Anionic lipids activate group IVA cytosolic phospholipase A_2 via distinct and separate mechanisms

Preeti Subramanian,* Mohsin Vora,† Luciana B. Gentile,* Robert V. Stahelin,[1,2,†,§] and Charles E. Chalfant[1,2,*,,††]**

Department of Biochemistry,* Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0614; Department of Biochemistry and Molecular Biology,† Indiana University School of Medicine, South Bend, IN 46617; Department of Chemistry and Biochemistry and Walther Center for Cancer Research,§ University of Notre Dame, South Bend, IN 46617; Research and Development,** Hunter Holmes McGuire Veterans Administration Medical Center, Richmond, VA 23249; and The Massey Cancer Center,†† Richmond, VA 23298

Abstract Previously, ceramide-1-phosphate (C1P) and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] were demonstrated to be potent and specific activators of group IVA cytosolic phospholipase A_2 (cPLA$_2\alpha$). In this study, we hypothesized that these anionic lipids functionally activated the enzyme by distinctly different mechanisms. Indeed, surface plasmon resonance and surface dilution kinetics demonstrated that C1P was a more potent effector than PI(4,5)P_2 in decreasing the dissociation constant of the cPLA$_2\alpha$-phosphatidylcholine (PC) interaction and increasing the residence time of the enzyme on the vesicles/micelles. PI(4,5)P_2, in contrast to C1P, decreased the Michaelis-Menten constant, increasing the catalytic efficiency of the enzyme. Furthermore, PI(4,5)P_2 activated cPLA$_2\alpha$ with a stoichiometry of 1:1 versus C1P at 2.4:1. Lastly, PI(4,5)P_2, but not C1P, increased the penetration ability of cPLA$_2\alpha$ into PC-rich membranes. Therefore, this study demonstrates two distinct mechanisms for the activation of cPLA$_2\alpha$ by anionic lipids. First, C1P activates cPLA$_2\alpha$ by increasing the residence time of the enzyme on membranes. Second, PI(4,5)P_2 activates the enzyme by increasing catalytic efficiency through increased membrane penetration.—Subramanian, P., M. Vora, L. B. Gentile, R. V. Stahelin, and C. E. Chalfant. **Anionic lipids activate group IVA cytosolic phospholipase A_2 via distinct and separate mechanisms. *J. Lipid Res.* 2007.** 48: **2701–2708.**

Supplementary key words ceramide-1-phosphate • ceramide kinase • eicosanoids • prostaglandins • inflammation • surface dilution kinetics • surface plasmon resonance • phosphatidylinositol-4,5-bisphosphate

Group IVA cytosolic phospholipase A_2 (cPLA$_2\alpha$) is the initial rate-limiting enzyme in eicosanoid biosynthesis in response to many inflammatory agonists (1, 2). The cellular activation of cPLA$_2\alpha$ requires Ca^{2+}-dependent membrane translocation of the enzyme, which is mediated by the N-terminal C2 domain (1–4). Cell-specific and agonist-dependent events coordinate the translocation of cPLA$_2\alpha$ to the nuclear envelope, endoplasmic reticulum, and Golgi apparatus via this domain (1–8). At these membranes, cPLA$_2\alpha$ hydrolyzes membrane phospholipids to produce arachidonic acid (AA), which initiates pathways leading to eicosanoid synthesis (1–8).

Two anionic lipids have been demonstrated to activate cPLA$_2\alpha$: ceramide-1-phosphate (C1P) and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] (5, 9, 10). C1P has been defined as the membrane lipid that enhances the association of the C2 domain of cPLA$_2\alpha$ with membranes at submicromolar calcium concentrations (11). Recent reports from our laboratory have demonstrated that ceramide kinase is an upstream mediator of calcium ionophore- and interleukin-1β-induced AA release and eicosanoid synthesis. Further studies revealed that cPLA$_2\alpha$ was required for C1P to induce AA release (11). Subsequently, C1P was shown to be an allosteric activator of cPLA$_2\alpha$ by enhancing the in vitro interaction of the enzyme with its membrane substrate phosphatidylcholine (PC) at the mechanistic level (9).

Using surface dilution kinetics coupled with surface plasmon resonance (SPR) technology, we previously demonstrated the role of C1P in regulating the association of cPLA$_2\alpha$ with PC-rich micelles/vesicles via basic amino acids in the β-groove of the C2 domain that were shown to be critical for the C1P-cPLA$_2\alpha$ interaction. With regard to phosphoinositides, Channon and Leslie (5) and Balsinde et al. (10) first showed that PI(4,5)P_2 was a potent activator

Manuscript received 9 August 2007 and in revised form 20 September 2007.

Published, JLR Papers in Press, September 21, 2007.
DOI 10.1194/jlr.M700356-JLR200

Abbreviations: AA, arachidonic acid; cPLA$_2\alpha$, group IVA cytosolic phospholipase A_2; C1P, ceramide-1-phosphate; K_d, dissociation constant; OPPC, 1-oleoyl-2-palmitoyl-phosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine; PC, phosphatidylcholine; PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; SPR, surface plasmon resonance.

[1] R. V. Stahelin and C. E. Chalfant contributed equally to this article.
[2] To whom correspondence should be addressed.
e-mail: rstaheli@iupui.edu (R.V.S.);
cechalfant@vcu.edu (C.E.C.)

Downloaded from www.jlr.org by guest, on June 14, 2012

of cPLA$_2$α. Mosior, Six, and Dennis (12) also showed that cPLA$_2$α binds with increased affinity to surfaces with PI(4,5)P$_2$ at physiologic concentrations with an increase in the substrate hydrolysis. Also, studies by Das and Cho (13) have identified a cluster of four basic amino acids localized to the catalytic domain (K488, K541, K543, K544) critical for PI(4,5)P$_2$ binding. In the current study, we identified the mechanistic difference in the activation of cPLA$_2$α by anionic lipids C1P and PI(4,5)P$_2$. We demonstrate that C1P acts primarily by decreasing the dissociation constant of the enzyme and increasing its residence time. On the other hand, PI(4,5)P$_2$ acts to increase the membrane penetration and the rate of substrate hydrolysis of the enzyme. Thus, the two anionic lipids act via two distinct mechanisms.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine (PAPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and used without further purification. [^{14}C]PAPC was purchased from American Radiolabeled Chemicals. 1,2-Dipalmitoyl derivatives of PI(4,5)P$_2$ were purchased from Cayman Chemical Co. (Ann Arbor, MI). Octyl glucoside and CHAPS were from Fisher Scientific (Hampton, NH). The Pioneer L1 sensor chip was from Biacore AB (Piscataway, NJ). Triton X-100 was purchased from Pierce. Phospholipid concentrations were determined by a modified Bartlett analysis (14). Restriction endonucleases and enzymes for molecular biology were obtained from New England Biolabs (Beverly, MA). C1P was prepared according to the published method by direct phosphorylation of D-erythro-C18:1-ceramide in 37% yield and >95% purity as determined by thin-layer chromatography, ^{1}H-NMR, ^{31}P-NMR, and mass spectrometry analysis (15).

