Use of Polymerized Mixed Liposomes To Study Interactions of Phospholipase A_2 with Membranes

Shih-Kwang Wu and Wonhwa Cho*

Department of Chemistry (M/C 111), University of Illinois at Chicago, Chicago, Illinois 60607-7061

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ABSTRACT: Polymerized liposomes of thiol-based phospholipids, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (BLPC) and -phosphoglycerol (BLPG) were used to study interactions of several phospholipases A_2 (PLA_2) with membranes. Large liposomes (an average diameter of 100 ± 10 nm) prepared from BLPC or BLPG were readily hydrolyzed by PLA_2. Once polymerized, however, these liposomes were resistant to the PLA_2 hydrolysis. When liposomes were prepared from a mixture of 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC) (5 mol %) and BLPC, fluorescence measurements of resulting polymerized mixed liposomes showed that the pyrene-PC molecules exist solely as monomers without forming a patch and were selectively hydrolyzed by PLA_2. Progress of the hydrolysis can be readily monitored by measuring the change in fluorescence emission at 380 nm in the presence of bovine serum albumin. Rapid and selective hydrolysis of inserted phospholipids in polymerized mixed liposomes supports the notion that facile migration of a phospholipid substrate from membrane to the active site of enzyme is a critical step in the catalysis of PLA_2. On the basis of these findings, various combinations of polymerized mixed liposomes were prepared and their hydrolysis by PLA_2 measured. When compared to the substrate specificity of PLA_2s determined using Triton X-100/phospholipid mixed micelles, results from polymerized mixed liposomes indicate that electrostatic interactions between the interfacial binding site of PLA_2 and membrane surfaces play an important role in the determination of substrate specificity of PLA_2 and in the regulation of PLA_2 activities. Lastly, polymerized mixed liposomes can serve as a versatile and sensitive PLA_2 assay system in which one can readily modify the structure of polymerized matrix to create liposome surfaces ideal for a specific PLA_2.

Phospholipase A_2 (PLA_2; EC 3.1.1.4) catalyzes, mostly in a Ca^{2+}-dependent mode, the hydrolysis of the fatty acid ester in the 2-position of 3-*sn*-phospholipids [for reviews, see Dennis (1983) and Waite (1987)]. Unlike integral membrane proteins with transmembrane segments, most PLA_2s are water-soluble proteins that interact with phospholipids in an aggregated form, such as in micelles and in bilayers. Thus, the binding of PLA_2 to the membrane surface (interfacial binding), which is thought to be distinct from the binding of a phospholipid molecule to the active site (substrate binding), is a unique and critical step in the PLA_2 catalysis [for a review, see Jain and Berg (1989)]. Because PLA_2s play essential roles in many extra- and intracellular processes, including cellular signal transduction (Dennis et al., 1991; Nishizuka, 1992), it is important to understand the principles of membrane-PLA_2 interactions and the mechanism by which these interactions mediate the activity and the specificity of PLA_2. Although recent structural determinations of enzyme–inhibitor complexes for some forms of PLA_2 (Scott et al., 1990a, 1991; Thunnison et al., 1990; White et al., 1989) have yielded insights into PLA_2-substrate interactions in the active site (Scott et al., 1990b), the exact nature of the interactions of PLA_2 with the membrane is not fully understood due in part to difficulties inherent in determining the structure of membrane-bound proteins. In parallel to structural studies of PLA_2, a wide variety of model membranes have been devised and used to analyze interactions of PLA_2 with membranes. Polymerized liposomes were originally designed to improve the stability of liposomes as a drug carrier [for reviews, see Regen et al. (1987) and Ringsdorf et al. (1988)] and have been recently used to study intercellular communications (Dodrer & Regen, 1990; Krisovitch & Regen, 1991). Earlier, Buschl et al. (1982) reported that patches of natural phospholipids on the surface of photopolymerized liposomes were hydrolyzed by PLA_2 and that these patches could be used as "corks" for the selective opening of polymerized liposomes. In this report, we describe the use of polymerized mixed liposomes to study the interfacial catalysis of PLA_2. In this system, inserted phospholipids are evenly distributed over the polymerized phospholipids without forming patches. Studies described in this report demonstrate that polymerized mixed liposomes can serve as useful and convenient kinetic systems to evaluate the importance of interfacial binding in the regulation of substrate specificity and catalytic activity of PLA_2.

MATERIALS AND METHODS

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), -ethanolamine (POPE), and -glycerol (POPG) were purchased from Avanti Polar Lipids. 1-Hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC), -ethanolamine (pyrene-PE), -glycerol (pyrene-PG), and 1-pyrenedecanoic acid were obtained from Molecular Probes. Triton X-100 was from Pierce. Large unilamellar liposomes were prepared by multiple extrusion through 0.1-μm polycarbonate

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[1] Abbreviations: App-D49, Asp-49 PLA_2 from *A. p. piscivorus*; BLPC, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine; BLPG, 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphoglycerol; BSA, bovine serum albumin; PC, phosphatidylcholine; PLA_2, phospholipase A_2; PLD, phospholipase D; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; pyrene-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; pyrene-PE, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine; pyrene-PG, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol;.

