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ORIGINAL CONTRIBUTION

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Product-retardation and -activation of catalytic hydrolysis by phospholipase D in small unilamellar vesicles of egg yolk phosphatidylcholine

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Abstract We evaluated the hydrolysis of egg yolk phosphatidylcholine (PC) by phospholipase D from *Streptomyces chromofuscus* (PLD) in small unilamellar vesicles (SUV) in presence of 50 μM Ca^{2+}. After initial choline production (hydrolysis of 1.5% of the PC at the outer leaflets of the vesicle bilayers), the hydrolysis was reduced to 5% of the initial velocity. The kinetic behavior in SUV of premixed PC and a low percentage of the hydrolysis product, phosphatidic acid (PA), was similar to that of PC SUV. The reduced velocity disappeared when the membrane structure was disintegrated by means of a nonionic surfactant. In the retardation phase, the partially hydrolyzed vesicles (postsubstrates) had much higher affinity for PLD than fresh PC SUV. These results indicated that small clusters of the product, PA, at the vesicle surface were responsible for the reduced velocity of hydrolysis.

The initial velocity increased in a biphasic manner with the substrate concentration. At a PC concentration range up to 4 mM, the experimental data fit Michaelis–Menten kinetics. At concentrations above 6 mM, the velocity again markedly increased. Negatively charged mixed vesicles of PC and PA did not have such kinetics. Furthermore, adding PC SUV to the postsubstrates, where the fraction of free PLD was less than 0.05, induced steep choline production. These results showed that PLD bound to vesicles had higher activity than free PLD. We speculated that PLD bound to vesicles collided with and was directly transferred to PC SUV when the fraction of free PLD in aqueous medium was very small.

Key words Phospholipase D – egg yolk phosphatidylcholine – small unilamellar vesicles – product-retardation

Introduction

Phospholipase D (E.C. 3.1.4.4.) from *Streptomyces chromofuscus* (PLD) has been purified from the culture supernatant, suggesting that it is soluble [1]. Detailed characteristics of the enzyme remain to be clarified. The enzyme mimics some cellular reactions. For example, exogenous PLD has a similar activity to endogenous phospholipase D in ovarian granulosa cell culture [2]. Exogenous PLD added to the medium of vascular smooth muscle induces marked DNA synthesis with the formation of choline and phosphatidic acid [3]. PLD also substitutes for serum, which is required for the invasion of cultured mesothelial cell monolayers by MM1 and OC10 cells [4]. Various physical and chemical properties of the membrane, such as lipid packing [5, 6], lateral lipid distribution

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and phase separation [7–9] influence phospholipase activities. For example, product clusters produced in PC SUV complicates the time course of phospholipase A_2 hydrolysis [10, 11].

We have found that (1) PLD hydrolyzes PC SUV in a Ca^{2+}-dependent manner. Both the apparent maximum velocity and the reciprocal of the apparent Michaelis constant increase with the Ca^{2+} concentration [12], and that (2) incorporation of neutral lipids such as diacylglycerol, into PC SUV, enhances the catalytic activity [13, 14]. In this study, we investigated (a) the retardation of PLD action by the hydrolysis product, PA, at the PC SUV surface and the formation of postsubstrates, as well as (b) the effects of negatively charged PA SUV and postsubstrates on the enzymatic hydrolysis. Both PA SUV and postsubstrates had higher affinity for PLD than substrate vesicles. These results are discussed in terms of the membrane structure and the distribution and binding mechanism of PLD to membranes.

Materials and methods

Materials

Egg yolk phosphatidylcholine (PC) was provided by Asahi Kasei (Tokyo). The purity (over 99%) was determined by thin-layer chromatography (TLC). Egg yolk phosphatidic acid (PA) and egg yolk lysophosphatidylcholine (LPC) obtained from Sigma (St. Louis, MO) were resolved as a single spot on TLC, respectively. Heptaethyleneglycol dodecylether (HED), a nonionic surfactant, was obtained from Nikko chemicals (Tokyo; purity above 99%). All other chemicals from Wako Pure Chemicals. (Osaka) were of special grade. Buffer was prepared with water distilled twice from a glass still. PLD obtained form Sigma was as described in Refs. [12–14].

