Purification and Characterization of Two Immunologically Distinct Phosphoinositide-specific Phospholipase C from Bovine Brain*

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We previously reported (Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G., and Rhee, S. G. (1986) Biochem. Biophys. Res. Commun. 141, 137–144) that cytosolic fractions of bovine brain contain two phosphoinositide-specific phospholipase C (PLC), PLC-I and PLC-II. In this paper, purification procedures and properties of these two forms of enzyme are presented. The two enzymes exhibit similar substrate specificity. Both PLC-I and PLC-II catalyze the hydrolysis of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP2). Yet, they respond differently to activators such as Ca2+ and nucleotides and to inhibitory divalent metal ions such as Hg2+ and Cd2+. In addition, they are immunologically distinct as evidenced by the fact that monoclonal antibodies directed against either enzyme do not cross-react with the other. Their activities are Ca2+ concentration-dependent. PIP and PIP2 are better substrates than PI for both PLC-I and PLC-II when the concentration of Ca2+ is in the micromolar range.

Study of the effect of nucleotides, such as GTP, guanosine 5′-[(3-O-thio)triphosphosphate, guanylyl-5′-yl imidodiphosphate, and ATP, on the activities of both isoforms with PIP2 as substrate revealed that (i) in the absence of Ca2+, PLC-I activity is enhanced by 400% by either GTP or ATP. In the presence of Ca2+ (a condition in which PLC-I exhibits much higher activity), the activation factor by nucleotides is diminished to ~140%. (ii) without Ca2+, PLC-II activity is too low to measure with or without added nucleotides. The effect of nucleotides on PLC-II activity is trivial in the presence of Ca2+. In addition, studies on the effect of metal ions on PI hydrolysis showed that the activities of both PLC-I and PLC-II are not affected by 50 μM of Mg2+, Mn2+, Ca2+, or Ni2+. However, Hg2+, Zn2+, and Cu2+ inhibited both PLC-I and PLC-II, with PLC-II exhibiting much higher sensitivity to these metal ions than PLC-I. For example, the value of I50 for Hg2+ inhibition is 0.2 μM for PLC-II and 1 μM for PLC-I. Cd2+ selectively inhibits PLC-II with a 50% value of 5 μM. Most of these metal ions’ inhibition can be overcome by either dithiothreitol or EDTA.

It has been well documented that hydrolysis of phosphoinositides is accelerated as a result of binding of agonists to receptors and generates two second messenger molecules, diacylglycerol and inositol 1,4,5-triphosphate (reviewed in Refs. 1–3). The hydrolysis proceeds through activation of phosphoinositide-specific phospholipase C (PLC).1 The activation process is believed to be mediated by unspecified guanine nucleotide-binding proteins (4–17). PLC activity has been demonstrated in various mammalian tissues (18–21) and has been shown by a number of workers can be resolved into several peaks in many tissue extracts (22–25). The best-characterized enzymes are from sheep seminal vesicular glands which contain two immunologically distinct enzymes (26, 27). One of these enzymes has been purified to homogeneity. The molecular weight was 70,000 when measured by gel filtration and 65,000 by SDS-PAGE. The other form was partially purified and its apparent Mw was estimated by gel filtration to be 85,000. The molecular weights of the seminal vesicle enzymes are significantly different from the bovine platelet enzyme (143,000 by SDS-PAGE), another PLC enzyme purified to homogeneity (28), alluding to the fact that different tissues might contain different forms of phospholipase C. In this paper, we described in detail procedures used to purify two immunologically distinct PLC enzymes from bovine brain and their molecular and catalytic properties. Although the two enzymes are hardly distinguishable on the basis of substrate specificity and Ca2+ dependence, they differ with regard to stimulation by nucleotides and inhibition by several heavy metals.

EXPERIMENTAL PROCEDURES

Materials

Horseradish peroxidase-conjugated goat anti-mouse IgG antibody and goat anti-mouse IgG-agarose were obtained from HyClone Laboratory. Other materials were as described previously (29).

Phospholipase C Assay

For the measurement of specific activity during purification of PLC-I and PLC-II, substrate was prepared in the presence of deoxycholate. Assays were performed in a 200-μl reaction mixture containing 20,000 cpm of [3H]PI, 300 μM soybean PI, 0.1% sodium deoxycholate, 3 mM CaCl2, 1 mM EGTA, 50 mM Hepes, pH 7.0, and a source of enzyme. For the measurement of Ca2+-dependent activities, unilamellar vesicles of [3H]PI, [3H]PIP, and [3H]PIP2 were prepared as described in Ref. 30, and the assay procedure is described in the figure legend. All assays were run at 27 °C and terminated as described previously.

