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Identification in Bovine Liver Plasma Membranes of a Gq-Activatable Phosphoinositide Phospholipase C

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ABSTRACT: Phosphoinositide phospholipase C (PLC) activity extracted from bovine liver plasma membranes with sodium cholate was stimulated by GTPγS-activated $G\alpha_q/G\alpha_{11}$, whereas the enzyme from liver cytosol was not. The membrane-associated PLC was subjected to chromatography on heparin–Sepharose, Q Sepharose, and S300HR, enabling the isolation of the G-protein stimulated activity and its resolution from PLC-γ and PLC-δ. Following gel filtration, two proteins of 150 and 140 kDa were found to correspond to the activatable enzyme. These proteins were identified immunologically as members of the PLC-β family and were completely resolved by chromatography on TSK Phenyl 5PW. The 150-kDa enzyme was markedly responsive to GTPγS-activated α-subunits of $G\alpha_q/G\alpha_{11}$ or to purified G_q/G_{11} in the presence of GTPγS. The response of this PLC was of much greater magnitude than that of the 140-kDa enzyme. The partially purified 150-kDa enzyme showed specificity for PtdIns(4,5)P_2 and PtdIns4P as compared to PtdIns and had an absolute dependence upon Ca^{2+}. These characteristics were similar to those of the brain PLC-β_1. The immunological and biochemical properties of the 150-kDa membrane-associated enzyme are consistent with its being the PLC-β isozyme that is involved in receptor–G-protein-mediated generation of inositol 1,4,5-triphosphate in liver.

It is well documented that calcium mobilizing agonists induce the rapid hydrolysis of phosphatidylinositol (4,5)-diphosphate (PtdIns4,5P_2) by a specific phospholipase C (PLC)[1] (Berridge & Irvine, 1984). Breakdown of this phospholipid results in the formation of two second messengers: inositol 1,4,5-triphosphate and diacylglycerol (Majerus et al., 1986), which amplify the initial signal and ultimately regulate cellular events.

Over the past 6 years, many different PLCs have been purified and characterized, and in most tissues, the enzyme has been shown to be heterogeneous [see Meldrum et al. (1991) for a review]. In 1987, three cytosolic PLCs were purified from bovine brain and were found to be discrete gene products, namely PLC-β1, PLC-γ1, and PLC-δ1 (Ryu et al., 1987; Katan & Parker, 1987). They have since been cloned and sequenced (Suh et al., 1988; Stahl et al., 1988; Katan et al., 1988) and have become representatives of three principal classes of phospholipase C isozymes: PLC-β (150 000–154 000), PLC-γ (145 000–148 000) and PLC-δ (85 000–88 000) according to the nomenclature proposed by Rhee et al. (1989). More recently, a cytosolic phospholipase of 85 kDa that is immunologically distinct from PLC-δ has been desig-

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[1] Abbreviations: PLC, phosphoinositide-specific phospholipase C; $G_q(G\alpha_q)/G_{11}(G\alpha_{11})$, a class of pertussis toxin insensitive G-proteins (and their α-subunits) defined by Strathmann and Simon (1990); PtdIns4P, phosphatidylinositol 4-phosphate; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; APMFS, (4-amidinophenyl)methanesulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; FPLC, fast protein liquid chromatography.

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nated PLC-ϵ (Thomas et al., 1991). The structure of the PLC-α isozymes is presently unclear. However, it has been suggested that PLC-α isozymes of 60–68 kDa are the predominant form of the enzyme in liver (Crooke & Bennett, 1989). The diversity of PLC isozymes has led to the idea that each may be regulated in a different manner.

The observation that tyrosine residues of PLC-γ_1 are phosphorylated following growth factor stimulation of fibroblasts and other cell types indicates a mode of regulation of this PLC isozyme (Wahl et al., 1990; Nishibe et al., 1990). In contrast, many membrane receptors do not exhibit protein tyrosine kinase activity but are coupled to PLC via G-proteins (Uhing et al., 1985; Litosch et al., 1985; Cockcroft & Gomperts, 1985).

The search for the G-proteins specifically involved in the inositol phospholipid signal transduction process proved to be very difficult. However, Taylor et al. (1990) obtained a highly purified G-protein α-subunit preparation that activated PLC and was subsequently found to contain $G\alpha_q$ and $G\alpha_{11}$ (Strathmann & Simon, 1990; Taylor et al., 1991; Taylor & Exton, 1991). These α-subunits were purified from bovine liver plasma membranes, which were first incubated with GTPγS, by reconstituting G-protein containing fractions with a crude preparation of PLC from bovine liver membranes. Concurrently, Smrcka et al. (1991) purified $G\alpha_q$ from bovine brain and showed that it also activated a crude PLC preparation containing PLC-β_1, as demonstrated by Western blotting with monoclonal antibodies. Carter et al. (1990) identified a 155-kDa isozyme (termed PLC-β_m) in rabbit brain membranes and obtained indirect evidence that it was regulated by a G-protein-linked system. In addition, a 150-kDa PLC purified from turkey erythrocytes was shown to be G-protein-regulated by reconstitution with turkey erythrocyte plasma membranes (Morris et al., 1990; Waldo et al., 1991). To date, however, there has been but one direct demonstration of the reconstitution of different PLC isozymes with purified $G\alpha_q/G\alpha_{11}$ (Taylor et al., 1991), and this study employed G-proteins purified from bovine liver and PLCs purified from a soluble fraction of bovine brain.

Since the G-proteins that regulate PLC are of the heterotrimeric type and couple to receptors in the plasma membrane and since PtdIns(4,5)P_2 is located in this membrane, association of PLC with the plasma membrane is a prerequisite for signal transduction by this system. Accordingly, the identification, by reconstitution, of the PLC isozyme that is regulated by G-proteins is best carried out using membrane-associated PLCs. In addition, the G-proteins that regulate PLCs show tissue differences; i.e., in some tissues they are pertussis toxin sensitive (e.g., $G\alpha_o$ and $G\alpha_i$) (Moriarty et al., 1990), whereas in others they are not (Uhing et al., 1986; Cockcroft, 1987; Pang & Sternweiss, 1989; Strathmann et al., 1989). For this reason, the identification of G-protein-regulated PLC isozymes by reconstitution is also best performed in a homogeneous system, i.e., one in which all proteins are derived from the same species, tissue type, and subcellular fraction.

