Purification and Characterization of Phosphatidylcholine Phospholipase D from Pig Lung* 

(Received for publication, August 16, 1994)

Shin-ichi Okamura and Satoshi Yamashita†

*From the Department of Biochemistry, Gunma University School of Medicine, Maebashi 371, Japan

Phospholipase D, which mediates phosphatidylcholine hydrolysis in response to agonist stimulation, is an important component of signal transduction. We now report the purification of this enzyme to homogeneity from pig lung microsomes. The enzyme was solubilized with heptylthiogluconolide and purified 2,200-fold by successive chromatography on sulfate-Celulofine, ether-Toyopearl, chelate-Toyopearl, Q-Sepharose, heparin-Toyopearl, and hydroxyapatite. The final enzyme preparation gave a single protein band of Mr = 190,000 on SDS-polyacrylamide gel electrophoresis. The enzyme hydrolyzed phosphatidylcholine but not lysophosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Optimum pH was 6.6. Half-maximal activity was obtained at 0.8 mM dipalmitoylglycerophosphocholine. The products were identified as phosphatidic acid and choline, but in the presence of ethanol, phosphatidylethanol was produced at the expense of phosphatidic acid. Ethanolamine and serine were not utilized as the phosphatidyl acceptor. Although not obligatory, Ca2+ and Mg2+ were stimulatory at high concentrations. The enzyme was markedly stimulated by unsaturated fatty acids in the presence of Mg2+ but not in its absence or by saturated fatty acids. N-Ethylmaleimide and detergents were inhibitory. Sucrose monolaurate had an aberrant effect on enzyme activity.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) in response to a variety of hormones, neurotransmitters, and growth factors and is believed to play a fundamental role in signal transduction of cells (for reviews, see Pelech and Vance (1989), Billah and Anthes (1990), and Exton (1990, 1994)). The primary lipid product of PLD is phosphatidic acid (PA), and a major metabolic fate of PA is PA-phosphatase-mediated dephosphorylation to form diacylglycerol (DAG) (Smith et al., 1987; Billah et al., 1989; Gruchalla et al., 1990; Qian and Drewes, 1990; Siddiqui and Exton, 1992; Kanoh et al., 1992), representing an alternative pathway for DAG formation to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (Thompson and Dawson, 1964). DAG formation from PC causes a delayed, prolonged increase in DAG as compared with an early but transient increase in DAG produced by hydrolysis of inositol phospholipids (Wright et al., 1988; Motozaki and Williams, 1989; Nakashima et al., 1991; Leach et al., 1991; van Blitterswijk et al., 1991). Thus the agonist-induced stimulation of PLD elicits prolonged activation of specific isoforms of protein kinase C and thereby plays a role in long term cellular responses, such as proliferation and differentiation (Asaoka et al., 1992; Nishizuka, 1992). Furthermore, recent studies demonstrated that PA, the direct product of PLD, acts as an activator for protein kinase. Bocckino et al. (1991) demonstrated PA-dependent protein phosphorylation with rat tissue extracts and suggested the existence of a PA-dependent protein kinase(s). A DAG-independent isoform of protein kinase C, θ, was purified and shown to be activated by PA (Nakanishi and Exton, 1992). More recently, Khan et al. (1994) partially purified and characterized a new protein kinase from human platelets, which was also activated by PA. The kinase was different from any of the currently identified protein kinase C isoforms since it did not cross-react with antibodies raised against them. The identification of PA-dependent protein kinases has suggested a new role for PLD in the protein kinase cascade of cell signaling.

