Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 5540–5544, August 1987
Biochemistry

Phospholipase C associated with particulate fractions of bovine brain

(signal transduction/cytosolic phospholipase C/membrane-bound phospholipase C)

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Communicated by E. R. Stadtman, April 8, 1987

ABSTRACT We previously reported that cytosolic fractions of bovine brain contain two immunologically distinct phosphoinositide-specific phospholipases C (PLCs), PLC-I and PLC-II. In this report the subcellular distribution of PLC-I and PLC-II in brain homogenates was measured using RIA. Significant differences were found in the distribution of the two forms of PLC in 100,000 × *g* supernatants (cytosolic fraction) of brain homogenized in hypotonic buffer and 2 M KCl extracts of washed pellets (particulate fraction). More than 90% of PLC-II was found in the cytosolic fractions, whereas the PLC-I-like molecules were equally distributed between cytosolic and particulate fractions. Purification of PLC enzyme to near homogeneity from the particulate fractions yielded two PLC enzymes, both of which could be recognized by anti-PLC-I antibodies but not by anti-PLC-II antibodies. Their M_r values, determined under denaturing conditions, were 150,000 and 140,000. The polypeptide of the enzyme of M_r 150,000 seems to be the same as that of the cytosolic enzyme PLC-I: their M_r values were identical, and their trypsin-digested peptides yielded a similar elution profile on a C_{18} reverse-phase column. We propose, therefore, that PLC-I and its truncated form are weakly associated with membranes.

Interaction of ligands with Ca^{2+}-mobilizing receptors stimulates the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdInsP_2) and generates two second-messenger molecules, diacylglycerol and inositol-1,4,5-trisphosphate (InsP_3) (reviewed in refs. 1–3). Diacylglycerol remains in the plasma membrane and activates protein kinase C (2), whereas InsP_3 causes the release of Ca^{2+} from endoplasmic reticulum, probably by binding to a specific intracellular receptor (1, 4).

Recent studies suggest that the coupling of receptor function to PLC is mediated by unspecified guanine nucleotide-binding protein(s) (5–18). This signal transduction model is analogous to the modulation of adenylate cyclase in which two guanine nucleotide-binding proteins, G_i and G_s, couple the receptor function to the synthesis of a second-messenger molecule, cAMP (19). In addition, because adenylate cyclase and the modulating G proteins are all membrane-bound proteins, the association of PLC with plasma membrane has been considered a prerequisite for the functional response of PLC to the signal transduced across the cell membrane. PLC activity has been demonstrated in various membrane preparations (10–12, 20–26). Nevertheless, all PLC enzymes purified until now are from cytosolic fractions, and they include two PLC isozymes from sheep seminal vesicular gland (27), platelet (28–30), and liver (31). Recently, we purified two immunologically distinct enzymes, PLC-I and PLC-II, from bovine brain cytosol (32, 33). Here, we report the purification of two PLC enzymes from particulate fractions of bovine brain, one of which appears to be identical to cytosolic PLC-I and the second form appears to be a truncated form of PLC-I.

MATERIALS AND METHODS

Materials. Soybean PtdIns was purchased from Sigma, heparin-agarose from Bethesda Research Laboratories, Mono Q HPLC column from Pharmacia, and TSK DEAE-5PW and TSK phenyl-5PW columns were from Beckman. ^{3}H-labeled PtdIns (^{3}H-PtdIns) was obtained from New England Nuclear.

Phospholipase C Assay. Assays were done in a 200-μl reaction mixture of 300 μM soybean PtdIns/0.1% sodium deoxycholate/3 mM $CaCl_2$/1 mM EGTA/50 mM Hepes, pH 7.0, containing 20,000 cpm of ^{3}H-PtdIns and a source of enzyme. All assays were run at 37°C and were terminated as described (32).

