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Composition Effect of the Outer Layer on the Vesicle Fusion Catalyzed by Phospholipase D

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Phospholipase D (PLD) catalyzed the generation of phosphatidic acid (PA) from phosphatidylcholine (PC) at the outer layer of the vesicles prepared through layer by layer via a double emulsion technique. The generation induced a curvature change in the vesicles, which eventually led them to fuse each other. The ratio of two-fatty-acid-tail ethanolamine (PE) to one-fatty-acid-tail ethanolamine (PE) was found to acquire the condition where the mixed-phospholipid vesicles were stable identically with pure two-fatty-acid-tail PC. The effect of the outer-layer mixture on the PLD-induced vesicle fusion was investigated using the fluorescence intensity change. 8-Aminonaph- thalene-1,3,6-trisulfonic acid disodium salt (ANTS) and *p*-Xylene-bis(*N*-pyridinium bromide) (DPX) were encapsulated in the vesicles, respectively, for the quantification of the fusion. The fluorescence scale was calibrated with the fluorescence of a 1/1 mixture of ANTS and DPX vesicles in NaCl buffer taken as 100% fluorescence (0% fusion) and the vesicles containing both ANTS and DPX as 0% fluorescence (100% fusion), considering the leakage into the medium studied directly in a separate experiment using vesicles containing both ANTS and DPX. The fusion data for each composition were acquired with the subtraction of the leakage from the quenching. From the monitoring, the vesicle fusion caused by the PLD reaction seems dominantly to occur rather than the vesicle lysis, because the composition effect on the fusion was observed identically with that on the change in the vesicle structure. Furthermore, the diameter measurements also support the fusion dominancy.

Key Words : Vesicle, Composition of outer layer, Phospholipase D, Fusion

Introduction

PLD has been recognized a signaling enzyme with its transphosphatidylation activity, which enables the synthesis of various phospholipids for the various cell-processes, including membrane/vesicle trafficking, actin cytoskeleton rearrangements, glucose transport, superoxide production, secretion, cellular proliferation, and apoptosis.[1,2] Therefore, PLD activation is implicated in a range of diseases including cancer, inflammation, and myocardial disease.[3-6] PLD uses water as a nucleophile to hydrolyse phosphatidylcholine (PC) to generate the membrane lipid phosphatidic acid (PA, a potent mitogen) that may be important for the formation of certain types of transport vesicles or may be constitutive vesicular transport to signal transduction pathways. With the reaction, the components of membranes are changed and the change could also play a role. PLD function may be involved in the further metabolism of PA to diacylglycerol (DAG) and lysophosphatidic acid.[1,6]

Membrane fusion is critical to various biochemical processes such as cell fusion, exocytosis, and endocytosis. Since lipid bilayers provides a barrier between extracellular and intracellular compartments of a biological cell, the layers are widely used as a models for cellular membrane-related-study. The fusion was greatly studied using the lipid layer to continuously monitor the mixing of the encapsulated aqueous contents and the merging of bilayers.[7-15] Two types of assays are complementary, because the layer fusion is the concomitant mixing of bilayers and aqueous contents. The lipid mixing assay provides the information at the same time for both fusion and case where the liposomes lyse first and the bilayer mix later. Additionally, it must be excluded that the lipid probe merely exchanges between liposomes without the merging of the bilayers. Therefore, the aqueous-space fusion assay is essential to confirm the fusion of the layers.[16,17]

The transphosphatidylation catalyzed by PLD leads the vesicle layers to fuse each other, and the fusion is central for the cellular processes. Due to the transphosphatidylation, the change occurs to the composition of the membranes. The change in the composition correlates to that in the geometry of the vesicle, because the transphosphatidylation results in the generation of the smaller headgroups at the outer layer of the vesicles. Recently, it has been investigated how the vesicle behavior was influenced by PLD with respect to the phase, asymmetry, and headgroup composition of the vesicle layers.[18-21] However, little is found how the vesicle fusion by PLD can be quantitatively adjusted with the phase of the mixed-phospholipid layers. The investigation of the fusion may have a contribution to understanding the physicochemical behavior of these enzymes under quantitative analysis. In this work, we aim to investigate the phase effect of the layers on the vesicle fusion.

