Effect of Glycerophospholipid on Oleate-Dependent Phospholipase D in Mammalian Source

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Effects of exogenous glycerophospholipids on oleate-dependent phospholipase D (PLD) activity were studied in lymphocytic mouse leukemia L1210 cells and in solubilized microsomal phospholipase D of rat brain. Among the phospholipids tested phosphatidic acid had the most stimulatory effects on both PLD activities up to about 3 folds. Lysophosphatidic acid also showed promoting effect on microsomal PLD activity but much less on L1210 cells compared to that of phosphatidic acid. While phosphatidylethanolamine increased PLD activity slightly, phosphatidylinositides were nearly ineffective in the tested sources. The stimulatory effect of phosphatidic acid observed can be utilized to improve the *in vitro* assay system for oleate-dependent PLD in mammalian sources.

Introduction

Phospholipase D (E.C. 3.1.4.4. PLD) catalyzes the hydrolysis of the terminal diester bond of phospholipids producing phosphatidic acid (PA) and the free polar head group.[1] In the presence of a primary alcohol the enzyme can also catalyze a transphosphatidyl reaction. Recently there is a surge of information on the involvement of mammalian PLD in cellular signal transduction.[2] The generation of PA by PLD or an addition of PA exerts a variety of biological response such as mitogenesis,[3] increase of intracellular $[Ca^{2+}]$,[4] release of insulin,[5] and causes the activation or modulation of various enzymes such as phosphatidylinositol 4-kinase,[6] phospholipase A_2,[7] a novel PA-activated kinase[8] and neutrophil NADPH oxidase.[9] All these reports suggest a strong possibility of PA role as a second messenger in cellular signaling.[10]

PLD has different isoforms and appears to be expressed as more than one form in mammals.[2] Two isoforms of PLD identified reveal that PLD1 localized in perinuclear is dependent on ADP-ribosylation factor (ARF) and phosphatidylinositol 4,5-diphosphate (PIP_2), whereas PLD2 in plasma membrane is regulated by PIP_2 but not by ARF.[11] Another form of PLD known to be activated by oleate was separated from rat brain membranes[12] and was purified from pig lung microsomes.[13] However, in spite of isolation of oleate-dependent PLD from various sources, the main property observed so far was limited to the activational effect of unsaturated fatty acid added exogenously.[14]

In this study, we examined effects of various glycerophospholipids on oleate-PLD activity in L1210 lymphocytic mouse leukemia cell and in solubilized microsomal PLD of rat brain. Our results indicate that effect of PA is particularly stimulative to the oleate-dependent PLD activity present in mammalian sources.

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Experimental

Materials. Radioactive 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine (specific activity 111 mCi/mmole) was purchased from Amersham (Aylesbury, England). Sodium oleate, decanoyl-N-methylglucamide (MEGA-10), Sephacryl S-300, HEPES, lysophosphatidic acid (LPA), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylinositol 4,5-diphosphate (PIP_2) were obtained from Sigma Co. (St. Louis, U.S.A.). Crude egg yolk phosphatidylcholine (PC) from Sigma was purified by aluminium oxide column. Phosphatidic acid (PA) and phosphatidylethanolamine (PE) were prepared from PC using cabbage PLD according to the procedure described previously.[15] Precoated silica gel 60 TLC plate was purchased from Merck (Darmstadt, Germany). And all the other chemicals used in this study were reagent grade commercially available.

Preparation of enzyme source. L1210 cells were grown routinely at 37 °C in a humidified 5% CO_2 incubator with RPMI-1640 medium containing 10% fetal bovine serum, 50 μM β-mercaptoethanol, 100 U/mL penicilline, 100 μg/mL streptomycin, 25 mM sodium bicarbonate, and 20 mM HEPES buffer (pH 7.0). Cells were subcultured in every 2-3 days. Cultured cells were washed with phosphate buffered saline (PBS) and centrifuged at 1000×g for 10 min. Precipitated cells were disrupted in 50 mM HEPES (pH 6.5) by sonication using Branson ultrasonicator. The cell homogenates were used routinely as the enzyme source.

The Wistar rats (4 weeks old) supplied by the Animal Breeding Laboratory of Seoul National University were decapitated and the brain was homogenized in ten volume of 10% sucrose solution by Potter Elvojem homogenizer with five strokes. The homogenate was centrifuged at 10,000×g for 10 min and the supernatant was recentrifuged at 100,000×g for 1 hr at 4 °C. The microsomal pellet was solubilized with MEGA-10 and was applied on sephacryl S-300 column.[16] The peak of maximum PLD activity (PLD II) was pooled and used as the solubilized brain PLD source.

