

Membrane Penetration of Cytosolic Phospholipase A₂ Is Necessary for Its Interfacial Catalysis and Arachidonate Specificity[†]

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Received April 20, 1998; Revised Manuscript Received July 1, 1998

ABSTRACT: To determine the mechanism of calcium-dependent membrane binding of cytosolic phospholipase A₂ (cPLA₂), we measured the interactions of cPLA₂ with phospholipid monolayers and polymerizable mixed liposomes containing various phospholipids. In the presence of calcium, cPLA₂ showed much higher penetrating power than secretory human pancreatic PLA₂ toward anionic and electrically neutral phospholipid monolayers. cPLA₂ also showed ca. 30-fold higher binding affinity for nonpolymerized 2,3-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-1-phosphoglycerol (D-BLPG) liposomes than for polymerized ones where the membrane penetration of protein is significantly restricted. Consistent with this difference in membrane binding affinity, cPLA₂ showed 20-fold higher activity toward fluorogenic substrates, 1-*O*-(1-pyrenedecyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine, inserted in nonpolymerized D-BLPG liposomes than the same substrate in polymerized D-BLPG liposomes. Furthermore, cPLA₂ showed much higher *sn*-2 acyl group specificity (arachidonate specificity) and headgroup specificity in nonpolymerized D-BLPG liposomes than in polymerized D-BLPG liposomes. Finally, diacylglycerols, such as 1,2-dioleoyl-*sn*-glycerol, selectively enhanced the membrane penetration, hydrophobic membrane binding, and interfacial enzyme activity of cPLA₂. Taken together, these results indicate the following: (1) calcium not only brings cPLA₂ to the membrane surface but also induces its membrane penetration. (2) This unique calcium-dependent membrane penetration of cPLA₂ is necessary for its interfacial binding and substrate specificity. (3) Diacylglycerols might work as a cellular activator of cPLA₂ by enhancing its membrane penetration and hydrophobic membrane binding.

Phospholipases A₂ (PLA₂; EC 3.1.1.4)¹ are a large family of lipolytic enzymes that catalyze the hydrolysis of the fatty acid ester at the *sn*-2 position of phospholipids (1, 2). The PLA₂-catalyzed hydrolysis of some membrane phospholipids liberates arachidonic acid, which can be converted to potent inflammatory lipid mediators, collectively known as eicosanoids (including prostaglandins, thromboxanes, leukotrienes

and lipoxins), through the cyclooxygenase or lipoxygenase pathways (3, 4). Arachidonic acid also functions as a cellular regulator of other proteins including protein kinase C (5) and phospholipase C (6). Mammalian cells contain various forms of PLA₂s including 14 kDa secretory PLA₂ (7, 8), 85 kDa cytosolic PLA₂ (cPLA₂) (9, 10), and calcium-independent PLA₂ (11). Among these PLA₂s, cPLA₂ has unique specificity for the arachidonoyl moiety in the *sn*-2 position of phospholipids (12, 13) and is generally thought to play a crucial role in maintaining cellular arachidonic acid levels (14). cPLA₂ is therefore an attractive target for developing specific inhibitors that can be used as a novel antiinflammatory drugs. cPLA₂ binds to membranes in the presence of micromolar Ca²⁺ via its C2 domain which contains calcium and membrane binding sites (12, 13). Also, phosphorylation of certain residues including Ser-505 has been shown to activate cPLA₂ in cells (15, 16). Besides this information, however, little is known about the catalytic mechanism and the Ca²⁺-dependent membrane-binding mechanism of cPLA₂. Since all PLA₂s act on phospholipids in membranes or in other aggregated forms, the reaction cycle includes the interfacial binding which is distinct from the binding of a phospholipid molecule to the active site (17). Recent structure–function studies on several secretory PLA₂s (18–21) have shown that the interfacial binding of these PLA₂s is driven by electrostatic and hydrophobic interactions mediated by surface cationic and hydrophobic residues of proteins and relative importance of these interactions varies

[†] This work was supported by National Institutes of Health Grants GM52598 and GM53987.

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¹ Abbreviations: 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; cPLA₂, cytosolic phospholipase A₂; DOG, 1,2-dioleoyl-*sn*-glycerol; D-BLPC, 2,3-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-1-phosphocholine; D-BLPG, 2,3-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-1-phosphoglycerol; D-POPC, 2-palmitoyl-3-oleoyl-*sn*-glycero-1-phosphocholine; D-POPG, 2-palmitoyl-3-oleoyl-*sn*-glycero-1-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); hpPLA₂, human pancreatic PLA₂; PLA₂, phospholipase A₂; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; PyAPC, 1-*O*-(1-pyrenedecyl)-2-acyl-*sn*-glycero-3-phosphocholine; PyArPC, 1-*O*-(1-pyrenedecyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PyArPE, 1-*O*-(1-pyrenedecyl)-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine; PyArPG, 1-*O*-(1-pyrenedecyl)-2-arachidonoyl-*sn*-glycero-3-phosphoglycerol; PyOPC, 1-*O*-(1-pyrenedecyl)-2-oleoyl-*sn*-glycero-3-phosphocholine; PyPPC, 1-*O*-(1-pyrenedecyl)-2-palmitoyl-*sn*-glycero-3-phosphocholine; [¹⁴C]SAPC, 1-stearoyl-2-[¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine.

with the type of PLA₂. Most of mammalian secretory PLA₂s cannot effectively penetrate into compactly packed electrically neutral membranes and hydrolyze them (19, 22, 23). A recently determined crystal structure of the C2 domain of cPLA₂ revealed a group of hydrophobic residues which might be involved in membrane penetration, thereby suggesting that cPLA₂ might have a unique interfacial binding mode which involves a significant degree of membrane penetration (24). To determine how cPLA₂ interacts with membranes and how its interfacial binding mode affects its interfacial catalytic properties including arachidonate specificity, we measured the interactions of human recombinant cPLA₂ with phospholipid monolayers and polymerizable mixed liposomes containing various phospholipids. In this report, we describe results from these studies showing that cPLA₂ has a unique ability to penetrate into the phospholipid bilayer which is in turn necessary for its interfacial catalysis and arachidonate specificity.

