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Mammalian phospholipase D: Phosphatidylethanolamine as an essential component

SHUN-ICHI NAKAMURA*, YOSHIMOTO KIYOHARA*, HITOSHI JINNAI*, TOMOHIRO HITOMI*, CHIAKI OGINO†, KIMIHISA YOSHIDA*, AND YASUTOMI NISHIZUKA*‡

*Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan; and ‡Biosignal Research Center, Kobe University, Kobe 657, Japan

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ABSTRACT **Bovine kidney phospholipase D (PLD) was assayed by measuring the formation of phosphatidylethanol from added radioactive phosphatidylcholine (PtdCho) in the presence of ethanol, guanosine 5′-[γ-thio]triphosphate, ammonium sulfate, and cytosol factor that contained small GTP-binding regulatory proteins. The PLD enzyme associated with particulate fractions was solubilized by deoxycholate and partially purified by chromatography on a heparin-Sepharose column. This PLD preferentially used PtdCho as substrate. After purification, the enzyme *per se* showed little or practically no activity but required an additional factor for the enzymatic reaction. This factor was extracted with chloroform/methanol directly from particulate fractions of various tissues, including kidney, liver, and brain, and identified as phosphatidylethanolamine (PtdEtn), although this phospholipid did not serve as a good substrate. Plasmalogen-rich PtdEtn, dioleoyl-PtdEtn, and L-α-palmitoyl-β-linoleoyl-PtdEtn were effective, but dipalmitoyl-PtdEtn was inert. Sphingomyelin was 30% as active as PtdEtn. The results suggest that mammalian PLD reacts nearly selectively with PtdCho in the form of mixed micelles or membranes with other phospholipids, especially PtdEtn.**

Mammalian phospholipase D (PLD) has been found in diverse tissues. Because of the potential role of PLD in transduction of signals, particularly those from some growth factors, extensive studies are being made to clarify the mechanism of this enzyme activation (for reviews, see refs. 1 and 2). PLD is associated tightly with particulate fractions, most likely membranous structures. Cell-free preparations of PLD have been obtained from several tissues and cell types (for reviews, see refs. 1 and 2). Two major forms of PLD have been partially purified from rat brain membranes (3). One form is activated by ADP-ribosylation factor and guanosine 5′-[γ-thio]triphosphate (GTP[γS]), and reacted preferentially with phosphatidylcholine (PtdCho) in the presence of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) as described (4, 5). The other form depends on unsaturated fatty acids such as sodium oleate and specifically hydrolyzes PtdCho as described (6). The latter form of PLD has been purified extensively from pig lung microsomes and shown to be stimulated by Ca^{2+} and Mg^{2+} (7).

A preceding report from this laboratory has shown that under normal conditions the activity of mammalian PLD is mostly latent, but it exhibits significant activity in the presence of a high concentration of ammonium sulfate, although the mechanism of this salt effect is unclear (8). This PLD requires a nonhydrolysable GTP analogue together with a cytosol fraction that contains ADP-ribosylation factor. The results presented herein will show that, with a PLD preparation partially purified from bovine kidney particulate fractions, a lipid factor is also needed to reveal the enzyme activity. This lipid factor is identified as phosphatidylethanolamine (PtdEtn), which *per se* does not serve as a good substrate. PtdInsP_2 shows no effect. Kinetic properties of this PLD activation by PtdEtn will be described.

MATERIALS AND METHODS

Materials. L-α,β-Dipalmitoyl[dipalmitoyl-1-^{14}C]phosphatidylcholine ([^{14}C]PtdCho, 115 mCi/mmol: 1 Ci = 37 GBq), L-α-palmitoyl-β-[^{14}C]linoleoyl-PtdEtn ([^{14}C]PtdEtn, 57 mCi/mmol), and L-α-stearoyl-β-[^{14}C]arachidonyl-phosphatidylinositol ([^{14}C]PtdIns, 43 mCi/mmol) were purchased from DuPont/NEN. L-α,β-Dioleoyl-phosphatidyl-[3-^{14}C]serine (Ptd[^{14}C]Ser, 51 mCi/mmol) was purchased from Amersham. Phosphatidylethanol (PtdEtOH) and dioleoyl-PtdEtn were purchased from Avanti Polar Lipids. Dipalmitoyl-PtdCho, dipalmitoyl-PtdEtn, and L-α-palmitoyl-β-linoleoyl-PtdEtn were obtained from Sigma. Phosphatidylserine (PtdSer), PtdEtn, phosphatidylinositol (PtdIns), phosphatidic acid, PtdInsP_2, sphingosine, ceramide, sphingomyelin, cholesterol, and plasmalogen-rich PtdEtn (60% plasmalogen) were purchased from Serdary Research Laboratories (Englewood Cliffs, NJ). GTP[γS] was obtained from Boehringer Mannheim. The Hi-Trap heparin-Sepharose column (16 × 25 mm; 5 ml) was obtained from Pharmacia. Other chemicals were of analytical grade.