In the present study, we utilized this approach to identify the G_q/G_{11} activatable PLC isozymes in bovine liver membranes. We isolated two proteins (150 and 140 kDa) which showed immunoreactivity to anti-PLC-β monoclonal antibodies. The higher molecular weight enzyme was markedly activated by α-subunits of G_q/G_{11}.

Experimental Procedures

Materials. All chemicals purchased were of the highest grade of purity. PtdIns(4,5)P_2 was prepared from a mixture of brain phosphoinositides (Sigma) as described previously (Taylor & Exton, 1987). PtdIns4P was recovered separately in the same preparation. PtdEtn and PtdIns were obtained from Avanti Polar Lipids.

Phosphatidyl[^{3}H]inositol(4,5)P_2 was purchased from New England Nuclear or American Radiolabelled Chemicals.

GTPγS, [Arg^8]-vasopressin, AMP-PNP, carrier-fixed α_2 macroglobulin, and APMFS were obtained from Boehringer Mannheim.

Heparin–Sepharose, Q Sepharose Fast Flow, and Sephacryl S300 HR Hi-Load were purchased from Pharmacia. TSK Phenyl-5PW was obtained from Beckman, and sodium cholate was from CalBiochem.

Bovine brain PLC-β_1, PLC-γ_1, and PLC-δ_1 isozymes were purified to homogeneity in the laboratory of Dr. S. G. Rhee, NIH, Bethesda, MD, and were kindly provided by Dr. Rhee.

Antisera. Anti-PLC-β, -γ, and -δ mixed monoclonal antisera were prepared according to the method of Suh et al. (1988) in the laboratory of Dr. S. G. Rhee and were generous gifts from Dr. Rhee. In a few experiments, antibodies obtained from Upstate Biotechnology Inc., Lake Placid, NY, were used.

Preparation of Bovine Liver Plasma Membranes. A fraction enriched in plasma membranes was prepared from bovine liver using the method of Taylor et al. (1990). All buffers used to prepare the fraction were supplemented with 10 μg/mL leupeptin, 10 μg/mL antipain, 10 μg/mL aprotinin, and 5 μg/mL APMFS. The membrane pellet was resuspended in 200 mL of a buffer consisting of 25 mM HEPES (pH 7.5) and 1 mM EGTA and was used immediately or stored at −70 °C. Prior to solubilization, further additions were made to give a final composition of 25 mM HEPES (pH 7.5), 1 mM EGTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 5 μg/mL APMFS (buffer A) along with 10 mM $MgCl_2$, 50 mM NaCl, 25 μM GTPγS, 300 μM AMP-PNP, and 3 μM vasopressin in a total of 250 mL. The mixture was incubated with shaking at 30 °C for 30 min. This step was included to prepare, from the same membranes, PLC and GTPγS-activated G-proteins.

A further 50 mL of ice-cold buffer A containing 6% sodium cholate was then added to give a final cholate concentration of 0.8–1%, and the mixture was stirred on ice for 1 h. The resultant extract was carefully decanted and incubated with 20 mL of 35% (v/v) carrier-fixed α_2 macroglobulin in buffer A to decrease protease activity. The mixture was shaken gently for 30 min at 4 °C and filtered through a chromatography column with a wide bed support to remove the carrier. The filtrate was used directly for isolation of PLC.

Preparation of Bovine Liver Cytosolic Fraction. A cytosolic fraction of bovine liver was prepared from the 1500*g* supernatant (approximately 4.81) obtained during the plasma membrane preparation.

A portion of this crude material was centrifuged at 100000*g* for 1 h. DDT (0.5 mM final concentration) and leupeptin and aprotinin (10 μg/mL final concentrations) were added to the supernatant, which was used directly.

Preparation of Bovine Brain Plasma Membrane and Cytosolic Fractions. Bovine brain was obtained from a local slaughterhouse and kept on ice. The brainstem was dissected away and the dural membrane removed. The cerebral tissue (approximately 350 g) was used to prepare the plasma membrane and cytosolic fractions in the manner described for bovine liver.

Assay of PIP_2 Phospholipase C Activity. PLC activity of column fractions was determined as described by Taylor and Exton (1987) using 100 μM [^{3}H]PIP_2 (400–600 cpm/nmol) in the presence of 400 μM PtdEtn.

Unless otherwise stated in the figure legends, all reactions were performed at 37 °C for 10 min in a 200-mL assay mix (pH 7.0) containing a free Ca^{2+} concentration of 219 nM, maintained by a Ca^{2+}-EGTA (2 mM) buffer.

G-Protein activatable PLC activity was determined by reconstituting column fractions with preparations of PLC "activator"[2] which had been purified to different chromatographic stages (Taylor et al., 1990). The total volume of activator plus the PLC fraction was 20 μL, and the final concentration of cholate was <0.08%.

Isolation of Phospholipase C. All purification steps were performed at 4 °C using a FPLC system (Pharmacia). Membrane cholate extract (approximately 320 mL; 4 g of protein after α_2 macroglobulin treatment) was loaded at 3 mL/min onto a 120-mL heparin–Sepharose column (2.6 × 30 cm) previously equilibrated with buffer A containing 0.8% (w/v) sodium cholate, 0.5 mM DTT (buffer B), and 0.05 M NaCl. The column was washed with buffer A until the absorbance at 280 nm (A_{280}) approached baseline. It was then developed with a linear gradient of 0.05–1 M NaCl over 1052 mL. Fractions (12 mL) were collected at a flow rate of 3 mL/min. Those that were stimulated by activator were pooled, concentrated 8-fold, and diluted 12.5-fold with buffer B until the NaCl concentration reached approximately 40 mM. The pool (108 mL) was then loaded at 3 mL/min onto a 100-mL Q Sepharose column (2.6 × 19 cm), equilibrated with buffer B containing 0.05 M NaCl, and the column was washed with the same buffer until the A_{280} returned to baseline.