Only limited information is available about the molecular properties of PLD because the enzyme has never been highly purified. Taki and Kanfer (1979) purified PLD 240-fold from freeze-dried rat brain after solubilization with Mircanol H2M and cholate. The specific activity of their preparation was very low, 2 nmol min⁻¹ mg protein⁻¹, partly because the activator of PLD was not known at that time. Their preparation utilized not only PC but also phosphatidylethanolamine (PE) with pH optimum at 6.0. Later, Chalifour and Kanfer (1982) identified unsaturated fatty acid as an activator of PLD in rat brain microsomes. Although the enzyme was located in the membrane fraction in rat tissues (Chalifour and Kanfer, 1982; Kobayashi and Kanfer, 1987) and Madin-Darby canine kidney cells (Huang et al., 1992), Wang et al. (1991) demonstrated that in bovine tissues a majority of enzyme activity was cytosolic. The cytosolic enzyme was purified 20-fold and shown to hydrolyze various phospholipids in the order of PE > PC > PI. The enzyme had a high Kᵦ for the PE species whereas the membrane-bound enzyme was specific to PC with a low Kᵦ. The cytosolic and membrane-bound PLDs are believed to be different isoforms.

The present study was undertaken to obtain a highly purified preparation of membrane-bound PLD. We used pig lung as the enzyme source because lung contains the highest PLD activity (Chalifour and Kanfer, 1982; Kobayashi and Kanfer, 1987; Wang et al., 1991). The purified PLD was a 190-kDa protein. It catalyzed not only hydrolysis but also transphosphatidylation and selectively utilized PC as substrate. Unsaturated fatty acids were a potent activator of the enzyme. Unlike base-exchange enzyme (Kanfer, 1972), PLD catalyzed transphosphatidylation in the absence of Ca²⁺.

* This work was supported in part by grants-in-aid for scientific research and cancer research from the Ministry of Education, Science, and Culture, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel: 81-272-20-7940; Fax: 81-272-20-7946.
Materials—Sulfate-Cellulofine and Gigapite (hydroxyapatite) were purchased from Seikagaku Kogyo (Tokyo, Japan). Ether-Toyopearl and heparin-Toyopearl were obtained from Toso (Tokyo, Japan), and Q-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Sucrose monolaurate, n-heptyl-β-o-thioglucoside, n-octyl-β-D-glucoside, and other detergents except for Triton X-100 were from Dojindo Laboratories (Kumamoto, Japan). Ether-Toyopearl, chelate-Toyopearl, and Gigapite were obtained from Toso (Tokyo, Japan). Ether-Toyopearl, chelate-Toyopearl, and heparin-Toyopearl were obtained from Toso (Tokyo, Japan), and Q-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Sucrose monolaurate, n-heptyl-β-o-thioglucoside, n-octyl-β-D-glucoside, and other detergents except for Triton X-100 were from Dojindo Laboratories (Kumamoto, Japan). Ether-Toyopearl, chelate-Toyopearl, and Gigapite were obtained from Toso (Tokyo, Japan).

EXPERIMENTAL PROCEDURES

PLD Assay—1,2-Dipalmitoyl-[methyl-3H]GPC was mixed with 1,2-dipalmitoyl-GPC, suspended in 0.05% Triton X-100 to 10 mM by sonication, and used as the substrate. The standard assay mixture contained 50 mM sodium diethylglutarate, pH 7.0, 1 mM dithiothreitol, and enzyme in a total volume of 0.1 ml. Reaction was started by the addition of 1,2-dipalmitoyl-GPC, allowed to proceed for 20 or 40 min at 30 °C, and terminated by the addition of 3 ml of chloroform/methanol (2:1) and 0.6 ml of saline. The mixture was shaken and centrifuged at 600 × g for 5 min. The upper layer was quantitatively transferred to a tube, shaken with 2 ml of butyronitrile containing 30 mg/ml tetraphenylboron and 0.5 ml of 70 mM sodium phosphate, pH 7.2, and centrifuged at 600 × g for 5 min. The upper phase containing the released choline was transferred to a vial, and radioactivity was counted in a toluene/Triton X-100 scintillator with a Beckman LS7000 liquid scintillation spectrometer.

Purification of PLD from Pig Lung—All procedures were carried out at 4 °C. Pig lung was minced and homogenized in 3 volumes of 0.25 M sucrose containing 5 mM Hepes-HCl, pH 7.2, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1,000 × g for 10 min to remove cell debris and then at 12,000 × g for 20 min. The supernatant was further centrifuged at 100,000 × g for 60 min. The resultant pellet (microsomes) was suspended in a minimal volume of the homogenizing buffer and kept at −80 °C until use.