Recombinant expression of cPLA$_2$α

Recombinant human cPLA$_2$α was expressed in Sf9 cells with a 6XHis tag using a baculovirus expression system and purified using a modified protocol as described previously (16, 17). Briefly, Sf9 cells were grown in suspension culture and infected with high-titer recombinant baculovirus at a multiplicity of infection of 10 for 72 h after infection. The cells were then harvested and resuspended in 10 ml of extraction buffer (50 mM Tris, pH 8.0, 200 mM KCl, 5 mM imidazole, 10 μg/ml leupeptin, and 1 mM PMSF) using a hand-held homogenizer. The cells were broken by 20 strokes with a Dounce homogenizer. The cell lysate was clarified by centrifugation at 100,000 *g* for 45 min at 4°C. The cleared lysate was batch-bound to 10 ml of nickel-nitrilotriacetic acid agarose for 30 min in a column. Once this solution passed through, the column was washed with 15 ml of buffer 1 (50 mM Tris, pH 7.2, 0.2 M KCl, 10 mM imidazole, and 10% glycerol). Subsequently, the column was washed with 15 ml of buffer 2 (50 mM Tris, pH 8.0, 0.1 M KCl, 15 mM imidazole, and 10% glycerol). Third, the column was washed with 15 ml of buffer 3 (50 mM Tris, pH 8.0, 0.1 M KCl, 20 mM imidazole, and 10% glycerol) The protein was eluted in 1 ml fractions using 10 ml of buffer 4 (50 mM Tris, pH 8.0, 0.1 M KCl, 250 mM imidazole, and 10% glycerol). The enzyme fractions were monitored using SDS-PAGE, and fractions containing significant amounts of cPLA$_2$α were pooled, concentrated, and desalted in a Ultracel YM-50 centrifugal filter device. Protein concentration was determined by the bicinchoninic acid method, and aliquots of 0.1 μg/μl were made using storage buffer (50 mM Tris, pH 7.4, 0.1 M KCl, and 30% glycerol).

SPR analysis

All SPR measurements were performed at 25°C. A detailed protocol for coating the L1 sensor chip has been described elsewhere (18, 19). Briefly, after washing the sensor chip surface, 90 μl of vesicles containing various phospholipids (**Table 1**) was injected at 5 μl/min to give a response of 6,500 resonance units. An uncoated flow channel was used as a control surface. Under our experimental conditions, no binding was detected to this control surface beyond the refractive index change for either the C2 domain or cPLA$_2$α (9, 18, 20). Each lipid layer was stabilized by injecting 10 μl of 50 mM NaOH three times at 100 μl/min. Typically, no decrease in lipid signal was seen after the first injection. Kinetic SPR measurements were done at a flow rate of 30 μl/min. A total of 90 μl of protein in 10 mM HEPES, pH 7.4, containing 0.16 M KCl, 5% glycerol, and 10 μM Ca^{2+} was injected to give an association time of 90 s, whereas the dissociation was monitored for 500 s or more. The lipid surface was regenerated using 10 μl of 50 mM NaOH. After sensorgrams were obtained for five different concentrations of each protein within a 10-fold range of the dissociation constant (K_d), each of the sensorgrams was corrected for refractive index change by subtracting the control surface response from it. The association and dissociation phases of all sensorgrams were globally fit to a 1:1 Langmuir binding model: protein + (protein binding site on vesicle) ↰ (complex), using BIAevalutation 3.0 software (Biacore) as described previously (9, 18, 20). The K_d was then calculated from the equation $K_d = k_d/k_a$. k_d is the membrane dissociation rate constant, and k_a is the membrane association constant. K_d is the dissociation constant calculated from the equation. A minimum of three data sets was collected for each protein. Equilibrium (steady-state) SPR measurements were performed with a flow rate

TABLE 1. Binding parameters for group IVA cytosolic phospholipase A$_2$ determined from surface plasmon resonance analysis

Lipid	k_a	k_d	K_d
	$M^{-1}\ s^{-1}$	s^{-1}	M
POPC	$(1.2 \pm 0.2) \times 10^5$	$(4.7 \pm 0.2) \times 10^{-3}$	$(3.9 \pm 0.7) \times 10^{-8}$
POPC/C1P (97:3)	$(2.4 \pm 0.3) \times 10^5$	$(1.4 \pm 0.3) \times 10^{-3}$	$(5.8 \pm 1.4) \times 10^{-9}$
POPC/C1P (95:5)	$(2.8 \pm 0.4) \times 10^5$	$(9.1 \pm 0.7) \times 10^{-4}$	$(3.3 \pm 0.5) \times 10^{-9}$
POPC/PI(4,5)P$_2$	$(1.7 \pm 0.2) \times 10^5$	$(3.0 \pm 0.3) \times 10^{-3}$	$(1.8 \pm 0.3) \times 10^{-8}$
POPC/PI(4,5)P$_2$	$(1.9 \pm 0.3) \times 10^5$	$(2.5 \pm 0.2) \times 10^{-3}$	$(1.3 \pm 0.2) \times 10^{-8}$

C1P, ceramide-1-phosphate; k_a, membrane association constant; k_d, membrane dissociation rate constant; K_d, dissociation constant; PI(4,5)P$_2$, phosphatidylinositol-4,5-bisphosphate. Values represent means ± SD from three determinations. All of the measurements were performed in 10 mM HEPES, pH 7.4, containing 0.16 M KCl, 10 μM Ca^{2+}, and 5% glycerol.

Downloaded from www.jlr.org by guest, on June 14, 2012

of 5 μl/min to allow sufficient time for the R values of the association phase to reach saturating response values (R_{eq}). R_{eq} values were then plotted versus protein concentrations (C), and the K_d value was determined by a nonlinear least-squares analysis of the binding isotherm using the equation $R_{eq} = R_{max}/(1 + K_d/C)$. Mass transport was not a limiting factor in our experiments, because change in flow rate (from 2 to 80 μl/min) did not affect the kinetics of association and dissociation. After curve-fitting, residual plots and Chi-square values were checked to verify the validity of the binding model. Each data set was repeated three times to calculate a standard deviation value.

Monolayer penetration analysis

The monolayer penetration of cPLA$_2$α into the phospholipid monolayer of different lipid compositions was measured in terms of the change in surface pressure (π) using a 1 ml circular Teflon trough and wire probe connected to a Kibron MicroTrough X (Kibron, Inc., Helsinki, Finland). A lipid monolayer containing various combinations of phospholipids was spread onto the subphase composed of either 10 mM HEPES, pH 7.4, containing 0.16 M KCl and 10 mM Ca^{2+} or 0.1 mM EGTA until the desired initial surface pressure (π_0) was reached. The signal stabilized quickly (~5 min), and 5 μg of protein was injected into the subphase through a hole in the wall of the trough. The surface pressure change (Δπ) was monitored for 45 min. The Δπ value reached a maximum after 25 min in all experiments.

