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Identification of Lipid Inhibitor of Mammalian Phospholipase D[1]

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Phospholipase D (PLD) is implicated in important cellular processes, such as hormone action, inflammation, secretion, mitogenesis, and neural activity. Recent studies using cell-free systems have shown that the enzyme activity is modulated by both positive and negative regulators. During an attempt to purify PLD from pig colon mucosa, we noted the presence of a PLD inhibitor in the tissue extract. The inhibitor was purified and identified as comprising lysophosphatidylserine, phosphatidylinositol, and lysophosphatidylinositol, of which lysophosphatidylserine was the most potent. These lipids affected all of the PLD isoforms examined, oleate-dependent PLD, ARF-dependent PLD (PLD1a, PLD1b), and phosphatidylinositol 4,5-bisphosphate-dependent PLD (PLD2), in the concentration range of the 1 or 10 μM order. In contrast to lysophosphatidylserine, the diacyl counterpart phosphatidylserine was without effect in the same concentration range. PLD inhibition by lysophosphatidylserine could not be reversed by an increase in the concentration of the substrate phosphatidylcholine or activator phosphatidylinositol 4,5-bisphosphate.

Key words: inhibitor, lysophosphatidylinositol, lysophosphatidylserine, phosphatidylinositol, phospholipase D.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid and choline. A variety of signal molecules, such as hormones, neurotransmitters, growth factors, cytokines, and antigens induce the activation of PLD in various tissues and cells. The product phosphatidic acid is known to produce various biological effects, such as mitogenesis (*1*), cytoskeletal rearrangement (*2*), coatmer assembly (*3*), and modulation of the activities of a number of key enzymes (*4-12*). Furthermore, phosphatidic acid is metabolized to well known biomodulators, diacylglycerol (*13*) and lysophosphatidic acid (*14*). Thus, PLD has been implicated in a broad spectrum of physiological and pathological processes, including hormone action, inflammation, secretion, mitogenesis, oncogenesis, neural, and cardiac stimulation, and senescence.

Although the signalling pathway whereby receptor stimulation leads to the activation of PLD is not well understood, activation of PLD enzyme *in vitro* is effected by unsaturated fatty acid (*15, 16*), small GTP-binding proteins (G proteins) such as ARF (*17, 18*), RhoA (*19, 20*), Rac1 (*20-22*), and RalA (*23*), phosphatidylinositol 4,5-bisphosphate (PIP_2) (*24*), and protein kinase C (*22, 25-27*). Furthermore, the occurrence of negative regulators of PLD was recently described. The PLD inhibitors reported include not only such proteins as fodrin (*28, 29*), synaptojanin (*30, 31*), 30-kDa protein (*32*), and clathrin assembly protein 3 (*33*), but also some lipids. Massenburg *et al.* (*34*) showed that the unsaturated fatty acid that is required for the activity of an isoform of PLD (oleate-dependent PLD) inhibited ARF-dependent PLD, phospholipase D1 (PLD1) (*35*), whereas PIP_2 which is a potent activator of PLD1 (*35*) was a potent inhibitor of oleate-dependent PLD (*36*). Ceramide was shown to prevent the protein kinase C-mediated activation of PLD extracted from HL-60 cells (*37*). Thus, the regulation of PLD activity may include not only positive, but also negative mechanisms.

During an attempt to purify oleate-stimulated PLD from pig colon, we observed a marked increase in the total activity of PLD after sulfate-Cellulofine chromatography, suggesting the removal of an inhibitor by the chromatography. We purified this inhibitor from pig colon and identified it as consisting of lysophosphatidylserine (lysoPS), lysophosphatidylinositol (lysoPI), and phosphatidylinositol (PI). These phospholipids were found to inhibit different types of PLD, oleate-stimulated PLD (*16*), ARF-stimulated PLDs (PLD1a, PLD1b) (*22, 35*), and PIP_2-stimulated PLD (PLD2) (*38, 39*). LysoPS strongly inhibited PLD, whereas phosphatidylserine (PS) was without effect. PI and its deacylated form, lysoPI, were inhibitory to PLD, although PIP_2 is an obligatory activator of PLDs (PLD1, PLD2). The results obtained here are consistent with the

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Abbreviations: PLD, phospholipase D; PC, phosphatidylcholine; G protein, GTP-binding protein; ARF, ADP-ribosylation factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD1, phospholipase D1; lysoPS, lysophosphatidylserine; lysoPI, lysophosphatidylinositol; PI, phosphatidylinositol; PLD2, phospholipase D2; PS, phosphatidylserine.

view that conversion of PIP_2 to PI and lysoPI, and of PS to lysoPS have negative effects on PLD activity.