MATERIALS AND METHODS

Materials. 1,2-Dioleoyl-*sn*-glycerol (DOG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Fatty acid-free bovine serum albumin (BSA) was from Bayer Inc. (Kankakee, IL). 1-Stearoyl-2-[¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine ([¹⁴C]SAPC) (specific activity, 55 mCi/mmol) and 9,10-[³H]DOG were from Amersham (Arlington Heights, IL) and American Radiolabeled Chemical Co. (St. Louis, MO), respectively.

Enzymes. Recombinant human cytosolic PLA₂ (cPLA₂) was expressed in Sf-9 insect cells using a baculovirus expression vector generously provided by Dr. Brian Kennedy of Merck Frosst Co. (25) and purified as described previously (25, 26) with some modifications. For protein expression, cells were grown to 2×10^6 cells/mL in 700 mL suspension cultures and infected with high titer recombinant baculovirus at the multiplicity of infection of 10. The cells were then incubated for 3 days at 27 °C. For harvesting, cells were centrifuged at 1000g for 10 min and resuspended in 35 mL of extraction buffer containing 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM EDTA, 20% (v/v) glycerol, 10 µg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride. Suspension was homogenized in a hand-held homogenizer chilled on ice. The extract was centrifuged at 100000g for 1 h at 4 °C. The supernatant was loaded onto a HiLoad 16/10 Q Sepharose column (Pharmacia). After extensive washing with buffer A (20 mM Tris-HCl, pH 8.0, 0.1 M KCl, 1 mM EGTA, 1 mM EDTA, and 5% glycerol), the column was eluted with 100 mL of linear salt gradient to 1 M KCl in buffer A. Active cPLA₂ fractions were pooled, adjusted to 2 M KCl and loaded onto a 10 mL Poros 20 PE column (Boehringer Mannheim) with a flow rate of 4 mL/min. A linear salt gradient from 2 to 0 M KCl in buffer A (total volume, 60 mL) was applied. Active cPLA₂ fractions were concentrated in an Ultrafree-15 centrifugal filter device (Millipore) and applied to a HiLoad 16/60 Superdex 200 HR column (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.15 M KCl. The column was eluted with the same buffer at the flow rate of 0.4 mL/min. cPLA₂ peak emerged at ca. 60 mL of elution volume. Active fractions were concentrated as above and stored at -20 °C in 20 mM

Tris buffer, pH 8.0, 0.15 M KCl, and 30% glycerol. cPLA₂ showed a single band with an apparent molecular mass of 100 kDa on a SDS-polyacrylamide electrophoresis gel. Human pancreatic PLA₂ (hpPLA₂) was expressed in *Escherichia coli* and purified as described (27). PLA₂ from the venom of *Agkistrodon piscivorus piscivorus* was purified as described (20). Protein concentrations were determined by bicinchoninic acid method (Pierce, Rockford, IL).

Phospholipid synthesis. 1,2-Bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (BLPC) and -phosphoglycerol (BLPG) were synthesized as described elsewhere (28). 2,3-Bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-1-phosphocholine (D-BLPC) and -glycerol (D-BLPG) were synthesized by the same protocol except that D-α-glycerophosphocholine (Biochemisches Labor, Bern, Switzerland) was used as a starting material instead of a L-isomer. 2-Oleoyl-3-palmitoyl-*sn*-glycero-1-phosphocholine (D-POPC) was synthesized from 1-palmitoyl-*rac*-glycero-3-phosphocholine (Sigma) as described (29). 2-Oleoyl-3-palmitoyl-*sn*-glycero-1-phosphoglycerol (D-POPG) was prepared from D-POPC by phospholipase-D-catalyzed transphosphatidylolation as described (30). Purity of all D-phospholipids was verified by measuring their specific rotation using corresponding L-phospholipids as standards. 1-*O*-(1-Pyrenedecyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PyArPC) was prepared from 10-(1-pyreno)decanoic acid by a five-step synthesis as described elsewhere (31). 1-*O*-(1-Pyrenedecyl)-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PyArPE) and -phosphoglycerol (PyArPG) were prepared from PyArPC by phospholipase-D-catalyzed transphosphatidylolation.

Preparation of Liposomes. Large unilamellar liposomes were prepared by multiple extrusion of lipid dispersion through 100 nm polycarbonate filter in a Liposofast microextruder (Avestin, Ottawa, Ontario). Sucrose-loaded liposomes were prepared as described (21). Liposomes were either used immediately within several hours after preparation or polymerized as described (32). Extrusion of lipid inserts (e.g., DOG) during polymerization was less than 5% of total inserts when measured with radiolabeled inserts. Phospholipid concentrations were determined by phosphate analysis (33).

Monolayer Measurements. Surface pressure (π) of solution in a circular Teflon trough was measured using a du Nouy ring attached to a computer-controlled Cahn electrobalance (model C-32) as described previously (29, 34). The trough (4 cm diameter \times 1 cm deep) has a 0.5 cm deep well for magnetic stir bar and a small hole drilled at an angle through the wall to allow an addition of protein solution. Five to 10 microliters of phospholipid solution in ethanol/hexane [1:9 (v/v)] or chloroform was spread onto 10 mL of subphase (10 mM HEPES, pH 8.0, 0.1 M KCl, and 0.5 mM CaCl₂ or 0.5 mM EGTA) to form a monolayer with a given initial surface pressure (π_0). The subphase was continuously stirred at 60 rpm with a magnetic stirrer. Once the surface pressure reading of monolayer had been stabilized (after ca. 5 min), the protein solution (typically 50 µL) was injected to the subphase and the change in surface pressure ($\Delta\pi$) was measured as a function of time at room temperature. Typically, the $\Delta\pi$ value reached a maximum after 20 min. At a given π_0 of phospholipid monolayer, the maximal $\Delta\pi$ value depended on the protein concentration in the subphase and reached a saturation when the protein concentration was

above a certain value (e.g., 1 $\mu\text{g/mL}$ of cPLA₂ and 1.5 $\mu\text{g/mL}$ of hpPLA₂ at $\pi_0 = 5 \text{ dyn/cm}$). Protein concentrations in the subphase were therefore maintained above those values to ensure that observed $\Delta\pi$ values represent maximal ones at given π_0 values.