Mixed-micelle assay for cPLA$_2$α

cPLA$_2$α activity was measured in a PC mixed-micelle assay in a standard buffer composed of 80 mM HEPES (pH 7.5), 150 mM NaCl, 10 μM free Ca^{2+}, and 1 mM DTT. The assay also contained 0.3 mM PAPC with 250,000 dpm [^{14}C]PAPC, 2 mM Triton X-100, 26% glycerol, and 500 ng of purified cPLA$_2$ protein in a total volume of 200 μl. To prepare the substrate, an appropriate volume of cold PAPC in chloroform, the indicated phospholipids, and [^{14}C]PAPC in toluene-ethanol (1:1) solution were evaporated under nitrogen. Triton X-100 was added to the dried lipid to give 4-fold concentrated substrate solution (1.2 mM PAPC). The solution was probe-sonicated on ice (1 min on, 1 min off for 3 min). The reaction was initiated by adding 500 ng of the enzyme and was stopped by the addition of 2.5 ml of Dole reagent (2-propanol, heptane, and 0.5 M H_2SO_4, 400:100:20, v/v/v). The amount of [^{14}C]AA produced was determined using the Dole procedure as described previously (21). All assays were conducted for 45 min at 37°C. Triton X-100 micelles provide an inert surface for cPLA$_2$ containing an average of 140 molecules per micelle (molecular weight = 95,000). Therefore, 1 mol% of the lipid is equivalent to 1.4 molecules per micelle and each micelle interacts with 2 molecules of enzyme in a ratio of 1:2. Statistical and kinetic analyses were performed using Sigma-Plot Enzyme Kinetics software, version 1.1, from SYSTAT Software, Inc.

Mixed-vesicle assay for cPLA$_2$α

cPLA$_2$α activity was measured in vesicles composed of 30 μM PAPC alone or 10 μM PAPC and 100 μM 1-oleoyl-2-palmitoylphosphatidylcholine (OPPC) in a standard assay buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 50 μM free Ca^{2+} with 250,000 dpm [^{14}C]PAPC. The lipid substrates were evaporated under nitrogen. The dried lipids were then brought up to the desired concentration in 50 mM Tris-HCl and sonicated on ice (1 min on, 1 min off for 3 min). The reaction was initiated by adding 500 ng of the enzyme and was stopped by the addition of 2.5 ml of Dole reagent (2-propanol, heptane, and 0.5 M H_2SO_4, 400:100:20, v/v/v). The amount of [^{14}C]AA produced was determined using the Dole procedure as described previously (21). All assays were conducted for 10 min at 37°C.

RESULTS

Activation of cPLA$_2$α in response to the anionic lipids C1P and PI(4,5)P$_2$

We first determined the number of molecules of the anionic lipids required for maximal activation of the enzyme in the mixed-micelle system. As shown in **Fig. 1A**, as low as 0.5 mol% of PI(4,5)P$_2$ achieved the maximal 15-fold activation of the enzyme. In contrast, between 2 and 3 mol% of C1P was required for the maximal 8-fold activation of the enzyme (Fig. 1B). These data correlate to a stoichiometry of ~1:1 for PI(4,5)P$_2$ per molecule of

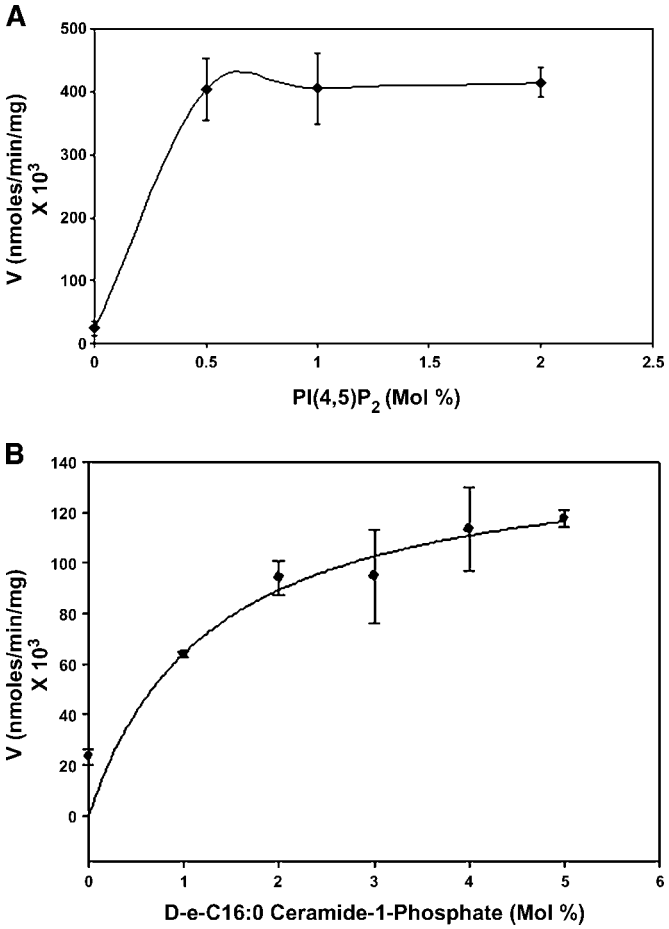

Fig. 1. Activation of group IVA cytosolic phospholipase A$_2$ (cPLA$_2$α) by ceramide-1-phosphate (C1P) and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$]. Recombinant cPLA$_2$α activity (0.5 μg) was assayed for 45 min at 37°C at 15 mol% of phosphatidylcholine (PC) [Triton X-100 + PC + PI(4,5)P$_2$/C1P]. A: cPLA$_2$α activity was measured at varying mol% of PI(4,5)P$_2$ ([PI(4,5)P$_2$]/[Triton X-100 + PC + PI(4,5)P$_2$]). B: cPLA$_2$α activity was measured at varying mol% of D-erythro-C16-C1P ([C1P]/[Triton X-100 + PC + C1P]). Data are presented as cPLA2α activity measured as nmoles of arachidonic acid produced/milligram of recombinant cPLA2α ± SEM. Data are representative of six separate determinations on three separate occasions.

Downloaded from www.jlr.org by guest, on June 14, 2012

$cPLA_2\alpha$ and ⩾2 molecules of C1P per molecule of $cPLA_2\alpha$. Thus, both C1P and $PI(4,5)P_2$ induce dramatic increases in the activity of the enzyme but different stoichiometries.

Effects of $PI(4,5)P_2$ and C1P on the membrane dissociation rate of $cPLA_2\alpha$

To demonstrate the effects of $PI(4,5)P_2$ and C1P on the dissociation rate constant or membrane residence time, we measured the kinetics and affinity of $cPLA_2\alpha$ binding with lipid vesicles using SPR. Both C1P and $PI(4,5)P_2$ increased the affinity of $cPLA_2\alpha$ for the membrane (6.7- and 3-fold, respectively), but C1P was a more potent effector (Table 1). The affinity increase observed in the interaction of C1P and $PI(4,5)P_2$ was mainly attributable to a decrease in k_d or an increase in membrane residence time (Table 1). Thus, C1P is more potent in decreasing the K_d of $cPLA_2\alpha$ for PC-rich membranes.

