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Bovine brain cytosol contains three immunologically distinct forms of inositolphospholipid-specific phospholipase C

(isozymes/signal transduction)

SUNG HO RYU, PANN-GHILL SUH, KEY SEUNG CHO*, KEE-YOUNG LEE, AND SUE GOO RHEE†

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **We previously reported that cytosolic fractions of bovine brain contain two immunologically distinct forms of phospholipase C (PLC), PLC-I and PLC-II. We now report the purification of another form of inositolphospholipid-specific phospholipase C from bovine brain cytosol, designated PLC-III, and the comparison of the catalytic properties of the three isozymes. Approximately 450 μg of pure PLC-III was obtained from 36 bovine brains, and it had a final specific activity of 30–40 μmol of phosphatidylinositol hydrolyzed per min per mg of enzyme in the presence of 0.1% deoxycholate. PLC-III exhibited an apparent M_r of 85,000 in NaDodSO$_4$/PAGE, which is considerably smaller than the M_r of 150,000 for PLC-I and 145,000 for PLC-II. Neither of the two mixtures of monoclonal antibodies nor the rabbit polyclonal antibodies directed against either PLC-I or PLC-II cross-reacted with PLC-III. The catalytic properties of the three isozymes were studied by using small unilamellar vesicles prepared from either phosphatidylinositol (PtdIns) or phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) as substrates. Hydrolysis of both PtdIns and PtdInsP_2 by the three enzymes was dependent on Ca^{2+}. However, at low Ca^{2+} concentration, PtdInsP_2 was the preferred substrate for all three enzymes. When PtdIns was the substrate, the three enzymes exhibited similar specific activities at their optimum pH, which was 4.8 for PLC-I, 5.0 for PLC-II, and 5.5 for PLC-III. But at neutral pH, the order of specific activity was PLC-III > PLC-II > PLC-I. In contrast, the order of specific activity was PLC-I > PLC-III > PLC-II for PtdInsP_2 hydrolysis, which means that PLC-I is the most specific for PtdInsP_2. The three enzymes were affected differently by bovine serum albumin: inhibition of PLC-I and activation of PLC-III were observed, whereas PLC-II was unaffected. This observation suggests that any putative protein effectors for PLC should be critically scrutinized.**

The hydrolysis of inositolphospholipids by phospholipase C is a major pathway of signal transduction during the response of many cells to stimulation by hormones, peptide growth factors, neurotransmitters, and other regulatory ligands (reviewed in refs. 1–4). The inositolphospholipid-specific phospholipase C (PLC) is present in most cell types and, as has been demonstrated by a number of workers, can be resolved into several active fractions in many crude tissue extracts (5, 6) and in partially purified preparations (7–9). Multiple activity peaks might be derived from proteolytic cleavage or multiple oligomeric states of a PLC enzyme (10). However, the presence of PLC isozymes was clearly demonstrated in sheep seminal vesicular glands and bovine brain.

Hofmann and Majerus (11) purified one enzyme of M_r 65,000 and partially purified another enzyme with a M_r of 85,000 from seminal vesicular glands; they showed that antibodies raised against the M_r 65,000 enzyme do not react with the other. Recently, two immunologically distinct PLC enzymes were purified to homogeneity in our laboratory (10). The M_r of the first enzyme, designated PLC-I, was 150,000 under denaturing conditions, and the purified PLC-I existed mainly in a dimeric form and in a tetrameric form to a smaller extent. The second enzyme, designated PLC-II, had a M_r of 145,000 and was present predominantly as a monomer and to a smaller extent as a dimer. Both polyclonal and monoclonal antibodies directed against either enzyme did not cross-react with the other (10, 12). It is clear from the molecular weights of the purified enzymes and from the immunoblot experiment that the two forms of PLC identified from bovine brain are different from the two forms from seminal vesicles. In addition, studies with antibodies indicate that PLC-I is highly localized in brain, whereas PLC-II is widely distributed in various tissues (P.-G.S., W. C. Choi, K.-Y.L., S.H.R., and S.G.R., unpublished results). Nevertheless, the reasons for the existence of multiple forms of PLC and for the tissue-specific distribution remain unclear.

When cytosolic fractions of bovine brain were eluted through an immunoaffinity gel to which antibodies specific to PLC-I and antibodies specific to PLC-II were attached, 20–30% of the PLC activity consistently was not bound. This led us to look for another form of PLC in the bovine brain cytosolic fraction. Here, we report the purification of a third form of PLC, which is immunologically and catalytically distinct from the other two. The catalytic properties of the three enzymes are compared in this report.