Kinetic Measurements. The kinetic measurement and analysis of the hydrolysis of polymerized mixed liposomes by secretory PLA₂ was described in detail elsewhere (18–21). The activity of cPLA₂ was measured at 37 °C using as a substrate mixed liposomes of PyAPC/D-BLPG (1:99 in mol ratio). The assay mixture contained 20 mM HEPES, pH 8.0, 5 μM liposomes, 1 mM CaCl₂, and 10 μM BSA (added in this particular order). Reaction was initiated by adding an aliquot of enzyme to the final concentration of ca. 20 nM. The progress of hydrolysis was monitored as an increase in fluorescence emission at 380 nm (F_{380}) using Hitachi F4500 fluorescence spectrometer with excitation wavelength set at 345 nm. Spectral bandwidth was set at 5 nm for both excitation and emission. Kinetics of vesicle hydrolysis by cPLA₂ has been shown to be complex due to rapid enzyme inactivation during catalysis (35). To avoid this complication, relative activity of cPLA₂ toward various mixed liposome substrates was determined from the initial rate of hydrolysis. The initial rate was determined from an initial linear portion of progress curve which varied with the enzyme concentration and the nature of substrate. Within the range of enzyme concentration used (5–50 nM), the initial rate was directly proportional to cPLA₂ concentration. The fluorescence intensity of a reaction product, 1-*O*-(1-pyrenedecyl)-2-hydroxy-*sn*-glycero-3-phosphocholine, which was measured in the same assay mixture minus enzyme, gave a linear response to its concentration in the range 1–50 nM (total lipid concentration of 0.1–5 μM) (data not shown). Thus, the initial rate was converted to specific activity ($\mu\text{mol/min/mg}$) using this linear correlation curve.

Binding of cPLA₂ and hpPLA₂ to Sucrose-Loaded Liposomes. For binding measurements, 100 μM of polymerized or nonpolymerized sucrose-loaded D-BLPG liposomes were incubated with 10–160 nM of cPLA₂ (30–300 nM of hpPLA₂) in 10 mM HEPES buffer, pH 7.5, containing 0.1 M KCl and 0.5 mM CaCl₂ for 20 min at 25 °C and then pelleted at 100000g and at 25 °C for 30 min. Controls contained the same mixtures minus liposomes. After centrifugation, aliquots of supernatants from both controls and binding mixtures were assayed for PLA₂ activity using as substrates [¹⁴C]SAPC/DOG liposomes (2:1 in molar ratio) for cPLA₂ (36) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol/BLPG (1:99) polymerized mixed liposomes for hpPLA₂ (27). Concentration of bound enzyme ($[E]_b$) was calculated from the difference in PLA₂ activity between controls and binding mixtures. Parameters n and K_d were determined by nonlinear least-squares analysis of the $[E]_b$ versus total enzyme concentration ($[E]_T$) plot using a standard binding equation:

$$[E]_b = \frac{1}{2} \{ [E]_T + K_d + [PL]_T/n - \sqrt{([E]_T + K_d + [PL]_T/n)^2 - 4[E]_T[PL]_T/n} \} \quad (1)$$

where $[PL]_T$ represents total phospholipid concentration. The above equation assumes that each enzyme binds indepen-

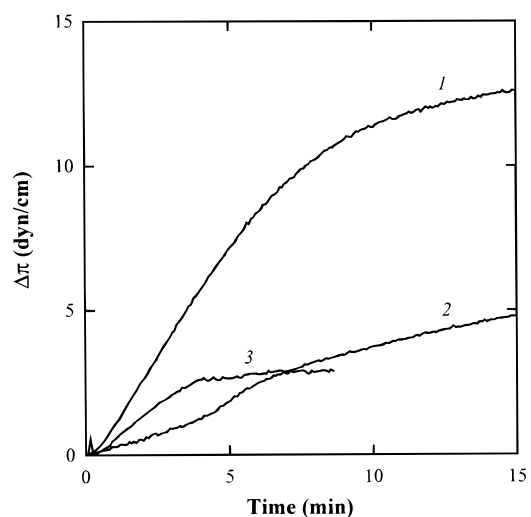


FIGURE 1: Progress curves of penetration of PLA₂ into the D-POPG monolayer. Subphase contained 10 mM HEPES, pH 8.0, and 0.1 M KCl. The enzyme concentration in the subphase was 1.5 $\mu\text{g/mL}$ for both enzymes and the initial surface pressure (π_0) of D-POPG monolayer was 10 dyn/cm. Curve 1, cPLA₂ in the presence of 0.5 mM Ca²⁺; curve 2, cPLA₂ in the presence of 0.5 mM EGTA; curve 3, human pancreatic PLA₂ in the presence of 0.5 mM EGTA. Although Ca²⁺ has a condensing effect on anionic monolayers, this effect could be fully screened by 0.1 M KCl used in this study.

dently to a site on the interface composed of n phospholipids with dissociation constant of K_d .