Preparation of vesicles

Egg yolk phosphatidylcholine (PC), egg yolk phosphatidic acid (PA) and mixtures of both were dissolved in chloroform. The solvent was evaporated under reduced pressure and the residual film was dried *in vacuo* overnight to ensure complete solvent removal. The lipid film was hydrated with a buffer which contained 10 mM Tris, 150 mM NaCl and 50 μM Ca^{2+} (pH 8.0) unless otherwise noted. The lipid suspension was dispersed by vortex and sonication for 30 min under a nitrogen stream at 5 °C using a UD-200 probe-type sonicator (Tomy Seiko, Tokyo) as described in Refs. [12–14].

Hydrolysis reaction of SUV by PLD

PLD catalyzes the hydrolysis of PC in SUV with the formation of PA and choline. The choline concentration was monitored using a choline electrode [15, 16]. The initial velocity was determined from the slope of the choline production within 5 min. The principle of the choline assay has been described in detail [13, 14]. The dissociation of PLD SUV complexes was separately estimated by an ultrafiltration method as before [12].

Results

Retardation of choline production

Figure 1 shows the time courses of choline production in 1 and 10 mM PC SUV in 150 mM NaCl, 50 μM Ca^{2+}, 10 mM Tris-HCl at PH 8.0. The enzymatic hydrolysis rate of PC declined progressively and was 5–6% of the initial value after a reaction time of 30 min. The reaction velocity was retarded after the conversion of about 1% of PC SUV (corresponding to about 1.5% of PC at the outer leaflets of SUV) to PA, irrespective of the SUV and Ca^{2+} concentrations. Adding more Ca^{2+} ($\sim$ 0.3 mM) or choline did not significantly influence the retardation (data not shown). The initial high velocity was not recovered by adding Ca^{2+} after it was eliminated from the reaction mixture (45 min incubation with 100 μM EDTA in a reaction mixture in the retardation phase, followed by adding 150 μM Ca^{2+} to reactivate PLD). These results showed that after the rapid conversion of a specific amount of PC to PA, PC SUV did not serve as an effective substrate and became to "postsubstrate". The susceptibility to PLD was recovered only when it was solubilized by an excess of the nonionic surfactant, HED (data not shown).

Adding fresh substrates (PC SUV) to the reaction mixture during the retardation phase (postsubstrate) induced abrupt choline production, also following the retardation phase after the conversion of about 1.5% PC at outer leaflets of the fresh SUV (Fig. 1). The enzyme itself was highly active only for fresh substrates and the hydrolysis product, PA of about 1.5 mol%, was responsible for the diminished activity of the enzyme. SUV of mixed PC and PA (molar ratio: 9.5/0.5) were examined (Fig. 2).The homogeneously dispersed PA in SUV [23] did not affect the time course of choline production. This figure also showed that PC molecules solubilized in nonionic micelles, HED micelles (PC:HED = 1:10), were hydrolyzed about 4 times faster than those in SUV. Furthermore, the enzymatic reaction in the micelles was not retarded. These observations indicated that the organized structures formed by the hydrolysis product, PA, at the

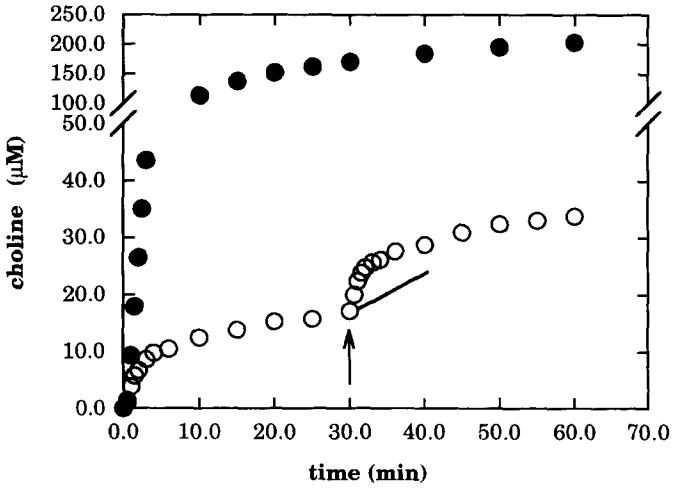

Fig. 1 Reaction curves for the hydrolysis of PC SUV by PLD pH 8.0 and 30 °C. Concentration of PC SUV: open circles, 1 mM; closed circles, 10 mM. The arrow shows the addition of fresh substrate (1 mM PC SUV) into the reaction mixture. The solid line was evaluated on the assumption that PLD competitively binds to fresh substrates and postsubstrates