Thawed microsomes were adjusted to 10 mg of protein/ml with 10 mM Hepes-HCl, pH 7.2, containing 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.02% NaN3. Following the addition of heptylthiogalactoside to a final concentration of 2%, the mixture was gently stirred for 30 min and then centrifuged at 100,000 × g for 60 min. The supernatant was carefully withdrawn, leaving the fluffy layer behind, and then applied to a sulfate-Cellulofine column (2.5 × 5 cm) equilibrated with buffer A containing 1.6 M ammonium sulfate. The column was eluted with a reverse gradient of ammonium sulfate from 1.6 to 0 M in 120 ml of buffer A and subsequently with 30 ml of buffer A. Elute containing activity was

---

**FIG. 1.** Chromatography of pig lung phospholipase D. A, sulfate-Cellulofine; B, chelate-Toyopearl; C, Gigapite. PLD activity was assayed as described under “Experimental Procedures.” The thick lines above the activity peaks indicate the fractions that were pooled. D, SDS-PAGE. Fractions from Gigapite column chromatography were subjected to SDS-PAGE on a 10% gel under the reducing conditions, followed by silver staining. Lanes 1–3, neighboring fractions from Gigapite with activity peak at lane 2. Lane 4, molecular mass markers (from top to bottom): myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).
adjusted to pH 8.0 with 1 mM K$_2$HPO$_4$ and applied to a zinc-treated chelate-Toyopearl column (1 x 5 cm) equilibrated with buffer A that contained 10 mM Tris·HCl, pH 8.0, instead of potassium phosphate buffer, and 0.5 mM NaCl. Enzyme was eluted with a 60-ml linear histidine gradient (0–25 mM) constructed in the same buffer, diluted with 2 volumes of buffer B (20 mM Tris·HCl, pH 8.0, 0.06% sucrose monolaurate, and 0.02% NaN$_3$), and then adsorbed to a Q-Sepharose column (0.7 x 5 cm) equilibrated with buffer B containing 0.15 mM NaCl. The column was eluted with a 30-ml linear NaCl gradient (0.15–1 M) in buffer B. Active fractions were combined, diluted with 1 volume of buffer A, applied to a heparin-Toyopearl column (0.7 x 7 cm) equilibrated with buffer A containing 0.2 mM NaCl, and then eluted with a 40-ml linear NaCl gradient (0.2–1.6 M) in buffer A. Eluted enzyme was adsorbed to a 0.8-ml Gigapite column. The loaded column was sequentially eluted with 16 ml linear gradient of potassium phosphate (0.04–1 M) in buffer A and then with 5 ml of 1 M potassium phosphate in buffer A to obtain the purified enzyme.

Identification of the Reaction Products—Enzyme was incubated with 1,2-dipalmitoyl-[methyl-3H]GPC or 1-palmitoyl-2-[3H]palmitoyl-GPC under the standard assay conditions. The reaction mixture was mixed with 3 ml of chloroform/methanol (2:1) and shaken with 0.6 ml of saline. When 1,2-dipalmitoyl-[methyl-3H]GPC was used as substrate, the upper phase was saved for the analysis of the water-soluble product, concentrated, and separated by thin-layer chromatography (TLC) on a Silica Gel 60 plate (Merck, Darmstadt, Germany) with methanol, 0.6% acetic acid; PEt, phosphatidylethanol. The numbers below the lanes indicate the fraction numbers from the Gigapite column. Control indicates the mock reaction without enzyme.