MATERIALS AND METHODS

Materials. Bovine serum albumin (A0281 or A7906) was purchased from Sigma, and the Mono S column was from Pharmacia. All other materials were as described (12).

Phospholipase C Assay. During the purification of PLC, the phosphatidylinositol (PtdIns) hydrolyzing activity was measured with Ptd[2-^{3}H]Ins in the presence of 0.1% deoxycholate as described (10). For the measurement of Ca^{2+} dependence, pH dependence, and the bovine serum albumin effect on the PLC activity, unilamellar vesicles of Ptd[2-^{3}H]Ins and phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) containing [2-^{3}H]inositol (Ptd[2-^{3}H]InsP_2) were separately prepared as described (13). All assays were run at 37°C and terminated as described (11).

Purification Procedure. *Step 1: Preparation of brain extracts.* Cytosolic fractions were prepared from 12 bovine

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Abbreviations: PtdIns, phosphatidylinositol; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; PLC, PtdIns-specific phospholipase C; BSA, bovine serum albumin.

*Present address: Department of Chemistry, Han Yang University, Seoul, Korea.

†To whom reprint requests should be addressed at: National Institutes of Health, 9000 Rockville Pike, Building 3, Room 203, Bethesda, MD 20892.

brains, and proteins were precipitated at pH 5.0 as described (12). The protein precipitates were dissolved in 1 liter of 20 mM Tris·HCl, pH 7.4/5 mM EGTA/2 mM phenylmethylsulfonyl fluoride/0.1 mM dithiothreitol/0.5 mM diisopropyl fluorophosphate. The turbid solution was centrifuged at 150,000 × *g* for 45 min.

Step 2: Ion-exchange chromatography on DE-52 DEAE-cellulose. The supernatant from step 1 was applied on a DE-52 DEAE-cellulose column (8 × 40 cm), which had been equilibrated with 20 mM Tris·HCl, pH 7.6/1 mM EGTA/0.1 mM dithiothreitol. The column was eluted with a 6-liter linear KCl gradient from 0 to 225 mM KCl in 50 mM Tris·HCl, pH 7.6/1 mM EGTA/0.1 mM dithiothreitol. Three PLC activity peaks were eluted. The peak fractions were pooled separately as indicated in Fig. 1. The first peak, which contained PLC-III, was further purified immediately in the next step, whereas the second and third peaks were stored frozen for purification of PLC-I and PLC-II, respectively, by the method described earlier (12).

Step 3: Heparin-agarose chromatography. The PLC-III fractions pooled from the previous step (750 ml) were directly applied to a heparin-agarose column (5 × 15 cm) equilibrated with 20 mM Hepes, pH 7.0/0.1 mM dithiothreitol/1 mM EGTA. The column was eluted with a 1.8-liter linear gradient of NaCl from 100 to 700 mM NaCl in equilibration buffer. The peak fractions (240 ml) were pooled as indicated in Fig. 2*A*, concentrated to ≈10 ml in an Amicon filtration apparatus, and stored frozen to be combined with concentrated fractions of PLC-III from two other identical preparations.

Step 4: Reverse-phase chromatography on TSK phenyl-5-PW. Solid KCl was added to the concentrated fractions (35 ml) from step 3 to give a final concentration of 3 M, and the mixtures were centrifuged to remove denatured proteins. The supernatants were applied at a flow rate of 5.0 ml/min to a HPLC preparative TSK phenyl-5-PW column (21.5 × 150 mm; Bio-Rad) equilibrated with 20 mM Hepes, pH 7.0/3 M KCl/1 mM EGTA/0.1 mM dithiothreitol. Elution was continued at 5.0 ml/min with a decreasing KCl gradient from 3 M to 1.2 M KCl for 10 min and with a decreasing KCl gradient from 1.2 M to 0 M KCl for 20 min. Fractions (25 ml) containing PLC activity were pooled and washed in an Amicon filtration apparatus with 20 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 5.7/0.1 mM dithiothreitol/1 mM EGTA and finally concentrated to ≈10 ml.