RESULTS

Monolayer Penetration. To see if the penetration of cPLA₂ into the membrane is involved in its interfacial binding, we measured the interactions of cPLA₂ with various phospholipid monolayers and compared its properties with those of secretory hpPLA₂. Lipid monolayers have proven to be a sensitive tool for measuring lipid–protein interactions (37, 38). In this system, the penetration of a protein into a phospholipid monolayer at the air–water interface can be sensitively monitored at constant area or at constant surface pressure. In these studies, a phospholipid monolayer of a given initial surface pressure (π_0) was spread at constant area and the change in surface pressure ($\Delta\pi$) was monitored after the injection of the protein into the subphase. All monolayer measurements were performed with D-isomers of phospholipids to prevent the hydrolysis of phospholipids by PLA₂ during measurements. To ensure that neither cPLA₂ nor hpPLA₂ distinguishes D- and L-isomers of phospholipids, the following control experiments were performed. For cPLA₂, which has negligible activity toward L-POPC and L-POPG monolayers (L.L., and W.C., unpublished observation), the penetration of the enzyme into these phospholipid monolayers could be measured without detectable hydrolysis. For hpPLA₂, on the other hand, the penetration of the enzyme into both isomers of POPC and POPG was measured in the absence of calcium. In all these cases, both cPLA₂ and hpPLA₂ showed essentially identical penetration patterns into both isomers of phospholipids (data not shown), indicating that their penetration does not depend on the chirality of phospholipid monolayers. To see the differences between cPLA₂ and hpPLA₂, we first compared their penetration into the D-POPG monolayer at a given π_0 . Figure 1 shows the time course of the penetration of cPLA₂ and hpPLA₂ into

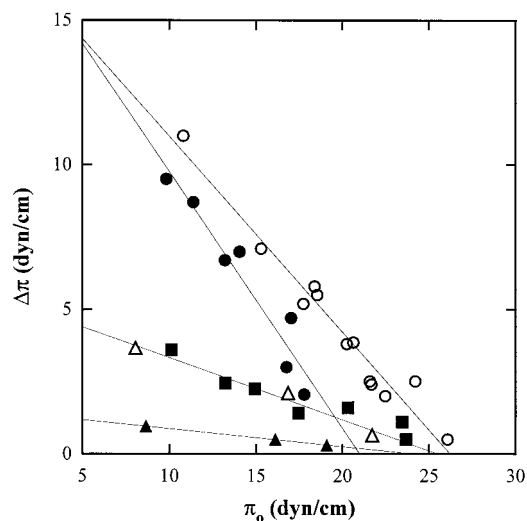


FIGURE 2: Penetration of cPLA₂ (circles) and human pancreatic PLA₂ (triangles) into the D-POPG (open symbols) and D-POPC (filled symbols) monolayer as a function of initial surface pressure of monolayers. Subphase contained 10 mM HEPES, pH 8.0, 0.1 M KCl, and 0.5 mM CaCl₂ (for cPLA₂) or 0.5 mM EGTA (for hpPLA₂). Also shown is the penetration of cPLA₂ into D-POPG monolayer with 0.5 mM EGTA in the subphase (■). Solid lines were obtained by the linear regression of experimental data.

the D-POPG monolayer at $\pi_0 = 10$ dyn/cm. From curves 1 and 2, it is evident that Ca²⁺ has a large effect on the monolayer penetration of cPLA₂. In the absence of Ca²⁺, the magnitude of cPLA₂ penetration was comparable to that of hpPLA₂ and the kinetics of penetration showed a complex pattern. In the presence of 0.5 mM Ca²⁺, cPLA₂ showed a much higher degree of penetration ($\Delta\pi \approx 12$ dyn/cm) the kinetics of which followed a monophasic increase. Although 0.5 mM Ca²⁺ is higher than required for cellular activation of cPLA₂, this concentration was used to facilitate the binding of enzyme to monolayers. Lower Ca²⁺ concentrations (e.g., 5–50 μ M) resulted in essentially the same degree of $\Delta\pi$ but at slower rates (data not shown). Ca²⁺ had no significant effect on the monolayer penetration of hpPLA₂. Thus, these data indicate that Ca²⁺ specifically enhances the rate of membrane binding of cPLA₂ as well as its membrane penetrating ability. Then, we measured the penetration of cPLA₂ and hpPLA₂ into anionic D-POPG and electrically neutral D-POPC monolayers as a function of their initial surface pressure. As shown in Figure 2, Ca²⁺-dependent penetration of cPLA₂ generally resulted in higher $\Delta\pi$ values than the penetration of hpPLA₂ at a given π_0 . Again, the penetration of cPLA₂ into the D-POPG monolayer in the absence of Ca²⁺ was comparable to that of hpPLA₂. The phospholipid composition of monolayer had different effects on the penetration of cPLA₂ and hpPLA₂. hpPLA₂ showed essentially no penetration into the D-POPC monolayer, implying that the initial electrostatic adsorption of protein to the monolayer surface is essential for the subsequent partial monolayer penetration of protein. This notion is consistent with our previous structure–function studies on bovine pancreatic PLA₂ (18, 19) which showed that electrostatic interactions are a driving force for its membrane binding and that its minor hydrophobic interactions are mediated by a partial membrane penetration of surface hydrophobic residues. In contrast to hpPLA₂, cPLA₂ in the presence of Ca²⁺ showed only modestly reduced $\Delta\pi$ values for the electrically

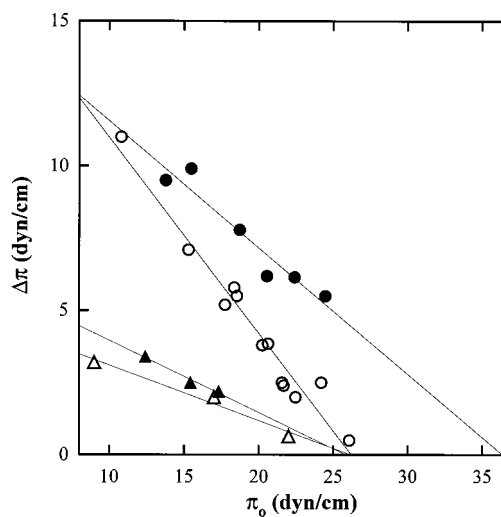


FIGURE 3: Effects of DOG on the penetration of cPLA₂ (circles) and human pancreatic PLA₂ (triangles) into the D-POPG monolayers. Subphase contained 10 mM HEPES, pH 8.0, 0.1 M KCl, and 0.5 mM CaCl₂ (for cPLA₂) or 0.5 mM EGTA (for hpPLA₂). The monolayer contained either D-POPG (open symbols) or D-POPG/DOG (95:5 in mol ratio) (filled symbols). Solid lines were obtained by linear regression of experimental data.

neutral D-POPC monolayer, which indicates that hydrophobic interactions make significant contributions to Ca²⁺-dependent interfacial binding of cPLA₂. This finding also suggests that the role of Ca²⁺ is not only to bring cPLA₂ to membrane surfaces but also to enhance its membrane penetration and hydrophobic interfacial binding.

