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# Effect of Mixed-Phospholipid Layer on Phospholipase D Reaction-induced Vesicle Rupture

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**Abstract** Spherical phospholipid bilayers, or vesicles, were prepared layer by layer using a double-emulsion technique, which allows the outer layer of the vesicles to be formed with two phospholipids that have different head groups: phosphatidylcholine (PC) and phosphatidylethanolamine. At the outer layer of the vesicles, the phospholipase D (PLD) catalyzed for the conversion of PC to phosphatidic acid. The reaction caused by PLD induced the curvature change of the vesicles, which eventually led to the rupture of the vesicles. Before the investigation, the ratio of dioleoylphosphatidylethanolamine to oleoylhydroxyphosphatidylethanolamine was found as a condition such that the vesicles made with the mixed lipids were as stable as those made with pure dioleoylphosphatidylcholine. Response time from the PLD injection to vesicle rupture was monitored by the composition of the outer layer by the fluorescence intensity change of pH-sensitive dye encapsulated in the vesicles. The response time began to be slowed at approximately 30 % PC. The response times for the compositions were associated with the surface density of PC at the outer layer. These results also seem to be determined by the size of PLD, specifically the PLD active site.

**Keywords** Biophysics · Fluorescence techniques · Lipid protein interactions · Measurement · Membrane biophysics · Membrane vesicles

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Phospholipase D (PLD) is membrane-active enzyme involved in a variety of cellular function, including membrane/vesicle trafficking, actin cytoskeleton rearrangements, glucose transport, superoxide production, secretion, cellular proliferation, and apoptosis (McDermott et al. 2004; Exton 2002). As a result, PLD is implicated in a range of diseases, including cancer, inflammation, and myocardial disease (Huang and Frohman 2007; Tappia et al. 2006; Scott et al. 2009; Brown et al. 2007). By PLD action upon phosphatidylcholine (PC), PC is cleaved into alcohol and phosphatidic acid (PA, a potent mitogen), which may be essential for the formation of certain types of transport vesicles or which may be constitutive vesicular transport-to-signal transduction pathways. This hydrolysis leads to changes in the lipid content of membranes that could also play a role. PLD may have a physiological function through the further metabolism of PA to diacylglycerol and lysophosphatidic acid (McDermott et al. 2004; Brown et al. 2007).

Lipid layers are widely used models for cell-surface analyses and for investigating molecular events in membranes because the preparation methodology for the layers has been well established, and sensitive analytical techniques can be applied to investigate the events (New 1990; McConnell et al. 1986; Sackmann 1996; Brian and McConnell 1984). The lipid layers have been applied to many areas of biomedical research, such as cell recognition, membrane-mediated catalysis, effects of anesthetics, and antimicrobial peptides (Giesen et al. 1991; Mou et al. 1994; Miszta et al. 2008; Fang et al. 2000; Cheow and Hadinoto 2011). This layer has been used to investigate the phospholipases, i.e., the effect of the enzymes on the wetting properties of the lipid layers and the configuration of the layers, as well as the activity of the enzymes (Jurak and Chibowski 2010; Chen et al. 2009; Chemburu et al. 2008).

Hydrolysis triggered by PLD is a critical step for the fusion essential for the cellular processes. This hydrolysis is found to induce a change in the composition of the membranes that eventually induces the rupture of the vesicles. The change in composition means that the geometry of the vesicle components is changed: the head groups of the components become smaller. Therefore, hydrolysis leads to a decrease in curvature, and rupture is finally reached. Recently, it has been found how the PLD-induced-vesicle rupture occurred with respect to the phase of the vesicle layers (Park 2011). However, little is known about how the composition of the outer layer affects the properties of the biological membranes caused by hydrolysis. Investigating this effect may contribute to an understanding of the physical behavior of these enzymes in terms of quantitative analysis. In this work, we aim to investigate the effect of the composition of the lipid outer layer on vesicle rupture.

