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

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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 PL A2 toward anionic and electrically neutral phospholipid monolayers. cPLA2 also showed 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-glycero-3-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) 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-dependent 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 arachidonate 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
Membrane Penetration of cPLA2

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-glycerol-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-glycerol-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 Frost 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 phenylmethylsulfonyl fluoride. Suspension was homogenized in a hand-held homogenizer chilled on ice. The extract was centrifuged at 10000g 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 (2 M KCl, 1 mM EDTA) to form a monolayer with a given surface pressure (σ0). 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 into the subphase and the change in surface pressure (Δτ) was measured as a function of time at room temperature. Typically, the Δτ value reached a maximum after 20 min. At a given σ0 of phospholipid monolayer, the maximal Δτ value depended on the protein concentration in the subphase and reached a saturation when the protein concentration was

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 (hPLA2) 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[2-(lipoxyloxy)docosanoyl]-sn-glycero-3-phosphocholine (BLPC) and -phosphoglycerol (BLPG) were synthesized as described elsewhere (28). 2,3-Bis[2-(lipoxyloxy)docosanoyl]-sn-glycero-1-phosphocholine (D-BLPC) and -glycerol (D-BLPG) were synthesized by the same protocol except that α-α-glycerophosphocholine (Biochemisches Labor, Bern, Switzerland) was used as a starting material instead of a l-isofrom. 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-glycerol-1-phosphoglycerol (D-POPG) was prepared from D-POPC by phospholipase-D-catalyzed transphosphatidylation as described (30). Purity of all δ-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 1-palmitoyl-rac-glycerol-3-phosphocholine (Sigma) 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 transphosphatidylation.

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 (σ) of solution in a circular Teflon trough was measured using a du Nouy ring attached to a computer-controlled Cahn electrabelance (model C-32) as described previously (29, 34). The trough (4 cm diameter × 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 (σ0). 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 (Δτ) was measured as a function of time at room temperature. Typically, the Δτ value reached a maximum after 20 min. At a given σ0 of phospholipid monolayer, the maximal Δτ 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 µg/mL of cPLA₂ and 1.5 µg/mL of hpPLA₂ at τ₀ = 5 dyn/cm). Protein concentrations in the subphase were therefore maintained above those values to ensure that observed Δτ values represent maximal ones at given τ₀ 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 (F₃₈₀) 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 (µmol/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 pelletted 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-pyrenecanoyl)-sn-glycero-3-phosphoglycerol/BLPG (1:99) polymerized liposomes for hpPLA₂ (27). Concentration of bound enzyme ([E]₀) was calculated from the difference in PLA₂ activity between controls and binding mixtures. Parameters n and Kd were determined by nonlinear least-squares analysis of the [E]₀ versus total enzyme concentration ([E]₅₀) plot using a standard binding equation:

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where [PL]₅₀ represents total phospholipid concentration. The above equation assumes that each enzyme binds indepen-

