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ORIGINAL ARTICLES

Effect of Phospholipid Bilayer Phase Asymmetry on Phospholipase D Reaction-Induced Vesicle Rupture

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Abstract Spherical phospholipid bilayers, vesicles, were formed with respect to phase of each layer via a double emulsion technique. At the outer layer of the vesicles, phospholipase D catalyzed for the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA). The reaction caused by phospholipase D (PLD) induced a curvature change in the vesicles, which eventually led them to rupture. Response time from the PLD injection to the rupture was monitored for the phase of each layer by using fluorescence intensity changes of pH-sensitive dye encapsulated in the vesicles. It was found that low ionic strength and asymmetric phase retarded response time. The retardation seems to be related to the stability of the vesicles, which is due to the interaction between the lipid molecules. In the liquid phases of the outer lipid layers, the unexpected slow response time may be attributed either to the fast lateral diffusion, which relieves the curvature change of the vesicles, or to the low concentration of PCs, which are less for the reaction compared to the solid phase of the outer lipid layer, rather than the stability.

Keywords Biophysics · Membrane biophysics · Membrane electrostatics · Membrane fusion · Membrane structure · Vesicle interaction

Phospholipase D (PLD) is membrane-active enzyme that is involved in a variety of cellular functions, 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). PLD acts on phosphatidylcholine (PC) to form phosphatidic acid (PA, a potent mitogen) that is believed to be the second messenger involved. This hydrolysis leads to the changes in the lipid content of membranes, which could also play a role. PLD may have a physiological function through the further metabolism of PA to diacylglycerol (DAG) and lysophosphatidic acid (McDermott et al. 2004; Brown et al. 2007).

Supported lipid layers are known as well-defined models for cell surface and for investigating molecular events in membranes because very sensitive analytical techniques can be applied to investigate the events (McConnell et al. 1986; Sackmann 1996; Brian and McConnell 1984). The supported lipid layers have been used in 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). 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, and the activity of the enzymes (Jurak and Chibowski 2010; Chen et al. 2009; Chemburu et al. 2008).

The hydrolysis triggered by PLD is known to cause fusion essential for cellular processes. The hydrolysis induces a change in the composition of the membranes, which results in the rupture of the vesicles. However, little is known about how the phase asymmetry of the vesicles affects the properties of the biological membranes caused by the reactions. An investigation of the effect may

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contribute to our understanding the physical behavior of these enzymes in terms of quantitative analysis. We thus investigated the systematic effect of phase asymmetry on vesicle rupture.

Experiments

Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidic acid (DPPA), and dioleoylphosphatidic acid (DOPA) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The lipids of either DPPA or DOPA were dissolved in 10 ml of *tert*-butyl methyl ether at 10 mg/ml, followed by adding 100 µl distilled water of 5 mM pyranine at pH 9.0. Therefore, the micelles with either DPPA or DOPA were prepared by extrusion through the 50 nm pores of 78 mm diameter PTFE membranes above the transition temperature of the desired lipid. Several drops (less than 10 µl) of the micelle solution and *tert*-butyl methyl ether solution of 10 mg/ml either DPPC or DOPC were continuously added through a 22 gauge needle inserted into the 10 ml aqueous solutions at pH 5.0, respectively (aqueous solutions: pure distilled water, 50 mM NaCl, 100 mM NaCl, 300 mM NaCl, 1 mM $CaCl_2$, 5 mM $CaCl_2$, 50 mM NaCl and 1 mM $CaCl_2$, 100 mM NaCl and 1 mM $CaCl_2$, 300 mM NaCl and 1 mM $CaCl_2$, 50 mM NaCl and 5 mM $CaCl_2$, 100 mM NaCl and 5 mM $CaCl_2$, and 300 mM NaCl and 5 mM $CaCl_2$). The final lipid concentration of the aqueous solution was 1 mg/ml. During the addition, the distilled water was magnetically stirred and nitrogen stream was injected into the water. These procedures are well known as a way to prepare vesicles (New 1990). To confirm the asymmetry of the vesicles, the diameter of the micelles was measured with a spectrometer (ELS-8000; Otsuka Electronics, Osaka, Japan) before they were transferred to the distilled water. For the measurement, the viscosity and the refractive index of the *tert*-butyl methyl ether are 0.23 cP and 1.3686, respectively (Lide 2005). The diameter of the micelles was 75 $\pm$ 10 nm. After the vesicles were formed, the diameter of the vesicles became 80 $\pm$ 10 nm, which was expected from the lipid layer formed on the micelle surface. In addition to the change in the diameter during the vesicle formation from the micelles, no leakage of the pyranine molecules indicated that the asymmetry was not disturbed. Otherwise, the fluorescence intensity at 510 nm would be greatly changed when several drops of distilled water at pH 3.0 were added to the vesicle solution.

