-kDa protein and PLC activity coalesced into a single symmetrical peak clearly distinguishable from the peak of the 77-kDa protein and shifted at least one fraction from the peak of the 101-kDa band. The 88-kDa protein was enriched greater than 30-fold relative to the other protein bands between the hydroxylapatite chromatography and the glycerol gradient steps of the purification procedure, whereas both the 77- and 101-kDa bands were substantially depleted. When the peak fractions from the glycerol gradient were pooled and rechromatographed on PI-Sepharose the 101-kDa band was removed. Moreover, by substituting deoxycholate for octyl glucoside during the application of the sample to the PI-Sepharose column both the 77- and 101-kDa protein bands were removed. Thus, we concluded that the 88-kDa protein is PLC-III. Its migration on SDS-PAGE and its behavior during sedimentation on glycerol gradients suggests a monomeric structure.

Thus far, two phosphoinositide-specific PLC proteins have been purified to homogeneity. Takenawa and Nagai (12) isolated a 70-kDa enzyme from rat liver cytosol which required calcium and was stimulated by arachidonic acid. Hoffmann and Majerus (13) purified a soluble 65-kDa PLC from sheep seminal vesicles (PLC-1). In the same study, Hoffmann and Majerus also reported the partial purification (2600-fold) of a second distinct phosphoinositide-specific PLC with an estimated molecular weight of 85 kDa. Both enzymes were subsequently shown to hydrolyze PIP₂ and PIP as well as PI (14). It is unlikely that the enzyme we have isolated from brain is related to the 65-kDa seminal vesicle PLC because antibody elicited to the latter protein failed to precipitate any PLC activity from sheep brain extracts, although this antibody precipitated PLC activity from several other tissues (13). Moreover, PI-Sepharose-purified PLC-III had a significantly higher specific activity for PIP₂ and PIP (73 and 114 pmol/min/mg of protein, respectively) than the pure seminal vesicle protein (less than 30 pmol/min/mg of protein). On the other hand, the 85-kDa seminal vesicle PLC activity (PLC-II) may be related to the enzyme we have purified. Both enzymes have approximately the same molecular weights and behave similarly on hydroxylapatite chromatography. A specific antibody to PLC-III will be needed to explore this possibility.

PLC-III accounted for about 15% of the total PLC activity measured in brain cytosol. Low and co-workers (8) have carefully compared the molecular weights by gel filtration of phosphoinositide-specific PLC activities from a number of different tissues. They found that a 96-kDa PLC comprised 12 and 20% of the total soluble PI-PLC activity in rat and rabbit brain, respectively, a result consistent with the relative content of PLC-III in bovine brain reported here.

The PI-Sepharose-purified PLC specifically cleaved only the phosphoinositides PIP₂, PIP, and PI. Neither PC, PE, nor PS were significantly hydrolyzed. The rate of hydrolysis of each phosphoinositide was influenced by the nature of the detergent used to solubilize the substrate. In the presence of octyl glucoside, a neutral detergent, the rates of hydrolysis of PIP₂ and PIP were greater than they were in buffer containing the anionic detergent deoxycholate. In contrast, hydrolysis of PI was stimulated by deoxycholate and inhibited by octyl glucoside. The effect of substrate concentration upon enzyme activity was determined under conditions which best supported each reaction, that is, PIP₂ and PIP hydrolysis were measured in the presence of octyl glucoside, and PI hydrolysis was determined in the presence of deoxycholate. Under these conditions, at saturating substrate concentrations, hydrolysis of PIP₂ was approximately 36 times greater than PI. The relative rates of hydrolysis of the phosphoinositides at 1 mM concentrations were in the following order PIP₂ > PIP > PI. Even when assayed in the presence of deoxycholate, PIP₂ was hydrolyzed at about three times the rate of PI. Thus, PLC-III hydrolyzed PIP₂ faster than PI under all conditions. The substrate turnover numbers for PIP₂ and PIP, assayed in buffer containing octyl glucoside, were 47,000 and 74,000 mol/min/mg of enzyme, respectively, higher than any phosphoi-
nositide-specific PLC isolated thus far. These data suggest
that this PLC may selectively hydrolyze PIP$_2$ and PIP in vitro.
The possibility that PLC-III has an intrinsically greater se-
llectivity for PIP$_2$ than other phosphoinositide-PLC activities
in brain is supported by our finding that the ratio of PIP$_2$-PLC
activity to PI-PLC activity increased approximately 10-
fold during the isolation of PLC-III.

