Ceramide-1-phosphate Binds Group IVA Cytosolic Phospholipase A₂ via a Novel Site in the C2 Domain*

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Previously, ceramide-1-phosphate (C1P) was demonstrated to be a potent and specific activator of group IV cytosolic phospholipase A₂ (cPLA₂) via interaction with the C2 domain. In this study, we hypothesized that the specific interaction site for C1P was localized to the cationic β-groove (Arg⁵⁷, Lys⁵⁸, Arg⁶⁹) of the C2 domain of cPLA₂. In this regard, mutants of this region of cPLA₂ were generated (R⁵⁷A/K⁵⁸A/R⁵⁹A, R⁵⁷A/R⁵⁹A, K⁵⁸A/R⁵⁹A, R⁵⁷A/K⁵⁸A, R⁵⁷A, K⁵⁸A, and R⁵⁹A) and examined for C1P affinity by surface plasmon resonance. The triple mutants (R⁵⁷A/K⁵⁸A/R⁵⁹A), the double mutants (R⁵⁷A/R⁵⁹A, K⁵⁸A/R⁵⁹A, and R⁵⁷A/K⁵⁸A), and the single mutant (R⁵⁹A) demonstrated significantly reduced affinity for C1P-containing vesicles as compared with wild-type cPLA₂. Examining these mutants for enzymatic activity demonstrated that these five mutants of cPLA₂ also showed a significant reduction in the ability of C1P to: 1) increase the Vₘₐₓ of the reaction; and 2) significantly decrease the dissociation constant (Kₐ) of the reaction as compared with the wild-type enzyme. The mutational effect was specific for C1P as all of the cationic mutants of cPLA₂ demonstrated normal basal activity as well as normal affinities for phosphatidylcholine and phosphatidylinositol-4,5-bisphosphate as compared with wild-type cPLA₂. This study, for the first time, demonstrates a novel C1P interaction site mapped to the cationic β-groove of the C2 domain of cPLA₂.

Group IV cytosolic phospholipase A₂ (cPLA₂) is the initial rate-limiting enzyme in eicosanoid biosynthesis in response to many inflammatory agonists (1, 2). The cellular activation of cPLA₂ requires Ca²⁺-dependent membrane translocation of the enzyme, which is mediated by the N-terminal C2 domain (1–4). Cell-specific and agonist-dependent events coordinate translocation of cPLA₂ to the nuclear envelope, endoplasmic reticulum, and Golgi apparatus via this domain (1–8). At these membranes, cPLA₂ hydrolyzes membrane phospholipids to produce arachidonic acid, which initiates pathways leading to eicosanoid synthesis (1–8).

C2 domains were originally described in protein kinase C (9) and since have been identified in numerous proteins involved in lipid signaling. C2 domains are composed of about 120 amino acids forming a common fold of eight-stranded anti-parallel β-sandwich. Most C2 domains bind to the membranes in a Ca²⁺-dependent manner via the three calcium binding regions (CBRs) that are located at one end of the β-sandwich. These C2 domains are known to exhibit different Ca²⁺ binding affinities, which can be modulated by the presence or absence of phospholipids. Also, most of the C2 domains contain a cationic patch in the concave face of the β-sandwich, known as the β-groove (48). The C2 domain of cPLA₂ binds two calcium ions via the hydrophobic calcium binding regions (CBR1 and CBR3) that are also critical to membrane binding and membrane penetration (10, 11). Recently, ceramide-1-phosphate (C1P) has been defined to be the membrane lipid that enhances the association of C2 domain of cPLA₂ with membranes at lower calcium concentration (e.g. submicromolar) (12).