Step 5: Ion-exchange chromatography on a Mono S column. The washed protein solution (≈10 ml) from step 4 was applied at a flow rate of 1.0 ml/min to a Mono S column (7 × 6 mm) equilibrated with 20 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.7/0.1 mM dithiothreitol/1 mM EGTA. Elution was continued at 1.0 ml/min with an NaCl gradient from 0 to 300 mM NaCl for 20 min and from 300 mM to 1 M for 10 min. Peak fractions (1.2 ml) were collected manually, diluted with 2 ml of 20 mM Hepes (pH 7.0), concentrated in a Centricon microconcentrator (Amicon) to ≈0.5 ml, separated into aliquots, and stored at −20°C.

Enzyme Concentration. The concentrations of PLC-I, -II, and -III were estimated using an average protein absorptivity of $A_{280}^{0.1\%} = 1.14$.

Other Methods. PAGE and immunoblotting with rabbit antiserum or murine monoclonal antibodies were as described (12). The preparation and properties of the monoclonal antibodies will be described elsewhere.

RESULTS

Purification of PLC-III. Chromatography of the 150,000 × *g* supernatants of bovine brain cytosolic fractions on a DEAE-cellulose column yielded three PLC activity peaks, PLC-III, PLC-I, and PLC-II, which eluted sequentially as shown in Fig. 1. In the previous paper reporting the purification of PLC-I and PLC-II (12), cytosolic fractions were centrifuged at 13,000 × *g*. The resulting turbid supernatants were adsorbed on a thin bed of DEAE-cellulose on a sintered glass funnel, and the bed was washed with a low ionic strength buffer to remove turbid lipid material before the washed DEAE-cellulose was poured onto a column already containing a layer of DEAE-cellulose. The column was then eluted with a KCl gradient. This procedure yielded only two activity peaks: the first peak was PLC-I and the second was PLC-II. This was probably because PLC-III was washed off during the batch-type DEAE chromatography procedure as evidenced by a low level of PLC activity detected in the unbound flow-through fractions. In the current procedure, after ultracentrifugation the supernatants were directly applied to a DEAE column equilibrated with a low ionic strength buffer. Consequently, PLC-III was retained and was eluted as shown in Fig. 1.

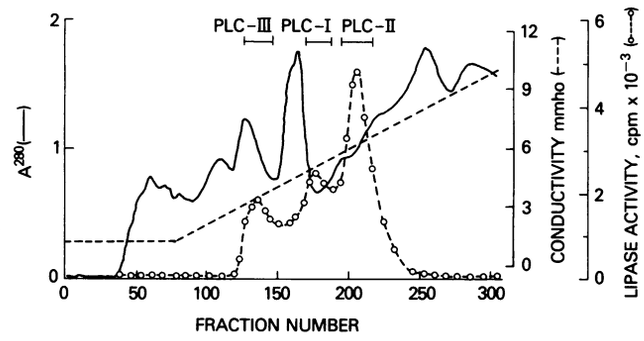

FIG. 1. DEAE-cellulose chromatography. Cytosolic fractions of 12 bovine brains were applied and eluted, and fractions containing PLC-I, -II, and -III were pooled separately as indicated by the bars.

Next, three purification steps—chromatography on heparin-agarose gel (Fig. 2*A*), an HPLC phenyl column (Fig. 2*B*), and cation-exchange resin Mono S (Fig. 2*C*)—allowed us to obtain 450 μg of homogeneous PLC-III from 36 bovine brains. The purified PLC-III had a final specific activity of 30–40 μmol of PtdIns cleaved per min per mg of enzyme in the presence of 0.1% deoxycholate. A summary of the purification is presented in Table 1. In addition, we also purified PLC-I and PLC-II from the corresponding peak fractions of the DEAE column.

Polyacrylamide Gel Analysis and Immunoblot. Fig. 3 shows the results of the analysis of the three purified PLC enzymes on a $NaDodSO_4$/polyacrylamide gradient gel and the immunoblots of the same gel. The M_r of the newly purified PLC-III is 85,000 (Fig. 3*A*, lane 3), as compared to 150,000 for PLC-I (Fig. 3*A*, lane 1) and 145,000 for PLC-II (Fig. 3*A*, lane 2). Immunoblots were carried out using a mixture of three murine monoclonal antibodies that recognize different epitopes of PLC-I (Fig. 3*B*). Similarly, a mixture of six different monoclonal anti-PLC-II antibodies were employed in Fig. 3*C*. As already reported, PLC-II was not recognized by anti-PLC-I antibodies, and PLC-I was not recognized by anti-PLC-II antibodies (12). Furthermore, PLC-III could not be detected by either of the two hybridoma proteins, indicating that PLC-III is another immunologically independent enzyme. Rabbit antibodies raised against either PLC-I or PLC-II also did not react with PLC-III (data not shown). The results of analysis of the three purified enzymes on nondenaturing polyacrylamide gradient gels are shown in Fig. 4. As reported previously (10), undamaged PLC-I exists mainly in a dimeric form and in a tetrameric form to a smaller extent, whereas PLC-II is predominantly monomeric and, to a smaller extent, dimeric. Two broad bands were observed for PLC-III. The estimated M_r values are 150,000–170,000 for the intense band and ≈250,000 for the faint band in lane 3 of

