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TWO FORMS OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C FROM BOVINE BRAIN

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SUMMARY: Two immunologically distinct forms of phosphoinositide-specific phospholipase C were purified to near homogeneity from bovine brain. Their molecular weights determined by SDS-PAGE are 150,000 (enzyme I) and 145,000 (enzyme II), respectively. Under a nondenaturing condition, purified enzyme I exists mainly in dimeric form and as tetramer to a small extent, while enzyme II is predominantly in monomer, to a small extent as dimer and to a very small extent as trimer. Multiple forms of phosphoinositide-specific phospholipase C in brain tissue described in the literature might be, therefore attributed to the oligomerization of the two independent forms.

Phosphoinositide-specific phospholipase C (PLC) plays a crucial role in initiating the surface receptor mediated signal transduction by generating two second messenger molecules, diacylglycerol and inositol 1,4,5-triphosphate (1, review 2-4). Phospholipase C from many tissues as shown by a number of workers can be resolved into several peaks by various biochemical separation techniques (5-12). However, none of these studies demonstrated unambiguously the existence of multiple isozymes. Hofmann and Majerus (9) have shown that sheep seminal vesicular glands contain two distinct forms of PLC. However, only one form was purified to homogeneity, preventing direct comparison of the two.

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The abbreviations used are: PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-diphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride; DIFP, diisopropyl fluorophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; PLC, PI-specific phospholipase C; HPLC, high pressure liquid chromatography.

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In this paper we report that PLC from bovine brain consists of two immunologically distinct forms, both of which have been purified to near homogeneity. The state of aggregation as well as the monomeric molecular weights under denaturing conditions were examined. Antibody was prepared against the 145 KDa PLC polypeptide. From the immuno-blots and the comparison of the M_r of PLC polypeptides, we concluded that the two PLC enzymes of brain are different from those isolated from seminal vesicular glands (9), liver (13), and platelets (12, 14).

MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphoric acid, [^{3}H-inositol]PI, [^{3}H-inositol]PIP and [^{3}H-inositol]PIP_2 were obtained from New England Nuclear. Soybean PI, brain PIP, brain PIP_2, brain PE, and brain PC were purchased from Sigma Chemical. [^{32}P]Phospholipids were extracted from yeast grown on a medium containing [^{32}P]orthophosphate (15), and [^{32}P]PI, [^{32}P]PC and [^{32}P]PE were separated on high-performance thin-layer chromatography plates (16). AG1-X2 anion exchange resin (formate form) was from Bio-Rad Laboratory, Matrex green gel from Amicon Corp, Heparin agarose from Bethesda Research Lab, Mono Q HPLC column from Pharmacia, and TSK DEAE-5PW and TSK phenyl-5PW HPLC columns were from Beckman.

Phospholipase C Assay. For the measurement of specific activity during purification, substrate was prepared in the presence of deoxycholate. Assays were performed in a 200 µl reaction mixture containing 20,000 cpm of [^{3}H]PI, 300 µM soybean PI, 0.1% sodium deoxycholate, 3 mM $CaCl_2$, 1 mM EGTA, 50 mM Hepes, pH 7.0, and a source of enzyme. Another assay procedure using small unilamellar vesicles is described in the figure legend. All assays were run at 37°C and terminated as described previously (18).

Preparation of Brain Extracts. Bovine brains were freshly obtained from a local slaughter house and 400 g of cerebra were homogenized in a Waring blender with 1 liter of buffer containing 10 mM Tris-HCl, pH 7.2, 4 mM EGTA, 2 mM PMSF, 0.1 mM DTT, and 0.5 mM DIFP. The homogenate was centrifuged for 30 minutes at 13,000 xg. The supernatant was adjusted to pH 5.0 with 1 M acetic acid. After 30 minutes at 4°C, precipitates were collected by centrifugation and dissolved in 200 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 4 mM EGTA, 2 mM PMSF, and 0.5 mM DIFP. This redissolved protein solution was designated as brain extracts.

Other Methods. Analytical and preparative polyacrylamide gel electrophoreses were performed by the method of Laemmli (19) in 6 or 10% vertical slab gels containing sodium dodecyl sulfate. Nondenaturing gradient (4-16%) slab gel electrophoresis employed the discontinuous buffer system as described by Chrambach et al. (20). Electrophoretic transfer of protein from slab gels to nitrocellulose sheets, and subsequent immunoblotting using peroxidase-conjugated sheep anti-rabbit IgG, were as described in (21).