MATERIALS AND METHODS

Preparation of Pig Colon Microsomes—All procedures were carried out at 4°C. A fresh pig colon was obtained from a local slaughterhouse. Colon mucus was removed and washed with distilled water. The mucosa was 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 with a Polytron blender (Kinematica, Littau, Switzerland), and centrifuged at 10,000 × *g* for 30 min. The supernatant was further centrifuged at 100,000 × *g* for 60 min. The resultant pellet was suspended in a minimal volume of the homogenizing buffer and kept at −80°C until use.

Isolation and Identification of PLD Inhibitor from Colon Microsomes—Thawed microsomes were adjusted to 10 mg protein/ml with 10 mM Hepes-HCl, pH 7.2, containing 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3. Following the addition of sucrose monolaurate to a final concentration of 2%, the mixture was gently stirred for 60 min, and then centrifuged at 100,000 × *g* for 60 min. The supernatant was carefully withdrawn and applied to a Q-Sepharose column (1.5 × 12 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, 0.06% sucrose monolaurate, 0.02% (w/v) NaN_3, and 0.2 mM phenylmethylsulfonyl fluoride. The inhibitor was eluted with a 90-ml linear NaCl gradient (0-2 M) in the same solution. Inhibitory fractions were combined, and extracted with a chloroform-methanol mixture by the method of Bligh and Dyer (*40*). The lower phase was concentrated and separated by TLC with chloroform/methanol/acetic acid/water (50:25:4:8, by volume). The lipid bands were scraped off the plate and lipids were extracted with a chloroform-methanol mixture. A portion was dispersed in distilled water using a Bransonic 220 sonicator (Branson Ultrasonic, Danbury, CT, USA) and assayed for PLD inhibitory activity. The inhibitory lipids were further fractionated by TLC with tetrahydrofuran/acetone/methanol/water (50:20:40:8, by volume) or chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5, by volume) and identified using reference markers.

Assays of PLD and PLD Inhibitor—Oleate-dependent PLD was assayed by measuring the release of [^{14}C]choline from 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*methyl*-^{14}C]-choline (297 dpm/nmol, Amersham International, Amersham, Bucks, UK) as described previously (*16*) except that the extraction with butyronitrile was omitted from the choline release assay. PLD1 and PLD2 activities were assayed by measuring the formation of [^{14}C]phosphatidylethanol from 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (130 dpm/pmol, Amersham International) as described previously (*38*). PLD inhibitory activity was assayed by measuring the capacity of the inhibitor to inhibit purified or expressed PLD enzymes under the standard conditions. Unless otherwise indicated, PLD inhibitor was dispersed in distilled water by sonication and added to the PLD assay mixture. Assay was started by the addition of the enzyme. All assays were performed at least twice, and the representative data were presented.

Preparation of PLD—PLD1a, PLD1b, and PLD2 were expressed in *Schizosaccharomyces pombe* cells transfected with respective cDNAs [pREP4KS-rPLD1a, pREP4KS-rPLD1b, and pREP3X-rPLD2 cloned in the *S. pombe* expression plasmid (*41*) as described previously (*38, 42*)]. The transformed cells were disrupted by vortexing with glass beads and centrifuged at 2,000 × *g* for 10 min. The membrane fraction was sedimented by centrifuging the supernatant at 100,000 × *g* for 60 min, suspended in 50 mM Hepes-NaOH, pH 7.0, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 2 mM *p*-amidinophenylmethanesulfonyl fluoride, and 300 mM sucrose, and used as the enzyme. Oleate-dependent PLD was prepared from pig lung microsomes as described by Okamura and Yamashita (*16*).

