Specific inhibition of rat brain phospholipase D by lysophospholipids

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Abstract **Although the importance of phospholipase D (PLD) in signal transduction in mammalian cells is well documented, the negative regulation of PLD is poorly understood. This is primarily due to a lack of known specific inhibitors of PLD. We herein report that the activity of partially purified rat brain PLD is inhibited by certain lysophospholipids, such as lysophosphatidylinositol, lysophosphatidylglycerol, and lysophosphatidylserine in a highly specific manner. Inhibition of PLD by lysophospholipids was dose-dependent: the concentration of lysophosphatidylinositol required for half-maximal inhibition was about 3 μm. An analysis of the enzyme-kinetics suggested that lysophospholipids act as non-competitive inhibitors of PLD activity. As expected, PLD activity was stimulated by ADP-ribosylation factor (Arf) and phosphatidylinositol 4,5-bisphosphate (PIP_2). The inhibition of PLD by lysophospholipids, however, was not affected by the presence or absence of Arf or by an increase in PIP_2 concentration. A protein-binding assay suggested that lysophospholipids bind directly to PLD. These results indicate that the observed inhibition of PLD by lysophospholipids is due to their direct interaction rather than to an interaction between lysophospholipids and either Arf or PIP_2. The present study suggests that certain lysophospholipids are specific inhibitors of rat brain PLD in a cell-free system and may provide the new opportunities to investigate mechanisms by which PLD is regulated by lysophospholipids, presumably liberated by phospholipase A_2 activation, in mammalian cells.**—Ryu, S. B. and J. P. Palta. **Specific inhibition of rat brain phospholipase D by lysophospholipids.** ***J. Lipid Res.*** **2000.** 41: **940–944.**

Supplementary key words bioactive phospholipids • PLD regulation • phospholipase A_2 • LPE • LPI • LPS • LPG • LPC

Phospholipase D (PLD) has been implicated in signal transduction, regulation of inflammatory and immune responses, cellular trafficking, and cell growth (1–5). PLD catalyzes the hydrolysis of phospholipids to yield phosphatidic acid (PA) and the free polar headgroups. PA has been implicated as a biologically active molecule and has been found to be metabolized by a PA phosphohydrolase to form diacylglycerol, a protein kinase C activator (2, 6–10). When added exogenously to cells, PA has been found to stimulate phospholipase A_2 activity (11, 12).

Several studies have focused on how PLD activity is regulated. While PLD-activating mechanisms have been widely studied, the negative regulation of PLD is poorly understood. This is primarily due to the lack of known specific inhibitors of PLD (13). The existence of PLD-inhibitory factors has been found in several mammalian tissues (14–19). Some of the protein factors in brain cytosol that exhibited a potent inhibitory action on PLD have been identified: fodrin (20), synaptojanin (21), and clathrin assembly protein 3 (22). A ceramide was also reported to inhibit PLD activation by phorbol myristate acetate by interfering with protein kinase C (18). ADP-ribosylation factor (Arf)-related protein was recently found to inhibit Arf-dependent activation of PLD by binding to the Arf-specific guanine nucleotide exchange factor cytohesin (23).

We recently found that lysophospholipids such as lysophosphatidylethanolamine (LPE) and lysophosphatidylinositol (LPI) inhibit plant PLD in a highly specific manner (24). Furthermore, the lysophospholipids were found to function as lipid-derived growth regulators involved in retarding senescence of plant tissues (25, 24) and in stimulating plant cell growth (26, 27). Another recent study observed the presence of PLD inhibitors in pig colon microsome extract, and these were identified to be lysophosphatidylserine (LPS), LPI, and phosphatidylinositol (28). The recent results from plant and animal systems led us to investigate if lysophospholipds might be specific inhibitors of mammalian PLD. In this present study we report certain lysophospholipids to be specific inhibitors of rat brain PLD, which directly interact with PLD enzyme.

MATERIALS AND METHODS

Materials

Bovine brain PC and PE were obtained from Avanti Polar Lipids, Inc., guanosine 5′-*O*-(3-thio)-triphosphate (GTPγS) and

Abbreviations: Arf, ADP-ribosylation factor; GTPγS, guanosine 5′-*O*-(3-thio)-triphosphate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP_2, phosphoinositol 4,5-bisphosphate; PLD, phospholipase D.