Tissue Fractionation. The bovine kidney was obtained at a local slaughterhouse and stored at −80°C until use. All procedures were carried out at 4°C. The tissue was minced and homogenized with a glass/Teflon homogenizer in 5 vol of buffer A (50 mM Hepes·NaOH, pH 7.5/1 mM EDTA/2 mM $MgCl_2$/1 mM dithiothreitol) containing 0.25 M sucrose, 20 μg/ml leupeptin, 10 μg/ml aprotinin, and 50 μM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF). The homogenate was centrifuged for 10 min at 1000 × *g* to remove nuclei and tissue debris. The postnuclear supernatant fraction was centrifuged for an additional 60 min at 100,000 × *g*. The pellet containing PLD was washed three times, each time by suspending it in buffer A, containing 10 μM *p*-APMSF, followed by centrifugation, and was taken up in the same buffer at a protein concentration of 20–25 mg/ml. The preparation was stored at −80°C and used as particulate fraction.

Enzyme Assay. PLD activity was determined by measuring the formation of PtdEtOH from [^{14}C]PtdCho in the presence of ethanol under the conditions described previously (8). The reaction mixture (100 μl) contained 0.1–0.5 μg of heparin-Sepharose-purified PLD (see below), 100 μg of cytosol factor

Abbreviations: PLD, phospholipase D; GTP[γS], guanosine 5′-[γ-thio]triphosphate; PtdCho, phosphatidylcholine; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; [^{14}C]PtdCho, L-α,β-dipalmitoyl[dipalmitoyl-1-^{14}C]phosphatidylcholine.

†On leave from: Graduate School of Science and Technology, Kobe University, Kobe 657, Japan.

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containing small guanine-nucleotide-binding regulatory proteins (see below), 2 nmol of [^{14}C]PtdCho (13,750 dpm/nmol), lipid fraction as indicated, 34.2 μmol of ethanol, 160 μmol of ammonium sulfate, 10 nmol of GTP[γS], and 2 μmol of Hepes·NaOH at pH 7.4. As 1-octyl β-D-glucopyranoside was highly inhibitory for the reaction, the final concentration of this detergent did not exceed 0.01%.

PLD Preparation. The particulate fraction (500 mg protein) was thawed and suspended in 50 ml of buffer A, containing 10 μg/ml leupeptin and 10 μg/ml aprotinin. Then, deoxycholate was added to reach a final concentration of 1%. The mixture was stirred gently on ice for 30 min and centrifuged for 60 min at 100,000 × *g*. To precipitate the enzyme, magnesium acetate was added slowly to the supernatant while stirring to a final concentration of 100 mM, and the mixture was centrifuged for 5 min at 10,000 × *g*. The pellet was suspended in 20 ml of buffer B (50 mM Hepes·NaOH, pH 7.5/1 mM EDTA/2 mM $MgCl_2$/1 mM dithiothreitol/0.25 M sucrose/10 μM *p*-APMSF/1% 1-octyl β-D-glucopyranside). Insoluble materials were removed by centrifugation for 30 min at 300,000 × *g*. The supernatant (70 mg protein) that contained PLD was applied to a fast protein liquid chromatography system (Pharmacia) equipped with a Hi-Trap heparin-Sepharose column (16 × 25 mm; 5 ml) that had been equilibrated with buffer B. After being washed with 75 ml of buffer B, PLD was eluted with a 100-ml linear concentration gradient (0–1.5 M) of NaCl in buffer B at a flow rate of 1 ml/min. Fractions (5 ml each) were collected. The enzyme eluted at 0.8–1.2 M NaCl was used for the present studies (see below).

