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Effects of α-tocopherol and its acetate on the hydrolytic activity of phospholipase D in egg yolk phosphatidylcholine bilayers

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Abstract: Effects of α-tocopherol (Toc) and α-tocopherol acetate (TocA) on the hydrolysis activity of phospholipase D (from *Streptomyces chromofuscus*) were studied in small unilamellar vesicles (SUV) of egg yolk phosphatidylcholine (PC). Choline produced in the reaction was monitored by use of a choline oxidase – oxygen electrode. Addition of 18 mol% Toc into SUV (2 mM PC) brought about a twofold rate of choline production. On the other hand, the effect of 18 mol% TocA in SUV was very small. The apparent maximum velocity, V_{max}(app), increased by addition of Toc in SUV. The apparent Michaelis constant, K_m(app), was unchanged by addition of Toc and TocA in SUV. The Toc and TocA molecules did not have significant effects when PC was solubilized in the micelles of heptaethylene glycol dodecyl ether. The effects of Toc and TocA are, therefore, not due to specific ones on the enzyme itself, but rather upon the bilayer-organization of the substrate. Measurements of spreading pressure showed complete miscibility of PC and Toc, and limited mutual solubility of PC and TocA, suggesting stronger attractive interactions between Toc and PC than those between TocA and PC in the bilayers.

Key words: Phospholipase D – α-tocopherol – α-tocopherol acetate – egg yolk phosphatidylcholine

Introduction

α-Tocopherol (Toc) is predominantly located in cellular and subcellular membranes. Recent research has focused on the effects of Toc on structural and dynamic properties of membrane because there can be found several physiological effects of Toc apart from its antioxidant effects. Deficiency of Toc in animal cells changes the fluidity of membrane lipids [1] and activities of membrane-bound enzymes [2, 3]. α-Tocopherol also changes the permeability and the fluidity of phospholipid bilayers [4–8]. Cushley et al. have demonstrated that addition of Toc increases the acyl chain motion of sn-2-substituted [$^2H_{31}$] palmitoylphosphatidylcholine bilayers below the transition temperature, but decreases the motion above the temperature [9]. α-Tocopherol acetate (TocA) is an analogue of Toc: The hydroxyl group of Toc is replaced by an acetate group. Effects of TocA on the fluidity, permeability, and antioxidant behavior of lipid membrane are different from those of Toc [7].

Phospholipase D (PLD) activity has been found to be associated with either membrane [10] or cytosol [11]. Billah et al. have observed that the main substrate for PLD is phosphatidylcholine [12]. Previously, we demonstrated that the structural changes of egg yolk phosphatidylcholine (PC) bilayers induced by diacylglyceride led to the activation of PLD. In the present work, we studied the effects of Toc and TocA in PC bilayers on the PC hydrolysis by PLD.

Experimental

Materials

Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei Company (Tokyo). The

purity (over 99%) was determined by thin layer chromatography (TLC) (Iatroscan Analyzer MK-5 from Iatron Laboratories Co. Ltd., Tokyo, solvent: chloroform/methanol = 3/1 v/v). DL-α-Tocopherol (Toc) and DL-α-tocopherol acetate (TocA) were purchased from Nacalai Tesque (Kyoto, Japan). Each showed a single spot on TLC (solvent: cyclohexane/chloroform = 2/1 v/v). Egg yolk lysophosphatidylcholine (lyso PC) and a nonionic surfactant, heptaethylene glycol dodecyl ether (HED), were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and Nikko Chemicals Co. Ltd. (Tokyo), respectively.

Phospholipase D (E.C.3.1.4.4.) (PLD) from *Streptomyces chromofuscus* was purchased from Sigma Chemicals Co. (St. Louis, MO). The molecular weight determined by SDS – polyacrylamide gel electrophoresis was in agreement with the reported value [13]. Choline oxidase (E.C.1.1.3.17.) from *Alcaligenes* sp. was obtained from TOYOBO Co., Ltd. (Osaka).

Measurement of spreading pressure

Neutral lipid (Toc or TocA), PC, or mixtures of the neutral lipid and PC were dissolved in benzene. After evaporation of the solvent, the residue was dried in vacuum overnight. Portions of the dried lipid mixture were supplied on the double-distilled water in a Teflon-coated Duralumin trough. Spreading pressures of lipid mixtures at an air/water interface (surface pressure of the mixed monolayer in equilibrium with the bulk lipid mixture) were obtained from the steady value of surface pressure at 1–2h after addition of the lipid or the lipid mixture on water. Spreading pressures were determined at 25 °C, and at this temperature, PC on water is hydrated within a few minutes. The details of the monolayer techniques have been described elsewhere [14, 15].