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filter (Millipore) in a microextruder Liposofast (Avestin, Ottawa, Ontario) (MacDonald et al., 1991). Phospholipid concentrations of liposomes were determined by phosphate analysis (Kate et al., 1986). Mixed micelles of Triton X-100 and phospholipids were prepared as described (Dennis, 1973). The mean hydrodynamic radius of liposomes was determined by dynamic light scattering measurement as described (Budzynski et al., 1992). Monomeric (D-49) PLA_2 from *A. p. piscivorus* (App-D49) (Maraganore et al., 1984) and dimeric PLA_2 from *Crotalus atrox* (Hachimori et al., 1977) were purified to homogeneity from lyophilized snake venom (Sigma) as described. Porcine pancreatic PLA_2 was purchased from Sigma as a suspension in a 3.2 M $(NH_4)_2SO_4$ solution and used after dialysis and lyophilization. Phospholipase D (PLD) from cabbage was purchased from Boehringer Mannheim Co. and used without further purification. Fatty acid-free bovine serum albumin (BSA) was from Miles Inc. Protein concentrations were determined by the micro bicinchoninic acid (BCA) method (Smith et al., 1985) (Pierce). Elemental analyses were performed by Midwest Microlab (Indianapolis, IN).

Synthesis of Phospholipids. 1,2-Bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (BLPC) was synthesized as described (Sadownik et al., 1986). 1,2-Bis[12-(lipoyloxy)-dodecanoyl]-*sn*-glycero-3-phosphoglycerol (BLPG) was prepared by PLD-catalyzed transphosphatidylation (Comfurius & Zwaal, 1977) of BLPC with glycerol and purified by flash chromatography (Merck silica gel grade 60, $CHCl_3$–CH_3OH–H_2O, 65:30:5 (v/v), R_f 0.5). ^1H NMR (determined on a Bruker 400-MHz instrument in $CDCl_3$): δ 1.25 (s, 28H), 1.4–2.0 (m, 20H), 2.3 (t, 8H), 2.5 (m, 4H), 3.16 (m, 4H), 3.58 (m, 2H), 3.70–3.76 (m, 2H), 3.90–4.05 (m, 5H), 4.06 (t, 4H), 4.17 (m, 1H), 4.40 (m, 1 H), 5.25 (m, 1H). Anal. Calcd for $C_{46}H_{82}O_{14}PS_4$: C, 54.25; H, 8.12; N, 0. Found: C, 52.60; H, 7.91; N, 0.

Kinetic Measurements. All the kinetic experiments were performed at 37 °C and at pH 7.4. Fluorescence spectra of pyrene-PC, pyrene-PE, and pyrene-PG in liposomes were obtained at 37 °C in 10 mM HEPES (pH 7.4) buffer containing 10 mM $CaCl_2$ and 0.16 KCl with a Perkin-Elmer Model LS-5B luminescence spectrometer. The sample was contained in a thermostated 1-cm path length quartz cuvette. The excitation wavelength was 345 nm, and emission spectra were measured. Spectral band width was set at 5 nm for both excitation and emission. Kinetics of PLA_2-catalyzed hydrolysis of pyrene-PC, -PE and -PG (5 mol %) in polymerized liposomes were measured by monitoring fluorescence emission at 380 nm in the presence of 20 μM pyrene-containing phospholipid, 10 μM BSA (Kupferberg et al., 1981; Radvanyi et al., 1989), 0.16 M KCl, 0.1 mM EDTA, and 10 mM $CaCl_2$. Typically, a reaction mixture containing liposomes and enzyme in the buffer solution minus $CaCl_2$ was incubated for 5 min and the enzymatic reaction initiated by adding an aliquot of concentrated $CaCl_2$ solution to the final concentration of 10 mM. This particular order of addition was designed to circumvent a short but definite latent period which was observed during the hydrolysis of pyrene-containing phospholipids in polymerized liposomes of BLPG when PLA_2 was added last to initiate the reaction. For the hydrolysis of pyrene-containing phospholipids in polymerized liposomes of BLPC, we observed a consistent kinetic pattern regardless of the order of addition. PLA_2-catalyzed hydrolyses of POPC liposomes and nonpolymerized liposomes of BLPC and BLPG were performed with 0.5 mM phospholipid, 0.1 mM BSA, 0.16 M KCl, 0.1 mM EDTA, and 10 mM $CaCl_2$ and monitored with a Metrom pH stat. PLA_2-catalyzed hydrolyses of mixed micelles (2 mM Triton X-100 and 0.5 mM phospholipids) were performed in a solution containing 0.1 mM EDTA and 10 mM $CaCl_2$, and monitored with a Metrom pH stat. For porcine pancreatic PLA_2, deoxycholic acid was added to the mixed micelles (2 mM Triton X-100, 2 mM deoxycholic acid and 0.5 mM phospholipids) to circumvent the anomalous kinetic behavior due to product activation. Under these defined conditions, all the PLA_2-catalyzed reactions followed first-order kinetics. Within the range of enzyme concentration used, pseudo-first-order rate constants were directly proportional to enzyme concentrations; apparent specificity constants $((k_{cat}/K_m)_{app})$ were calculated by dividing the pseudo-first-order rate constants by enzyme concentrations. Enzyme concentration for each reaction was adjusted so as to keep the half-life of reaction below 5 min.