Fractionation of Soluble and Particulate Forms of PLC Enzymes. Bovine brain proteins were extracted sequentially in five steps: (*i*) Bovine brain was freshly obtained from a local slaughterhouse. Fifty grams of cerebra were homogenized in 200 ml of homogenization buffer (10 mM Tris·HCl buffer, pH 7.2/0.1 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride, 0.5 mM diisopropyl fluorophosphate/5 mM EGTA containing 0.125 μg of leupeptin per ml) in a Potter–Elvehjem homogenizer and then centrifuged for 1 hr at 100,000 × *g*. (*ii*) The pellet from step *i* was rehomogenized in 200 ml of homogenization buffer and then centrifuged for 1 hr at 100,000 × *g*. (*iii*) Step *ii* was repeated. (*iv*) The thoroughly homogenized and washed pellet was suspended in 50 ml of 2 M KCl in homogenization buffer and was stirred for 2 hr at 4°C. The suspension was centrifuged for 30 min at 200,000 × *g*. (*v*) Step *iv* was repeated. Supernatants from steps *iv* and *v* were dialyzed separately against homogenization buffer to remove KCl before the measurement of the PLC enzymes.

Purification of Membrane-Associated PLC. (*i*) *Extraction of PLC from particulate fractions.* Twelve bovine brains (3.3 kg) were homogenized in a Waring blender with 6.6 liters of homogenization buffer. The homogenate was centrifuged for 30 min at 13,000 × *g*. The precipitate was resuspended in the same buffer (6.6 liters) and homogenized again to ensure complete breakage of cells. The homogenate was centrifuged for 30 min at 13,000 × *g*. The washed pellet was suspended in 2 M KCl in homogenization buffer and stirred for 2 hr at 4°C. The suspension was then centrifuged for 90 min at 13,000 × *g*. The supernatant was brought to 60% $(NH_4)_2SO_4$ saturation by adding solid salt. This suspension was centrifuged for 30 min at 13,000 × *g*, and the pellet was suspended in 500 ml of homogenization buffer; the suspension was

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Abbreviations: PtdIns, phosphatidylinositol; PtdInsP_2, phosphatidylinositol-4,5-diphosphate; PLC, PtdIns-specific phospholipase C.

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dialyzed overnight against the homogenization buffer. Dialyzed solution was centrifuged for 30 min at 13,000 × g to remove insoluble particles, and the supernatant, still very turbid, was kept at −20°C to be combined with the dialyzed solutions from two other identical preparations. *(ii) Ion-exchange chromatography on DEAE-cellulose.* Because of turbidity, combined protein solution from step *i* could not be chromatographed on a DEAE column directly. Therefore, two stages of DEAE chromatography, a batch procedure followed by a column step, were employed as described (33). In the batch step, the proteins were adsorbed on 2 liters of DEAE; then unbound proteins and microsomes were washed off. The washed DEAE was placed on the top of a 10-cm-high bed of DEAE in a column (8 × 45 cm). The column was eluted with a KCl gradient as described (33). *(iii) Heparin-agarose chromatography.* The pooled fraction from step *ii* (600 ml) was applied to a heparin-agarose column (5 × 25 cm) equilibrated with 20 mM Hepes, pH 7.0/100 mM NaCl/0.1 mM dithiothreitol/1 mM EGTA. The column was eluted with a linear gradient from 100 mM to 500 mM NaCl in 1.5 liters of equilibrium buffer. Peak fractions (310 ml) were pooled and concentrated on an Amicon filter to 27 ml. *(iv) Reverse-phase chromatography on TSK phenyl-5PW column.* Solid KCl was added to the concentrated fractions from step *iii* to give a concentration of 3 M, and the mixtures were centrifuged to remove denatured proteins. The supernatants were applied at a flow rate of 5 ml/min to a HPLC preparative phenyl column (21.5 × 150 mm) equilibrated with 20 mM Hepes, pH 7.0/3 M KCl/1 mM EGTA/0.1 mM dithiothreitol. Elution was continued at 5 ml/min with a decreasing KCl gradient from 3 M to 1.2 M for 15 min and with a decreasing gradient from 1.2 M to 0 M for 20 min. Then the column was washed with a KCl-free buffer. Fractions containing each of the two peaks of protein (15 ml for fraction M1 and 13 ml for fraction M2) were collected separately. The pooled solutions were washed with a KCl-free 20 mM Hepes, pH 7.0, and were concentrated to 5 ml in an Amicon filter concentrating procedure. *(v) Ion-exchange chromatography on Mono Q HPLC column.* The washed protein solutions M1 and M2 (5 ml each) were applied separately to a Mono Q HPLC column. This elution procedure was the same as described (33).