Phospholipid Determination—Phospholipid was colorimetrically assayed by determining the phosphate concentration by the method of Bartlett (*43*) after ashing the samples as described by Ames and Dubin (*44*). Absorbance at 830 nm was measured using a Hitachi U-1100 spectrophotometer (Tokyo).

Preparation of PS, PI, LysoPS, and LysoPI from Pig Colon—Colon mucosa (90 g) was minced and homogenized with a Polytron blender in 120 ml of 0.1 M potassium phosphate buffer, pH 7.2, and then mixed with 300 ml of methanol and 150 ml of chloroform. After shaking the mixture vigorously, 150 ml of 0.1 M potassium phosphate buffer, pH 7.2, was added and the mixture was again shaken. The lower phase was filtered, concentrated, and applied to a Silica Gel 60 column (2.5 × 18 cm) (Merck, Darmstadt, Germany) equilibrated with chloroform. The column was subjected to stepwise elution with chloroform, chloroform/methanol (95:5, by volume), chloroform/methanol (4:1, by volume), chloroform/methanol (3:2, by volume), and chloroform/methanol (1:4, by volume). The fractions containing PS (chloroform/methanol, 4:1, by volume) and PI (chloroform/methanol, 3:2, by volume) were applied to Silica Gel 60 plates (Merck) and developed in two steps with chloroform/methanol/acetic acid/distilled water (50:25:4:8, by volume) and tetrahydrofuran/acetone/methanol/distilled water (50:20:40:8, by volume) to thoroughly separate PS and PI. LysoPS and lysoPI were prepared from PS and PI by the action of *Trimeresurus flavoviridis* phospholipase A_2. The purity of PS, PI, lysoPS, and lysoPI was confirmed by two-dimensional TLC using two different solvent systems: tetrahydrofuran/acetone/methanol/distilled water (50:20:40:8, by volume) and chloroform/acetone/methanol/acetic acid/distilled water (50:20:10:15:5, by volume); and chloroform/methanol/water (65:25:4, by volume) and *n*-butanol/acetic acid/water (60:20:20, by volume).

RESULTS

Purification and Identification of PLD Inhibitor from Pig Colon Mucosal Membranes—During an attempt to purify oleate-dependent PLD from pig colon mucosal microsomes, we noted that the total activity of cholate-solubilized pig colon microsomes increased 3.3-fold after chromatography on a sulfate-Cellulofine column. We hypothesized that an inhibitory component had been removed from the enzyme. To test this possibility, we examined each chromatographic fraction from the sulfate-Cellulofine column for the capacity to inhibit PLD activity and confirmed the presence of inhibitory activity in the flow-through fraction (Fig. 1). We

initially assumed the inhibitor to be a protein and so tested several chromatographic columns as means to purify it. The inhibitor could be adsorbed onto and eluted from various columns, such as ion exchange, hydrophobic, chelate, blue-dye, and hydroxyapatite columns. However, after purifying the inhibitor by sequential use of Q-Sepharose, chelate-Cellulofine, Toyopearl HW-55F gel filtration, and DEAE-Cellulofine, we found that the inhibitory activity was extractable with acetone from the inhibitory fractions of the column, indicating the lipid nature of the inhibitor. To confirm this and identify the inhibitor, we decided to extract the inhibitor in a larger scale. Colon microsomes were solubilized with 2% sucrose monolaurate and chromatographed on Q-Sepharose with a linear NaCl gradient (Fig. 2). Inhibitory activity appeared from the column after the major protein peak, and the inhibitory fractions were combined and treated with a chloroform-methanol mixture by the method of Bligh and Dyer (*40*). Over 95% of the inhibitory activity of the combined fractions determined before the chloroform-methanol treatment was recovered in the lower phase, confirming the lipid nature of the inhibitor. The extracted lipids were then separated on Silica Gel 60 plates with chloroform/methanol/acetic acid/distilled water (50:25:4:8, by volume) as the solvent, and lipid bands were located with iodine vapor. Six discernible bands were scraped from the plate, extracted, and assayed for PLD inhibitor activity using purified oleate-dependent PLD (*16*). The lowest band containing lysophosphatidylcholine and the uppermost band containing free fatty acid were without inhibitory effect (data not shown). The inhibitory activities of the other four bands were compared at the phosphate concentration of 30 μM (Table I). Band 1 containing lysoPS and lysoPI and band 3 containing PS and PI strongly inhibited oleate-dependent PLD. In contrast, bands 2 and 4 containing PC and phosphatidylethanolamine, respectively, exhibited negligible inhibitory effects on the enzyme at this concentration. Bands 1 and 3 were further separated by TLC, and individual phospholipids were examined for PLD inhibitory activity. LysoPS was a potent inhibitor of oleate-dependent PLD, but PS was not. PI and lysoPI were similarly inhibitory (data not shown). Thus the PLD inhibitor endogenously present in colon mucosa was thought to consist of lysoPS, PI, and lysoPI. This was confirmed by the following experiments.

