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ACTIVATION OF PHOSPHOLIPASE A_2 BY FRESHLY ADDED LYSOPHOSPHOLIPIDS

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Reaction progress curves for the hydrolysis of dimyristoylphosphatidylcholine by pig pancreatic phospholipase A_2 exhibits a latency phase. Addition of l-palmitoyllysophosphatidylcholine to the preformed vesicles reduces the latency phase and enhances the binding of phospholipase A_2 to the vesicles. In contrast, the binary codispersions prepared from diacylphospholipids premixed with lysophosphatidylcholine do not exhibit such enhanced susceptibility to the phospholipase. This effect appears to be due to organizational defects created by asymmetrical incorporation of lysophospholipid molecules into the outer monolayer of the vesicles, and the action of phospholipase is not observed when the additive is equilibrated in both the monolayers of the vesicles.

Factors governing the action of phospholipase A_2 on phospholipids in bilayers are poorly understood [1]. Incorporation of the enzyme into bilayers is thought to be the first step in the overall reaction cycle, whereas the catalytic turnover takes place without enzyme leaving the bilayer. Elsewhere we have shown that the pig pancreatic phospholipase A_2 does not bind to bilayers of ditetradecylphosphatidylcholine, and that the hydrolysis of dimyristoylphosphatidylcholine proceeds with a long latency phase, suggesting that this enzyme does not readily penetrate the bilayer interface [2]. However, the ternary codispersions containing dialkylphospholipids with lysophospholipid and fatty acid bind the enzyme, and the ternary codispersions containing diacylphospholipids as substrate are hydrolyzed readily [3]. In contrast, the binary codispersions prepared from diacylphospholipids premixed with fatty acid or lysophospholipid do not act as substrates for binding or for catalysis [2,3]. In this paper we report that lysophosphatidylcholine freshly added to preformed vesicles of diacylphosphatidylcholine facilitates incorporation of pig pancreatic phospholipase A_2 and the hydrolysis of the substrate. This effect lasts about 8 h, that is, approximately the time required for transmembrane equilibration of lysophospholipids; and the effect appears to be due to organizational defects created by asymmetrical incorporation of lysophospholipid molecules into the outer monolayer of the vesicles. This phenomenon represents yet another type of organizational perturbation by a zwitterionic additive that modulates the quality of the bilayer interface conducive to the incorporation of pig pancreatic phospholipase A_2. Such factors are important in understanding the secondary substrate specificity of phospholipase A_2.

Materials and Methods

Lysophospholipids were prepared by hydrolysis of appropriate diacylphosphatidylcholines by

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Crotalus venom phospholipase A_2, or they were purchased from Calbiochem (San Diego), Medmark (Munich), or Senn Chemicals (Basel). All these compounds were judged to be pure by thin-layer chromatography, and used without further purification. All other chemicals were best available grades.

All procedures used in this study have been described in detail elsewhere. Kinetics of phospholipase action on sonicated dimyristoylphosphatidylcholine vesicles was followed by pH-stat titration of the released protons [3]. Binding of phospholipase A_2 to ditetradecylphosphatidylcholine vesicles was followed by monitoring the tryptophan fluorescence change [2]. All experiments were performed at 25°C, pH 8.0 in 100 mM KCl, 10 mM $CaCl_2$. For binding experiments the solution was buffered with 20 mM Hepes. Specific experimental details are given in the figure captions.

Results and Discussion

The reaction progress curve for the action of pig pancreatic phospholipase A_2 on dimyristoylphosphatidylcholine vesicles exhibits a long latency phase, the duration of which depends upon all the factors that regulate the mole fraction of lysophosphatidylcholine and fatty acid in the substrate vesicles [2,3]. For example, with annealed vesicles made from pure dimyristoylphosphatidylcholine the latency period may be up to several hours depending upon the experimental conditions [3], and the latency period completely disappears when the products are preincorporated in these vesicles. Thus the duration of the latency phase is an indication of the presence of organizational defects, which are the putative sites for the binding of the pig pancreatic phospholipase A_2 on the bilayer of the vesicles. Elsewhere we have shown that lysophosphatidylcholine preincorporated into dimyristoylphosphatidylcholine vesicles does not induce such defects, and therefore the latency period is very long in this system [3]. The experiments presented here demonstrate that lysophospholipids, freshly added to the preformed substrate vesicles facilitate, incorporation and catalytic action of the pig pancreatic phospholipase A_2.

