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Regulation of phospholipase D activity by neutral lipids in egg-yolk phosphatidylcholine small unilamellar vesicles and by calcium ion in aqueous medium

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Abstract

Hydrolysis activity of phospholipase D from *Streptomyces chromofuscus* (PLD) was studied in small unilamellar vesicles (SUV) of egg yolk phosphatidylcholine (PC). The enzyme was associated with PC-SUV in a Ca^{2+}-dependent manner. Both apparent maximum velocity, V_{max}(app), and reciprocal of apparent Michaelis constant, i.e., apparent binding constant, $1/K_m$(app), increased with Ca^{2+} concentration, and the maximum values of these kinetic parameters were obtained at about 20 μM Ca^{2+}. Incorporation of 1,2-diacylglycerol (DAG), cholesterol (Chol) or α-tocopherol (Toc) into PC-SUV induced shift of the antisymmetric PO_2^- stretching band of PC to lower frequency. The neutral lipids in SUV brought about increase of the V_{max}(app) value (Yamamoto et al. (1993) Biochim. Biophys. Acta 1145, 293–297). On the basis of these findings we discussed the regulation of PLD activity in terms of the Ca^{2+}-dependent complex formation of PLD with SUV, and the enhancement of susceptibility of the P-O bond in PC molecule by neutral lipids.

Keywords: Phospholipase D; Calcium ion; 1,2-Diacylglycerol; Cholesterol; α-Tocopherol; FTIR

1. Introduction

Phospholipase D (EC 3.1.4.4) activity has been observed in both membrane [1] and cytosolic [2] fractions of mammalian cells and has been argued in connection with intracellular signal transduction [3–6]. Activation of phospholipase D in Ca^{2+}-permeabilized HL60 cells requires the ion of micromolar concentration range in medium [7,8]. A cell-permeable diacylglycerol, 1-oleoyl-2-acetylglycerol, and calcium ionophore, A23187, are also potent inducers of the enzyme activity in HL60 cells [9]. Attempts to purify the membrane-associated or the cytosolic phospholipases D have been disappointing [10], and the exact regulation mechanism remains to be clarified. A main substrate for the enzyme is phosphatidylcholine in membrane [6]. Huang et al. have demonstrated that the enzyme activity is more dependent on the structure of the phosphatidylcholine derivative than on the source of the enzyme [11].

Phospholipase D from *Streptomyces chromofuscus* (PLD) has been purified from the culture supernatant [12], suggesting a soluble enzyme. Phospholipase D from *Streptomyces chromofuscus* mimics some reactions in cells. For example, the exogenous PLD has a similar activity to endogenous phospholipase D in ovarian granulosa cell culture [13]. Exogenous addition of PLD to the medium of vascular smooth muscle cells induces a marked DNA synthesis with formation of choline and phosphatidic acid [14]. Phospholipase D from *Streptomyces chromofuscus* also substitutes for serum which is required for invasion of cultured mesothelial cell monolayers by MM1 and OC10 cells [15]. We have observed that incorporation of some neutral lipids (1,2-diacylglycerol, cholesterol and DL-α-tocopherol) into egg-yolk phosphatidylcholine bilayers enhances the catalytic activity of PLD [16,17]. In the present work, we investigated: (1) Ca^{2+}-dependence of PLD activity and (2) correlation between enhanced catalytic activity of PLD and modification of bilayer surface structure induced by neutral lipids. The modification was estimated by infrared measurements of the PO_2^- antisymmetric stretching band of PC and LPC.

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2. Materials and methods

2.1. Materials

Egg-yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei (Tokyo). The purity (over 99%) was determined by thin-layer chromatography (TLC) (Iatroscan Analyzer MK-5 from Iatron Laboratories, Tokyo, solvent: chloroform/methanol (3:1, 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. Cholesterol (Chol) and DL-α-tocopherol (Toc) were purchased from Sigma (St. Louis, MO) and Nacalai Tesque (Kyoto, Japan), respectively. Each sample showed a single spot on TLC (solvent: chloroform/acetone/methanol/acetic acid/water (45:20:12:10:5, v/v) for Chol, cyclohexane/chloroform (2:1, v/v) for Toc). Egg-yolk lysophosphatidylcholine (LPC) was purchased from Sigma.