Other Methods. Determination of protein concentration, PAGE, and immunoblotting using murine monoclonal antibodies are as described (33); preparation and properties of these monoclonal antibodies were also described in ref. 33.

RESULTS

Keough and Thompson (21) showed that bovine brain contains membrane-bound PLC enzyme that could be solubilized by a 2 M KCl buffer. Therefore, to identify the membrane-bound enzyme in this report, brain tissue was homogenized and extracted three times in a KCl-free buffer and twice in a 2 M KCl buffer; PLC-I and PLC-II in supernatants from each extraction step were quantitated using RIA. The sum of PLC-I-like and PLC-II-like proteins detected in the five supernatants derived from 50 g of bovine brain was 440 μg and 231 μg, respectively. As summarized in Table 1, in the case of PLC-I-like protein, a total of 46% was found in the supernatant of the KCl-free extracts (from steps *i*, *ii*, and *iii*) and a total of 54% in the 2 M KCl extract (from steps *iv* and *v*). In contrast, almost all (92%) of the PLC-II-like protein was found in the KCl-free extract, and only 8%, which may have been extracted by additional washes with the KCl-free buffer, was found in the 2 M KCl extract.

The cytosolic proteins (from step *i*) and particulate proteins (from step *iv*) were analyzed on a HPLC DEAE column as shown in Fig. 1, and the PtdIns-hydrolyzing activity was measured. Although the protein elution profiles were different, two PLC activity peaks, a minor peak† centered at fraction number 57 and a major peak† centered at fraction number 72, were observed for both cytosolic and particulate proteins. The main peak fractions (71–73) were analyzed on $NaDodSO_4$/PAGE and immunoblotted. As shown in Fig. 1*A Inset*, the band blotted by anti-PLC-II antibodies (lane 2) was approximately twice as intense as the band blotted by anti-PLC-I antibodies (lane 1). However, if one remembers that the intensity of immunoblot observed with PLC-I was approximately one-half that of PLC-II when equal amounts of enzymes were used (see Fig. 3), it could be concluded that the cytosolic proteins contained approximately equal amounts of PLC-I and PLC-II. This is consistent with the result obtained using RIA (Table 1) and also with the fact that almost equal quantities of PLC-I and PLC-II could be purified from bovine brain cytosol (33). However, the particulate proteins yielded two intense bands of 150 kDa and 140 kDa recognized by anti-PLC-I antibodies (Fig. 1*B Inset*, lane 1) and a low-intensity band of 145 kDa recognized by anti-PLC-II antibodies (Fig. 1*B Inset*, lane 2).

PLC enzymes were purified in large scale from the washed particulate fractions by a slight modification of the procedure used for the purification of cytosolic enzyme. Chromatography of the dialyzed 2 M KCl extracts on a DEAE column yielded two activity peaks (Fig. 2*A*), a major peak centered at fraction 115 and a minor peak centered at fraction 140. The minor peak contained PLC-II that probably had been trapped

Table 1. Determination of PLC-I-like and PLC-II-like proteins in soluble and particulate fractions from 50 g of bovine brain

Step	Vol, ml	Total protein, mg	PLC-I		PLC-II	
			μg	%	μg	%
i	190	570	166	38	164	71
ii	188	94	24	5	34	15
iii	187	37	11	3	15	6
iv	62	93	188	43	14	6
v	60	36	51	11	4	2
		Total	440	100	231	100