The column was subsequently developed with a gradient of 0.05–0.8 M NaCl over 570 mL, and fractions of 6 mL were collected at a flow rate of 3 mL/min. Activator-responsive fractions (54 mL) were pooled and concentrated by ultrafiltration to a final volume of 7 mL using an Amicon Centricon concentrator fitted with a YM30 membrane. The concentrate was loaded at 0.5 mL/min onto a 337-mL Sephacryl S300HR column (2.6 × 63.5 cm) equilibrated with buffer B containing 0.15 M NaCl at a flow rate of 0.5 mL/min. Fractions (4 mL) were collected, and those that responded to activator were pooled (44 mL) and diluted 8-fold with buffer A containing 2.85 M NaCl. The final salt concentration of the pooled fractions was approximately 3 M, and the sodium cholate concentration was less than 0.1%.

This pool was loaded at a flow rate of 5 mL/min onto a TSK Phenyl 5PW hydrophobic interaction HPLC column (2.15 × 15 cm) previously equilibrated with buffer A containing 3 M NaCl.

The column was developed with a decreasing gradient of 3–0 M NaCl over 200 mL. Fractions (3 mL) were collected at a flow rate of 5 mL/min. Two regions of PLC activity were pooled separately, aliquoted, frozen in liquid N_2, and stored at −70 °C for further study.

Other Methods. Calibration of the Sephacryl S300HR column was performed using a range of high and low molecular weight protein standards (Pharmacia). Protein concentrations of samples in the 0.1–1.0 mg/mL range were estimated using the bicinchoninic (BCA) protein reagent (Pierce). Protein concentrations in the range 10 ng–0.1 mg/mL were estimated using the Amido black assay (Schaffner & Weissman, 1973). Sodium cholate concentrations were determined by thin-layer chromatography using a solvent system composed of ethyl acetate, isooctane, and acetic acid (45:25:10 v/v). The plates were charred using a solution of 5% (w/v) $CuSO_4$ and 4% (w/v) phosphoric acid and scanned by laser densitometry.

[2] We use the term "activator" to describe the GTPγS-activated form of $G\alpha_q/G\alpha_{11}$ (Taylor et al., 1990).

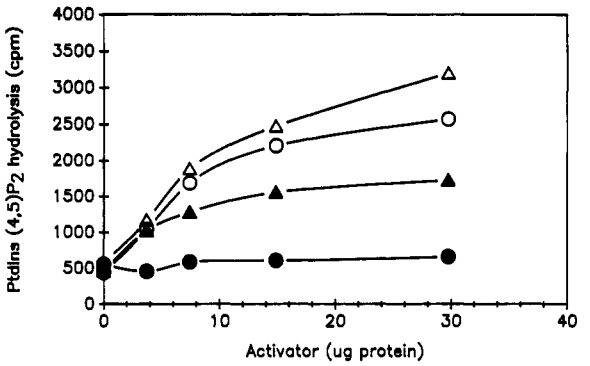

FIGURE 1: Effect of activator on PLC activity in cytosol and plasma membrane extract of bovine liver and bovine brain. Plasma membranes obtained from bovine liver or bovine brain were solubilized with sodium cholate as described under Experimental Procedures but were not preactivated with GTPγS. Cytosolic fractions were prepared exactly as described. Bovine brain membranes (open triangles) or bovine liver cytosol (solid circles) was diluted 1:6, and bovine liver membranes (open circles) or bovine brain cytosol (solid triangles) was diluted 1:3. Ten microliters of each was incubated for 10 min at 37 °C in the absence (data were normalized to approximately 450 cpm in all cases) or presence of increasing amounts of activator.

SDS–PAGE was performed on precast 8 or 10% acrylamide gels (Novex, Encinitas, CA) according to the method of Laemmli (1970). Proteins were visualized by silver staining or transferred to Immobilon P membranes (Millipore Corp.) for Western blotting using a semidry transfer apparatus (Bio-Rad; 15 V for 1.9 h). Following transfer, nonspecific binding sites were blocked for 1 h with 1% (w/v) bovine serum albumin and 1% (v/v) horse serum.

Blots were incubated overnight with primary antibody and developed using a Vectastain alkaline phosphatase ABC kit (Vector Labs). PLC isozymes identified by the Western blotting technique were quantitated, where applicable, by scanning laser densitometry, after the blots were made translucent by soaking in 50 mL of a DMSO/ethanol solution (2:1).

Results

Effect of Activator on PLC Activity in Cytosolic and Plasma Membrane Fractions of Bovine Liver and Bovine Brain. PLC activities associated with crude subcellular fractions of both bovine liver and bovine brain were stimulated to different extents by a partially purified preparation of activator[3] (Figure 1). The plasma membrane fractions of each tissue were more responsive to increasing amounts of activator than were the cytosolic fractions. In the presence of 15 μg of activator preparation, both bovine brain and bovine liver membranes were stimulated approximately 5-fold. The cytosolic fraction of bovine brain could be activated 3.4-fold, whereas the bovine liver cytosol was unresponsive. These results thus indicate that a PLC that can be stimulated by $G\alpha_q/G\alpha_{11}$ is present in the plasma membranes of both tissues and in the cytosol of bovine brain.

Partial Purification of Bovine Liver Membrane-Associated PtdIns(4,5)P_2 Phospholipase C. PLC activity was solubilized

[3] Figures 1, 8, and 9 utilized the same activator preparation. This was obtained at the S300HR gel filtration step (Taylor et al., 1990) and was selectively pooled following reconstitution with purified bovine brain cytosolic PLC-β_1. The pool was enriched in $G\alpha_q/G\alpha_{11}$, had a protein concentration of 14.9 mg/mL, and contained approximately 0.8% sodium cholate. Figures 2–4 and 6 utilized different activator preparations. Thus, these data could not be quantitated in terms of fold activation of PLC. However, the profiles of activation obtained at each chromatographic step were qualitatively identical irrespective of the degree of purity of the activator.

