

# Ceramide 1-Phosphate Acts as a Positive Allosteric Activator of Group IVA Cytosolic Phospholipase A<sub>2</sub>α and Enhances the Interaction of the Enzyme with Phosphatidylcholine\*<sup>§</sup>

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Previous findings from our laboratory have demonstrated that cPLA<sub>2</sub>α is directly activated by the emerging bioactive sphingolipid, ceramide 1-phosphate (C-1-P) (1). In this study, a Triton X-100/phosphatidylcholine (PC) mixed micelle assay was utilized to determine the kinetics and specificity of this lipid-enzyme interaction. Using this assay, the addition of C-1-P induced a dramatic increase in the activity of cPLA<sub>2</sub>α (>15-fold) with a K<sub>a</sub> of 2.4 mol % C-1-P/Triton X-100 micelle. This activation was highly specific as the addition of other lipids had insignificant effects on cPLA<sub>2</sub>α activity. Studies using surface-dilution kinetics revealed that C-1-P had no effect on the Michaelis-Menten constant, K<sub>m</sub><sup>B</sup>, but decreased the dissociation constant (K<sub>s</sub><sup>A</sup>) value by 87%. Thus, C-1-P not only increases the membrane affinity of cPLA<sub>2</sub>α but also may act as an allosteric activator of the enzyme. Surface plasmon resonance analysis of the C-1-P/cPLA<sub>2</sub>α interaction verified a decrease in the dissociation constant, demonstrating that cPLA<sub>2</sub>α bound PC vesicles containing C-1-P with increased affinity (5-fold) compared with PC vesicles alone. The effect on the dissociation rate of cPLA<sub>2</sub>α was also found to be lipid-specific with the exception of phosphatidylinositol 4,5-bisphosphate, which caused a modest increase in vesicle affinity (2-fold). Lastly, the binding site for C-1-P was determined to be within the C2-domain of cPLA<sub>2</sub>α, unlike phosphatidylinositol 4,5-bisphosphate. These data demonstrate a novel interaction site for C-1-P and suggest that C-1-P may function to recruit cPLA<sub>2</sub>α to intracellular membranes as well as allosterically activate the membrane-associated enzyme.

Group IVA cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>α)<sup>1</sup> was first characterized in platelets and macrophage cells and subsequently cloned from a macrophage cDNA library (2–8). The cDNA of cPLA<sub>2</sub>α encodes a 85-kDa protein, and the mRNA for cPLA<sub>2</sub> is widely expressed in brain, lung, kidney, heart, and spleen (2–8). cPLA<sub>2</sub>α is the major phospholipase that regulates eicosanoid synthesis in response to inflammatory agonists (2, 3). *In vitro*, cPLA<sub>2</sub>α is activated by Ca<sup>2+</sup>; however, the addition of salt at physiologic concentrations will also induce enzyme activation and thus the catalytic activity of cPLA<sub>2</sub>α is not dependent on Ca<sup>2+</sup> (2, 9, 10). The cellular activation of cPLA<sub>2</sub>α requires Ca<sup>2+</sup>-dependent membrane translocation of the enzyme, which is mediated by the N-terminal C2 domain (2–5). Cell-specific and agonist-dependent events coordinate translocation of cPLA<sub>2</sub>α to the nuclear envelope, endoplasmic reticulum, and Golgi apparatus via this domain (2–7, 9, 10). At these membranes, cPLA<sub>2</sub>α hydrolyzes membrane phospholipids to produce arachidonic acid, initiating the eicosanoid synthetic pathways (2–7, 9, 10). However, the specific membrane lipids that regulate the association of this domain with membranes, especially at low cellular calcium concentration (*e.g.* submicromolar), have yet to be defined (4).

One possible candidate for an activator of cPLA<sub>2</sub>α was hypothesized from an unlikely source. The main component of the venom from *Loxosceles reclusa* (brown recluse spider) is the enzyme sphingomyelinase D (11), which hydrolyzes sphingomyelin to produce ceramide 1-phosphate (C-1-P). The pathology of a wound generated from the bite of this spider consists of an intense inflammatory response mediated by AA and prostaglandins (12–14). Thus, the production of endogenous C-1-P by the action of sphingomyelinase D correlated with phospho-

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains Supplemental Fig. 1.

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<sup>1</sup> The abbreviations used are: cPLA<sub>2</sub>α, group IVA cytosolic phospholipase A<sub>2</sub>; C-1-P, ceramide 1-phosphate; AA, arachidonic acid; IL-1β, interleukin-1β; PAPS, 1-palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine; PG, phosphatidylglycerol; CER, ceramide; S-1-P, sphingosine-1-phosphate; DAG, *sn*-1,2-dioleoylglycerol; LPA, lysophosphatidic acid; mol %, mole percentage of mixed-micelle; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PA, phosphatidic acid; PI, phosphoinositide; PS, phosphatidylserine; PtdIns-3-P, phosphatidylinositol-3-phosphate; PtdIns-4-P, phosphatidylinositol-4-phosphate; PtdIns-5-P, phosphatidylinositol-5-phosphate; PtdIns-3,4-P<sub>2</sub>, phosphatidylinositol-3,4-bisphosphate; PtdIns-4,5-P<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PtdIns-3,4,5-P<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; SPR, surface plasmon resonance.

lipase A<sub>2</sub> activation, suggesting that C-1-P may act as an endogenous and proximal activator of a PLA<sub>2</sub> species.

C-1-P is a new addition to a growing group of bioactive sphingolipids, which include ceramide and sphingosine-1-phosphate. C-1-P is generated in mammalian cells by the phosphorylation of ceramide at the 1-position by the enzyme ceramide kinase, and recently, our laboratory reported (15) that ceramide kinase is an upstream mediator of calcium ionophore- and IL-1β-induced AA release. In a separate study, the mechanism by which C-1-P induced AA release was determined. Our laboratory showed that C-1-P activated cPLA<sub>2</sub>α in cells inducing the translocation of the enzyme to the Golgi apparatus and perinuclear membranes (1). Further studies disclosed that cPLA<sub>2</sub>α was required for C-1-P to induce AA release (1). Lastly, C-1-P was shown to directly interact with cPLA<sub>2</sub>α *in vitro* (1).

In this study, the interaction between cPLA<sub>2</sub>α and C-1-P was investigated at the mechanistic level. C-1-P was found to specifically and dramatically enhance the activity of cPLA<sub>2</sub>α, acting as a positive allosteric activator. Surface dilution kinetics coupled with surface plasmon resonance (SPR) technology also demonstrated that C-1-P regulated the association of cPLA<sub>2</sub>α with PC-rich micelles/vesicles. Furthermore, the presented study clearly shows that C-1-P interacts with cPLA<sub>2</sub>α via a previously undescribed binding site, thereby validating C-1-P as a novel activator of cPLA<sub>2</sub>α.