C1P is a new addition to a growing group of bioactive sphingolipids, which include ceramide and sphingosine-1-phosphate. Recent reports from our laboratory have shown ceramide kinase to be an upstream mediator of calcium ionophore- and interleukin-1β-induced arachidonic acid

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1 The on-line version of this article (available at http://www.jbc.org) contains two supplemental figures and a supplemental table.
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3 The abbreviations used are: cPLA₂, group IVA cytosolic phospholipase A₂; C1P, ceramide-1-phosphate; CerK, ceramide kinase; PAPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; mol %, molar percentage of mixed-micelle; CHAPS, (3-(3-cholamidopropyl) dimethylammonio)-1-propane-sulfonate; PtdIns(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; SPR, surface plasmon resonance; PC, phosphatidylcholine; CBR, calcium binding region; D-E-C₁₈-₁, dextro-erythro-C₁₈₁.
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release and eicosanoid synthesis. Further studies revealed that cPLA₁α was required for C1P to induce arachidonic acid release (12). In a more recent study, we have shown that C1P allosterically activates cPLA₁α and enhances the in vitro interaction of the enzyme with its membrane substrate phosphatidylcholine (PC) at the mechanistic level. Using surface dilution kinetics coupled with surface plasmon resonance (SPR) technology, C1P was demonstrated to regulate the association of cPLA₁α with PC-rich micelles/vesicles via a novel undescribed site in the C2 domain. The current study identified this novel site to be on the β-groove of cPLA₂α and identified critical amino acids in this region required for the interaction of this bioactive sphingolipid with the enzyme. Importantly, this is the first study to map a site for interaction of C1P with a target protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (PAPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. [14C]PAPC was purchased from American Radiolabeled Chemicals. A 1,2-dipalmitoyl derivative of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Octyl glucose and (3-(3-cholamidopropyl) dimethylammonio)-1-propane-sulfonate (CHAPS) were from Fisher Scientific. 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 (13). Restriction endonucleases and enzymes for molecular biology were obtained from New England Biolabs (Beverly, MA). Ceramide-1-phosphate was prepared according to the published method by direct phosphorylation of D-erythro-C₁₈:₁-ceramide in 37% [³¹P]H₃PO₄ at 85% for each cPLA₁α (see supplemental Fig. 1).

**Construction of cPLA₁α Mutants**—The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce mutations in the pVL1393 vector with a His₆ tag engineered to rected mutagenesis kit (Stratagene) was used to introduce

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**Construction of cPLA₁α Mutants**—The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce mutations in the pVL1393 vector with a His₆ tag engineered to the C-terminus of cPLA₁α gene. The three basic amino acids in the C2 domain of cPLA₁α were mutated in combination to generate triple, double, and single mutants. Temperature cycling was performed according to manufacturer’s instructions using Pfu DNA polymerase, which replicates both strands with high fidelity and without displacing the mutagenic primers. This generates a mutated plasmid containing staggered nicks. The product was treated with DpnI endonuclease, which specifically digests methylated and hemimethylated parent DNA template and selects for mutations containing synthesized DNA. The nicked vector DNAs containing the desired mutations were then transformed into Escherichia coli XL-10 Gold cells. The mutated vectors were sequenced to ensure the presence of only the desired mutation.

**Recombinant Expression of cPLA₁α**—Recombinant human cPLA₁α was expressed in SF9 cells with a His₆ tag using a baculovirus expression system and purified using a modified protocol as described previously (10, 15). 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, 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 100,000 × g for 45 min at 4°C. The cleared lysate was batch-bound to 10 ml of nickel-nitrotriacetic 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 mM KCl, 10 mM imidazole, and 10% glycerol). Secondly, the column was washed with 15 ml of Buffer 2 (50 mM Tris, pH 8.0, 0.1 mM KCl, 15 mM imidazole, and 10% glycerol). Thirdly, the column was washed with 15 ml of Buffer 3 (50 mM Tris, pH 8.0, 0.1 mM 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 mM KCl, 250 mM imidazole, and 10% glycerol). The enzyme fractions were monitored using SDS-PAGE, and fractions containing significant amounts of cPLA₁α were pooled, concentrated, and desalted in an 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 mM KCl, and 30% glycerol). The recombinantly expressed enzyme was analyzed by SDS-PAGE and Coomassie Brilliant Blue staining, demonstrating a purity of ~85% for each cPLA₁α (see supplemental Fig. 1).