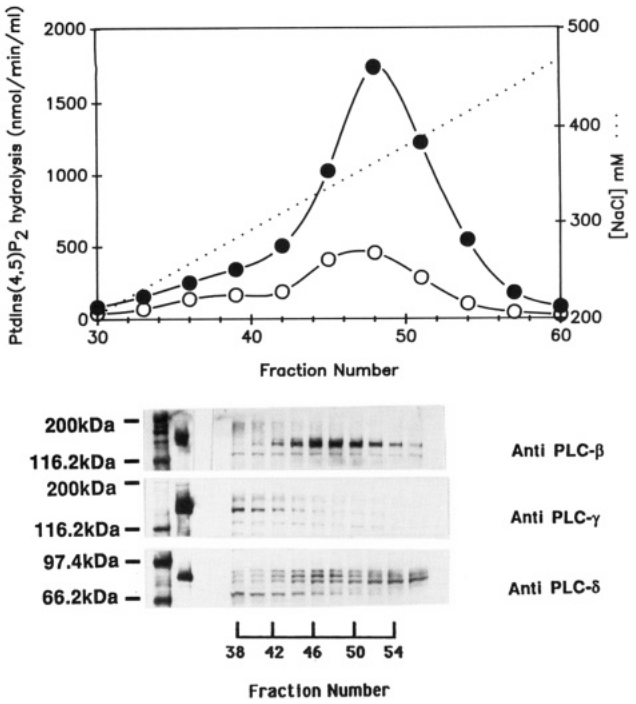

FIGURE 2: Affinity chromatography of PLC on heparin–Sepharose. (A, top) A sodium cholate extract obtained from the particulate fraction of the bovine liver was applied to a heparin–Sepharose column (Experimental Procedures). Aliquots (0.5 μL) of the column fractions were assayed in the presence of 100 μM PtdIns(4,5)P_2 for PLC activity. Open circles represent basal PLC activity. Solid circles represent PtdIns(4,5)P_2 hydrolysis in the presence of 1 μL of activator. The linear salt gradient is represented by the dotted line. Fractions 44–52 were pooled. (B, bottom) Aliquots (20 μL) of appropriate fractions were diluted with an equal volume of Laemmli SDS-containing sample buffer. Samples were loaded onto 10% acrylamide gels, transferred to an Immobilon membrane, and incubated with the appropriate monoclonal antisera as described under Experimental Procedures. Biotinylated molecular weight markers are rabbit muscle myosin (100 000), β-galactosidase (116 200), phosphorylase *b* (97 400), and bovine serum albumin (66 200).

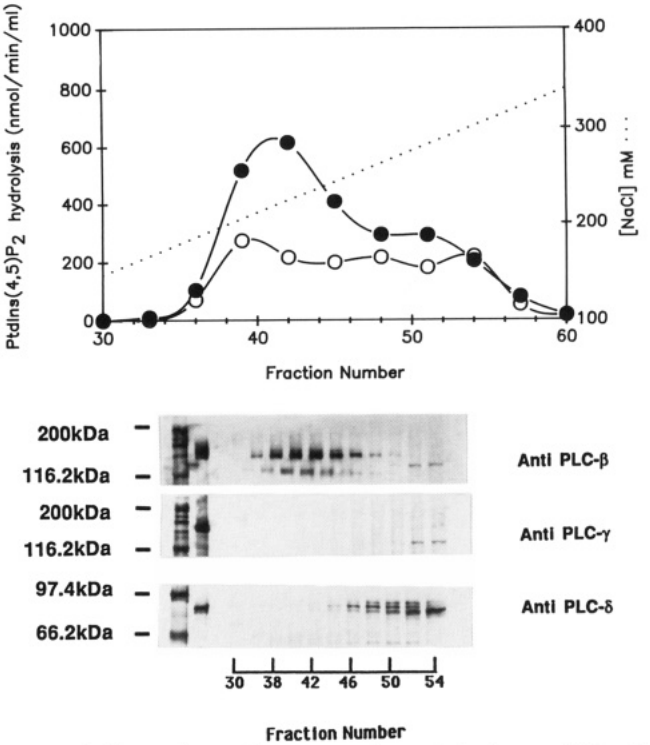

FIGURE 3: Ion-exchange chromatography on Q Sepharose. (A, top) A preparation of PLC purified by heparin–Sepharose was subjected to chromatography on Q Sepharose as described under Experimental Procedures. The fractions (0.5-μL aliquots) were assayed for PLC activity as described in the legend to Figure 2A, using 1 μL of a 1:3 dilution of activator. Open circles represent basal PLC activity, and solid circles represent G_q/G_{11}-activatable activity. Fractions 37–45 were pooled for further purification. (B, bottom) Two hundred microliters of fractions was prepared for electrophoresis as described in the legend to Figure 2B and subsequently immunoblotted with monoclonal antibodies to either PLC-β, PLC-γ, or PLC-δ.

from bovine liver plasma membranes with 0.8–1% sodium cholate as described under Experimental Procedures. The activity was subsequently purified on the basis of reconstitution with activator.

A representative purification of the activatable phospholipase C activity is shown. Although the magnitude of the activation varied with different G-protein preparations, the profiles of activation obtained at the various chromatographic steps were very similar.

(*a*) *Heparin–Sepharose Affinity Chromatography*. The α_2 macroglobulin-treated cholate extract from the bovine liver membranes was applied to a heparin–Sepharose affinity column which was subsequently washed with low-salt buffer as described under Experimental Procedures. This procedure removed the activator which does not absorb to the column. The PLC bound to the column was eluted with a linear salt gradient. The profile of enzyme activity is shown in Figure 2A.

Activatable PLC was present predominantly in fractions 42–54 (peak 48), between 325 and 425 mM NaCl. Fractions were immunoblotted with antisera against PLC-β, PLC-γ, and PLC-δ (Figure 2B). The results showed that all three isozymes were present, with PLC-γ eluting earliest in the gradient followed by PLC-β and then PLC-δ. The fractions exhibiting the greatest activation contained predominantly PLC-β. The heparin–Sepharose column was therefore able to partially resolve the three isozymes for which antibodies were available.