In general, the $\Delta\pi$ value is inversely proportional to the π_0 value and the extrapolation of the $\Delta\pi$ vs π_0 plot yields the critical surface pressure (π_c) which specifies an upper limit of π_0 of the monolayer that a protein can penetrate into (38). Figure 2 shows that the effect of Ca²⁺ on the penetration of cPLA₂ becomes less pronounced with the increase of π_0 , indicating that Ca²⁺ greatly enhances the penetrating power of cPLA₂ into loosely packed membranes but not into compactly packed ones. As a result, π_c values for cPLA₂ and D-POPG monolayer are 27 dyn/cm in the presence of Ca²⁺ and 25 dyn/cm without Ca²⁺, respectively. Since the estimated surface pressure of biological membranes is ca. 31 dyn/cm (39–42), cPLA₂ might not be able to penetrate into compactly packed biological membranes to perform its interfacial catalysis. To see if any lipid cofactors could help cPLA₂ overcome this potential problem, we measured the effect of DOG on the monolayer penetration of cPLA₂. Diacylglycerols including DOG have been shown to increase the activity of cPLA₂ for vesicle substrates, but the mechanism of activation is not fully understood (36, 43). We reasoned that diacylglycerols might be able to enhance the penetration of cPLA₂ by acting as a spacer between phospholipids based on its unique structure lacking a bulky phospholipid headgroup. As shown in Figure 3, the addition of 5 mol % of DOG into D-POPG monolayer dramatically increased the membrane penetrating power of cPLA₂. The comparison of $\Delta\pi$ vs π_0 plots in the presence and absence of DOG revealed the shift of π_c value from 27 dyn/cm to 37 dyn/cm. A similar result was obtained for D-POPC monolayer (increase in π_c value from 21 to 28 dyn/cm; data not shown). However, DOG did not have any effect on either the penetration of hpPLA₂ (Figure 3) or the penetration

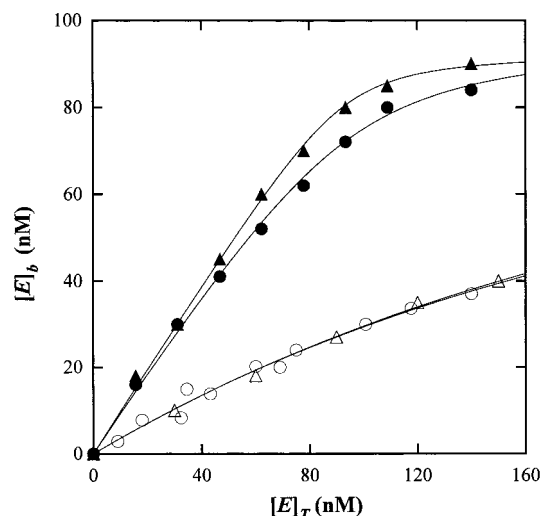


FIGURE 4: Binding of cPLA₂ to sucrose-loaded liposomes. Liposomes were polymerized D-BLPG (○), polymerized D-BLPG/DOG (99:1) (△), nonpolymerized D-BLPG (●) and nonpolymerized D-BLPG/DOG (99:1) (▲). Total lipid concentrations were kept at 100 μ M. The binding mixture contained 10 mM HEPES buffer, pH 7.5, 0.1 M KCl, and 0.5 mM CaCl₂. Each data represents an average of at least three independent measurements. Solid lines indicate theoretical curves constructed using eq 1 with n and K_d values determined from the nonlinear least-squares analysis. See under Materials and Methods for the determination of free and bound enzyme concentrations.

of cPLA₂ in the absence of Ca²⁺ in the subphase (data not shown). Thus, DOG selectively enhances the Ca²⁺-dependent membrane penetration of cPLA₂, thereby enabling it to penetrate into compactly packed membranes. This enhanced penetration would in turn increase hydrophobic interactions between cPLA₂ and membranes.

Binding to Nonpolymerized versus Polymerized Liposomes. To evaluate the importance of the membrane penetration of cPLA₂ and consequent hydrophobic interactions in its interfacial binding, we measured the equilibrium binding of cPLA₂ to sucrose-loaded polymerized and nonpolymerized D-BLPG liposomes. We previously showed that the polymerization of BLPG molecules in liposomes prevented their out-of-surface and lateral movement (28) and thereby greatly reduced the penetration of PLA₂ into the hydrophobic core of lipid bilayers (29). Since BLPG molecules in nonpolymerized liposomes are hydrolyzed by PLA₂ (28), we synthesized a nonhydrolyzable D-BLPG isomer and prepared its nonpolymerized and polymerized liposomes. Binding isotherms for cPLA₂ are shown in Figure 4 and the binding parameters calculated from the nonlinear least-squares analysis of data using Eq 1 are summarized in Table 1. K_d values showed that cPLA₂ bound nonpolymerized D-BLPG liposomes ca. 30 times more tightly than polymerized ones.

In addition, similar n values for polymerized and nonpolymerized liposomes revealed that the polymerization of liposomes did not change the liposome binding mode of cPLA₂. From these data, the contribution of hydrophobic interactions to the free energy of binding of cPLA₂ to nonpolymerized liposomes can be estimated using the equation $\Delta\Delta G^\circ = RT \ln(K_d \text{ for nonpolymerized liposomes} / K_d \text{ for polymerized liposomes})$. Under standard conditions with the concentration of free phospholipid set at 1 M, this corresponds to ca. -2.0 kcal/mol at 25 $^\circ$ C. Note that this value only represents a lower estimate since the polymerization would not completely block the penetration and hydrophobic interactions. These results thus indicate that the membrane penetration of cPLA₂ makes a significant contribution to its interfacial binding. The importance of hydrophobic interactions in the interfacial binding of cPLA₂ is further supported by the effect of DOG on the vesicle binding affinity of cPLA₂. As shown in Figure 4 and Table 1, cPLA₂ bound nonpolymerized BLPG vesicles containing 5 mol % DOG ca. three times more tightly than nonpolymerized BLPG vesicles alone. In contrast, DOG had no significant effect on the binding of cPLA₂ to polymerized BLPG vesicles. In conjunction with our monolayer data, it is thus evident that DOG increases the membrane affinity of cPLA₂ by enhancing its membrane penetration and hydrophobic interactions with membranes. Finally, vesicle polymerization had essentially no effect on the vesicle binding affinity of hpPLA₂ (Table 1), again indicating that hydrophobic interactions are not important in the interfacial binding of hpPLA₂. This also argues against the possibility that lower affinity of cPLA₂ for polymerized liposomes was due to anomalous physical properties of polymerized liposomes.