Solubilized enzyme was first separated on a sulfate-Cellulofine column (Fig. 1A). Over 99% of protein passed through the column while about 40% of the PLD activity was adsorbed to the column. PLD was eluted with a NaCl gradient, resulting in 64-fold purification with 32% yield. The eluted enzyme was applied to a hydrophobic column, ether-Toyopearl. The use of hydrophobic adsorbents with larger aliphatic or aromatic hydrocarbon chains resulted in low yields. The ammonium sulfate eluate was then loaded onto a chelate column that had been pretreated with ZnCl$_2$ (panel B). PLD could be eluted by a gradient increase in histidine concentration. Probably because of inhibition by contaminated zinc (see below), total activity was decreased to 15% but was restored considerably after Q-Sepharose chromatography. As the final step, we used hydroxyapatite (panel C). Overall, PLD was purified 2,200-fold from pig lung microsomes. When examined at a fixed microsomal protein concentration of 10 mg/ml, this nonionic detergent was most effective at 2% with a recovery of 70–100% PLD activity in the 100,000 x g supernatant. Octylglucoside, octylthioglucoside, and Mega-9 (n-nonanoyl-N-methylglucamide) gave less satisfactory results. Triton X-100, CHAPS, CHAPSO, Mega-8 (n-octanoyl-N-methylglucamide), and Mega-10 (n-decanoyl-N-methylglucamide) yielded poor results. After solubilization with heptylthioglucoside, PLD was subjected to sequential column chromatographic procedures. To keep enzyme soluble and minimize nonspecific adsorption, we included a low concentration of sucrose monolaurate (0.06%) in all solutions used for column chromatography.
and the radioactive lipids were separated by TLC. As shown in
Fig. 2A, the radioactive lipid produced in the absence of ethanol
comigrated with authentic PA. In the presence of ethanol, how-
ever, the same fractions catalyzed the formation of phosphati-
dylethanol at the expense of PA. It should be noted that phos-
phatidylethanol formation occurred in the absence of Ca²⁺ (see
"Experimental Procedures"). These results provide conclusive
evidence for the widely accepted view that mammalian PLD
catalyzes not only hydrolysis but transphosphatidylation as
well (Kobayashi and Kanfer, 1987; Gustavsson and Alling,
1987; Bocckino et al., 1987b). Unlike transphosphatidylation
catalyzed by base-exchange enzyme (Kanfer, 1972), ethanol-
amine and serine were not utilized as the phosphatidyl accep-
tors (data not shown).

To identify the water-soluble product, purified PLD was in-
cubated with 1,2-dipalmityl-[methyl-³H]GPE, the upper layer
of chloroform/methanol extraction was separated by TLC, and
the chromatogram was analyzed for radioactive product as de-
scribed under "Experimental Procedures." Radioactivity comi-
grated with authentic choline, and no radioactivity was asso-
ciated with phosphocholine and GPC (data not shown). Thus,
choline was the sole water-soluble product. In the routine PLD
assay, released choline was extracted from the upper layer of
chloroform/methanol extraction with butyronitrile in the pres-
ence of tetraphenyl-boron (Murray et al., 1990) and counted
(see "Experimental Procedures"). Taken together, these results
demonstrate that the purified enzyme catalyzed the hydrolysis
of PC to PA and choline and, in the presence of ethanol, the
formation of phosphatidylethanol.

Properties of Purified PLD—The molecular mass of purified
PLD was estimated to be 190 kDa by SDS-PAGE under the
reducing conditions (Fig. 1D). SDS-PAGE performed under the
nonreducing conditions gave the same results (data not shown).
PLD showed an anomalous behavior in gel filtration matrices,
such as Sephacryl S-200, S-300, and S-400 (Pharmacia), Toyop-
pearl HW65 (Toso), and Protein Pak G-300 (Waters, Milford,
MA). Its elution was markedly retarded in these gels probably
because of interaction with the gels. Thus it was difficult to
determine the native molecular mass by gel filtration. As
shown in Fig. 3, PLD showed a rather sharp pH profile with
maximum activity at pH 6.6 in sodium dimethylglutarate
buffer. A similar pH profile was obtained with sodium phos-
phate buffer. Activities measured in these two buffers were not
significantly different. Similar pH optima were reported for
partially purified rat brain PLD (Taki and Kanfer, 1979) and
detergent-solubilized bovine lung PLD (Wang et al., 1991). Chol-
ine formation was proportional to the incubation time up to 80
min under the standard assay conditions. Fig. 4 shows the
effect of varying concentrations of dipalmityl-GPC on enzyme
activity. PLD did not follow a normal saturation kinetics. The
substrate dependence curve was sigmoidal as previously noted
with rat brain synaptosomes (Kobayashi and Kanfer, 1987).
Half-maximal activity was attained at 0.8 mM dipalmityl-
GPC. This value was close to the Kₘ value measured with
partially purified rat brain enzyme, 0.75 mM (Taki and Kanfer,
1979).