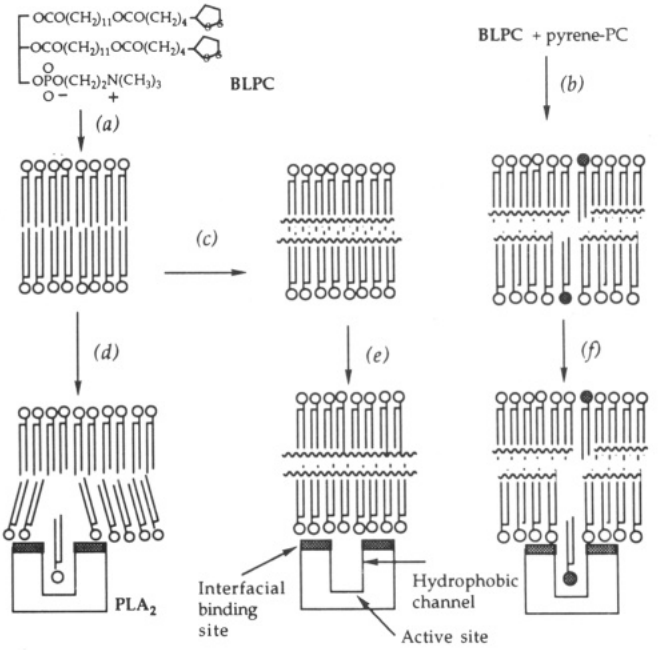

FIGURE 1: Schematic representation of the interaction of PLA_2 with polymerized mixed liposomes of BLPC. Phospholipids with a head group shown in filled circle indicate inserted pyrene-PC. (a) Formation of liposomes. (b) Formation of mixed liposomes and polymerization. (c) Polymerization. (d) Extraction of a phospholipid by PLA_2 and protein penetration into phospholipid bilayers. (e) No extraction and no penetration. (f) Extraction of an inserted phospholipid without penetration.

RESULTS

Effects of Polymerization on Properties of BLPC Liposomes. We selected for our studies thiol-based polymerizable phospholipids, BLPC and BLPG, for two reasons; they are readily available by chemical and enzymatic synthesis and they polymerize under mild conditions once they form liposomes (Sadownik et al., 1986). Essentially complete polymerization (>99%) and cross-linking was routinely achieved at room temperature after treatment with dithiothreitol for 30 h at pH 8.7, as evidenced by TLC and by the loss of the UV absorption at 333 nm (see the inset of Figure 2). Polymerization did not change physical properties of BLPC liposomes to an appreciable extent; e.g., mean hydrodynamic diameter (100 ± 10 nm) of BLPC liposomes determined by dynamic molecular scattering remained unchanged (100 ± 10 nm) after polymerization. In order to investigate the effect of phospholipid polymerization on the PLA_2-catalyzed hydrolysis of BLPC liposomes, we measured the kinetics of

Table 1: Specificity Constant $((k_{cat}/K_m)_{app})^a$ of the PLA_2-Catalyzed Hydrolysis of Various Liposomes

	liposomes					
PLA_2	POPC ($10^3 \times M^{-1} s^{-1}$)	*nonpoly* BLPC[b] ($10^4 \times M^{-1} s^{-1}$)	*poly* BLPC[c]	pyrene-PC + *poly* BLPC ($10^3 \times M^{-1} s^{-1}$)	*nonpoly* BLPG[d] ($10^6 \times M^{-1} s^{-1}$)	*poly* BLPG[e]
App-D49	7.1 ± 0.1	1.87 ± 0.10	ND[f]	5.5 ± 0.2	2.80 ± 0.05	ND
porcine PLA_2	0.4 ± 0.06	0.08 ± 0.01	ND	0.32 ± 0.01	0.48 ± 0.02	ND
C. *atrox* PLA_2	20.2 ± 1.5	7.90 ± 0.40	ND	20.0 ± 0.3	1.50 ± 0.02	ND

[a] See Materials and Methods for experimental conditions and methods to calculate rate constants. Values of $(k_{cat}/K_m)_{app}$ represent mean values ± standard errors determined from a minimum of three measurements. [b] Nonpolymerized liposomes of BLPC. [c] Polymerized liposomes of BLPC. [d] Nonpolymerized liposomes of BLPG. [e] Polymerized liposomes of BLPG. [f] ND, not detectable.