Bovine brain proteins were extracted sequentially as described. The extraction buffer contained no KCl for steps *i*, *ii*, and *iii*, and 2 M KCl for steps *iv* and *v*. Purified monoclonal antibodies recognizing different antigenic sites on each enzyme were used for the radioimmune determination of PLC-I-like and PLC-II-like proteins in the dialyzed supernatants from each extraction step. For determination, 96-well microtiter plates were first coated with anti-PLC-I antibody K-32-3 or anti-PLC-II antibody F-7-2. The dialyzed supernatants were added at several different concentrations to immobilize the PLC enzyme. After several washings the immobilized enzyme was quantitated by adding an ^{125}I-labeled antibody (hybridoma protein K-92-3 for PLC-I and D-7-3 for PLC-II). The values of ^{125}I-radioactivity were converted to ng of PLC enzyme per well from the standard curves established using purified PLC-I or PLC-II. This sensitive assay was possible because monoclonal antibodies prepared against either PLC-I or PLC-II did not cross-react with the other. The properties of monoclonal anti-PLC antibodies and the procedure of RIA will be presented elsewhere.

†When cytosolic fractions of bovine brain were chromatographed on a conventional DEAE-cellulose A-52 column, two partially resolved peaks of PLC activity were eluted; the purified enzymes from the first and second peaks were named PLC-I and PLC-II, respectively. Because one usually obtains better separation of proteins with a HPLC DEAE column, we had mistakenly assumed that the peak centered at fraction 57 was due to PLC-I and the peak centered at fraction 72 was due to the PLC-II of ref. 32. However, current results indicate that the fraction 72 peak contains both PLC-I and PLC-II and that the fraction 57 peak is due to another PLC enzyme of ≈85 kDa. This third enzyme was recognized by neither anti-PLC-I nor anti-PLC-II antibodies. Purification and properties of this 85-kDa polypeptide will be reported elsewhere.

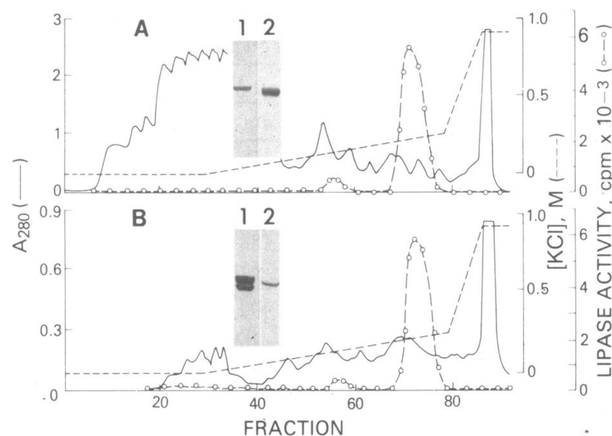

FIG. 1. Ion-exchange chromatography on TSK-DEAE-5PW column. Proteins from 50 g of bovine cerebra were extracted three times with a KCl-free buffer in steps *i*, *ii*, and *iii* and twice with a 2 M KCl buffer in steps *iv* and *v* as described. (*A*) One-half (95 ml) of the cytosolic protein from (*i*) was applied at a flow rate of 5 ml/min, onto a HPLC DEAE-5PW column (21.5 × 150 mm) previously equilibrated with 50 mM Tris·HCl, pH 7.6/1 mM EGTA. Elution was continued at 5 ml/min with KCl gradient, 0–250 mM for 50 min, and 250–900 mM for 5 min. Fractions of 5.0 ml were collected. ^{3}H-PtdIns-hydrolyzing activity was measured in the presence of 0.1% sodium deoxycholate as described in ref. 32. (*B*) Particulate protein extracted with 2 M KCl in step *iv* was dialyzed to remove KCl, and its volume was adjusted to 95 ml before it was applied to the HPLC column. All other chromatography procedures were the same as in *A*. (*Inset*) Pools of the main peak fractions (71–73) were analyzed on a 6% NaDodSO$_4$/polyacrylamide gel, electrophoretically transferred to nitrocellulose paper, and immunoblotted with anti-PLC-I antibodies (lane 1) or anti-PLC-II antibodies (lane 2).