1 The abbreviations used are: PLC, PI-specific phospholipase C; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP2, phosphatidylinositol-4,5-bisphosphate; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Triton X-100, nonionic detergent; DTT, dithiothreitol; GTPγS, guanosine 5′-[(3-O-thio)triphosphate; GppNp, guanylyl-5′-yl imidodiphosphate; PBS, phosphate-buffered saline containing 10 mM phosphate, pH 7.2, 150 mM NaCl; HPLC, high pressure liquid chromatography.
in Ref. 26. Under these assay conditions, the PLC activities were linear with respect to time and protein concentration.

**Purification Procedure**

**Step 1. Preparation of Brain Extracts—**Sixteen bovine brains were freshly obtained from a local slaughter house and the cerebella (4.5 kg) were homogenized in a Waring blender with 9.5 liters of buffer containing 10 mM Tris-HCl, pH 7.2, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT, and 0.6 mM diisopropyl fluorophosphate. The homogenate was centrifuged for 30 min at 13,000 × g. The supernatant was adjusted to pH 5.0 with 1 M acetic acid. After 30 min at 4 °C, precipitates were collected by centrifugation and dissolved in 3 liters of buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT, and 0.5 mM diisopropyl fluorophosphate. The insoluble materials were pelleted by centrifuging for 30 min at 13,000 × g, and the turbid supernatant was removed for chromatography as described.

**Step 2. Ion Exchange Chromatography on DEAE-Cellulose—**The supernatant from Step 1 was chromatographed in two stages, a batch procedure followed by a column step. For the batch procedure, 2.5 liters of DEAE (equilibrated with 20 mM Tris-HCl buffer at pH 7.6 containing 5 mM EGTA and 0.1 mM DTT) was added into the supernatant from Step 1, equilibrated for 15 min with stirring, and then the DEAE was collected in a 4-liter sintered glass (coarse) filter funnel, mixed with the equilibration buffer, and poured onto a column containing 10 mM Tris-HCl, pH 7.2, 1 mM EGTA, and 0.1 mM DTT. The proteins were eluted at a flow rate of 1.0 ml/min by successive application of KCl gradients from 0 to 0.3 M for 20 min and from 0.3 to 1.0 M for 10 min. A major protein peak coinciding with PLC activity emerged at 20 min (Fig. 5). Peak fractions (3 ml) were pooled, washed with Tris-HCl buffer, pH 7.6, concentrated to ~1 ml, separated into aliquots, and stored at ~20 °C.

**Step 3. Affinity Chromatography on Matrix Green Gel—**A total of 600 ml of PLC-I solution from Step 2 (the combined sample from three DEAE preparations from a total of 48 brains) was applied to a Matrix Green gel column (4 × 20 cm) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 1 mM EGTA. A 600-ml KCl gradient (0.1–1 M) was used to elute PLC-I. The PLC-I solution obtained from a single DEAE preparation (18 brains) was applied to a smaller Matrix Green gel column (2 × 17 cm) equilibrated with the same buffer. The PLC-I containing fractions from each run (120 ml for PLC-I and 88 ml for PLC-II) were pooled and concentrated 3-fold. The PLC-I solution was carried directly to Step 4 and the concentrated PLC-I solution was kept frozen to be combined with concentrated fractions from two additional identical preparations of PLC-I.

**Step 4. Heparin-Agarose Chromatography—**Fractions from Step 3 were diluted with 20 mM Hepes buffer, pH 7.0, containing no NaCl, to a conductivity equivalent to that of 100 mM NaCl buffer and applied separately to heparin-agarose columns (3 × 17 cm for PLC-I and 5 × 17 cm for PLC-II) equilibrated with 20 mM Hepes, pH 7.0, containing 50 mM NaCl, 1 mM EGTA, and 0.1 mM DTT. The columns were eluted with a linear gradient from 0 to 500 mM NaCl present in the equilibration buffer (400 ml for PLC-I and 1 liter for PLC-II). The peak of PLC-I was eluted at 0.42 M NaCl (Fig. 3A) and PLC-II at 0.5 M NaCl (Fig. 3B). Peak fractions (125 ml for PLC-I and 154 ml for PLC-II) were pooled and concentrated 5-fold.