Effects of $PI(4,5)P_2$ and C1P on the K_d and the Michaelis-Menten constant of the reaction

In an effort to kinetically decipher the differences in the mechanisms of C1P and $PI(4,5)P_2$ interactions with $cPLA_2\alpha$, we used a surface dilution model of enzyme kinetics (22–26). Previous studies from our laboratory have demonstrated that C1P acts by decreasing the dissociation constant (K_s^A) of the enzyme by 80% and increasing the V_{max} of the reaction by ~8- to 10-fold (9). The dissociation constant, K_s^A, is equal to k_{-1}/k_1 and expressed in bulk concentration terms describing the interaction of the enzyme with the mixed micelles in the first binding step of the surface dilution model. In this study, the effect of $PI(4,5)P_2$ on the K_s^A of the reaction was examined using the same methodology. $PI(4,5)P_2$ induced a dramatic 15-fold increase in the V_{max} of the reaction but had only a small effect on the K_s^A of the reaction (**Fig. 2A**). This suggests that $PI(4,5)P_2$ does not have as great a role as C1P in mediating the membrane residence time of $cPLA_2\alpha$, corroborating the SPR data discussed above.

We also previously showed that C1P had no effect on the Michaelis-Menten constant (K_m^B) of the reaction, which is the second step of the surface dilution model (9). In this study, we found that with an increasing mole fraction of PC, $PI(4,5)P_2$ induced a 30–40% decrease in the K_m^B of the reaction, thereby increasing substrate hydrolysis (Fig. 2B).

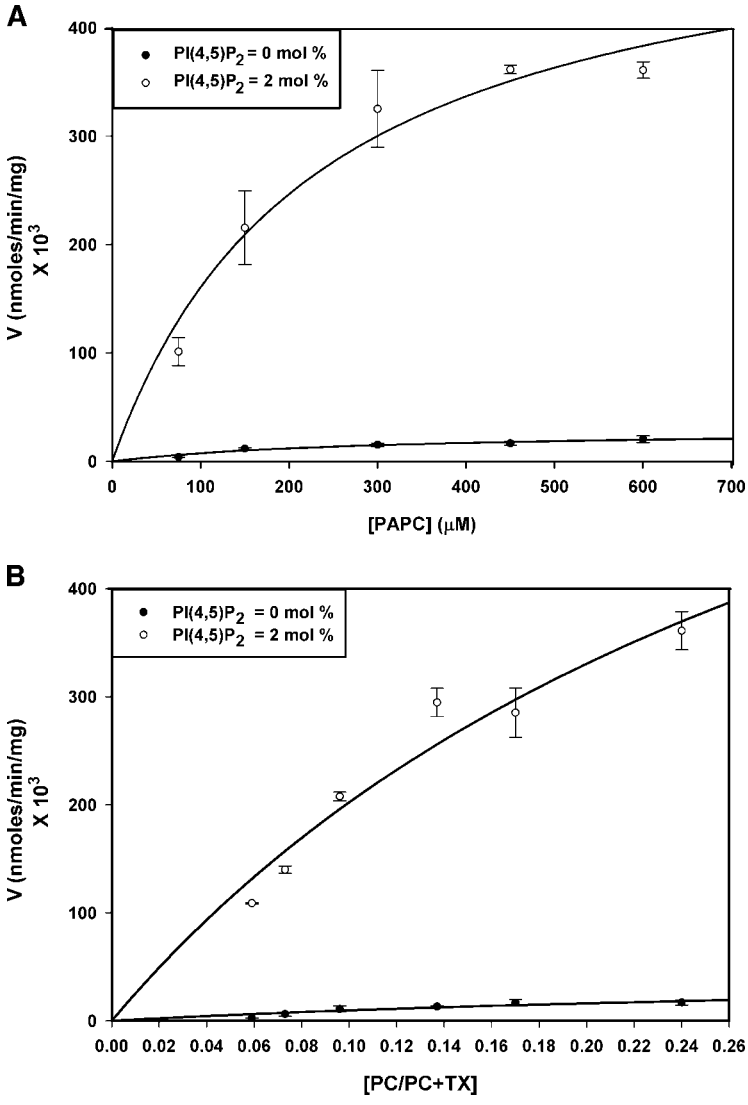

Fig. 2. Effects of $PI(4,5)P_2$ on the kinetic behavior of $cPLA_2\alpha$. A: The effect of $PI(4,5)P_2$ on the dissociation constant of $cPLA_2\alpha$. $cPLA_2\alpha$ activity was measured as a function of PC molar concentration in the absence (closed squares) and presence (open squares) of 2 mol% $PI(4,5)P_2$ for 45 min at 37°C. The PC mole fraction was held constant at 0.137. Data are presented as $cPLA_2\alpha$ activity measured as nmol arachidonic acid (AA) produced/min/mg recombinant $cPLA_2\alpha$ ± SEM. Data are representative of six separate determinations on three separate occasions. PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine. B: The effect of $PI(4,5)P_2$ on the Michaelis-Menten constant of $cPLA_2\alpha$. $cPLA_2\alpha$ activity was measured as a function of the mole fraction of PC ([PC]/[PC + Triton X-100 + $PI(4,5)P_2$]) in the absence (closed squares) and presence (open squares) of 2 mol% $PI(4,5)P_2$ for 45 min at 37°C. Data are presented as $cPLA_2\alpha$ activity measured as nmol AA produced/min/mg recombinant $cPLA_2\alpha$ ± SEM. Data are representative of six separate determinations on three separate occasions.

Downloaded from www.jlr.org by guest, on June 14, 2012

Thus, the two anionic lipids activate cPLA$_2\alpha$ via different mechanisms: C1P having an effect on K_s^A, and PI(4,5)P$_2$ having an effect on the K_m^B of the reaction.

Effects of C1P and PI(4,5)P$_2$ on the membrane penetration of cPLA$_2\alpha$

A number of studies have demonstrated the ability of cPLA$_2\alpha$ to penetrate zwitterionic membranes using hydrophobic residues in the Ca^{2+} binding loops of the C2 domain (16, 27, 28). Additionally, Das and Cho (13) suggested that exposed hydrophobic residues on the rim of the catalytic domain near the active site of cPLA$_2\alpha$ might also contribute to the penetration of the enzyme. Our finding that C1P and PI(4,5)P$_2$ bind to distinct sites in the C2 and catalytic domains (29), respectively, suggests that these lipids may have distinct roles in either inducing or enhancing the membrane penetration of cPLA$_2\alpha$. To assess the roles of these anionic lipids in membrane penetration, we used monolayer penetration analysis using a Kibron MicroTrough (Kibron, Inc.). Because the surface pressures of cell membranes and large unilamellar vesicles have been estimated to be in the range of 30–35 mN/m (30–32), for a protein to penetrate bilayer membranes its π_c value should be >30 mN/m.