RESULTS AND DISCUSSION

About 80% of PLC activity could be precipitated with concomitant 2-fold purification when the pH of brain homogenate was lowered to 5.0. Redissolved precipitates yielded two peaks, PLC-I and PLC-II, upon chromatography on an

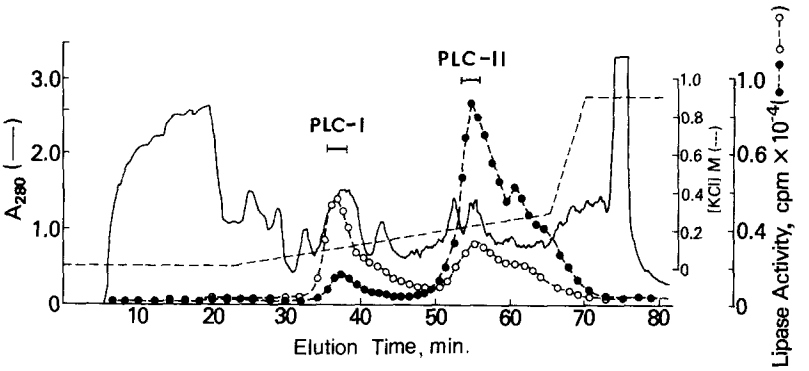

Fig. 1. Ion exchange chromatography on TSK DEAE-5PW. Bovine brain extracts (80 ml) prepared as described in Materials and Methods section was applied at a flow rate of 5 ml/min, onto a HPLC DEAE column (21.5 X 150 mM, purchased from Beckman Instruments) previously equilibrated with 50 mM Tris-HCl, pH 7.6, 1 mM EGTA. Elution was continued at 5 ml/min with KCl gradient, 0 to 250 mM for 50 min, and from 250 to 900 mM for 5 min. Fractions of 5.0 ml were collected. [^{3}H]PI hydrolyzing activity was measured either by incubating 10 µl of eluant for 10 min at pH 5.3 (--●--●--) or by incubating 50 µl of eluant for 10 min at pH 7.2 (--O--O--). Substrate was prepared as small unilamellar vesicles as described previously (15). An assay mixture contained 30,000 cpm of [^{3}H]PI, 450 µM soybean PI, 1.05 mM Ca^{2+}, 1.0 mM EGTA, 50 mM NaCl, 60 mM Tris-maleate (pH 5.3 or 7.2) and a source of enzyme in a total volume of 200 µl.

HPLC-DEAE column (Figure 1). Similar elution profiles were observed without the pH 5.0 precipitation. The two enzymes exhibited different catalytic properties when their activities were measured at pH 5.3 and 7.2. It can be seen from Figure 1 that activity measured at pH 5.3 is greater in fraction II than in fraction I, while fraction I is more active at the pH 7.2. Control experiments (data not shown) indicate that the different responses of fraction I and fraction II to pH are not due to the slightly higher concentration of KCl in fraction II. The water-soluble reaction products from incubation of PLC-I and PLC-II with small vesicles containing [^{3}H-inositol]PI, [^{3}H-inositol]PIP or [^{3}H-inositol]PIP_2 were analyzed on an AG1-X2 anion exchange resin. This chromatographic procedure resolves the various phosphorylated inositol species (18). Each of the three substrates gave rise to the phosphorylated inositol product predicted for PLC-mediated hydrolysis. When a substrate containing a 1:1 mixture of [^{3}H]PI and [^{32}P]PC or of [^{3}H]PI and [^{32}P]PE was used, ^{3}H-radioactivity was detected only in the product, indicating that PLC-I and PLC-II are specific for the hydrolysis of phosphoinositides.