Experimental

Dioeloylphosphatidylcholine (DOPC), Dioeloylphosphatidic acid (DOPA), Dipalmitoylphosphatidylcholine (DPPC), Dipalmitoylphosphatidic acid (DPPA), Dioeloylphosphatidylethanolamine (DOPE), Dipalmitoylphosphatidylethanolamine (DPPE), Oleoylhydroxyphosphatidylethanolamine (OHPE), and Palmitoylhydroxyphosphatidylethanolamine (PHPE) were purchased from Avanti Polar Lipids (Alabaster, AL), and used without further purification. ANTS and DPX were from Sigma Aldrich (St. Louis, MO). The DOPA (or DPPA) was dissolved in 10 ml of *tert*-butyl methyl ether at 10 mg/mL, followed by adding 100 μL DI water of 25 mM ANTS, 40 mM NaCl, 10 mM Tris-HCl at pH 7.4 for one population of vesicles and 100 μL DI water of 90 mM DPX, 10 mM Tris-HCl at pH 7.4 for the other population of vesicles. Therefore, the micelles were formed by extruding the DOPA (or DPPA) solution through the 50 nm pores of 78 mm diameter PTFE membranes above the transition temperature of the DOPA (or DPPA). As a reference for quenching effect, 12.5 mM ANTS, 20 mM NaCl, 45 mM DPX, 10 mM Tris-HCl at pH 7.4 was used as an aqueous solution to prepare micelles. Less than 10 μL of the micelle solution was added drop by drop through 22 gauge needle immersed into the 10 mL aqueous solutions of 10 mM Tris-HCl at pH 3.0, followed by dropping the 10 mg/mL *tert*-butyl methyl ether solution of a desired ratio of DOPC, DOPE, OHPE (or DPPC, DPPE, PHPE) continuously. After the centrifugation (3700 μg) of the solution was performed to precipitate the phospholipids that were not components of the vesicles, the supernatant of the solution was the liposome solution. These procedures are a well-established method for vesicle preparation.[22] The lipid composition of the layer was found using the HPLC analyzer with a Gel Silica 60 column (particle size 5 μL; ID 47 mm; Length 15 cm; Tosoh Co., 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 wave length detector and a Type 740 data module. The peaks for the lipid components were distributed identically with those previously published.[20]

The diameter measurements of the micelles and the vesicles were, respectively, conducted using ELS-8000 (Otsuka Electronics Co. Ltd, Osaka, Japan) so that the formation of the vesicles could be confirmed. The diameters of the micelles and the vesicles were 75 ± 10 nm and 80 ± 10 nm. The viscosity and the refractive index of the *tert*-butyl methyl ether are 0.23 cP and 1.3686, respectively.[23] Besides the measurement of the diameters, no leakage of the ANTS molecules indicated that the structure of each layer was conserved. Otherwise, the fluorescence intensity at 530 nm would be changed tremendously with the addition of several drops of aqueous solutions of 10 mM Tris-HCl at pH 3.0 into the vesicle solution (excitation at 384 nm and emission at 530 nm). The tremendous change in the intensity was observed only with detergent (Tween 20) treatment. Without the treatment, no change was found after the addition of pH 3 DI water drops. Therefore, the encapsulation was successfully achieved.

Phospholipase D (PLD) was purchased from Sigma Aldrich (St. Louis, MO), and 1 nM was used from the results investigated previously.[18] The release of the ANTS encapsulated in the vesicles was monitored in real time using a Wallac Victor3 multi-well fluorometer (Perkin Elmer, Waltham, MA). The significant difference in the intensity between the PLD injection and only the buffer solution injection means either fluorescence quench between ANTS and DPX or fluorescence leakage into the medium, which was caused by the PLD reaction. Therefore, the effect of the leakage only on the fluorescence intensity was monitored directly in a separate experiment using vesicles containing both ANTS and DPX. For the preparation of the third vesicle population, it was used to add 100 μL DI water of 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, 10 mM Tris-HCl at pH 7.4.

