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Interaction of phospholipase D to vesicles induces membrane fusion

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Abreviations: PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; DG, diacylglycerol; TLC, thin layer chromatography; DPPC, dipalmitoylphosphatidyl choline; R-18, octadecyl rhodamine B chloride; LUV, large unilamellar vesicle; GDI, GDP dissociation inhibitor; GDS, GDP dissociation stimulator

Abstracts

It was reported that membrane fusion was induced by phospholipase D (PLD) in the presence of calcium ion. Initial fusion rate of vesicles in the presence of Ca^{2+} and PLD was much faster than the expected value when only phosphatidic acid (PA) produced by PLD was taken into account. To elucidate the mechanism of membrane fusion induced by PLD, the interaction of PLD to vesicles and fluorescence changes of PLD were studied. It was found the rate of membrane fusion was much faster than that of membrane aggregation, suggesting PLD did not play a role in vesicle aggregation. The fluorescence of PLD was changed in the presence of vesicle membrane even without Ca^{2+}, indicating that the structural changes of PLD without Ca^{2+} was not sufficient for membrane fusion. PLD was bound to vesicles irreversibly in the presence of Ca^{2+} which was essential for induction of membrane fusion. The induction of membrane fusion by PLD may be due to the interaction of PLD itself to vesicles as well as asymmetric distribution of PA on the membranes produced by PLD.

Keywords: phospholipase D, Ca^{2+}, membrane fusion

Introduction

Secretion from animal cells can be either constitutive or regulated. The constitutive secretory pathway is present in most cells and provides a generalized route for transporting proteins to the cell surface. In addition to its housekeeping function of maintaining the plasma membranes, this pathway is used for unregulated secretion of enzymes, growth factor and extracellular matrix components. By contrast, specialized cells use the regulated secretory pathway for rapid release of stored hormones, neurotransmitters, and enzymes in response to physiological needs (Miller and Moore, 1990).

Ca^{2+}-induced membrane fusion plays a central role in many biological phenomena such as exocytosis. A necessary condition for the Ca^{2+}-induced fusion is that membranes and vesicles contain anionic phospholipids (Sundler *et al.*, 1981). The interaction between Ca^{2+} and vesicles containing anionic phospholipids has been the subject of extensive study in recent years (Smaal *et al.*, 1987). In recent investigation, Park *et al.* (1992, 1993) have studied Ca^{2+}-induced fusion of vesicles containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as these phospholipids are being converted into phosphatidic acid (PA) by phospholipase D (PLD, EC 3.1.4.4) which has been implicated as an important signal transducing enzymes in a variety of cells and tissues (Billah *et al.*, 1989). In those studies, it was insisted that the differential PA distribution between the two monolayers rather than absolute PA content of the outer monomlayer was the factor contributing to the destabilization of vesicles which leads to the fusion. The interaction of PLD to vesicles and fluorescence changes of PLD cuased by structural change were examined in detail to elucidate the mechanism of membrane fusion induced by PLD in the presence of Ca^{2+} in this paper.

Materials and Methods

Materials

PC, PE from egg yolk and PA hydrolyzed from egg yolk PC were purchased from Avanti Polar Lipids. Octadecyl Rhodamine B chloride (R-18) was from Molecular Probe. EDTA, L-histidine, bovine serum albumin (BSA), N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), PLD (from streptomyces chromofuscus) were from Sigma. Thin layer chromatography plates and $CaCl_2 \cdot 4H_2O$ (suprapure) were from Merck.