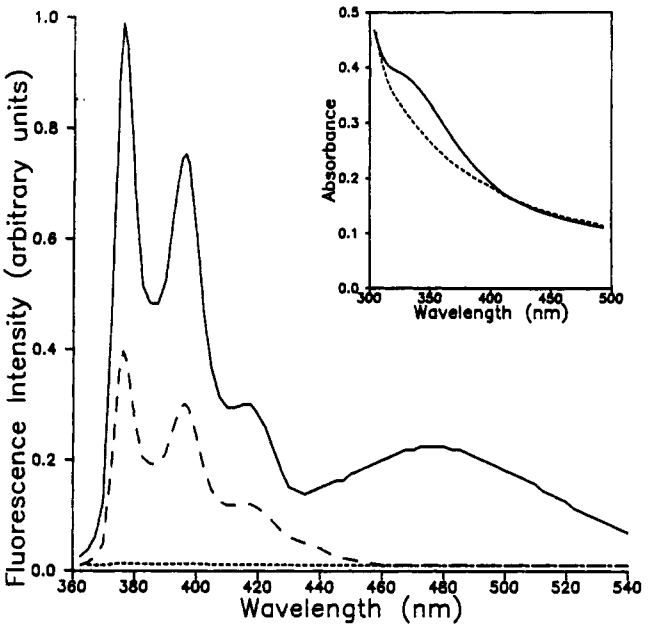

FIGURE 2: Fluorescence emission spectra of pyrene-containing phospholipids inserted in various liposomes. For all the measurements, pyrene-containing phospholipids in mixed liposomes were maintained at 5 mol %. A solid line represents the spectrum of pyrene-PC (0.5 μM) in POPC liposomes (9.5 μM). A long dashed line represents the spectra of pyrene-PC (5 μM), -PE (5 μM), and -PG (5 μM), respectively, in polymerized mixed liposomes of BLPC (95 μM). Three spectra are identical. A short dashed line at the bottom represents the spectrum of pyrene-PC (5 μM) in nonpolymerized mixed liposomes of BLPC (95 μM). Because of low fluorescence intensity of pyrene-PC in BLPC liposomes, a higher concentration of pyrene-PC was used to provide a better illustration of spectra. A figure in the inset represents absorption spectra of BLPC liposomes (0.25 mM) before (solid line) and after (broken line) polymerization.

reaction before and after polymerization of BLPC. As summarized in Table 1, nonpolymerized BLPC liposomes were hydrolyzed by all three forms of PLA_2s as readily as POPC liposomes were. Presumably, an additional ester function in each acyl group of BLPC (see the structure of BLPC in Figure 1) confers the fluidity on the phospholipid bilayer as a *cis*-unsaturation in the *sn*-2 acyl group of POPC does. No PLA_2, however, exhibited any detectable activity toward polymerized liposomes of BLPC. Also, polymerized liposomes of BLPG showed similar behaviors (Table 1); i.e., rapid hydrolysis of nonpolymerized liposomes by PLA_2, no change in size of liposomes after polymerization, and inertness of polymerized liposomes to PLA_2 activity. All the PLA_2s catalyzed the hydrolysis of nonpolymerized BLPG liposomes much faster than nonpolymerized BLPC liposomes because of favorable electrostatic interactions between cationic interfacial binding site of PLA_2 and anionic interfaces (see Figure 3 and the accompanying text in Discussion).

Polymerized Mixed Liposomes Containing Hydrolyzable Phospholipids. Based on the properties of polymerized liposomes of BLPC and BLPG described above, we explored a possibility of using polymerized liposomes as an inert host in which hydrolyzable phospholipids are imbedded. The resulting polymerized mixed liposomes could be extremely useful for studying the interfacial catalysis of PLA_2 if inserted phospholipids are uniformly distributed in the polymerized matrix and they are selectively hydrolyzed by PLA_2. In order to measure the distribution of inserted phospholipids, we inserted a pyrene-containing phospholipid, pyrene-PC, into BLPC liposomes and determined its distribution using fluorometry before and after polymerization. In fluid, liquid-crystalline membranes, pyrene-PC molecules exist both as monomers with fluorescence emission peaks at 380 and 398 nm and as intermolecular excimers with a peak at 480 nm (Radvanyi et al., 1989; Galla & Sackmann, 1974) (Figure 2). In general, the formation of pyrene excimer is a diffusion-controlled process and the excimer-to-monomer ratio of the pyrene probe is dependent on its concentration within the membrane, its lateral diffusion coefficient, and the fluorescence lifetime of the excimer (Galla & Sackmann, 1974). At 5 mol % of pyrene-PC in POPC liposomes, the excimer-to-monomer ratio, estimated from the ratio of fluorescence intensities at 480 nm (I_{480}) and 380 nm (I_{380}), was 0.23. As shown in Figure 2, 5 mol % of pyrene-PC in nonpolymerized liposomes of BLPC did not show any detectable emission at either 380 or 480 nm due to strong fluorescence quenching by lipoic acid rings in BLPC. Polymerization and concomitant ring opening, however, resulted in a loss of UV absorption at 333 nm which, in turn, significantly increased the fluorescence emission of pyrene-PC at 380 and 389 nm; yet no emission was observed at 480 nm. It should be noted that the absorbance of polymerized liposomes of BLPC at 380 nm, although relatively weak, is still stronger than that at 480 nm (Figure 2). Because the fluorescence quenching of pyrene-PC is due to the light absorption of BLPC, I_{480}/I_{380} value of pyrene-PC is an upper estimate of excimer-to-monomer ratio and should be even smaller in the absence of fluorescence quenching. These results, thus, show that inserted pyrene-PC molecules exist solely as isolated monomers and do not form a patch in polymerized liposomes of BLPC. Because polymerization of BLPC would not significantly change lifetime of the excimer, the lack of excimer formation is due to reduced lateral mobility of pyrene-PC in the polymerized matrix. Lastly, we measured the effect of the pyrene-PC concentration on the excimer-to-monomer ratio in polymerized liposomes of BLPC. The excimer emission became appreciable only when the mol % of pyrene-PC was above 15; I_{480}/I_{380} reached 0.18 at 20 mol % of pyrene-PC. To ensure that all the pyrene-containing phospholipids exist as monomers, their concentrations in polymerized mixed liposomes were maintained at 5 mol % for all the kinetic studies.