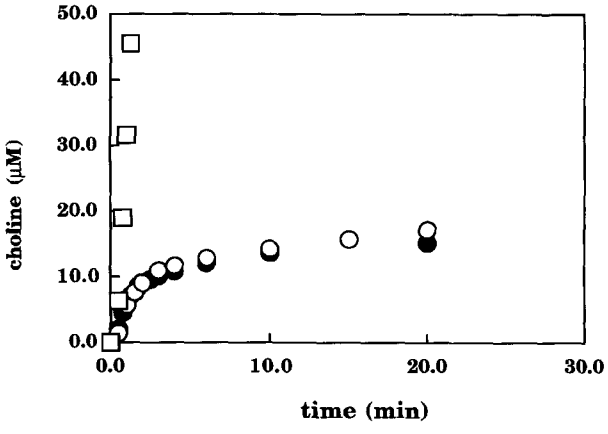

Fig. 2 Reaction progress curves of PC hydrolysis by PLD for various substrate preparation. Open circles, 1 mM PC SUV; closed circles, PC/PA mixed SUV (0.95 and 0.05 mM, respectively); open squares, solubilized PC in micelles (PC:HED = 1 mM:10 mM)

surface of substrate vesicle participate in the retardation phase.

The dissociation coefficient of the PLD–PC SUV complex was estimated by means of ultrafiltration as a function of reaction time. Table 1 shows that at the initial stage of high PLD activity (~5 min), the coefficient was 0.64 mM, and that the affinity of PC SUV to PLD increased remarkably at the retardation phase (postsubstrates) as shown by the value of 0.17 mM. Table 1 also shows that the dissociation coefficient for mixed SUV of PC and PA lowered rapidly around a PA molar fraction of 0.7, indicating that

Table 1 Dissociation coefficient of PLD to PC/PA-SUV at 30 °C

PC/PA (mole ratio) in SUV	Dissociation coefficient [mM]	
	< 5 min	> 60 min
10/0	0.64 ± 0.1	0.17 ± 0.05
5/5	0.53 ± 0.05	—
3/7	0.26 ± 0.03	—
2/8	0.20 ± 0.03	—
1/9	0.21 ± 0.02	—
0/10	0.20 ± 0.03	0.13 ± 0.02

Values are represented as means ± S.D. of two or three independent experiments.

closely neighboring PA molecules at the vesicle surface of postsubstrates are concerned with the high affinity for PLD and the retardation of hydrolysis.

Figure 1 shows about 1.5% conversion of PC to PA after 30 min irrespective PC SUV concentration (the PLD concentration was about 40 nM, whereas the vesicle (particle) concentration was 300–3000 nM). These facts suggest that the enzyme was exchanged among substrate particles and that each particle was similarly hydrolyzed.

Biphasic enzymatic reaction

The substrate concentration (S)–initial velocity (v) profiles for PC SUV and PC/PA (5/5 molar ratio) SUV are presented in Fig. 3. The enzymatic reaction velocity for PC SUV increased in a biphasic manner. At a PC concentration range up to 4 mM, the experimental data fit Michaelis–Menten kinetics and gave an apparent maximum velocity, V max(app), and an apparent Michaelis constant, Km(app) of 8.2 μM/min and 0.67 mM, respectively (Table 2). The latter value (0.67 mM) agreed with the dissociation coefficient obtained by ultrafiltration (0.64 mM). When the PC concentration was above 6 mM, the velocity again increased markedly, where the fraction of free PLD in the medium was less than 0.1, indicating further activation of PLD at the PC SUV surface. This type of increase in the enzymatic reaction was not observed in the negatively charged mixed SUV of PC and PA (5/5) (Fig. 3). The value of Km(app) for the mixed SUV (0.56 mM) agreed with the dissociation coefficient obtained by ultrafiltration (0.53 mM).