Three different conditions were considered for the PLD reaction: concentrations of 1, 3, and 10 nM. Because it is known that the reaction causes the rupture of the vesicles and the pyranine (pH-sensitive fluorescent dye) was encapsulated inside of the vesicles, fluorescence intensity was monitored in real time with a Wallac Victor3 multiwell fluorimeter (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 changes in the differences in intensity between the vesicle solution with the PLD injection and with buffer-only solution injection meant that the vesicles ruptured. Therefore, an observation of the intensity was conducted to investigate the effect of the phase asymmetry on the PLD reaction with respect to various conditions of PLD concentration and ionic strength. From our observations for each condition, we then measured when rupture occurred after injection of the PLD molecules.

Results and Discussion

For the investigation of the PLD reaction on the lipid layer, pyranine molecules were dissolved inside of the vesicles. The encapsulation of the molecules was confirmed with a fluorometer (Fig. 1). The fluorescence intensity was changed with Tween 20 treatment. Without this treatment, the intensity did not vary after the addition of distilled water drops at pH 3. Thus, encapsulation was successfully achieved.

Apart from each lipid layer phase, we considered two factors for the PLD reaction: the PLD concentration and the ionic strength. The concentration of the phospholipids was not varied, and it is known that 10 mg/ml of the phospholipids corresponds to the number of vesicles (10^7–10^8) (Park 2007). At this phospholipid concentration, the PLD reaction was carried out at various biomimetic conditions, as follows: NaCl 50–300 mM, $CaCl_2$ 1–5 mM,

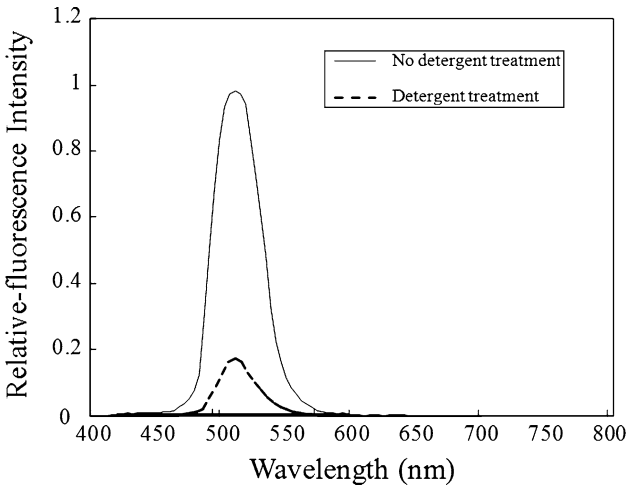

Fig. 1 Fluorescence intensity change after the addition of pH 3 distilled water drops

and PLD 1–10 nM (Brown et al. 2007; Webb et al. 2010; Cheow et al. 2010; Furt and Moreau 2009). The phase of the lipid layers was made with the phospholipids whose transition temperature was considered (Park and Ahn 2008). The PLD reaction was performed at room temperature. Dioleoyl lipids were used for the liquid phase since their transition temperature was much lower than room temperature. However, dipalmitoyl lipids were for solid at room temperature. Phase of each layer was adjusted, and the asymmetric phase of the layers was made. Therefore, four types of vesicles were prepared (Fig. 2).