(*b*) *Q Sepharose Anion-Exchange Chromatography*. Fractions exhibiting the greatest activation from heparin–Sepharose contained approximately 350 mM NaCl, and this pool of activity was diluted and applied to Q Sepharose. PLC activity eluted as a diffuse peak around 225 mM NaCl (Figure 3A) using a gradient of increasing NaCl.

At this stage, PLC-γ was no longer detected by Western blotting (Figure 3B) and the activatable enzyme was present mainly in fractions 38–46 corresponding to PLC-β immunoreactivity. PLC-δ eluted from Q Sepharose later in the gradient, i.e., at 250–300 mM NaCl (fractions 46–54), and was largely resolved from PLC-β. Q Sepharose, therefore, not only eliminated PLC-γ but resolved PLC-β from PLC-δ.

(*c*) *S300HR Gel Filtration Chromatography*. Fractions representing the peak of activatable activity were pooled, concentrated, and applied to a Sephacryl S300HR gel filtration column. A single peak of activatable activity was obtained following elution with 144–164 mL of buffer (fractions 36–41) as shown in Figure 4A. Selected fractions were immunoblotted with antisera against PLC-β, PLC-γ, and PLC-δ (Figure 4B). As expected, PLC-γ was not detected, whereas PLC-δ was only weakly detected in fractions 36–38 and PLC-β was most abundant in fractions 36–40. Following calibration of the column, the PLC-β type enzyme was found to have an apparent mass of 151 kDa.

Close examination of the immunoblots (Figure 4B) revealed the presence of two distinct proteins (approximately 140 and 150 kDa) in fractions 36–42. The 150-kDa species comigrated with purified bovine brain PLC-β_1, which was used as a molecular weight marker. The ratio of staining intensity of the

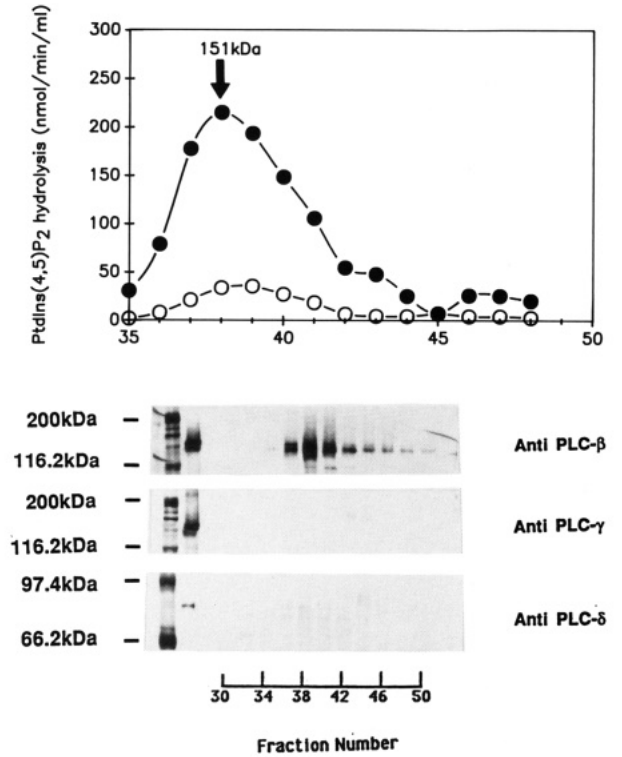

FIGURE 4: Gel filtration chromatography on S300HR. (A, top) Fractions exhibiting activatable PLC activity from the Q Sepharose column were pooled and concentrated as described under Experimental Procedures and loaded onto an S300HR column. Aliquots (2 μL) of fractions were assayed in the absence (open circles) or presence of 1 μL of activator (solid circles). (B, bottom) Samples of appropriate fractions were prepared using Laemmli SDS sample buffer and immunoblotted with monoclonal antibodies to isozymes of PLC as described in the legend to Figure 2B.

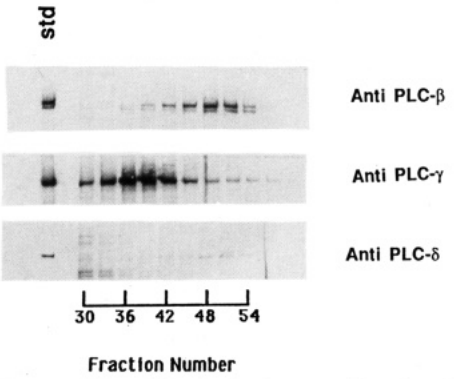

FIGURE 5: Western blots of bovine brain cytosol fractions following chromatography on heparin–Sepharose. Twenty microliters of every second fraction 30–60 from heparin–Sepharose was prepared for SDS–PAGE using 10% acrylamide gels as described in the legend to Figure 2B. The gels were subsequently used for Western blotting against anti-PLC-β, γ, and -δ monoclonal antibodies as described under Experimental Procedures.

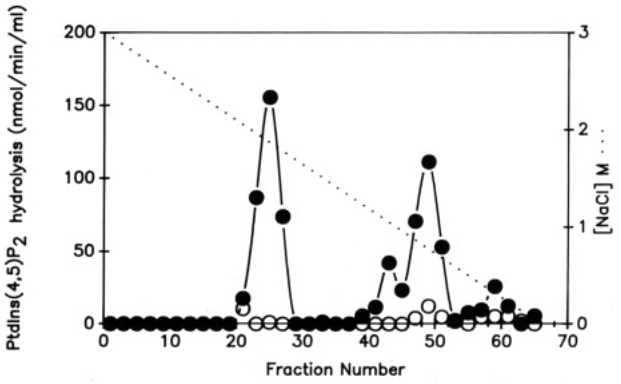

FIGURE 6: Hydrophobic interaction chromatography on TSKL Phenyl 5PW. A preparation of PLC purified by gel filtration on S300HR was diluted into a high-salt buffer and applied to an HPLC Phenyl column as explained in the text. Aliquots (2 μL) of the column fractions were assayed for PtdIns(4,5)P_2 hydrolysis in the absence (open circles) and presence of 10 μL of activator (solid circles). Fractions 22–26 and 47–51 were pooled separately.