The fluorescence scale was calibrated with the fluorescence of a 1/1 mixture of ANTS and DPX vesicles in NaCl buffer set as 100% fluorescence (0% fusion) level. On the other hand, 0% fluorescence (100% fusion) was set with the residual fluorescence of vesicles containing both 12.5 mM ANTS and 45 mM DPX. The vesicles containing both 12.5 mM ANTS and 45 mM DPX initially emit 4% of the fluorescence of the lysed vesicles, and this intensity is set to 0% leakage, while the fluorescence of the vesicles lysed with Triton X-100 is set to 100% leakage. Therefore, the fusion is equal to 100 minus the recorded fluorescence, which is equal to the percentage of ANTS that is quenched by DPX at that time. Additionally, the fusion is modified with the fluorescence acquired from the leakage effect experiment with the third vesicle population. The diameter measurements of the vesicles were also conducted using ELS-8000 to monitor the behavior of the vesicles simultaneously.

Results and Discussion

Towards the investigation of the vesicle fusion induced by PLD, the quenching of ANTS fluorescence by DPX was used. ANTS was encapsulated in one population of vesicles and DPX in another, and the mixing of aqueous contents was monitored as a decrease in the fluorescence intensity. Therefore, the quantification of the fusion was found with the percentage of ANTS quenched at a given time. The number of fused vesicles increases with time; however, the percentage of ANTS quenched by DPX at a given time depends up on both the fusion kinetics of the vesicles and the leakage kinetics of the fused products. The exclusion of the leakage signal was performed with the fluorescence intensity for the lysed vesicles containing both ANTS and DPX.

The vesicle fusion induced by the PLD reaction is influenced by more than several factors - the vesicle number, the vesicle radius, the PLD concentration, the ionic concentration of the vesicle solution, the vesicle stability, and the phases of the vesicle layers, and the lipid composition at the outer layer. The determination of these factors was explained, as follows. The phospholipid concentration and the

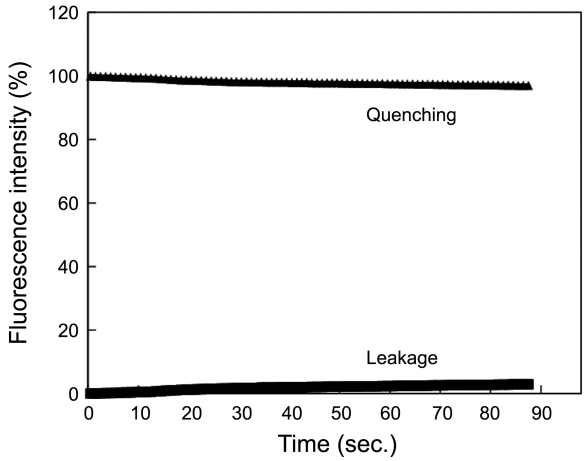

Figure 1. Fluorescence intensity with respect to time for the solid phase of the inner layer.

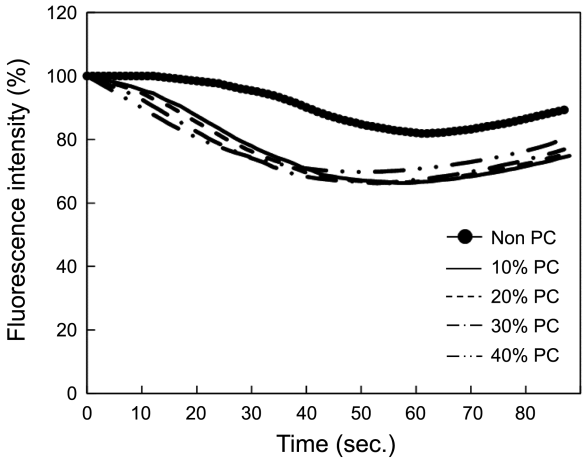

Figure 2. Quenching results according to the composition of the liquid phase outer-layer.

vesicle radius were invariant in this research. The other factors were found from the previous research except the lipid composition.[19] At the 1 mg/mL solution of phospholipids, 1 to 10 nM PLD concentration induced little change in the reactivity to the vesicles. Fifty mM NaCl and 1 mM $CaCl_2$, one of biomimetic conditions, was selected for the ionic concentration, because the reactivity was influenced by the ionic strength at lower than the concentration.[6,24-26] Under the condition described above, it was found that the fusion behavior was independent of the inner-layer phase. At the solid phase of the inner layer, it was observed that none of vesicles fused each other (Fig. 1).