Preparation of Cytosol Factor. Cytosol factor was prepared from the soluble fraction of rat kidney as described earlier (8). This factor appears to consist of multiple components as described (9).

Lipid Extraction and Fractionation. Lipids were extracted from tissue sources with chloroform and methanol (8). The chloroform phase was applied to TLC plates and developed with the upper phase of a mixture of ethyl acetate, 2,2,4-trimethylpentane, acetic acid, and water (13:2:3:10) as a solvent. Lipids were visualized under UV light after spraying of 0.01% primuline in 80% acetone. Lipids were eluted from the TLC plates with chloroform/methanol (95:5), and aliquots were dried under vacuum. The residue was weighed and suspended in buffer B by sonication and assayed for its activity to enhance PLD reaction. For further purification, the lipid was chromatographed on a μBondasphere 5-μm NH_2, 100-Å column (3.9 × 150 mm, Waters) that had been equilibrated with acetonitrile/methanol/0.2% trimethylamine at pH 3.0 (64:28:8) using an HPLC system (600E, Waters). Fractions (1 ml each) were collected at a flow rate of 1 ml/min, and an aliquot (100 μl) of each fraction was assayed for PLD activation as above.

Other Procedure. Protein was determined by the method of Bradford (10).

RESULTS

PLD and Lipid Factor. PLD associated primarily with particulate fractions was solubilized and chromatographed on a heparin-Sepharose column as described in *Materials and Methods*. When each fraction was assayed for PLD, a weak activity was detected at 0.8–1.2 M NaCl (fractions 24–28) with a recovery of less than 5% (Fig. 1). The flow-through fraction contained no detectable enzymatic activity. If, however, an aliquot of the flow-through fraction (fractions 4–10) was added to the reaction mixture, the PLD activity in fractions 24–28 was markedly enhanced (5- to 10-fold).

The stimulatory factor in the flow-through fraction was heat-stable. Fractions 4–10 from the heparin-Sepharose column were combined and extracted directly with chloroform-methanol. Most of the factor to enhance the PLD activity was recovered in the chloroform layer. A similar factor to enhance PLD appeared to be distributed widely in particulate fractions, presumably in membranes, of various tissues, including kidney, liver, and brain.

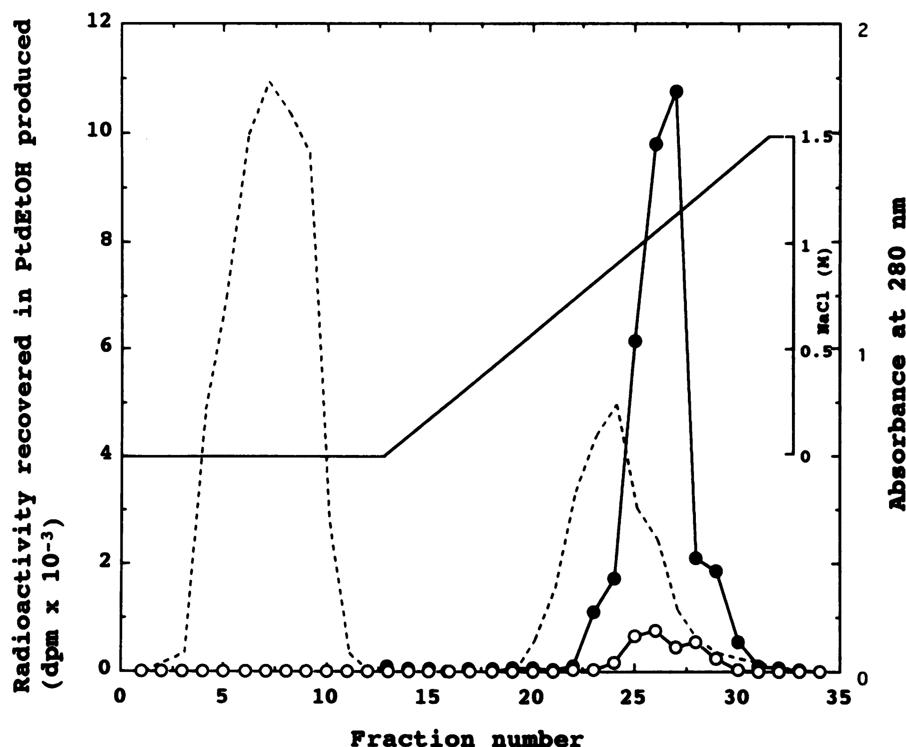

FIG. 1. Resolution of PLD and lipid factor by heparin-Sepharose column. Solubilized bovine kidney particulate fraction was chromatographed on a heparin-Sepharose column, and PLD activity of each fraction was assayed as described in *Materials and Methods*. ●, PLD activity assayed with flow-through fractions; ○, PLD activity assayed without flow-through fractions; ----, absorbance at 280 nm; —, NaCl concentration.