two immunoreactive proteins in the column fractions was estimated to be approximately 1:1 (lower band/upper band) using scanning laser densitometry. The purified bovine brain PLC-β_1 (obtained from Dr. Rhee) also appeared to consist of two immunoreactive proteins with a staining intensity of 2:1 (upper band/lower band). When bovine brain cytosol was subjected to heparin–Sepharose chromatography under conditions identical to those used for the bovine liver plasma membrane preparation, Western blotting of selected fractions with anti-PLC-β monoclonal antibodies (Figure 5) also identified two immunoreactive proteins, both of which comigrated on SDS–PAGE with those of the purified PLC-β_1 enzyme.

(d) TSK Phenyl Hydrophobic Interaction Chromatography. Fractions 37–40 from the gel filtration column were pooled and diluted with a high-salt buffer to maximize binding of PLC to the hydrophobic column. Since the sodium cholate concentration was above 0.3% at this stage, the pool of activity was diluted to approximately 0.1% cholate as determined by thin-layer chromatography. The diluted sample was applied at a flow rate of 5 mL/min onto the column, and under these conditions, the A_{280} (not shown) revealed that most of the protein had bound to the column.

Two peaks of activatable PLC activity were eluted during the salt gradient, the first peak at approximately 1.8 M NaCl and the second at 0.8 M NaCl (Figure 6). The first peak of activity (fractions 22–26) showed almost complete dependence on activator, whereas the second (fractions 47–51) showed detectable activity in the absence of activator. The two peaks were pooled separately, and 6 mL of each was concentrated to approximately 100 μL using an Amicon Centricon concentrator (45 min at 3500*g*). The remainder of each pool was immediately aliquoted and frozen at −70 °C.

Western blotting and silver staining of the concentrated pools (Figure 7) revealed a complete separation of the upper and lower bands of PLC-β. The first peak contained the upper of the two protein bands associated with PLC-β as identified by Western blotting using anti-PLC-β antibodies. The mass of this protein [designated PLC-M_1 according to the study of Lee et al. (1987)] corresponded to that of the purified cytosolic PLC-β_1 from bovine brain (Ryu et al., 1986). Its apparent molecular mass as determined by SDS–PAGE was 150 kDa (Figure 4B). The second peak contained the lower of the two protein bands (designated PLC-M_2) with an apparent molecular mass of 140 kDa.

Effect of Activator on PLC-M_1, PLC-M_2, and PLC-β_1 Activities. The activities of both PLC-β pools obtained from the TSK Phenyl column were studied in the absence and presence of activator (Figure 8), and the results were compared with those obtained with purified PLC-β_1. The activator stimulated all of the enzymes, but the different scales of the ordinates should be noted. Under the conditions of the assay, the basal activity of PLC-M_1 was 144 pmol min^{-1} mL^{-1}, but in the presence of increasing amounts of activator it rose to approximately 4800 pmol min^{-1} mL^{-1}. In comparison, the activator was less effective on PLC-M_2 and PLC-β_1; i.e., it only maximally increased the PLC-M_2 from 77 to approximately 500 pmol min^{-1} mL^{-1} and that of PLC-β_1 from 391

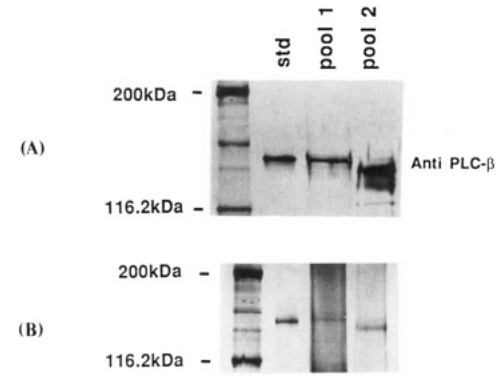

FIGURE 7: SDS–PAGE and immunoblot of two pools of PLC activity from TSK Phenyl 5PW. Six milliliters of both pools of PLC activity obtained from the hydrophobic interaction column were concentrated to 100 μL, and an aliquot (50 μL) was mixed with an equal volume of Laemmli SDS buffer. Twenty microliters of each sample was subjected to electrophoresis on 8% acrylamide gels as described under Experimental Procedures. The separated proteins were then (A) blotted against anti-PLC-β mixed monoclonal antibodies or (B) stained with silver. A sample of PLC-β_1 purified from a cytosolic fraction of bovine brain (supplied by Dr. S. G. Rhee) was run under the same conditions. The weight of this protein on SDS–PAGE has been reported to be 154000 (Ryu et al., 1987). The relative positions of molecular weight markers, rabbit muscle myosin (200000) and β-galactosidase (116200), are shown.

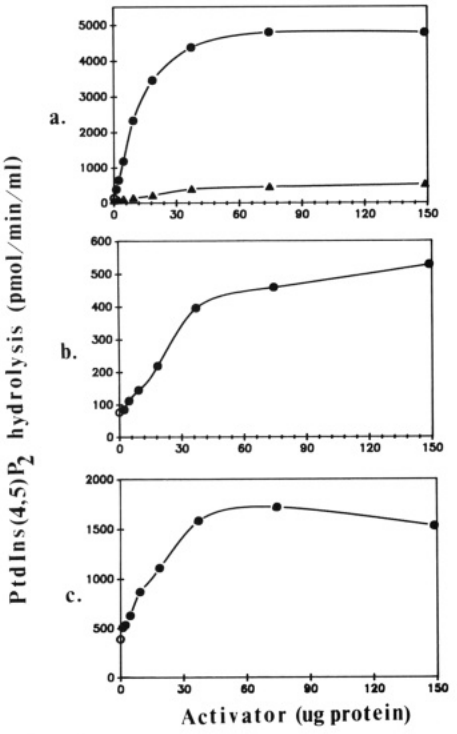

FIGURE 8: Effect of activator on PtdIns(4,5)P_2 hydrolysis by PLC-M_1, PLC-M_2, and PLC-β_1. Five microliters of concentrated PLC-M_1 pool (panel a, circles) from the TSK Phenyl column, 5 μL of a 1:5 dilution of concentrated PLC-M_2 pool (panel a, triangles; panel b, circles), or 1 ng of purified PLC-β_1 (panel c, circles) was incubated in the presence of 10 μL of increasing amounts of activator as indicated. Reaction mixtures included 100 μM PtdInd(4,5)P_2 and 1 mM Ca^{2+}. Incubations were at 37 °C for 10 min.