In order to purify the two forms of phospholipase C, brain extracts were prepared from 4.8 Kg of cerebra as described in Material and Methods and chromatographed on a DE-52 ion exchange column by eluting with a KCl gradient (0-0.3 M) in 20 mM Tris buffer, pH 7.6. The activity peak of PLC-I overlapped slightly with that of PLC-II, but they could be pooled without significant cross contamination. Thereafter, fractions containing PLC-I and PLC-II were purified sequentially on the following columns: Matrex green affinity gel eluted with a KCl (0-1 M) gradient in 20 mM Hepes, pH 7.0; heparin agarose gel eluted with a NaCl (0.1-0.5 M) gradient in 20 mM Hepes, pH 7.0; TSK phenyl-5 PW HPLC hydrophobic column eluted with a reverse KCl (3-0 M) gradient in 20 mM Hepes, pH 7.0. During these three chromatography steps, PLC-I and PLC-II always eluted at different positions, indicating that these two forms have different physical properties. PLC-II was further purified on a Mono Q HPLC ion exchange column by eluting with a KCl (0-0.5 M) gradient in 20 mM Tris buffer, pH 7.6. All buffer solutions used in these chromatography steps except for the Matrex green gel included 0.1 mM DTT and 1 mM EGTA. For the Matrex green gel, DTT was omitted. Detailed purification procedures will be published elsewhere.

At the final steps, the PLC activity peaks coincided with the main protein peaks, and nearly homogeneous preparations of PLC-I (0.4 mg) and PLC-II (0.6 mg) were obtained as shown by SDS-PAGE (Fig. 2). Specific activity of the purified enzyme was 11 ~ 15 μmole/min/mg for PLC-I and 21 ~ 26 μmole/min/mg for PLC-II, when measured by the deoxycholate assay as described in Materials and Methods. Although the preparations of PLC-I and PLC-II still contained minor impurity bands (particularly on the dye front), the 150K Da band (Fig. 2A, lanes 1 and 2B) and the 145K Da band (Fig. 2A, lane 3) could be assigned as these polypeptides, because these were the only bands whose intensities were closely correlated with the increase in PLC activity during the purification steps. Apparent sizes of the two forms were clearly different as evidenced by two separate bands observed with their mixture (Fig. 2A, lane 2). The 105K Da band in the PLC-I

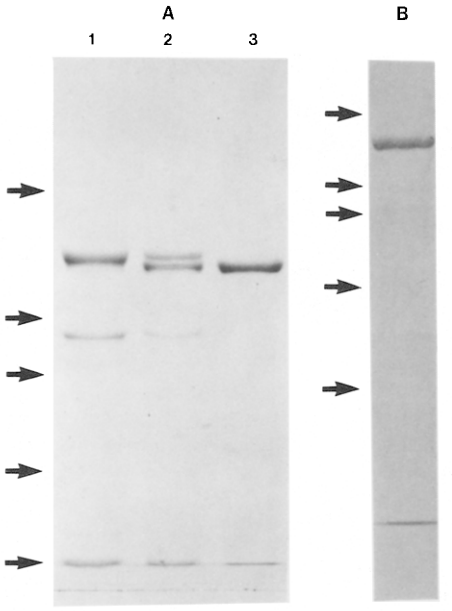

Fig. 2. SDS-PAGE analysis of PLC-I and PLC-II. In panel A (6% gel), lane 1, PLC-I after repeated freezing and thawing; lane 2, mixture of PLC-I and PLC-II; lane 3, PLC-II. In panel B (8% gel), PLC-I immediately after purification. Arrows indicate molecular weight standards from top, myosin (200,000), β-galacsidase (116,000), phosphorylase b (94,000) bovine serum albumin (67,000) and ovalbumin (45,000).

preparation seems to be a fragment derived from the 150K Da polypeptide: the fragment band could not be clearly seen in freshly prepared enzyme but became signficant upon aging.

The preparations of PLC-I and PLC-II were also analyzed on native gradient (4-16%) polyacrylamide gel. Two bands with apparent M_r of 280,000 and 560,000 were prominent in the freshly prepared PLC-I (Fig. 3B). In the aged PLC-I, another band with an apparent M_r of 130,000 was visible (Fig. 3A, lane 1). It seems, therefore, that PLC-I exists mainly in dimeric form (M_r 280,000) and as tetramer (560,000) to a small extent. The 130K Da band is likely due to the monomeric form of 105K Da fragment. Under native conditions, PLC-II separated into three bands (Fig. 3A, lane 2); one major band of M_r 190,000, and two minor bands (M_r 390,000 and 560,000) with M_r corresponding to dimer and trimer of the 190K Da protein. The apparent M_r of PLC-II polypeptide was 145,000 when estimated from 6% (Fig. 2, lane 3) and 10% (figure not shown) SDS-PAGE, and no other band except one on the dye