in the particulate fraction. Note that the specific activity of PLC-II is twice that for PLC-I under the assay condition (33). Further purification of the major peak proteins on a heparin-agarose column (Fig. 2*B*) and then on a HPLC phenyl-5PW column (Fig. 2*C*) yielded two partially resolved protein peaks with PLC activity, designated by PLC-M1 and PLC-M2. In the final step in the purification, PLC-M1 and PLC-M2 were separately chromatographed on a Mono Q HPLC column. Fig. 2*D1* shows the elution profile of PLC-M1, which is similar to the profile for PLC-M2 shown in Fig. 2*D2*. In each elution profile, five protein peaks, designated as peaks a, b, c, d, and e, were observed. The five protein peaks were pooled separately, and each was subjected to PLC assay and PAGE. For both PLC-M1 and PLC-M2, the PLC activity detected in peak a was negligible, and peak b contained several protein bands when subjected to NaDodSO$_4$/PAGE analysis. On the other hand, PLC-M1 enzymes in peaks c, d, and e all showed a single band with a M_r equivalent to 150,000 on a NaDodSO$_4$/PAGE gel as exemplified in Fig. 3, lane 3. On the same NaDodSO$_4$/PAGE gel, the PLC-M2 enzymes in peaks c, d, and e all exhibited a major protein band of 140 kDa and a minor band of 150 kDa (Fig. 3*A*, lane 4); the minor band of 150 kDa must be due to the contamination by PLC-M1. Apparent sizes of PLC-M1 and PLC-M2 were clearly different as evidenced by two separate bands observed with their mixtures (Fig. 3*A*, lane 6). These results indicate that the phenyl-5PW HPLC column partially separated PLC-M1 and PLC-M2 into polypeptide sizes of 150 kDa and 140 kDa, respectively. However, the reproducible separation of four protein peaks—b, c, d, and e—by the Mono Q HPLC column cannot be explained. As summarized in Table 2, the specific PtdIns-hydrolyzing activity measured in the presence of 0.1% deoxycholate was similar for enzymes c, d, and e, ranging from 20 to 22 μmol/min/mg for PLC-M1 and 22 to 26 μmol/min/mg for PLC-M2. Fig. 3 shows the results of the protein staining of NaDodSO$_4$/PAGE gel and the immuno-