Effects of LysoPS, LysoPI, PS, and PI on PLD Isoforms—To confirm and characterize the inhibitory effects of the identified phospholipids, we prepared the phospholipids from pig colon mucosa. PS and PI were prepared by silicic acid chromatography of chloroform-methanol extracts (*40*) from pig colon mucosa, followed by two-step developing TLC to separate them. The lipids thus obtained were treated with snake venom phospholipase A_2 to prepare lysoPS and lysoPI. The purity and identity of the prepared lipids was confirmed by two-dimensional TLC by referring to authentic lipids using chloroform/methanol/water (65:25:4, by volume) and chloroform/acetone/acetic acid/methanol/water (10:4:2:2:1, by volume) (Fig. 3). The purity was also confirmed using a different solvent system, chloroform/methanol/water (65:25:4, by volume) and *n*-butanol/acetic acid/water (60:20:20, by volume) (data not shown). As shown in Fig. 4, lysoPS was the most potent inhibitor of oleate-dependent PLD, effective at concentrations of 10^{-5} M. In contrast, PS was without effect in this

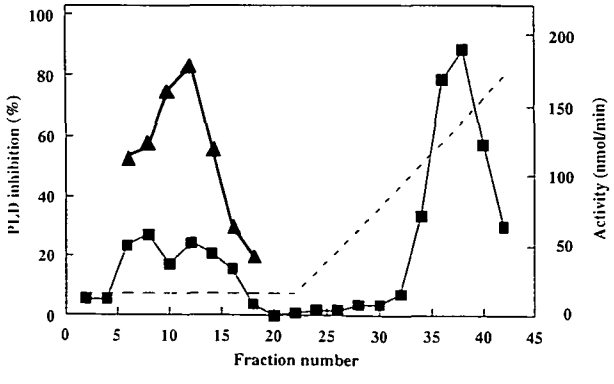

Fig. 1. **Sulfate-Cellulofine chromatography of pig colon oleate-dependent PLD.** Pig colon microsomes were solubilized with 2% sodium cholate and applied to a sulfate-Cellulofine column (1.6×4.0 cm) equilibrated with 40 mM potassium phosphate, pH 7.2, containing 0.08% Triton-X100, and 0.02% NaN_3. The column was eluted with a 20-ml linear NaCl gradient (0.2-2 M) (--) in the same solution. Oleate-dependent PLD activity (■) and PLD inhibitor activity (▲) were assayed as described in "MATERIALS AND METHODS." The inhibitory activity was determined with sulfate-Cellulofine-purified oleate-dependent PLD (*16*).

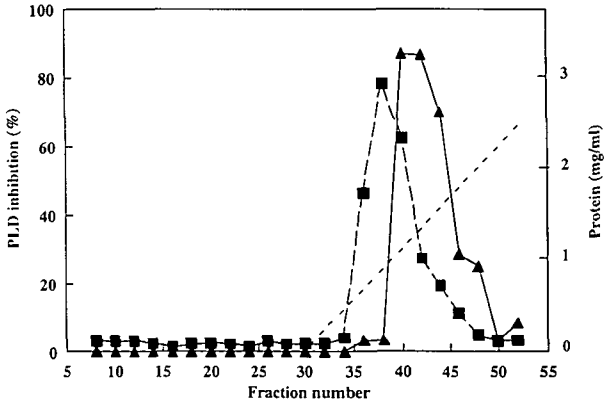

Fig. 2. **Q-Sepharose chromatography of PLD inhibitor.** Pig colon microsomes were solubilized with 2% sucrose monolaurate, and applied to a Q-Sepharose column (2.5×12 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.06% sucrose monolaurate, 0.02% NaN_3, and 0.2 mM phenylmethylsulfonyl fluoride. PLD inhibitor was eluted with a NaCl gradient (0-2 M) (--) in the same solution. Inhibition of oleate-dependent PLD [sulfate-Cellulofine step enzyme (*16*)] (▲) and protein (■) were determined as described in "MATERIALS AND METHODS."