Preparations of Vesicles and Mixed Micelles

Neutral lipid (Toc or TocA) and PC were dissolved in chloroform. The solvent was evaporated under reduced pressure, and the residue was dried in vacuum overnight to ensure complete solvent removal. The lipid or lipid mixture was hydrated in a buffer consisting of 10 mM Tris-HCl and 150 mM NaCl (pH 8.0), 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 Company Ltd. The lipid dispersion (small unilamellar vesicles, SUV or emulsions) was centrifuged at 3000 rpm for 10 min to remove titanium dust. Size of dispersed vesicles was measured at 25 °C by dynamic light scattering (DLS) (Photal LPA-3000/3100). The weight-averaged vesicle size of each sample was 30 ± 15 nm. The effects of neutral lipids on the size were small.

Lipid (PC, PC/Toc, or PC/TocA) and HED were mixed in chloroform. After complete removal of the solvent, the mixture was dissolved in the Tris-HCl buffer to give mixed micellar solutions. Egg yolk phosphatidylcholine in SUV, emulsions and micellar solutions was assayed by the method of Bartlett [16].

Determination of the solubility of TocA into PC bilayers

A lipid mixture composed of PC and TocA (PC/TocA = 2/8 in mole ratio) was prepared as described above. The mixture was hydrated in the Tris-HCl buffer and vortexed for 1 h. The dispersion was ultracentrifuged at 150 000 × g for 14 h (at 25 °C). The supernatant was removed and the precipitate was resuspended in the Tris-HCl buffer. The procedure was repeated until little floating oil was detected. The composition of the finally precipitated bilayers (TocA/PC ratio) was determined by TLC (solvent: cyclohexane/chloroform = 2/1 v/v).

Assay for the phospholipase D activity

The enzymatic reaction was started by the injection of PLD (in the Tris-HCl buffer) into a 50 ml sample solution (SUV) maintained at 30 °C. The concentration of choline produced was monitored by a choline oxidase-oxygen electrode. A phospholipase D activity was checked by measuring the hydrolysis activity in a standard lyso-PC micelle solution. The procedure of PLD preparation and the principle of choline assay have been described in detail elsewhere [17].

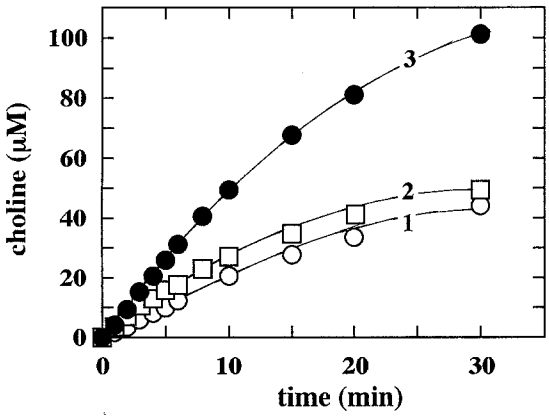

Fig. 1. Effects of α-tocopherol (Toc) and α-tocopherol acetate (TocA) on the choline production by PLD in small unilamellar vesicles (SUV). 1: PC-SUV; 2: PC/18 mol% TocA-SUV; 3: PC/18 mol% Toc-SUV. SUVs were prepared in a buffer consisting of 10 mM Tris-HCl and 150 mM NaCl (pH 8.0) by sonication. The PC concentration in SUV is 2 mM. Choline concentration was monitored with a choline oxidase-oxygen electrode, at 30 °C

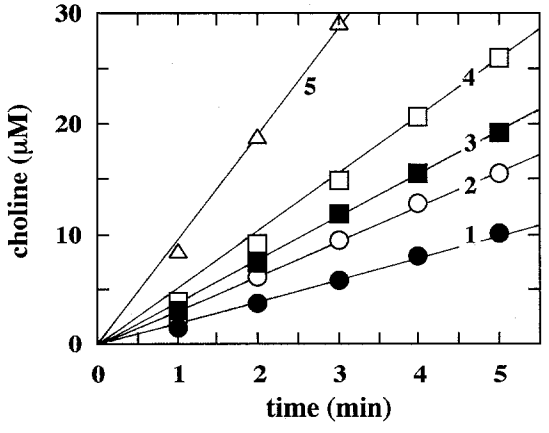

Fig. 2. Hydrolysis of PC by PLD in PC/Toc-SUV at the initial stage. The standard error bars are included in the symbols for the experimental values. Toc (in mol%), 1: 0; 2: 6; 3: 12; 4: 18; 5: 24. The PC concentration in SUV is 2 mM

