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Structural Stability of Lecithin Liposomes as Improved by Adding an Artificial Boundary Lipid

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Enzymatic lyses of phosphatidylcholine liposomes by phospholipase A_2 and D are significantly depressed by adding an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC). Most interestingly, DDPC was not the substrate even for phospholipase D and phospholipase A_2 as well.

Naturally occurring phospholipids are, in general, easily decomposed by enzymes and/or chemicals. Liposomes as formed by such naturally occurring lipids also are, therefore, easily destructed by external stimuli such as changes of osmotic pressure, temperature, ionic strength, pH, and/or polarity of medium. In addition, the *in vivo* stabilization of liposomes significantly decreases upon interaction with serum and/or plasma.[1] Especially, liposomes interacting with high density lipoprotein (HDL) undergo lysis upon abstraction of liposomal lipids by HDL.[1,2] Such lipid abstraction by HDL is relatively depressed by cholesterol and/or sphingomyelin added for making liposomes structurally tough.[2,3] As reported by Gupta and Bali,[4,5] liposomes which are prepared with an artificial lipid, 1,2-dipentadecanylcarbamoyloxyglycero-3-phosphatidylcholine, capable to provide an intermolecular hydrogen bonding in membrane are, for example, rather stable even in serum. Sunamoto and his coworkers have recently developed an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) for improving both the structural stability of lipid bilayer membrane and the reconstitution efficiency of cell membrane proteins in liposome.[6-10] In this communication, we would like to report the improved stability of egg PC or DMPC liposomes against enzymatic lyses by adding the artificial lipid, DDPC.

First of all, hydrolysis of phospholipids by phospholipase A_2 (PLA_2) (Bee Venom, 560 U/mg, Sigma) in a homogeneous system was investigated. To a given amount of phospholipid (15 μmol equiv.) dissolved in 1.5 ml of ether-ethanol (95 : 5 by vol) were added 200 μl of an aqueous PLA_2 solution (20 μg of the lipase into 0.2 ml of an aqueous buffered (pH 8.5) solution containing 10 mM Tris·HCl, 10 mM $CaCl_2$, and 0.9% NaCl). Hydrolysis of phospholipids was semiquantitatively followed by thin layer chromatographic (TLC) method. At a given time interval, an aliquot (15 μl) was withdrawn and submitted to TLC analysis developed by chloroform : methanol : water (65 : 25 : 4 by vol) and the reaction products were detected by exposing the chromatogram to iodine vapor. 30 % of egg PC were hydrolyzed, even just after adding the enzyme, and most of the original lipids disappeared and were converted to lysolecithins (approx. 30 %) and fatty acids after 5 h. Meanwhile, DDPC was not hydrolyzed at all even after 5 h under the same conditions.

Secondly, hydrolysis of phospholipids by phospholipase D (PLD) (a kind gift of Toyo Jyozo Co., Ohito) was investigated using TLC. A given amount of the lipids (16 μmol) was dissolved in 1 ml of ether-chloroform (1 : 1 by vol). To this solution were added 0.25 ml of 1 M $CaCl_2$ and then 1.25 ml of 0.1 M acetate buffer (pH 5.6). One ml of an aqueous PLD solution (4 mg/ml, 250 U/mg) made by the same buffer was added under stirring, and the hydrolyzates produced at a given time interval were analyzed on TLC similarly to the case of PLA_2 lysis. As expected, approximately 80 % of egg PC were hydrolyzed after 4 h, and most of the egg PC were converted to phosphatidic acids after 24 h. Contrary to the case of egg PC, DDPC was completely silent to PLD even 24 h later.