Phospholipase D (EC 3.1.4.4) from *Streptomyces chromofuscus* (PLD) (obtained from Sigma) was the same as in the previous work [16]. The specimen had two bands on SDS-polyacrylamide gel electrophoresis (molecular masses: 56 and 42 kDa). The molecular mass of 56 kDa agreed with the reported value for PLD [12]. The peptide of 42 kDa was separated from SUV-associating peptides by ultrafiltration in the presence of 10 mM PC-SUV and 50 μM Ca^{2+}. It was concluded that the peptide of 42 kDa did not associated with SUV. No choline production was detected by an injection of the peptide into PC-SUV (PC: 8 mM) containing 50 μM Ca^{2+}, suggesting no PLD activity in the peptide. Furthermore, augmentation of the peptide in the specimen did not affect the enzyme activity (data not shown).

2.2. Preparations of vesicles and micellar solution

Cholesterol (Chol), 1,2-diacylglycerol (DAG), DL-α-tocopherol (Toc) and egg-yolk phosphatidylcholine (PC) were mixed in chloroform and the solvent was evaporated under reduced pressure. After drying under vacuum overnight, the lipid film was hydrated with a Tris-HCl buffer which contained 10 mM Tris, 150 mM NaCl and desired amount of $CaCl_2$ or 1 mM EDTA. Calcium ion concentration in buffer was determined by an EDTA titration with fra-2 as an indicator [18]. The lipid dispersion was 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 (Tokyo Japan). The vesicle suspension (small unilamellar vesicles, SUV) was centrifuged at 3000 rpm for 10 min to remove titanium dust and stored at 25°C. No free fatty acids or phosphatidic acids resulting from the decomposition of PC during sonication were detected by TLC. Dynamic light scattering (DLS) of SUV was measured with a Photal LPA-3000/3100. The weight-averaged vesicle size of each sample was 30 ± 15 nm. Effects of neutral lipids on the vesicle size were small. Lysophosphatidylcholine (LPC) was dissolved in chloroform/methanol (2:1, v/v). After complete removal of the solvent, the residue was dissolved in the Tris-HCl buffer to give a micellar solution. Phospholipid in SUV and micellar solution was assayed by the method of Bartlett [19].

2.3. FT-IR spectroscopy

Samples for infrared spectroscopic analysis were prepared in 50 μm thick cells with CaF_2 windows. The infrared spectra were measured with a Nicolet 205 Fourier transform infrared (FT-IR) spectrometer equipped with an Hg-Cd-Te detector. The temperature of sample was controlled by means of a block assembly through which water circulated and monitored by a thermosensor placed at the edge of the cell window. The 256 interferograms collected were analyzed by use of Nicolet SX software on a 620 workstation. The resolution was 4 cm^{-1}. The subtraction of spectra in buffer was carried out to remove the contribution from water bands. The accuracy of the frequency reading was better than ± 0.1 cm^{-1}.

2.4. Assay for the hydrolysis of PC by PLD

Phospholipase D from *Streptomyces chromofuscus* catalyzes the hydrolysis of PC in SUV with formation of choline and phosphatidic acid. The enzymatic reaction was started by the injection of PLD into a sample solution (SUV or micellar solution) maintained at 30°C. The choline concentration was monitored by use of a choline oxidase-oxygen electrode. The PLD activity was checked by measurement of the hydrolysis activity in a standard LPC micellar solution. The principle of choline assay has been described in detail elsewhere [16].

