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Effects of 1,2-diacylglycerol and cholesterol on the hydrolysis activity of phospholipase D in egg-yolk phosphatidylcholine bilayers

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Effects of cholesterol (Chol) and 1,2-diacylglycerol (DAG) on the hydrolysis activity of phospholipase D (from *Streptomyces chromofuscus*) were studied in small unilamellar vesicles (SUV) of egg-yolk phosphatidylcholine (PC). 1,2-Diacylglycerol used here is derived from PC. Choline produced in the reaction was monitored by using a choline oxidase-oxygen electrode. Addition of 18.3 mol% Chol into SUV (2 mM PC) led to a small increase in the reaction rate. On the other hand, 18.3 mol% DAG in SUV brought about a 5–6-fold rate of choline production. The apparent maximum velocity, V_{max}(app), increased by addition of DAG and Chol in SUV. In PC/Chol-SUV, the effect of increase in V_{max}(app) was largely compensated by the increase in the apparent Michaelis constant, K_m(app). The Chol and DAG molecules did not have significant effects on the kinetic parameters, when PC was solubilized in the micelles of heptaethylene glycol dodecyl ether. The effects of Chol and DAG are, therefore, not due to specific ones on the enzyme itself, but rather upon the bilayer-organization of the substrate. We discuss the activation of phospholipase D in terms of the influences of DAG and Chol on the structure of hydrophilic region and fluidity of the bilayers.

Introduction

Cholesterol is a ubiquitous component of eukaryotic cell membranes. Physicochemical studies have suggested a cholesterol-dependent modulation of membrane fluidity as a mechanism for regulating the activity of other membrane-associated components [1]. ^{2}H-NMR and IR spectroscopy has shown the effect of cholesterol (Chol) on the acyl chain order of phosphatidylcholine (PC) in bilayers [2,3]. At a temperature above the gel-liquid crystalline transition point, Chol inhibits *gauche* rotamer formation of the acyl chains, thus demonstrating a strong ordering effect in regions of the bilayers where the sterol rings insert. The ability of Chol to order the acyl chains is much reduced near the ω-methyl-position [2,3].

The effect of 1,2-diacylglycerols (DAG) on bilayer membrane structures is also a topic of interest because of their role in signal transduction in cells. 1,2-Diacylglycerols enhance the hydrolysis by phospholipases A_2, C and D [4–6]. The ^{31}P- and ^{13}C-NMR spectroscopy of DAG-PC mixtures shows that the carbonyl groups of DAG are proximal to the aqueous interface, the DAG molecule orients along the normal to the bilayer, and the glycerol backbone is perpendicular to the bilayer plane [7]. Further addition of DAG (> 15–20 mol%) causes the formation of inverse hexagonal and inverse face-centered cubic phases [8,9].

1,2-Diacylglycerols are presumed to be potent inducers of phospholipase D (PLD) in HL-60 granulocytes [5] and NG 108–15 neuroblastoma X glioma hybrid cells [6]. Exogenous PLD from *Streptomyces chromofuscus* has a similar activity to endogenous PLD in ovarian granulosa cell culture [10]. Phospholipase D catalyzes the hydrolytic formation of phosphatidic acid, which may be cleaved to DAG, an activator of protein kinase C [11].

In the present work, we investigate the effects of Chol and DAG on the activity of PLD from *Streptomyces chromofuscus* in egg-yolk phosphatidylcholine (PC) bilayers.

Materials and Methods

Materials

Egg-yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei (Tokyo). The purity (over 99%) was determined by thin layer chromatography (TLC)

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(Iatroscan Analyzer MK-5 from Iatron Laboratories, Tokyo, solvent: chloroform/methanol (3 : 1, v/v)). Cholesterol (Chol) purchased from Sigma (St. Louis, MO) showed a single spot on TLC (solvent: chloroform/acetone/methanol/acetic acid/water (45 : 20 : 12 : 10 : 5, v/v)). 1,2-Diacylglycerol derived from egg-yolk phosphatidylcholine (DAG) was obtained from Serdary Research Laboratories (London, Canada). The purity was determined to be 94% by TLC (solvent: heptane/isopropyl ether/acetic acid (60 : 40 : 4, v/v)). The small amount of impurity (6%) was identified as the 1,3-isomer. Egg-yolk lysophosphatidylcholine (lyso-PC) and a nonionic surfactant, heptaethylene glycol dodecyl ether (HED), were purchased from Serdary Research Laboratories and Nikko Chemicals (Tokyo), respectively.