#### EXPERIMENTAL PROCEDURES

**Materials**—1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), cardiolipin, cholesterol, sphingosine-1-phosphate (S-1-P), and *sn*-1,2-dioleoylglycerol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. The purity of the lipids used was >99%. 1,2-Dipalmitoyl derivatives of phosphatidylinositol 3-phosphate (PtdIns-3-P), phosphatidylinositol 4-phosphate (PtdIns-4-P), phosphatidylinositol 5-phosphate (PtdIns-5-P), phosphatidylinositol 3,4-bisphosphate (PtdIns-3,4-P<sub>2</sub>), phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P<sub>2</sub>), and phosphatidylinositol 3,4,5-trisphosphate PtdIns-3,4,5-P<sub>3</sub> were a kind gift from Dr. Karol S. Bruzik and synthesized as described elsewhere (16). Triton X-100, octyl glucoside, and CHAPS were from Sigma and Pierce. Phospholipid concentrations were determined by a modified Bartlett analysis (17). [ $\gamma$ -<sup>32</sup>P]ATP (3 Ci/ $\mu$ mol) was from Amersham Biosciences. Restriction endonucleases and enzymes for molecular biology were obtained from New England Biolabs (Beverly, MA). Pioneer L1 sensor chip was from Biacore AB (Piscataway, NJ). Ceramide 1-phosphate 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, <sup>1</sup>H NMR, <sup>31</sup>P NMR, and mass spectrometry analysis (18).

**Recombinant Expression of cPLA<sub>2</sub>α**—Recombinant human cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>α) was expressed in Sf9 cells with a His<sub>6</sub> tag using a baculovirus expression system and purified using a modified protocol as previously described (19, 20). Sf9 cells were grown in suspension culture and infected with high titer recombinant baculovirus at a multiplicity of infection of 10 and 72 h post-infection. The cells were then harvested and resuspended in 1160 ml of extraction buffer (50 mM Tris, pH 8.0, 200 mM KCl, 5 mM imidazole, 10  $\mu$ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) using a hand-held homogenizer. The cells were broken by 20 strokes with a Dounce homogenizer. The cell lysate was clarified by centrifugation at 85,000  $\times$  g for 45 min at 4 °C. The cleared lysate was batch-bound to 80 ml of nickel-nitrilotriacetic acid-agarose for 60 min. The resin was captured over a Buchner funnel and suspended in 80 ml of Buffer 1 (50 mM Tris, pH 8.0, 0.2 M KCl, 10 mM imidazole, 10% glycerol) and packed in a 5.0-cm-diameter column at 8.0 ml/min. The column was washed with 455 ml of Buffer 1 at 8.0 ml/min followed by wash with 360 ml of Buffer 2 (50 mM Tris, pH 8.0, 0.1 M KCl, 15 mM imidazole, 10% glycerol) at 8 ml/min and a final wash with 270 ml of Buffer 3 (50 mM Tris, pH 8.0, 0.1 M KCl, 20 mM imidazole, 10% glycerol) at 8 ml/min. The column was then eluted with 200 ml of Buffer 4 (50 mM Tris, pH 8.0, 0.1 M KCl, 250 mM imidazole, 10% glycerol) at 8 ml/min. The fractions were analyzed by SDS-PAGE, and fractions containing significant amounts of cPLA<sub>2</sub>α were pooled and

dialyzed against storage buffer (50 mM Tris, pH 7.4, 0.1 M KCl, 30% glycerol). The dialyzed material was then centrifuged at 39,000  $\times$  g for 30 min to remove precipitate and filtered through a 0.2- $\mu$ m syringe filter and dispensed in 5-ml aliquots.

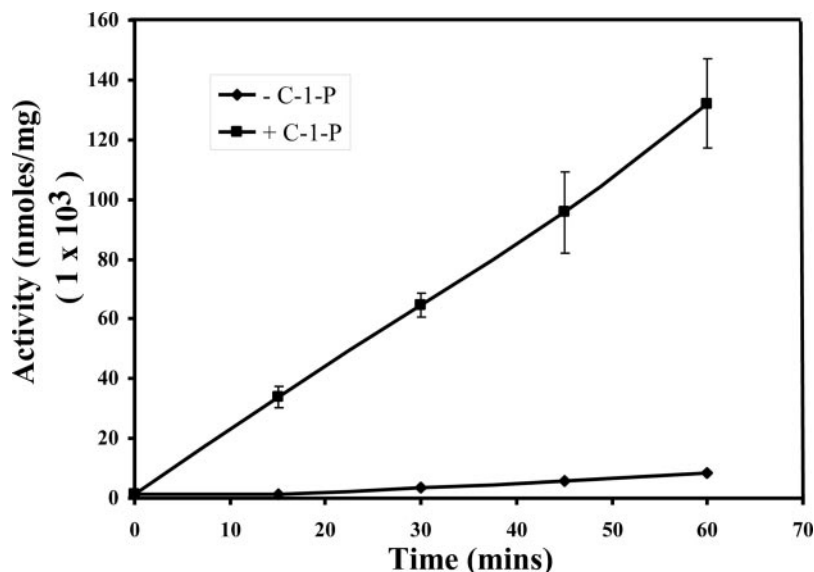
**Mixed Micelle Assay for cPLA<sub>2</sub>α**—cPLA<sub>2</sub>α activity was measured in a PC-mixed micelle assay in a standard buffer composed of 80 mM HEPES, pH 7.5, 150 mM NaCl, 10  $\mu$ M free Ca<sup>2+</sup>, and 1 mM dithiothreitol. The assay also contained 0.3 mM 1-palmitoyl-2-arachidonoylphosphatidylcholine (PAPC) with 250,000 dpm of [<sup>14</sup>C]PAPC, 2 mM Triton X-100, 26% glycerol, and 500 ng of purified cPLA<sub>2</sub>α protein in a total volume of 200  $\mu$ l. To prepare the substrate, an appropriate volume of cold PAPC in chloroform, indicated phospholipids, and [<sup>14</sup>C]PAPC in toluene/ethanol 1:1 solution were evaporated under nitrogen. Triton X-100 was added to the dried lipid to give a 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, 0.5 M H<sub>2</sub>SO<sub>4</sub>; 400:100:20, v/v/v). The amount of [<sup>14</sup>C]AA produced was determined using the Dole procedure as previously described (21). All of the assays were conducted for 45 min at 37 °C with the exception of those presented for various times in Fig. 1. Statistical and kinetic analysis was performed using Sigma-Plot Enzyme Kinetics software, version 1.1, from SYSSTAT software, Inc.