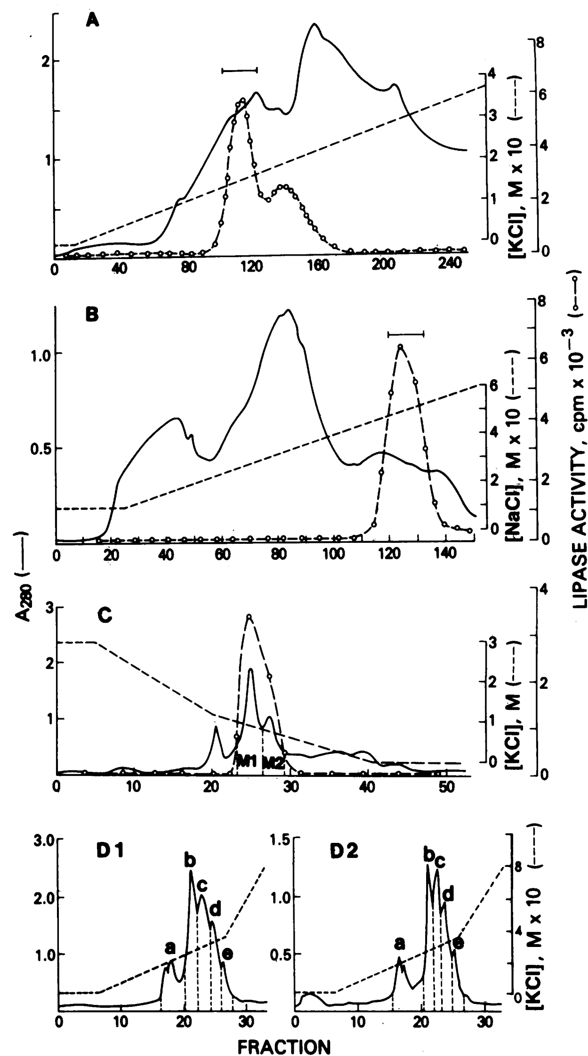

FIG. 2. Large-scale purification of phospholipase C (PLC-M) from 2 M KCl extracts of bovine brain particulate fractions. Detailed procedures on column chromatography steps are described. (*A*) DEAE-cellulose chromatography. Well-washed particulate fractions were extracted with 2 M KCl solution. Proteins in the extracts were precipitated by adding $(NH_4)_2SO_4$; then the proteins were redissolved and dialyzed before being loaded onto a DEAE-cellulose column. (*B*) Heparin-agarose chromatography. (*C*) Reverse-phase chromatography on TSK-phenyl-5PW column. In this step, proteins containing PLC activity were partially resolved into two peaks, PLC-M1 and PLC-M2. (*D*) Ion-exchange chromatography on Mono Q column of PLC-M1 (*D1*) and PLC-M2 (*D2*). From each chromatography step, five protein peaks, designated a, b, c, d, and e, were collected manually.

blot of PLC-I, PLC-II, PLC-M1, and PLC-M2. The apparent sizes of PLC-I and PLC-M1 were identical, whereas the M_r values of the two cytosolic enzymes PLC-I and PLC-II were different. Indeed, the mixture of PLC-M1 and PLC-I was shown as a single band in Fig. 3*A*, lane 5. In the immunoblot, a mixture of four monoclonal antibodies derived against PLC-I recognized PLC-I, PLC-M1, and PLC-M2, but not PLC-II. On the other hand, anti-PLC-II was specific to PLC-II.

The preparations of PLC-M1 and PLC-M2 were also analyzed on native gradient polyacrylamide gel. Immediately after purification, the PLC-M1 yielded two bands with M_r values of 280,000 and 560,000 regardless of protein peaks c, d, and e (data not shown). This electrophoretic pattern was very similar to that of PLC-I, in which the 280- and 560-kDa bands were assigned to dimeric and tetrameric forms of PLC-I, respectively (32). The PLC-M2 also contained dimer as a major component and tetramer to a lesser extent for all

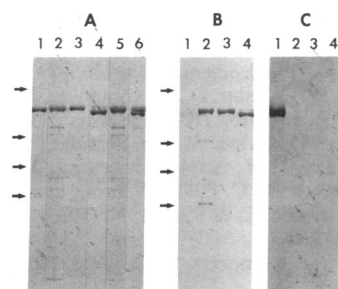

FIG. 3. NaDodSO$_4$/PAGE and immunoblots of various PLC enzymes. Two cytosolic enzymes (PLC-I and PLC-II) and two particulate enzymes (PLC-M1 and PLC-M2) were subjected to NaDodSO$_4$/polyacrylamide gradient (6–16%) gels and either stained with Coomassie blue (*A*) or immunoblotted with anti-PLC-I antibodies (*B*) or immunoblotted with anti-PLC-II antibodies (*C*). (*A–C*) Lanes 1, PLC-II; lanes 2, PLC-I; lanes 3, PLC-M1; and lanes 4, PLC-M2. (*A*) Lane 5, mixture of PLC-I and PLC-M1; and lane 6, mixture of PLC-M1 and PLC-M2. Arrows, M_r standards (from top) myosin (200,000), β-galactosidase (116,000), phosphorylase *b* (94,000), and bovine serum albumin (67,000).

three protein peaks, c, d, and e (data not shown). However, during storage at −20°C for several weeks, both PLC-M1 and PLC-M2 oligomerized, but to a different degree (Fig. 4). In aged PLC-M1, despite the formation of new bands with higher M_r values, the protein bands due to dimer and tetramer remained dominant. On the other hand, PLC-M2 oligomerized so extensively that a ladder of bands was observed extending to the gel top.

All four monoclonal anti-PLC-I antibodies (33), which recognize different antigenic sites on the PLC-I polypeptide, cross-reacted with PLC-M1. These same antibodies also cross-reacted with PLC-M2 (data not shown). In addition, the elution profiles on a C_{18} column of tryptic digests derived from PLC-I and PLC-M1 were similar, as shown in Fig. 5. It seems, therefore, that the enzyme activities of PLC-I and PLC-M1 are due to the same polypeptide of 150 kDa and that PLC-M2 is a truncated fragment of PLC-M1.