Interfacial Activity of cPLA₂ in Polymerizable Mixed Liposomes. To understand how the unique interfacial binding mode of cPLA₂ affects its interfacial catalysis, we measured the hydrolysis of cPLA₂ substrates inserted in nonpolymerized and polymerized D-BLPG liposomes. cPLA₂ has both PLA₂ activity and lysophospholipase activity, and as a result, it hydrolyzes both *sn*-1 and *sn*-2 acyl esters if 1,2-diacyl-*sn*-glycero-3-phospholipids are used as a substrate (44). PyAPC which has a nonhydrolyzable ether linkage at *sn*-1 position was thus used as a fluorogenic substrate for exclusively evaluating PLA₂ activity of cPLA₂. We have demonstrated the advantages of the kinetic system using BLPG-containing polymerized mixed liposomes in structure–function studies of secretory PLA₂ (18–21) and the determination of substrate specificity (27, 45). In this kinetic system, products of PLA₂ hydrolysis, fatty acid and lysophospholipid, are rapidly extracted from the liposome surfaces by BSA which results in a large fluorescence

Table 1: Binding of cPLA₂ and hpPLA₂ to Sucrose-Loaded Polymerized and NonPolymerized D-BLPG Liposomes^a

liposomes	cPLA ₂		hpPLA ₂	
	K_d (nM)	n	K_d (nM)	n
nonpolymerized D-BLPG	7.3 \pm 1.3	1100 \pm 90	17 \pm 5	300 \pm 50
polymerized D-BLPG	200 \pm 60	950 \pm 80	15 \pm 3	230 \pm 40
nonpolymerized D-BLPG/DOG	2.2 \pm 0.5	1100 \pm 100	ND	ND
polymerized D-BLPG/DOG	170 \pm 80	1000 \pm 100	ND	ND

^a See Materials and Methods for experimental conditions and methods to calculate binding constants. Values of n and K_d represent (best-fit values \pm standard errors) determined from nonlinear least-squares analyses of data. ND, not determined.

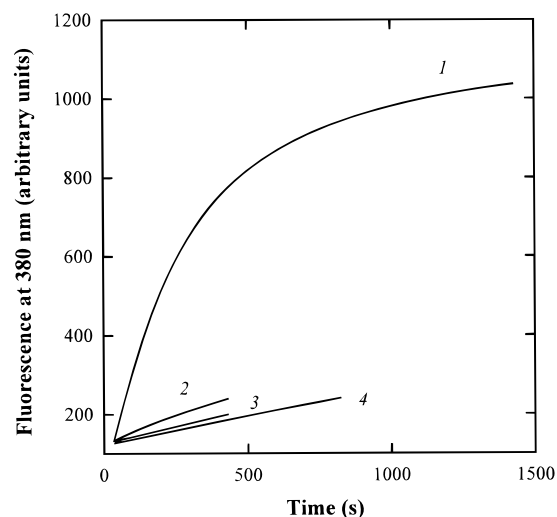


FIGURE 5: Time course of cPLA₂-catalyzed hydrolysis of 1 mol % PyArPC and PyOPC substrates inserted in nonpolymerized and polymerized D-BLPG liposomes. Curve 1. PyArPC/D-BLPG nonpolymerized mixed liposomes. Curve 2. PyOPC/D-BLPG nonpolymerized mixed liposomes. Curve 3. PyArPC/D-BLPG polymerized mixed liposomes. Curve 4. PyOPC/D-BLPG polymerized mixed liposomes. Total phospholipid concentrations were 5 μ M. Assay mixtures contained 20 mM HEPES, pH 8.0, 1 mM CaCl₂, and 10 μ M BSA. Reactions were initiated by adding enzyme (20 nM final concentration) to the mixtures.

emission intensity of pyrene moiety. In control experiments, it was shown that 1 mol % of 1-*O*-(1-pyrenedecyl)-2-hydroxy-*sn*-glycero-3-phosphocholine inserted in polymerized (or nonpolymerized) BLPG was rapidly removed from the liposome surfaces by BSA and that a secretory PLA₂ from *A. p. piscivorus* and hpPLA₂ readily hydrolyzed 1 mol % PyAPC inserted in polymerized (or nonpolymerized) BLPG under a standard kinetic condition described in the Materials and Methods section (data not shown). Furthermore, both hpPLA₂ and *A. p. piscivorus* PLA₂ showed comparable activities toward PyArPC in polymerized and nonpolymerized liposomes (data not shown), again demonstrating the lack of anomalous structural changes of liposomes due to polymerization, which would have resulted in slower extraction of PyAPC from the membrane surface by PLA₂ or slower removal of product from the membrane surface by BSA. Having established the feasibility of PyAPC/BLPG mixed liposomes as a PLA₂ assay system, we then measured the activity of cPLA₂ toward nonpolymerized and polymerized PyAPC/BLPG liposomes. The hydrolysis curves illustrated in Figure 5 demonstrate that cPLA₂ has much higher activity toward PyArPC in nonpolymerized mixed liposomes than toward the same substrate in polymerized mixed liposomes. As described in the Materials and Methods, relative activity of cPLA₂ toward different substrates was determined by comparing initial rates of hydrolysis. As summarized in Table 2, cPLA₂ was 20-fold less active toward PyArPC in polymerized mixed liposomes than toward the same substrate in nonpolymerized mixed liposomes. This difference compares well with 30-fold difference in binding affinity for nonpolymerized and polymerized D-BLPG liposomes. Thus, these data clearly indicate that the membrane penetration of cPLA₂ is an essential step not only in its interfacial binding but also in its interfacial catalysis and interactions with substrates.