**Step 5. Ion Exchange Chromatography on TSK Phenyl-SPW—**Solid KCl was added to the concentrated fractions from Step 4 to give a concentration of 5 M, and the mixtures were centrifuged to remove the precipitate formed. The supernatants were applied at a flow rate of 1 ml/min to HPLC phenyl column (4.6 × 75 mm) equilibrated with 50 mM Tris-HCl, pH 7.0, 3 mM KCl, 1 mM EGTA, and 0.1 mM DTT. The proteins were eluted at a flow rate of 1 ml/min, by successive application of (i) the equilibration buffer for 5 min, (ii) a decreasing KCl gradient from 3 to 1.2 M for 10 min, and (iii) a decreasing KCl gradient from 1.2 to 0 M for 20 min. Then the column was washed with a 50 mM Tris-HCl buffer for 30 min. Two milliliters of 1 M KCl wereeluted in a peak centered at 23 min (Fig. 4A) and PLC-II in a peak centered at 30 min (Fig. 4B). Nearly homogeneous PLC-I enzyme emerged from the column. Three milliliters of PLC-I fractions were pooled, washed in a centricron microconcentrator (Amicon) with a 100-kD filter (Tris-HCl, pH 7.6, 1 mM EGTA, and 0.1 mM DTT) and concentrated to ~1 ml, separated into aliquots, and stored at ~20 °C. Peak fractions of PLC-II were pooled, washed with the same buffer used (5 ml) for PLC-I, and further purified.

**Step 6. Ion Exchange Chromatography of PLC-II on MonoQ Column—**The washed PLC-II (5 ml) were applied at a flow rate of 1 ml/min to a MonoQ column (7 × 60 mm) equilibrated with 20 mM Tris-HCl, pH 7.6, 1 mM EGTA, and 0.1 mM DTT. The proteins were eluted at a flow rate of 1.0 ml/min by successive application of KCl gradients from 0 to 0.3 M for 20 min and from 0.3 to 1.0 M for 10 min. A major protein peak coinciding with PLC activity emerged at 20 min (Fig. 5). Peak fractions (3 ml) were pooled, washed with Tris-HCl buffer, pH 7.6, concentrated to ~1 ml, separated into aliquots, and stored at ~20 °C.

**Preparation and Characterization of Anti-PLC-I and Anti-PLC-II Antibodies—**Hybridomas monoclones producing anti-PLC-I antibodies and anti-PLC-II antibodies were obtained by the fusion of Sp2/0-Ag14 mouse myeloma cells with spleen cells derived from BALB/c mice immunized with purified PLC-I or PLC-II. Standard fusion, screening, and cloning procedures were followed. To ensure monoclonality, the cloning procedure was repeated. In this way, four hybridomas monoclones for PLC-I and PLC-II were established. The hybridoma cells were grown as ascites tumors in BALB/c mice primed with pristane. Monoclonal antibodies, precipitated from the ascites fluids with 45% ammonium sulfate and subsequently dialyzed against PBS, were used in the present study. Subclass typing was done by immunodiffusion analysis with rabbit antisera (MES) specific for mouse γ, α1, γ2A, γ2B, and γ3 immunoglobulin chains.

The four monoclonal anti-PLC-I antibodies were divided into three groups based on whether or not they bind to the same epitope on PLC-I molecule. This grouping was achieved by the competition experiment described below. In this competition experiment, four monoclonal antibodies were labeled separately with 125I in the presence of lodo-Gen (Pierce Chemical Co.) according to the instructions of the manufacturer. A 96-well microtiter plate was coated with an antibody (primary coating antibody) by incubating for 2 h at room temperature with 15 µg of antibody in 100 µl PBS/well. Four plates were prepared, one each for the four antibodies. The antibody solutions were removed by flicking, and 200 µl of 2% bovine serum albumin in PBS was added to block the unsaturated plastic surface. After 30 min at room temperature, the bovine serum albumin solution was removed, and 50 ng of PLC-I in 100 µl of PBS was added to each well and incubated for 2 h at room temperature (17 cm for PLC-I and 154 ml for PLC-II). The incubation was stopped by washing three times with PBS buffer. Then, the four 125I-labeled antibodies (~300,000 cpm/well) were added separately to the wells, so that every combination of pairs of the primary antibodies and the secondary 125I-labeled antibodies were mixed, and incubated for 2 h at room temperature. After three washes with PBS, 125I radioactivity of each well was measured. Radioactivity in control experiments, where the primary coating antibody and the secondary 125I-labeled antibody were the same, was ~2,000 cpm. Therefore, a pair of primary and secondary antibodies yielding this baseline radioactivity was considered to be competing for the same epitope. On the other hand, a pair yielding 25,000–40,000 cpm was considered as antibodies which bind to two separate sites on PLC-I. There was no ambiguity in differentiating a competing pair from the noncompeting pairs. From this experiment, the four monoclonal antibodies could be divided into three groups which recognize different epitopes. Representatives of these groups are K-32-3 (IgG2a), K-82-3 (IgG1), and K-92-3 (IgG1). By following a similar procedure, the 23 anti-PLC-II antibodies were divided into six groups, each of which is represented by A-2-5 (IgG1), E-8-4 (IgG2a), B-12-5 (IgG2d), D-7-3 (IgG1), and E-9-4 (IgG2d).
columns, 12 µg of PLC-I or PLC-II in 200 µl of PBS buffer was loaded. After 20 min at room temperature, unbound enzyme was eluted with 1 ml of PBS buffer, and the PI hydrolyzing activity in the eluent was measured.