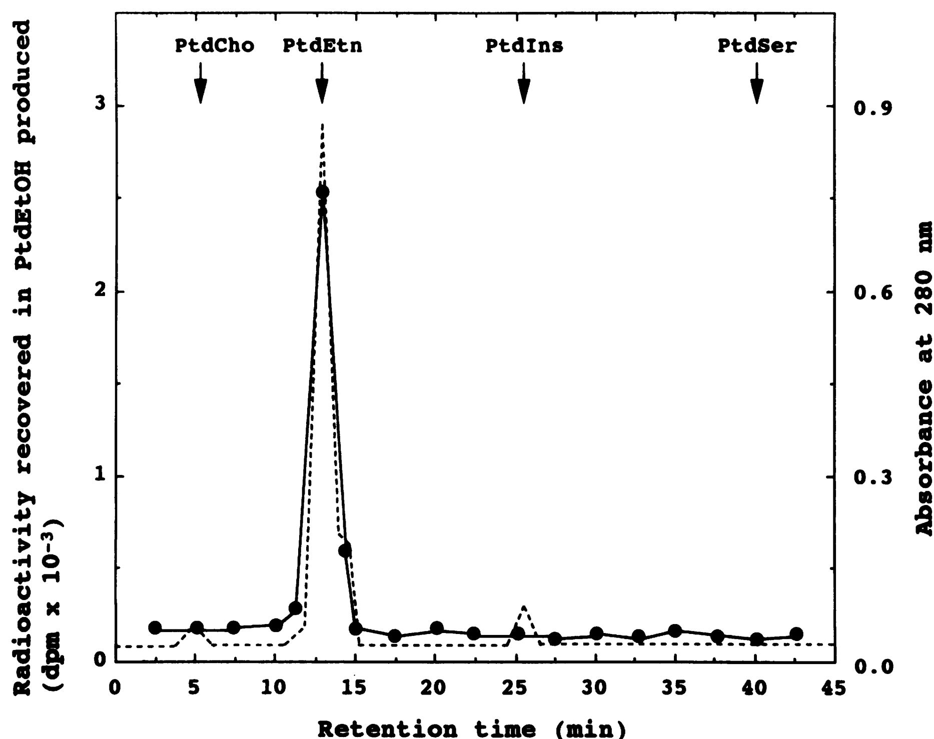

FIG. 2. Purification of lipid factor on μBondasphere, 5-μm NH_2, 100-Å column. The TLC-separated lipid factor was chromatographed on a μBondasphere, 5-μ NH_2, 100-Å column equipped with an HPLC system. An aliquot of each fraction was assayed for the ability to enhance PLD activity as described in *Materials and Methods*. ●, PLD activity; ----, absorbance at 205 nm. Arrows indicate the positions of marker phospholipids that were run in parallel experiments.

Lipid Factor as PtdEtn. The lipid fraction obtained as described above was subjected to TLC. The activity to enhance PLD was recovered from the spot that comigrated with PtdEtn. This active component was purified further by HPLC on a μBondasphere, 5-μm NH_2, 100-Å column. The peak of the activity coincided with that of PtdEtn (Fig. 2). The fractions corresponding to PtdCho, PtdIns, and PtdSer were ineffective. The recovery of this active principle was nearly 100%. In another set of experiments with authentic samples of various lipids, PtdEtn indeed enhanced the transphosphatidylation reaction from PtdCho to ethanol.