**Surface Plasmon Resonance Analysis**—All of the SPR measurements were performed at 23 °C. A detailed protocol for coating the L1 sensor chip has been described elsewhere (22, 23). After washing the sensor chip surface, 90  $\mu$ l of vesicles containing various phospholipids were injected at 5  $\mu$ l/min to give a response of 6000 resonance units (RU). 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<sub>2</sub>α (24, 25). Each lipid layer was stabilized by injecting 10  $\mu$ l of 50 mM NaOH three times at 100  $\mu$ l/min. Typically, no decrease in lipid signal was seen after the first injection. Kinetic SPR measurements were done at the flow rate of 30  $\mu$ l/min. 90  $\mu$ l of protein in 10 mM HEPES, pH 7.4, containing 0.16 M KCl and varying [Ca<sup>2+</sup>] 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  $\mu$ l of 50 mM NaOH. After sensorgrams were obtained for five different concentrations of each protein within a 10-fold range of *K<sub>d</sub>*, 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 of the sensorgrams were globally fit to a 1:1 Langmuir binding model: protein + (protein binding site on vesicle)  $\leftrightarrow$  (complex) using BIAevaluation 3.0 software (Biacore) as described previously (22, 24, 26). The dissociation constant (*K<sub>d</sub>*) was then calculated from the equation, *K<sub>d</sub>* = *k<sub>d</sub>*/*k<sub>a</sub>*. Equilibrium (steady-state) SPR measurements were performed with the flow rate of 5  $\mu$ l/min to allow sufficient time for the *R* values of the association phase to reach saturating response values (*R<sub>eq</sub>*). *R<sub>eq</sub>* values were then plotted *versus* protein concentrations (*C*), and the *K<sub>d</sub>* value was determined by a nonlinear least-squares analysis of the binding isotherm using an equation, *R<sub>eq</sub>* = *R<sub>max</sub>*/(1 + *K<sub>d</sub>*/*C*). Mass transport (27, 28) was not a limiting factor in our experiments, because change in flow rate (from 2 to 60  $\mu$ l/min) did not affect kinetics of association and dissociation. After curve fitting, residual plots and  $\chi^2$  values were checked to verify the validity of the binding model. Each data set was repeated three times to calculate a standard deviation value. The interaction of cPLA<sub>2</sub> with PC and PC/C-1-P was independent of the His<sub>6</sub> tag as determined by SPR (see Supplemental Table I).

#### RESULTS

**Ceramide 1-Phosphate Is a Potent Activator of cPLA<sub>2</sub>α**—Our laboratory has previously shown that C-1-P activated cPLA<sub>2</sub>α *in vitro* using a mixed vesicle-based assay (1). A main problem with this mixed vesicle-based assay was the complexity of kinetics that hampered the robust determination of kinetic parameters in the absence and presence of C-1-P. Therefore, it was difficult to quantitatively assess the effect of C-1-P on cPLA<sub>2</sub>α activity. To overcome this problem, we employed a mixed micelle assay in this study. There are several other rationales for now using the detergent mixed micelle assay. First, Triton X-100 micelles provide an inert surface for cPLA<sub>2</sub>α containing an average of 140 molecules/micelle, *M<sub>r</sub>* = 95,000. The size of the micelles is relatively independent of ionic

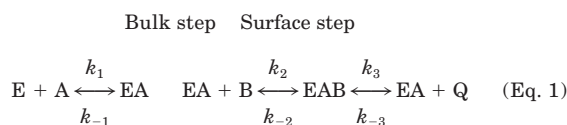
FIG. 1. C-1-P enhances the enzymatic activity of cPLA<sub>2</sub>α. Recombinant cPLA<sub>2</sub>α (0.5 μg) was assayed in the absence (◆) or presence (■) of 4 mol % D-e-C<sub>18:1</sub> ceramide 1-phosphate as described under "Experimental Procedures" for the indicated times. Data are presented as cPLA<sub>2</sub>α activity measured as nanomoles of arachidonic acid produced per milligram of recombinant cPLA<sub>2</sub>α ± S.E. Data are representative of 12 separate determinations on 6 separate occasions.



strength and temperature within the physiological range (29–32). Mixed micelles up to 20 mol % phospholipids are similar in structure to pure Triton X-100 micelles but proportionally larger (29, 30, 32). Furthermore, pure Triton X-100 micelles do not co-exist with mixed micelles. Lastly, a clear advantage of this kinetic system is that cPLA<sub>2</sub>α activity remains linear for at least 60 min (Fig. 1). The introduction of 5 mol % D-e-C<sub>18:1</sub> ceramide 1-phosphate into the Triton X-100 micelles induced a dramatic increase in cPLA<sub>2</sub>α activity (>15-fold) without affecting the linearity of the mixed-micelle assay (Fig. 1). Thus, ceramide 1-phosphate is a potent activator of cPLA<sub>2</sub>α, dramatically increasing the catalytic activity of the enzyme.

**The Stoichiometry of the cPLA<sub>2</sub>α/Ceramide 1-Phosphate Interaction**—A mixed micelle assay also has another advantage over mixed-vesicle assays in that mixed micelles provide a homogeneous, physically defined system to investigate stoichiometry and specificity of cPLA<sub>2</sub>α activation by lipids. Taking advantage of this system, Fig. 2 demonstrates that the activation of cPLA<sub>2</sub>α by ceramide 1-phosphate was saturatable and significant activation was observed with as little as 0.5 mol % D-e-C<sub>18:1</sub> ceramide 1-phosphate. Maximal activation of cPLA<sub>2</sub>α was observed with micelles containing >4.0 mol % D-e-C<sub>18:1</sub> ceramide 1-phosphate (>5.6 molecules of C-1-P/micelle) with a  $K_a$  of 2.4 mol % (3.4 molecules of C-1-P/micelle). Comparing the number of micelles to the number of molecules of cPLA<sub>2</sub>α in the reaction revealed a ratio of 1 micelle/2 molecules of cPLA<sub>2</sub>α. This implies that cPLA<sub>2</sub>α interacts with two or more molecules of ceramide 1-phosphate to achieve full activation.

**C-1-P Lowers the Dissociation Constant ( $K_s^A$ ) of the Micelle/cPLA<sub>2</sub>α Interaction but Has No Effect on the Michaelis-Menten Constant ( $K_m^B$ ) of the Reaction**—To study the kinetic behavior of the C-1-P/cPLA<sub>2</sub>α interaction, we used the surface dilution model of enzyme kinetics (33–37). This model takes into account both two-dimensional surface interaction and three-dimensional bulk interaction between an interfacial enzyme and lipid substrates. The principle of the surface dilution model is presented in Equation 1.



The equation demonstrates the action of enzyme ( $E$ ) in two consecutive steps. First, the enzyme interacts noncatalytically with the surface of detergent/lipid micelles ( $A$ ). Subsequently,