Competitive binding of PLD to vesicles

We examined the competitive binding effects of postsubstrates and PA SUV on the enzymatic hydrolysis of PC and PC/PA mixed SUV, respectively. PA SUV and postsubstrates had high affinity for PLD, with a dissociation

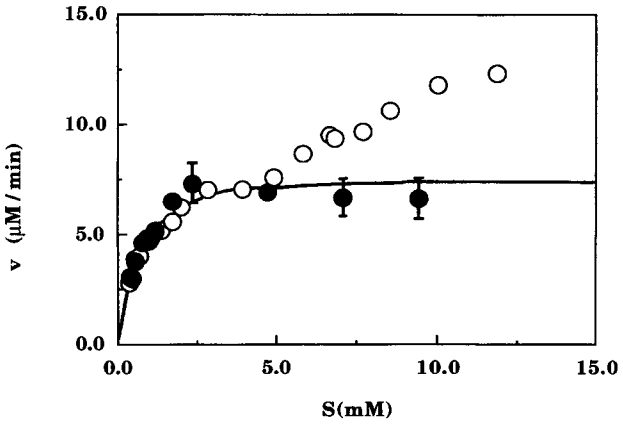

Fig. 3 Dependence of initial velocity (< 5 min) of hydrolysis on the substrate concentration. Open circles, PC SUV; closed circles, PC/PA (5/5) SUV. Solid curve represents the calculated value with the kinetic parameters, V max(app) and Km(app), obtained for PC SUV in the lower concentration region ($S < 4$ mM)

constant of 0.13–0.17 mM. Figure 4A shows the S–v profiles of the hydrolysis of negatively charged SUV (PC/PA = 5/5) by PLD in the presence and absence of 2 mM PA SUV. The presence of PA SUV in the reaction mixture, markedly decreased the hydrolysis velocity. The double reciprocal ($1/S$–$1/v$) plots presented in Fig. 4B gave similar V max(app) values with and without 2 mM PA SUV (Table 2).

For the competitive binding of PLD to the substrate and PA vesicles, the initial velocity is presented as

$$v = \frac{V\,\mathrm{max(app)} \cdot S}{(1 + I/K_I)\mathrm{Km(app)} + S}. \quad (1)$$

Here, the parameters, V max(app) and Km(app) were obtained from the kinetic analysis without PA SUV, and the dissociation coefficient of PLD and PA SUV, K_I, was obtained by the binding experiment. I is the concentration of PA SUV. The solid curve in Fig. 4A represents the v values calculated by Eq. (1) for hydrolysis of the mixed SUV in the presence of PA SUV. The experimental and calculated values for V max(app) and $(1 + I/K_I)$Km(app) are compared in Table 2. The agreement between the experimental and calculated values suggested that the enzyme competitively distributes between the substrate SUV and PA SUV throughout the aqueous phase.

When PC/PA mixed SUV were replaced by PC SUV as substrates, PA vesicles did not function as effective competitive inhibitors, giving a severalfold higher PLD activity than that predicted by Eq. (1) (data not shown).

As shown in Fig. 1, adding fresh substrates into the reaction mixture at the retardation phase (postsubstrates) induced abrupt choline production. The velocity was much higher than that calculated by Eq. (1) for the simple competitive binding of PLD to postsubstrates and fresh substrates (the solid line in Fig. 1). In Fig. 5 the effects of postsubstrates on the enzymatic reaction of PC SUV are presented. Incubating PC SUV with PLD for 1 h converted the SUV to postsubstrates with high binding affinity to the enzyme but they did not work as an effective substrates as seen in Figs. 1 and 2. Various amounts of fresh substrates (PC SUV) were added to the incubation mixture and the choline production was monitored. The v values calculated by Eq. (1) for the simple competitive inhibition model (solid line) were much lower than those observed (Fig. 5A). Despite the high affinity of postsubstrates to PLD, they did not competitively inhibit, but rather enhanced the enzymatic activity above the substrate concentration of 2 mM, where the fraction of free PLD in the medium was less than 0.05 of the total PLD. The double reciprocal plot, being linear, gave a V max(app) value of 24.8 μM/min, which was 3 times larger than that (8.2 μM/min) for the fresh substrates without postsubstrates (Fig. 5B). The observed value of Km(app) was 6.0 mM, which agreed with the calculated value 8.6 mM within the experimental error (Table 2). These results