Assay of phospholipase D. *In vitro* PLD activity was determined essentially according to Taki and Kanfer[17] except the detergent. The oleate-dependent PLD was measured by the radioactivity of hydrolysis and transphosphatidylation products of 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine in the presence of ethanol. The standard reaction mixture consisted of 60 μM PC with 0.1 μCi 1,2-di[1-^{14}C]palmitoylphosphatidylcholine, 50 mM HEPES buffer (pH 6.5), 5 mM $CaCl_2$, 10 mM KF, 0.1% (W/V) MEGA-10, 1.5% ethanol, 3 mM oleate, various concentrations of phospholipids, and the enzyme preparation in a total volume of 100 μL. In case of solubilized brain PLD fractions of S-300 column, pH 7.0 HEPES buffer (50 mM) and 2 mM oleate were used. All phospholipids were dissolved in chloroform and the solvent was evaporated. Into this dried sample, HEPES buffer containing all the other reagents was added and then sonicated for 1 min. The reaction was initiated by the addition of enzyme solution and incubated for 2 hr at 30 °C. The reaction mixture of zero time incubation or boiled enzyme was taken as the blank. The reaction was terminated by the addition of chloroform: methanol: 1 N HCl (100:50:3 by volume) solution. The extracted phospholipid solution (organic layer) was washed twice with 0.1 M KCl solution and then dried. The dried lipids were redissolved in chloroform and spotted on the silica gel 60 TLC plate. The extracted phospholipids were separated on TLC plate employing an organic solvent system of ethyl acetate : isooctane : acetic acid : water (130 : 20 : 30 : 100 by volume). The spot of PA and phosphatidylethanol (PEt) were identified by staining the plate with iodine vapor and the radioactivities of scraped PA and PEt were quantitated by liquid scintillation counting with an efficiency of 85%.

PLD activity in intact cells was assayed according to the method described by Dubyak *et al.*[18] Cells were labelled with [^{3}H]myristic acid (1-2 μCi/mL) for 3 hr according to the method described by Yeo and Exton.[19] Harvested ^{3}H-labelled cells were washed twice with PBS and were resuspended in an assay medium containing 20 mM HEPES (pH 7.2), 137 mM NaCl, 2.7 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM EGTA, and 1 mg/mL BSA. After preincubation for 10 min at 37 °C, a portion (100 μL) was diluted (1 : 2) by a prewarmed assay medium containing 3% ethanol. The assay medium was incubated for 20 min. Assays were carried out in duplicate and each set of experiments was repeated 2 to 3 times with similar results. Some data were presented as averages of two experimental points, which usually agreed closely within 10% range.

Results and Discussion

In the course of characterization of oleate-dependent PLD in brain microsomes, we came upon an unexpected stimulatory effect of PA on the PLD activity when PA was added exogenously in the reaction mixture. This observation prompted us to investigate effectiveness of other glycerophospholipids on activating oleate-dependent PLD and extended the lipid effect to L1210 cells which was presumably another source for oleate-dependent PLD. Figure 1 shows the oleate-dependency of PLD in L1210 cells and solubilized brain microsomal fraction in a concentration dependent manner. An optimal concentration of oleate was found to be 3 mM for L1210 cells and 2 mM for the solubilized brain PLD. While, for the first time in L1210 cells, the high level of PLD activity which depended on oleate was observed, the oleate-dependent PLD in brain microsomes was consistent with the previous reports that oleate at millimolar concentration could activate PLD in various mammalian tissues.[12,13,20]

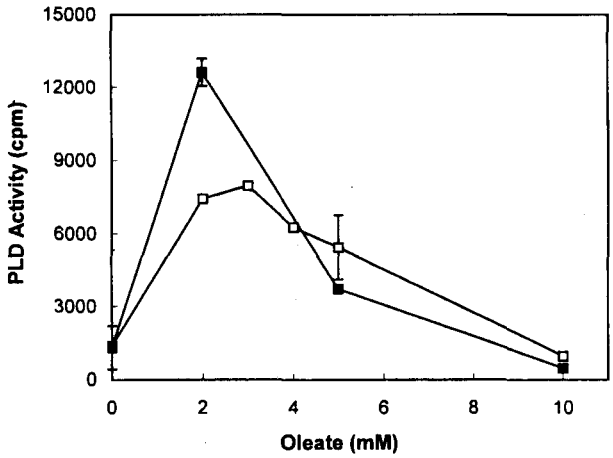

Figure 1. Oleate-dependence of PLD activity in homogenate of L1210 cells and in solubilized microsomal fraction of rat brain. The reaction mixtures containing 60 μM PC with 0.1 μCi [^{14}C] PC were incubated for 2 hr at 30 °C. The products, [^{14}C]PA and [^{14}C]PEt, were measured as described under "Experimental" section. PLD activity in L1210 cells was presented as a specific activity per mg protein, whereas microsomal PLD activity was presented as a total cpm per 5 μg protein of solubilized source. □, *L1210 cell homogenate*; ■, *solubilized brain microsomes*. The data are presented as means of two separate experiments each performed in duplicate.