Results

Enzymatic hydrolysis of PC

The enzymatic hydrolysis of PC was influenced by addition of a neutral lipid, Toc or TocA, into the PC bilayers (PC-SUV). Figure 1 shows the time-course of choline production by PLD. The choline produced in PC-SUV (PC 2 mM) was

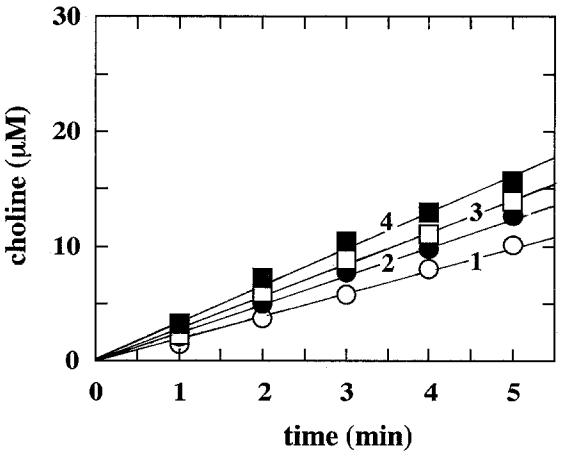

Fig. 3. Hydrolysis of PC by PLD in PC/TocA-SUV at the initial stage. TocA (in mol%), 1: 0; 2: 6; 3: 12; 4: 18. The PC concentration in SUV is 2 mM

45 μM for 30 min (control). In PC/18 mol% Toc-SUV, the choline production was twice higher than the control value. The effect, however, was half that of diacylglyceride [17]. The addition of 18 mol% TocA gave only a small increase on the choline production in comparison with the control value.

Figures 2 and 3 show the effects of Toc and TocA contents on the choline production in SUV (PC 2 mM) at the initial stage (~ 5 min). The choline concentration proportionally increased with time. This result suggested the establishment of the steady state for enzyme-substrate complexes. Increase in the mol% of Toc in SUV led to the higher velocity (v) of PC hydrolysis (Fig. 2). On the other hand TocA had a little effect on the velocity (Fig. 3). The double reciprocal plots, $1/v$ vs. $1/s$ (Lineweaver–Burk plots) were all linear. Similar correlations have been observed for the reaction of phospholipase A_2 in PC-SUVs [20]. Figure 4 illustrates the linear plots for PC-SUV and PC-SUVs containing 18 mol% Toc and TocA. The apparent maximum velocity, V_{max}(app), and the apparent Michaelis constant, K_m(app), values obtained are shown in Table 1. The K_m(app) and V_{max}(app) values in PC-SUV were 0.844 mM and 3.05×10^{-3} mM/min, respectively. Increasing mol% of Toc in PC-SUV gave the higher V_{max}(app) value, and the value for PC/24 mol% Toc-SUV was fivefold higher than the control value, while little change in the

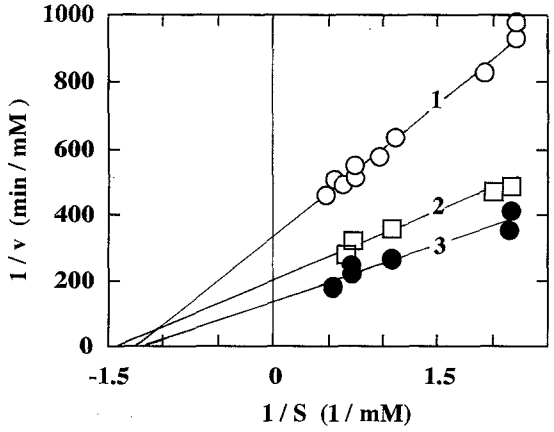

Fig. 4. Lineweaver–Burk plots for PC-SUV (1), PC/18 mol% TocA-SUV (2) and PC/18 mol% Toc-SUV (3)