Effects of Divalent Cations and Detergents—Ca²⁺ has been
implicated in the activation of PLD (Augert et al., 1989; Huang
et al., 1991; Kanaho et al., 1992). In HL-60 cells and neu-
rophils, Ca²⁺ was required for G protein-mediated activation
of PLD (Antal et al., 1991; Olson et al., 1991; Geny et al., 1993;
Brown et al., 1993; Cockcroft et al., 1994). Ca²⁺ requirement
was also demonstrated in the protein kinase C-dependent ac-
tivation, for example, of CCL39 cell membrane PLD (Conricode
et al., 1994). We were interested in examining whether Ca²⁺
had a direct effect on PLD enzyme. As shown in Fig. 5, purified
enzyme exhibited no absolute requirement for Ca²⁺ and Mg²⁺.
EDTA had no effect. Low Ca²⁺ and Mg²⁺ had no significant
effect, but higher Ca²⁺ and Mg²⁺ increased PLD activity to a
maximum of 2.5- and 1.7-fold at 1 and 2 mM, respectively. Zn²⁺
was inhibitory. Other divalent cations, such as Cu²⁺, Co²⁺, and
Ni²⁺, were also inhibitory (data not shown). These results are
fairly well consistent with the observations obtained with par-
tially purified rat brain PLD and synaptosomal membrane PLD
(Taki and Kanfer, 1979; Chalifa et al., 1990).

Several reports showed the stimulation of PLD activity by
detergents (Chalifour and Kanfer, 1982; Kobayashi and Kanfer,
1987; Kanoh et al., 1991; Huang et al., 1992). We examined the
effects of some detergents on purified enzyme. Although purified
enzyme already contained 0.06% sucrose monolaurate, its
final concentration in the assay mixture was calculated to be
0.012%, a concentration considerably lower than its critical
micellar concentration, 0.021%. Thus the effect of the endoge-
Purification of Phospholipase D

The optimal oleate concentration varied somewhat with the concentration of PC and amount of enzyme protein present in the assay mixture. In Table II the effects of various fatty acids were compared at fixed concentrations of 2 mM. The data show that all the unsaturated fatty acids examined were effective, whereas all the saturated fatty acids were ineffective. Arachidonic acid was the most effective fatty acid. Interestingly, Mg²⁺ was needed for maximal activation of PLD by fatty acid (Fig. 7). The stimulatory effects of Mg²⁺ and Ca²⁺ (see above) required the presence of fatty acid (data not shown).

We next examined the effects of various complex lipids on PLD activity by adding the lipids at a concentration of 0.1 mM to the standard reaction mixture instead of oleic acid. Whereas lyso-PC, PE, PI, PA, and DAG were inhibitory to the enzyme, lyso-PA and Pip₂ slightly stimulatory (about 20% increase in activity). This weak stimulatory effect of Pip₂ was unexpected because Brown et al. (1993) reported that HL-60 PLD was strongly stimulated by this phospholipid. This discrepancy may be caused by different isoforms of PLD present in HL-60 cells and lung or simply by different assay conditions. Phosphatidylserine had no effect.

Substrate Specificity—We examined the activity of purified PLD toward different phospholipids. Enzyme was incubated with 1 μM 1-palmitoyl-2-[1-¹⁴C]palmitoyl-GPC, 1-acyl-2-[1-¹⁴C]-arachidonoyl-sn-glycero-3-phosphoethanolamine, 1-1-¹⁴C-palmitoyl-GPC, and 1,2-diacyl-sn-glycero-3-phosphoil-⁴H]inositol under the standard assay conditions, and we examined

**Fig. 5. Effects of divalent cations.** Assay was carried out under the standard assay conditions except that Mg²⁺ (●), Ca²⁺ (○), or Zn²⁺ (◊) of the indicated concentrations was present in the assay mixture.