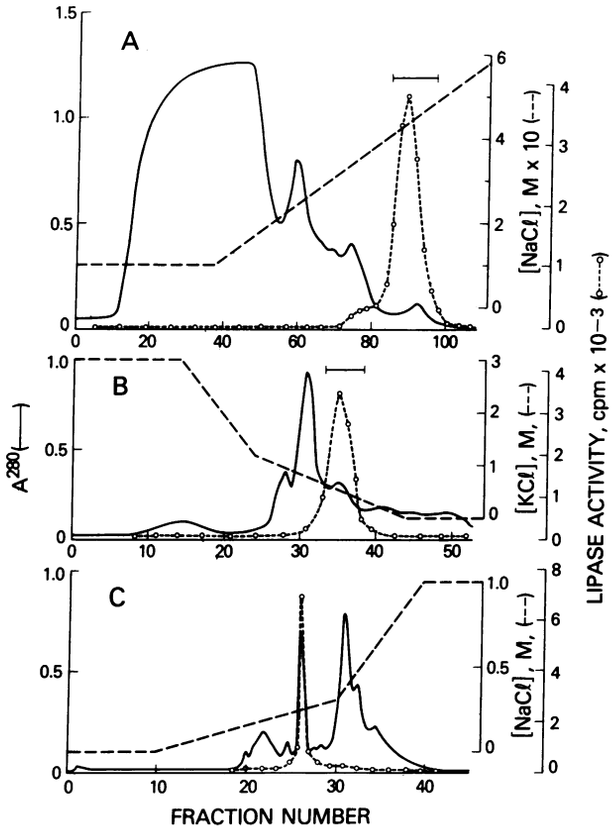

FIG. 2. Purification of PLC-III on heparin-agarose gel (*A*), TSK phenyl-5-PW HPLC column (*B*), and Mono S column (*C*).

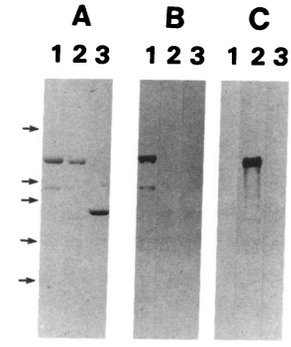

FIG. 3. $NaDodSO_4$/PAGE and immunoblot analysis. PLC-I, -II, and -III were subjected to $NaDodSO_4$/polyacrylamide gradient (6–16%) gels and either stained with Coomassie blue (*A*), immunoblotted with monoclonal anti-PLC-I antibodies (*B*), or immunoblotted with monoclonal anti-PLC-II antibodies (*C*). Lanes: 1, PLC-I; 2, PLC-II; 3, PLC-III. Arrows indicate M_r standards from top to bottom: myosin (200,000), β-galactosidase (116,000), phosphorylase b (94,000), bovine serum albumin (BSA; 67,000), and ovalbumin (45,000).

Fig. 4. It seems, therefore, that PLC-III exists mainly in a dimeric form.

pH Dependence. The effect of pH on the Ptd[^{3}H]Ins hydrolyzing activity measured by using unilamellar vesicles of pure PtdIns in the presence of 1 mM free Ca^{2+} is shown in Fig. 5. All three enzymes exhibited pH optima at an acidic pH, ≈4.8 for PLC-I, 5.0 for PLC-II, and 5.5 for PLC-III. As the pH was increased, PLC-I activity decreased rapidly, and PLC-II activity decreased less rapidly, whereas PLC-III activity decreased by only 30% and remained constant between pH 7.0 and 8.5. The specific activities of the three enzymes were similar at their optimal pH. However, the order of specific activity was PLC-III > PLC-II > PLC-I at neutral pH.