Having established a uniform distribution of pyrene-PC in polymerized liposomes of BLPC, we then measured the hydrolysis of pyrene-PC by *C. atrox* PLA_2 which is highly

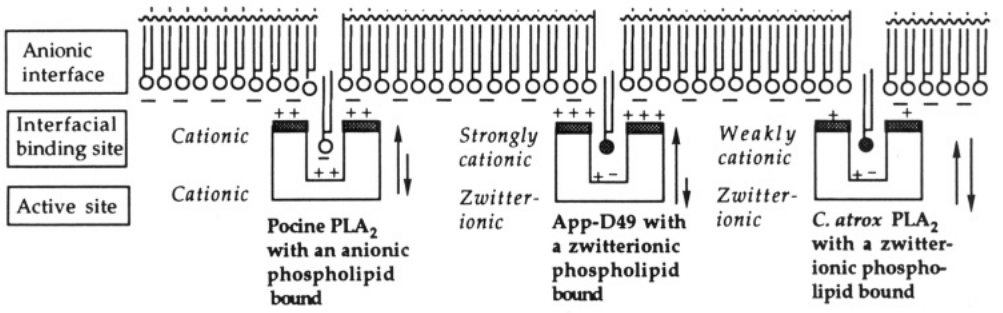

FIGURE 3: Schematic representation of the interfacial binding and the substrate binding in the active site of PLA_2. Electrostatic properties of interfacial binding site and active site are roughly described in terms of the number of positive or negative signs (the number does not indicate the exact net charge). Cross-linked phospholipids indicate polymerized BLPG. Head groups of inserted phospholipids are shown either in open circle for anionic phospholipids or in filled circle for zwitterionic phospholipids. The arrows illustrate the equilibrium position of PLA_2 between the interface and the bulk phase.

Table 2: Specificity constant $((k_{cat}/K_m)_{app})^a$ Determined from the PLA_2-Catalyzed Hydrolysis[a] of Different Phospholipids in Triton X-100 Mixed Micelles and in Polymerized Mixed Liposomes

	in Triton X-100 mixed micelles ($\times 10^5$ M^{-1} s^{-1})			in *poly* BLPC[b] ($\times 10^3$ M^{-1} s^{-1})			in *poly* BLPG[c] ($\times 10^6$ M^{-1} s^{-1})		
enzyme	POPC	POPE	POPG	pyrene-PC	pyrene-PE	pyrene-PG	pyrene-PC	pyrene-PE	pyrene-PG
App-D49	3.70 ± 0.20 (1.0)	3.33 ± 0.10 (0.9)	3.40 ± 0.16 (0.9)	5.50 ± 0.20 (1.0)	7.20 ± 0.30 (1.3)	16.50 ± 0.60 (3.0)	26.00 ± 2.20 (1.0)	28.00 ± 2.70 (1.1)	32.00 ± 4.90 (1.2)
Porcine PLA_2	0.15 ± 0.01 (1.0)	0.44 ± 0.03 (2.9)	1.40 ± 0.09 (9.3)	0.32 ± 0.01 (1.0)	0.42 ± 0.02 (1.3)	0.51 ± 0.02 (1.6)	0.12 ± 0.01 (1.0)	0.40 ± 0.02 (3.2)	1.40 ± 0.10 (11.6)
C. *atrox* PLA_2	5.60 ± 0.20 (1.0)	6.72 ± 0.04 (1.2)	5.04 ± 0.04 (0.9)	20.00 ± 0.30 (1.0)	24.00 ± 0.70 (1.2)	30.00 ± 0.90 (1.5)	1.10 ± 0.03 (1.0)	1.10 ± 0.03 (1.0)	1.40 ± 0.10 (1.3)

[a] See Materials and Methods for experimental conditions and methods to calculate rate constants. Values of $(k_{cat}/K_m)_{app}$ represent mean values ± standard errors determined from a minimum of three measurements. Values in parenthesis represent relative activity of each enzyme toward PE and PG compared to PC in each of three systems. [b] Polymerized liposomes of BLPC. [c] Polymerized liposomes of BLPG.

active toward phosphatidylcholine (PC) bilayers (see Table 2). Because fluorescence emission of pyrene moieties at 380 nm in polymerized liposomes of BLPC is strongly quenched, removal of pyrene groups from the bilayer would result in a large increase in I_{380}. BSA is known to selectively bind 1-pyrenedecanoic acid molecules present in liposomes of pyrene-PC (Radvanyi et al., 1989) and extract them from the bilayer to solution. On the basis of these observations, we monitored the change in I_{380} during the course of PLA_2-catalyzed hydrolysis of pyrene-PC in polymerized liposomes of BLPC in the presence of BSA. Indeed, we observed a 25-fold increase in I_{380} during the catalysis, which followed first-order kinetics (data not shown). The increase in I_{380} was solely due to the hydrolysis of pyrene-PC because I_{380} remained constant in the absence of cofactor Ca^{2+}. To rule out a possibility that extraction of hydrolyzed products by BSA from the bilayer is rate-limiting, we prepared a solution of polymerized liposomes of BLPC containing 1-pyrenedecanoic acid (5 mol %) and measured the change in I_{380} upon adding BSA. BSA resulted in an immediate (within a few seconds) increase in I_{380}, the magnitude of which was proportional to BSA concentration and eventually reached a saturating value when the mole ratio of BSA/1-pyrenedecanoic acid exceeded 0.25. To ensure complete extraction of 1-pyrenedecanoic acid by BSA during our kinetic measurements, we maintained mole ratio of BSA/1-pyrenedecanoic acid at 0.5. Under this condition, an increase in I_{380} during the hydrolysis of pyrene-PC by *C. atrox* PLA_2 was consistent with the value expected from the migration of the same concentration of 1-pyrenedecanoic acid out of the outer monolayer (data not shown). Thus, it is evident that the pyrene-PC molecules in (the outer monolayer of) polymerized liposomes of BLPC are completely hydrolyzed by PLA_2, and the rate of change in I_{380} is governed by the rate of hydrolysis. Rate constants of reactions were determined from the nonlinear least-squares analysis of the I_{380} vs time plot using a first-order rate equation and are summarized in Table 1. For all the three PLA_2s used, $(k_{cat}/K_m)_{app}$ values for the hydrolysis of pyrene-PC in the polymerized liposomes of BLPC are comparable to those for the hydrolysis of nonpolymerized BLPC liposomes and for the hydrolysis of POPC liposomes. This indicates that pyrene-PC/BLPC polymerized mixed liposomes are just as good a substrate for PLA_2 as conventional liposomes.