## Experiments

Dioleoylphosphatidylethanolamine (DOPE), oleoylhydroxyphosphatidylethanolamine (OHPE), dioleoylphosphatidylcholine (DOPC), and dipalmitoylphosphatidic acid (DPPA) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The DPPA was dissolved in 10 ml of *tert*-butyl methyl ether at 10 mg/ml, followed by adding 100 µl DI water of 5 mM pyranine, 50 mM NaCl, and 1 mM $CaCl_2$ at pH 9.0. Therefore, the micelles with DPPA were prepared by extrusion through the 50 nm pores of 78-mm diameter PTFE membranes above the transition temperature of the DPPA. Several drops (<10 µl) of the micelle solution and the 10 mg/ml *tert*-butyl methyl ether solution of a desired ratio of DOPC, DOPE, or OHPE were continuously added through a 22-gauge needle inserted into 10 ml aqueous solutions of 50 mM NaCl and 1 mM $CaCl_2$ at pH 9.0. The final lipid concentration of the aqueous solution was 1 mg/ml. During the addition, the aqueous solution was magnetically stirred, and a nitrogen stream was injected into the aqueous solution. The solution was centrifuged ($3700\times g$) to remove the phospholipids that did not form the vesicles. The liposome solution was acquired from the supernatant of the solution. These procedures are well known as a methodology to prepare vesicles (New 1990). For the confirmation of the vesicle formation, the diameter of the micelles was measured (ELS-8000; Otsuka Electronics, Osaka, Japan) before the micelles were transferred to the aqueous solution. For the measurement, the viscosity and the refractive index of the *tert*-butyl methyl ether were 0.23 cP and 1.3686, respectively (Lide 2005). After the transfer of the micelles into the aqueous solution, the diameter of the vesicles was measured. The diameters of the micelles and the vesicles were 75 ± 10 and 80 ± 10 nm, respectively. These results were consistent with the expectation that the lipid layer formed on the micelle surface. Besides the change in the diameter, no leakage of the pyranine molecules indicated that each layer was not disturbed. Otherwise, the fluorescence intensity at 510 nm would be changed tremendously when several drops of aqueous solutions of 50 mM NaCl and 1 mM $CaCl_2$ at pH 3.0 were added to the vesicle solution.

Phospholipiase D (PLD) was purchased from Sigma Aldrich (St. Louis, MO). One nanomole was selected as a PLD concentration because recent results indicated that more than 1 nM of PLD concentration had little effect on the rupture's response time (Park 2011). Because it was known that the reaction causes the rupture of the vesicles and the pyranine (pH-sensitive fluorescence dye) was encapsulated inside the vesicles, the fluorescence intensity was monitored in real time with a Wallac Victor3 multiwell fluorometer (Perkin Elmer, Waltham, MA). Because the pyranine molecules have different fluorescence intensities when they are exposed to the different pH solutions within the rupture, the tremendous change in the intensity difference between the vesicle solution with the PLD injection and with only the buffer solution injection means that the vesicles rupture. Therefore, the intensity was observed to investigate the effect of the composition on the vesicle rupture on the PLD reaction. For each phospholipid ratio, we measured when the rupture occurred from the injection of the PLD molecules.

The high-performance liquid chromatography (HPLC) analyzer consisted of a Gel Silica 60 column (particle size 5 µl; inner diameter 47 mm; length 15 cm; Tosoh, Tokyo, Japan) and an HPLC system (Waters Associates, Milford, MA) containing a type 600 solvent delivery system, a type U6K injector, a type 490 variable wavelength detector, and a type 740 data module. Elution was performed with a solution of acetonitrile–methanol–85 % phosphoric acid (130:5:1.7, v/v/v) at a flow rate of 1 ml/min at room temperature. Phospholipid solutions were dissolved in 100 µl of methylene chloride, and 10 µl of the solution was injected into the HPLC system.

## Results and Discussion

For the investigation of the PLD reaction on the lipid layer, the pyranine molecules were dissolved only inside of the vesicles. The encapsulation of the molecules was confirmed via fluorometry (Perkin Elmer, Boston, MA) with 460 nm excitation and 520 nm emission wavelengths. The fluorescence intensity was changed with Tween-20 treatment. Without the treatment, the intensity was not changed after the addition of pH 3 DI water drops. The results are

provided in Fig. 1. Encapsulation was successfully achieved.