A slight change in the intensity of the quenching, found in Figure 1, indicates only a little of leakage at the solid phase of the inner layer. This analysis is supported with the intensity results from the separate leakage experiments. Therefore, DOPA was used instead of DPPA as an inner-layer component at room temperature. The phase of the lipid layer was determined by the phospholipids' transition temperatures.[27] Since dioleoyl-phospholipids had the transition temperature lower than 0 °C, the lipids were suitable for the liquid phase at room temperature. The stability was adjusted identically for the vesicles of each component, as suggested in the previous research.[18] The suitable ratio of DOPE to OHPE was 15:1, and the vesicles made with DOPC, DOPE, and OHPE at this ratio were as stable as those with pure DOPC. Except the composition of the outer layer, the determination of all the factors was completed.

As an inert component for the outer layer, PE was selected because the transphosphatidylation of PC only was facilitated by PLD. In terms of the relative amount of PC, 11 conditions (pure PE to pure PC by 10%) were considered. At these conditions, the fluorescence intensity was monitored with respect to time for the quenching and leakage. The intensity change was observed up on the PC composition. The composition of the change occurrence was varied with the phase of the outer layer. At the liquid phase, the intensity results were shown in Figure 2 and Figure 3 (next page) that are the quenching and the leakage. As presented, the intensity curve was invariant from 100% DOPC up to 40%, but became slower with the less DOPC from 30% to non-PC.

However, at the solid phase of the outer layer, the intensity was not changed up to 30%. The fusion data for each composition were acquired with the subtraction of the leakage from the quenching. The curves, presented at Figure 4 (next page), were from the subtraction and represent the fusion behavior for the liquid phase of the outer layer. The fusion showed the trend identical with the leakage. These results seem to relate to the reactivity of PLD to the lipid layer, because the fusion is facilitated much faster by the PLD reaction. These results are consistent with the research performed previously for the change in the structure of the vesicles by the PLD reaction.[19] Therefore, as expected, the fusion is definitely related to the structural change of the vesicles, which is preceded. In the previous research, only the release of the encapsulated contents was focused to monitor the structural change of the vesicles, which was not involved in the fusion between the vesicles. In this research, the phenomena following the change were monitored. The change can be followed by either vesicle-lysis or vesicle-

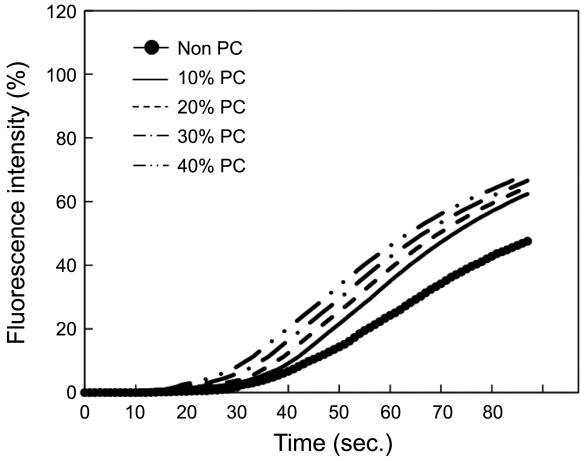

Figure 3. Leakage results according to the composition of the liquid phase outer-layer.