Ca^{2+} Dependence. The Ca^{2+} dependence of hydrolysis of Ptd[^{3}H]Ins and Ptd[^{3}H]InsP_2 by the three enzymes is shown in Fig. 6. With unilamellar vesicles of PtdIns as substrate, the hydrolysis rates of PLC-II and PLC-III increased with increasing Ca^{2+} concentration up to 10 mM, whereas the activity of PLC-I increased slowly until it reached a maximum at 6 mM Ca^{2+} and then decreased. Although the PtdIns hydrolyzing specific activities of the three enzymes were similarly low below 10 μM Ca^{2+}, the difference became significant at higher concentrations of Ca^{2+}. For example, the specific activities observed with PLC-I, -II, and -III were 2, 8, and 24 μmol·min^{-1}·mg^{-1}, respectively, at 10 mM Ca^{2+}. With PtdInsP_2 vesicles as substrate, the rates for the three enzymes increased with increasing Ca^{2+} concentration until they reached maxima at 10–100 μM. Ca^{2+} at concentrations higher than 100 μM inhibited the activities, perhaps owing to precipitation of the substrate. At all Ca^{2+} concentrations, the specific activities were in the order PLC-I > PLC-III > PLC-II. At 0.1–1 μM Ca^{2+}, the PtdInsP_2 hydrolyzing activities of PLC-I and PLC-III were much higher than their PtdIns hydrolyzing activities. For example, at 1 μM Ca^{2+}, the PtdInsP_2 hydrolysis rates were 5.0 and 2.8 μmol·min^{-1}·mg^{-1} for PLC-I and PLC-III, respectively, while the PtdIns hydrolysis rate was 0.3 μmol·min^{-1}·mg^{-1} for both enzymes. The rate of PtdInsP_2 hydrolysis by PLC-II was 0.35 μmol·min^{-1}·mg^{-1} at 0.1 μM Ca^{2+}, which is ≈3 times the PtdIns hydrolysis rate at the same Ca^{2+} concentration; but at 1 μM, the rates for both inositolphospholipids were nearly the same.

Table 1. Purification of PLC-III from 36 bovine brains

Purification step	PLC	Total protein, mg	Total activity, μmol·min^{-1}	Specific activity, μmol·min^{-1}·mg^{-1}	Fold purification	Yield, %
Homogenate	I	81,000	533	0.007	1	
	II		1206	0.015	1	
	III		402	0.005	1	100
Precipitate at pH 5.0	I	45,000	420	0.009	1.4	
	II		967	0.021	1.4	
	III		323	0.007	1.4	80
DEAE-cellulose	I	1,600	203	0.13	19	
	II	1,850	549	0.30	20	
	III	1,900	186	0.10	20	46
Heparin-agarose	III	57.3	80.2	1.4	280	19.8
Phenyl-5-PW	III	6.8	43.0	6.3	1260	10.6
Mono S	III	0.45	15.3	34.0	6800	3.8

The PtdIns hydrolyzing activities of PLC-I, -II, and -III were separated by DEAE chromatography, and the relative contribution of each enzyme to the total amount of activity in the homogenate and in the pH 5.0 precipitate was estimated from the area of each activity in Fig. 1.

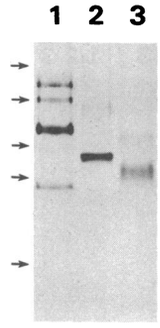

FIG. 4. Polyacrylamide gradient (4–16%) gel analysis. Lane 1, PLC-I. The tetramer of M_r 150,000 PLC-I, the tetramer of M_r 100,000 fragment, the dimer of M_r 150,000 PLC-I, and the monomer of M_r 100,000 fragment are visible from top to bottom. The PLC-I preparation contained a M_r 100,000 fragment derived from the M_r 150,000 enzyme as shown in Fig. 3 and in ref. 10. Lane 2, PLC-II. Monomeric (intense band) and dimeric (faint band) forms are visible. Lane 3, PLC-III. A broad band with M_r of 150,000–170,000 and a faint band with M_r of ≈250,000 are observed. The faint band is also likely due to an oligomer of PLC-III because the PLC-III preparation exhibited no visible impurity bands when analyzed by NaDodSO$_4$/PAGE. Arrows indicate M_r standards from top to bottom: thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and BSA (67,000). Native polyacrylamide gel analysis of PLC-I and PLC-II has been described previously (10).