Purification of bacterial PLD

PLD from *Streptomyces chromofuscus* was purified using HPLC with hydrophobic affinity column following as described (Park *et al.*, 1992). Original bacterial PLD powder was dissolved in 1.7 M ammonium sulfate, 50 mM phosphate, pH 7.0. After brief centrifugation at 4°C,

the supernatant was injected into a phenyl-Superose column HR5/5 (LKB) preequilibrated with the same buffer. Proteins were eluted gradiently with 50 mM phosphate buffer (pH 7.0) and subsequently with 50% ethyleneglycol, 25 mM phosphate buffer (pH 7.0). Active bacterial PLD fractions were collected and desalted with Bio-Gel P6 column (Bio Rad). Purified bacterial PLD was freeze-dried and stored at -20°C. PLD activity was assayed by identifying PA spot on TLC (Siddiqi, 1987).

Determination of PA by using TLC

PA production by PLD was quantitated by TLC. Reaction mixtures were stopped by adding 2 vol. of chloroform/methanol (2:1). After vigorous stirring and centrifugation, the lower chloroform layer was obtained and dried with a stream of nitrogen gas. Small aliquot of chloroform/methanol (2:1) solution were added to dissolve the lipids and spotted on a TLC plate precoated with silica gel-60. The plate was developed in chloroform/methanol/water/ammonia (90:54:5.5:5.5) for 20 min (Kruijff and Baken, 1978). The plate was air-dried and stained by dipping in solution containing 0.05% Coomasie blue R-250, 30% methanol and 100 mM NaCl (Boccking *et al.*, 1987) for 30 min. The plate was scanned using a densitometer (Helena Lab). The standard phospholipids on TLC plate showed linear relation between peak area and the amount present. The amounts of phospholipids were calculated from standard curve after staining with Coomassie blue.

Preparation of vesicles

Large unilamellar vesicles (LUV) were prepared by the reverse-phase evaporatrion technique (Szoka and Papahadjopoulos, 1978) with a minor modification (Hong *et al.*, 1982). Phospholipids (10 μmol) was dissolved in 1 ml of diethylether and sonicated under nitrogen gas for 2.5 min at 20°C after 0.3 ml of the aqueous solution to be encapsulated was added. Vesicles were separated from nonencapsulated material by gel filtration on a Sephadex G-75 column using an elution buffer containing 100 mM NaCl, 2 mM Tes, 1 mM EDTA, pH 7.0. To remove external EDTA, vesicles were rechromatographed on the Sephadex G-75 column with the above elution buffer without EDTA.

Assay of vesicle fusion

Membrane fusion was measured by dilution of lipid probe (R-18). R-18 fluoresence (excitation and emission wavelength of 560 nm and 590 nm, respectively) was continuously monitored for dilution of R-18 because of lipid mixing (Hoekstra *et al.*, 1984). The 0% fusion was determined from 1:4 mixture of 4 mol% R-18 containing liposomes and R-18 free liposomes. Liposome mixture (1:4) diluted independently by addition of 1% of Triton X-100 (v/v, final concentration) was taken as the 100% fluorescence level (or 100% fusion). The final incubation volume was 2.5 ml (total 50 μM phospholipid). The aggregation and fusion of vesicles were also measured in terms of 90° light scattering at 430 nm using spectrofluorometer (Wilschut *et al.*, 1980).

Lipid phase separation

Cation- or PLD-induced phase separation was monitored continuously by using vesicles containing 4 mol% of fluorescent dye R-18 in PC/PE/PA. The method is based on the self-quenching of R-18 fluorescence that ocurrs when the local concentration of R-18 lipid in the bilayer increases during segregation of membrane lipids into discrete domains in the plane of the bilayer (Hoektra, 1984). Unquenched fluorescence was taken as 100% when R-18 containing vesicles (50 μM phospholipids) was suspended in 2.5 mM buffer A (100 mM NaCl, 2 mM Tes, 2 mM His, and 0.1 mM EDTA, pH 7.0) without either Ca^{2+} or PLD. The change of fluorescence intensity with time was monitored after addition of Ca^{2+} and/or PLD. The excitation of R-18 was made at 560 nm and fluorescence was measured at 590 nm.