The activity of PtdEtn itself was too low to serve as substrate for PLD (see below). With a small amount (1.1 μM) of radioactive PtdCho as substrate, a large excess (up to 150 μM) of nonradioactive PtdEtn increased the radioactivity recovered in PtdEtOH produced, whereas the addition of nonradioactive PtdCho reduced it simply because of the dilution of the specific activity of radioactive PtdCho substrate (Fig. 3*A*). Inversely, with a small amount (2.2 μM) of radioactive PtdEtn as substrate, the radioactivity recovered in PtdEtOH produced was markedly reduced by the addition of nonradioactive PtdCho (Fig. 3*B*). In the presence of increasing amounts of

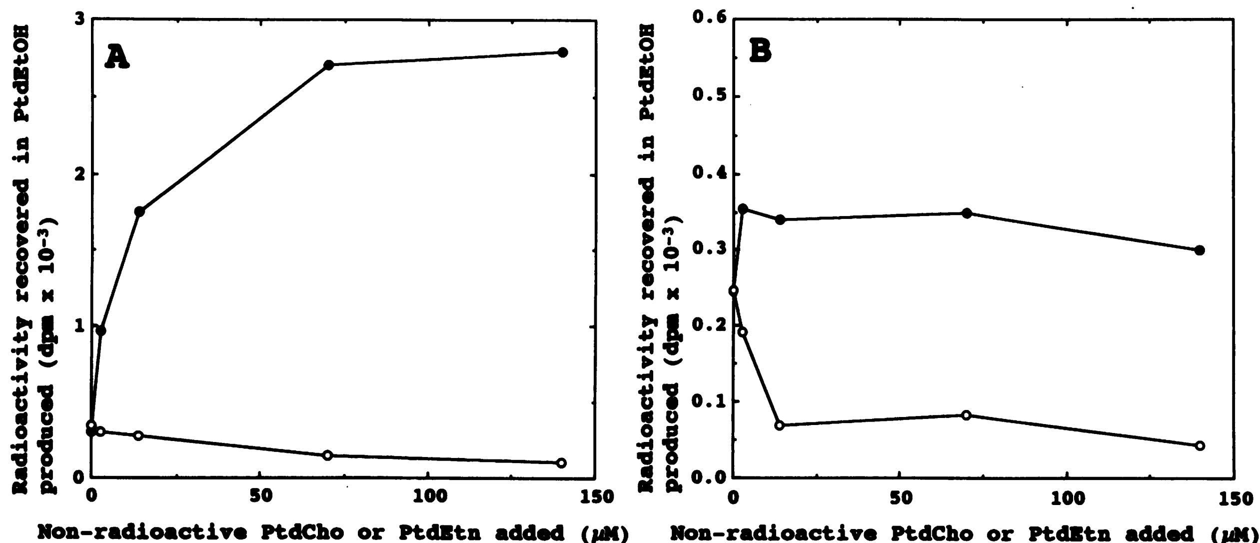

FIG. 3. Reaction velocity of PLD in coexistence of two phospholipids. Dipalmitoyl-PtdCho and L-α-palmitoyl-β-linoleoyl-PtdEtn were dried under nitrogen gas, suspended with buffer B, and sonicated for 2 min. Various concentrations of each phospholipid were added to the reaction mixture containing 1.1 μM [^{14}C]PtdCho (27,500 dpm/0.11 nmol) (*A*) or 2.2 μM [^{14}C]PtdEtn (27,500 dpm/0.22 nmol) (*B*) as radioactive substrate. Other conditions were as described in *Materials and Methods*. ●, With L-α-palmitoyl-β-linoleoyl-PtdEtn; ○, with dipalmitoyl-PtdCho.