**Protein Concentration**—The concentrations of PLC-I and PLC-II were estimated using an average protein absorbivity of 0.281 mg/ml.

**Other Methods**—Analytical polyacrylamide gel electrophoresis was performed by the method of Laemmli (31) in gradient (6–12%) slab gels containing sodium dodecyl sulfate. Electrophoretic transfer of protein from slab gels to nitrocellulose sheets and subsequent immunoblotting using peroxidase-conjugated goat anti-mouse IgGs were as described (32).

**Inhibition of PLC Enzymes by Heavy Metal Ions**—Immediately before inhibition studies, the PLC enzymes, which had been stored frozen, were thawed and washed twice in a centrifuge microcentrifuge with 50 mM Hepes buffer (Chelex 100 treated), pH 7.0, to remove DTT. Properly diluted enzyme solution (~0.5 µg in 20 µl of 50 mM Hepes buffer, pH 7.0) was mixed with 40 µl of 10 mM CaCl₂, 20 µl of various metal ion solutions, and 20 µl of water. The mixture was incubated at room temperature for 2 min and the PI-hydrolyzing activities were measured at 37°C by adding 100 µl of substrate solution containing 150 mM of PI, 30,000 cpm of [3H]PI, 2 mM/ml sodium deoxycholate, and 50 mM Hepes, pH 7.0.

To evaluate whether DTT or EDTA could reverse the inhibition by metal ions, PLC enzyme solutions were incubated with various metal ions as described above except that 20 µl of water was omitted. After 2 min at room temperature, 20 µl of either water, 1 mM DTT, or 2.5 mM EDTA was added. The mixture was further incubated for 2 min at 37°C at which time 190 µl of PI substrate solution was added to measure the PLC activity.

**RESULTS**

**Purification of PLC Enzymes**—Bovine brain cytosol contains more PLC-II enzyme activity than PLC-I (Fig. 1). To maximize the recovery of PLC-I, a large number of fractions were pooled in DEAE chromatography resulting in significant contamination of the PLC-I pool by PLC-II. Nevertheless, since PLC-I and PLC-II were eluted from a Matrex green gel at different KCl concentration (PLC-I at 0.5 M and PLC-II at 0.75 M), the contaminating PLC-II could be removed easily as evidenced by the smaller activity peak of PLC-II eluted after the major peak of PLC-I in Fig. 2A. In addition, during the next chromatography step on heparin-agarose gel (Fig. 3), PLC-I was eluted at a higher NaCl concentration than PLC-II at 0.42 M and PLC-II at 0.50 M. The heparin-agarose step was very effective for the purification of PLC-I.

A fraction pooled from this step yielded the PLC-I enzyme as the major protein peak when chromatographed on a phenyl-5PW HPLC column (Fig. 4A). The PLC-I activity peak did not coincide exactly with the protein peak in Figure 4A because the HPLC column was eluted with a decreasing KCl gradient and the PI-hydrolyzing activity was inhibited to different extents by different concentrations of KCl in the PLC-I fractions (data not shown).

On the other hand, the PLC-II activity peak came out among the bulk of proteins from the heparin column (Fig. 3B). And further purification on phenyl-5PW column yielded a PLC-II activity peak coinciding with one of several overlapped protein peaks. Therefore, it was necessary to proceed with one more purification step on a MonoQ ion exchange column from which the PLC-II emerged as a major peak (Fig. 5). Different physical properties of PLC-I and PLC-II were also manifested by the fact that they were eluted at signifi-
rime monoclonal antibodies directed against PLC-I and PLC-II were prepared. The competition experiments described under "Experimental Procedures" showed that anti-PLC-I antibodies, K-32-3, K-82-3, and K-92-3, recognize different epitopes on PLC-I and that anti-PLC-II antibodies, A-2-5, B-12-5, E-3-4, B-16-5, D-7-3, and E-9-4, bind to different sites on PLC-II. Specificity of these antibodies to either PLC-I or PLC-II could be shown by binding experiments with immunoaaffinity gels. Each of the three immunoaffinity gels prepared separately from the anti-PLC-I antibodies retained >95% of PLC-I activity while less than 5% of PLC-II activity was retained by them. On the other hand, the immunoaaffinity gels prepared separately from the six anti-PLC-II antibodies could retain PLC-II (~100%) but not PLC-I.