Factors Governing Substrate Specificity of PLA_2. To test if polymerized mixed liposomes composed of other derivatives of pyrene-PC and BLPC behave similarly, we prepared a wide variety of polymerized mixed liposomes and measured their properties. First of all, we measured the distribution and the hydrolysis of pyrene-PE and pyrene-PG in polymerized liposomes of BLPC. Both pyrene-PE and pyrene-PG molecules exist as monomers in polymerized liposomes of BLPC (Figure 2) and their hydrolysis can be reasily measured by monitoring I_{380} as a function of time in the presence of BSA. Similarly, all three types of pyrene-containing phospholipids exist as monomers in polymerized liposomes of BLPG (data not shown) and were hydrolyzed by PLA_2 (Table 2). All the known phosphatidylglycerols with acyl chains longer than 14 carbon atoms form the bilayer structure at a neutral pH and at room temperature. The hexagonal phase has been observed only at high temperature in the presence of 1 M $CaCl_2$ (Cullis et al. 1991). We did not use a PE-derivative of BLPC in this study because of its tendency to form a hexagonal phase instead of the bilayer phase. Sizes of all the polymerized mixed liposomes of BLPG were essentially the same as those of BLPC within the range of experimental error of determination (100 ± 10 nm). Thus, the structural integrity of polymerized mixed liposomes is maintained when the chemical nature of both the inert polymerized matrix and hydrolyzable inserted phospholipids is varied. This property of polymerized mixed liposomes should allow one to systematically measure the effect

of structural variation of phospholipids on the interfacial catalysis of PLA_2 without having to deal with changes in the physical state of interface.

On the basis of this premise, we performed a systematic analysis of factors governing the substrate specificity (phospholipid head group specificity) of PLA_2. Table 2 summarizes the specificity constants determined for several PLA_2s using polymerized mixed liposomes and Triton X-100/phospholipid mixed micelles. Triton X-100 mixed micellar system has been widely used for measuring substrate specificity of phospholipases (Roberts et al., 1978) because it offers a relatively well-defined, inert matrix. Because measuring the hydrolysis of mixed micelles by a pH stat requires a relatively large amount of phospholipids, we used POPC, POPE, and POPG instead of pyrene-containing phospholipids in this system. For the reactions catalyzed by the porcine PLA_2, the anionic detergent deoxycholic acid (44 mol %) was added to mixed micelles in order to circumvent anomalous kinetics due to product activation by fatty acids (Pluckthun & Dennis, 1985). Substrate specificities of PLA_2s (see the ratios in parenthesis in Table 2) determined using mixed micelles are consistent with previously reported data (Roberts et al., 1978). Briefly, PLA_2s from snake venom, App-D49 and *C. atrox* PLA_2, showed a broad specificity while the porcine PLA_2 preferred POPG (and POPE) to POPC. When specificities of the above PLA_2s were determined using polymerized mixed liposomes of BLPC, all the enzymes slightly favored pyrene-PG over pyrene-PE and -PC. In general, PLA_2s cannot hydrolyze the compactly-packed phospholipids, such as large liposomes, as efficiently as loosely-packed phospholipids, such as micelles and mixed micelles (Waite, 1987). Difference in catalytic activity among PLA_2s appeared to reflect difference in their intrinsic activity toward PC bilayers (see Table 1). Compared to polymerized mixed liposomes of BLPC, all the enzymes showed enhanced activities toward polymerized mixed liposomes of BLPG. When the rate constants for pyrene-PC were compared, however, the magnitude of rate increase varied greatly. This difference can be accounted for in terms of surface electrostatics of individual enzymes (Ward & Pattabiraman, 1990). App-D49 that strongly favors the anionic interface of pyrene-PC/BLPG polymerized mixed liposomes (4700-fold increase) contains several lysines (Lys-7, -10, and -15) sequestered in one face of the amino-terminal helix that has been proposed to be involved in interfacial binding. *C. atrox* PLA_2 that shows a marginal 50-fold increase contains only one arginine (Arg-15) in this region and does not form any noticeable cationic patch on the molecular surface (Ward & Pattabiraman, 1990). Porcine pancreatic PLA_2 that shows a relatively high preference for the anionic interface (380-fold increase) possesses a cationic surface patch formed by Arg-6, Lys-10, and His-17 (Jain & Berg, 1989; Ward & Pattabiraman, 1990). Preference of App-D49 for pyrene-PG to pyrene-PC in polymerized mixed liposomes of BLPC appears to be due to local anionic interfaces provided by inserted pyrene-PG. This preference was not manifested in polymerized mixed liposomes of BLPG because local anionic interfaces provided by pyrene-PG would not contribute significantly to the overall surface charge of largely anionic BLPG liposomes. Intriguingly, the porcine PLA_2 is the only enzyme that preferred an anionic pyrene-PG inserted in the anionic polymerized matrix of BLPG. These results indicate that all the three PLA_2s contain cationic interfacial binding sites but that only the porcine enzyme contains a cationic substrate-binding site in the active site.