Phospholipase D (EC 3.1.4.4) from *Streptomyces chromofuscus* was purchased from Sigma. The molecular weight determined by SDS-polyacrylamide gel electrophoresis is in agreement with the reported value [12]. The enzyme was dissolved in a buffer consisting of 10 mM Tris-HCl and 150 mM NaCl (pH 8.0), and the solution was centrifuged (10000 rpm) at 4°C for 30 min. 3-ml aliquots of the supernatant were stored at 5°C. The phospholipase D (PLD) activity in each aliquot was checked by measuring the enzymatic choline production in a standard lyso-PC solution before use. Decrease in the activity was less than 10% after 1 month storage. Choline oxidase (EC 1.1.3.17) from *Alcaligenes* sp. was obtained from Toyobo (Osaka).

Preparations of vesicles and mixed micelles

1,2-Diacylglycerol (DAG), cholesterol (Chol) and egg-yolk phosphatidyl-choline (PC) were mixed in chloroform and the solvent was evaporated under reduced pressure. After drying in vacuum overnight, the lipid film was hydrated with the Tris-HCl buffer, vortexed and sonicated for 40 min under a nitrogen stream at 4°C. The probe-type sonicator used was a UD-200 from Tomy Seiko. The vesicle suspension (small unilamellar vesicles, SUV) was centrifuged at 3000 rpm for 10 min to remove titanium dust and stored at 25°C. Dynamic light scattering (DLS) of PC-, PC/DAG- and PC/Chol-SUVs was measured with a Photal LPA-3000/3100. The weight-averaged vesicle size of each sample was 30 ± 15 nm. The effects of mixing of Chol or DAG with PC on the vesicle size were small.

Lipid (PC, PC/DAG or PC/Chol) and HED were mixed in chloroform. After evaporating the solvent and drying in vacuum overnight, the mixture was dissolved in the Tris-HCl buffer to give mixed micellar solutions. Phospholipid in SUV and micellar solutions was assayed by the method of Bartlett [13].

Assay for the phospholipase D activity

The phospholipase D activity was measured by monitoring the concentration of choline produced using a choline oxidase-oxygen electrode at 30°C. The principle of choline assay has been described elsewhere [14,15]. Choline is oxidized to betaine by the choline oxidase with oxygen consumption. The oxygen consumed was monitored by use of a Clark oxygen electrode.

$$\text{PC} + \text{H}_2\text{O} \xrightarrow{\text{PLD}} \text{choline} + \text{phosphatidic acid}$$

$$\text{choline} + 2\text{O}_2 \xrightarrow{\text{choline oxidase}} \text{betaine} + 2\text{H}_2\text{O}_2$$

Choline oxidase was immobilized in a cellulose triacetate-membrane by the method of Campanella et al. [16]. The choline oxidase membrane was mounted on the top of a Clark oxygen electrode (Horiba, Kyoto) and covered with a cellophane film and O-rings. The choline electrode was stored in a 0.1 M glycine-buffer (pH 9.0) at 5°C, and calibrated with standard choline chloride solutions before use. The enzymatic reaction was started by the injection of 540 μl PLD solution (450 units; one unit liberates 1.0 μmol of choline from egg-yolk L-α-phosphatidylcholine per h at pH 8.0 and 30°C) into a 50 ml sample solution. The PLD activity was assayed within 10 h after the sample preparation.

Results

Enzymatic hydrolysis of phosphatidylcholine

The enzymatic hydrolysis of PC was influenced by adding a neutral lipid, Chol or DAG, into the PC bilayers (PC-SUV). Fig. 1 shows the productions of choline by PLD. The choline produced in PC-SUV (2 mM) was 45 μM for 30 min (control). Addition of 18.3 mol% Chol into PC-SUV slightly increased the choline production at the early stage. The choline concentration at 30 min, however, was similar to the control value. On the other hand, the addition of 18.3 mol% DAG enhanced the enzymatic production of choline remarkably (5-fold choline concentration at 30 min).