The desired ratio of the phospholipids at the outer layer was confirmed by HPLC. The peak area of each component was compared for the solution before centrifugation and for the supernatant after centrifugation (Fig. 2). Although the areas for every component after the centrifugation were reduced except PA, the degree of the decrease was proportional. The proportional decrease means that the ratio of the phospholipids at the outer layer are still almost identical with that of the DOPC, DOPE, and OHPE solution added for liposome preparation. As expected, the retention time for OHPE was longest, the result of its molecular weight. It was also expected that the more negative component would correspond to the shorter retention as a result of the surface interaction with the silica spheres, the surfaces of which had inherently negative dipoles. The order of the peak for each component was consistent with that previously described (Kurumi et al. 1991; Singleton and Stikeleather 1995). PA peak after centrifugation varied little. From this invariance, we found that the inner layer made with PAs remained during the centrifugation. This result is consistent with a confirmation of encapsulation.

Apart from the composition, there are other factors that influence the reaction—for example, the vesicle number, the PLD concentration, the ionic concentration of the vesicle solution, the phase of the inner layer, the vesicle radius, and the vesicle stability. Therefore, these factors were determined before the composition experiments. The concentration of the phospholipids and the radius of the vesicles were constant in this research, and it is known that 10 mg/ml of the phospholipids correspond to the $10^7$–$10^8$ quantity of the vesicles (Park 2007). Previous research found effects of the other factors (Park 2011). At the 1 mg/ml solution of phospholipids, 1–10 nM PLD concentration led to little change in the reactivity. Fifty micromoles of NaCl and 1 mM $CaCl_2$, one of the biomimetic conditions, were selected for the ionic concentration because the reactivity was dependent on a concentration less than that (Brown et al. 2007; Webb et al. 2010; Cheow et al. 2010; Furt and Moreau 2009). Under the conditions described above, it was found that the reactivity was independent of the inner-layer phase. Because the solid phase is known to be more stable, DPPA was used instead of DOPA at room temperature (Park 2010a). The phase of the lipid layers was made with the phospholipids whose transition temperature was considered (Park and Ahn 2008). Dioleoyl lipids were used for the liquid phase because their transition temperature was much lower than room temperature. However, dipalmitoyl lipids were solid at room temperature. The stability was adjusted identically for the vesicle of each composition. Stability depended on the composition of the vesicles. Except for the composition of the outer layer, all the factors were decided.

As components for the outer layer, PC and phosphatidylethanolamine (PE) were selected because only PC was