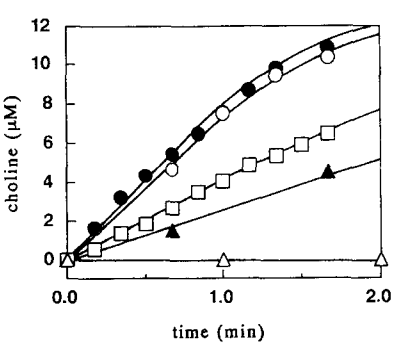

Fig. 1. Effect of Ca^{2+} concentration on the choline production following hydrolysis of PC by PLD. PC-SUV (2 mM) was prepared in a buffer containing 10 mM Tris, 150 mM NaCl (pH 8) and the indicated $CaCl_2$ or 1 mM EDTA. Open triangles: 1 mM EDTA. Ca^{2+} concentration (μM): closed triangles, 7.8; open squares, 15; open circles, 23; closed circles, 50.

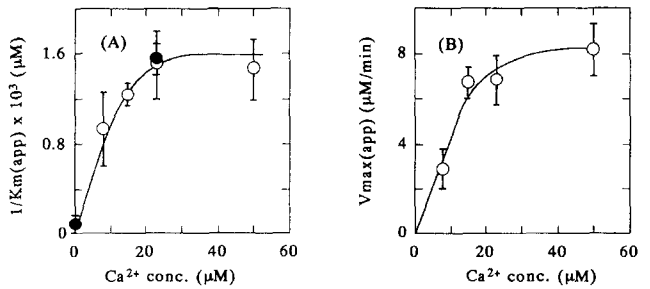

Fig. 2. Dependence of Ca^{2+} concentration on the $1/K_m$(app) (A) and V_{max}(app) (B) values. The open circles represent the values determined from PLD reactions and the closed circles from binding (ultrafiltration) experiments. Results are expressed as means ± S.E.

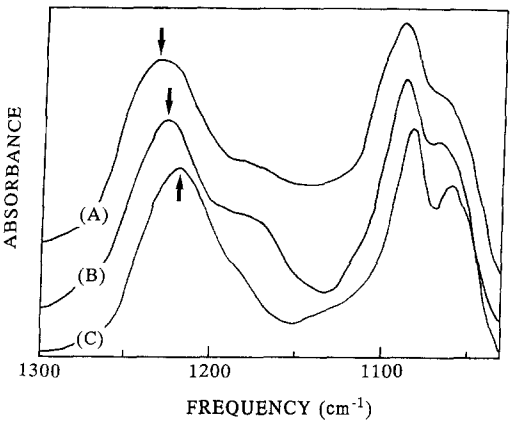

Fig. 3. Infrared spectra of PC vesicles and LPC micelles for the PO_2^- double bond stretching region at 30°C. Arrows show the maximum frequencies of the antisymmetric stretching band (ν_{max}). (A) PC-SUV, (B) PC/18.3 mol% DAG-SUV, (C) LPC micelles.

2.5. Ultrafiltration

Phospholipase D from *Streptomyces chromofuscus* was incubated with SUV in the Tris-HCl buffer. Free PLD in the incubation medium was separated from SUV-associating PLD by ultrafiltration. The ultrafiltration membrane used was rated at 100 000 molecular weight cutoff (YM100 DIAFLO ultrafilters, W.R. Grace). The operating pressure was about 10 psi. The free PLD amount in the filtrate was determined by measurement of the enzyme activity in a standard LPC micellar solution.