In the presence of optimal concentration of oleate, effects of various types of glycerophospholipids on the PLD activity in both mammalian sources were examined. As observed in preliminary experiment, PA exerted stimulatory effect on both PLD sources (Figure 2a). The activation reached plateau at 0.3-1 mM of PA and decreased thereafter. The PLD activity was enhanced at least 3-fold over the control values. The activation of oleate-PLD by PA seems to be unusual considering PA is one of the end product of PLD action. However, there was another report that PLD activity can be stimulated by PA in rat fibroblast where PA was considered as a mitogenic lipid.[21] LPA, known as another mitogenic lipid,[22] was examined for its effect on the PLD activity (Figure 2b). LPA stimulated significantly the oleate-PLD in solubilized brain microsomes (approximately 2.5-fold) but barely exerted its effect on the PLD in L1210 cells, indicating LPA acted differently in different PLD sources. When PE was added in the reaction mixtures, the PLD activity increased slightly in both sources at 0.5 mM concentration (Figure 3). On the other hand, addition of PS did not affect the PLD activity (data not shown). Since a certain type of phosphatidylinositides, particularly PIP_2 has been known to be a major regulatory factor for ARF-PLD, we examined effects of PI and PIP_2 on both PLD sources (Figure 4a and b). It turned out that there was no ac-

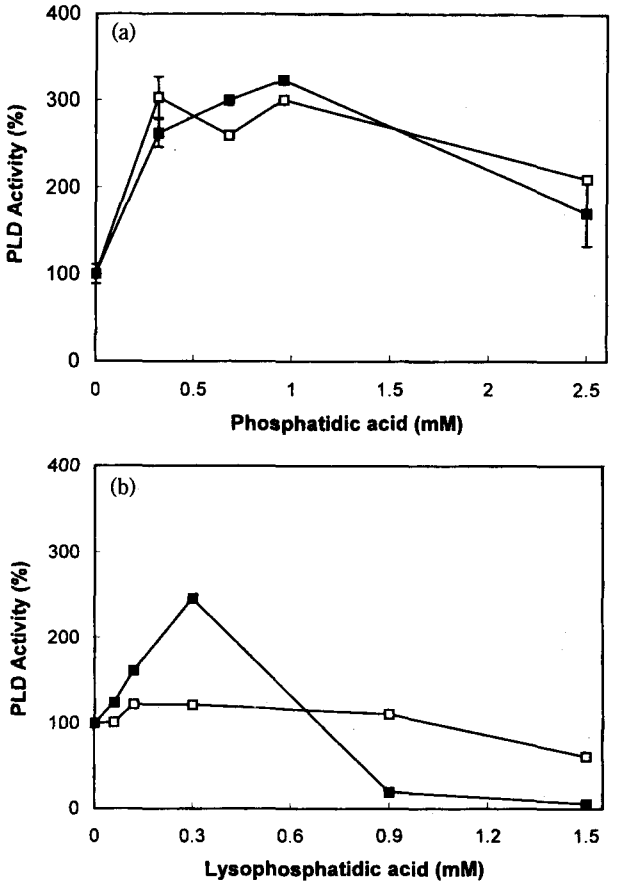

Figure 2. Effects of phosphatidic acid (a) and lysophosphatidic acid (b) on oleate-dependent PLD activity. □, L1210 cell homogenate; ■, solubilized brain microsomes.

tivational effect on the oleate-dependent PLDs by any phosphatidylinositides under the experimental conditions observed. However in the presence of ARF and GTPγS, the stimulational effect of PIP_2 on PLD in solubilized brain microsomes was observed as previously reported[12] but not with L1210 cells (data not shown). Contrary to our results, there was one report that all acidic phospholipids including

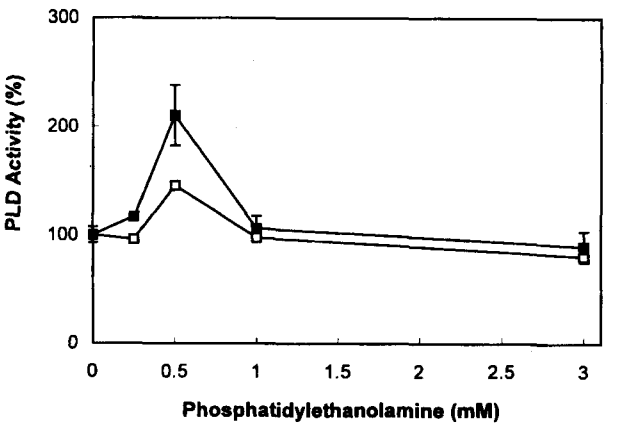

Figure 3. Effect of phosphatidylethnolamine on oleate-dependent PLD activity. □, L1210 cell homogenate; ■, solubilized brain microsomes.