As shown in Fig. 1, upon addition of 1-palmitoyl-2-deoxylysophosphatidylcholine (35 μM) to dimyristoylphosphatidylcholine vesicles (400 μM), the latency period in the reaction progress curve decreases to less than 2 min, compared to the latency period of about 30 min in the absence of any additive to the vesicles under the otherwise identical solutions. This effect of the addition of lysophospholipid to the preformed vesicles does not depend upon the sequence of mixing of the enzyme, vesicles, and lysophospholipid. Thus, the steady-state rate of hydrolysis and the latency phase remain the same whether the lysophospholipid is added simultaneously with the enzyme or several minutes before or after the addition of the enzyme. These observations suggest that the rate of mixing of lysophospholipid to the bilayers is fast. This is also demonstrated by the direct binding experiments. For example, on mixing pig pancreatic phospholipase A_2 with ditetradecylphosphatidylcholine vesicles, there is a slight (about 3%) increase in intrinsic tryptophan fluorescence intensity, suggesting that the enzyme does not bind significantly to these vesicles [2]. However as shown in Fig. 2, upon addition of lysophosphatidylcholine the fluorescence intensity increases rapidly. While the increase in fluorescence intensity does not change significantly with

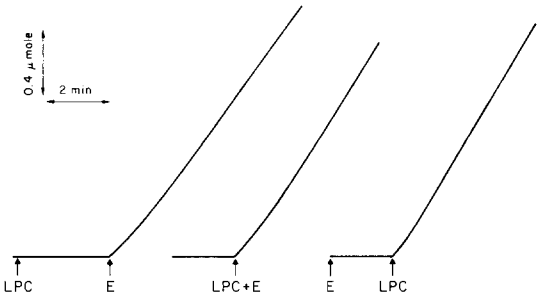

Fig. 1. Reaction progress curves for the hydrolysis of dimyristoylphsophatidylcholine vesicles (400 μM) by pig pancreatic phospholipase A_2 (E, 1 μg) in the presence of 1-palmitoyl-2-deoxylysophosphatidylcholine (LPC, 24 μM). The reaction mixture (5 ml) contained 100 mM KCl and 10 mM $CaCl_2$ at pH 8 and 25°C. The latency period is measured by extrapolating the steady-state portion of the reaction progress curve. In these curves the latency period is about 1 min. The ordinate is given in arbitrary units, however, the specific activity of the enzyme under these conditions at steady-state is about 350 IU.

the mole fraction of the additive at constant ditetradecylphosphatidylcholine concentration (Fig. 2), it changes substantially with the concentration of the diether vesicles containing a constant mole fraction of lysophosphatidylcholine (Fig. 3). These observations demonstrate that the binding of the enzyme to the vesicles containing freshly added lysophosphatidylcholine is rapid (halftime < 5 min, depending upon the mole fraction of added lysophosphatidylcholine). Also, the fraction of the enzyme bound to the vesicles at equilibrium does not change significantly with the mole fraction of lysophospholipids in the presence of excess ditetradecylphosphatidylcholine vesicles. However, as shown in Fig. 3, the fraction of the enzyme bound to the vesicles changes with the increasing concentration of the vesicles containing a constant mole fraction of lysophospholipid to ditetradecylphosphatidylcholine. By curve fitting the hyperbolic binding isotherm [2] shown in Fig. 3, we can calculate the dissociation constant for the protein lipid complex, K_d (0.4 μM), and the lipid/protein stoichiometry in the complex, n (= 112). The covariance was < 0.7 and standard deviation $\pm$22% [2]. The apparent dissociation constant for the complex would be $n \cdot K_d$ (= 50 μM), and it should

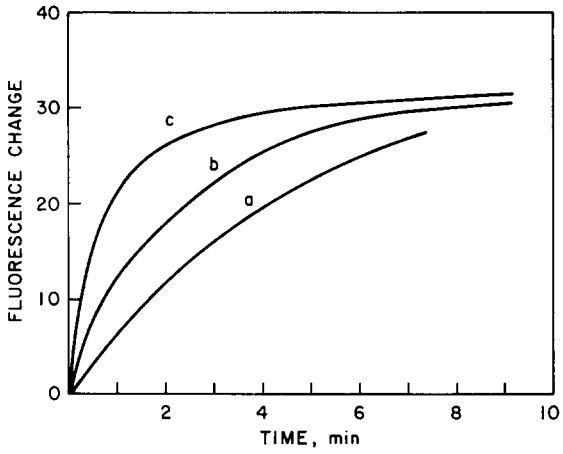

Fig. 2. The change in the intrinsic tryptophan fluoresence intensity of a mixture of pig pancreatic phospholipase A_2 (5 μM) and ditetradecylphosphatidylcholine vesicles (1.0 mM) upon addition of (a) 40 μM, (b) 120 μM, and (c) 240 μM 1-palmitoyl-2-deoxylysophosphatidylcholine. The mixture (2.5 ml) contained 100 mM KCl, 10 mM $CaCl_2$, 20 mM Hepes, pH 8.0, at 25°C. Excitation at 280 nm, emission at 328 nm. Fluorescence intensity is given in arbitrary units.