In order to quantitatively investigate the effect of addition of DDPC on PLA_2 lyses of both DMPC multilamellar liposomes and neutral micelles, hydroxamic acid-Fe^{2+} method was employed.[11] For the following three samples, DMPC (10 mg) (sample a), a mixture of DMPC (10 mg) and DDPC (2.5 mg) (sample b), and a mixture of DMPC (10 mg) and DDPC (10 mg) (sample c), lipid thin films were respectively prepared in a round bottomed flask according to the authorized method.[8] After the thin film so formed was swollen with 3.5 ml of 0.5 M Tris-HCl buffer (pH 8.0), ultrasonication was carried out at 37.0 °C for 20 min under nitrogen atmosphere using a Tomy ultrasonifier UR-200P. Liposomal suspension so obtained was diluted with 0.1 ml of the same buffer. In order to prepare a micellar suspension, to 0.5 ml of a liposomal suspension was added 0.1 ml of 1 % (w/v) aqueous Triton X-100 solution. Then, 0.1 ml of 7 mM EDTA containing 0.2 mM $CaCl_2$ were added to the liposomal and micellar suspensions obtained above, and 20 μl of aqueous PLA_2 (3.72 μg) solution was added at 37. 0 °C after preincubation for 15-20 min. Reaction was carried out for 10 min at the same temperature. To the reaction mixture were added 2 ml of ethanol, 1 ml of 1 mM aqueous hydroxylamine solution containing 1.75 mM NaCl , and the reaction was continued for another 30 min at the same temperature. 0.6 ml of 3.6 M HCl, and 0.5 ml of $FeCl_3$ reagent (50 g of $FeCl_3 \cdot 6H_2O$ dissolved in 10 ml of 3.6 M HCl) were then added to give a colored hydroxamic acid-Fe^{2+} complex, and intensity of the absorption at 520 nm was determined on a Hitachi 220A spectrophotometer. Results are given in Table 1.

Table 1. PLA_2 lysis (%) of liposomal and micellar DMPC in the presence of DDPC (at 37 °C for 10 min)

	Sample a	Sample b	Sample c
Micellar system	100.0	97.0	84.0
Liposomal system	94.6	91.2	62.0

In both micellar and liposomal systems, the addition of DDPC decreased the hydrolytic lysis of DMPC. It is of interest that this effect of DDPC is more drastic in the liposomal system than that in the micellar system. By adding DDPC which is essentially nonspecific to PLA_2, even the specific substrate DMPC became harder to be hydrolyzed.

The effect of addition of DDPC on permeability of egg PC small unilamellar liposome(SUV) was investigated by leakage of carboxyfluorescein (CF) from the liposomes as induced upon the PLA_2 lysis. For both egg PC (28 mg) and a mixture of egg PC (22 mg, 80 mol% equiv.) and DDPC (5 mg, 20 mol% equiv.), lipid thin film

was respectively prepared according to basically the same method adopted above. The lipid thin film so obtained was dispersed in an aqueous buffered solution (pH 8.6) containing 200 mM CF according to an authorized method.[8] To a given amount of SUV suspension (1.0 ml, 1.0×10^{-4} M as lipid concentration) were added 100 μl of an aqueous PLA_2 solution (1.0 mg in 10 ml of Tris·HCl buffer), and CF leakage was monitored.[8] Though conventional egg PC liposomes showed a very rapid CF-release upon the addition of PLA_2, the addition of DDPC to egg PC resulted in a significant decrease of the CF release (Fig. 1). When 0.1 ml of 500 mM EDTA were added to the system for eliminating calcium ion which had been added to attain the enzyme activity, the CF release drastically decreased (though data were not shown). This means that the CF release in the presence of PLA_2 was not simply caused by adsorption of the protein onto the liposomal membrane and subsequent physicochemical perturbation accompanied by a decrease in the permeability of the membrane, but a result of enzymatic lysis of egg PC.

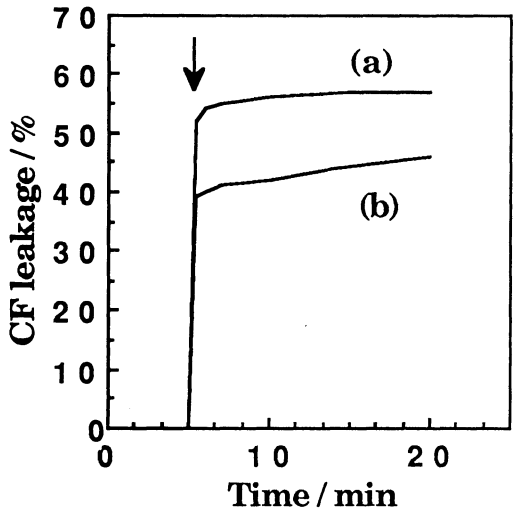

Fig. 1. CF-release from liposome accompanied by the PLA_2 lysis at 37.0 °C. Arrow indicates the point that the enzyme was added.
(a), egg PC liposome
(b), egg PC(80 mol%)-DDPC(20 mol%) liposome