3. Results

3.1. Calcium ion-dependent hydrolysis of PC by PLD

The enzymatic hydrolysis of PC by PLD was influenced by Ca^{2+} concentration in medium. Fig. 1 shows the time courses of choline production in PC-SUV by PLD at various Ca^{2+} concentration. At 7.8 μM Ca^{2+} the initial velocity, v, of the choline production was 2.34 μM/min where PC-SUV concentration, s, was 2 mM. The initial velocity increased with Ca^{2+} concentration in medium and it was 6.89 μM/min at 50 μM Ca^{2+}. In the buffer containing 1 mM EDTA, choline production was not detected. Ultrafiltration experiments showed that PLD was not bound to SUV in the Ca^{2+}-eliminated medium. The double-reciprocal plots, $1/v$ vs. $1/s$ (Lineweaver-Burk plots) were all linear (data not shown). Fig. 2A and 2B shows the effects of Ca^{2+} ion on the kinetic parameters, the reciprocal of apparent Michaelis constant, $1/K_m$(app), and the apparent maximum velocity, V_{max}(app), respectively. The kinetic parameters increased with Ca^{2+} concentration in medium. The maximum activity of PLD was given at about 20 μM Ca^{2+}. At 0 and 23 μM Ca^{2+}, the $1/K_m$(app) values determined by the enzymatic reaction were in agreement with those estimated by the binding (ultrafiltration) experiments (the closed circles in Fig. 2A).

3.2. FTIR spectra for antisymmetric PO_2^- stretching region of PC

Fig. 3 presents infrared spectra of PC-SUV, PC/18.3 mol% DAG-SUV and LPC micellar solution for the PO_2^- double bond stretching bands (1000--1300 cm^{-1}) at 7.8 μM Ca^{2+}. The assignment of spectra was found in Ref.

Table 1
Infrared frequencies of the PO_2^- antisymmetric stretching band of PC in small unilamellar vesicles containing various neutral lipids and of LPC in micelle solution

Bilayers		PO_2^- antisymmetric frequency (ν_{max}) (cm^{-1})	$\Delta\nu_{max}$ (ν_{max} shift from PC-SUV (cm^{-1}))
PC-SUV		1231.7 ± 0.8	0
+ DAG	6.3%	1229.9 ± 0.5	1.8
	9.0% [a]	1228.4 ± 1.3	3.3
	18.3%	1226.8 ± 0.2	4.9
+ Chol	9.6%	1230.4 ± 1.3	1.3
	18.3%	1229.6 ± 0.7	2.1
	26.0%	1228.6 ± 0.5	3.1
	47.0%	1227.1 ± 0.1	4.6
+ Toc	12.0% [b]	1229.7 ± 0.5	2.0
LPC micelle [c]		1220.6 ± 0.1	11.1

Data represented are the means ± S.D. of two or three independent experiments. V_{max}(app) value: [a] 7.42 ± 0.68 μM/min, [b] 5.85 ± 0.60 μM/min (Ref. [17]), [c] 37.4 ± 7.0 μM/min (unpublished data)

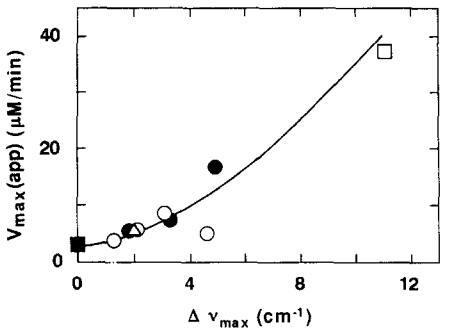

Fig. 4. Relationship between the V_{max}(app) value and the $\Delta\nu_{max}$. The V_{max}(app) values (determined from the enzymatic reaction) are seen in Ref. [16] and in Table 1. Closed squares, PC-SUV; open squares, LPC micelles; closed circles, DAG/PC-SUV; open circles, Chol/PC-SUV; open triangles, Toc/PC-SUV.