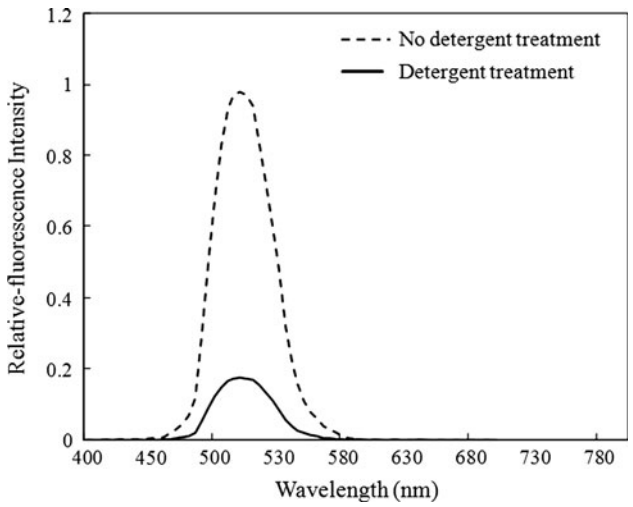


**Fig. 1** Fluorescence intensity change after the addition of pH 3 DI water drops

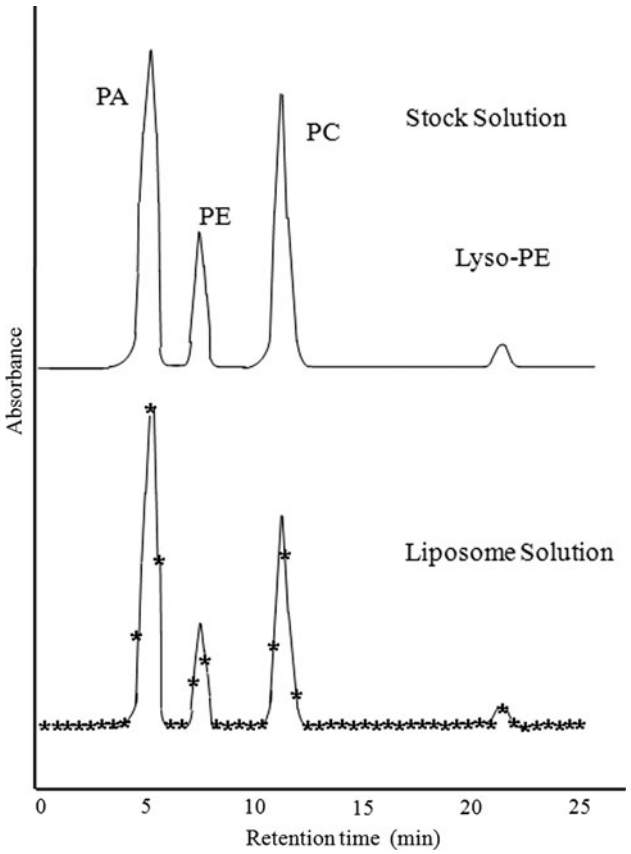


**Fig. 2** HPLC peaks for each component: lipid solution before centrifugation (*top*) and supernatant of solution after centrifugation (*bottom*)

reacted by PLD. However, if DOPC was replaced with DOPE only, the radius of the vesicles would be increased as a result of the difference in the geometries between DOPC and DOPE. Therefore, OHPE, lyso-PE, was utilized for the vesicle preparation with DOPC and DOPE so that the radius could be kept constant. The schematic diagrams for the vesicles made with different components are presented in Fig. 3. Because the PLD reaction was based on the curvature change of the vesicles, it was essential that the radius remain constant. The introduction of OHPE allowed only the composition effect on the PLD reaction found. In terms of the PE:PC ratio that was important for the PLD reaction, 11 conditions (pure PE to pure PC by 10 %) were considered. However, at a specific PE, it was also necessary to determine the amounts of DOPE and OHPE that might influence the stability of the vesicles. Therefore, the composition of the mixed outer layer was adjusted because the vesicles of each composition were necessarily found to be as stable as the vesicles made with pure DOPC. To find out the composition resulting in identical stability, we observed permeability at 5:1, 10:1, and 15:1 ratios of DOPE to OHPE for each PE condition, measuring the fluorescence intensity change with respect to time (Fig. 4). The ratio of PE to PC at a specific ratio of DOPE to OHPE had little effect on the stability. It was found that the stability of the vesicles at 15:1 of DOPE to OHPE was almost identical with that of the vesicles made with pure DOPC. This result was consistent with the expectation from the geometries of the lipids in that the ratios of lipid volume to head group area and lipid length were 0.5–1, 1, and less than 0.33333 for DOPC, DOPE, and OHPE, respectively (Cevc and Marsh 1987).

Reactivity was monitored at the various composition conditions by fluorescence response time. The time of pure DOPC vesicles was consistent with the previous result (Park 2011). A 30 % PC to non-PC condition led to a gradual increase in the response time (Table 1), which means that the reactivity was decreased gradually from a 30 % PC to a non-PC condition. Because the vesicles of the outer layer made with only PE included OHPE of less than 7 %, the mean molecular area of the outer layer was presumably $\sim$0.8 nm$^2$ (Park and Ahn 2008; Park 2010b). Therefore, the density of PC on the outer layer made with 30 % PC was one PC molecule per 2.7 nm$^2$. Considering that the PLD gyration radius is known to be approximately 4 nm, the value of the PLD area calculated with the radius is much higher than the area necessary to find one PC molecule in the case where the outer layer of the vesicles was made with even 10 % PC (Brown et al. 1995). The density of PC on the outer layer made with 10 % PC was one PC molecule per 8 nm$^2$. Therefore, it was expected