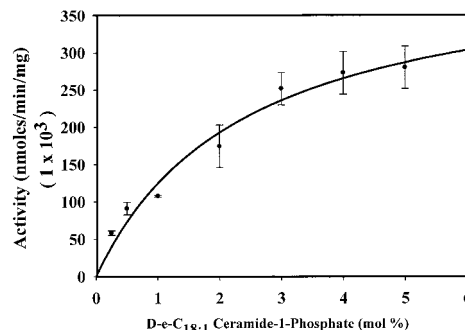


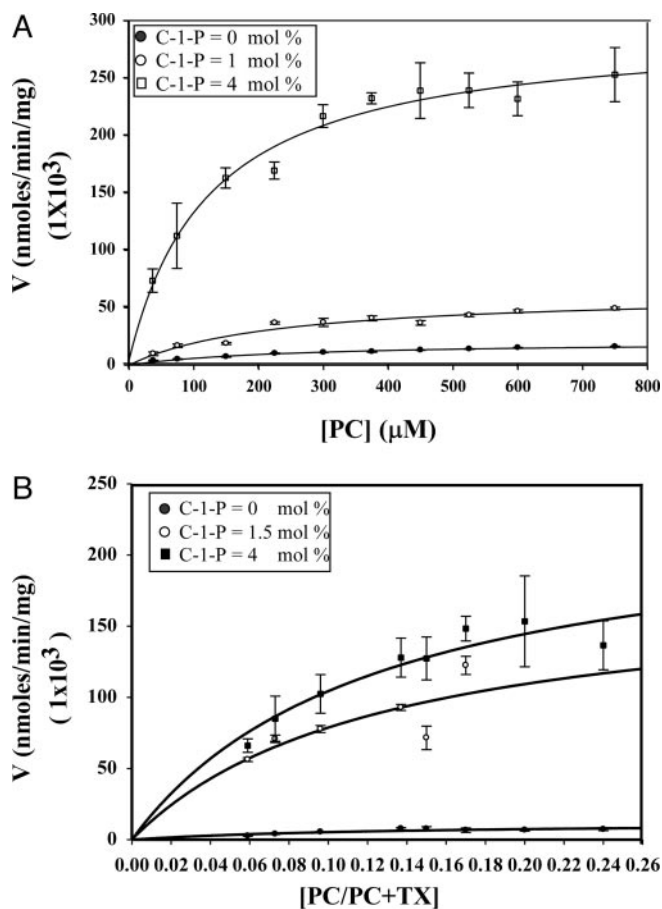
FIG. 2. The stoichiometry of the activation of cPLA<sub>2</sub>α by C-1-P. Recombinant cPLA<sub>2</sub>α (0.5 μg) was assayed in the presence of various mol % D-e-C<sub>18:1</sub> ceramide 1-phosphate ([C-1-P]/[Triton X-100 + PC + C-1-P]) for 45 min at 37 °C as described under "Experimental Procedures." The mol % PC was fixed at 15 mol% [Triton X-100 + PC + C-1-P]. Data are presented as cPLA<sub>2</sub>α activity measured as nanomoles of arachidonic acid produced/minute/milligram of recombinant cPLA<sub>2</sub>α ± S.E. Data are representative of six separate determinations on three separate occasions.

the enzyme-mixed micelle complex ( $EA$ ) binds to an individual substrate ( $B$ ) at its catalytic site, which leads to the hydrolysis of substrate to product ( $Q$ ). Here,  $A$  represents the sum of the molar concentrations of detergent and lipid substrate and  $B$  is the mole fraction of lipid substrate in the mixed micelle. The rate expression for surface dilution kinetic model (33, 34, 36) is shown in Equation 2.

$$V = \frac{V_{\max}(A)(B)}{K_s^A K_m^B + K_m^B(A) + (A)(B)} \quad (\text{Eq. 2})$$

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.  $K_m^B$  is equal to  $(k_{-2} + k_3)/k_2$  and defines the interfacial Michaelis-Menten constant for the second binding step and is expressed in surface concentration units such as mole fraction.  $V_{\max}$  is the maximal velocity at an infinite mole fraction and an infinite bulk concentration of phospholipid substrate.

First, the effects of C-1-P on the first step of the reaction for cPLA<sub>2</sub>α were examined by varying the concentration of the substrate, 1-palmitoyl, 2-arachidonyl-phosphatidylcholine (PAPC) (and the number of total mixed micelles), while keeping the mole fraction of PAPC (e.g. [PAPC]/[Triton X-100 + PAPC + C-1-P]) constant. The addition of ceramide 1-phosphate not only increased the  $V_{\max}$  of the reaction by 10-fold



**FIG. 3. Effect of C-1-P on the kinetic behavior of cPLA<sub>2</sub>α.** Panel A, the effect of C-1-P on the dissociation constant,  $K_s^A$ , of cPLA<sub>2</sub>α. cPLA<sub>2</sub>α activity was measured as a function of PC molar concentration in the absence (●) and presence (○) of 1 mol % D-e-C<sub>18:1</sub> C-1-P or (□) 4 mol % D-e-C<sub>18:1</sub> C-1-P for 45 min at 37 °C. The PC mole fraction was held constant at 0.137. Data are presented as cPLA<sub>2</sub>α activity measured as nanomoles of arachidonic acid produced/minute/milligram of recombinant cPLA<sub>2</sub>α ± S.E. Data are representative of six separate determinations on three separate occasions ( $R^2$  value for 0 mol %, 1.0 mol %, and 4 mol % are 0.94, 0.97, and 0.95, respectively). Panel B, the effect of C-1-P on the Michaelis-Menten constant,  $K_m^B$ , of cPLA<sub>2</sub>α. cPLA<sub>2</sub>α activity was measured as a function of mole fraction of PC ([PC]/[PC + Triton X-100 + C-1-P]) in the absence (●) and presence (○) of 1.5 mol % D-e-C<sub>18:1</sub> C-1-P or 4 mol % D-e-C<sub>18:1</sub> C-1-P for 45 min at 37 °C. Data are presented as cPLA<sub>2</sub>α activity measured as nanomoles of arachidonic acid produced/minute/milligram of recombinant cPLA<sub>2</sub>α ± S.E. Data are representative of six separate determinations on three separate occasions ( $R^2$  value for 0, 1.5, and 4 mol % are 0.97, 0.94, and 0.94, respectively).

but also lowered the apparent dissociation constant ( $K_s^A$ ) from 590.2 to 124.9 μM (e.g. a 5-fold increase in apparent affinity) (Fig. 3A and Table I). Thus, C-1-P increases the activity of cPLA<sub>2</sub>α by enhancing both surface affinity and catalytic activity of the enzyme.

To examine the effect of C-1-P on the catalytic ability/specificity of cPLA<sub>2</sub>α, (second step of the reaction after the enzyme associates with the micelle), we kinetically analyzed cPLA<sub>2</sub>α activity under varying mole fractions of PAPC (e.g. [PC]/[Triton X-100 + PAPC + C-1-P]) while keeping the total lipid concentration (e.g. [PAPC + Triton X-100 + C-1-P]) constant. The addition of C-1-P again dramatically increased the  $V_{max}$  of the reaction from 12.7 to 234.6 nmol/min/mg but interestingly had no effect on the apparent  $K_m^B$  of the reaction (Fig. 3B and Table II). Thus, it would seem that C-1-P does not directly effect the binding of the substrate to the active site of cPLA<sub>2</sub>α. Collectively, these kinetic studies demonstrate that C-1-P acts in two distinct ways. First, C-1-P acts to increase the association of

**TABLE I**  
 $K_s^A$  is the dissociation constant that is expressed in bulk concentration terms

$V_{max}$  is the true  $V_{max}$  at an infinite bulk concentration of lipid substrate.