**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 (τ₀) was spread at constant area and the change in surface pressure (Δτ) was monitored after the injection of the protein into the subphase. All monolayer measurements were performed with D- and L-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, 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 τ₀. Figure 1 shows the time course of the penetration of cPLA₂ and hpPLA₂ into...
the D-POPG monolayer at $\pi_0 = 10$ dyn/cm. From curves 1 and 2, it is evident that Ca$^{2+}$ has a large effect on the monolayer penetration of cPLA$_2$. In the absence of Ca$^{2+}$, the magnitude of cPLA$_2$ penetration was comparable to that of hpPLA$_2$ and the kinetics of penetration showed a complex pattern. In the presence of 0.5 mM Ca$^{2+}$, cPLA$_2$ 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 Ca$^{2+}$ is higher than required for cellular activation of cPLA$_2$, this concentration was used to facilitate the binding of enzyme to monolayers. Lower Ca$^{2+}$ concentrations (e.g., 5–50 $\mu$M) resulted in essentially the same degree of $\Delta \pi$ but at slower rates (data not shown). Ca$^{2+}$ had no significant effect on the monolayer penetration of hpPLA$_2$. Thus, these data indicate that Ca$^{2+}$ specifically enhances the rate of membrane binding of cPLA$_2$ as well as its membrane penetrating ability. Then, we measured the penetration of cPLA$_2$ and hpPLA$_2$ into anionic D-POPG and electrically neutral D-POPC monolayers as a function of their initial surface pressure. As shown in Figure 2, Ca$^{2+}$-dependent penetration of cPLA$_2$ generally resulted in higher $\Delta \pi$ values than the penetration of hpPLA$_2$ at a given $\pi_0$. Again, the penetration of cPLA$_2$ into the D-POPG monolayer in the absence of Ca$^{2+}$ was comparable to that of hpPLA$_2$. The phospholipid composition of monolayer had different effects on the penetration of cPLA$_2$ and hpPLA$_2$. hpPLA$_2$ 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 PLA$_2$ (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 hpPLA$_2$, cPLA$_2$ in the presence of Ca$^{2+}$ showed only modestly reduced $\Delta \pi$ values for the electrically neutral D-POPC monolayer, which indicates that hydrophobic interactions make significant contributions to Ca$^{2+}$-dependent interfacial binding of cPLA$_2$. This finding also suggests that the role of Ca$^{2+}$ is not only to bring cPLA$_2$ 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 $\pi_0$ value and the extrapolation of the $\Delta \pi$ vs $\pi_0$ plot yields the critical surface pressure ($\pi_c$) which specifies an upper limit of $\pi_0$ of the monolayer that a protein can penetrate into (38). Figure 2 shows that the effect of Ca$^{2+}$ on the penetration of cPLA$_2$ becomes less pronounced with the increase of $\pi_0$, indicating that Ca$^{2+}$ greatly enhances the penetrating power of cPLA$_2$ into loosely packed membranes but not into compactly packed ones. As a result, $\pi_c$ values for cPLA$_2$ and D-POPG monolayer are 27 dyn/cm in the presence of Ca$^{2+}$ and 25 dyn/cm without Ca$^{2+}$, respectively. Since the estimated surface pressure of biological membranes is ca. 31 dyn/cm (39–42), cPLA$_2$ might not be able to penetrate into compactly packed biological membranes to perform its interfacial catalysis. To see if any lipid cofactors could help cPLA$_2$ overcome this potential problem, we measured the effect of DOG on the monolayer penetration of cPLA$_2$. Diacylglycerols including DOG have been shown to increase the activity of cPLA$_2$ 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 cPLA$_2$ 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 cPLA$_2$. The comparison of $\Delta \pi$ vs $\pi_0$ plots in the presence and absence of DOG revealed the shift of $\pi_c$ value from 27 dyn/cm to 37 dyn/cm. A similar result was obtained for D-POPC monolayer (increase in $\pi_c$ value from 21 to 28 dyn/cm; data not shown). However, DOG did not have any effect on either the penetration of hpPLA$_2$ (Figure 3) or the penetration...
of cPLA2 in the absence of Ca\(^{2+}\) in the subphase (data not shown). Thus, DOG selectively enhances the Ca\(^{2+}\)-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.

\textbf{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 lipidosome 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 \(\Delta G = RT \ln(K_d)\) for nonpolymerized liposomes/ \(K_d\) 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 °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.

\textbf{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>
<thead>
<tr>
<th>liposomes</th>
<th>(K_d) (nM)</th>
<th>(n)</th>
<th>(K_d) (nM)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonpolymerized D-BLPG</td>
<td>7.3 ± 1.3</td>
<td>1100 ± 90</td>
<td>17 ± 5</td>
<td>300 ± 50</td>
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<tr>
<td>polymerized D-BLPG</td>
<td>200 ± 60</td>
<td>950 ± 80</td>
<td>15 ± 3</td>
<td>230 ± 40</td>
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<tr>
<td>nonpolymerized D-BLPG/DOG</td>
<td>2.2 ± 0.5</td>
<td>1100 ± 100</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>polymerized D-BLPG/DOG</td>
<td>170 ± 80</td>
<td>1000 ± 100</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(a\) See Materials and Methods for experimental conditions and methods to calculate binding constants. Values of \(n\) and \(K_d\) represent (best-fit values ± standard errors) determined from nonlinear least-squares analyses of data. ND, not determined.
Membrane Penetration 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. cPLA2 has much higher activity toward PyArPC in nonpolymerized mixed liposomes than toward the same substrate in polymerized 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**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>sn-2 acyl group specific activity in nonpolymerized mixed liposomes</th>
<th>sn-2 acyl group specific activity in polymerized mixed liposomes</th>
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</thead>
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<tr>
<td>PyPPC palmitoyl</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>PyOPC oleoyl</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>PyArPC arachidonoyl</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>PyArPE arachidonoyl</td>
<td>170</td>
<td>7</td>
</tr>
<tr>
<td>PyArPG arachidonoyl</td>
<td>730</td>
<td>8</td>
</tr>
</tbody>
</table>

*Relative activities were calculated as percentage of the specific activity for PyArPC/D-BLPG nonpolymerized liposomes (2.0 ± 0.5 amol/min/mg). Specific activity was determined as described in the Materials and Methods.
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 Δπ value represents the maximal value for either enzyme at a given πo value. The measurements were designed this way to ensure that the Δπ versus πo 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 hpPLA2. 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, cPLA3 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., πo ≥ 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 πo 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.
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 amnion 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.

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REFERENCES


BIBLIOGRAPHY