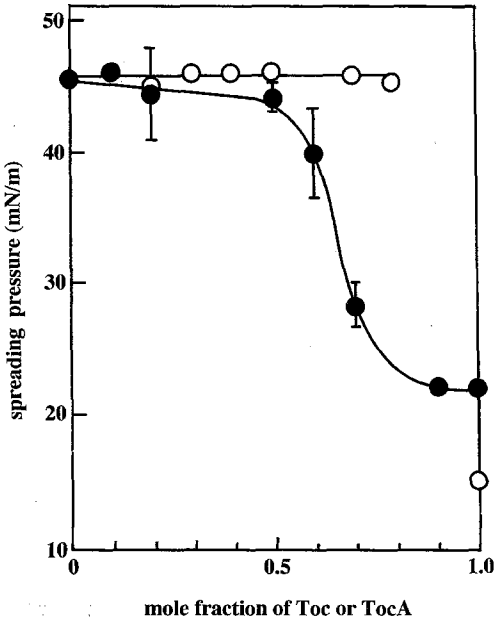

Fig. 5. Spreading pressures of lipid mixtures at an air/water interface at 25 °C. ● PC/Toc mixtures ○ PC/TocA mixtures

K_m(app) value was observed for PC/Toc-SUV. These results suggested that the incorporation of Toc increased the reactivity of PLD on the SUV-surface, but did not change the binding ability into the surface. On the other hand, incorporation of TocA into PC-SUV caused small increase in the V_{max}(app) value, and did not influence the K_m(app) value.

Hydrolysis of PC in the HED micelles was also examined (PC/HED = 1/10 in mole ratio). The concentration of HED was sufficient to preclude interactions between PC molecules in the micelles. The addition of Toc and TocA (PC/Toc or TocA = 1/0.22 in mole ratio) did not change the kinetic parameters of PC hydrolysis by PLD in the micelles (data not shown).

Table 1. The K_m(app) and V_{max}(app) values for the hydrolysis of PC by PLD

Bilayers		K_m(app) (mM)	V_{max}(app) × 10^3 (mM/min)[a]
PC-SUV		0.844 ± 0.137	3.05 ± 0.32
PC/Toc-SUV			
Toc;	12 mol%	0.852 ± 0.147	5.85 ± 0.60
	18	0.871 ± 0.299	7.43 ± 1.55
	24	0.796 ± 0.201	14.9 ± 1.90
PC/TocA-SUV			
TocA;	6 mol%	0.781 ± 0.168	3.86 ± 0.48
	12	0.789 ± 0.211	4.46 ± 0.69
	18	0.617 ± 0.105	4.74 ± 0.37
	14.9[b]	0.812 ± 0.318	4.64 ± 1.17

[a] 1 mM/min = 111 nmol/min unit

[b] SUV was prepared with the PC and TocA mixture (i.e., bilayers of PC saturated with TocA) separated from excess TocA by the ultracentrifugation procedure. The composition of the mixture was determined by TLC. (see text) Values are expressed as mean ± S.E.

Spreading pressure of PC/Toc or TocA Mixtures

Figure 5 shows spreading pressures of PC/Toc and PC/TocA mixtures. Spreading pressure of hydrated PC (lamellar bilayers of PC) was 45.6 mN/m. Those of Toc and TocA were 22 mN/m and 15 mN/m, respectively. Spreading pressure of a lipid mixture is dependent on the miscibility of the lipids in bulk phases [15, 21–23]. Spreading pressure of PC/Toc mixture varied with the mole fraction of Toc, while that of PC/TocA mixture remained constant at 46 mN/m over the mole fraction range of TocA 0–0.8. On the basis of the surface phase rule [15, 24], it was concluded that PC and Toc were miscible in the bulk phases (bilayer and hexagonal phases [25]), and that PC and TocA were partially miscible in the bulk phases (PC bilayer and TocA liquid phases). The bilayers separated from the PC-TocA dispersion (2/8 in mole) by ultracentrifugation (i.e. bilayers of PC saturated with TocA) contained 14.9 ± 0.3 mol% TocA and 85.1 ± 0.3 mol% PC. The enzymatic kinetic parameters for the bilayers saturated with TocA (SUV) are shown in Table 1.