Table 2 Kinetic parameters obtained for the hydrolysis of PC SUV and PA/PC SUV by PLD

Substrate	Additive	V max(app) [μM/min] Observed	Calculated	Km(app) [mM] Observed	Calculated[c]
PC SUV		8.2 ± 1.0[b]	—	0.67 ± 0.13[b]	—
	2mM PC SUV[a]	24.8 ± 7.6	8.2 ± 1.0	6.0 ± 2.0	8.6 ± 3.0
PA/PC SUV		7.6 ± 1.0	—	0.56 ± 0.10	—
	2mM PA SUV[d]	7.3 ± 0.58	7.6 ± 1.0	12 ± 4.4	9.2 ± 3.7

[a] PC SUV was incubated with PLD for 1 h (postsubstrates) and added to fresh PC SUV (substrate).
[b] PC SUV ≤ 4 mM.
[c] $(1 + I/K_I)$Km(app) (see Eq. (1)).
[d] PA SUV was incubated with PLD for 1 h and added to fresh PA/PC SUV (the final concentration of PA SUV, 2 mM; the PLD concentration was identical with that of reaction mixtures without PA SUV).

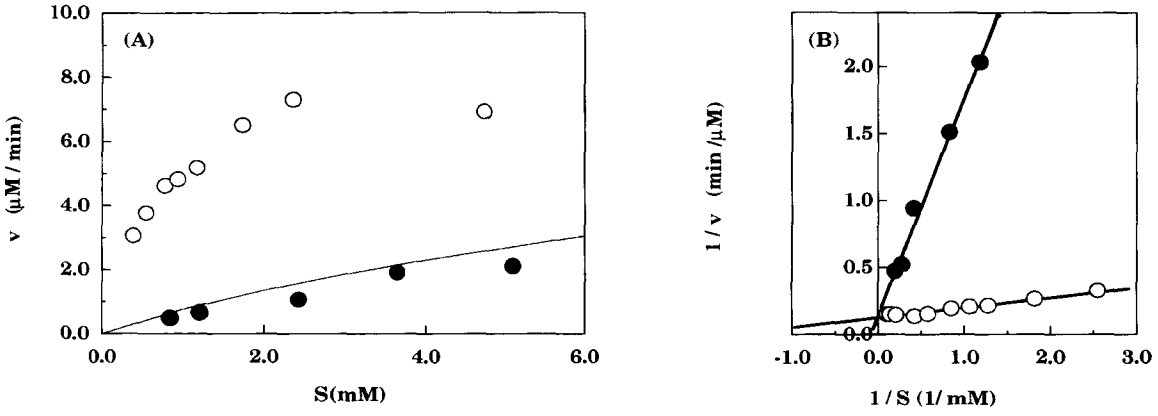

Fig. 4 Dependence of initial velocity (< 5 min) of PC-hydrolysis on the vesicle concentration (**A**) and the Lineweaver Burk plots (**B**). The substrate was PC/PA (5/5) SUV and the reaction was initiated by adding PLD (open circles) or the enzyme pre-equilibrated with PA SUV for 1 h (final concentration of PA SUV was 2 mM; closed circles). The linear plots (**B**) gave an apparent maximal velocity (*V* max(app)), and an apparent Michaelis constant (Km(app)) as shown in Table 2. Solid curve in the figure (**A**) was calculated using Eq. (1)