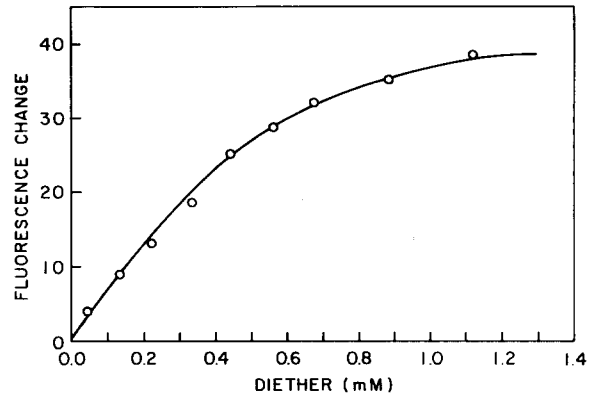

Fig. 3. The change in fluorescence intensity as a function of ditetradecylphosphatidylcholine concentration at constant mole fraction of 2-deoxylysophosphatidylcholine (0.12). Conditions are given in the legend of Fig. 2. The isotherm was curve fitted to compute values of K_d and n as outlined elsewhere [2].

be comparable to the Michaelis-Menten constant (K_m), if the interfacial K_m * and k_{cat} are significantly smaller. As shown below, the apparent dissociation constant for the complex is indeed comparable to the kinetic constant, apparent K_m (200 μM), thus suggesting that k_{cat} is small.

Elmahrani and Blume [4] have shown that the halftime for the mixing of lysophospholipids with vesicles is less than 15 s. Thus following the incorporation of lysophospholipids, incorporation of the enzyme in the vesicle is rapid, as is found to be the case with the ternary codispersions [2]. It should also be noted that following the addition of lysophospholipid the vesicles undergo aggregation/fusion, the halftime for which is of the order of 20 min under these conditions [4]. Since the rate of incorporation of the enzyme in the bilayer is of the order of a few seconds, it would imply that a transient state formed during the aggregation/fusion process is not involved in the enzyme binding. However, both the enzyme binding and the aggregation/fusion processes probably depend on a common organizational feature that is induced in the bilayer of the vesicles by freshly added lysophosphatidylcholine.

Binary codispersions formed from premixed lysophospholipid and diacylphosphatidylcholine are not susceptible to the action of the phospholipase nor do they undergo aggregation/fusion. This

suggests that the action of freshly added lysophospholipid is transient. Indeed, as shown in Fig. 4, the latency period increases if the mixture of freshly added lysophospholipid to dimyristoylphosphatidylcholine vesicles is incubated for several hours. After, incubation of the mixture for 48 h in latency period is about 30 min, which is the same as observed with vesicles formed from the premixed components.

The kinetic and binding experiments described above show that the activating effect of freshly added lysophospholipid is due to a transient state that dissipates with a halftime of several hours. The rate of mixing of lysophospholipid with vesicles is much faster [4], and the rate of lateral diffusion of lysophosphatidylcholine in the vesicles is also expected to be rapid. Therefore, it appears that immediately after mixing, lysophosphatidylcholine is incorporated in the outer monolayer of the vesicles, and the equilibration of lysophosphatidylcholine between the inner and outer monolayers of the vesicle (flip-flop) is the slow step with the halftime of several hours. Direct experiments to demonstrate this are contemplated, however, the slow rate of dissipation of the transient state (cf. Fig. 4) is consistent with the observed slow rates for flip-flop of phospholipids [5] and lysophospholipids [6,7] in bilayers. This mechanism would imply that the asymmetric distribution of lysophosphospholipid molecules in vesicles creates organizational defects that act as sites for incorporation of the enzyme in the bilayer. The alternative possibility is that the slow dissipation of the transient state conduvice to the binding of the enzyme to the vesicles is due to a decrease in the surface area of the vesicles due to fusion. This can be ruled out because the rate of fusion (halftime < 20 min) is much faster than the rate of dissipation of the transient state (halftime 8 h). Other mechanisms invoking an enhanced rate of exchange of diacylphospholipid between the bilayer and the enzyme in the aqueous phase were not taken into consideration on the basis of the plausible mechanisms for the interfacial activation of phospholipase A_2 [1].