PLD binding to vesicles

Reaction mixtures of vesicles and purified PLD in buffer A were incubated at 27°C for 10 min. The vesicles with bound PLD were precipitated by ultracentrifugation (100,000 *g*) at 4°C for 1 h with TLA-100.3 rotor (Beckman). Protein concentration in the supernatant was determined by using Coomassie Brilliant blue-G with BSA as a standard (Bradford, 1976).

Fluorescence of PLD

PLD in 2 ml of buffer A was excited at 280 nm and emission fluorescence was measured at 320 nm at 27°C for 5 min with fluorescence spectrophotometer (Kontron, SFM25). For scanning, emission spectra from 305 nm to 400 nm was recorded when the protein solution was excited at 280 nm.

Results and Discussion

Purification of PLD

To exclude the effects of other proteins in PLD powder from *Streptomyces* on subsequent experiments, PLD was purified by using hydrophobic affinity column (phenyl-Sepharose HR5/5) (Figure 1). Single band on SDS-PAGE after Coomassie blue staining (Figure 2) was obtained whereas the original source gave 7 major protein bands. Gel filtration with Superose-12 column or ion exchange chromatography with Mono-Q coulumn could not separate the PLD from other proteins (data not shown).

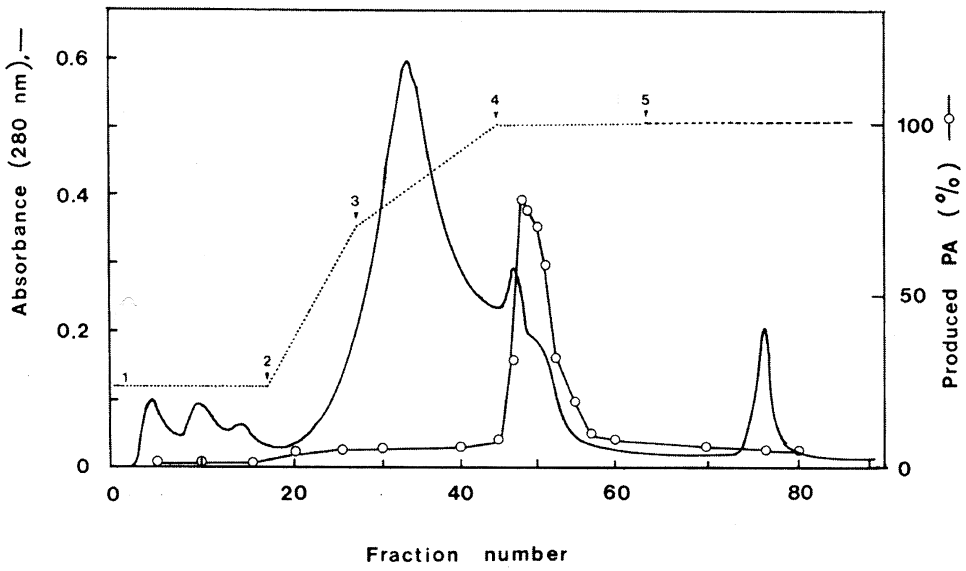

Figure 1. Purification of PLD from *Streptpmyces chromofuscus* using HPLC with hydrophobic affinity column. The column was eluted gradiently (···) with buffer exchange from buffer A (1.7 M $(NH_4)_2SO_4$, 50 mM phosphate, pH 7.0) (1), 0% buffer B (50 mM phosphate, pH 7.0) (2), 70% buffer B (3), 100% buffer B (4), and 100% buffer C (25 mM phosphate, 50% ethyleneglycol, pH 7.0) (5). PLD activity was assayed by determination of PA by TLC (o).