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PIP_2 from Sigma, and 1,2-di[1-^{14}C]palmitoyl PC from Amersham, respectively. Partially purified PLD (classified as PLD 1) from rat brain membranes and Arf purified from rat brain cytosol were gifts from Dr. Sue Goo Rhee (National Institutes of Health), and myristoylated Arf and rabbit PLD-antibodies were generously provided by Dr. Sung Ho Ryu (Pohang University of Science and Technology, Korea). Unless stated otherwise, standard assay included Arf purified from rat brain cytosol. Lysophospholipids such as egg lysophosphatidylcholine (LPC), egg LPE, brain LPE, LPG (18:0), liver LPI, and brain LPS were obtained from Avanti Polar Lipids, Inc. All other reagents were purchased from Sigma (St. Louis, MO).

Measurement of PLD activity

PLD activity was measured according to optimum conditions previously well established (17) with a minor modification. Substrate lipid mixture of bovine brain PE, PIP_2, and bovine brain PC in a molar ratio of 16:1.4:1 with 1,2-di[1-^{14}C]palmitoyl PC to yield 40,000 dpm per assay was emulsified in chilled water by sonication. To a standard enzyme assay mixture containing 50 mm Hepes (pH 7.5), 3 mm EGTA, 80 mm KCl, 2.5 mm $MgCl_2$, 2 mm $CaCl_2$, and 1% ethanol were added 100 nm Arf (rat brain), 5 μm GTPγS, 3 μl of PLD (58 ng), and 25 μl substrate (62.6 μm phospholipids) in a total volume of 150 μl. Assays were incubated at 37°C for 30 min in a circulating water bath. The reaction was stopped by adding 750 μl chloroform–methanol 1:2. Chloroform (200 μl) was then added to the mixtures followed by 200 μl of KCl (2M). After vortexing, chloroform and aqueous phases were separated by centrifugation at 12,000 *g* for 5 min. The chloroform phase was collected and dried under nitrogen gas. The samples were redissolved in 45 μl of chloroform and lipid standards were added before being spotted onto a thin layer chromatography plate (silica gel G). The plate was developed with a solvent containing chloroform–methanol–NH_4OH 65:35:5. Lipids on thin layer chromatography plates were visualized by exposure to iodine vapor and the spots corresponding to lipid standards were scraped into scintillation vials. Radioactivity in the scraped spots was quantitated by scintillation spectroscopy. The radioactivities of both products of transphosphatidylation activity (phosphatidylethanol) and hydrolysis activity (PA) were combined to represent the activity of PLD (24).

Treatment of PLD inhibitors

Lysophospholipids and free fatty acid (18:1) were dissolved in chloroform–methanol 95:5 (v/v). After water was added, the organic solvents were removed by flowing nitrogen gas. Stock solution concentrations were adjusted to 250 μm with water before being added to the reaction mixture (24). The range of the final concentrations of lysophospholipids ran from 40 nm to 20 μm. The control was assayed by adding solution, which had been prepared by expelling organic solvent in water. Headgroups such as choline, glycerol, inositol, and serine as well as triton X-100 were directly dissolved in water before being added to the reaction mixture. The results of PLD activity inhibition were expressed as a percentage of the control.

Measurement of binding of lysophospholipids to PLD

The binding ability of lysophospholipids to PLD was estimated by precipitation assay followed by immunoblot. The final assay volume was 0.3 ml containing 50 mm Hepes (pH 7.5), 3 mm EGTA, 80 mm KCl, 2.5 mm $MgCl_2$, 2 mm $CaCl_2$, 1% ethanol, 5 μm GTPγS, 33 nm myristoylated Arf, 300 μg of BSA, 200 ng of rat brain PLD and 1 mm lysophospholipid or phospholipid vesicles. After incubation at 37°C for 10 min, the mixture was cooled in ice water for 30 min and centrifuged at 17,000 *g* for 30 min at 4°C. The precipitant of lysophospholipid vesicles containing bound PLD in the excess of BSA (1,500-fold than rat PLD) was resuspended in 20 μl PBS (pH 7.5). The bound PLD was separated by 8% SDS-PAGE without reducing agent (2-mercaptoethanol) in sample loading buffer and transferred onto immunoblot membrane. The membrane was blotted with rabbit-PLD antibodies that detect both PLD 1 and PLD 2 isozymes. The PLD-antibody complex was visualized by staining alkaline phosphatase conjugated to a second antibody (Bio-Rad).