As shown in **Fig. 3A**, 3 mol% of C1P did not significantly influence the monolayer penetration of cPLA$_2\alpha$ to PC monolayers, as both with and without C1P the π_c value (x-intercept) was ~28 mN/m. However, when 3 mol% of PI(4,5)P$_2$ was added to the monolayer, cPLA$_2\alpha$ penetration increased significantly to a π_c of ~34 mN/m. PI(4,5)P$_2$ could increase the penetration of cPLA$_2\alpha$ by enhancing the penetration of the C2 domain, the catalytic domain, or both. To assess these possibilities, we monitored the penetration of hydrophilic mutants in the C2 domain (V97A) and the catalytic domain (I399/L400A) in the full-length enzyme. Both mutations reduced penetration to PC monolayers and PC monolayers containing 3 mol% C1P (Fig. 3B). However, I399/L400A but not V97A displayed reduced penetration in the presence of PI(4,5)P$_2$ (Fig. 3C). This demonstrates that PI(4,5)P$_2$ binding to the catalytic domain enhances/induces the penetration of hydrophobic residues around the rim of the active site to enhance the activity of cPLA$_2\alpha$.

Comparison of the effects of C1P versus PI(4,5)P$_2$ on surface dilution in a vesicle-based assay

Traditionally, cPLA$_2\alpha$ activity has been measured using a vesicle-based assay (5). This assay requires 1,2-dioleoyl glycerol or ceramide for cPLA$_2\alpha$ activation, both of which act by increasing the spacing of the substrate and easier membrane penetration of the enzyme. Under these conditions, C1P only induced a 2-fold increase in the activity (**Fig. 4A**). We manipulated the substrate presentation in the vesicle-based system to demonstrate the role of C1P in increasing the residence time of the enzyme. We generated vesicles containing the substrate PAPC mixed with excess OPPC, which diluted the substrate in the vesicle. Then, upon the addition of 20 mol% of C1P to the vesicle, there was a 5-fold increase in the activity. PI(4,5)P$_2$ induced a 2-fold increase in the activity irrespective of the substrate dilution, confirming a different mode of action for both anionic lipids (Fig. 4B). Thus, C1P more significantly enhances the binding of the enzyme to vesicles, in contrast to the PI(4,5)P$_2$ interaction.

C1P competes with PI(4,5)P$_2$ for activation of cPLA$_2\alpha$

We measured the activity of cPLA$_2\alpha$ in response to both C1P and PI(4,5)P$_2$ in the same micelle at the mol% of maximal activation of each phospholipid. As shown in **Fig. 5**, C1P alone showed an 8-fold increase in the activity and PI(4,5)P$_2$ showed a 16-fold increase in the activity. However, upon the addition of both C1P and PI(4,5)P$_2$ to the micelle, there was no synergistic or additive increase in the activity of the enzyme. Furthermore, addition of PI(4,5)P$_2$ to the C1P-containing micelles did not further increase the activity of the enzyme than that with only C1P. The lack of a synergistic or additive effect was also observed using the vesicle-based assay (data not shown). These data suggest that C1P competes with PI(4,5)P$_2$ for cPLA$_2\alpha$ activation as a result of the higher affinity for C1P.

DISCUSSION

In this study, for the first time, we have explored the mechanistic differences in the activation of cPLA$_2\alpha$ by the anionic lipids C1P and PI(4,5)P$_2$. We have confirmed our previous findings that C1P acts by decreasing the dissociation of the enzyme from the membrane and, thereby, increasing the residence time of the enzyme on membranes. We have also expanded these studies using a new surface dilution model in a vesicle-based assay. Importantly, this study demonstrated that PI(4,5)P$_2$ acts by increasing the membrane penetration and catalytic efficiency of the enzyme, thus increasing substrate hydrolysis. Therefore, the two anionic lipids act via distinct mechanisms for the activation of cPLA$_2\alpha$.

Our first indicator of two distinct mechanisms for C1P and PI(4,5)P$_2$ was the stoichiometry of the activation of cPLA$_2\alpha$ by PI(4,5)P$_2$. PI(4,5)P$_2$ only required ~0.5 mol% to achieve maximal activation. Thus, the interaction of PIP$_2$ and cPLA$_2\alpha$ is in a stoichiometry of 1:1. As shown previously by our laboratory, C1P required ⩾2 molecules of C1P per molecule of cPLA$_2\alpha$ to achieve maximal activation. This stoichiometry of C1P suggests that C1P interacts with cPLA$_2\alpha$ differently than PI(4,5)P$_2$, possibly as a dimeric/multimeric complex analogous to phosphatidylserine and protein kinase C (33).

Building upon these contrasting stoichiometries, further studies found that C1P differed from PI(4,5)P$_2$ on the actual mechanism of activation. Specifically, PI(4,5)P$_2$ failed to induce a dramatic effect on the K_s^A of the reaction (only a 30% decrease), whereas previous studies from our laboratory demonstrated that C1P decreased the K_s^A of the reaction by 80%. In contrast, PI(4,5)P$_2$ decreased the K_m^B of the reaction by 30–40%, whereas C1P did not have any effect on the K_m^B of the reaction (9). Examining the mechanism by which PI(4,5)P$_2$ affected the catalytic effi-