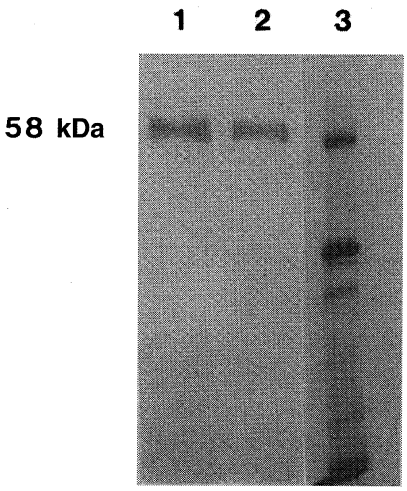

Figure 2. SDS-PAGE of purified PLD. Purified PLD of fraction number 49 (lane 1), 51 (lane 2) in Figure 1, and original PLD powder from Sigma (lane 3) were analyzed on SDS-PAGE. Gel was stained with Coomassie blue R-250.

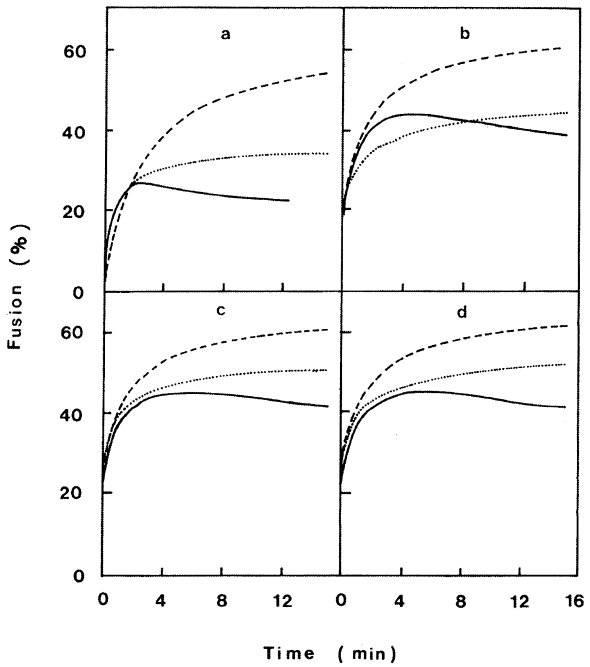

Figure 3. Time course of fusions of vesicle I with PLD or without PLD, and vesicle II without PLD. Fusion was started by adding of Ca^{2+} at 2 mM (a), 4 mM (b), 5 mM (c), and 10 mM (d) to vesicle I with PLD (—, 0.125 μg/ml) or without PLD (···), and vesicle II without PLD (---). Total reaction volumes were 2 ml. Fluorescences were measured as lipids of two populations of membranes were mixed. The ratio of non-labeled vesicle/labeled vesicles with R-18 (4 mol%) was 4:1.

Fusion and aggregation of membranes with or without PLD

Figure 3 shows time course of fusion of vesicle I (PC/PE/PA = 20: 50: 30, with or without PLD) and vesicle II (PC/PE/PA = 15:35:50, without PLD) at sevral Ca^{2+} concentration assayed by lipid mixing. The rate of fusion when assayed by lipid mixing was larger than those assayed by Tb/DPA content mixing (Park *et al.*, 1992). In contrast to the values of membrane fusion assayed with content mixing, the initial fusion rate of vesicle I induced by PLD in the presence of Ca^{2+} assayed with lipid mixing was less than that of vesicle II induced by Ca^{2+} alone. To examine whether fluorescence quenching caused by lipid phase separation induced the discrepancy, fluoroscence quenching was recorded with time after adding Ca^{2+}