Our results on the relative rates of hydrolysis of the phos-
phoinositides are qualitatively similar to those reported by
others. Rittenhouse et al. (31) found that the platelet cytosol PLC
activity, assayed in buffer containing deoxycholate, cleaved
PIP and PIP$_2$ faster than PI. Irvine and co-workers (32)
demonstrated that crude soluble brain PLC hydrolyzed PIP$_2$
many times faster than PI when the substrates were presented
to the enzyme as a mixture of phosphoinositide and PE.
Wilson et al. (14) have carefully studied the effects of the
phospholipid composition of small unilamellar vesicles con-
taining phosphoinositide on the rates of hydrolysis of PIP$_2$,
PIP, and PI by two distinct PLC enzymes isolated from sheep
seminal vesicles. They found that when presented with sub-
strate contained in PE vesicles, both enzymes hydrolyzed
PIP$_2$ and PIP faster than PI. Inclusion of PC in the vesicles
caus ed a much greater inhibition of PI than of polyphospho-
nositide hydrolysis suggesting that the selectivity of PLC
might be altered by the presentation of the substrate. Thus,
the immediate environment of the phosphoinositide in mem-
branes may be a key factor in controlling the apparent select-
ivity of the PLC isoforms for a specific substrate. In this
regard, Wilson and co-workers (14) have reported that both
PLC activities purified from sheep seminal vesicles hydrolyze
PIP and PIP$_2$ faster than PI in artificial membranes that
simulate the phospholipid composition of the plasma mem-
brane inner leaflet.

Calcium is essential for activity of PLC-III. The minimum
concentration required for PLC activation was 300 nM, well
above the free calcium concentration typically found in cyto-
sol (33). The enzyme was stimulated over a wide range of free
calcium concentrations, from $3 \times 10^{-7}$ through $3 \times 10^{-5}$ M, in
buffers that simulate cytosolic ionic conditions. The activa-
tion curve appeared somewhat biphasic with half-maximal
stimulations occurring at 2 and 300 $\mu$M. It may be that PLC-
III contains more than one calcium-binding site. It is also
possible that the activation seen at high calcium concentra-
tions may reflect the binding of calcium to the phospholipid
substrate. The results suggest that at resting cytosolic calcium
levels this enzyme exists in an inactive state. It is worth
noting that several recent studies have demonstrated that at
resting cytosolic calcium levels the receptor-regulated PLC is
inactive (see Refs. 6, 7, 18). These studies concluded that the
mechanism of PLC activation by receptors may involve a
GTP-binding protein which increases the sensitivity of the
PLC to activation by calcium.

In contrast to the results reported here, the PLC activities
isolated from sheep seminal vesicles were maximally stimu-
lated by very low levels of calcium (less than 10 $\mu$M) and
were active in the presence of EGTA alone (14). However, in that
study, magnesium, a cation known to decrease the sensitivity
of PLC to activation by calcium (32), was not included in the
buffer. Moreover, the enzymes were assayed using vesicles
composed of the phosphoinositides and PS, a phospholipid
known to increase the sensitivity of PLC to activation by
calcium (32). Hence, one cannot directly compare the calcium
activation data obtained for the seminal vesicle enzymes and
the brain enzyme reported herein.

We have observed that the principal PLC solubilized from
fibroblast membranes behaves like PLC-III on hydrophobic
and ion exchange chromatography and during sedimentation
on glycerol gradients. Since it is likely that this membrane-
associated fibroblast enzyme is the PLC regulated in vitro by
thrombin, GTP, and calcium (18), it is possible that the
enzyme we have isolated from brain might also serve as a
receptor-regulated lipase. The fact that it is isolated as a
soluble enzyme may not affect its distribution in intact cells.
It could, for example, be bound in a relatively inactive state
to PIP$_2$ which is rapidly degraded during disruption of cells.
Alternative possibilities include translocation of PLC-III from
the cytosolic to the plasma membrane compartments during
stimulation by calcium-mobilizing ligands. The various prop-
erties described for PLC-III including the level of calcium
required for activation, the substrate specificity, the relatively
low abundance and high specific activity are all consistent with
a role for this enzyme in amplifying the initial trans-
membrane signals that promote cellular calcium mobilization.
Reconstitution of PLC-III with a suitable receptor and/or
GTP-binding protein will be necessary to evaluate this possi-

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