Downloaded from www.jlr.org by guest, on June 14, 2012

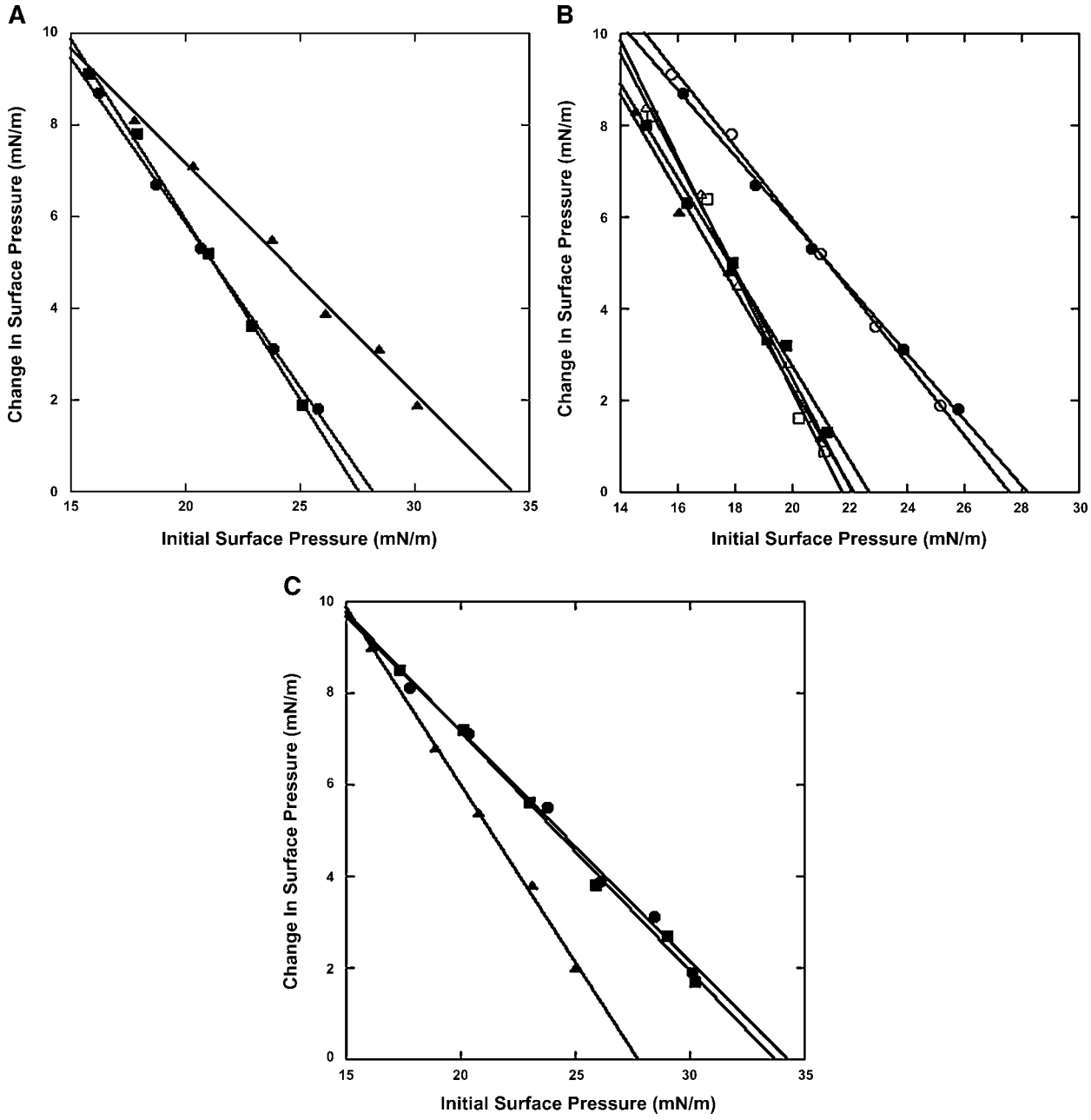

Fig. 3. Monolayer penetration of cPLA$_2\alpha$ with PI(4,5)P$_2$ and C1P. A: Penetration of wild-type cPLA$_2\alpha$ into a POPC (circles), POPC/C1P (97:3) (squares), or POPC/PI(4,5)P$_2$ (triangles) monolayer was monitored as a function of π_0. The subphase was 10 mM HEPES, pH 7.4, containing 0.16 M KCl and 10 μM Ca^{2+}. B: Penetration of wild-type cPLA$_2\alpha$ (circles), V97A (squares), and I399/L400A (triangles) was monitored into a POPC monolayer and then a POPC/C1P monolayer (open symbols). The subphase was 10 mM HEPES, pH 7.4, containing 0.16 M KCl and 10 μM Ca^{2+}. C: Penetration of cPLA$_2\alpha$ (circles), V97A (squares), and I399/L400A (triangles) was monitored into a POPC/PI(4,5)P$_2$ (97:3) monolayer. The subphase was 10 mM HEPES, pH 7.4, containing 0.16 M KCl and 10 μM Ca^{2+}.

ciency of cPLA$_2\alpha$, the enzyme demonstrated increased membrane penetration in the presence of PIP$_2$, whereas C1P was ineffective. Thus, PIP$_2$ activates cPLA$_2\alpha$ by enhancing the catalytic ability of the enzyme once bound to the membrane/vesicle/micelle through enhanced membrane penetration. On the other hand, C1P enhances the activity of cPLA$_2\alpha$ by increasing the residence time of the enzyme on the membrane (increased time to identify and hydrolyze substrate).

The effects of C1P and PI(4,5)P$_2$ on the activation of cPLA$_2\alpha$ was further demonstrated using a newly developed surface dilution assay, which uses vesicles in place of micelles. Using this assay, we modified the substrate presentation of cPLA$_2\alpha$ in the vesicle by increasing the dilution of the substrate, PAPC, with OPPC. This approach diluted the substrate without significantly affecting the PC content of the vesicle [more similar to a Golgi membrane in composition (34)]. We found that upon diluting the

Downloaded from www.jlr.org by guest, on June 14, 2012

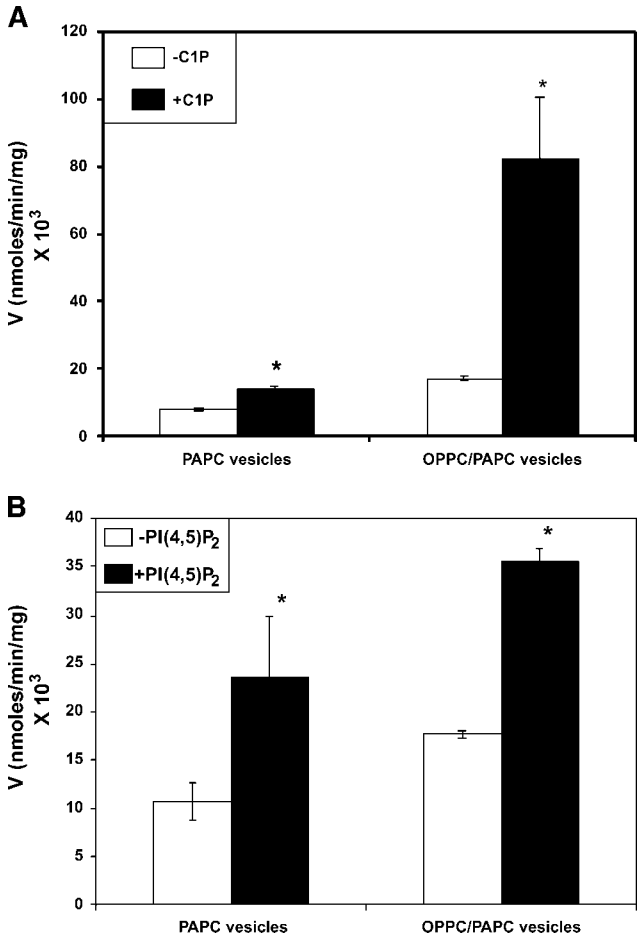

Fig. 4. Effects of C1P versus $PI(4,5)P_2$ on surface dilution in a vesicle-based assay. Recombinant $cPLA_2\alpha$ (0.5 μg) activity for 10 min at 37°C was assayed in vesicles containing PAPC alone or PAPC/1-oleoyl-2-palmitoyl-phosphatidylcholine (OPPC). A: $cPLA_2\alpha$ activity in the absence (open squares) and presence (closed squares) of 20 mol% of CIP. B: $cPLA_2\alpha$ activity in the absence (open squares) and presence (closed squares) of 20 mol% of $PI(4,5)P_2$. Data are presented as $cPLA_2\alpha$ activity measured as nmol AA produced/min/mg recombinant $cPLA_2\alpha$ ± SEM. Data are representative of six separate determinations on three separate occasions. * $P < 0.05$, by two-tailed *t*-test.

substrate PAPC with OPPC, the activity of $cPLA_2\alpha$ was increased by 5-fold in the presence of C1P versus an 80% increase observed using vesicles composed of only PAPC. $PI(4,5)P_2$ under these conditions increased the activity of the enzyme by 2-fold regardless of the vesicle composition. Furthermore, addition of both C1P and $PI(4,5)P_2$ together to the same vesicle did not give a synergistic increase in the activity, suggesting a different mechanism for both anionic lipids. These findings further demonstrate that $PI(4,5)P_2$ activates $cPLA_2\alpha$ at the membrane and C1P recruits the enzyme and increases residence time. This is supported by the notion of increased membrane penetration of the catalytic domain in response to $PI(4,5)P_2$ but not C1P. Instead, C1P increases the residence time through electrostatic interactions with cationic residues in the C2 domain (29).