C-1-P	Approximate $V_{max}$	Approximate $K_s^A$
	nmol/min/mg	μM
0 mol %	28.2 ± 1.56	590.2 ± 65.9
1.0 mol %	63.2 ± 6.7	228.6 ± 32.3
4 mol %	294.9 ± 13.6	124.9 ± 21.8

**TABLE II**  
 $K_m^B$  is the interfacial Michaelis constant expressed in surface concentration unit, mole fraction

$V_{max}$  is the true  $V_{max}$  at an infinite surface concentration of lipid substrate.

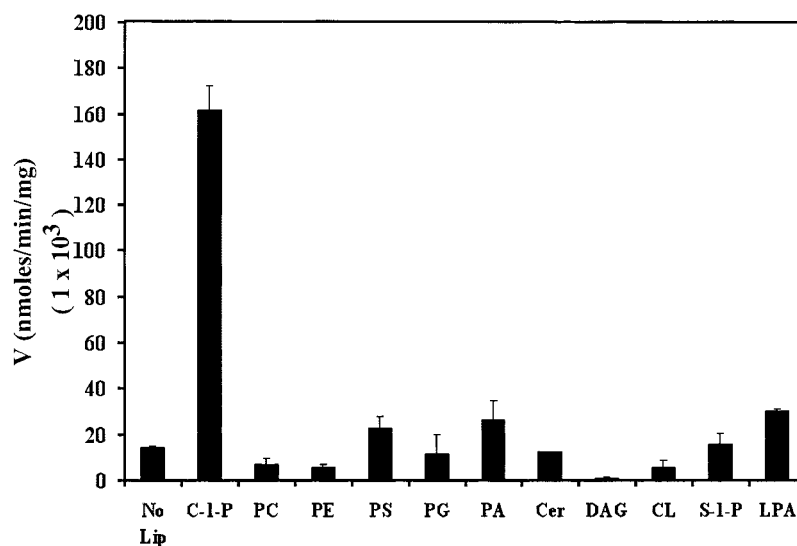
C-1-P	Approximate $V_{max}$	Approximate $K_m^B$
	nmol/min/mg	mole fraction
0 mol %	12.7 ± 15.27	0.129 ± 0.31
1.5 mol %	178.6 ± 49.6	0.126 ± 0.06
4 mol %	234.6 ± 50.6	0.124 ± 0.05

cPLA<sub>2</sub>α to membranes, and second, C-1-P enhances the catalytic ability of the enzyme acting as a positive allosteric activator once cPLA<sub>2</sub>α is associated with the membrane.

**The Activation of cPLA<sub>2</sub>α by C-1-P Is Specific**—To determine whether the activation of cPLA<sub>2</sub>α by C-1-P is a specific effect, the kinetic assay was repeated using various lipids including phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylglycerol (PG) (all at 4.0 mol %). Furthermore, closely related lipids and metabolites were also examined (4.0 mol %) that included D-e-C<sub>18:1</sub> ceramide (CER), D-e-S-1-P, (1-palmitoyl-2-oleoyl) diacylglycerol (DAG), lysophosphatidic acid (LPA), and (1-palmitoyl-2-oleoyl) phosphatidic acid (PA). Fig. 4 demonstrates that, in sharp contrast to ceramide 1-phosphate, the addition of PS, PC, PE, PG, DAG, LPA, S-1-P, PA, or CER had no significant effect on the enzymatic activity of cPLA<sub>2</sub>α. PIP<sub>2</sub> induced a calcium-independent (data not shown) increase in cPLA<sub>2</sub>α activity (18-fold) in accord with previous reports (39, 41, 42). Thus, although PIP<sub>2</sub> activates cPLA<sub>2</sub>α to a similar extent, C-1-P is the most potent activator of cPLA<sub>2</sub>α in a calcium-dependent manner under our kinetic conditions (see Supplemental Fig. 1) (1).

**C-1-P Specifically Increases the Membrane Affinity of cPLA<sub>2</sub>α for Lipid Vesicles**—Recently, C-1-P has been shown to induce the translocation of cPLA<sub>2</sub>α to PC-rich membranes (1). Furthermore, surface dilution kinetics showed that C-1-P affected the affinity of the enzyme for PC-rich micelles. To demonstrate that C-1-P directly enhances the membrane affinity of cPLA<sub>2</sub>α, we employed SPR analysis, which offers a great advantage over other methods in that the effects of lipids on membrane  $k_a$  and  $k_d$  rate constants can be directly determined (24, 28, 38). Additionally, we have recently characterized the binding of cPLA<sub>2</sub>α and its C2 domain to a variety of lipid vesicles by SPR analysis (19, 24, 25). First, we measured the binding of cPLA<sub>2</sub>α to POPC vesicles in the absence and presence of 5 mol % C-1-P at 50 μM Ca<sup>2+</sup>. cPLA<sub>2</sub>α bound to PC vesicles with 13 nm affinity. More importantly, the addition of 5 mol % C-1-P in the vesicle increased the affinity of cPLA<sub>2</sub>α by 5-fold (2.7 nm) (see Table III). This increased affinity was primarily due to slower  $k_d$ , whereas the  $k_a$  was not significantly influenced (see Table III). Typically, a slower dissociation rate is attributed to a specific interaction at the membrane interface such as recognition of a PI (26, 38) or increased hydrophobic interactions achieved by membrane penetration (22, 25, 26). It is unclear whether or not C-1-P increases the hydrophobic interaction by

**FIG. 4. The activation of cPLA<sub>2</sub>α by C-1-P is lipid-specific.** The activity of cPLA<sub>2</sub>α was assayed in the presence of 4 mol% of the indicated lipids for 45 min at 37 °C as described under "Experimental Procedures." Lipids examined were POPC, POPG, POPA, POPE, dioleoyl-cardiolipin (CL), POPS, S-1-P, CER, DAG, LPA, and C-1-P. Data are presented as cPLA<sub>2</sub>α activity measured as nanomoles of arachidonic acid produced/minute/miligram of recombinant cPLA<sub>2</sub>α ± S.E. Data are representative of four separate determinations on three separate occasions.



**TABLE III**  
Binding parameters for cPLA<sub>2</sub>α determined from SPR analysis

Values represent the mean ± S.D. from three determinations. All of the measurements were performed in 10 mM HEPES, pH 7.4, containing 0.16 M KCl and indicated 50 μM Ca<sup>2+</sup>.