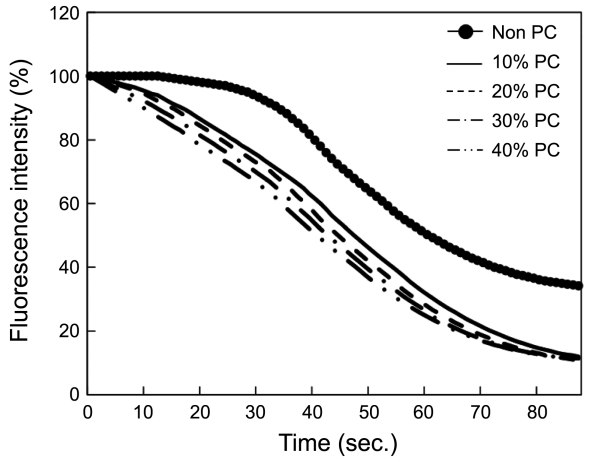

Figure 4. Fusion results according to the composition of the liquid phase outer-layer.

fusion. Surprisingly, in the condition where the vesicles were well dispersed with the concentration of 10 mg/mL lipid, the fusion was found significantly dominant rather than the lysis. Otherwise, the composition for the structural change would not be identical with that for the fusion.

The fusion behavior seems to be interpreted with the analysis performed in the previous research.[19] The composition effect on the fusion may be estimated with the surface density on the outer layer. The effect is also different up on the phase of the outer layer, which cause the variation in the density. Besides the density, the size of the PLD active-site is the other factor to determine the effect.

Using ELS-8000, the diameters of the vesicles were monitored at each composition of the layer. By the PLD reaction, the distribution of the vesicle diameters was changed from one peak with 150 nm to the several peaks with intervals and intensities in a reproducible fashion (Fig. 5). No change in the distribution occurred according to the change in the composition. The locations of the diameter peaks appear to mean the sizes of the vesicles generated from the fusion induced by the PLD reaction. This analysis is predicted from the calculation with the fusions of 2, 3, or 4 vesicles. The results of the diameter measurements support

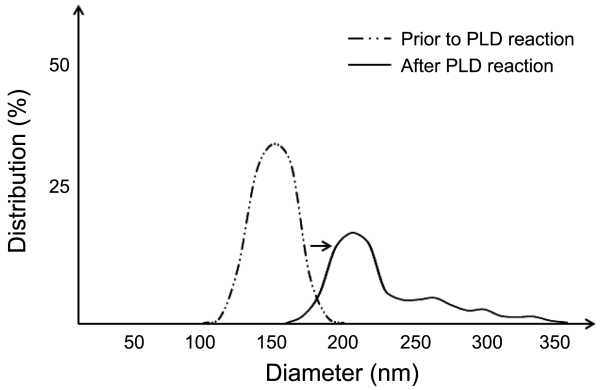

Figure 5. Change in the distribution of the vesicle diameters caused by the PLD reaction.

the fusion data acquired from the fluorescence measurements, which indicate that the reaction facilitated by the PLD induce mostly the vesicle fusion rather than the vesicle lysis.

In conclusion, in this study, the composition effect of the outer layer on the vesicle fusion catalyzed by PLD was investigated up on the phase of the outer layer using the fluorescence intensity change. Prior to the investigation, the ratio of dioleoylPE to oleoylhydroxyPE was found to acquire the condition where the mixed-phospholipid vesicles were stable identically with pure-dioleoylPC vesicles. For the quantification of the fusion, the fluorescence scale was calibrated with the fluorescence of a 1/1 mixture of ANTS and DPX vesicles in NaCl buffer taken as 100% fluorescence (0% fusion). The 0% fluorescence (100% fusion) was taken with the residual fluorescence of the vesicles containing both 12.5 mM ANTS and 45 mM DPX. To remove the leakage signal, the release is studied directly in a separate experiment using vesicles containing both ANTS and DPX. The fusion data for each composition were acquired with the subtraction of the leakage from the quenching. From the monitoring, the vesicle fusion caused by the PLD reaction seems dominantly to occur rather than the vesicle lysis, because the composition effect on the fusion was observed identically with that on the change in the vesicle structure. Furthermore, the diameter measurements also support the fusion dominancy.

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