RESULTS AND DISCUSSION

Inhibition of rat brain PLD activity by lysophospholipids

Different lysophospholipids were tested for their effects on the activity of partially purified rat brain PLD. Among the lysophospholipids tested, LPI, LPG, and LPS had a potent inhibitory effect on PLD activity at 10 μm concentration, while LPC and brain LPE (bLPE) showed some inhibitory effect (**Fig. 1**). PLD activity was 23.3, 24.9, 30.3, 61.0, and 70.5% of the control in the presence of LPI, LPG, LPS, LPC, and bLPE, respectively. The PLD activity of the control was 25.3 nmol min^{-1} mg^{-1} protein. The ratio of two hydrolysis products (PA vs. phosphatidylethanol) was 0.83. The rat brain PLD activity was comparable to those reported previously in crude extract (0.3 pmol min^{-1} mg^{-1} protein) by Provost et al. (29) and in purified recombinant PLD 1 (100–200 nmol min^{-1} mg^{-1} protein) by Hammond et al. (30). Brain LPE is composed of LPE (50%) and plasmalogen LPE (50%). When egg LPE (eLPE) was tested for the inhibition of PLD, rat brain PLD activity was not inhibited but rather slightly stimulated (Fig. 1). This result indicates that rat brain PLD may be inhibited by plasmalogen LPE, but not by LPE. Rat brain PLD was not stimulated by lysophosphatidic acid at the concentra-

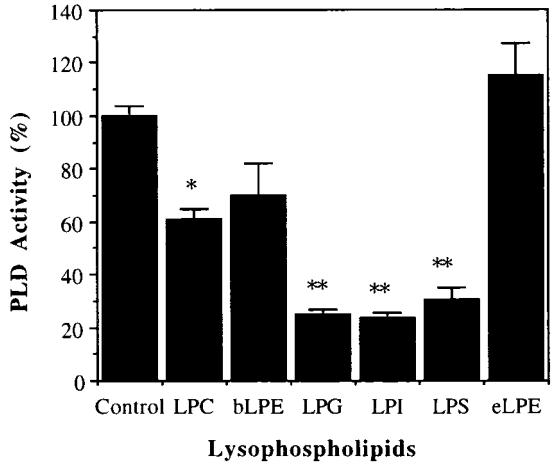

Fig. 1. Effect of lysophospholipids on rat brain PLD activity. The activity of partially purified PLD from rat brain membrane was assayed by the standard reaction procedures as described under Materials and Methods in the presence of different compounds of which final concentration was 10 μm. The standard reaction mixture contained PLD stimulators such as 4.8 μm PIP_2, 100 nm Arf, and 5 μm GTPγS. bLPE, brain LPE; eLPE, egg LPE. Data points were mean ± SEM of three separate experiments. * $P < 0.01$; ** $P < 0.005$, with respect to control.

tions of 10 and 20 μm (data not shown). LPA was previously found to stimulate PLD activity in PC-3 cells in vivo (31). However, our assay mixture contains other PLD stimulators Arf and PIP_2. This result may suggest that PLD activity in vitro was not stimulated by LPA over and above the stimulation by Arf and PIP2.

Counter to the results with rat brain PLD presented here, the activity of PLD from plant tissues was not significantly inhibited by LPC, LPG, or LPS, but only by LPE and LPI (24). This difference suggests that animal and plant PLDs are regulated differently.

