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Effect of Vesicle Curvature on Phospholipase D Reaction-Induced-Rupture

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Spherical phospholipid-bilayers, vesicles, were prepared using the layer-by-layer double emulsion technique, which allows the bilayer to be formed asymmetrically. On the outer layer of the vesicles, the phospholipase D (PLD) reacted to convert phosphatidylcholine (PC) to phosphatidic acid (PA). The reaction induced the curvature change of the vesicles, which eventually led to rupture. The response time from the time of PLD injection to the time of rupture was measured against different vesicle curvatures and the outer layer phase, using the fluorescence intensity change of a pH-sensitive dye encapsulated within the vesicles. The effect of the vesicle curvature on the response was observed to be more significantly dramatic at the solid phase, compared to the liquid phase. Furthermore, in the solid phase, the response time was faster for 80 and 155 nm vesicles and, slower for 605 nm vesicles than similarly sized vesicles in the liquid phase vesicles. This difference in the response time was thought to result from the configuration determined by the phase difference and the PLD behavior.

Key Words : Vesicle, Phospholipase D, Curvature, Phase asymmetry

Introduction

Phospholipase D (PLD), as one of membrane-active enzymes, is related to various cellular functions, including membrane/vesicle trafficking, actin cytoskeleton rearrangements, glucose transport, superoxide production, secretion, cellular proliferation, and apoptosis.[1,2] Therefore, PLD is implicated in a range of diseases such as cancer, inflammation, and myocardial disease.[3-6] The reaction of PLD on phosphatidylcholine (PC) converts PC into an alcohol and phosphatidic acid (PA, a potent mitogen) that may be essential for the formation of certain types of transport vesicles or may initiate vesicular transport to signal transduction pathways. The conversion that induces changes in the composition of membranes could also play a role. PLD may have an additional physiological function in the reaction that breaks down PA to diacylglycerol (DAG) and lysophosphatidic acid.[1,6]

Lipid layers are widely-used as a model of the cell surface and also for investigating molecular events in membranes, because the preparation methodology for lipid bilayers has been well established and highly sensitive analytical techniques can be applied to investigate the events.[7-10] These lipid layers have also been useful for the biomedical research of cell recognition, membrane-mediated catalysis, anesthetic effect, and antimicrobial peptide activity.[11-15] The actions of the phospholipases have also been investigated using these bilayers, to study the effect of the enzymes on the wetting properties and the configuration of the bilayers, as well as the activity of the enzymes.[16-18]

The hydrolysis triggered by PLD is a critical step for membrane fusion, which is an essential process of cells. The hydrolysis is found to induce changes in the composition of the membranes, which eventually induces vesicle rupture. This composition change caused by hydrolysis results in the occurrence of smaller headgroups at the outer layer of the vesicles and correlates to a change in the geometry of the vesicle. Therefore, the hydrolysis leads to a curvature decrease that creates the rupture to occur. Recently, the PLD-induced-vesicle rupture has been characterized with respect to the phase of the vesicle layers.[19] However, little is known how the curvature with the different phases of the outer layer affects the PLD-induced vesicle rupture. The investigation of this effect may contribute to the understanding of the physical behavior of these enzymes under quantitative analysis. In this work, we aim to investigate the effect of the curvature with the different phases on vesicle rupture.

Experimental

Dioeloylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), oleoylhydroxyphosphatidylcholine (OHPC), palmitoylhydroxyphosphatidylcholine (PHPC), palmitoylhydroxyphosphatidic acid (PHPA), and dipalmitoylphosphatidic acid (DPPA) were purchased from Avanti Polar Lipids (Alabaster, AL), and were used without further purification. The PHPA and DPPA, mixed to a desired ratio, were dissolved in 10 mL of *tert*-butyl methyl ether to achieve 10 mg/mL. Next, 100 μL DI water of 5 mM pyranine, 50 mM NaCl, and 1 mM $CaCl_2$ at pH 9.0 was added to the lipid solution. The micelles were acquired by extrusion above their transition temperature through the 78 mm diameter PTFE membranes that have a specified pore size. For pore sizes, 50, 100, 200, and 500 nm were selected. Several drops (less than 10 μL) of the micelle solution were added using 22-gauge needle inserted into the 10 ml aqueous solutions of