Table 2: Relative Activity of cPLA₂ toward Various Pyrene-Labeled Substrates in Polymerized and Nonpolymerized D-BLPG Liposomes^a

substrates	<i>sn</i> -2 acyl group	in nonpolymerized mixed liposomes	in polymerized mixed liposomes
PyPPC	palmitoyl	8	2
PyOPC	oleoyl	8	2
PyArPC	arachidonoyl	100	5
PyArPE	arachidonoyl	170	7
PyArPG	arachidonoyl	730	8

^a Relative activities were calculated as percentage of the specific activity for PyArPC/D-BLPG nonpolymerized liposomes (2.0 ± 0.5 μ mol/min/mg). Specific activity was determined as described in the Materials and Methods.

Substrate Specificity of cPLA₂ in Polymerizable Mixed Liposomes. Two types of substrate specificity, *sn*-2 acyl group specificity and headgroup specificity, were determined in nonpolymerized and polymerized liposomes. When the *sn*-2 acyl group specificity was measured in nonpolymerized mixed liposome system, cPLA₂ exhibited about 12-fold higher activity toward PyArPC than toward PyOPC and PyPPC, which is comparable to reported arachidonate specificity of cPLA₂ determined in other kinetic systems (12, 46–48). In polymerized mixed liposome system, however, cPLA₂ hydrolyzed PyArPC only 2.5 times faster than PyOPC and PyPPC. Thus, cPLA₂ exhibited the pronounced arachidonate specificity only when its membrane penetration was allowed. Then, the activity of cPLA₂ was measured toward PyArPC and its PE (PyArPE) and PG (PyArPG) derivatives in nonpolymerized and polymerized D-BLPG liposomes. As listed in Table 2, cPLA₂ showed significant preference (4–7-fold) for anionic PG headgroup over electrically neutral ones in nonpolymerized mixed liposomes: between PC and PE, PE was hydrolyzed ca. two times faster. As is true for the *sn*-2 acyl specificity, cPLA₂ had much reduced overall activity and headgroup specificity for pyrene-labeled substrates inserted in polymerized D-BLPG liposomes. This again shows that the substrate specificity of cPLA₂ is fully expressed only when the enzyme is allowed to penetrate into membranes. Taken all together, these results indicate that the hydrolysis of phospholipids by cPLA₂ involves the penetration of enzyme into the bilayer and that the substrate specificity of cPLA₂ depends on this unique property of cPLA₂.

DISCUSSION

All data presented herein, the monolayer penetration data in particular, show that interfacial binding of cPLA₂ involves a larger degree of membrane penetration and hence a larger degree of hydrophobic interactions than that of secretory hpPLA₂. It should be noted that concentrations of monolayer-bound proteins were not determined in these studies due to experimental difficulties involved in the radiolabeling of the protein and counting of the monolayer-bound protein. Therefore, data presented herein could not provide quantitative information about the area (or surface pressure) change per bound molecule. As a result, our surface pressure measurements could not distinguish whether a larger surface pressure change for cPLA₂ than for hpPLA₂ under a given condition is due to a larger population of monolayer-bound cPLA₂ molecules or due to a larger degree of penetration

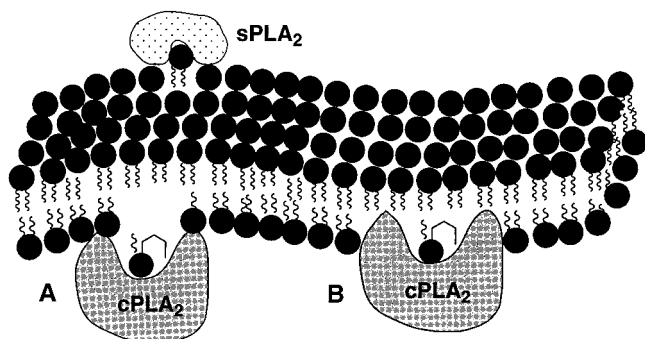


FIGURE 6: Hypothetical models of the membrane binding of hpPLA₂ and cPLA₂. hpPLA₂ and other secretory PLA₂s bind to membrane surface mainly by electrostatic interactions and only a part of phospholipid substrate molecule migrates into the active site, which explains the lack of *sn*-2 acyl specificity. In contrast, cPLA₂ can bind a larger portion of the substrate including four *cis* double bonds in the *sn*-2 arachidonoyl group. To achieve this binding, cPLA₂ can either bind to the membrane surface as many secretory PLA₂ do and extract a substrate into its active site (model A) or penetrate into the membrane to capture the substrate molecule in its active site (model B). Note that model B does not necessarily assume that the active site of the enzyme also penetrates into the phospholipid bilayer.

per bound cPLA₂ molecule. Due to this limitation, all monolayer penetration measurements were performed in the presence of saturating concentrations of cPLA₂ and hpPLA₂ in the subphase. That is, each $\Delta\pi$ value represents the maximal value for either enzyme at a given π_0 value. The measurements were designed this way to ensure that the $\Delta\pi$ versus π_0 plot reflects the intrinsic penetrating power of the protein as a function of monolayer packing density. Thus, the observed differences between cPLA₂ and hpPLA₂ are due more likely to the higher penetrating power of monolayer-bound cPLA₂ than to the larger population of monolayer-bound cPLA₂. In that many secretory PLA₂s, including mammalian pancreatic PLA₂s, bind to the membrane surface mainly by electrostatic interactions without significant membrane penetration (18, 20, 21), this finding represents the first experimental demonstration that cPLA₂ has a unique interfacial binding mechanism which is correlated with its unique interfacial enzymatic properties including arachidonate specificity. All known secretory PLA₂s show no significant *sn*-2 acyl specificity. Structures of several secretory PLA₂-inhibitor complexes show (49–53) that only about nine carbons in the *sn*-2 acyl chain are bound to the active site, and this provides an explanation for a lack of *sn*-2 acyl group specificity of secretory PLA₂s. Because of larger molecular size, cPLA₂ is likely, although not necessarily, to have a larger active-site cavity than secretory PLA₂s. Thus, if cPLA₂ binds to the membrane in the same manner as many secretory PLA₂s do (Figure 6, model A), the arachidonate specificity of cPLA₂ might derive primarily from its large active site that can fully accommodate a bulkier phospholipid molecule and interact favorably with the *sn*-2 arachidonoyl group. Smaller phospholipids would have lower binding affinity due to the lack of these interactions. This kind of binding mode, however, might not be energetically favorable because it entails a large degree of out-of-plane migration of a bulky phospholipid. Alternatively, cPLA₂ could penetrate into the membrane to bring its active site closer to (or within) the phospholipid bilayer, thereby facilitating the migration of an arachidonoyl-containing