Specificity of PLD inhibition by lysophospholipids

To resolve whether lysophospholipids inhibit rat brain PLD activity because of their detergent-like characteristics, the detergent triton X-100 was tested for its effect on PLD activity. Triton X-100 did not inhibit PLD activity, but did have a slight stimulatory effect (6–9%) when present at 10 μm concentration (data not shown). We next tested different headgroups and an acyl chain, subcomponents of lysophospholipids. Free fatty acid (oleate) showed a slightly inhibitory effect (10%) at 20 μm concentration while significant inhibition of PLD by oleate (20%) was observed at 40 μm concentration (data not shown). Hammond et al. (32) found that PLD1 was dramatically inhibited by oleate already at 10 μm concentration in absence of Arf and PIP_2. Thus the lack of dramatic inhibition by oleate in our experiments is due to different assay conditions. None of the headgroups such as choline, glycerol, inositol, or serine significantly inhibited PLD activity at 10 μm concentration (data not shown). These results demonstrate that rat brain membrane PLD is inhibited by lysophospholipids in a highly specific manner.

Dose dependency and kinetics of the PLD inhibition by lysophospholipids

The activity of rat brain PLD was inhibited by lysophospholipids in a concentration-dependent manner. For example, the inhibitory effect of the lysophospholipids, LPI, and LPS, on PLD activity was dose-dependent in the range of concentrations from 160 nm to 20 μm (**Fig. 2**). LPI was a slightly more effective inhibitor than LPS as it was able to inhibit 19% of PLD activity at 160 nm and 27% at 640 nm. Half-maximal inhibition of PLD activity was observed at 3 μm LPI and 6 μm LPS.

We constructed a Lineweaver-Burk plot to determine the effect of substrate concentration on the inhibition of PLD by lysophospholipids. The K_m for PLD was 33.3 μm PC and V_{max} was 166.7 pmol/h (**Fig. 3**). The K_m did not change in the presence of the inhibitor LPI (4 μm) or LPS (4 μm); however, the V_{max} decreased to 121.5 pmol/h in the presence of LPS, and to 76.9 pmol/h in the presence of LPI (Fig. 3). These results suggest that these lysophospholipids are non-competitive inhibitors of PLD.

Inhibition of PLD by lysophospholipids is independent of PIP_2 and Arf

To address whether or not Arf is involved in the inhibition of rat brain PLD activity by lysophospholipids, the effect of lysophospholipids on PLD activity in the absence of

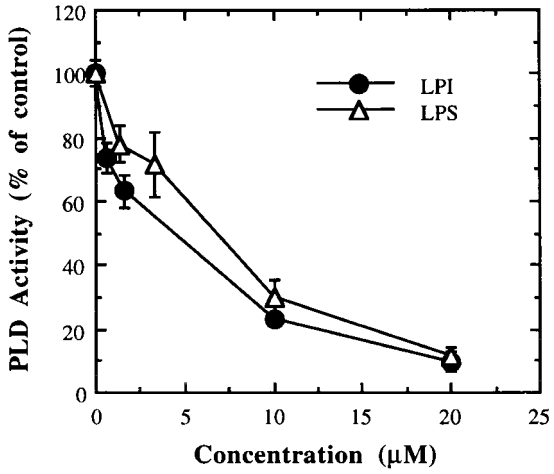

Fig. 2. Inhibition of rat brain PLD activity as a function of lysophospholipid concentrations. The activity of partially purified brain PLD was assayed by standard reaction procedures as described under Materials and Methods in the presence of different concentrations of LPI (●) and LPS (△). Half-maximal inhibition of PLD activity was observed at 3 μm LPI and 6 μm LPS. Data points are mean ± SEM of three separate experiments.

Arf was examined. In the absence of Arf, PLD activity decreased to 72% of the control with Arf (**Fig. 4A**). Adding the inhibitor LPI (4 μm) in the absence of Arf to the reaction mixture further reduced the PLD activity to 33% of the control (Fig. 4A). Since rat brain PLD activity can be inhibited by LPI in the presence or absence of Arf, the inhibition may not be caused by an interference of LPI with Arf stimulation. In an earlier study, a protein factor from bovine brain cytosol was found to suppress PLD activity