50 mM NaCl and 1 mM $CaCl_2$ at pH 5.0, followed by dropping the 10 mg/mL *tert*-butyl methyl ether solution containing the desired ratio of DOPC and OHPC (or DPPC and PHPC) continuously. The final lipid concentration of the aqueous solution was 1 mg/mL. During the addition, the aqueous solution was magnetically stirred and nitrogen stream was injected into the aqueous solution. After the solution underwent the centrifugation (3700 × g) to remove the phospholipids that did not form the outer layer of the vesicles, the vesicle solution was extracted from the supernatant of the solution. These procedures are part of a well known methodology used to prepare vesicle solutions.[7]

For the confirmation of the vesicle formation, the diameter of the micelles was measured using the ELS-8000 (Otsuka Electronics Co. Ltd, Osaka, Japan) before they were transferred to the aqueous solution. The viscosity and the refractive index of the *tert*-butyl methyl ether, required for the measurements, were 0.23 cP and 1.3686, respectively.[20] The average diameter of the micelles filtered through the membranes was 75, 150, 280, and 600 nm, respectively. After the transfer of the micelles into the aqueous solution, the average diameter of the vesicles was measured. The average diameter of the vesicles was 80, 155, 285, and 605 nm for each pore size, and had no change in variance from the variance values of the micelles. These diameters indicated that a single lipid layer was formed, as an outer layer of a vesicle, on the micelle surface. In addition to the change in the diameter, there was no leakage of the pyranine molecules, which indicated that each layer was not disturbed. If there were such a disturbance, the fluorescence intensity at 510 nm would change tremendously with the addition of several drops of aqueous solutions of 50 mM NaCl and 1 mM $CaCl_2$ at pH 3.0 into the vesicle solution.

Phospholipiase D (PLD), purchased from Sigma Aldrich (St. Louis, MO, USA), was dissolved in the vesicle solution with a 1 nM concentration, because the recent results showed that more than a 1 nM concentration of PLD had little effect on the response time.[19] Because the pyranine(pH-sensitive fluorescence dye) was encapsulated within the vesicles, the vesicle rupture was monitored in real time using a Wallac Victor3 multi-well fluorimeter (Perkin Elmer, Waltham, MA, USA). The tremendous difference in the change in intensity between the vesicle solution with the PLD injection and the solution that underwent only the buffer solution injection means the rupture of the vesicles, which was caused by PLC reaction. Therefore, the change in intensity change was observed to investigate the effect of the curvature and phase on the vesicle rupture. For the different curvatures with each phase of the outer layer, the intensity was measured when the rupture occurred after the injection of the PLD molecules. The measurement was performed more than 5 times at each condition.

Results and Discussion

For the investigation of the PLD reaction on the lipid layer, the pyranine molecules were encapsulated inside of vesicles with different curvatures. The encapsulation of the molecules was confirmed using fluorometer (Perkin Elmer, Boston, MA, USA) with 460 nm excitation and 520 nm emission wavelengths. The change in the fluorescence intensity of the vesicle solution was observed with detergent (Tween 20) treatment, after the addition of pH 3 DI water drops. Without the treatment, a change in intensity was not observed after the addition (Figure 1). Therefore, the encapsulation was successfully achieved.

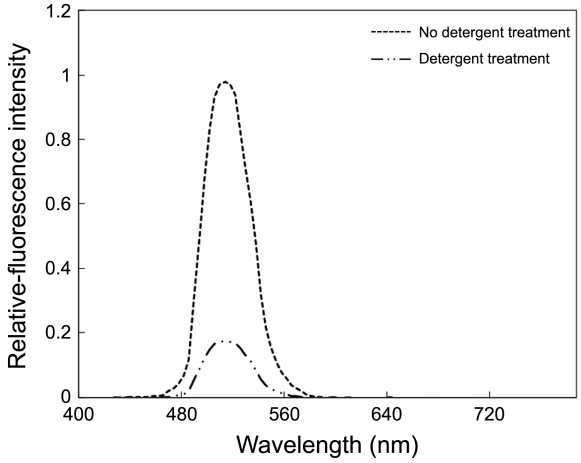

Figure 1. Fluorescence intensity change after the addition of pH 3 DI water drops.