DISCUSSION

In the previous report, we showed that cytosolic fractions of bovine brain contain two immunologically distinct phospholipase C enzymes, PLC-I and PLC-II. Our present results indicate that PLC-II exists mainly in cytosol, whereas PLC-I is found in both cytosolic and particulate fractions. Furthermore, microscopic studies on rat brain slices stained using peroxidase-conjugated anti-PLC-I antibodies showed that cytoplasm and plasma membranes, but not nuclear membranes, of certain populations of neuronal cells are densely populated by PLC-I-like molecules (C. R. Gerfen, W.C.C., P.-G.S., S.G.R., unpublished results).

The PLC enzymes from particulate fractions could be separated into two enzymes, PLC-M1 consisting of a 150-kDa polypeptide and PLC-M2 of a 140-kDa polypeptide. Then, it could be concluded that both the membrane-bound PLC-M1 and the cytosolic PLC-1 contain an identical polypeptide based on the following observations: (*i*) the M_r values for these enzymes measured under denaturing conditions were identical; (*ii*) both exist mainly in dimeric form and in tetramer to a small extent; (*iii*) all the monoclonal antibodies prepared against the cytosolic PLC-I recognized the mem-

Table 2. Purification of PLC-M1 and PLC-M2 from 36 bovine brains

	Total protein, mg	Total activity,* μmol/min	Specific activity,* (μmol/min)/mg	Yield, %
$(NH_4)_2SO_4$ ppt	14,000	1,593	0.11	
DEAE-cellulose	1,490	670	0.45	100
Heparin-agarose	105	502	4.8	75
Phenyl-5PW				
PLC-M1	15	316	21	47
PLC-M2	8	105	13	16
Mono Q				
PLC-M1 b	3.1	28.4	9	20†
c	2.9	58.1	20	
d	1.7	37.0	22	
e	0.6	12.5	21	
PLC-M2 b	1.4	12.8	9	11†
c	1.3	24.9	20	
d	0.9	22.3	25	
e	0.4	10.4	26	

ppt, precipitate.
*^{3}H-PtdIns-hydrolyzing activity was measured in the presence of 0.1% sodium deoxycholate.
†Yield for the sum of enzyme activity in peaks b, c, d, and e.

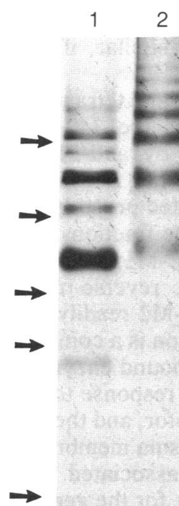

FIG. 4. Polyacrylamide gradient gel PLC-M1 (lane 1) and PLC-M2 (lane 2) that had been stored at −20°C for several weeks were subjected to polyacrylamide gradient (4–16%) gel under a nondenaturing condition. Arrows, M_r standards (from top) thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000).