to 1700 pmol min^{-1} mL^{-1}. When the amounts of enzyme added were varied, PLC-M_1 still showed the greatest fold response to activator (data not shown). With all of the enzymes, maximal activation was produced by approximately 40 μg of activator protein (Figure 8).

The much greater activation of PLC-M_1 compared with that of PLC-M_2 was verified in subsequent experiments (data not shown) which utilized 100 μM GTPγS and purified G_q/G_{11}

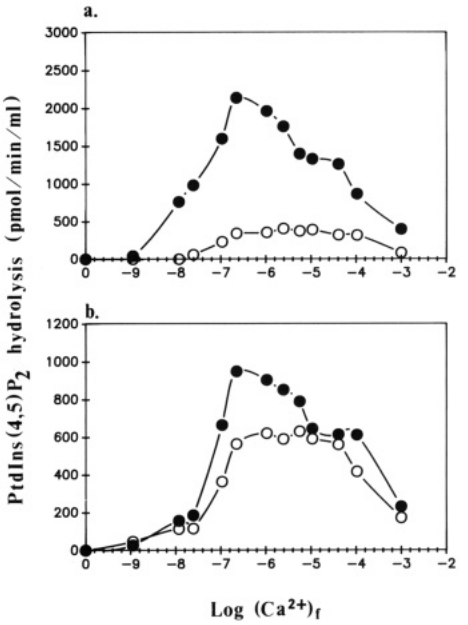

FIGURE 9: Calcium ion requirement of PLC-M_1 and PLC-β_1. The calcium dependence of PLC-M_1 and purified PLC-β_1 in the presence of 15 μM PtdIns(4,5)P_2 and 60 μM PtdEtn was determined using Ca^{2+}-EGTA buffers to vary the free Ca^{2+} concentration. (a) Five microliters of concentrated PLC-M_1 from the TSK Phenyl column was used over the range 0–219 nM Ca^{2+}; 2.5 μL of the same enzyme pool was used for higher Ca^{2+} concentrations. (b) One nanogram of PLC-β_1 was assayed over the free Ca^{2+} range 0–219 nM, and 0.5 ng was assayed over higher Ca^{2+} concentrations. Incubations were performed in the absence (open circles) or presence (solid circles) of 1 μL of a 1:4 dilution of activator.

heterotrimer (Blank et al., 1991). In these experiments, the activation of PLC-M_1 was 45-fold compared with 9-fold for PLC-M_2. These results indicate that there is a selective activation of the PLC-M_1 isozyme by G_q/G_{11} and their α-subunits.

Substrate Specificity of PLC-M_1. The relative rates of hydrolysis of PtdIns(4,5)P_2 and PtdIns4P by the partially purified PLC-M_1 were measured in the presence of 1 mM Ca^{2+} and a 4-fold molar excess of PtdEtn.

When PtdIns(4,5)P_2 was the substrate, enzyme activity was maximal over the range 15–50 μM. The presence of the activator did not influence the substrate concentration yielding a half-maximal rate of hydrolysis (data not shown) but greatly increased the maximal rate. These results suggest that the activator exerts its effect on the V_{max} of the reaction.[4]

When PtdIns4P was used as substrate, enzyme activity was maximal over the range 5–30 μM and, again, the activator increased the maximal activity without altering the substrate concentration for half-maximal hydrolysis (not shown). To directly compare the effect of PLC-M_1 on the rate of hydrolysis of both PtdIns(4,5)P_2 and PtdIns4P in the same assay, the same amount of PLC-M_1 was incubated with 15 μM of each substrate at a Ca^{2+} concentration of 1 mM. Under these conditions, PtdIns4P was hydrolyzed at about half the rate of PtdIns(4,5)P_2. In the presence of activator, there was a 6-fold increase in PLC activity with either substrate. These results indicate that activation of PLC-M_1 by the G-protein does not alter its substrate specificity.

PtdIns was also tested as a substrate in these experiments, but its rate of hydrolysis was extremely low, even in the

[4] The activity of PLC-M_1 was inhibited by concentrations of PtdIns(4,5)P_2 in excess of 50 μM and by PtdIns4P concentrations greater than 30 μM. Consequently, reliable K_m and V_{max} values could not be calculated.

presence of higher Ca^{2+} concentrations (data not shown).

Calcium Ion Requirement of PLC-M_1. Partially purified PLC-M_1 had an absolute dependence upon Ca^{2+} for activity. Figure 9a shows the rate of hydrolysis of PtdIns(4,5)P_2 at different concentrations of free Ca^{2+}. These concentrations were maintained by Ca^{2+}-EGTA buffers and were calculated using a metal ion–ligand calculation program (COMICS). Hydrolysis of PtdIns(4,5)P_2 was maximal at submicromolar concentrations of Ca^{2+}. In the presence of activator, there was a marked increase in hydrolytic activity of the enzyme over the entire range of free Ca^{2+} concentrations. The half-maximal rate of PtdIns(4,5)P_2 hydrolysis by PLC-M_1 (1068 pmol min^{-1} mL^{-1}) was observed with approximately 100 nM free Ca^{2+} in the absence of activator and with 25 nM free Ca^{2+} in its presence.