[20]. In PC-SUV the symmetric and antisymmetric stretching modes had the maximum frequencies at 1088.4 cm^{-1} and 1231.7 cm^{-1}, respectively. These values are consistent with those of dipalmitoylphosphatidylcholine vesicles in the liquid-crystalline state [21]. Incorporation of DAG into PC-SUV shifted the PO_2^- antisymmetric stretching band to lower frequency. The bandwidth appeared to slightly decrease. However an accurate estimation of the bandwidth was difficult because of overlapping with the C-O stretching band of the ester group at approx. 1170 cm^{-1} [20]. In LPC micellar solution, the PO_2^- antisymmetric stretching band was observed at 1220.6 cm^{-1}. Table 1 shows the effects of neutral lipid in PC-SUV on the maximum frequency of PO_2^- antisymmetric stretching band (ν_{max}). Addition of the neutral lipid into PC-SUV led to decrease in the frequency. Incorporation of 18.3 mol% DAG into PC-SUV brought about a 5 cm^{-1} shift to lower frequency. A remarkable shift of about 11 cm^{-1} was found for the LPC micellar solution. On the other hand Ca^{2+} did not lead to any detectable frequency shift of PO_2^- in the concentration range for assay of the enzymatic reaction (0–50 μM) (data not shown).

3.3. Relationship between IR spectral change and catalytic activity

The enzymatic hydrolysis of PC is influenced by incorporation of neutral lipids (DAG, Chol and Toc) into PC-SUV [16,17]. 1,2-Diacylglycerol and α-tocopherol in PC-SUV increases the V_{max}(app) value, however, dose not influence the K_m(app) value. On the other hand, addition of Chol up to 26.0 mol% increases both the K_m(app) and V_{max}(app) values [16]. Table 1 shows infrared frequencies of the PO_2^- antisymmetric band of PC in mixed SUV and of LPC in micelles (ν_{max}), and the shift from that in PC-SUV ($\Delta\nu_{max}$). Fig. 4 represents the correlation between $\Delta\nu_{max}$ and V_{max}(app), showing that the catalytic activity of PLD increases with the lower shift of the antisymmetric stretching band of PC except for 47.0 mol% Chol/PC-SUV.

4. Discussion

4.1. Regulation of PLD activity by Ca^{2+} ion

Phospholipase D excreted from *Streptomyces chromofuscus* (PLD) was associated with PC-SUV in a Ca^{2+}-dependent fashion in the Tris-HCl buffer. The Ca^{2+}-dependent association was similar to that of cytosolic phospholipase A_2 (cPLA$_2$), which translocates to synaptosomal- [22] and phosphatidylcholine- [23] membranes in a Ca^{2+}-dependent manner. When our PLD was preincubated with 1 mM EDTA and was injected to a PC-SUV solution containing 100 μM $CaCl_2$, the choline production started after a lag time of about 2 min. On the other hand the PLD preincubated in 23 μM $CaCl_2$ immediately started the hydrolysis (data not shown). Ultrafiltration experiments showed that Ca^{2+} was essential for the PLD-binding to PC-SUV. These results suggest that the binding of Ca^{2+} to PLD is followed by a conformational change of PLD, and then the enzyme likely associates with PC-SUV. Details for PLD and Ca^{2+} interactions are not discussed in the present work, and we assayed the hydrolysis reaction with PLD preincubated in Ca^{2+}-containing buffer.

Calcium ion increased both the binding ability of PLD to SUV ($1/K_m$(app)) and the catalytic activity (V_{max}(app)) (Fig. 2), and the maximal values of these parameters were obtained at about 20 μM Ca^{2+}. The Ca^{2+} requirement was similar to that for activation of phospholipase D in HL60 cells [7,8] and of cPLA$_2$ in human monocytic cell line [24] and in rabbit platelet cytosol [25]. The free Ca^{2+} concentration is about 10^{-7} M in the cytosol and cell activations elevate it to 10^{-6}–10^{-4} M by influx of extracellular Ca^{2+} or by intracellular release of Ca^{2+} [26]. The Ca^{2+}-dependence of PLD suggests that the translocation and the catalytic activation is possibly regulated by physiological alternation in the Ca^{2+} level of the cytosol. However, we do not know if our results with a bacterial phospholipase D are relevant to physiological roles of cytosolic phospholipase D in mammalian cells.