DISCUSSION

The interfacial catalysis by PLA_2 is sensitive to the physical state of phospholipid substrates; i.e., PLA_2 recognizes not only the chemical structure of phospholipid but also the physical structure of the aggregate it forms. This unique property of PLA_2 makes it difficult to accurately determine the substrate specificity of this enzyme and, more importantly, to understand factors governing the specificity. For instance, it is not fully understood whether high specificity of a recently purified cytosolic PLA_2 (Clark et al., 1991; Sharp et al., 1991) for arachidonyl-containing phospholipids originates from a preference of its active site for these phospholipids or from the ability of its interfacial binding site to recognize a unique local membrane structure induced by polyunsaturated arachidonyl moiety (Scharkwijk et al., 1990). Unfortunately, it is difficult to sort out these two factors in conventional kinetic systems using micelles or liposomes. In our kinetic system using polymerized mixed liposomes, one can unambiguously designate those phospholipids which interact with the active site of PLA_2 and those which interact with the interfacial binding site of PLA_2. In addition, one can systematically vary the chemical structure of both hydrolyzable phospholipids and an inert polymerized host while the defined physical structure of liposomes is maintained. Also, uniform and sparse distribution of inserted phospholipids in a rigid polymerized matrix essentially rules out a possibility that inserted phospholipids induce a significant change in local membrane structure.

Physical properties of liposomes of BLPC and BLPG are similar to those of POPC liposomes of the same size. They do not undergo any phase transition in the temperature range of 10 to 50 °C (data not shown). PC liposomes in the gel phase are extremely poor substrates for PLA_2 and show anomalous kinetic patterns during hydrolysis (Biltonen et al., 1990). Thus, relatively facile hydrolysis of BLPC (and BLPG) by PLA_2 which follows a simple first-order kinetics indicates that they are in the liquid-crystalline phase at 37 °C at which all the kinetic measurements were made. Polymerization of BLPC (and BLPG) liposomes could be confined within each monolayer of the bilayer or occur across the bilayer to tie together the inner and outer monolayer. Freeze-fracture electron micrographs of polymerized liposomes of 1,2-bis(11-mercaptoundecanoyl)-*sn*-glycero-3-phosphocholine indicated that the polymerization was confined within each monolayer (Samuel et al., 1985). Because this phospholipid is structurally similar to BLPC, it is likely that polymerization of BLPC (and BLPG) liposomes is largely confined within each monolayer. In any event, the ability of a BLPC (and BLPG) molecule to form a cross-linkage using two lipoic acid moieties with their neighboring molecules would restrict the diffusion of phospholipids within and out of each monolayer. Indeed, polymerization severely restricted the lateral diffusion of phospholipids in the bilayer as witnessed by fluorescence spectra of pyrene-containing phospholipids inserted in the polymerized liposomes of BLPC and BLPG. In addition, polymerized liposomes of BLPC and BLPG were inert to PLA_2 catalysis presumably due to the restricted motion of phospholipids out of the bilayer. Polymerization of BLPC and BLPG did not change the size of liposomes. Also, polymerized mixed liposomes of BLPC and BLPG showed the same average size as polymerized liposomes of BLPC and BLPG. Thus, all the polymerized (mixed) liposomes appear to be structurally similar to conventional liposomes and their unique mode of interactions with PLA_2 are mainly caused by the restricted movement of phospholipids within and outside the bilayer.

We have exclusively used large polymerized mixed liposomes for our kinetic studies because they are more stable than smaller liposomes and a better model for biological membranes (New, 1990).

Structural analyses (Scott et al., 1990a,b, 1991; Thunnison et al., 1990; White et al., 1989) of several PLA_2-inhibitor complexes have shown the mode of interactions between an active-site-bound phospholipid analog and catalytic machinery of PLA_2. On the basis of the structural comparison of the enzyme–inhibitor complex with the uninhibited enzyme, Scott et al. (1990b) have proposed that facilitated diffusion of a phospholipid substrate from membrane surfaces to the active site of the enzyme accounts for optimal catalysis of PLA_2 at the lipid–water interface. As schematically illustrated in Figure 1, this notion is supported by our results showing the inertness of polymerized liposomes to PLA_2 catalysis and the rapid and selective hydrolysis of inserted phospholipids in polymerized mixed liposomes. Independently, it has been proposed by Demel et al. (1975) that the ability of PLA_2 to hydrolyze compactly-packed phospholipid bilayers including erythrocyte membranes depends on the membrane-penetrating capacity of enzyme. Since the protein penetration would require lateral movement of phospholipids (Figure 1), this process is expected to be significantly inhibited by the polymerization of liposomes. Our results suggest, however, that the penetration and concomitant hydrophobic interactions between PLA_2 and phospholipids might not be important in the interfacial binding.