SDS-PAGE Analysis and Immunoblot—Purified PLC enzymes exhibited protein bands corresponding to a Mr of 150,000 and 145,000 for PLC-I and PLC-II, respectively, when analyzed on a SDS-polyacrylamide gradient (6–16%) gel (Fig. 6A). The apparent sizes of the two forms were clearly different as evidenced by two separate bands observed with their mixture (Fig. 6A, lane 2). In addition, when heavily loaded, several additional minor bands were also visible. The PLC-I preparation contained a 100- and a 55-kDa band, while the PLC-II preparation contained bands of 80, 68, 54, and 32 kDa. Immunoblots of the SDS-PAGE gel were carried out by using individual monoclonal antibodies as well as their mixtures. Each of the three anti-PLC-I antibodies, K-32-3, K-82-3, and K-92-3, independently recognized 150-kDa PLC-I polypeptide but not 145-kDa PLC-II. Similarly, each of the six anti-PLC-II antibodies, A-2-5, B-12-5, E-3-4, B-16-5, D-7-3, and E-9-4, was specific to 145-kDa PLC-II polypeptide. Shown in Fig. 6B are the results of immunoblots obtained with the mixture of the three anti-PLC-I antibodies (lanes 1 and 2) and with the mixture of the six anti-PLC-II antibodies (lanes 3 and 4). As expected, the PLC-I antibody mixture recognized only 150-kDa PLC-I polypeptide while PLC-II antibody was specific to PLC-II. Additionally, the 100-kDa band in PLC-I was also blotted by anti-PLC-I antibody, indicating that the 100-kDa peptide is a fragment derived from the 150-kDa PLC-I polypeptide. In the previous communication (29), we reported that the intensity of this 100-kDa band increased when a PLC-I sample was repetitively frozen and thawed. The remaining minor bands in Fig. 6A are likely due to the presence of other isoforms.

**Effect of Ca**^{2+} **on Enzyme Activity**—As reported in a previous communication, both PLC-I and PLC-II hydrolyzed all three phosphoinositides—PI, PIP, and PIP_2—but did not hydrolyze other phospholipids such as phosphatidylethanolamine and phosphatidylcholine (29). The hydrolysis rates measured as a function of Ca**^{2+}** concentration using a small unilamellar vesicle as substrate are shown in Fig. 7. With PI as substrate, the hydrolysis rate of PLC-I increased with increasing Ca**^{2+}** concentration up to 10 mM, while the activity of PLC-I increased until it reached a maximum at 6 mM Ca**^{2+}** and then decreased. However, PIP and PIP_2 were better substrates for both PLC-I and PLC-II when the concentration of Ca**^{2+}** was in the micromolar range. With these two substrates the rates of hydrolysis increased with increasing Ca**^{2+}** concentration until they reached maxima at 10–100 μM, while higher Ca**^{2+}** concentrations (i.e. 1 mM or higher) are rather inhibitory for both enzymes. In the absence of Ca**^{2+}** (but in the presence of 2 mM EGTA), the PIP_2-hydrolyzing activity by PLC-I was 1–2% of maximal rate, and all other activities for both PLC-I and PLC-II were less. However, when the PLC-I enzyme which had experienced freezing and thawing was assayed, its

**Monoclonal Anti-PLC-I and Anti-PLC-II Antibodies**—Monoclonal antibodies to PLC-I and PLC-II were prepared by immunizing several BALB/c mice with various preparations of these enzymes, followed by the antibody screening procedure described under "Experimental Procedures." Fractions of 1.0 ml were collected. The purified PLC-I (A) and PLC-II (B) were applied as described in Fig. 3. The PLC-I preparation contained a 100- and a 55-kDa band, while the PLC-II preparation contained bands of 80, 68, 54, and 32 kDa. Immunoblots of the SDS-PAGE gel were carried out by using individual monoclonal antibodies as well as their mixtures. Each of the three anti-PLC-I antibodies, K-32-3, K-82-3, and K-92-3, independently recognized 150-kDa PLC-I polypeptide but not 145-kDa PLC-II. Similarly, each of the six anti-PLC-II antibodies, A-2-5, B-12-5, E-3-4, B-16-5, D-7-3, and E-9-4, was specific to 145-kDa PLC-II polypeptide. Shown in Fig. 6B are the results of immunoblots obtained with the mixture of the three anti-PLC-I antibodies (lanes 1 and 2) and with the mixture of the six anti-PLC-II antibodies (lanes 3 and 4). As expected, the PLC-I antibody mixture recognized only 150-kDa PLC-I polypeptide while PLC-II antibody was specific to PLC-II. Additionally, the 100-kDa band in PLC-I was also blotted by anti-PLC-I antibody, indicating that the 100-kDa peptide is a fragment derived from the 150-kDa PLC-I polypeptide. In the previous communication (29), we reported that the intensity of this 100-kDa band increased when a PLC-I sample was repetitively frozen and thawed. The remaining minor bands in Fig. 6A are likely due to the presence of other isoforms.

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activities were often enhanced by ~50% and, in particular, thePIP2-hydrolyzing activity in the absence of Ca2+ often increased to a level equivalent to 5% of maximal rate. However, quantitative estimation of the activation was not possible because of the variation from batch to batch.