Lipids	$k_a$ $M^{-1} s^{-1}$	$k_d$ $s^{-1}$	$K_d$ $M$	Fold increase in $K_d^a$
PC	$(2.8 \pm 0.5) \times 10^5$	$(3.5 \pm 0.6) \times 10^{-3}$	$(1.3 \pm 0.3) \times 10^{-8}$	5
PC:C-1-P <sup>b</sup>	$(3.0 \pm 0.4) \times 10^5$	$(8.0 \pm 0.8) \times 10^{-4}$	$(2.7 \pm 0.4) \times 10^{-9}$	1
PC:PS	$(2.4 \pm 0.3) \times 10^5$	$(6.0 \pm 0.7) \times 10^{-3}$	$(2.5 \pm 0.4) \times 10^{-8}$	9
PC:PG	$(2.6 \pm 0.4) \times 10^5$	$(5.6 \pm 0.6) \times 10^{-3}$	$(2.2 \pm 0.4) \times 10^{-8}$	8
PC:PI	$(2.3 \pm 0.5) \times 10^5$	$(5.1 \pm 0.6) \times 10^{-3}$	$(2.2 \pm 0.5) \times 10^{-8}$	8
PC:PA	$(2.2 \pm 0.3) \times 10^5$	$(5.4 \pm 0.7) \times 10^{-3}$	$(2.5 \pm 0.5) \times 10^{-8}$	9
PC:PtdIns-3-P	$(2.9 \pm 0.4) \times 10^5$	$(4.8 \pm 0.6) \times 10^{-3}$	$(1.7 \pm 0.3) \times 10^{-8}$	6
PC:PtdIns-4-P	$(2.7 \pm 0.3) \times 10^5$	$(4.1 \pm 0.5) \times 10^{-3}$	$(1.5 \pm 0.2) \times 10^{-8}$	6
PC:PtdIns-5-P	$(2.6 \pm 0.4) \times 10^5$	$(3.9 \pm 0.5) \times 10^{-3}$	$(1.5 \pm 0.3) \times 10^{-8}$	6
PC:PtdIns-3,4-P <sub>2</sub>	$(2.9 \pm 0.4) \times 10^5$	$(3.7 \pm 0.5) \times 10^{-3}$	$(1.3 \pm 0.3) \times 10^{-8}$	5
PC:PtdIns-4,5-P <sub>2</sub>	$(3.0 \pm 0.5) \times 10^5$	$(1.9 \pm 0.3) \times 10^{-3}$	$(6.3 \pm 1.0) \times 10^{-9}$	2
PC:PtdIns-3,4,5-P <sub>3</sub>	$(2.7 \pm 0.3) \times 10^5$	$(5.1 \pm 0.6) \times 10^{-3}$	$(1.9 \pm 0.3) \times 10^{-8}$	7
PC:S-1-P	$(3.1 \pm 0.5) \times 10^5$	$(4.5 \pm 0.6) \times 10^{-3}$	$(1.5 \pm 0.3) \times 10^{-8}$	6
PC:cardiolipin	$(2.1 \pm 0.4) \times 10^5$	$(5.1 \pm 0.7) \times 10^{-3}$	$(2.4 \pm 0.6) \times 10^{-8}$	9
PC:DAG	$(2.8 \pm 0.2) \times 10^5$	$(3.3 \pm 0.4) \times 10^{-3}$	$(1.2 \pm 0.2) \times 10^{-8}$	4
PC:cholesterol	$(2.6 \pm 0.6) \times 10^5$	$(2.9 \pm 0.5) \times 10^{-3}$	$(1.1 \pm 0.3) \times 10^{-8}$	4

<sup>a</sup> Fold increase in  $K_d$  relative to cPLA<sub>2</sub> binding to PC:C-1-P (95:5).

<sup>b</sup> All vesicles (excluding POPC alone) contained 95 mol % POPC and 5 mol % of other lipid.

inducing membrane penetration of cPLA<sub>2</sub>α, but it seems clear that C-1-P forms specific interactions with the cPLA<sub>2</sub>α molecule.

We also measured the binding of cPLA<sub>2</sub>α to POPC vesicles in the presence of other lipids to verify the specific nature of the C-1-P effect. As shown in Table III, bulk lipids such as PS, PG, PI, and PA had no effect on membrane affinity (*i.e.*  $K_d$ ) and actually reduced affinity by nearly 50%. Similarly, S-1-P and cardiolipin slightly reduced membrane affinity, whereas DAG or cholesterol had little effect. Furthermore, all of the phosphoinositides with the exception of PtdIns-4,5-P<sub>2</sub> exhibited little change in membrane affinity (see Table III). The modest 2-fold increase in vesicle affinity by PtdIns-4,5-P<sub>2</sub> was not unexpected and was consistent with a previous report (39, 42). Thus, the C-1-P effects on membrane affinity are highly specific.

**C-1-P Binds Directly and Specifically to the C2 Domain of cPLA<sub>2</sub>α**—Our recent report suggested that C-1-P binds to the C2 domain of cPLA<sub>2</sub>α (1). To quantitatively assess the affinity of the cPLA<sub>2</sub>α-C2 domain for C-1-P-containing membranes, we employed the SPR analysis again. We first measured the binding of the C2 domain to POPC vesicles and POPC vesicles in the absence and presence of 5 mol % C-1-P. The C2 domain bound with a  $K_d = 12$  nm to POPC vesicles, whereas 5 mol %

C-1-P caused a 7-fold increase in affinity ( $K_d = 1.8$  nm) (see Table IV). As with cPLA<sub>2</sub>α, the increase in affinity was primarily attributed to a smaller  $k_d$ . This indicates that the specific C-1-P binding site in cPLA<sub>2</sub>α is located in the C2 domain. To validate the  $K_d$  values determined from the kinetic SPR analysis, we also determined  $K_d$  by equilibrium SPR analysis (see Fig. 5). The  $K_d$  value ( $12 \pm 0.5$  nm for POPC;  $1.9 \pm 0.3$  nm for POPC/C-1-P (95:5)) calculated from the equilibrium binding isotherm agreed well with  $K_d$  values determined from the kinetic analysis ( $K_d = 12 \pm 3$  nm for POPC;  $1.8 \pm 0.4$  nm for POPC/C-1-P (95:5)).

We also measured the effects of other anionic phospholipids on the vesicle affinity of the C2 domain of cPLA<sub>2</sub>α. As was the case with cPLA<sub>2</sub>α, 5 mol % anionic phospholipids such as PS, PG, PI, and PA actually reduced affinity ~50% (see Table IV). Similarly, S-1-P, cardiolipin, DAG, and cholesterol had little effect on membrane binding (see Table IV). Furthermore, all of the PIs including PtdIns-4,5-P<sub>2</sub> reduced affinity by at least 50% for the isolated C2 domain. Given the modest enhancing effect of PtdIns-4,5-P<sub>2</sub> on the vesicle affinity of the full-length cPLA<sub>2</sub>α, this indicates that PtdIns-4,5-P<sub>2</sub> interaction site resides outside the C2 domain, possibly in the catalytic domain as previously suggested (40).

TABLE IV  
Binding parameters for cPLA<sub>2</sub>α-C2 determined from SPR analysis

Values represent the mean ± S.D. from three determinations. All of the measurements were performed in 10 mM HEPES, pH 7.4, containing 0.16 M KCl and indicated 50 μM Ca<sup>2+</sup>.