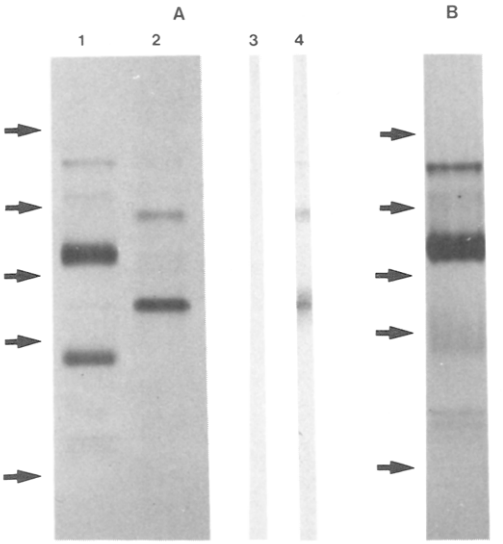

Fig. 3. Polyacrylamide gradient (4-16%) gels and immunoblots of PLC-I and PLC-II. PLC-I and PLC-II were subjected to gradient PAGE and either strained with Coomassie blue (lanes 1 and 2 in panel A and panel B) or immunoblotted with anti-PLC-II antibody (lanes 3 and 4 in panel A). Lane 1, PLC-I after repeated freezing and thawing. This is the same protein used in lane 1 of Fig. 2; lane 2, PLC-II; lane 3, PLC-I; lane 4, PLC-II; panel B, PLC-I immediately after purification. Arrows indicate molecular weight standards from top, thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), BSA (67,000).

front was visible on both gels. It is, therefore, likely that the significant discrepancy between molecular weights measured under native (190,000) and denaturing (145,000) conditions is not due to the association of another polypeptide with a M_r in the range of 45,000. Rather, the difference is probably due to the unusually nonglobular shape of the PLC-II molecule. However, the possibility that PLC-II consists of several small polypeptides in addition to the 145K Da protein cannot be eliminated.

To obtain antibodies against PLC-II of the highest purity, the band corresponding to M_r = 145,000 was sliced from a preparative SDS-PAGE gel and electrophoretically eluted to yield antigen to be used for the immunization of a rabbit. Using standard techniques, we obtained anti-PLC-II IgG. Immunoblots experiments with either SDS-PAGE (data not shown) or native-PAGE (Fig. 3, lanes 3 and 4) revealed that anti PLC-II antibody recognized PLC-II but not PLC-I. The immunoblotting intensity observed with the monomeric, dimeric and trimeric forms of PLC-II is proportional to the amount of protein

in each band. We next prepared immunoaffinity gel by incubating anti-PLC-II antibody with protein-A sepharose and evaluated the reactivity toward PLC-I and PLC-II. Only PLC-II was selectively retained by this affinity gel (data not shown). These results suggest that PLC-I and PLC-II are isozymes. Previously, Hofmann and Majerus demonstrated that sheep seminal vesicle contains two immunologically distinct PLC enzymes (9). One of these enzymes was purified to homogeneity with a M_r of 65,000 by SDS-PAGE. The other form was partially purified and its apparent M_r estimated by gel filtration was 85,000. This result indicates that bovine brain PLC enzymes are different from the two enzymes of sheep seminal vesicle, discounting the suggestion (9) that the 85K Da PLC is the major form of PLC in brain. The discrepancy in M_r is not due to species variation because antibody derived against the bovine brain PLC-II recognizes 145K Da peptides in homogenates of rat and human brain (figure not shown). Additionally, it has been shown that platelet cytosol contains a PLC of M_r = 140,000 (12, 14). Despite its similarity to bovine PLC-II in M_r, no cross-reactivity to anti-PLC-II antibody could be demonstrated by immuno-affinity column and immuno-blot experiments. It has been suggested that the function of PLC is influenced by unspecified guanine nucleotide binding protein (22-24). There are several well characterized guanine nucleotide binding proteins (25). Therefore, it is tempting to speculate that different PLC isozymes couple to different guanine nucleotide binding proteins in a tissue- and hormone-specific manner.

Previously, PLC activities in rat brain homogenates had been resolved into four peaks by isoelectric focusing gels and chromatofocusing columns (5). In an independent experiment, the molecular weights of two fractions containing rat brain PLC enzymes were estimated by gel filtration chromatography to be 150,000 and 290,000 in homogenates (8), and by other workers to be 250,000 and 300,000 in partially purified preparations (10). Perhaps these multiple forms can be attributed to the oligomerization of two isozymes, PLC-I and PLC-II. Studies on the kinetic and catalytic properties of these isozymes are in progress.

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