TABLE I. **TLC of PLD inhibitor.** Q-Sepharose column fractions were extracted with a chloroform-methanol mixture as described by Bligh and Dyer (*40*) and separated by TLC with chloroform/methanol/acetic acid/distilled water (50:25:4:8, by volume). Lipids were located with iodine vapor, lipid bands were scraped off the plate, and lipids were extracted with a chloroform-methanol mixture and assayed for PLD inhibitory activity with 1.5 μg of partially purified oleate-dependent PLD [ether-Toyopearl step enzyme (*16*)] as described in "MATERIALS AND METHODS." Each band was assayed at the concentration of 30 μM phosphate.

TLC band	Lipid	PLD inhibition (%)
1	lysoPS, lysoPI	88
2	PC	1.9
3	PS, PI	78
4	phosphatidylethanolamine	16

concentration range. PI and lysoPI were also inhibitory to the enzyme, but less so than lysoPS. To examine whether these phospholipids also inhibit other PLD isoforms, namely, ARF-dependent PLD (PLD1a, PLD1b) (*22, 35*) and PIP_2-dependent PLD (PLD2) (*38, 39*), rat PLD1a, PLD1b, and PLD2 cDNA were expressed in the fission yeast *S. pombe* cells as described (*38, 42*). PLD1a and b are ARF- and PIP_2-dependent, while PLD2 is small G protein-independent, but PIP_2-dependent. Both PLD1 and PLD2 were inhibited by oleate, thus differing from oleate-dependent PLD (*38, 42*). These PLDs were also highly sensitive to lysoPS, but virtually insensitive to PS at concentrations of the μM order (Fig. 4). Like oleate-dependent PLD, these isoforms were also inhibited by PI and lysoPI, although less strongly than by lysoPS. Table II summarizes the IC_{50} values of lysoPS, PI, and lysoPI for inhibition of each type of PLD. In every case, the inhibitory capacity of the lipid was in the order of lysoPS, PI, and lysoPI. However, we noted considerable differences in the sensitivity to inhibi-

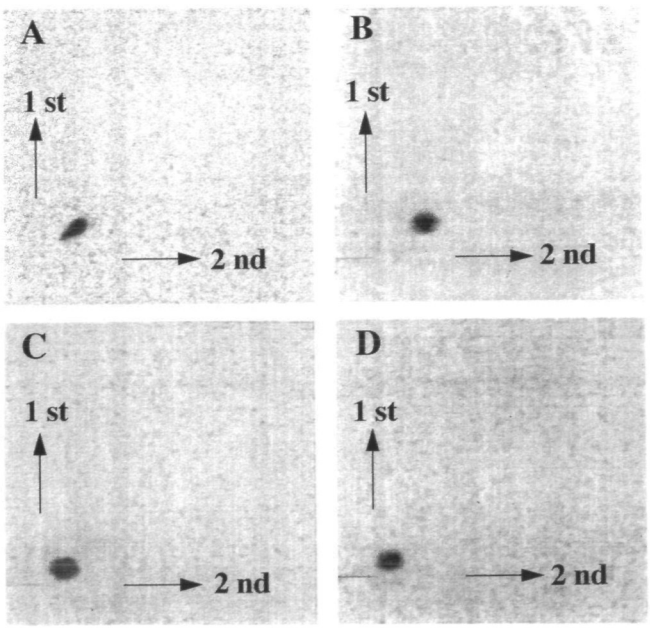

Fig. 3. **Two-dimensional TLC of the purified phospholipids.** The purity of the PI (A), PS (B), lysoPI (C), and lysoPS (D) prepared from pig colon was examined by two-dimensional TLC using chloroform/methanol/water (65:25:4, by volume) in the first dimension and chloroform/acetone/acetic acid/methanol/water (10:4:2:2:1, by volume) on a silica gel H plate (Merck).