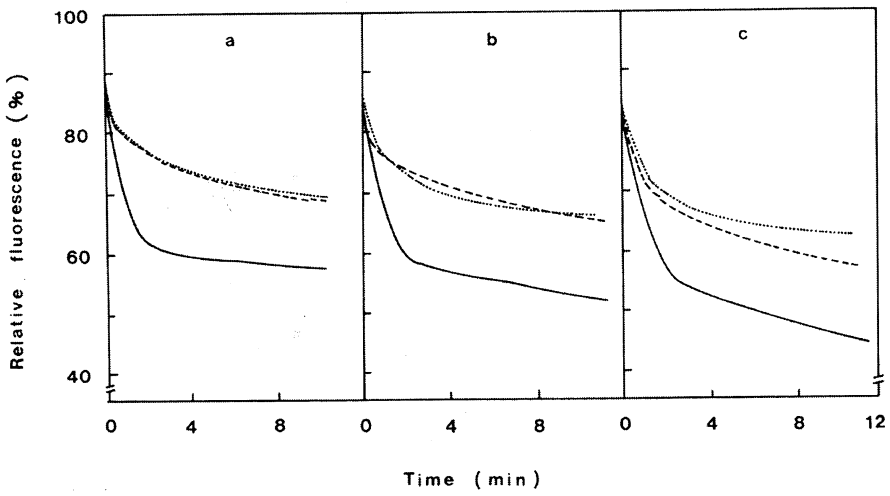

Figure 4. Time-course of fluorescence quenching of vesicle I with or without PLD, and vesicle II without PLD. Time course of fluorescence quenching of vesicle I with PLD (—, 0.125 μg/ml) or without PLD (···), and vesicle II without PLD (---) were monitored after addition of 2 mM (a), 5 mM (b), and 10 mM (c) Ca^{2+}.

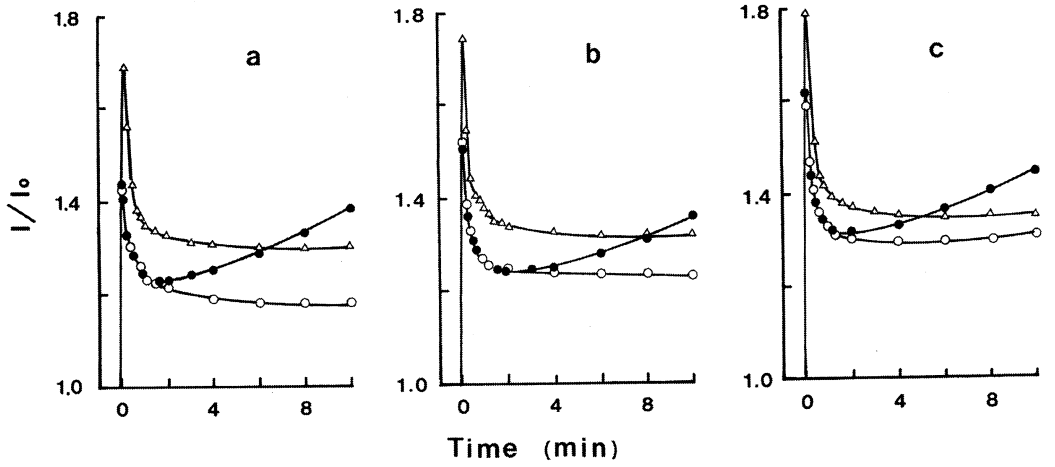

Figure 5. Light scattering of vesicles. Light scatterings of vesicle I with PLD (●, 0.125 μg/ml) or without PLD (○), and vesicle II without PLD (△) at 5 mM (a), 10 mM (b), and 20 mM of Ca^{2+} were measured with fluorescence fsectrophotometer. Excitation and emission wavelength was fixed at 430 nm at 90° direction.