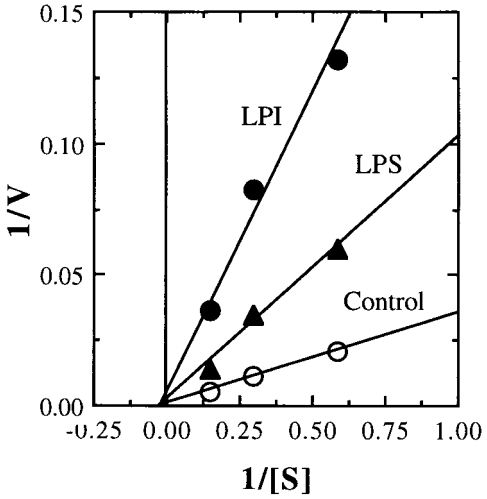

Fig. 3. Lineweaver-Burk plot suggesting noncompetitive inhibition of rat brain PLD by lysophospholipids. Lineweaver-Burk plot was obtained by measuring the activity of partially purified rat brain PLD at different concentrations of a substrate lipid mixture of PE, PIP_2, and PC in a molar ratio of 16:1.4:1 in the absence (○) and presence of inhibitors such as 4 μm LPI (●) and 4μm LPS (▲). V indicates reaction velocity (pmol/h) and [S] a substrate concentration (μm) of PC. Results are shown as data points representing three separate experiments.

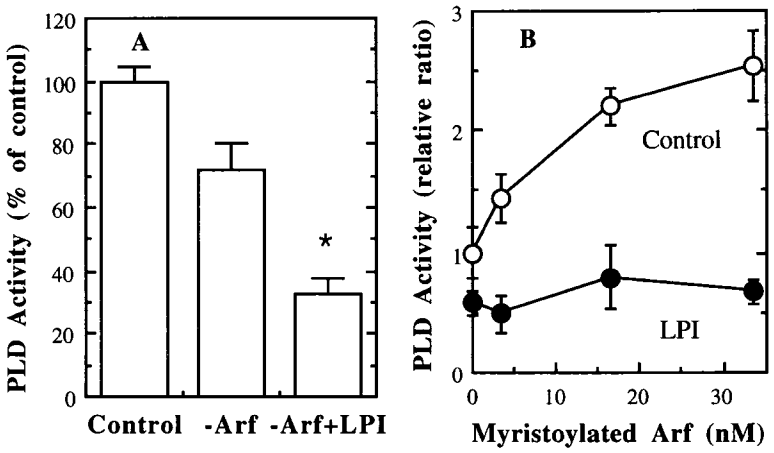

Fig. 4. Inhibition of rat brain PLD by lysophospholipids is not due to their interaction with Arf. A: In the control, the activity of partially purified rat brain PLD was assayed by standard reaction procedures as described under Materials and Methods, while "−Arf" indicates that the PLD activity was assayed in the absence of Arf from the standard reaction mixture. "−Arf + LPI" represents that the PLD activity was assayed in the absence of Arf but in the presence of the inhibitor, LPI (4 μm). B: In the control (○), the activity of partially purified rat brain PLD was assayed by standard reaction procedures as described under Materials and Methods using the different concentrations of myristoylated Arf. "LPI" (●) indicates that the PLD activity was assayed in the presence of the inhibitor, LPI (15 μm). Data points are mean ± SEM of three separate experiments. * $P <$ 0.01, with respect to control.

and this suppression was largely eliminated by the addition of Arf, suggesting that this protein inhibitor interacts with Arf (16). When rat brain Arf (100 nm) in a standard PLD assay of this study was substituted with 33 nm myristoylated Arf (16), PLD activity dramatically increased to 250% of the control (Fig. 4B). However, in the presence of LPI (15 μm), PLD activity was not significantly changed by the addition of myristoylated Arf, indicating that Arf could not nullify the inhibition of PLD by LPI.

Since PLD activity requires PIP_2 as a co-factor, another possible means by which lysophospholipids can inhibit PLD is by interacting with PIP_2. For example, *Clostridium difficile* toxin B (33) and synaptojanin (21) were found to inhibit PLD activity by depleting PIP_2. Simply by increasing the concentration of PIP_2, PLD activity could be restored (33). When the concentration of PIP_2 in the reaction mixture was tripled, the PLD inhibition by LPI (4 μm) was not significantly changed (**Fig. 5**). When this experiment was repeated using LPS (12 μm), PLD inhibition by LPS was similar regardless of PIP_2 concentrations (data not shown). These results suggest that inhibition of PLD by lysophospholipids is independent of PIP_2.