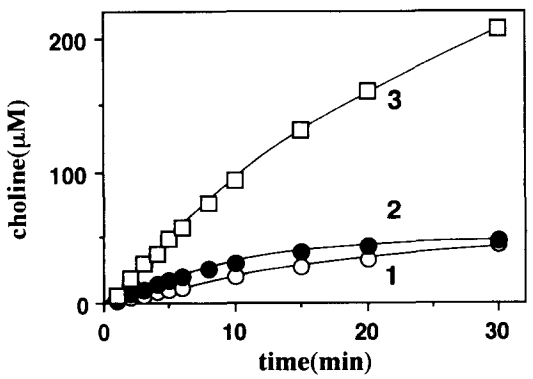

Fig. 1. Effects of Chol and DAG on the choline production by PLD in SUV. 1, PC-SUV; 2, PC/18.3 mol% Chol-SUV; 3, PC/18.3 mol% DAG-SUV. The PC concentration in SUV is 2 mM in Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0). Choline concentration was monitored with a choline oxidase-oxygen electrode, at 30°C.

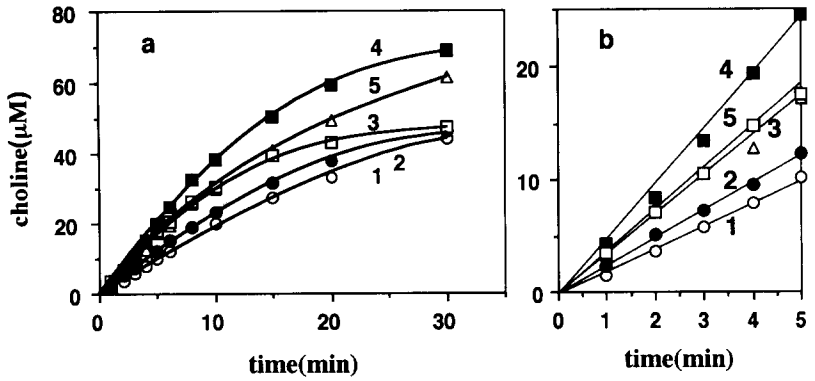

Fig. 2. (a) Hydrolysis of PC by PLD in PC/Chol-SUV. (b) Hydrolysis of PC by PLD in PC/Chol-SUV at early stage. The standard error bars are included in the symbols for the experimental values in Fig 2b. Chol (in mol%), 1, 0; 2, 9.6; 3, 18.3; 4, 26; 5, 47. The PC concentration in SUV is 2 mM.

Figs. 2 and 3 show the effects of the neutral lipid composition on the choline production in SUV. Increase in the mol% of DAG greatly enhanced the hydrolysis of PC in SUV (Fig. 3). The addition of more than 20 mol% of DAG perturbs the bilayer structure largely and induces the formation of non-bilayer structures; hexagonal and cubic phases of inverse topology [8,9]. The incorporation of Chol into PC-SUV gave smaller effects on the choline production than that of DAG.

Kinetics of PLD hydrolysis

The reaction velocity, v, was determined from an initial slope of the enzymatic production of choline (5 min) with changing initial substrate concentration, s, (in mM of PC in SUV) at a fixed neutral lipid composition. The double-reciprocal plots, $1/v$ vs. $1/s$ were all linear. Fig. 4 illustrates the linear plots for PC-, PC/18.3 mol% Chol- and PC/18.3 mol% DAG-SUVs. The Lineweaver-Burk plots gave the V_{max}(app) (mM/min) and K_m(app) (mM) values as shown in Table I. V_{max}(app) and K_m(app), here, are apparent maximum

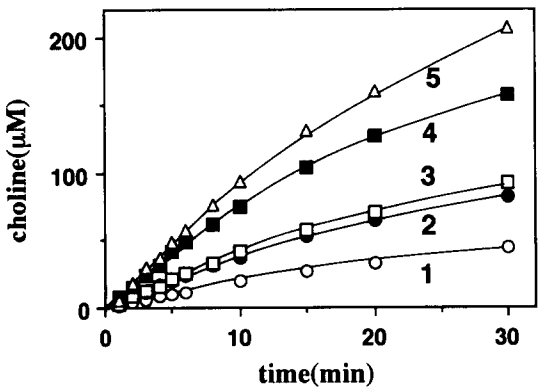

Fig. 3. Hydrolysis of PC by PLD in PC/DAG-SUV. DAG (in mol%), 1, 0; 2, 6.3; 3, 9.3; 4, 14.8; 5, 18.3. The PC concentration in SUV is 2 mM.