Substrate specificities of several PLA_2s determined using polymerized mixed liposomes reveal an intimate interplay between the interfacial binding and the substrate binding in the active site. As schematically illustrated in Figure 3, PLA_2 with a cationic interfacial binding site favorably recognizes anionic interfaces but its phospholipid head group specificity is largely governed by electrostatic properties of its active site. The interfacial binding site of PLA_2 is generally thought to consist of cationic residues located at one side of protein that makes a contact with membrane surfaces (Jain & Berg, 1989; Scott et al., 1990b; Dijkstra et al., 1981). Consequently, most PLA_2s prefer anionic interfaces (see Table 1 for the comparison of rate constants for nonpolymerized liposomes of BLPC and BLPG). In particular, PLA_2s including App-D49 and the porcine PLA_2 that contain a large number of cationic residues in the interfacial binding site have a marked preference for anionic interfaces. In an active site of conventional Ca^{2+}-dependent PLA_2, a bound Ca^{2+} (and other residues) provides a cationic substrate-binding site that interacts with a phosphate anion of bound phospholipid. Recent structural and mutational studies on both bovine (Noel et al., 1990, 1992) and porcine (Lugtigheid et al., 1993) pancreatic PLA_2 have shown that two specific lysines in the active site, Lys-53 (Arg-53 for porcine enzyme) and Lys-56, interact with the phospholipid head group. These two cationic residues are either missing or replaced by anionic (or neutral) residues for snake venom PLA_2s (Van den Bergh et al., 1989). It should be emphasized, however, that specificity of the porcine PLA_2 for anionic substrates is manifested only when phospholipids are distributed in anionic interfaces including deoxycholic acid-containing mixed micelles and polymerized mixed liposomes of BLPG; i.e., when the enzyme is tightly bound to the interface. Thus, the active site of PLA_2 cannot fully express its affinity for a specific phospholipid head group if the interfacial binding is rate-limiting as in the case of the hydrolysis of polymerized mixed liposomes of BLPC. The notion that electrostatic properties of the interface regulate the mode of interfacial catalysis of PLA_2 has been elaborated by Jain and Berg (1989) in their kinetic system utilizing liposomes of dimyristoylphosphatidylmethanol. In this system, PLA_2 has been shown to catalyze the hydrolysis of phospholipid in the "scooting mode" in which all the enzyme molecules are tightly bound to the interface. Although we have not tested if PLA_2 catalyzes the hydrolysis of polymerized mixed liposomes of BLPG in the scooting mode, it is evident that the interfacial binding, electrostatic interactions in particular, plays an important role in determining substrate specificity of PLA_2. Obviously, there are other factors, such as a steric factor and the hydrogen bond, that also govern the substrate specificity of PLA_2 (Ortiz et al., 1992). We are currently investigating the importance of these factors using various forms of polymerizable mixed liposomes.

In summary, polymerized mixed liposomes provide a simple and versatile kinetic system with which one can systematically evaluate the interfacial binding and the substrate binding in the active site of PLA_2. In combination with site-directed mutagenesis of PLA_2, this system should be particularly useful for identifying amino acid residues involved in these interactions. In addition, polymerized mixed liposomes of BLPG can serve as a sensitive fluorescence PLA_2 assay system because all the PLA_2s tested show high activities toward these liposomes. Furthermore, one can readily modify the structure of polymerized matrix to create liposome surfaces ideal for a specific PLA_2. We did not include other derivatives (e.g., phosphatidylserine) of BLPC in this study but they should be readily available by PLD-catalyzed transphosphatidylation. Compared to the kinetic system using liposomes made entirely of pyrene-containing phospholipids (Radvanyi et al., 1989), our kinetic system requires only a small amount of relatively expensive pyrene-containing phospholipids. Although this study mainly concerns the use of polymerized mixed liposomes in studying the substrate specificity of PLA_2, our system is applicable to the study of other proteins that interact with biological membranes. Our preliminary results indicate that polymerized liposomes of BLPC are inert to PC-specific phospholipase C from *Bacillus cereus* and PLD from various plants. With an appropriate selection of inserted phospholipid which allows a sensitive monitoring of hydrolysis by these phospholipases, polymerized mixed liposomes could be used to study the interfacial catalysis of these enzymes. Also, inertness of polymerized liposomes to phospholipase hydrolysis makes them suited for the determination of equilibrium dissociation constants for phospholipase–liposome complexes. In that polymerized liposomes would largely restrict hydrophobic interactions between protein and phospholipids, comparison of dissociation constants determined using polymerized liposomes and liposomes made of nonhydrolyzable phospholipids, such as 2,3-diacyl-*sn*-glycero-1-phospholipids (the D-isomer), would reveal relative importance of hydrophobic vs electrostatic interactions in interfacial binding. Lastly, one can study the importance of phospholipid segregation (Bazzi & Nelsestuen, 1991) in membrane-protein interactions based on the fact that the lateral diffusion of inserted phospholipids is restricted in polymerized mixed liposomes. Comparison of the binding isotherms determined from nonpolymerized mixed liposomes and from polymerized mixed liposomes would provide insights into how the segregation of phospholipid influences membrane–protein interactions.

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