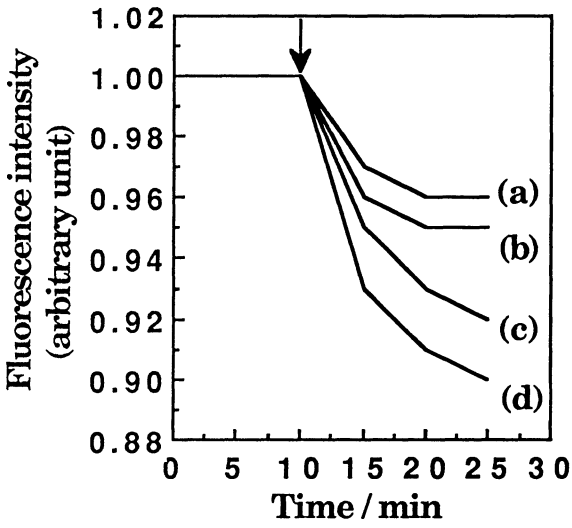

Fig. 2. Quenching of ANS emission accompanied PLD lysis of liposome at 37.0 °C. Arrow indicates the point that the enzyme was added. (a), DMPC (50 mol%)-DDPC (50 mol%); (b), DMPC (65)-DDPC (35); (c), DMPC (80)-DDPC (20); and (d), DMPC (100)

An anionic fluorescent probe, ANS (sodium 1-anilino-8-naphthalenesulfonate), binds to the positive choline moiety of phosphatidylcholines in liposomal membrane and emits relatively strong fluorescence in a less polar environment. If PLD hydrolyzed lipids and the choline moiety was released from lecithins in liposomal membranes upon the enzymatic cleavage, ANS also is released into bulk aqueous phase together with choline freed from the membrane and strongly quenched upon hydration.[12] Using this methodology, effect of the

addition of DDPC on the PLD lysis of DMPC liposome(SUV) was investigated. For DMPC (10.0 mg, 1.5×10^{-5} mol) (sample a), a mixture of DMPC (10 mg) and DDPC (2.5 mg) (sample b), a mixture of DMPC (10.0 mg) and DDPC(5.0 mg) (sample c), and a mixture of DMPC (10.0 mg) and DDPC (10.0 mg) (sample d), SUVs were respectively prepared by the same method adopted above. After encapsulation of a given amount ANS (0.417 mmol equiv.) into liposomes, 1 ml of a liposomal suspension was placed in a cuvette cell, 20 μl of an aqueous PLD solution (185 U mg^{-1} ml^{-1}) and 50 μl of 1M $CaCl_2$ were added, and a decrease in the emission of ANS was followed at 490 nm. Results are shown in Fig. 2. Even conventional DMPC liposome(SUV) gains resistance against PLD lysis by adding DDPC. The lipid synthesized by Gupta and Bali has been found not to be hydrolyzed by phospholipase A but C.[4,5] 1-Octanoyl-2-octanoylamido-2-deoxyglycero-3-phosphatidylcholine prepared by Bonsen et al shows a phospholipase A-resisting property.[13] The present lipid DDPC also was not hydrolyzed by PLA_2. If we understand that PLA_2 specifically cleaves only the carboxy ester bond at C-2 position of glycero skeleton, these results about DDPC obtained in this work seem reasonable.

The most interesting finding in this work is that, contrary to what would be expected, DDPC is not hydrolyzed even by PLD which is considered to specifically eliminate the choline moiety of lecithins. Taking account of a previous result of Chen and Barton,who have reported that an ether lipid (1,2-dioctadecylglycero-3-phosphatidylcholine) is not hydrolyzed by PLD,[14] it seems reasonable to consider that PLD recognizes not only the phosphatidylcholine moiety, but the glycero skeleton of the lecithins also.

Judging from all the previous and present results, the improved resistance of DDPC-containing lecithin liposomes against the lipase lyses is ascribed to (i) the increased structural stability of the membrane by formation of hydrogen belt [8] and/or (ii) the competitive inhibition of DDPC in enzymatic lyses of regular lecithins.[13]

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