The PLC-β_1 enzyme also had an absolute dependence on Ca^{2+} (Figure 9b). The hydrolysis of PtdIns(4,5)P_2 measured over the same range of calcium concentrations exhibited a profile somewhat similar to that of PLC-M_1. The half-maximal rate of PtdIns(4,5)P_2 hydrolysis was 307 pmol min^{-1} mL^{-1}, corresponding to 79 nM free Ca^{2+}, and in the presence of activator this was reduced to 31 nM.

These studies confirm that the PLC-M_1 isozyme is most responsive to activator and indicate that the activator slightly reduces its calcium requirement.

Discussion

G-Proteins function as signal transducers for PLC activation by many Ca^{2+} mobilizing agonists. Analogues of GTP that are resistant to hydrolysis (e.g., GTPγS) stimulate basal PLC activity (Cockcroft & Gomperts, 1985; Uhing et al., 1985) and promote agonist activation of PLC in liver plasma membranes (Litosch et al., 1985; Uhing et al., 1986). Despite the fact that the interactions among receptors, G-proteins, and PLC occur in the plasma membrane, there have been relatively few studies dealing with the partitioning of PLC activity between plasma membranes and cytosol. There is evidence that some forms of PLC may be exclusive to either membranes or cytosol (Baldassare et al., 1989; Martin & Kowalchyk, 1990) and also for the "same" isozyme to be dispersed between both fractions (Lee et al., 1987; Behl et al., 1988). In view of this confusing situation, we began our study by determining the location of the $G\alpha_1/G\alpha_{11}$-activatable PLC in crude fractions of bovine liver. We found a marked difference in the responses of the liver cytosol and membranes. Although the basal activity of PLC in cytosol was higher than that in membranes, the cytosol was unresponsive to activation by $G\alpha_q/G\alpha_{11}$. From these data, it may be concluded that the PLC activity regulated by $G\alpha_q/G\alpha_{11}$ is probably exclusive to the membrane fraction.[5] We were able to further fractionate this activity into two forms by successive chromatography on heparin–Sepharose, Q Sepharose, and S300HR gel filtration.

A crucial step in resolution of these forms was hydrophobic chromatography on Phenyl 5PW. Phenyl-Sepharose has been successfully employed in a number of previous PLC purifications (Chau & Tai, 1982; Rebbechi & Rosen, 1987) in cytosolic fractions of different tissue types.

A major question is the identity of the two PLCs. The 150-kDa protein is probably the liver homologue of bovine brain PLC-β because of comigration with the brain enzyme, and the reactivity of the 140-kDa protein with antibodies to PLC-β would also place it in this family but its relationship to other PLC forms is unclear.

PLC-β_1 and a 140-kDa anti-PLC-β immunoreactive protein [termed PLC-M_2 by Lee et al. (1987)] are the only PLCs that have been purified from membrane fractions of bovine brain (Katan & Parker, 1987; Lee et al., 1987; Rhee et al., 1989). There is much confusion in the literature as to the differences between PLC-β_2 and PLC-M_2. PLC-β_2 has been cloned from an HL-60 library and is a discrete gene product with high sequence homology to PLC-β_1 in the putative catalytic domains (Kriz et al., 1990). Its deduced mass is 140 kDa.

By comparison, it has been suggested that the 140-kDa membrane-associated PLC-M_2 is either a proteolytic fragment of the 150-kDa protein or a product of alternative splicing (Lee et al., 1987). Since protease inhibitors were used extensively during the present purification procedure and since the 140-kDa protein was visible following SDS–PAGE of freshly prepared, solubilized membranes (unpublished observations), we suggest that it was not generated by proteolysis. In this regard, it should be noted that the 140-kDa protein was also present in bovine brain cytosol following fractionation on heparin–Sepharose (see Figure 5).

In the present study, we have tentatively identified the 140-kDa protein as PLC-M_2. However, since our sample was not purified to homogeneity and monoclonal antibodies specific for PLC-β_2 were not available, we could not ascertain its true identity.[6]

$G\alpha_q/G_{11}$ appeared to activate PLC-M_1 through an effect on the V_{max} of the reaction rather than the K_m and also did not alter the specificity of the enzyme for its phosphoinositide substrates. The data also indicate that $G\alpha_q/G\alpha_{11}$ slightly increases the Ca^{2+} sensitivity of PLC-M_1. This has been suggested previously (Taylor & Exton, 1987; Smrcka et al., 1991) for crude preparations of membrane-associated PLC. Additionally, Blank et al. (1991) demonstrated a small increase in Ca^{2+} sensitivity of brain cytosolic PLC-β_1 using GTPγS and purified G_q/G_{11} heterotrimer. The change in calcium sensitivity caused by interaction of PLC with the G-proteins could provide an additional mechanism by which the enzyme is activated by agonists in intact cells.

In line with previous studies on the characterization of PLC-β_1 (Ryu et al., 1987; Katan & Parker, 1987), PLC-M_1 was found to preferentially hydrolyze PtdIns(4,5)P_2 at low calcium concentrations. We were somewhat surprised that PtdIns was not hydrolyzed, but this is in agreement with other studies, e.g., Smrcka et al. (1991), using similar conditions.

The present findings identify PLC-M_1 as the PLC isozyme most responsive to G_q/G_{11} in bovine liver plasma membranes. It appears to be closely related to PLC-β_1 from bovine brain. A lower molecular weight isozyme of the PLC-β family is much less responsive to these G-proteins. It is possible that this isozyme responds better to other G-proteins, e.g., other members of the G_q family. It is also possible that one or more of the PLC isozymes in the cytosol responds to these or other G-proteins. Such questions await the availability of these novel G-proteins in purified form.

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[5] This does not exclude the possibility that cytosolic PLC(s) could respond to other G-proteins.

[6] Sequence information would confirm its identity with PLC-β_2, but this would require generation of the protein in much greater amounts and purity than presently obtainable.

arations and for their expert advise. We gratefully acknowledge technical assistance from Roosevelt Bryant and manuscript preparation by Judy Childs.

Registry No. PLC, 63551-76-8; Ca, 7440-70-2.

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