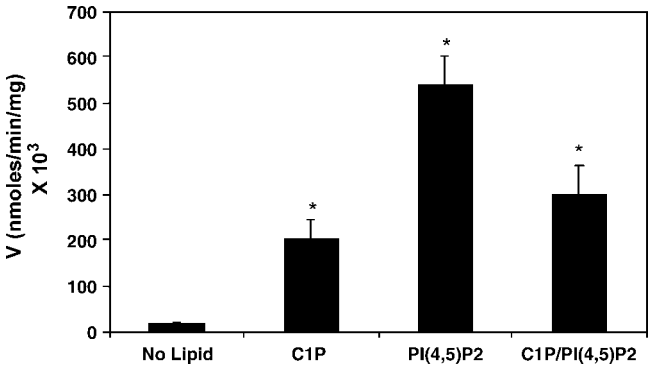

Fig. 5. C1P competes with $PI(4,5)P_2$ for activation of $cPLA_2\alpha$. Recombinant $cPLA_2\alpha$ was assayed in the presence of no lipid [Triton X-100 + PC], 4 mol% of C1P [Triton X-100 + PC + C1P], 2 mol% of $PI(4,5)P_2$ [Triton X-100 + PC + $PI(4,5)P_2$], and 4 mol% of C1P + 2 mol% of $PI(4,5)P_2$ [Triton X-100 + PC + C1P + $PI(4,5)P_2$] for 45 min at 37°C. The PC mol% for all reactions was held constant at 15 mol%. Data are presented as $cPLA_2\alpha$ activity measured as nmol AA produced/min/mg recombinant $cPLA_2\alpha$ ± SEM. Data are representative of six separate determinations on three separate occasions. * $P < 0.01$, by two-tailed *t*-test.

In the last part of this study, we explored the possibility that $PI(4,5)P_2$ and C1P act synergistically or additively on $cPLA_2\alpha$ activity. Surprisingly, the addition of both lipids to mixed micelles failed to activate the enzyme above the effect of C1P alone. Thus, C1P and $PI(4,5)P_2$ do not coordinately activate $cPLA_2\alpha$ in vitro. These data are also interesting given the observation that the addition of C1P negated the $PI(4,5)P_2$ activation of the enzyme. Therefore, C1P, having higher affinity for $cPLA_2\alpha$, recruited the enzyme to the micelle, not allowing any interaction with $PI(4,5)P_2$. These data suggest that the lipids may play different signaling roles in the context of cells. Another possibility is that C1P and $PI(4,5)P_2$ may be localized in lipid subdomains and require colocalization at a specific distance to activate $cPLA_2\alpha$ in a coordinated manner. With the binding sites for these anionic lipids in close proximity in three-dimensional space, this is a distinct possibility (29). Furthermore, randomized addition of these anionic lipids to vesicles or mixed micelles would not simulate this mechanism and would explain the lack of additive or synergistic effects.

In conclusion, this study demonstrates three important findings: *1*) $PI(4,5)P_2$ and C1P activate $cPLA_2\alpha$ via separate mechanisms; *2*) $PI(4,5)P_2$ activates $cPLA_2\alpha$ by increasing the enzyme's catalytic efficiency and membrane penetration once bound to the membrane; and *3*) C1P and $PI(4,5)P_2$ do not activate $cPLA_2\alpha$ in a coordinated manner in vitro. These studies open an entirely new avenue to investigate the nature and orientation of $cPLA_2\alpha$ at the C1P and $PI(4,5)P_2$ membrane interface through lipid penetration analysis (16), electron paramagnetic resonance (35–37), X-ray reflectivity studies (38), or molecular dynamics simulations (36). Furthermore, we can begin to ask questions such as: Do these lipids activate $cPLA_2\alpha$ in cells in a coordinated manner? How? Can we simulate this effect in vitro, or are these lipids important for entirely different signaling mechanisms?

Downloaded from www.jlr.org by guest, on June 14, 2012

This work was supported by grants from the Veteran's Administration (VA Merit Review I to C.E.C.), from the National Institutes of Health (Grants HL-072925 and CA-117950 to C.E.C.), and from the American Heart Association (Predoctoral Fellowship AHA 5-30693 to P.S.). This research was partially supported by an Indiana University Biomedical Research Grant and by American Heart Association National Scientist Development Grant 0735350N to R.V.S. The authors thank Dr. Darrell Peterson and Mario A. Saavedra for their help and support with the 6XHis tag purification.