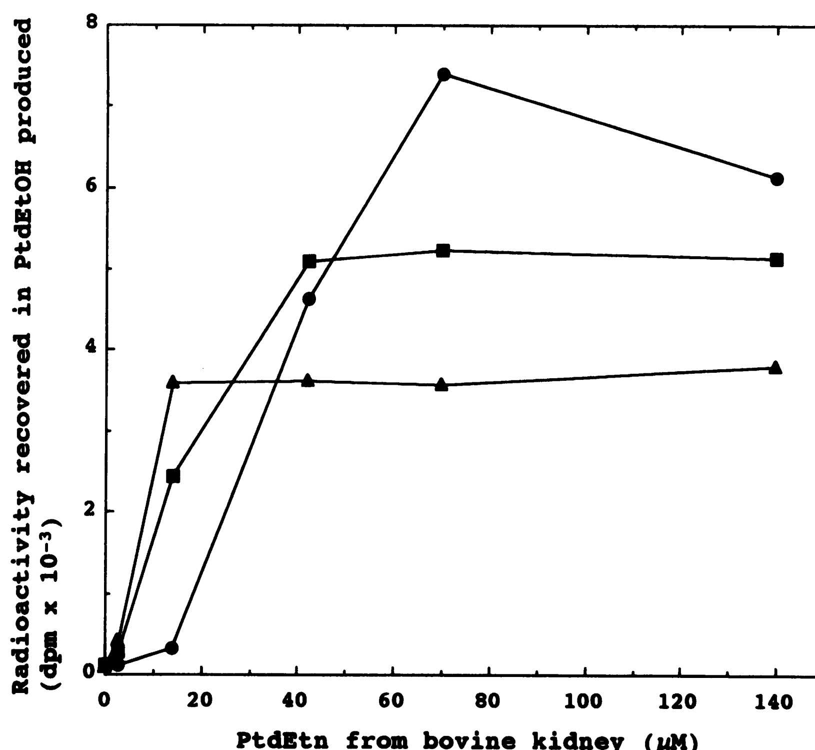

FIG. 4. Effect of various molar ratios of PtdCho and PtdEtn on reaction velocity of PLD. With three fixed concentrations of radioactive PtdCho as substrate, the reaction velocity of PLD was assayed in the coexistence of various concentrations of PtdEtn that was isolated from bovine kidney. ▲, With 20 μM PtdCho; ■, with 40 μM PtdCho; ●, with 60 μM PtdCho as substrate.

nonradioactive PtdEtn (up to 16 μM), the radioactivity recovered in PtdEtOH produced was apparently increased, probably because of the higher value (50 μM, see below) of K_m for the substrate, and then decreased (over 16 μM) because of the dilution of its specific activity (Fig. 3*B*).

The concentration of PtdEtn needed for this apparent enhancement of the transphosphatidylation reaction from PtdCho to ethanol depended on the ratio of these two phospholipids. At a given concentration of PtdCho, the maximum enhancement was observed in the presence of an approximately equimolar concentration of PtdEtn (Fig. 4). The maximum rate of reaction was obtained when [^{14}C]PtdCho and PtdEtn were mixed in chloroform before sonication to prepare the phospholipid micelles. If, however, the micelles of these two phospholipids were separately prepared and then added in the incubation mixture, the PLD reaction with PtdCho was still enhanced by the coexistence of PtdEtn to some extent (50–70%).

Several other lipids tested thus far were too inactive to enhance the PLD reaction, except for sphingomyelin, which was about 30% as active as PtdEtn (Table 1). PtdInsP_2 was too inactive to enhance the kidney PLD reaction when added alone or together with PtdCho, under the assay conditions employed for the PLD from HL-60 cells (4).

Table 1. Effect of various lipids on reaction velocity of PLD

Lipid added	PtdEtOH produced, dpm × 10^{-3}	Relative reaction velocity, %
None	0.6	100
Lipid factor	2.2	367
PtdEtn	1.9	310
PtdSer	0.6	97
PtdIns	0.6	95
PtdOH	0.5	83
PtdInsP_2	0.6	98
Sphingosine	0.6	96
Ceramide	0.6	95
Sphingomyelin	1.0	160
Cholesterol	0.6	95

The activity of PLD was assayed with [^{14}C]PtdCho (60 μM) as substrate in the coexistence of various lipids (70 μM each) indicated under the conditions described in *Materials and Methods*. Each lipid was sonicated and added to the reaction mixture. Lipid factor was isolated from bovine kidney as described in the text, and its concentration was equivalent to that of PtdEtn.