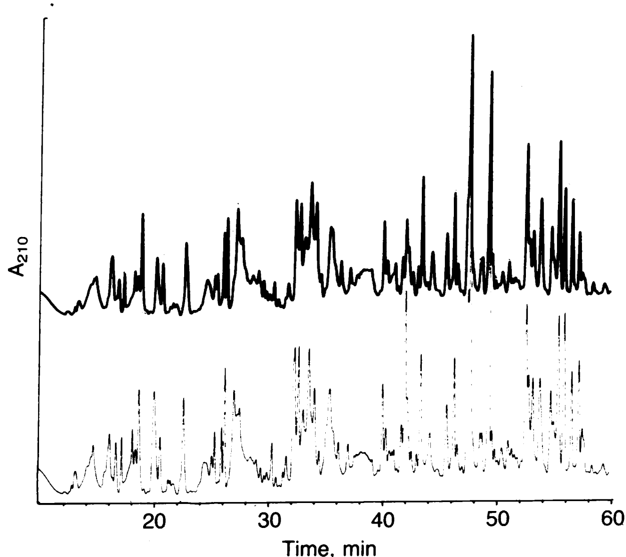

FIG. 5. HPLC of tryptic peptides. PLC-I (300 μg) (upper darker line) or PLC-M1 (lower lighter line) was first denatured by 6 M guanidine chloride and then precipitated by acetone. The protein precipitates were dispersed by brief sonication and digested with 6 μg of trypsin for 12 hr at 30°C and for another 8 hr after being supplemented with 6 μg of trypsin. The trypsin-digested peptides were chromatographed on a reverse-phase column (Vydac C_{18} column, 4.6 × 250 mm) by eluting with a linear gradient of 0–60% CH_3CN in 0.1% trifluoroacetic acid.

brane-bound enzymes; and (*iv*) the elution profiles of tryptic peptides of PLC-M1 and PLC-I were very similar, if not identical.

The PLC-M2 enzyme also assumed dimeric and tetrameric forms when freshly prepared. In addition, the 140-kDa polypeptide also could be immunoblotted by anti-PLC-I antibodies. Therefore, it is likely that PLC-M1 is derived from the 150-kDa enzyme, perhaps by a limited proteolysis. PLC-M2 was exclusively found in particulate fractions and appears to be more hydrophobic than PLC-M1: PLC-M2 eluted after the peak of PLC-M1 from the reverse-phase phenyl-5PW column, and the purified PLC-M2 readily oligomerized to form aggregates. Oligomerization is a common phenomenon seen with purified membrane-bound enzymes.

PLC enzymes act at a very early stage in response to the binding of a Ca^{2+}-mobilizing agonist to receptor, and the site of phosphoinositide breakdown is in the plasma membrane. Thus, it has been assumed that the PLC associated with membranes must be the enzyme responsible for the generation of the two second-messenger molecules. Indeed, various studies have been pursued using PLC enzyme activities associated with the membrane fractions obtained from mammalian brains (10, 20, 21), blow fly salivary gland (22), murine lymphocytes (26), rat liver (11, 24), and astrocytoma cell (12). However, all PLC enzymes purified have been from cytosol (27–32). In addition, several reports claimed that PLC enzyme activity detected in particulate fractions could be accounted for by the contaminating cytosolic enzyme (34, 35). This controversy probably arose because of the weak interaction of PLC-M1 (or PLC-M2) with plasma membranes as shown by the fact that these enzymes could easily be extracted by 2 M KCl solution. Our present data summarized in Table 1 clearly show that PLC-M detected in particulate fractions cannot be accounted for by trapped cytosolic enzymes: whereas the repeated sonication-washing using a salt-free buffer brought 92% of PLC-II to solution, only 46% of PLC-I-like enzymes were solubilized by the same procedure, and the remaining did not come into solution until the precipitate was treated with a strong hypertonic buffer. This assessment was made using a highly specific and reliable RIA, in which a pair of monoclonal antibodies recognizing two independent antigenic sites on the same polypeptide was used. Previous investigators relied on the assay of enzymic activity to estimate the subcellular distribution of PLC. Because PLC activity is critically affected by various lipids, detergents, and salts (36), the assay result could provide inaccurate information.

It remains unclear how PLC-I is associated with membranes. The enzyme can attach directly to lipid bilayer or through a membrane-binding protein, such as guanine nucleotide-binding protein (G protein). Recently, Majerus and coworkers (37) found that a partially purified PLC enzyme from cytosolic fractions of bovine brain could be activated by GTP, whereas homogeneous preparations of PLC were insensitive to the GTP stimulation. This result led them to suggest that a soluble G protein modulates PLC enzyme. All reported G proteins are oligomeric proteins with $\alpha\beta\gamma$-subunit structure (19). GTP promotes the dissociation of α subunit from $\beta\gamma$ complex. α subunit contains an active site for the GTPase, and the $\beta\gamma$ complex serves as anchor for the association of α subunits with membranes (38). In this connection, it can be speculated that under certain conditions, the $\beta\gamma$ complex is the binding site for the complex formed between PLC-I and α subunit of the putative G protein.

W.C.C. is the recipient of a John E. Fogarty International Fellowship.

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