Binding of lysophospholipids to rat brain PLD

In light of above experiments, the inhibition of PLD appears to be caused by the direct interaction of lysophospholipids with PLD rather than their disruption of PIP_2–PLD or Arf–PLD interactions. We therefore determined binding capability of lysophospholipids to PLD itself. In the presence of a 1,500-fold excess BSA, PLD proteins co-precipitated with brain LPE or LPI vesicles, but not with brain PE vesicles (**Fig. 6**). The distribution of precipitated protein bands on SDS-PAGE followed by Western blot with rabbit PLD 1 and 2 antibodies (Fig. 6) was similar to one of the original non-precipitated PLD preparation. Major band was detected at 120 kDa in the Western blot, indicating that the rat brain PLD preparation consists of PLD 1 but not PLD 2. The PLD preparation also contained some

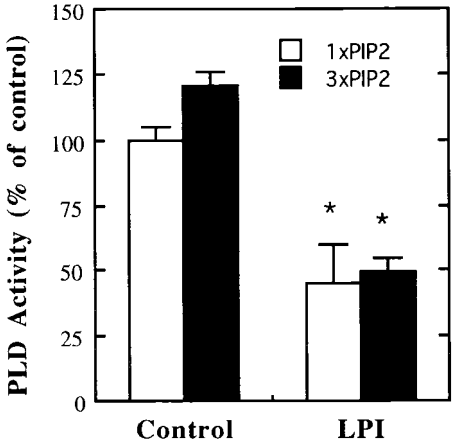

Fig. 5. Inhibition of rat brain PLD by lysophospholipids is not affected as the concentration of PIP_2 increases. In the control, the activity of partially purified rat brain PLD was assayed by standard reaction procedures as described under Materials and Methods in the presence of two different concentrations of PIP_2: 1 PIP_2 (4.8 μm) and 3 PIP_2 (14.4 μm). "LPI" indicates that the PLD activity was assayed in the presence of the inhibitor, LPI (4 μm). Data points are mean ± range of two separate experiments. * $P < 0.05$, with respect to control.

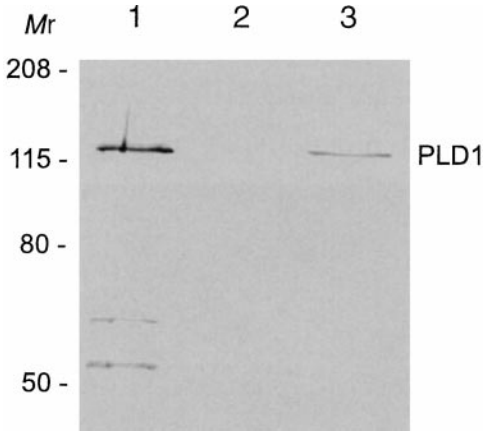

Fig. 6. Binding of rat brain PLD to lysophospholipid vesicles. Rat brain PLD protein was incubated with lysophospholipid or phospholipid vesicles such as brain LPE (lane 1), brain PE (lane 2), and LPI (lane 3) in the presence of an 1,500-fold excess of BSA as described under Materials and Methods. After centrifugation, the PLD protein co-precipitated with lipid vesicles was subjected to electrophoresis on an 8% SDS-PAGE. PLD was visualized by alkaline phosphatase-immunoblotting using rabbit PLD 1 and −2 antibodies.

lower molecular weight proteins that could be detected by the PLD antibodies, suggesting partial proteolysis of some of the PLD protein. The co-precipitation of PLD proteins by lysophospholipid vesicles suggests direct binding between PLD and lysophospholipids. The fact that this precipitation of PLD did not occur with the phospholipid vesicles further strengthens this argument.

In conclusion, these results provide in vitro evidence for specific inhibition of PLD by lysophospholipids, which directly interact with PLD. Among regulators that activate or inhibit PLD, only some of them, e.g., protein kinase C, Arf, and Rho family proteins, have been shown to interact directly with PLD in vitro (3). Cellular roles of lysophospholipids as specific inhibitors of PLD remain to be elucidated (34). Lysophospholipids are known to be generated in vivo by the action of phospholipase A_2, suggesting an interesting interplay between PLD and PLA_2.

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