Lipids	$k_a$ $M^{-1} s^{-1}$	$k_d$ $s^{-1}$	$K_d$ $M$	Fold increase in $K_d^a$
PC	$(2.8 \pm 0.4) \times 10^5$	$(3.5 \pm 0.6) \times 10^{-3}$	$(1.2 \pm 0.3) \times 10^{-8}$	7
PC:C-1-P <sup>b</sup>	$(3.0 \pm 0.5) \times 10^5$	$(5.5 \pm 0.7) \times 10^{-4}$	$(1.8 \pm 0.4) \times 10^{-9}$	1
PC:PS	$(2.2 \pm 0.3) \times 10^5$	$(6.3 \pm 0.8) \times 10^{-3}$	$(2.9 \pm 0.5) \times 10^{-8}$	16
PC:PG	$(2.5 \pm 0.4) \times 10^5$	$(6.1 \pm 0.9) \times 10^{-3}$	$(2.4 \pm 0.6) \times 10^{-8}$	13
PC:PI	$(2.2 \pm 0.5) \times 10^5$	$(6.5 \pm 0.8) \times 10^{-3}$	$(3.0 \pm 0.8) \times 10^{-8}$	17
PC:PA	$(2.1 \pm 0.3) \times 10^5$	$(5.8 \pm 0.5) \times 10^{-3}$	$(2.8 \pm 0.5) \times 10^{-8}$	16
PC:PtdIns-3-P	$(2.9 \pm 0.5) \times 10^5$	$(4.4 \pm 0.6) \times 10^{-3}$	$(1.5 \pm 0.3) \times 10^{-8}$	8
PC:PtdIns-4-P	$(2.7 \pm 0.4) \times 10^5$	$(4.9 \pm 0.7) \times 10^{-3}$	$(1.8 \pm 0.4) \times 10^{-8}$	15
PC:PtdIns-5-P	$(2.5 \pm 0.4) \times 10^5$	$(4.7 \pm 0.6) \times 10^{-3}$	$(1.8 \pm 0.4) \times 10^{-8}$	15
PC:PtdIns-3,4-P <sub>2</sub>	$(2.1 \pm 0.4) \times 10^5$	$(7.1 \pm 0.8) \times 10^{-3}$	$(3.4 \pm 0.8) \times 10^{-8}$	19
PC:PtdIns-4,5-P <sub>2</sub>	$(1.9 \pm 0.3) \times 10^5$	$(7.4 \pm 0.6) \times 10^{-3}$	$(3.9 \pm 0.7) \times 10^{-8}$	22
PC:PtdIns-3,4,5-P <sub>3</sub>	$(1.5 \pm 0.2) \times 10^5$	$(8.0 \pm 0.9) \times 10^{-3}$	$(5.3 \pm 0.9) \times 10^{-8}$	29
PC:S-1-P	$(2.6 \pm 0.5) \times 10^5$	$(5.1 \pm 0.8) \times 10^{-3}$	$(1.9 \pm 0.5) \times 10^{-8}$	11
PC:cardiolipin	$(1.9 \pm 0.5) \times 10^5$	$(5.4 \pm 0.7) \times 10^{-3}$	$(2.8 \pm 0.8) \times 10^{-8}$	16
PC:DAG	$(2.5 \pm 0.4) \times 10^5$	$(3.1 \pm 0.6) \times 10^{-3}$	$(1.2 \pm 0.3) \times 10^{-8}$	7
PC:cholesterol	$(2.3 \pm 0.5) \times 10^5$	$(2.7 \pm 0.4) \times 10^{-3}$	$(1.2 \pm 0.3) \times 10^{-8}$	7

<sup>a</sup> Fold increase in  $K_d$  relative to cPLA<sub>2</sub>α-C2 binding to PC:C-1-P (95:5).

<sup>b</sup> All vesicles (excluding POPC alone) contained 95 mol % POPC and 5 mol % of other lipid.

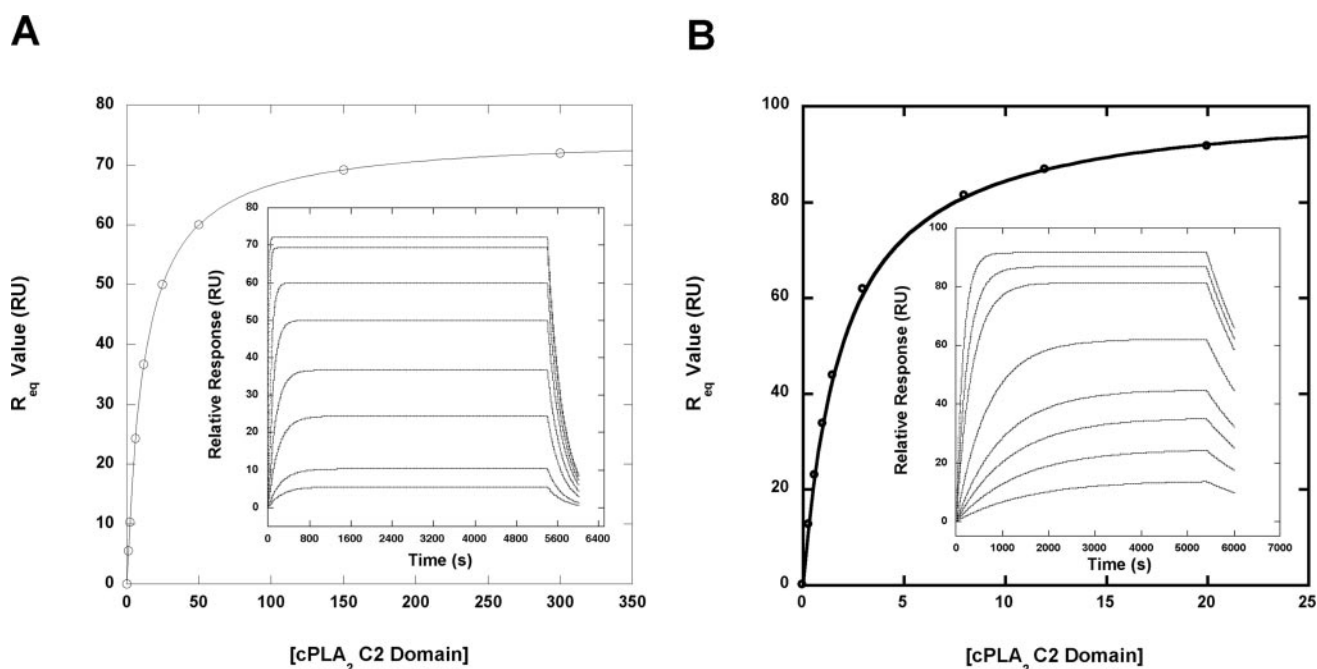


FIG. 5. SPR binding analysis of the cPLA<sub>2</sub>α C2 domain. Panel A, equilibrium SPR measurements of cPLA<sub>2</sub>α C2 domain to POPC vesicles. The C2 domain was injected at 1 μl/min at varying concentrations (1, 2, 6, 12, 25, 50, 150, and 300 nM), and  $R_{eq}$  values were measured (see inset). A binding isotherm was then generated from the  $R_{eq}$  versus the concentration of the C2 domain plot. A solid line represents a theoretical curve constructed from  $R_{max}$  ( $75 \pm 0.7$  relative units (RU)) and  $K_d$  ( $12 \pm 0.5$  nM) values determined by nonlinear least-squares analysis of the isotherm using equation:  $R_{eq} = R_{max}/(1 + K_d/C)$ . Panel B, equilibrium SPR measurements of cPLA<sub>2</sub>α C2 domain to POPC/C-1-P (95:5) vesicles. The C2 domain was injected at 1 μl/min at varying concentrations (0.3, 0.6, 1, 1.5, 3, 8, 12, and 20 nM) and  $R_{eq}$  values were measured (see inset). A binding isotherm was then generated from the  $R_{eq}$  versus the concentration of the C2 domain plot. A solid line represents a theoretical curve constructed from  $R_{max}$  ( $100 \pm 1$  RU) and  $K_d$  ( $1.9 \pm 0.3$  nM) values determined by nonlinear least-squares analysis of the isotherm using equation:  $R_{eq} = R_{max}/(1 + K_d/C)$ . 10 mM HEPES buffer, pH 7.4, with 0.16 M KCl and 50 μM Ca<sup>2+</sup> was used for both sets of measurements.