and/or PLD. Figure 4 shows the time course of fluorescence quenching by phase separation of vesicle I (with or wiothut PLD) and vesicle II at several Ca^{2+} concentrations. The extents of fluorescence quenching of vesicle I and II induced by Ca^{2+} alone were less than that of vesicle I induced by Ca^{2+} and PLD. It suggests that PLD drastically accelerates phase separation of vesicle I. After correction, during the first 4 min, the extent of fusion of vesicle I is 44%/0.7 = 63%, vesicle I plus PLD was 43%/0.57 = 75%, and vesicle II is 54%/0.73 = 74% at 5 mM Ca^{2+}. It suggests that the initial fusion rate of vesicle I (30% PA) induced by PLD was not less than that of vesicle II (50% PA) induced by Ca^{2+} alone. The results were in accord with previous studies (Park *et al.*, 1992, 1993). To examine whether PLD plays a role in membrane aggregation, turbidity of vesicle membranes was measured using a fluorescence spectrophotometer at fixed excitation and emission wavelength at 420 nm. Turbidity of veicle I induced with or without PLD for 2 min in the presence of Ca^{2+} were similar (Figure 5) whereas fusion rates were different. PA content produced by PLD for 2 min was found to be 2-3% (Park *et al.*, 1992). It was interesting that the PA on membranes elevated by PLD for 2 min did not have an effect on membrane aggregation but had a significant effect on the membrane fusion. It suggests that PLD induced membrane fusion not by increasing aggregation of vesicles, but by other action(s).

Interaction of PLD to vesicles and the changes of PLD fluorescence

To invesigate the role of PLD in membrane fusion induced by PLD, the interaction of PLD to vesicles and the change of PLD fluoresence were examined. First the binding of PLD to vesicles was determined by measuring the protein concentration of supernatant after ultracentrifugation of protein-vesicle mixtures (Table 1). Most PLD was bound to vesicles in the presence of Ca^{2+} whereas small amounts of PLD was

Table 1. Phospholipase D binding to vesicles. Phospholipid concentration of vesicles was 0.6 μmol/ml, PLD was 5 μg/ml and Ca^{2+} was 10 mM. Final volume was 0.2 ml. Reaction mixtures were incubated for 10 min at 27°C, then were ultracentrifuged for 1 h at 55,000 r.p.m., 4°C. Protein concentration of the supernatant was determined using Coomasie blue G-250. Independent 2 experiments were performed and values are average ± S.E.

Condition	Precipitated PLD (%)
Vesicle + PLD	13 ± 16
Vesicle + PLD + Ca^{2+}	96 ± 3
PLD + Ca^{2+}	8 ± 13
PLD	0

Table 2. Fluorescence change of PLD in the presence of vesicles. PLD, 0.3 μg/ml and [a]0.125 μg/ml, 5 mM Ca^{2+}, and 0.05 μmol/ml vesicles were used for measuring fluorescence changes. The samples were excited at 280 nm and emission fluorescences at 320 nm were measured.

Condition	F/Fo	
	1 st exp	2nd exp[a]
PLD	1	1
PLD + Ca^{2+}	0.78	0.9
PLD + vesicles	1.42	1.55
PLD + vesicles + Ca^{2}	1.45	1.42

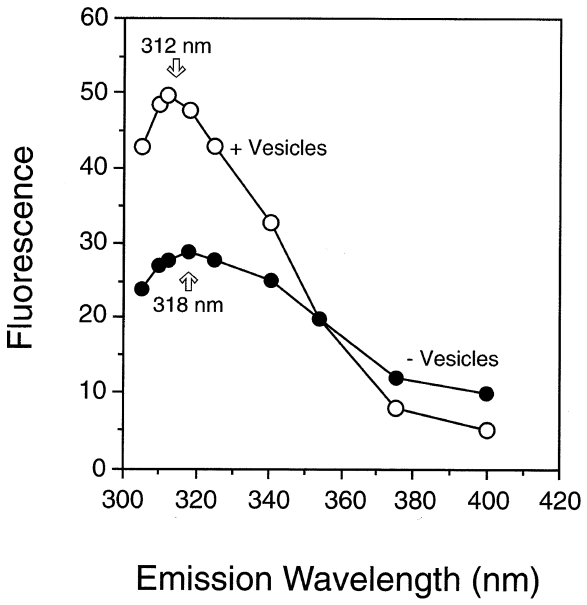

Figure 6. Fluorescence of PLD. PLD emmission fluorescence was scanned in the absence of vesicles (○) and in the presence of vesicles (●). Excitation wavelength was 280 nm. Ca^{2+} concentration was 5 mM. Other conditons were the same as in Figure 2.