Discussion

Kinetic parameters of PC Hydrolysis

The α-tocopherol and α-tocopherol acetate molecules in SUV influenced the kinetic parameters of PC hydrolysis by PLD (Table 1), while those in the micelle did not have any significant effect. We have reported similar results in PC/diacylglyceride (DAG)- and PC/cholesterol (Chol)-SUVs [17]. Thus, the effects of these lipids (Toc, TocA, DAG and Chol) were not due to specific influences on the enzyme itself, but rather upon the organization of substrate when it was dispersed in bilayer form. The indirect activation of PLD by Toc and DAG contrasted with the activation of cobra venom phospholipase A_2 toward phosphatidylethanolamine by phosphatidylcholine in mixed micelles where direct interactions between the enzyme and the activator played important rules [26,27].

α-Tocopherol and α-tocopherol acetate molecules had different effects on the PC hydrolysis (Table 1). In PC/Toc-SUV, the K_m(app) value was nearly constant and the increase in V_{max}(app) was directly related to the increase in the choline production. On the other hand, in PC/TocA-SUV, the K_m(app) and V_{max}(app) values were nearly constant. The results for PC/Toc-SUV were similar to those of PC/DAG-SUV [17]. The effect of DAG have been attributed to a change of membrane structure [17]. Figure 5 shows complete miscibility of Toc and PC, and partial miscibility of TocA and PC in the mixed bilayers, suggesting stronger attractive interactions between Toc and PC than those between TocA and PC. The different interactions of Toc and TocA were considered to induce their distinct effects on the PC hydrolysis. The solubility of TocA in the PC bilayers was not determined from the spreading pressure of PC/TocA mixture. The ultracentrifugation experiments presented the solubility of TocA as about 15 mol%. In PC-SUV containing more than 15 mol% TocA, the excess TocA separated from the bilayers and formed emulsion particles stabilized by PC (unpublished data). Such phenomena have been observed for weakly interacting lipid mixtures, such as PC and ubiquinone-10, cholesterylester, or triacylglyceride-mixtures [23, 28].

Effects of lipid interactions on PLD-activity

The addition of Toc to SUV increased the V_{max}(app) value. In contrast, TocA had a little influence on the V_{max}(app) value. The value is associated with the formation of the activated PC-PLD complex in the SUV surface and the decomposition of the complex into the products and PLD [17]. Spectroscopic and dielectric studies have shown that the $^-P–N^+$ dipoles of PC are almost parallel to the plane of the bilayers and form the two-dimensional regular structure [29]. ^{13}C-NMR studies show that the spin-lattice relaxation time for the α-CH_2 group of choline moiety increases by the incorporation of Toc into PC bilayers [4]. ^{31}P-NMR chemical shift anisotropy measurements indicate that the phosphate region of the polar head group in PC bilayers is disturbed by the incorporation of Toc [25]. In contrast, TocA has no effect on the ^{31}P-NMR chemical shift anisotropy in PC dispersion [25], and hardly influences the regular structure of PC bilayers. Intercalation of DAG or Chol between the PC molecules in the bilayers increases free space around a hydrophilic group of PC [30, 31], and increases the V_{max}(app) value [17]. The increase in

V_{max}(app) by DAG and Chol was closely correlated with the mode of the P = O antisymmetric stretching vibration of PC (unpublished data). We propose the orientational change of the P = O bond of PC as a possible mechanism for the enhanced activity of PLD by the addition of Toc.

We have shown that cholesterol with the intense ordering effect on the PC bilayers [31, 32] decreases the PLD affinity {i.e., increased in K_m(app) value} [17]. The addition of Toc and TocA scarcely influenced the K_m(app) value. FT-IR and ESR measurements have indicated that the incorporation of Toc broadens the gel to liquid-crystalline phase transition of L-α-dipalmitoylphosphatidylcholine (DPPC) bilayers, but TocA does not affect it appreciably [7, 33]. NMR studies have shown that the hydrocarbon chain of TocA is in fluid state in the liquid-crystalline phase of DPPC bilayers, but Toc is packed closer to the DPPC molecule [7]. The ordering effects of Toc are however much smaller than those of cholesterol [9, 31, 32]. Therefore, the addition of Toc and TocA did not influence the binding of PLD to the bilayers as the addition of Chol did [17].

α-Tocopherol activated the PC hydrolysis by PLD but TocA did not. This result indicates that the hydroxyl group of Toc plays important roles in the lipid interactions and consequently on the indirect activation of PLD. Further studies on the effects of Toc and TocA on the binding of PLD to SUV and susceptibility of the phosphorylcholine-bond are in progress and will be discussed in the near future.

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