#### DISCUSSION

In this study, the mechanism of C-1-P activation of cPLA<sub>2</sub>α was examined in depth. Several important aspects of this interaction were discovered. First, C-1-P was found to be both a potent and specific allosteric activator of cPLA<sub>2</sub>α. Second, our results revealed that C-1-P also greatly affects the affinity of the enzyme for PC membranes. Lastly, this study demonstrated that C-1-P specifically interacts with a novel site within the C2 domain of cPLA<sub>2</sub>α.

Previously, our laboratory demonstrated that C-1-P was a moderate activator of cPLA<sub>2</sub>α using a mixed vesicle-based as-

say (1). This study expanded on these previous studies using a more physically defined system (Triton X-100 mixed micelle assay) to determine the linear kinetics of PC hydrolysis as observed in the presence and absence of C-1-P. This allowed robust determination of various kinetic parameters under relatively well defined conditions and consequently quantitative assessment of the effect of C-1-P on the cPLA<sub>2</sub>α activity. Under this assay condition, C-1-P induced a dramatic (>15-fold) increase in cPLA<sub>2</sub>α activity. Furthermore, this study also demonstrated that the C-1-P/cPLA<sub>2</sub> interaction was specific as other lipids had little to no effect on the activity of cPLA<sub>2</sub>α.

Even the closely related lipids, PA, LPA, CER, and S-1-P, had insignificant effects on the activity and binding of cPLA<sub>2</sub>α. Only PtdIns-4,5-P<sub>2</sub>, which has been reported to activate cPLA<sub>2</sub>α in a calcium-independent manner (39, 41, 42), showed an activating effect in the presence and absence of calcium making ceramide 1-phosphate, to date, the most potent allosteric activator of cPLA<sub>2</sub>α requiring the presence of calcium (1).

Importantly, this study clearly demonstrates the mechanism by which C-1-P activates cPLA<sub>2</sub>α. First, C-1-P dramatically decreased in the dissociation constant of cPLA<sub>2</sub>α with PC-containing membranes as determined by two independent techniques, surface dilution kinetics and SPR analysis. Thus, C-1-P may function to recruit cPLA<sub>2</sub>α to PC-rich membranes correlating with our report (1) that treatment of cells with C-1-P induced the translocation of cPLA<sub>2</sub>α to the Golgi apparatus. Furthermore, a recent report showed (43) that the enzyme responsible for C-1-P generation, ceramide kinase, was localized to the Golgi apparatus, thus, placing the generation of C-1-P in the proper location for recruitment of cPLA<sub>2</sub>α. Second, the observation that C-1-P has no effect on the Michaelis-Menten constant,  $K_m^B$ , whereas inducing a large increase in the  $V_{max}$  suggests that C-1-P is a positive allosteric activator of the enzyme. The observation that binding of C-1-P to the C2 domain enhances the catalytic efficiency of cPLA<sub>2</sub>α (as seen in  $V_{max}$  effect) suggests that C-1-P might function as an allosteric activator that induces a conformation change of the protein. A similar mechanism has been proposed for the activation of cPLA<sub>2</sub>α by phosphorylation (19) and by PtdIns-4,5-P<sub>2</sub> (40). Thus, the generation of C-1-P within a PC-rich membrane (e.g. Golgi apparatus) in response to an inflammatory agonist (e.g. IL-1β) may first act to recruit cPLA<sub>2</sub>α to the membrane and then act to enhance the catalytic ability of the membrane-associated enzyme. This hypothesis is supported by our previous report that down-regulation of ceramide kinase and thus C-1-P levels potentially inhibited AA release in response to IL-1β and A23187 (15). Therefore, the recruitment and activation of cPLA<sub>2</sub>α by C-1-P may play an important role in the mediation of inflammatory responses.

Of further interest in this study was the demonstration that C-1-P acted to enhance the association of cPLA<sub>2</sub>α and its C2 domain to PC vesicles, thus not competing with PC for binding to the enzyme. Furthermore, unlike the full-length protein, the C2 domain did not show enhanced binding when phosphoinositides such as PIP<sub>2</sub> were incorporated with PC vesicles. Therefore, a novel lipid interaction site (or critical amino acids) for ceramide 1-phosphate exists within the C2 domain of cPLA<sub>2</sub>α. The localization of the C-1-P binding site within the C2 domain (calcium lipid binding domain) is very logical since our laboratory previously demonstrated that the activation of cPLA<sub>2</sub>α by C-1-P was dependent on the presence of calcium (see Supplemental Fig. 1) (1). Our laboratory also demonstrated that cPLA<sub>2</sub>α interacted with C-1-P at submicromolar concentrations of calcium (1). Furthermore, unpublished findings from our laboratory suggest that the intracellular activation of cPLA<sub>2</sub>α by C-1-P is more responsive in lower calcium concentrations and is partially dispensable in high calcium environments.<sup>2</sup> Thus, C-1-P may be a very important intracellular activator of cPLA<sub>2</sub>α in response to inflammatory agonists that induce a relatively small (more physiologic) or transient increase in intracellular calcium.

In conclusion, this study demonstrates that C-1-P is a potent allosteric activator of cPLA<sub>2</sub>α. Furthermore, this study shows that the C-1-P interaction site of cPLA<sub>2</sub>α is novel, not competing for PC or PIP<sub>2</sub>. Lastly, this study implicates a dual mechanistic role for C-1-P in the regulation of cPLA<sub>2</sub>α activation

response to inflammatory agonists. First, C-1-P acts to increase the association of cPLA<sub>2</sub>α with membranes followed by increasing the catalytic ability of the enzyme. Thus, not only would inhibitors of ceramide kinase be of possible therapeutic value but also the therapeutics based on inhibition of this interaction between C-1-P and cPLA<sub>2</sub>α. Therefore, it is a high priority to define the specific amino acids within the C2 domain of cPLA<sub>2</sub>α that are critical for this interaction.

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<sup>2</sup> P. Subramanian, R. V. Stahelin, Z. Szulc, A. Bielawska, W. Cho, and C. E. Chalfant, unpublished data.