Effect of BSA on PLC Activities. The Ca^{2+}-dependent activity profiles shown in Fig. 7 for the PtdIns hydrolysis by PLC-I and PLC-II are similar to those reported previously (12). However, the profiles for PtdInsP_2 hydrolysis were significantly different from the previous profiles in which PLC-I activities were much lower than those shown in Fig. 6. We found that this discrepancy resulted because BSA had been included at 0.15 mg/ml in the assay mixture, whereas it was absent in the current experiment. In the previous experiments, the enzymes had been diluted in a BSA solution. Therefore, the effect of BSA on the PLC activities was studied.

As shown in Fig. 7, the most prominent changes in response to BSA were observed in the PtdInsP_2 hydrolysis by PLC-I. Fifty percent inhibition occurred at 50 μg/ml (IC_{50} = 50 μg/ml). The PtdIns hydrolysis by PLC-I was also inhibited but at higher BSA concentration (IC_{50} ≈ 450 μg/ml). Both the PtdIns and PtdInsP_2 hydrolyzing activities of PLC-II were not significantly affected by BSA, whereas those of PLC-III exhibited biphasic responses: the activities were enhanced by BSA at lower concentration and then decreased slowly with further additions. These BSA effects were probably not due to small size contaminants such as metal ions and lipid molecules in BSA because similar results were obtained either with BSA (Sigma A7906) dialyzed against a buffer containing EGTA or with fatty acid free BSA (Sigma A0281).

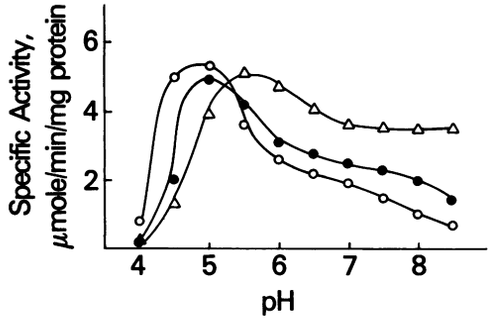

FIG. 5. Effect of pH on PLC activities. The PtdIns hydrolysis reactions were initiated by adding 0.075 μg of PLC-I (○), PLC-II (●), or PLC-III (△) to the reaction mixture containing 113 μM Ptd[^{3}H]Ins (30,000 cpm; 1 Ci = 37 GBq), 50 mM NaCl, 2 μM dithiothreitol, 2 mM EGTA, and 3 mM $CaCl_2$ in a total volume of 200 μl. The pH was varied by the addition of 50 mM (final concentration) Tris·HCl/maleate/NaOH buffers.

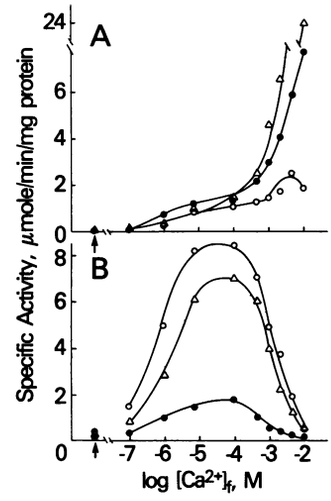

FIG. 6. Effect of Ca^{2+} on PLC activities. (*A*) PtdIns hydrolysis. The reactions were initiated by adding 0.05 μg of PLC-I (○), PLC-II (●), or PLC-III (△) to the reaction mixtures containing 113 μM Ptd[^{3}H]Ins (30,000 cpm), various amounts of $CaCl_2$, 2 mM EGTA, 100 mM NaCl, 3 mM $MgCl_2$, 2 μM dithiothreitol, 50 mM Hepes (pH 7.0) in a total volume of 200 μl. (*B*) PtdInsP_2 hydrolysis. The reaction condition was the same as in *A*, except that 113 μM Ptd[^{3}H]InsP_2 (30,000 cpm) was the substrate. The symbols for PLC-I, -II, and -III were also the same as in *A*. Free Ca^{2+} concentrations were calculated as described (12). Arrows (↑) indicate where no $CaCl_2$ was added.

DISCUSSION

Our current results clearly demonstrate that bovine brain contains at least three immunologically distinct PLC enzymes. The use of purified enzymes allowed us to compare their catalytic properties directly under identical experimental conditions. Similarly to PLC-I and PLC-II, PLC-III was specific to inositolphospholipids and did not utilize phosphatidylcholine and phosphatidylethanolamine as substrate