The PLD reaction are influenced by several factors such as the vesicle number, the PLD concentration, the ionic concentration of the vesicle solution, the phase of the inner layer, the vesicle curvature, and the vesicle stability. Therefore, the factors were determined prior to conducting the experiments for the curvature. The concentration of the phospholipids was kept constant throughout this experiment. It is well-known that a 10 mg/mL concentration of the phospholipids corresponds to 10^7~10^8 vesicles.[21] Results from previous research provided the information on the other factors.[20] For the 1 mg/ml solution of phospholipids, a PDL concentration of 1 to 10 nM caused little change in the reactivity. Fifty mM NaCl and 1 mM $CaCl_2$, which is one of biomimetic conditions, was selected as the ionic concentration, because the reactivity of PLD was dependent on the concentration only at lower concentrations.[6,22-24] Furthermore, for the conditions determined above, the PLD-induced hydrolysis was independent of the inner-layer phase. Because solid phase was known to be more stable, saturated PA was used instead of unsaturated PA at room temperature.[25] Controlling the phase of the lipid layers was achieved considering the transition temperatures.[26] Oleoyl lipids were used for the liquid phase experiments at room temperature due to their transition temperature that is lower than room temperature, while Palmitoyl lipids were selected for solid phase experiments at room temperature. The 75 nm micelle was prepared with DPPA only. The ratios of lyso-PA to DPPA were 1:15, 1:20, 1:25 for 150, 280, and 600 nm, respectively. At a specific phase, the stability was adjusted to be identical for each vesicle size at each composition, because stability

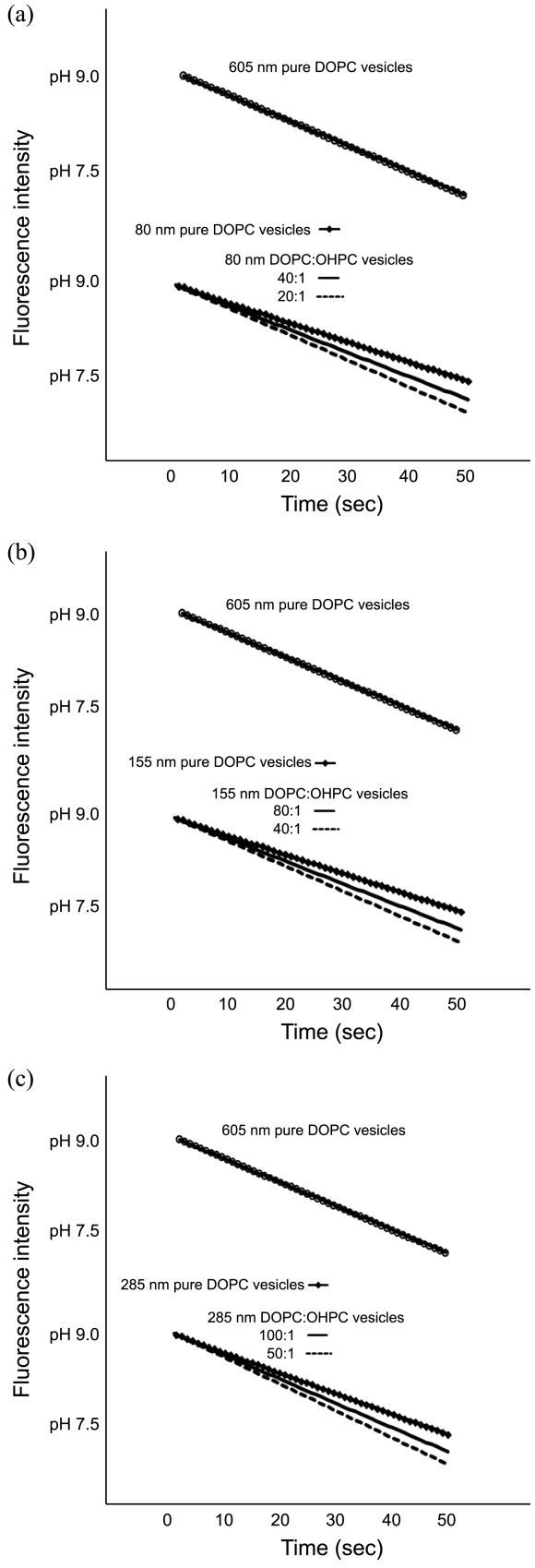

Figure 2. Fluorescence intensity change with respect to time for each diameter of vesicles, (a) 80 nm, (b) 155 nm, (c) 285 nm, prepared with the ratio of OHPC(lyso-PC) to DOPC(two fatty-acid tail PC) based on the 605 nm diameter vesicles with pure DOPC.

can vary with both the vesicle curvature and the vesicle component. Once all of factors were established, the curvature effect of each outer-layer phase was investigated.