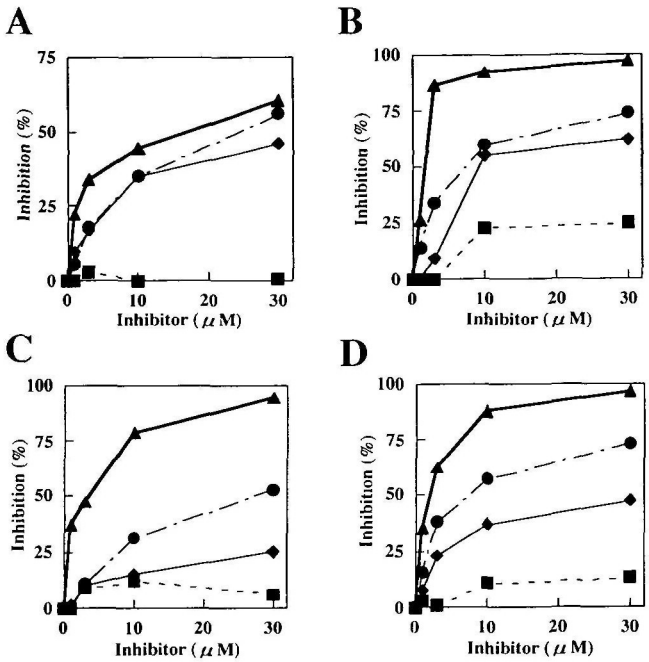

Fig. 4. **Effects of PLD inhibitor lipids on various PLD isoforms.** Activities of purified oleate-dependent PLD [Q-Sepharose step enzyme (*16*)] (A) and PLD2 (B), PLD1a (C), and PLD1b (D) expressed in *S. pombe* cells were determined in the presence of varying concentrations of PS (■), lysoPS (▲), PI (●), and lysoPI (◆) as described in "MATERIALS AND METHODS." The activities obtained in the absence of the inhibitors were 356 (A), 3.4 (B), 3.0 (C), and 0.46 pmol/min (D), respectively. The amounts of enzyme protein used were 1.5 (A), 2.0 (B), 2.0 (C), and 3.0 μg (D), respectively.

TABLE II. **IC_{50} values for PLD inhibitors.**

Enzyme	IC_{50} (μM)		
	LysoPS	PI	LysoPI
PLD1a	3.5	28	>30
PLD1b	2	7.4	>30
PLD2	1.9	7.2	9.2
Oleate-dependent PLD	17	25	>30

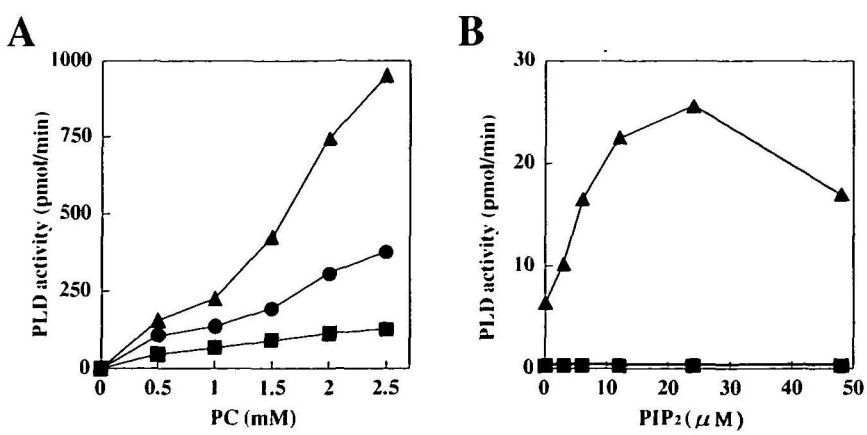

Fig. 5. **Effects of increasing concentrations of PC and PIP_2 on the inhibition of PLD by lysoPS.** Activities of oleate-dependent PLD (A) and PLD1a (B) were determined using increasing concentrations of PC (A) and PIP_2 (B) in the absence (▲) or presence of 15 μM (●) or 30 μM lysoPS (■) as described in "MATERIALS AND METHODS." The amounts of enzyme protein used were 2.6 (A) and 1.3 μg (B), respectively.

tors of the different PLD isoforms. Oleate-dependent PLD was relatively insensitive to lysoPS inhibition compared to PLD1a, PLD1b, and PLD2. PLD2 showed the highest sensitivity to PI among the various types of PLD.