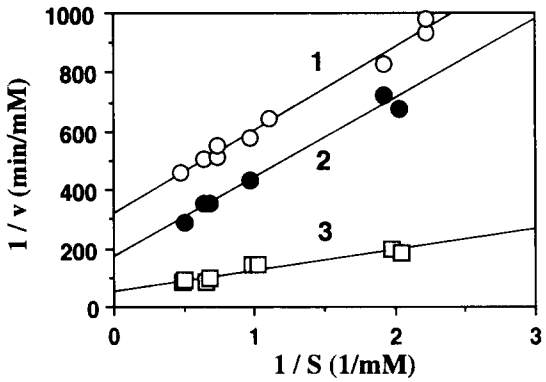

Fig. 4. Double-reciprocal plots, $1/v$ vs. $1/s$ for PC-SUV (1), PC/18.3 mol% Chol-SUV (2) and PC/18.3 mol% DAG-SUV (3).

TABLE I

The K_m(app) and V_{max}(app) values in the hydrolysis of PC by PLD

	K_m(app)×10 (mM)	V_{max}(app)×10^3 (mM/min) *
Bilayers		
PC-SUV	8.44 ± 1.37	3.05±0.32
PC/DAG-SUV		
DAG; 3.8 mol%	9.71 ± 1.89	4.90±0.50
6.3	8.51 ± 2.29	5.40±0.85
14.8	11.9 ± 4.2	12.7 ±2.8
18.3	11.5 ± 4.1	16.8 ±4.1
PC/Chol-SUV		
Chol; 9.6 mol%	9.95 ± 1.15	3.74±0.31
18.3	13.0 ± 5.9	5.87±0.97
22.0	15.1 ± 2.7	6.04±0.68
26.0	23.7 ±14.0	8.51±4.29
35.0	17.3 ± 3.3	6.48±0.85
47.0	13.0 ± 1.1	5.09±0.24
Micelles		
PC/HED (1:10)	1.10 ± 0.340	6.48±1.14
PC/DAG/HED (1:0.1:10)	0.847± 0.293	5.66±0.99
PC/Chol/HED (1:0.1:10)	0.977± 0.343	5.35±1.14

* 1 mM/min = 111 nmol/min per unit.
Values are expressed as means ± S.E.

velocity and apparent Michaelis constant for the PC hydrolysis, respectively.

The K_m(app) and V_{max}(app) values in PC-SUV were 0.844 mM and $3.05 \cdot 10^{-3}$ mM/min, respectively. The V_{max}(app) value increased with the mol% of DAG in PC/DAG-SUV, and was 6-fold the control value for PC/18.3 mol%DAG-SUV. The K_m(app) value was virtually independent of the DAG composition. In the Chol-containing SUV, both K_m(app) and V_{max}(app) doubled at 26 mol% of Chol. Further addition of Chol lowered both K_m(app) and V_{max}(app) values and decreased the rate of the PC hydrolysis (see PC/35 and 47 mol% Chol-SUVs in Fig. 2 and Table I).

The enzymatic hydrolysis of PC was also examined in the HED micelles (PC/HED = 1:10 in mol ratio). The concentration of HED is sufficient to largely preclude interactions between PC molecules in the micelle. The values of V_{max}(app) and K_m(app) in HED micelles are shown in Table I. The value of K_m(app) was smaller than those in SUV by one order of magnitude. The addition of DAG or Chol (10 mol% of PC) did not change the kinetic parameters of PC hydrolysis by PLD in the micelles.

Discussion

Kinetic parameters of the PC hydrolysis

The PC hydrolysis in heterogeneous interfacial system (SUV) proceeds as

$$e + s \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (es) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (es^*) \xrightarrow{k_3} e + p$$

Here, e, (es) and (es*) are the free PLD, the bound PLD in the non-activated and activated (complex) states, respectively.

The concentration of choline produced at early stage was proportional to reaction time (Figs. 1 and 2b), and suggests establishment of the steady state for enzyme-substrate complexes. The steady-state assumption for (es) and (es*) leads to the reaction velocity, v:

$$v = V_{max}(\text{app}) \cdot s / (K_m(\text{app}) + s) \quad (1)$$

Here, V_{max}(app) and K_m(app) are represented as

$$V_{max}(\text{app}) = (k_2/(k_2 + k_{-2} + k_3)) \cdot e_0 \cdot k_3$$
$$= ((es^*)/((es) + (es^*)))e_0 \cdot k_3 = f \cdot e_0 \cdot k_3 \quad (2)$$

$$K_m(\text{app}) = (k_{-1}(k_{-2} + k_3))/(k_1(k_2 + k_{-2} + k_3)) + k_2 \cdot k_3/(k_2 + k_{-2} + k_3)$$
$$= e \cdot s/((es) + (es^*)) \quad (3)$$