The response time from the PLD injection to the fluorescence intensity change is listed for each condition in Table 1. In the range of the PLD concentration 1–10 nM of the vesicle solution at 1 mg/ml lipid concentration, the response time was not varied. Obviously the time may be affected by the ratio of PLD molecules to number of liposomes. We found that the condition given with 1–10 nM PLD and 1 mg/ml phospholipid did not cause any change in the response time. With a change in ionic strength, however, response time was changed. We observed that the lower the ionic strength, the slower the response time. This observation seems to be related to the interaction between head groups of phospholipids. At a neutral-pH condition, zwitterionic PCs were converted into anionic PAs by the PLD reaction. If the ionic strength was insufficient, the

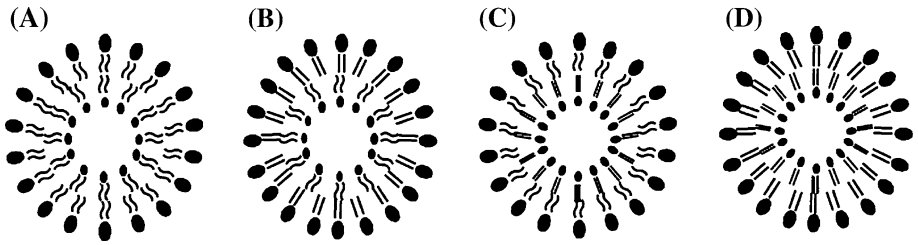

Fig. 2 Types of vesicles. *Straight line* corresponds to solid phase of the lipid layer, and *curved line* to its liquid phase. **a** Liquid phases for both layers. **b** Solid phase for outer layer and liquid phase for inner layer. **c** Liquid phase for outer layer and solid phase for inner layer. **d** Solid phase for both layers

Table 1 Response time (s) from PLD injection to vesicle rupture

Characteristic	Inner-layer solid phase			Inner-layer liquid phase		
	PLD concentration (nM)			PLD concentration (nM)		
	1	3	10	1	3	10
Outer-layer solid phase						
Distilled water	1	1	1	0.7	0.7	0.7
50 mM NaCl	0.7	0.7	0.7	0.5	0.5	0.5
100 mM NaCl	0.5	0.5	0.5	0.3	0.3	0.3
300 mM NaCl	0.5	0.5	0.5	0.3	0.3	0.3
1 mM $CaCl_2$	0.8	0.8	0.8	0.6	0.6	0.6
5 mM $CaCl_2$	0.5	0.5	0.5	0.3	0.3	0.3
50 mM NaCl, 1 mM $CaCl_2$	0.5	0.5	0.5	0.3	0.3	0.3
100 mM NaCl, 1 mM $CaCl_2$	0.5	0.5	0.5	0.3	0.3	0.3
50 mM NaCl, 5 mM $CaCl_2$	0.5	0.5	0.5	0.3	0.3	0.3
Outer-layer liquid phase						
Distilled water	1.5	1.5	1.5	1.7	1.7	1.7
50 mM NaCl	1.1	1.1	1.1	1.3	1.3	1.3
100 mM NaCl	0.7	0.7	0.7	0.7	0.7	0.7
300 mM NaCl	0.7	0.7	0.7	0.7	0.7	0.7
1 mM $CaCl_2$	1.3	1.3	1.3	1.5	1.5	1.5
5 mM $CaCl_2$	0.7	0.7	0.7	0.7	0.7	0.7
50 mM NaCl, 1 mM $CaCl_2$	0.7	0.7	0.7	0.7	0.7	0.7
100 mM NaCl, 1 mM $CaCl_2$	0.7	0.7	0.7	0.7	0.7	0.7
50 mM NaCl, 5 mM $CaCl_2$	0.7	0.7	0.7	0.7	0.7	0.7