In parallel, we tested a commercially available preparation of lysoPS (Avanti Polar Lipids, Alabaster, AL, USA) and obtained similar results (data not shown). As a control, the effects of these lipids on rat liver 24-kDa lysophospholipase (a gift from Dr. H. Sugimoto, Gunma University) were examined, but no inhibition was observed in the 10^{-5} M concentration range (data not shown). Pancreatic phospholipase A_2 (Boehringer Mannheim, Germany) was marginally inhibited by PI and lysoPI, but not affected by lysoPS in this concentration range (data not shown).

To examine lysoPS inhibition could be reversed by increasing the concentration of the substrate or activators, PLD inhibition by lysoPS was examined in the presence of increasing concentrations of PC and PIP_2 using oleate-dependent PLD and PLD1a, respectively, as the enzyme. As shown in Fig. 5, an increase in the concentrations of PC and PIP_2 did not prevent PLD inhibition by lysoPS. The substrate-activity plots did not follow simple Michaelis-Menten kinetics but showed sigmoidicity with a Hill coefficient of 2.8.

DISCUSSION

Kanfer *et al.* (*36*) examined the effects of various phospholipids, such as PIP_2, cardiolipin, PS, phosphatidylglycerol, and phosphatidic acid on the activity of oleate-dependent PLD of rat neural nucleus and showed that PIP_2 was a potent inhibitor of the PLD with an IC_{50} of 3.5-6 μM, although this lipid is known as an obligatory activator of the other forms of PLD (PLD1, PLD2). Cardiolipin was a second potent inhibitor with an IC_{50} of 17-20 μM. The other acidic phospholipids were also inhibitory, but less so. In the present study, the major lipid inhibitor of PLD present in the colon mucosa was identified as comprising lysoPS, PI, and lysoPI, which were not examined in the above study. Moreover, these three phospholipids inhibited all the PLD isoforms examined. Thus, PLDs are lipid-sensitive enzymes, activated by unsaturated fatty acid or PIP_2 depending on the isoform, but commonly inhibited by lysoPS, PI, and lysoPI. The mechanism of the PLD inhibition by lysoPS, PI, and lysoPI is not fully understood. The inhibitors may interact directly with the enzyme, modulate its conformation, and decrease its activity. Alternatively, they may change the physicochemical state of the substrate PC micelle and hinder its availability for the enzyme reaction. At present it is difficult to distinguish these two possibilities, but binding experiments should provide a clue to solve the question.

It is worthy of note that whereas PS did not affect PLD activity, the deacylated product lysoPS was a potent inhibitor of the enzyme with IC_{50} values of 1.9-3.5 μM for PLD1 and PLD2 and 17 μM for oleate-dependent PLD. LysoPS is a trace component of cellular phospholipid, but is highly inhibitory to PLD1 and PLD2. It is tempting to speculate that conversion of PS to lysoPS may participate in the signal transduction through the inhibition of PLD. The present results also suggest a role of PI in the regulation of PLD. Chung *et al.* (*31*) identified synaptojanin as a potent inhibitor of PLD and demonstrated that it catalyzed the conversion of PIP_2 to PI. The present work showed that the product PI is highly inhibitory to PLD. Thus, the inhibitory effect of synaptojanin on PLD activity may be due to not only the removal of the PLD activator PIP_2, but also the formation of the PLD inhibitor PI. On the contrary, the conversion of PI to PIP_2 by sequential action of PI 4-kinase and PI-4-phosphate 5-kinase, which are known to be regulated by tyrosine kinase (*45*), phosphatidic acid (*7*), and rho family G protein (*46*), would cause de-inhibition of PLD by the removal of PI and activation by an increase in PIP_2.

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