For the investigation of only the curvature effect, it was essential to exclude the stability effect. If only DPPC or DOPC was used for the vesicle preparation, the stability would vary with the curvature. Therefore, PC headgroup lipids with only one tail, lyso-PC, were used for this preparation. Because it is known that the ratio of the volume to the headgroup area times lipid length is 0.5-1 and 0.3 for two fatty-acid tail PC and lyso-PC, respectively, it is possible to calculate the amount of lyso-PC necessary to achieve identical stability that does not vary with change in vesicle size. From this calculation, it was expected that the stability levels of our vesicles of 80, 155, and 285 nm diameters with the ratios of lyso-PC to two fatty-acid tail PC of 1:20, 1:40, and 1:100, respectively, were identical to 605 nm diameter with pure two fatty-acid tail PC. This result was verified experimentally. Vesicles of each diameter were prepared using the ratios suggested above. The change in fluorescence intensity with respect to time was measured as the indicator for the stability. Figure 2 presents the results of the intensity change for each vesicle diameter of vesicles with a liquid phase outer-layer. At ratios other than those described above, the vesicle has different stabilities. For example, 80 nm vesicles with a 1:40 ratio were more stable than those with 1:20, and 155 nm vesicles with a 1:80 ratio were more than those with 1:40 (Figure 2(a) and (b)). For 285 nm vesicles with a 1:50 ratio, the stability was lower compared to 285 nm vesicles with 1:100 (Figure 2(c)). The condition for the constant stability with respect to the curvature was found, but the stability still changed in different phases.

After the condition for the stability was determined, the reactivity of PLD was monitored for each size and each phase of the outer layer using the fluorescence response time. At the solid phase of the outer layer, the response time increased as the curvature decreased, as summarized in Table 1. From this change in response time, it may be inferred that the reaction of PLD has a preferred configuration of the lipid layer, which is related to the curvature of the lipid layer. The preferred curvature may be approximately 0.01-0.02, considering the active area of PLD.[27] The other vesicle sizes, 285 and 605 nm, have curvatures much lower than 0.01. However, for the liquid phase outer layer, the results are slightly different from those at the solid phase (Table 1). For the 285 nm vesicle, the response time was identical to the vesicles with a diameter less than 285 nm. This tendency was not observed at the solid phase of the outer layer. The discrepancy between phases for the 285 nm vesicle seems to result from the phase difference and the PLD behavior. It is known that the enzyme reaction occurs through an induced fit (enfolding of enzyme to substrate).[28] The induced fit requires a change in the configuration, which is generated more easily in the liquid phase than in the solid phase at the lipid layer due to the elasticity of the lipid layer.[25] For the 605 nm vesicle, the response time of the liquid phase also became slower than the smaller diameter, as in the solid

Table 1. Response time from the time PLD injection to the time of vesicle rupture for each diameter for each phase of the vesicle outer-layer (s)

	80 nm	155 nm	285 nm	605 nm
Solid phase	0.5	0.5	0.7	1.1
Liquid phase	0.7	0.7	0.7	0.9

phase outer layer experiments.

Recently, it was found that the PLD reaction time was faster at the solid phase outer layer than the liquid phase outer layer.[19] This finding was interpreted in terms of the lipid density and the lateral diffusion of the lipid molecules. Furthermore, the response time found in this research was also faster at the solid phase, for 80 and 155 nm vesicles. However, for the 605 nm vesicles, the response time was unexpectedly slower for the solid phase rather than for the liquid phase. This phenomenon may result from the phase and the PLD behavior, as described in the previous paragraph. It may be concluded that the configuration is more important than the surface density and lateral diffusion of the lipid layer. From the results acquired in this experiment, the reaction with PLD was affected by the curvature of the lipid layer rather than from the density and lateral diffusion of the lipids, the lipid composition, the stability of the lipid layer, the ionic concentration of the vesicle solution, the phase asymmetry of the vesicle, and the ratio of the vesicle to PLD.[29,30]

In conclusion, the effect of vesicle curvature on the PLD-induced hydrolysis was investigated using the fluorescence intensity change measurements. Prior to this investigation, the ratio of two fatty-acid tail PC to lyso-PC was established for the vesicles with the different diameters to make them as stable as 605 nm vesicles made with two fatty-acid tail PC only. Response time from the PLD injection to the rupture was measured at different curvatures of the vesicles and at different outer layer phases, using the fluorescence intensity change of pH-sensitive dye encapsulated within the vesicles. The change in the response time caused by a change in the curvature was observed to be more significant in the solid phase than the liquid phase. Furthermore, in the solid phase, the response time was faster for 80- and 155 nm vesicles and slower for 605 nm vesicles than the response time for the liquid phase vesicles. This difference in the response time was interpreted to be caused by the configuration determined by the vesicle phase difference and the PLD behavior, which may be more important than the surface density and lateral diffusion of the lipid layer.

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