interaction would not be affected. Therefore, the repulsive interaction between PAs was stronger at the lower ionic strength. The repulsion appears to keep the curvature of the vesicles from the reduction. However, the increase in the ionic strength leads to the decrease in the curvature. At either 100 mM NaCl or 5 mM $CaCl_2$, the repulsion was almost negligible because the response time decreased very little. Calcium chloride ($CaCl_2$) led to the rupture of the vesicles at much lower concentrations compared to NaCl. In other words, Ca^{2+} seemed to induce other phenomena rather than the interference. Clearly, valency induces the other phenomenon that attracts PAs around the Ca^{2+}. The attraction may facilitate the rupture of the vesicles so that the response time decreases even at 1 mM $CaCl_2$.

For the solid phase of the outer layer, the response time was slower in the solid phase of the inner layer than in the liquid phase. This seems to be caused by the instability of the hydrophobic interior, which was formed with the tail groups of the phospholipids. The instability appears to be generated from the mismatches between the tail groups of each layer. Our findings are consistent with the mechanical properties described in the literature and supported by the response time for the liquid phase of the outer layer (Park 2010). Because it is known that mechanical properties of the lipid layers made with solid phase are stronger than those made with liquid phase (Park 2009), we expected that the response time would be faster for the liquid phase of the outer layer than for the solid phase. However, we found that the fluorescence intensity change was much slower for the liquid phase of the outer layer. This unexpected result means that the rupture of the vesicles appears to be related to other factors aside from the mechanical properties. Because the rupture is caused by the PLD reaction, the reaction rate needs to be considered. Obviously, the reaction occurs at the outer layer. The PCs are less dense for the liquid phase of the outer layer, compared to the solid phase. In terms of the quantitative concentration, it is known from the Langmuir isotherms that the density of the DPPC is almost two times higher than that of DOPC (Park and Ahn 2008). Therefore, the lower PC concentration of the liquid phase may induce the slower PLD reaction rate, which leads to increase in the response time.

The unexpected retardation in the response time for the liquid phase of the outer lipid layer may be interpreted from lateral diffusion. If the curvature change resulted from the PLD reaction is reduced by the motility of PCs around the reaction sites, the rupture event would be postponed. Because the liquid phase of planar lipid layers has 10–100 times more diffusivity than the solid phase (Schram et al. 1996), the response time may be slower for the liquid phase of the outer lipid layer even under the assumption that the PLD reaction rates would be identical for both the solid and liquid phases of the outer lipid layers.

Conclusion

In this study, the response time from the PLD injection to the rupture of the vesicle was monitored via fluorescence intensity change. The rupture caused by the PLD reaction was interpreted with respect to the ionic strength and the phase of each layer. We found that the ionic strength and asymmetric phase retarded response time. The retardation seems to be related to the stability of the vesicles, which is due to the interaction between the lipid molecules. Apart from the stability, the unexpectedly slow response time that we observed in the liquid phases of the outer lipid layers may be attributed either to a fast lateral diffusion that relieves the curvature change of the vesicles, or to the low concentration of PCs that are less for the reaction compared to the solid phase of the outer lipid layer.

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References

Brian AA, McConnell HM (1984) Allogeneic stimulation of cytotoxic T-cells by supported planar membranes. Proc Natl Acad Sci USA 81:6159–6163

Brown HA, Henage LG, Preininger AM, Xiang Y, Exton JH (2007) In: Brown HA (ed) Lipidomics and bioactive lipids: lipids and cell signaling. Academic Press, New York

Chemburu S, Ji E, Casana Y, Wu Y, Buranda T, Schanze KS, Lopez GP, Whitten DG (2008) Conjugated polyelectrolyte supported bead based assays for phospholipase a(2) activity. J Phys Chem B 112:14492–14499