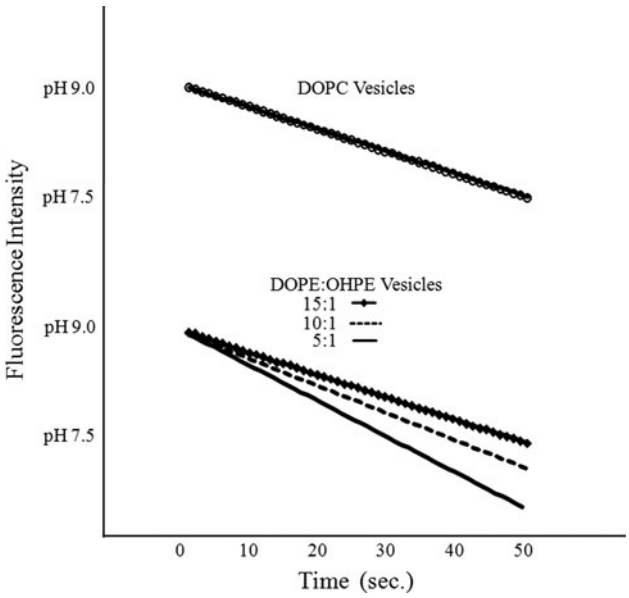


**Fig. 4** Fluorescence intensity change with respect to time for the vesicles of the outer layer made with pure DOPC (*top*) and made with a mixture of DOPC, DOPE, and OHPE at different DOPE:OHPE ratios (*bottom*)

**Table 1** Response time from PLD injection to vesicle rupture[a]

| DOPC and PE | Response time (s) |
|---|---|
| Pure DOPC | 0.7 |
| 90 % DOPC, 10 % PE | 0.7 |
| 80 % DOPC, 20 % PE | 0.7 |
| 70 % DOPC, 30 % PE | 0.7 |
| 60 % DOPC, 40 % PE | 0.7 |
| 50 % DOPC, 50 % PE | 0.7 |
| 40 % DOPC, 60 % PE | 0.7 |
| 30 % DOPC, 70 % PE | 0.9 |
| 20 % DOPC, 80 % PE | 1.3 |
| 10 % DOPC, 90 % PE | 2.7 |
| Pure PE | 50 |

[a] PE consisted of DOPE:OHPE at a 15:1 ratio

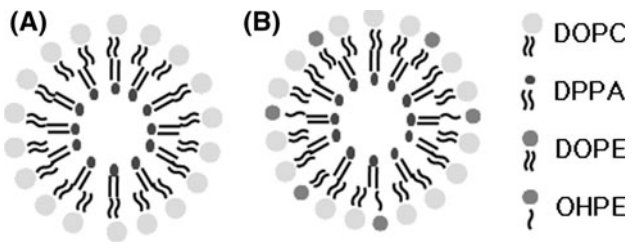


**Fig. 3** Schematic diagram for the vesicles made with different components. **a** Vesicles of the outer layer made with pure DOPC. **b** Vesicles made with mixture of DOPC, DOPE, and OHPE

that the response time would be little changed from the vesicles of the outer layer made with 10 % PC. This discrepancy might be due to the size of the PLD active site. Leiros et al. (2000) noted that PLD had a PC hydrolysis site with an area of 1.5–2 $nm^2$. Therefore, if the average area to find one PC molecule was decreased to less than 2 $nm^2$, the reactivity remained. From 30 % PC to non-PC, the reactivity began to be reduced.

In conclusion, in this study, the effect of mixed lipid layer on the PLD-induced hydrolysis was investigated by fluorescence intensity change. Before the investigation, the ratio of DOPE to OHPE was found to be a condition such that the vesicles made with the mixed lipids were as stable as those made with pure DOPC. Monitoring revealed that the rupture caused by the PLD reaction could be interpreted with respect to the composition of each layer, especially the PC density on the outer layer of the vesicles. It was observed that the vesicles made with more than 30 % PC at the layer showed identical reactivity as to PLD reaction. However, from 30 % PC to non-PC at the layer, the reactivity of the vesicles was reduced. These results seem to be caused by not only the density of PC on the layer, but also the size of PLD, specifically the PLD active site.

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