Effect of Deoxycholate on Enzyme Activity—In the presence of 3 mM Ca2+ and 1 mM EGTA, addition of up to 0.5 mg/ml deoxycholate did not have much affect on the PI-hydrolyzing activities of PLC-I and PLC-II (Fig. 8). However, the rates were enhanced greatly when the deoxycholate concentration reached 0.75—1.0 mg/ml and decreased rapidly as the deoxycholate concentration increased above 1.0 mg/ml.

Effect of Nucleotides on Enzyme Activity—The effects of GTP, GTP analogs, and ATP on the PIP2 hydrolysis were studied using small unilamellar vesicles as substrate, and the results are summarized in Table II. In the absence of Ca2+, the PIP2-hydrolyzing activity of the PLC-I used in this experiment was only 1.5% of the activity measured at optimal Ca2+ concentration. This Ca2+-independent activity was enhanced significantly by GTP in a concentration-dependent manner: 90, 175, and 285% enhancements were attained by 0.2, 1.0, and 2.0 mM of GTP, respectively. The GTP analogs (GTPyS and GppNp) and ATP were also as effective as GTP in stimulating PLC-I activity. In the absence of Ca2+, PLC-I1 reached 0.75-1.0 mg/ml and decreased rapidly as the deoxycholate concentration increased above 1.0 mg/ml.

Table I

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<th>Purification of PLC-I and PLC-II from 48 bovine brains</th>
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<td>The PI-hydrolyzing activities of PLC-I and PLC-II were separated by DEAE-cellulose chromatography as shown in Fig. 1, and the relative amount of activity contributed by PLC-I and PLC-II to the total amount of activity in the homogenate and in the pH 5.0 precipitate was estimated from the area of each activity peak in Fig. 1.</td>
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Fig. 6. SDS-PAGE and immunoblots of PLC-I and PLC-II. PLC-I and PLC-II were subjected to SDS-polyacrylamide gradient (6–16%) gels and either stained with Coomassie Blue (panel A) or immunoblotted with monoclonal anti-PLC-I antibodies (panel B, lanes 1 and 2) or with monoclonal anti-PLC-II antibodies (panel B, lanes 3 and 4). In panel A, lane 1, PLC-I; lane 2, mixture of PLC-I and PLC-II; lane 3, PLC-II. In panel B, lane 1, PLC-I; lane 2, PLC-II; lane 3, PLC-I; lane 4, PLC-II.

Fig. 7. Ca2+ dependence of phosphoinositide hydrolysis by PLC-I (open symbols) and PLC-II (closed symbols). Small unilamellar vesicles derived from [3H]PI (A), [3H]PIP2 (B), and [3H]PIP (C) were used as substrates. The reaction mixtures were incubated for 5 min at 37°C and included a mixture of 30,000 cpm of [3H]phosphoinositide and 113 μM unlabeled phosphoinositide, increasing amounts of CaCl2 in 200 μl of buffer containing 50 mM HEPES, pH 7.0, 2 mM EGTA, 100 mM NaCl, 3 mM MgCl2, and 0.15 mg/ml bovine serum albumin. The PI-hydrolyzing activities in this experiment were significantly lower than those values reported in Table II because of the absence of sodium deoxycholate which activates the PLC enzymes. Free Ca2+ concentrations were calculated using the following constants (33, 34): log K1 = 9.22 and log K2 = 10.01 for the association of M$+ and M2$; log K3 = 6.40 for the protonation of EGTA; log K = 16.00 for the association of Ca2+ and EGTA; log K = 16.00 for the association of Mg2+ and EGTA. Where indicated with an arrow (†), no CaCl2 was added.
TABLE II
Effects of nucleotides on the specific activity (S.A.) of purified PLC enzymes

<table>
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<tr>
<th>Nucleotide</th>
<th>0.5 μM Ca++</th>
<th>9.0 μM Ca++</th>
<th>0.5 μM Ca++</th>
<th>9.0 μM Ca++</th>
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<td>0.024</td>
<td>120</td>
<td>1.38</td>
<td>104</td>
</tr>
<tr>
<td>ATP⋅S</td>
<td>0.029</td>
<td>145</td>
<td>1.20</td>
<td>86</td>
</tr>
<tr>
<td>ATP⋅S⋅S</td>
<td>0.085</td>
<td>425</td>
<td>1.69</td>
<td>142</td>
</tr>
</tbody>
</table>

* ND, not detectable.

**Treated with Chelax-100 to remove any divalent cation contamination.

disappeared was less significant; noticeable activation of PLC-I was observed only with 2 mM GTP (35%) and 2 mM ATP (42%). PLC-II activity was less sensitive than PLC-I to the activation by nucleotides since 2 mM GTP enhanced PLC-I activity by only 12% and the enhancement by 2 mM ATP was 13%.