Substrate Specificity and Kinetic Properties. Several radioactive phospholipids were tested for their ability to serve as substrate for PLD in the presence of PtdEtn isolated from the bovine kidney (Table 2). It is shown that the PLD reacted preferentially with PtdCho, and PtdEtn itself was far less active as substrate. Neither PtdSer nor PtdIns could serve as substrates. The K_m value and relative reaction velocity for PtdEtn given in this table were obtained from the assay without other phospholipids. The coexistence of PtdCho in the reaction mixture markedly inhibited the enzymatic reaction, perhaps by competing with PtdEtn for the catalytically active site (Fig. 3*B*). In the absence of ethanol in the reaction mixture, the rate of hydrolysis of PtdCho to produce phosphatidic acid was

Table 2. Substrate specificity of bovine kidney PLD

Substrate	K_m, μM	Reaction velocity, pmol/min/tube
PtdCho	42	82.0
PtdEtn	50	8.5
PtdSer	—	ND
PtdIns	—	ND

The PLD activity was assayed as described in *Materials and Methods*, except that either [^{14}C]PtdCho, [^{14}C]PtdEtn, [^{14}C]PtdIns, or Ptd[^{14}C]Ser (1375–275,000 dpm/nmol) (100 μM each) was added in the presence of lipid factor (PtdEtn, 140 μM). When [^{14}C]PtdEtn was used, lipid factor was omitted. When Ptd[^{14}C]Ser was used, the PLD activity was determined by measuring the ethanol-dependent formation of radioactive serine. ND, not detected.

Table 3. Stimulatory effect of various molecular species of PtdEtn on bovine kidney PLD reaction

Additions	PtdEtOH produced, dpm × 10^{-3}	Relative reaction velocity, %
None	0.6	100
Plasmalogen-rich PtdEtn	2.8	467
Dioleoyl-PtdEtn	2.3	390
α-Palmitoyl-β-linoleoyl-PtdEtn	1.9	290
Dipalmitoyl-PtdEtn	0.5	83

Various molecular species of PtdEtn (70 μM each) were added to the reaction mixture, and PLD activity was measured with 60 μM [^{14}C]PtdCho as substrate as described in *Materials and Methods.*

approximately one-third of that of phosphatidyl transfer reaction to ethanol. The optimal pH for the PLD was 6.5–7.0. Ca^{2+} showed no effect, and Mg^{2+} was stimulatory at 1 mM, although not essential.

Active Molecular Species of PtdEtn. To enhance the PLD reaction with PtdCho as substrate, plasmalogen-rich PtdEtn was more potent than diacyl-type PtdEtn (Table 3). Among various PtdEtn molecules tested with different fatty acyl moieties, the phospholipids having at least one unsaturated fatty acid were active, but dipalmitoyl-PtdEtn was practically inactive.

DISCUSSION

By use of a unique assay procedure previously described (8) with high concentrations of ammonium sulfate, the results presented above show that PLD associated with bovine kidney particulate fractions uses PtdCho as nearly specific substrate in the presence of PtdEtn. PtdEtn itself is a poor substrate. Kinetic analysis suggests that PtdEtn does not serve simply as an activator of PLD, but modifies the micelle structure of PtdCho to facilitate the enzymatic reaction. Sphingomyelin is slightly as active as PtdEtn, but other lipids tested, including PtdSer and PtdIns, are inert. It seems plausible that the kidney PLD reacts selectively with PtdCho in the membrane lipid bilayer, and that PtdEtn serves as an essential component for the enzymatic reaction. An equimolar ratio of PtdCho to PtdEtn appears to be the most suitable for the reaction as assayed under the present conditions *in vitro*. This ratio differs from that generally found in natural membranes, and further analysis is needed to verify the above interpretation. Several reports from other laboratories have shown that PtdInsP_2 may act as an activator of PLD (4, 11, 12). In the reaction of bovine kidney PLD, PtdInsP_2 does not exert an effect in the presence or absence of either PtdEtn or other lipids, although the enzyme requires ADP-ribosylation factor and GTP[γS] for catalytic activity. It is suggestive that each member of the phospholipids in cell membranes shows different susceptibility to various phospholipases and plays a distinctly different role in cell signaling.

Mammalian PLD appears to be heterogeneous (3, 13, 14). Ca^{2+}-activated, unsaturated fatty acid-dependent PLD has been found in rat brain membranes (3, 6) and pig lung microsomes (7). A cytosolic PLD that hydrolyzes PtdCho as well as PtdEtn and PtdIns has been reported (14). It remains unclear whether these types of PLD occur in the bovine kidney.

Note Added in Proof. With a yeast PtdCho-specific PLD gene (*SPO14*) as a probe, a mammalian cDNA encoding PLD with a molecular mass of about 120 kDa has recently been isolated. The relationship between this enzyme and that described herein remains unexplored.

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