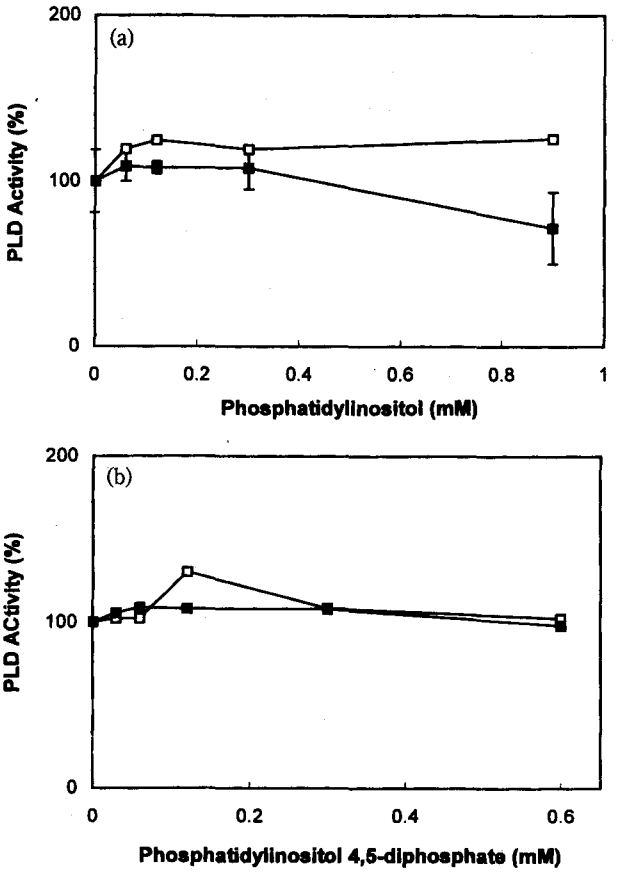

Figure 4. Effects of phosphatidylinositol (a) and phosphatidylinositol 4, 5-diphosphate (b) on oleate-dependent PLD activity. □, L1210 cell homogenate; ■, solubilized brain microsomes.

PA, PS, and PIP_2 were inhibitory to PLD activity in rat brain neuronal nuclei.[23]

The stimulatory PA effect observed *in vitro* assay system was further examined in intact L1210 cells (Figure 5). Interestingly, the PA added in cell culture medium stimulated the PLD activity even in intact cells. The maximum stimu-

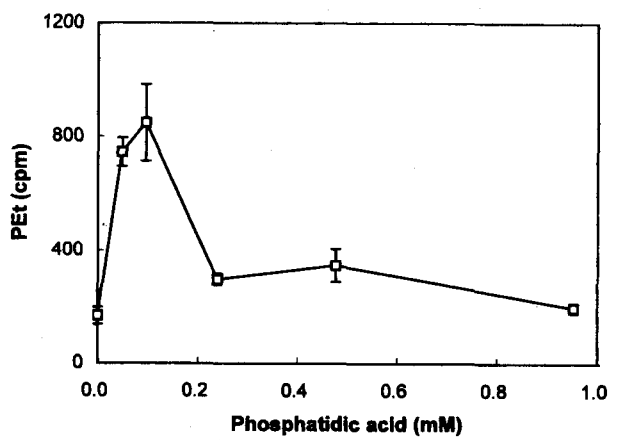

Figure 5. Effect of phosphatidic acid on PLD activity in intact L1210 cells. L1210 cells were radiolabelled with [^{3}H]myristic acid and assayed in the presence of various concentrations of phosphatidic acid as indicated. [^{3}H]PEt produced was measured as described under "Experimental" section.

lation was approximately 2-4 fold over the control values depending on experimental condition. This result implies that PA apparently act as a cellular messenger which eventually stimulate the PLD activity through probably some combination of signal pathways. Although any pathways involving PA signals have not been identified clearly yet, there are many reports on PA effects routinely speculating PA role as a second messenger.[10] In another point of view, which mainly focus the PA as a membrane component, the present data of PA activation could be interpreted in the following manner. PA might bind specifically to the protein of oleate-dependent PLD and thereby exerts its regulatory effect or PLD could be activated by means of membrane perturbation which can be brought by non-bilayer lipid action of PA. Recently there are numerous informations that non-bilayer favoring lipids are indicated to act as essential lipid components in various cellular events such as membrane transport,[24] fusion,[25] and pore formation.[26] As a matter of fact, direct binding of PA to Raf-1 kinase has been reported.[27] While comprehending the action of PA on oleate-PLD certainly requires further investigation, the 3-fold stimulatory effect of PA observed can be utilized fully in the assay system for oleate-PLD in mammalian sources.

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