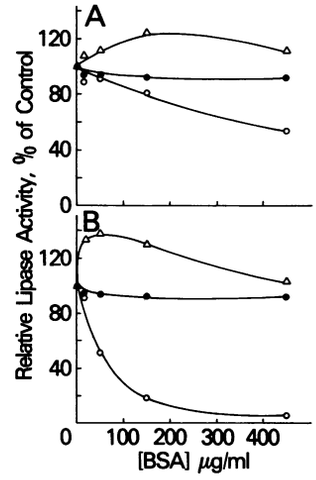

FIG. 7. Effect of BSA on PLC activities. Increasing amounts of BSA were added to the reaction mixture containing a fixed concentration of free Ca^{2+}, 1 mM for PtdIns hydrolysis (*A*) and 0.1 mM for PtdInsP_2 hydrolysis (*B*). Other reaction conditions were as in Fig. 5. The reactions were initiated by adding 0.075 μg of PLC-I (○), PLC-II (●), or PLC-III (△).

(data not shown). When vesicles derived from pure PtdIns were used as substrate, the three enzymes exhibited similar specific activities at their optimum pHs, which are all acidic but differ from one another. However, at neutral pH, PtdIns hydrolyzing activities were significantly different: PLC-III was the most active and PLC-I was the least active. This order of activity, PLC-III > PLC-II > PLC-I, could be observed at 0.5–10 mM Ca^{2+}. Their PtdIns hydrolyzing activities were low below 0.5 mM Ca^{2+} and nearly zero at 0.1 μM Ca^{2+}. The catalytic properties toward PtdInsP_2 were very different from those toward PtdIns. All three enzymes exhibited significantly higher specific activities toward PtdInsP_2 at 0.1–10 μM Ca^{2+}. Furthermore, the order of activity was different. At all Ca^{2+} concentrations, PLC-I and PLC-III were more active than PLC-II. Consequently, the ratios of PtdIns hydrolysis to PtdInsP_2 hydrolysis catalyzed by each enzyme were very different. PLC-I, which had been shown to exist in plasma membrane as well as in cytosol (14), showed the highest specificity for PtdInsP_2.

Our current studies were made with vesicles derived from pure PtdIns or pure PtdInsP_2 instead of employing a lipid mixture approximating the composition of brain plasma membranes. Nevertheless, the clearly different catalytic properties of the three enzymes described in this report encourage us to speculate that the three enzymes with isofunctions have different roles *in vivo* and are probably regulated differently. Additionally, the finding that PtdInsP_2 hydrolysis is favored over PtdIns hydrolysis at low Ca^{2+} concentrations is in good accord with the concept that inositol trisphosphate triggers a rise in intracellular Ca^{2+} (1, 2, 15–17). According to this hypothesis, occupancy of specific receptor by a Ca^{2+}-mobilizing agonist leads to PtdInsP_2 hydrolysis, which in turn raises cytoplasmic Ca^{2+}, thereby initiating PtdIns breakdown.

It is interesting to note that the PLC activities are affected differently by BSA: PLC-I was inhibited whereas PLC-III was activated by BSA at low concentrations, and PLC-II was not significantly affected. It had been well demonstrated that the presence of lipids such as phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and diacylglycerol in the substrate vesicles exerts a strong influence on PLC activities by changing the physical properties of the substrate (13, 18). Unlike this lipid effect, however, the BSA effect must be on the enzyme rather than on the substrate, because the three enzymes were affected differently. Purified PLC enzymes exist in different oligomeric states. It is therefore conceivable that the nonspecific interaction with BSA changed the equilibrium between different oligomeric states, thereby changing their specific activities. The BSA effect was also noticed during the study with H-*ras* p21 protein (H. K. Chung, J. C. Lacal, and S.G.R., unpublished results). The p21 protein had been suggested as a GTP-binding protein, which couples the receptor function to the stimulation of PLC (19, 20). Because the hydrophobic p21 protein was solubilized by the use of excess BSA, addition of p21 caused apparent inhibition of PLC-I, activation of PLC-III, and no change in PLC-II activity independently of guanosine-5′-*O*-(3-thiotriphosphate). In any event, these results suggest that any changes in PLC activity resulting from the addition of any putative effector protein should be scrutinized critically.