The activating effect of lysophosphatidylcholine depends upon its concentration. As shown in Fig. 5, the latency period decreases and the steady-state rate remains constant (not shown, however, see

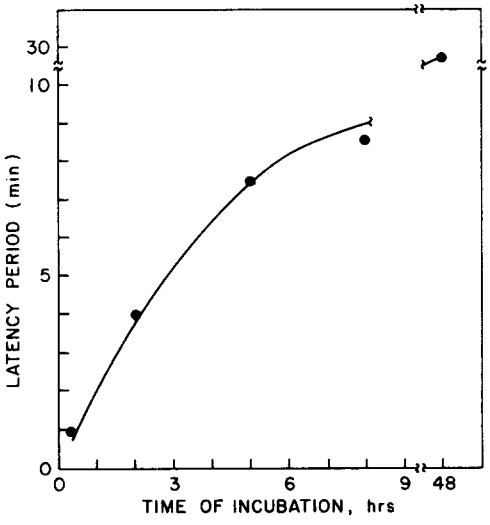

Fig. 4. Dependency of latency period for the hydrolysis by phospholipase A_2 upon the time of incubation of dimyristoylphosphatidylcholine (66 mM) with deoxylysophosphatidylcholine (4 mM). After an appropriate incubation period, the mixture was diluted (160-fold) with the salt solution and the reaction was initiated by adding phospholipase A_2 (1 μg). The final concentrations and the conditions for the reaction were the same as in the legend of Fig. 1.

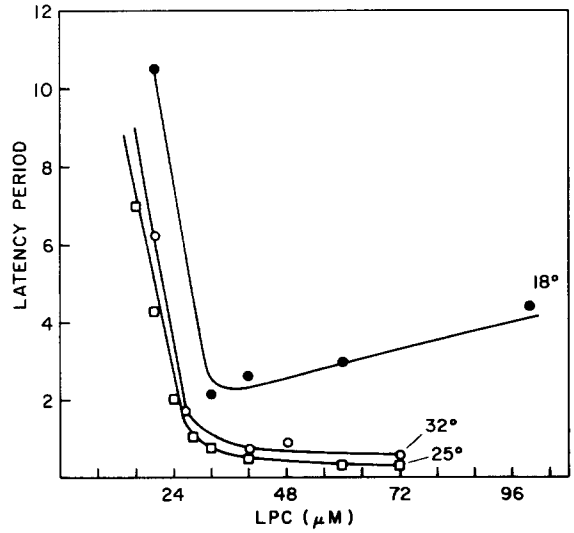

Fig. 5. Dependence of the latency period upon deoxylysophosphatidylcholine at constant dimyristoylphosphatidylcholine concentration (400 μM) at 18, 25, and 32°C. Other conditions as given in the legend of Fig. 1. The steady-state rates (not shown) changed only slightly as a function of lysophosphatidylcholine concentration.

Fig. 2) with increasing concentration of lysophosphatidylcholine at constant dimyristoylphosphatidylcholine vesicle concentration. Also, as shown in Fig. 6, increasing concentration of lysophosphatidylcholine at constant mole ratio (0.07) to dimyristoylphosphatidylcholine, does not change the latency period noticeably. This observation rules out the possibility that the effect of freshly added lysophosphatidylcholine is due to the detergent-like solubilizing effect on the vesicles. Indeed, this conclusion is also consistent with the observation that up to 0.5 mole fraction of lysophosphatidylcholine does not disrupt the bilayer organization [8,9]. As expected the steady-state rate of hydrolysis increases with increasing concentration of vesicles. This is due to an increase in the fraction of the bound enzyme. The apparent K_m calculated from this data is about 200 μM, which is within a factor of 4 to the apparent K_d value measured for the ditetradecylphosphatidylcholine system (cf. Fig. 3), as well as, the K_m and K_d values measured for the ternary codispersions [2,3].