Here, e_o is the total PLD concentration, f is the fraction of activated complex in the total bound PLD, and is given by the following equation,

$$f = (es^*)/((es) + (es^*)) \quad (4)$$

The f value is independent of the SUV concentration, but possibly depends upon the lipid composition of SUV. Eqn. 1 shows that the linear correlation between $1/v$ and $1/s$ is obtained for PLD reactions in the heterogeneous interfacial system as seen in Fig. 4. Similar correlations have been obtained for the reaction of phospholipase A_2 in PC-SUVs [17]. Eqn. 3 shows that K_m(app) in the heterogeneous interfacial system is equal to the apparent dissociation constant of the PLD-SUV complex. A Michaelis constant in a homogeneous solution, is expressed as $(e \cdot s/(es^*))$.

The cholesterol and 1,2-diacylglycerol molecules in the micelle did not have significant effects on the enzymatic reaction (Table I). Thus, the effects of Chol and DAG are not due to specific influences on the enzyme itself, but rather upon the organization of substrate when it is dispersed in bilayer form. Activation of cobra venom phospholipase A_2 toward phosphatidylethanolamine by phosphatidylcholine or sphingomyelin in mixed micelles is caused by a direct interaction between the enzyme and activators [18], and is in contrast to the PLD activation in this work.

Cholesterol and 1,2-diacylglycerol have different effects on the PC hydrolysis (Table I). In PC/Chol-SUV, the effects of the increase in V_{max}(app) are largely compensated by the increase in K_m(app), and the resultant choline production is a little enhanced with the Chol composition up to about 26 mol%. Further addition of Chol turns back the K_m(app) and V_{max}(app) values (see Fig. 2). In PC/DAG-SUV, the K_m(app) value is nearly constant, and the increase in V_{max}(app) leads to the straightforward increase in the choline production (see Fig. 3).

Effects of neutral lipids in SUV

The addition of Chol or DAG to SUV increased the V_{max}(app) value $(= f \cdot e_o \cdot k_3)$. The value is associated with the degree of activation of PC-PLD complex in the SUV surface (i.e., f) and the decomposition of the activated complex into choline, phosphatidic acid and PLD (k_3). Intercalation of the neutral lipids between the PC molecules in the bilayers increases space around a hydrophilic group of PC [2,7] and influences the orientation of the head group. The addition of Chol or DAG, thus, enhances the accessibility of PLD to the susceptible bond of PC, and/or promotes the release of the choline group. Similar effects of phospholipid packing on hydrolysis activity of phospholipase A have been known in lipid monolayer [22,23]. 1,2-Diacylglycerols are presented to be inducers of PLD in several cells [5,6]. Mechanisms of the activation of PLD

by DAG are little known. We propose the increase in space around the P-O bond of PC and the orientational change of the bond as a possible mechanism for the activation of PLD by DAG.

The addition of DAG and Chol gave the different effects on the K_m(app) value. The DAG molecules have an orientation similar to that of the PC molecules in bilayers. The acyl chains are roughly parallel to the acyl chains of PC. The glycerol backbone of DAG is approximately parallel to the acyl chains and the *sn*-2 carbonyl group is more close to the water phase than the *sn*-1 carbonyl group [7]. DAG used in this work is derived from egg-yolk phosphatidylcholine (PC) and has the same fatty acid composition with PC. Therefore, the effect of DAG on the fluidity of bilayers may be much smaller than those of Chol. The penetration of hydrophobic residues of PLD to SUV is considered to be little influenced by the DAG addition.

The Chol incorporation into SUV lowers *gauche* rotamer formation of the acyl chain of PC and enhances the order in bilayers [2,3]. The penetration of hydrophobic moieties of PLD into hydrophobic regions of the ordered bilayers is inhibited and the PLD affinity may decrease (i.e., increase in K_m(app) value). Similar effects of Chol have been observed in the interactions between bilayers and plasma proteins [19–21]. Physicochemical studies on PC-Chol mixtures have identified a liquid-disordered phase in the Chol composition range 0 to 23 mol% and a liquid-ordered phase in the range 25–33 to 50 mol% [2,3,24]. The phase change and phase separation probably cause the return of the kinetic parameters in SUVs with 35 and 47 mol% Chol (Table I). Further studies on the binding of PLD to SUV are in progress and will be discussed in the near future.

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