Chen CH, Malkova S, Pingali SV, Long F, Garde S, Cho W, Schlossman ML (2009) Configuration of PKC alpha-C2 domain bound to mixed SOPC/SOPS lipid monolayers. Biophys J 97:2794–2802

Cheow WS, Chang MW, Hadinoto K (2010) Antibacterial efficacy of inhalable antibiotic-encapsulated biodegradable polymeric nanoparticles against *E. coli* biofilm cells. J Biomed Nanotechnol 6:391–403

Exton JH (2002) Phospholipase D: structure, regulation and function. Rev Physiol Biochem Pharmacol 144:1–94

Fang HH, Chan PK-Y, Xu L-C (2000) Quantification of bacterial adhesion forces using atomic force microscopy (AFM). J Microbiol Methods 40:89–97

Furt F, Moreau P (2009) Importance of lipid metabolism for intracellular and mitochondrial membrane fusion/fission processes. Int J Biochem Cell Biol 41:1828–1836

Giesen PL, Willems GM, Hemker HC, Hermens WT (1991) Membrane-mediated assembly of the prothrombinase complex. J Biol Chem 266:18720–18725

Huang P, Frohman MA (2007) The potential for phospholipase D as a new therapeutic target. Expert Opin Ther Targets 11:707–716

Jurak M, Chibowski E (2010) Influence of (phospho)lipases on properties of mica supported phospholipid layers. Appl Surf Sci 256:6304–6312

Lide DR (2005) CRC handbook of chemistry and physics: a ready-reference book of chemical and physical data, 85th edn. CRC Press, Boca Raton

McConnell HM, Watts TH, Weis RM, Brian AA (1986) Supported planar membranes in studies of cell–cell recognition in the immune system. Biochim Biophys Acta 864:95–106

McDermott M, Wakelam MJ, Morris AJ (2004) Phospholipase D. Biochem Cell Biol 82:225–253

Miszta A, Machan R, Benda A, Ouellette AJ, Hermens WT, Hof M (2008) Combination of ellipsometry, laser scanning microscopy and Z-scan fluorescence correlation spectroscopy elucidating interaction of cryptdin-4 with supported phospholipid bilayers. J Pept Sci 14:503–509

Mou JX, Yang J, Huang C, Shao Z (1994) Alcohol induces interdigitated domains in unilamellar phosphatidylcholine bilayers. Biochemistry 33:9981–9985

New RRC (1990) Liposomes: a practical approach. Academic Press, New York

Park JW (2007) Nanoliter reactor arrays for antibiotic study. Bull Korean Chem Soc 28:1709–1714

Park JW (2009) Individual leaflet phase effect on nanometer-scale surface properties of phospholipid bilayers. Colloids Surf B 71:128–132

Park JW (2010) First-leaflet phase effect on properties of phospholipid bilayer formed through vesicle adsorption on LB monolayer. J Membr Biol 237:107–114

Park JW, Ahn DJ (2008) Temperature effect on nanometer-scale physical properties of mixed phospholipid monolayers. Colloids Surf B 62:157–161

Sackmann E (1996) Supported membranes: scientific and practical applications. Science 271:43–48

Schram V, Lin H-N, Thompson TE (1996) Topology of gel-phase domains and lipid mixing properties in phase-separated two-component phosphatidylcholine bilayers. Biophys J 71:1811–1822

Scott SA, Selvy PE, Buck JR, Cho HP, Criswell TL, Thomas AL, Armstrong MD, Arteaga CL, Lindsley CW, Brown HA (2009) Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. Nat Chem Biol 5:108–117

Tappia PS, Dent MR, Dhalla NS (2006) Oxidative stress and redox regulation of phospholipase D in myocardial disease. Free Radic Biol Med 41:349–361

Webb LM, Arnholt AT, Venable ME (2010) Phospholipase D modulation by ceramide in senescence. Mol Cell Biochem 337:153–158