**Fig. 6. Effects of detergents.** Purified enzyme (10 ng) was assayed in the presence of varying concentrations of detergents as described under “Experimental Procedures.” Detergents used were sucrose monolaurate (●) and Triton X-100 (○).

**Fig. 7. Effect of oleic acid.** The assay was carried out with 19 ng of enzyme as described under “Experimental Procedures” except that varying concentrations of oleic acid were added in the presence (●) and absence (○) of Mg²⁺.

**Table II**

| Fatty acid             | Relative activity |
|------------------------|-------------------|
| None                   | 100               |
| Palmitic acid          | 46                |
| Stearic acid           | 60                |
| Oleic acid             | 300               |
| Linoleic acid          | 182               |
| Arachidonic acid       | 490               |

The optimal oleate concentration varied somewhat with the concentration of PC and amount of enzyme protein present in the assay mixture. In Table II the effects of various fatty acids were compared at fixed concentrations of 2 mM. The data show that all the unsaturated fatty acids examined were effective, whereas all the saturated fatty acids were ineffective. Arachidonic acid was the most effective fatty acid. Interestingly, Mg²⁺ was needed for maximal activation of PLD by fatty acid (Fig. 7). The stimulatory effects of Mg²⁺ and Ca²⁺ (see above) required the presence of fatty acid (data not shown).

We next examined the effects of various complex lipids on PLD activity by adding the lipids at a concentration of 0.1 mM to the standard reaction mixture instead of oleic acid. Whereas lyso-PC, PE, PI, PA, and DAG were inhibitory to the enzyme, lyso-PA and Pip₂ slightly stimulatory (about 20% increase in activity). This weak stimulatory effect of Pip₂ was unexpected because Brown et al. (1993) reported that HL-60 PLD was strongly stimulated by this phospholipid. This discrepancy may be caused by different isoforms of PLD present in HL-60 cells and lung or simply by different assay conditions. Phosphatidylserine had no effect.

Substrate Specificity—We examined the activity of purified PLD toward different phospholipids. Enzyme was incubated with 1 μM 1-palmitoyl-2-[1-¹⁴C]palmitoyl-GPC, 1-acyl-2-[1-¹⁴C]-arachidonoyl-sn-glycero-3-phosphoethanolamine, 1-1-¹⁴C-palmitoyl-GPC, and 1,2-diacyl-sn-glycero-3-phosphoil-⁴H]inositol under the standard assay conditions, and we examined
whether radioactive PA or lyso-PA could be formed from the former three phospholipids by TLC and autoradiography. When 1,2-diacyl-sn-glycero-3-phospho(2-3H)inositol was used as substrate, the incubation mixture was shaken with 3 ml of chloroform/methanol (2:1) and 0.6 ml of saline, and the aqueous phase was used to examine whether or not radioactive inositol was formed. But the release of radioactive inositol could not be demonstrated (data not shown). As shown in Fig. 8, PA was liberated from PC but not from PE. Lyso-PA formation from lyso-PC could not be demonstrated. Thus PE, lyso-PC, and PI were not utilized by the enzyme. These results clearly show that the enzyme was specific to PC.

FIG. 8. **Substrate specificity.** Purified enzyme (19 ng) was incubated with the indicated substrate (1 mM), and the reaction products were identified by TLC and autoradiography as described under “Experimental Procedures.” Substrates used were phosphatidylcholine (lanes 1–3), phosphatidylethanolamine (lanes 4–6), and lyso phosphatidylcholine (lanes 7–9). Control experiments were performed without enzyme (lanes 3, 6, 9). Arrows indicate the locations of the authentic standards: PA, phosphatidic acid; LPA, lyso phosphatidic acid.