4.2. Susceptibility of the P-O bond of PC and LPC to PLD

The antisymmetric stretching band for the PO_2^- double bond of PC is useful to monitor the hydration state of the polar headgroup [27]. A frequency of approx. 1220 cm^{-1} characterizes a fully hydrated PO_2^- group, whereas the dehydration makes it appear at higher frequency (approx. 1240 cm^{-1}) [28]. The shift to lower frequency by incorporation of neutral lipids (Table 2) was therefore caused by an enhanced hydration of PO_2^- group of PC at membrane surface.

The rather small changes in ^{31}P chemical shift anisotropy of PC bilayers have demonstrated that the average orientation and motion of the headgroup segments are not significantly affected by incorporating DAG (< 20 mol%) [29] and Chol (< 50 mol%) [30]. ^{13}C-NMR spectroscopy

also shows that the DAG molecules have an orientation similar to that of the PC molecules in bilayers and that the glycerol backbone of DAG is approximately parallel to the acyl chains [29]. On the basis of the deuterium quadrupolar splitting of choline methylene groups of PC, Brown and Seelig have indicated that Chol in PC bilayers acts as spacer molecules in the polar headgroup region which increase the separation between headgroups [30]. Addition of Chol (up to 26.0 mol%) and DAG(up to 18.3 mol%), thus, induces increase in the space around the P-O bond and enhances the hydration of PO_2^- (i.e., shift of antisymmetric PO_2^- band to lower frequency). The ν_{max} value further decreased with increasing the Chol content to 47.0 mol% (Table 1). Physicochemical studies on PC-Chol mixtures shows phase change and phase separation around a Chol mol% of 25–30 [31,32]. The heterogeneity of bilayer surface is probably responsible for the return of kinetic parameters observed in the previous paper [16]. On the other hand, ^{31}P-NMR chemical shift anisotropy measurements have indicated that the phosphate region of the polar headgroup in PC bilayers is slightly perturbed by the incorporation of Toc (< 30 mol%) [33]. ^{13}C-NMR studies show that the spin-lattice relaxation time for the choline moiety increases by incorporation of Toc into PC bilayers [34]. In phosphatidylcholine bilayers the P^--N^+ dipoles align parallel to the plane of membrane surface and the unesterified phosphate oxygens form hydrogen bonds with adjacent molecules through water molecules [35]. Addition of Toc into PC bilayers may perturb the headgroup structure and enhance hydration of the lipid headgroup.

We have shown that addition of DAG, Chol and Toc into PC-SUV increases the V_{max}(app) value of PLD [16,17]. The increase in V_{max}(app) value was correlated with the $\Delta\nu_{max}$ which was caused by an increasing hydration of PO_2^- group of PC (Fig. 4). The increasing hydration implies the increased space around the P-O bond and the enhanced accessibility of the catalytic site of PLD to the susceptible bond by incorporating neutral lipids into PC-SUV. The PLD activity in HL60 cells is enhanced by a permeable diacylglycerol [9]. 1,2-Diacylglycerol added into SUV was as high as 18.3 mol%. Coorssen and Rand shows that Chol reduces the amount of DAG required to perturb the bilayer structure of PC [36]. Further work on the effects of DAG in Chol-containing PC-bilayers is required in connection with PLD activation in biological membrane.

In LPC micelle the hydrolysis by PLD was about 12-fold (V_{max}(app): 37.4 ± 7.0 μM/min) larger than that in PC-SUV. The ν_{max}, 1220.6 cm^{-1} was consistent with a frequency which characterizes a fully hydrated PO_2^- group [28]. LPC micelles have larger curvature than that of SUV. NMR studies have shown that LPC headgroup moves much more freely than that of a diacyl phospholipid (diacyl-PC) [37]. The majority of this increase in motion is attributed to rapid spinning around the *sn*-1 to *sn*-2 carbon-carbon bond of the glycerol backbone which occurs only in the LPC and not in the diacyl-PC molecules [38]. In addition LPC molecule probably has a larger headgroup area at the surface due to the additional hydroxy group [38]. These factors in LPC micelles lead to the extensive hydration and higher susceptibility to PLD.

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