bound to vesicles in the absence of Ca^{2+}. To study the change of PLD structure on the surface of vesicles, the fluorescence of PLD was measured. The fluorescence of PLD in the presence of vesicles was increased and the wavelength of emission for maximal fluorescence peak was shifted from 318 nm to 312 nm (Figure 6). This blue shift indicates that environment of fluorephore of PLD was changed more hydrophobically. The change of PLD fluorescence was measured in the presence of PLD and/or Ca^{2+} (Table 2). The fluorescence of PLD was increased in the presence of vesicles irrespective of Ca^{2+}. This suggests that PLD could interact with vesicles without Ca^{2+} and PLD structure was changed. However, the change of PLD structure by vesicle in the absence of Ca^{2+} did not induce membrane fusion. It was considerable that the change of PLD structure may not be a major factor for the induction of vesicle fusion by PLD because fusion does not occur in the absence of Ca^{2+}. Conclusively, it seems that irreversible binding of PLD to vesicles in the presence of Ca^{2+} in addition to the asymmetric PA distribution between the two monolayers was essential for induction of membrane fusion.

Physiological significance of PLD related to membrane fusion

Activation of PLD pathway is a widespread response when cells are activated by agonists that bind receptors on the cell surface. Recently extensive studies have been reported that PLD is essential for the membrane trafficking and fusion (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). PLD in human neutrophil lysates is activated by guanosine 5'-O-(3-thiotriphosphate) (GTPγS), implying the participation of a GTP-binding protein. Reconstitution of GTPγS-dependent activity requires protein factors in both the plasma membrane and the cytosol (Olson *et al.*, 1991). SmgGDS (small GTP-binding protein (smg) GDP dissociation stimulator (GDS)), which stimulates the exchange of GDP for GTP on a variety of smg, stimulates GTP-dependent PLD activity. RhoGDI, a regulatory protein that binds specifically to and inhibits the functions of Rho subfamily smg, inhibited GTPγS-dependent activity. Thus, neutrophil PLD is regulated by a membrane-associated smg protein, likely to be a member of the Rho family (Bowman *et al.*, 1993). RhoGDI rapidly extracts Rho, Cdc42 from membranes, but Rac1 is not extracted. Full reconsitution of GTPγS-stimulated PLD in RhoGDI-washed membranes is achieved with recombinant RhoA (Malcolm *et al.*, 1994). A cytosolic factor markedly enhanced PLD activity in membranes and was essential for GTPγS-dependent stimulation of an enriched preparation of PLD. The factor was purified to homogeneity from bovine brain cytosol and identified as a member of the ADP-ribosylation factor (ARF)

subfamily of smg. ARF proteins have been implicated recently as factors for regulation of intracellular vesicle traffic (Ostermann *et al.*, 1993). The current finding suggests that PLD activity plays a prominent role in the action of ARF and that ARF may be a key component in the generation of second messenger via PLD (Brown *et al.*, 1993). The combination of RhoA and ARF shows a synergistic effect. The data suggest the existence of at least two different PLD isozymes in HL60 cells (Siddiqi *et al.*, 1995). The well-established role of ARF in vesicle traffic would suggest that alterations in lipid content by PLD are an important determinant in vesicular dynamics (Cockcroft *et al.*, 1994).

It was reported PLD induces membrane fusion of liposome composed of PC/PE/PA (20:50:30) in the presence of Ca^{2+} (Park *et al.*, 1992). The differential PA distribution between the two monolayer rather than absolute PA content of the outer monolayer was important. In this paper, we suggested that PLD binding to vesicles may be important as well as PA distribution between two monolayers of vesicles. Membrane fusion induced by PLD *in vitro* may give a light to elucidating the mechanism of membrane fusion *in vivo* and the interaction of PLD to membranes and PA asymmetry of membrane bilayer induced by PLD may be important for membrane fusion *in vivo*.

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