REFERENCES

1. Leslie, C. C. 1997. Properties and regulation of cytosolic phospholipase A2. *J. Biol. Chem.* **272:** 16709–16712.
2. Clark, J. D., A. R. Schievella, E. A. Nalefski, and L. L. Lin. 1995. Cytosolic phospholipase A2. *J. Lipid Mediat. Cell Signal.* **12:** 83–117.
3. Clark, J. D., L. L. Lin, R. W. Kriz, C. S. Ramesha, L. A. Sultzman, A. Y. Lin, N. Milona, and J. L. Knopf. 1991. A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell.* **65:** 1043–1051.
4. Kramer, R. M., and J. D. Sharp. 1997. Structure, function and regulation of Ca^{2+}-sensitive cytosolic phospholipase A2 (cPLA2). *FEBS Lett.* **410:** 49–53.
5. Channon, J. Y., and C. C. Leslie. 1990. A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A2 with membrane in the macrophage cell line RAW 264.7. *J. Biol. Chem.* **265:** 5409–5413.
6. Nalefski, E. A., L. A. Sultzman, D. M. Martin, R. W. Kriz, P. S. Towler, J. L. Knopf, and J. D. Clark. 1994. Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain. *J. Biol. Chem.* **269:** 18239–18249.
7. Reynolds, L. J., L. L. Hughes, A. I. Louis, R. M. Kramer, and E. A. Dennis. 1993. Metal ion and salt effects on the phospholipase A2, lysophospholipase, and transacylase activities of human cytosolic phospholipase A2. *Biochim. Biophys. Acta.* **1167:** 272–280.
8. Sharp, J. D., D. L. White, X. G. Chiou, T. Goodson, G. C. Gamboa, D. McClure, S. Burgett, J. Hoskins, P. L. Skatrud, J. R. Sportsman, et al. 1991. Molecular cloning and expression of human Ca(2+)-sensitive cytosolic phospholipase A2. *J. Biol. Chem.* **266:** 14850–14853.
9. Subramanian, P., R. V. Stahelin, Z. Szulc, A. Bielawska, W. Cho, and C. E. Chalfant. 2005. Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2alpha and enhances the interaction of the enzyme with phosphatidylcholine. *J. Biol. Chem.* **280:** 17601–17607.
10. Balsinde, J., M. A. Balboa, W. H. Li, J. Llopis, and E. A. Dennis. 2000. Cellular regulation of cytosolic group IV phospholipase A2 by phosphatidylinositol bisphosphate levels. *J. Immunol.* **164:** 5398–5402.
11. Pettus, B. J., A. Bielawska, P. Subramanian, D. S. Wijesinghe, M. Maceyka, C. C. Leslie, J. H. Evans, J. Freiberg, P. Roddy, Y. A. Hannun, et al. 2004. Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. *J. Biol. Chem.* **279:** 11320–11326.
12. Mosior, M., D. A. Six, and E. A. Dennis. 1998. Group IV cytosolic phospholipase A2 binds with high affinity and specificity to phosphatidylinositol 4,5-bisphosphate resulting in dramatic increases in activity. J Biol Chem 273:2184–2191.
13. Das, S., and W. Cho. 2002. Roles of catalytic domain residues in interfacial binding and activation of group IV cytosolic phospholipase A2. *J. Biol. Chem.* **277:** 23838–23846.
14. Kates, M. 1986. Techniques of Lipidology. Elsevier Science Publishers, New York. 114–115.
15. Byun, H. S., R. K. Erkulla, and R. Bittman. 1994. Synthesis of sphingomyelin and ceramide-1-phosphate from ceramide without protection of the allylic hydroxyl group. *J. Org. Chem.* **59:** 6495–6498.
16. Bittova, L., M. Sumandea, and W. Cho. 1999. A structure-function study of the C2 domain of cytosolic phospholipase A2. Identification of essential calcium ligands and hydrophobic membrane binding residues. *J. Biol. Chem.* **274:** 9665–9672.
17. Das, S., J. D. Rafter, K. P. Kim, S. P. Gygi, and W. Cho. 2003. Mechanism of group IVA cytosolic phospholipase A(2) activation by phosphorylation. *J. Biol. Chem.* **278:** 41431–41442.
18. Stahelin, R. V., and W. Cho. 2001. Differential roles of ionic, aliphatic, and aromatic residues in membrane-protein interactions: a surface plasmon resonance study on phospholipases A2. *Biochemistry.* **40:** 4672–4678.
19. Stahelin, R. V., J. D. Rafter, S. Das, and W. Cho. 2003. The molecular basis of differential subcellular localization of C2 domains of protein kinase C-alpha and group IVa cytosolic phospholipase A2. *J. Biol. Chem.* **278:** 12452–12460.
20. Stahelin, R. V., F. Long, K. Diraviyam, K. S. Bruzik, D. Murray, and W. Cho. 2002. Phosphatidylinositol 3-phosphate induces the membrane penetration of the FYVE domains of Vps27p and Hrs. *J. Biol. Chem.* **277:** 26379–26388.
21. Ulevitch, R. J., Y. Watanabe, M. Sano, M. D. Lister, R. A. Deems, and E. A. Dennis. 1988. Solubilization, purification, and characterization of a membrane-bound phospholipase A2 from the P388D1 macrophage-like cell line. *J. Biol. Chem.* **263:** 3079–3085.
22. Carman, G. M., R. A. Deems, and E. A. Dennis. 1995. Lipid signaling enzymes and surface dilution kinetics. *J. Biol. Chem.* **270:** 18711–18714.
23. Deems, R. A., B. R. Eaton, and E. A. Dennis. 1975. Kinetic analysis of phospholipase A2 activity toward mixed micelles and its implications for the study of lipolytic enzymes. *J. Biol. Chem.* **250:** 9013–9020.
24. Hendrickson, H. S., and E. A. Dennis. 1984. Kinetic analysis of the dual phospholipid model for phospholipase A2 action. *J. Biol. Chem.* **259:** 5734–5739.
25. Hendrickson, H. S., and E. A. Dennis. 1984. Analysis of the kinetics of phospholipid activation of cobra venom phospholipase A2. *J. Biol. Chem.* **259:** 5740–5744.
26. Roberts, M. F., R. A. Deems, and E. A. Dennis. 1977. Human type 2 phosphatidic acid phosphohydrolases. Substrate specificity of the type 2a, 2b, and 2c enzymes and cell surface activity of the 2a isoform. *Proc. Natl. Acad. Sci. USA.* **74:** 1950–1954.
27. Lichtenbergova, L., E. T. Yoon, and W. Cho. 1998. Membrane penetration of cytosolic phospholipase A2 is necessary for its interfacial catalysis and arachidonate specificity. *Biochemistry.* **37:** 14128–14136.
28. Stahelin, R. V., and W. Cho. 2001. Roles of calcium ions in the membrane binding of C2 domains. *Biochem. J.* **359:** 679–685.
29. Stahelin, R. V., P. Subramanian, M. Vora, W. Cho, and C. E. Chalfant. 2007. Ceramide-1-phosphate binds group IVA cytosolic phospholipase A2 via a novel site in the C2 domain. *J. Biol. Chem.* **282:** 20467–20474.
30. Blume, A. 1979. A comparative study of the phase transitions of phospholipid bilayers and monolayers. *Biochim. Biophys. Acta.* **557:** 32–44.
31. Demel, R. A., W. S. Geurts van Kessel, R. F. Zwaal, B. Roelofsen, and L. L. van Deenen. 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. *Biochim. Biophys. Acta.* **406:** 97–107.
32. Marsh, D. 1996. Lateral pressure in membranes. *Biochim. Biophys. Acta.* **1286:** 183–223.
33. Bell, R. M., Y. A. Hannun, and C. R. Loomis. 1986. Mechanism of regulation of protein kinase C by lipid second messengers. *Symp. Fundam. Cancer Res.* **39:** 145–156.
34. Lamour, N. F., R. V. Stahelin, D. S. Wijesinghe, M. Maceyka, E. Wang, J. C. Allegood, A. H. Merrill, Jr., W. Cho, and C. E. Chalfant. 2007. Ceramide kinase uses ceramide provided by ceramide transport protein: localization to organelles of eicosanoid synthesis. *J. Lipid Res.* **48:** 1293–1304.
35. Frazier, A. A., M. A. Wisner, N. J. Malmberg, K. G. Victor, G. E. Fanucci, E. A. Nalefski, J. J. Falke, and D. S. Cafiso. 2002. Membrane orientation and position of the C2 domain from cPLA2 by site-directed spin labeling. *Biochemistry.* **41:** 6282–6292.
36. Jaud, S., D. J. Tobias, J. J. Falke, and S. H. White. 2007. Self-induced docking site of a deeply embedded peripheral membrane protein. *Biophys. J.* **92:** 517–524.
37. Malmberg, N. J., D. R. Van Buskirk, and J. J. Falke. 2003. Membrane-docking loops of the cPLA2 C2 domain: detailed structural analysis of the protein-membrane interface via site-directed spin-labeling. *Biochemistry.* **42:** 13227–13240.
38. Malkova, S., F. Long, R. V. Stahelin, S. V. Pingali, D. Murray, W. Cho, and M. L. Schlossman. 2005. X-ray reflectivity studies of cPLA2{alpha}-C2 domains adsorbed onto Langmuir monolayers of SOPC. *Biophys. J.* **89:** 1861–1873.

Downloaded from www.jlr.org by guest, on June 14, 2012