phospholipid molecule into the active site (Figure 6, model B). Our results from kinetics of liposome hydrolysis, equilibrium liposome binding, and monolayer penetration all point to this model. First of all, cPLA₂ demonstrates its unique arachidonate specificity and headgroup selectivity for its liposome substrates only if the penetration of cPLA₂ into the liposomes is allowed. When the penetration is restricted by the polymerization of liposomes, cPLA₂ shows not only reduced arachidonate specificity but also much less overall activity. Second, cPLA₂ has reduced binding affinity for polymerized liposomes, the degree of which is comparable to the decrease in activity toward polymerized mixed liposomes. Finally, cPLA₂ has much higher membrane penetrating power than secretory hpPLA₂ under the same condition.

Due to the lack of tertiary structural information about cPLA₂, it is difficult to explain how exactly cPLA₂ achieves the membrane penetration. A recent study on the Ca²⁺-dependent membrane binding of independently expressed C2 domain of cPLA₂ provides some insights into the membrane binding mechanism of cPLA₂ (54). This study showed that the binding of two calcium ions to the C2 domain resulted in conformational changes and the membrane anchoring of the protein. Our monolayer data yielded similar but more detailed information about the role of calcium ions in interfacial binding of cPLA₂; promoting the surface adsorption and enhancing the penetration power of cPLA₂. Thus, it appears that calcium ions not only bring cPLA₂ to membrane surfaces presumably by forming a cPLA₂-Ca²⁺-phospholipid complex (55, 56) but also induce conformational changes of cPLA₂ to expose its hydrophobic residues, which are then inserted into the hydrocarbon region of the membrane. Although these studies do not present any direct evidence for Ca²⁺-induced conformational changes, it is a most likely mechanism for Ca²⁺-induced membrane penetration of cPLA₂. For instance, it is less likely that Ca²⁺ simply enhances the membrane binding of cPLA₂ which is always in a membrane-penetrating conformation in solution because cPLA₂ with exposed hydrophobic surfaces will then aggregate and precipitate in the absence of membranes due to hydrophobic effect. A similar model has been proposed for the membrane binding of a conventional protein kinase C (i.e., protein kinase C- α) which shares traits with cPLA₂ (57). A main difference is, however, that protein kinase C- α is capable of penetrating into compactly packed monolayers (i.e., $\pi_0 \geq 30$ dyn/cm) under comparable conditions, which is correlated with its ability to penetrate into biological membranes and its physiological activity. Although this difference suggests that cPLA₂ might not be able to penetrate into biological membranes even in the presence of Ca²⁺, it should be noted that the surface pressure of biological membranes was estimated using erythrocyte membranes (39) and model membranes (40–42). Since cPLA₂ was shown to translocate from the cytosol to the nuclear envelope (58) and endoplasmic reticulum (59) in stimulated cells, the fact that π_c for cPLA₂ is below 30 dyn/cm does not necessarily mean that it cannot penetrate its target membranes to bind and hydrolyze arachidonoyl-containing phospholipids. It is possible that these membranes have lower packing density than erythrocyte cell membranes. Also, the presence of a bulky arachidonoyl-containing phospholipid itself could lower the local packing density of membranes. The latter

possibility is supported by our finding that cPLA₂ displayed high specificity for 1 mol % PyArPC inserted in large nonpolymerized D-BLPG liposomes the surface pressure of which should be comparable to that of biological membranes. Finally, diacylglycerol might act as a cellular activator of cPLA₂ in addition to Ca²⁺ and phosphorylation, based on the selective enhancing effect of DOG on the membrane penetration and hydrophobic interfacial binding of cPLA₂. Many protein kinases C including protein kinase C- α have a specific binding site for diacylglycerols, whose binding to these enzymes leads to their activation presumably by greatly enhancing hydrophobic membrane-protein interactions. (57, 60–62). As expected from the ability of protein kinase C- α to penetrate into compactly packed membranes by itself, however, diacylglycerols do not directly affect the membrane penetration of proteins (57, 60, 61). For cPLA₂, diacylglycerols do not appear to bind to a specific site in cPLA₂ but rather increase its hydrophobic interactions with membranes by improving its membrane penetration. Although DOG was shown to induce phase separation on membrane surfaces, this requires a high concentration, typically above 20 mol % (63, 64). Furthermore, Das and Rand showed that calcium stabilizes the lamellar bilayer structure of DOG/anionic phospholipid mixtures (64). Thus, at a lower concentration of DOG used in these studies (i.e., 5 mol %), DOG would act as a spacer between phospholipids to spread their polar headgroups, which would in turn help cPLA₂ penetrate into the membrane. The role of diacylglycerol as a cellular activator of cPLA₂ in human amnionic WISH cells has been recently suggested (65). In conclusion, these studies show that cPLA₂ has a unique ability to penetrate into membranes which is essential for its catalytic actions and substrate specificity. These studies also highlight the importance of physical properties of membranes in regulation of cPLA₂ activities and thereby suggest that lipid cofactors that affect the membrane surface properties, most notably diacylglycerols, might have significant effects on cellular cPLA₂ activities.

ACKNOWLEDGMENT

We thank Yana Snitko for liposome binding studies of hpPLA₂.

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BI980888S