The transient effect of the freshly added lysophosphatidylcholine depends upon the phase properties of the bilayer. As shown in Fig. 5 the dependence of the latency period on the concentration of lyosphosphatidylcholine is a function of temperature. Below the phase transition temperature the latency period reaches its minimum at higher mole fraction than it does above the phase transition temperature. This data further suggests that the activating effect is not directly related to the presence of coexisting gel and liquid-crystalline phases in the bilayer. By differential scanning calorimetry it can be shown [10] that dimyristoylphosphatidylcholine bilayers containing 5 to 10 mol% premixed lysophospholipid have the transition range of 21 to 26°C. However, the data shown in Fig. 5 demonstrates that the activating effect is seen both below and above this temperature range.

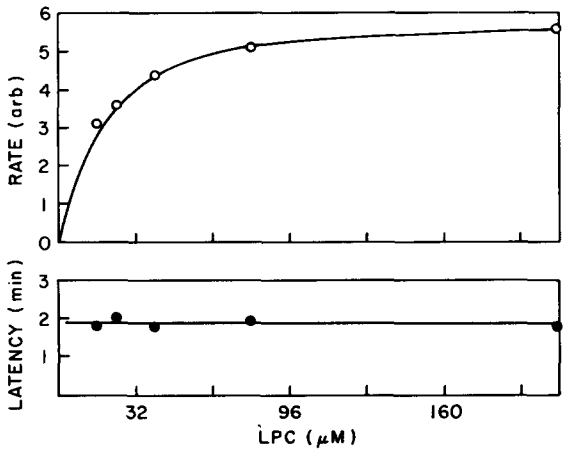

Fig. 6. Dependence of the latency period (filled circles) and the steady-state rate of hydrolysis (open circles) upon lysophosphatidylcholine concentration at constant mole fraction with dimyristoylphosphatidylcholine vesicles. Mole fraction of lysophosphatidylcholine was kept constant at 0.07.

Most of the experiments reported here are done with deoxylysophosphatidylcholine because it is more stable and thus it does not create complications due to the presence of fatty acid impurity. However, as summarized in Table I several other lysophospholipids exhibit the activating effect similar to that observed with 1-palmitoyl-2-deoxylysophosphatidylcholine. While qualitatively the same effect is exhibited by these structurally different analogs, significant quantitative differences may be noted. No obvious structure-activity correlation or a correlation to their critical micelle concentrations can be drawn from this data. It

TABLE I

THE MOLE FRACTIONS OF LYSOPHOSPHOLIPIDS IN DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES REQUIRED FOR LOWERING THE LATENCY PERIOD TO 3 MIN AT 25°C.

LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine.

Analog	Mole fraction
1-Lauroyl-LPC	0.12
1-Myristoyl-LPC	0.055
1-Palmimitoyl-LPC	0.065
1-Stearoyl-LPC	0.075
1-Elaedoyl-LPC	0.12
1-Hexadecyl-LPC	0.035
1-Palmitoyl-2-deoxy-LPC	0.05
1-Hexadecyl-2-methyl-PC	0.035
1-Hexadecyl-2-acetyl-PC	0.04
Hexadecylphosphorylcholine	0.04
Tetradecylphosphorylcholine	0.07
Octadecylphosphorylcholine	0.16
Palmitoylglycolphosphorylcholine	0.09
1-Hexadecyl-LPE	0.20

should, however, be noted that the analog 1-hexadecyl-2-methylphosphatidylcholine is effective at much lower mole fraction in the bilayer than 1-hexadecyllysophosphatidylethanolamine. The origin of this difference between the effects of the various lysophospholipid analogs is not known, however, it could be due to differential miscibility of these analogs in the bilayer.

The observations summarized above demonstrate that there is a significant correlation between the kinetics of hydrolysis and the binding of pig pancreatic phospholipase A_2 as measured by fluorescence enhancement of the tryptophan in the protein. Enhanced binding of the enzyme induced by freshly added lysophosphatidylcholine is intriguing not only because this additive has no net charge, but also because several analogs of lysophosphatidylcholine are able to induce a similar change. Such observations suggest that these additives induce a change in the organization of the outer monolayer of the vesicles, and the resulting changes not only enhance the binding of the enzyme but they also promote aggregation/fusion of the vesicles. Similar correlation between the binding of the phospholipase and the fusion/aggregation of the vesicles is also found in the ternary codispersions. Moreover, both of these effects are also inhibited by agents like butacaine and mepacrine in both the binary and the ternary codispersions (to be published). All these observations strongly suggest that similar features of bilayer organization (defect sites) may act as a common biophysical locus for fusion/aggregation, phospholipase A_2 action, and the effects of several drugs on lipid bilayers.

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