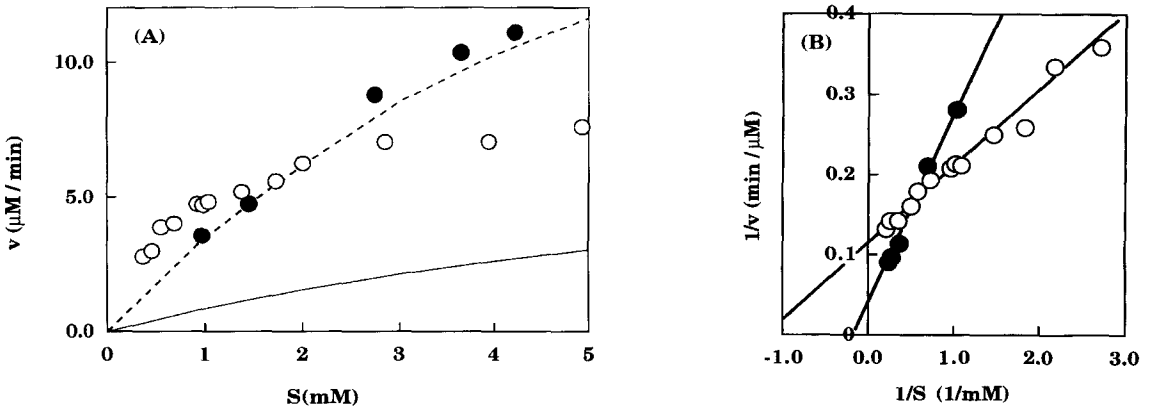

Fig. 5 Dependence of the initial velocity (< 5 min) of PC-hydrolysis on the vesicle concentration (**A**) and a Lineweaver Burk plot (**B**). The substrate was PC SUV and the reaction was initiated by adding PLD (open circles) or the enzyme equilibrated with PC SUV for 1 h beforehand (postsubstrates; closed circles). The final concentration of postsubstrates was 2 mM in the reaction mixture. The linear plots (**B**) gave the kinetic parameters shown in Table 2. The solid curve in the figure (**A**) was calculated using Eq. (1) for the competitive inhibition with the kinetic parameters, *V* max(app) and Km(app), obtained from the Lineweaver Burk plots in the lower concentration region of PC SUV ($S < 4$ mM). The broken curve in the figure (**A**) was calculated for the enhanced activity of PLD transferred from the postsubstrate to PC SUV with the kinetic parameters obtained from the double reciprocal plot in the figure (**B**)

suggested that the postsubstrates brought about an increase in the PLD activity for fresh PC SUV.

Discussion

Product-retardation of enzymatic hydrolysis

Bound PLD molecules to PC SUV diffuse laterally at the surface and hydrolyze closely neighboring PC molecules to form small clusters of PA. The results shown in Table 1 suggest that the cluster at the vesicle surface contains a high molar fraction of PA (> 0.7) and preferentially entraps PLD molecules. According to Gouy's theory, if the binding of PLD to SUV, is caused by electrostatic interaction, it would be roughly proportional to the surface charge density [18]. The variation of the dissociation coefficient with the PA fraction suggested that the interaction was not simply electrostatic, but rather specific. The enzyme entrapped at the PA cluster on vesicles is inactive to substrate molecules on the same vesicles because of a depression of the lateral diffusion.

The formation of PA clusters and the retarded hydrolysis seem to depend upon the bilayer-organization of

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PC molecules. Adding neutral lipids, diacylglyceride or α-tocopherol into PC SUV relaxed the product retardation [12, 13]. Neutral lipids intercalated between regularly arrayed phospholipid molecules at the vesicle surface interfered with the cluster formation of PA molecules produced by the PLD hydrolysis. Surfactant (HED) molecules added to postsubstrate vesicles disintegrated the bilayer structure and eliminated the retardation of hydrolysis.