It is an intriguing question to ask now how many different phosphatidylinositol-specific phospholipases does mammalian tissue contain and how specifically these enzymes are distributed in tissues. Radioimmunoassays of 12 different rat tissue homogenates with anti-PLC-I antibodies and anti-PLC-II antibodies revealed that PLC-II is distributed in most tissues including liver and seminal vesicles, whereas the PLC-I-like enzyme could not be found in significant amounts in any tissues we investigated (P.-G.S., W. C. Choi, K.-Y.L., S.H.R., and S.G.R., unpublished results). The molecular weight of the protein recognized by anti-PLC-II antibodies in various tissues was identical to that of brain PLC-II, indicating that smaller size PLC enzymes reported in the literature are not the proteolytic fragments of PLC-II. The M_r 65,000 enzyme purified by Hofmann and Majerus (11) may be the same as the enzymes purified from rat liver (M_r = 68,000) by Takenawa and Nagai (21) and partially purified from human platelet (M_r = 67,000) by Banno *et al.* (9). Hofmann and Majerus (11) also identified a M_r 85,000 enzyme as an immunologically distinct entity in partially purified seminal vesicle proteins. This M_r 85,000 enzyme could be the same as the PLC-III we are reporting. Preparation of anti-PLC-III antibodies will aid in further clarification of this matter. Two more distinct PLC enzymes were identified in platelets which have M_r values of 140,000 and 95,000 (8, 22). Although the M_r 140,000 platelet enzyme is similar in size to the brain enzymes PLC-I and PLC-II, none of the antibodies for the brain enzymes recognized the platelet enzyme in immunoblot experiments (10).

Therefore, we can count at least five distinct PLC enzymes in mammalian tissues: (*i*) M_r 150,000 PLC-I, which seems to be specifically localized in brain cells, (*ii*) M_r 145,000 PLC-II, which is widely distributed in various tissues, (*iii*) M_r 85,000 PLC-III, which might be the same as the enzyme identified in seminal vesicles, (*iv*) M_r 65,000 enzyme purified from seminal vesicles, liver, and platelets, and (*v*) M_r 140,000 platelet enzyme, which could not be recognized by either anti-PLC-I antibodies or anti-PLC-II antibodies.

1. Berridge, M. J. (1984) *Nature (London)* **312,** 315–321.
2. Litosch, I. & Fain, J. N. (1986) *Life Sci.* **39,** 187–194.
3. Nishizuka, Y. (1986) *Science* **233,** 305–312.
4. Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. & Wilson, D. B. (1986) *Science* **234,** 1519–1526.
5. Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1982) *Biochem. Biophys. Res. Commun.* **107,** 533–537.
6. Low, M. G., Carroll, R. C. & Weglicki, W. B. (1984) *Biochem. J.* **221,** 813–820.
7. Nakanishi, H., Nomura, H., Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1985) *Biochem. Biophys. Res. Commun.* **132,** 582–590.
8. Low, M. G., Carroll, R. C. & Cox, A. C. (1986) *Biochem. J.* **237,** 139–145.
9. Banno, Y., Nakashima, S. & Nozawa, Y. (1986) *Biochem. Biophys. Res. Commun.* **136,** 713–721.
10. Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G. & Rhee, S. G. (1986) *Biochem. Biophys. Res. Commun.* **141,** 137–144.
11. Hofmann, S. L. & Majerus, P. W. (1982) *J. Biol. Chem.* **257,** 6461–6469.
12. Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G. & Rhee, S. G. (1987) *J. Biol. Chem.*, in press.
13. Hofmann, S. L. & Majerus, P. W. (1982) *J. Biol. Chem.* **257,** 14359–14364.
14. Lee, K. Y., Ryu, S. H., Suh, P. G., Choi, W. C. & Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5540–5544.
15. Burgess, G. M., Irvine, R. F., Berridge, M. J., McKinney, J. S. & Putney, J. W., Jr. (1984) *Biochem. J.* **224,** 741–746.
16. Chueh, S. H. & Gill, D. L. (1986) *J. Biol. Chem.* **261,** 13883–13886.
17. Joseph, S. K. & Williamson, J. R. (1986) *J. Biol. Chem.* **261,** 14658–14664.
18. Irvine, R. F., Letcher, A. J. & Dawson, R. M. C. (1984) *Biochem. J.* **218,** 177–185.
19. Wakelam, M. J. O., Davies, S. A., Houslay, M. D., McKay, I., Marshall, C. J. & Hall, A. (1986) *Nature (London)* **323,** 173–176.
20. Chiarugi, V., Parciatti, F., Pasquali, F. & Bruni, P. (1985) *Biochem. Biophys. Res. Commun.* **132,** 900–907.
21. Takenawa, T. & Nagai, Y. (1981) *J. Biol. Chem.* **256,** 6769–6775.
22. Hakata, H., Kambayashi, J. & Kosaki, G. (1982) *J. Biochem. (Tokyo)* **92,** 929–935.