**DISCUSSION**

The present study has shown that PLD can be efficiently solubilized from lung microsomes with a nonionic detergent, heptylthioglycoside. The solubilized enzyme was purified to homogeneity by sequential chromatographic procedures in the presence of sucrose monolaurate. The molecular mass of the purified enzyme was considerably large, 190 kDa. A similar value, 200 kDa, was also reported for the molecular mass of rat brain PLD determined by gel filtration (Taki and Kanfer, 1979). The enzyme is Ca2+-dependent and cytosolic, thus different from the present PLD. These findings, together with the present results, suggest that there are several PLDs with different substrate specificities. The present enzyme should be adequately called phosphatidylcholine phospholipase D. An additional finding of some interest is the lack of activity toward lyso-PC in the purified enzyme. Recently, lyso-PA has attracted much attention as an intercellular signaling molecule (van Corven et al., 1989; van de Bend et al., 1993). This phospholipid is thought to be synthesized from PA by the action of a specific phospholipase A2 in platelets (Billah et al., 1981; Gerrard and Robinson, 1989) and secreted in response to stimuli (Eichholtz et al., 1993). Theoretically, sequential action of, first, phospholipase A2 and, second, PLD on PC could be considered as an alternative route for the synthesis of lyso-PA, but the present results seem to exclude this possibility.

**REFERENCES**

Anthes, J. C., Wang, P., Siegel, M. I., Egan, R. W., and Billah, M. M. (1991) Biochem. Biophys. Res. Commun. **175**, 236–243.

Assecia, Y., Nakamura, S., Yoshida, K., and Nishizuka, Y. (1992) Trends Biochem. Sci. **17**, 414–417.

Augert, G., Bocckino, S. B., Blackmore, P. F., and Exton, J. H. (1989) **J. Biol. Chem.** **264**, 21689–21698.

Balsinde, J., Diez, E., Fernandez, B., and Mollinedo, F. (1989) Eur. J. Biochem. **186**, 717–724.

Billah, M. M., Lapetina, E. G., and Cuatrecasas, P. (1981) J. Biol. Chem. **256**, 5399–5403.

Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. W., and Siegel, M. I. (1989) J. Biol. Chem. **264**, 17069–17077.

Billah, M. M., and Anthes, J. C. (1990) Biochem. J. **269**, 281–291.

Bocckino, S. B., Blackmore, P. F., Wilson, P. B., and Exton, J. H. (1987a) J. Biol. Chem. **262**, 15309–15315.

Bocckino, S. B., Wilson, P. B., and Exton, J. H. (1987b) FEMS Lett. **255**, 201–204.

Bocckino, S. B., Wilson, P. B., and Exton, J. H. (1991) Proc. Natl. Acad. Sci. U. S. A. **88**, 6210–6213.
Purification of Phospholipase D

31213

Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
Brown, H. A., Gutowski, S., Moonaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137-1144
Chalfie, V., Mohn, H., and Liscovitch, M. (1990) J. Biol. Chem. 265, 17512-17519
Chalifour, R., and Kanfer, J. N. (1982) J. Neurochem. 32, 299-305
Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, J., Hiles, I., Totty, N. F., Truong, O., and Hassan, J. J. (1994) Science 263, 523-526
Con ricorde, K. M., Smith, J. L., Burns, D. J., and Exton, J. H. (1994) FEBS Lett. 343, 149-153
Daniel, L. W., Waite, M., and Wykle, R. L. (1986) J. Biol. Chem. 261, 9126-9132
Deems, R. A., Eaton, B. R., and Dennis, E. A. (1975) J. Biol. Chem. 250, 9013-9020
Deems, R., Tally, T., Fahrenfort, I., and Modenaer, W. H. (1995) EMBO J. 14, 677-686
Exton, J. H. (1991) Annu. Rev. Physiol. 53, 661-683
Exton, J. H. (1991) Biochem. Biophys. Res. Commun. 182, 454-460
Guy, M. R., and Marchesi, V. (1995) Nature 375, 629-631