Interactions between PLD and closely neighboring PA molecules may be responsible for retaining the cluster at the SUV surface. In this study, however, the number of PLD molecules in the reaction mixture was too small to interact with all SUV particles simultaneously. The elimination of Ca^{2+} by EDTA in postsubstrates did not recover the susceptibility to PLD. Therefore, neither PLD nor Ca^{2+} directly participated in the cluster formation. PA analogs, such as alkylphosphates, form acid–soap clusters at pH 8.0 and also associate into separated patches from other lipid components in monolayers by hydrogen bonding [19–22]. Such interactions are likely to lead to the local clustering of nascent PA molecules at the SUV surface. The formation of local-patches enriched PA in membrane has been suggested by Liscovitch and Cantley in a description of PLD activation by a low molecular weight GTP-binding protein ADP-ribosylation factor (ARF) [23]. Products of phospholipase A_2 (fatty acid) also reportedly separate from the substrate, PC, and form clusters in vesicles [9]. Burack et al. have described that only 3% of the substrate is hydrolyzed in large unilamellar vesicles composed of dipalmytoylphosphatidylcholine and that the product clusters entrap the enzyme [11].

Competitive inhibitor and postsubstrates

Negatively charged PA SUV competitively inhibited the PLD-hydrolysis of PA/PC SUV (Fig. 4). In this reaction, PLD is transferred between the inhibitor (PA SUV) and substrate vesicles (PA/PC SUV) solely through the aqueous medium even at a high concentration of the latter, because collision between them is limited by electrostatic repulsion.

On the other hand, postsubstrates, despite a similar high affinity for PLD to PA SUV did not competitively inhibit the hydrolysis of PC-SUV (Fig. 5), but rather enhanced the enzymatic activity. The enzyme is entrapped at the PA cluster on postsubstrates, but itself has catalytic activity toward fresh PC SUV. Postsubstrates and the substrate vesicles can mutually collide because the electrostatic repulsion is not strong. That is, postsubstrates function as enzyme-donors by collision with the substrate vesicles. A similarly enhanced activity of PLD was also observed for the combination of PC SUV and PA SUV as substrates and competitive inhibitors, respectively. Here, the collision between the substrates and inhibitors was not completely suppressed as it was between PA/PC SUV and PA SUV.

The enzyme on postsubstrates had higher catalytic activity V max(app) = 24.2 μM/min) than that which was transferred through aqueous medium (V max(app) = 8.2 μM/min). When PLD is exchanged among vesicles through aqueous medium, conformational changes of the protein at the vesicle surface would appreciably affect restabilization. The lag in enzymatic reaction owing to the conformational rearrangement could be significant during residence at the SUV surface. The enzyme exchange between vesicles through the aqueous phase would involve an on- and off-step in each cycle, resulting in reduced catalytic activity. Jain et al. [24] have suggested that similar on- and off-steps play a critical role in the distinct interfacial activation between phospholipase A_2 and prophospholipase A_2. On the other hand, the enzyme on postsubstrates encounters substrate vesicles primarily by collision and the conformational rearrangement for the activation at vesicle surface is small, giving higher V max(app) and Km(app) values than those of PLD from the aqueous phase (Table 2).

Biphasic hydrolysis

The enzymatic hydrolysis of PC SUV depended on the substrate concentration in a biphasic manner. At low concentrations ($S < 4$ mM), the kinetic data at the initial stage were consistent with those predicted by the Michaelis–Menten equation. The apparent Michaelis constant agreed with the initial dissociation coefficient of the PLD SUV complex, demonstrating that the distribution of PLD between the SUV surface and aqueous medium played a critical role.

The initial velocity again increased at the higher concentration of PC SUV ($S > 6$ mM), where the fraction of free PLD in medium was small. In contrast with PC SUV, the negatively charged SUV did not undergo an anomalous biphasic reaction, but rather underwent hydrolysis according to Michaelis–Menten kinetics even at the higher concentration. Here, the electrostatic repulsive force suppressed the collision between vesicles. On the contrary, the weak repulsive force allows collisions between PC SUV. These results indicated that the hydrolysis of SUV by PLD proceeds by means of two processes: (1) the enzyme encounters substrate vesicles through aqueous medium, (2) the enzyme is exchanged between substrate vesicles by their collision. At lower concentrations of PC SUV, PLD desorbs from the vesicle surface before colliding with other vesicles. At higher concentrations of PC SUV, the second

process becomes more dominant. Further studies are required on the PA-cluster formation by PLD-reaction at PC SUV surface and on the detailed kinetic mechanism to clarify the effects of a high concentration of PC SUV and of postsubstrates.

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