Effects of Metal Ions on Enzyme Activity—The effects of various metal ions on the activity of PLC enzymes were studied. At a concentration of 50 μM, Hg+, Cu++, and Zn++ inhibited both PLC-I and PLC-II activities by more than 80% while Cd++ inhibited only PLC-II. Other metal ions such as Co++, Mn++, Mg++, and Ni++ were without effect on either PLC-I or PLC-II. Weak inhibitions of PLC-I and PLC-II were observed with 50 μM Fe++. Metal ion concentration dependence for PLC inhibition was studied for Hg++, Cd++, Zn++, and Cu++. (Fig. 9). PLC-II was extremely sensitive to Hg++ ion. Fifty percent inhibition occurred at 0.2 μM (I50 = 0.2 μM). PLC-I was less sensitive to Hg++ (I50 = 1 μM). Cd++ inhibited PLC-II (I50 = 5 μM) but not PLC-I. Inhibition by Zn++ and Cu++ was also more pronounced for PLC-II than for PLC-I; I50 of Zn++ was 4 μM for PLC-II and >10 μM for PLC-I; I50 of Cu++ was 2 μM for PLC-II and 10 μM for PLC-I. We further investigated whether the inactivation caused by heavy metals can be reversed either by DTT or EDTA. Table III shows that the activities of PLC-I and PLC-II could be restored completely if after inactivation with Hg++, they were incubated with DTT, while similar treatment with EDTA had no effect. During this inactivation with Hg++, incubation time was limited to 2 min because longer incubation (>10 min) led the enzymes to inactive forms which could not be reactivated by DTT. In contrast, the inhibition by Zn++ and Cd++ could be reversed completely by EDTA but not by DTT. The Cu++-mediated inhibition was partially reversed by either DTT or EDTA.

DISCUSSION
In the previous paper (29), we reported that (i) bovine brain contains two forms of PLC enzyme, PLC-I and PLC-II, whose molecular weights are 150,000 and 145,000, respectively, under a denaturing condition; (ii) PLC-I exists mainly in dimer
while PLC-II is predominantly in monomer under a non-denaturing condition; and (iii) rabbit antibodies prepared against PLC-II do not recognize PLC-I. These observations were reproduced with several independent preparations of bovine brain enzymes during the current study. In addition, the fact that PLC-I and PLC-II contain dissimilar antigenic sites was also confirmed by the use of various monoclonal antibodies in the binding of native enzymes to immunoaffinity gels and in the immunoblots of denatured enzymes. Nevertheless, the catalytic properties of PLC-I and PLC-II were similar in that they both hydrolyzed all three phosphoinositides (PI, PIP, and PIP_2) and showed no clear differences in Ca^{2+} dependence except that the PI-hydrolyzing activity of PLC-I was about half of the PLC-II activity. The specific activity of PLC-I was enhanced upon freezing and thawing. This is probably related to the formation of 100-kDa polyepitides or to the shift in equilibrium between dimer and tetramer (29).

Although the optimal Ca^{2+} concentration (10 μM to 1 mM) required for the hydrolysis of PIP and PIP_2 was lower than that for PI-hydrolysis, it was still much higher than physiological intracellular Ca^{2+} concentration (0.1–0.2 μM). This was probably because the PIP_2 substrate used in the assay and not contain any activator molecules such as phosphatidylinserine, diacylglycerol (30), and guanine nucleotide-binding protein (G protein). It has been demonstrated that G protein in polymorphonuclear leukocyte (7, 8) stimulates a PLC enzyme by reducing the Ca^{2+} requirement for PIP_2 hydrolysis from superphysiological to normal intracellular concentrations.

Two immunologically distinct forms of PLC enzyme previously purified from the seminal vesicles gland also exhibited indistinguishable catalytic properties (27). It seems, however, that the brain enzymes and seminal vesicle enzymes are clearly different since the molecular weights (150,000 and 145,000) of brain enzymes are different from those (65,000 and 85,000) of seminal vesicle enzymes (26) and, in the absence of Ca^{2+}, the brain enzyme exhibited negligible activity (<2%) toward the three phosphoinositides while seminal vesicle enzymes hydrolyzed PIP and PIP_2 with rates equivalent to 15–30% of maximal (27). With respect to the molecular weight and the dependence of activity on Ca^{2+}, brain enzymes were rather similar to a PLC enzyme purified from bovine platelets, both exhibiting negligible activity in the absence of Ca^{2+} (28). However, monoclonal antibodies derived against bovine brain enzymes failed to recognize the platelet enzyme. These results suggest that mammalian cells probably contain several types of PLC enzyme with isoformic properties. The reason behind the existence of isoforms is not clear at this moment. It may be that different receptor functions are coupled to different PLC enzymes and that the receptor-PLC enzyme couplings involve different G proteins. Based on the observations that either GTP or the nonhydrolyzable analogs of GTP could mimic the effect of adding an agonist in isolated membranes and permeabilized cells, and by analogy with the receptor-mediated control of adenylate cyclase, G proteins have been proposed as mediators for coupling between the receptor and the PLC enzyme (4–17). However, the putative G proteins involved in this process have not been identified. Attempts to identify them using pertussis and cholera toxins suggested that the G protein involved in coupling might be different for different cells (4, 10–17). Therefore, it is tempting to speculate that the multiplicity of PLC enzymes is related to the multiplicity of G proteins.

It has recently been observed that PLC activity in crude cytosolic fraction was activated by guanine nucleotides in vitro (35). Furthermore, Banno et al. (36) showed that partially purified cytosolic PLC enzyme from human platelets could be stimulated directly by GTP and GTPyS but not by ATP. In view of this finding, we tested the effects of GTP, nonhydrolyzable GTP analogs, and ATP on the PI_2-hydrolyzing activity of purified PLC-I and PLC-II. The addition of either GTP or ATP at the concentration of 2 mM resulted in a 300% enhancement of the PLC-I activity in the absence of Ca^{2+} and a 35–50% enhancement in the presence of 9 mM Ca^{2+}. On the contrary, the PLC-II activity was not affected significantly. The nonspecific activation of PLC-I by ATP and GTP is in contrast to specific activation of the human platelet enzyme (36). However, it has been reported that inositol triphosphate formation by a PLC enzyme in rat cerebral cortical membranes was enhanced by both ATP and GTP (9). Since PLC-I was activated nonspecifically at higher concentrations of nucleotide, it is difficult to envision any physiological significance of this nucleotide effect. This result thus warns us that positive effects of GTP or GTP analogs on the PLC activity should not automatically be taken as the indication of involvement of G proteins in the receptor-PLC coupling.

Hg^{2+}, Cd^{2+}, Zn^{2+}, and Cu^{2+} all show a strong affinity for ligands such as phosphates, cysteinyl, and histidyl side chains of proteins, purines, pteridines, and porphyrins (37). Hence, all four metal ions could act on a large number of macromolecules in mammalian cells. Hg^{2+} and Cd^{2+} are also known to interact with carboxyl groups, tryptophan, and tyrosine (38). However, the biochemical basis of toxicological effects of Hg^{2+} and Cd^{2+} are generally thought to be through their interaction with the —SH and S-S groups of proteins (37, 38).

Our present data show that PLC-II is more sensitive than PLC-I to inhibition by Hg^{2+}, Cd^{2+}, Zn^{2+}, and Cu^{2+}, and that, in particular, Hg^{2+} is a very potent inhibitor of PLC-II. In contrast to the strong inhibition of PLC enzymes by Hg^{2+} and Cd^{2+}, incubation with iodoacetamide (0.5 mM) did not affect the activity of either PLC-I or PLC-II (data not shown). Previously, Thompson and Dawson (39) demonstrated that PLC activity in rat brain homogenates could be inhibited by Hg^{2+} and Cu^{2+} but not by iodoacetate and iodoacetamide. It is therefore likely that interactions of these metals with side chain groups other than —SH in PLC enzymes are important factors in determining their effect on enzymic activities and that different metals interact somewhat differently with them. This is supported by the fact that PLC-I is inhibited by Hg^{2+} but not by Cd^{2+}. The selective inhibition of PLC-II by Cd^{2+} should be useful in quantitating the PLC-II type enzyme in various cell homogenates.

Hg^{2+} binds extremely tightly to cysteine ([log K = 45.4] compared to its binding to EDTA ([log K = 22.1]) (40). On the other hand, Zn^{2+} binds EDTA ([log K = 16.4] with higher affinity than cysteine ([log K = 46]) (40). Therefore, it is predictable that the Hg^{2+} inhibition is reversed by DTT but not by EDTA, while the Zn^{2+} inhibition is reversed by EDTA assuming that the major binding force for Hg^{2+} and Zn^{2+} is due to their chelation to a —SH group. The Cu^{2+}-induced inactivation could be reversed only partially by either EDTA or DTT. This is probably because Cu^{2+} binds PLC enzymes very tightly or because Cu^{2+} binding caused irreversible conformational damage. Irreversible inactivation could be seen with Hg^{2+} when incubated for longer times (>10 min).

The molecular basis of toxicity due to various heavy metals is not clearly known and there may of course be multiple effects. But any consideration of their toxicity must take into account the sensitivity of PLC enzymes, particularly due to

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the sensitivity of PLC-II to Hg²⁺, Cd²⁺, Zn²⁺, and Cu²⁺ and the pivotal role that these PLC enzymes play in receptor-mediated signal transduction.

REFERENCES