**Surface Plasmon Resonance Analysis**—All SPR measurements were performed at 25 °C. A detailed protocol for coating the L1 sensor chip has been described elsewhere (16, 17). Briefly, after washing the sensor chip surface, 90 μl of vesicles containing various phospholipids (see Table 1) was injected at 5 μl/min to give a response of 6500 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 cPLA₁α (16, 18, 19). 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 the flow rate of 30 μl/min. 90 μl of protein in 10 mM HEPES, pH 7.4, containing 0.16 mM KCl, 5% glycerol, and 10 mM Ca²⁺ 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 Kₐ, 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 fitted to a 1:1 Langmuir binding model: protein + (protein binding site on vesicle) ↔ (complex) using BIAevaluation 3.0 software (Biacore) as described previously (16, 18, 19). The dissociation constant (Kₐ) was then calculated from the equation, Kₐ = kₐ/k₉. A minimum of three data sets was collected for each protein. Equilibrium (steady-state) SPR measurements were performed with the flow rate of 5 μl/min to allow sufficient time...
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RESULTS

Structural analysis has shown that C2 domains have a common fold of conserved eight-stranded antiparallel β-sheet connected by surface loops (21–24). The surface loops are highly variable in terms of amino acid sequence and conformation and connect the β-strands in two different topologies. Interestingly, a large number of C2 domains, including cPLA2α, contain a cationic patch (cationic β-groove) (Fig. 1). Although the size and the electrostatic potential of the cationic β-groove vary widely among C2 domains, its presence in most C2 domains implies an essential structural or functional role. The presence of these cationic residues in the β-groove of cPLA2α was intriguing, as our previous data demonstrated that the C1P binding site resides in the C2 domain. To assess the importance of the β-groove residues in cPLA2α membrane binding (Fig. 1), we prepared the following mutations: R57A, K58A, R59A, R57A/K58A, R57A/R59A, K58A/R59A, and R57A/K58A/R59A for membrane binding and activation studies.

Identification of the C1P Binding Site of cPLA2α—Herein, we employed SPR analysis for monitoring the affinity of wild-type and mutant cPLA2α for C1P-containing membranes. We have quantitatively measured the binding of cPLA2α and its C2 domain to a variety of lipid vesicles by SPR analysis (16, 17, 19, 25). To delineate the C1P binding site in cPLA2α, first, we compared the binding of wild-type cPLA2α with POPC vesicles and POPC vesicles containing 3 mol % C1P at 10 μM Ca2+. Lower Ca2+ concentrations were employed than in our previous study (19) to maximize the affinity disparity for C1P-containing vesicles between wild-type and mutants. Wild-type cPLA2α bound to PC vesicles with 49 nM affinity, similar to previous reports (15, 25), whereas interestingly, 3 mol % C1P in the vesicle increased the affinity of cPLA2α by nearly 10-fold (5.0 nM). This increased affinity was primarily due to a 4.4-fold slower dissociation rate (kd), whereas the association rate (ka) constant increased by 2-fold (Table 1). Based on our previous results, a slower dissociation rate caused by C1P suggests specific interactions with C1P or C1P-induced membrane penetration of the C2 domain (18, 49). To validate the ka values determined from the kinetic SPR analysis, we also determined Kd by equilibrium SPR analysis (Fig. 2). The Kd value (44 ± 2.0 nM) calculated from the equilibrium binding isotherm agreed well with the Kd determined from the kinetic analysis (Kd = 49 ± 10 nM) for POPC vesicles, and that determined from equilibrium analysis with
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### TABLE 1
cPLA$_2$ and Mutant Membrane Binding Analysis

| Protein          | $k_a$ ($M^{-1} s^{-1}$) | $k_d$ ($s^{-1}$) | $K_d$ (nM) | Fold increase in $K_d$* |
|------------------|-------------------------|------------------|------------|------------------------|
| POPC             |                         |                  |            |                        |
| cPLA$_2$         | $(1.0 \pm 0.2) \times 10^2$ | $(4.9 \pm 0.4) \times 10^{-3}$ | $(4.9 \pm 1.0) \times 10^{-8}$ | 9.8 |
| R75A             | $(1.1 \pm 0.3) \times 10^2$ | $(5.1 \pm 0.3) \times 10^{-3}$ | $(4.6 \pm 1.2) \times 10^{-8}$ | 9.2 |
| K58A             | $(1.3 \pm 0.2) \times 10^2$ | $(5.2 \pm 0.6) \times 10^{-3}$ | $(4.0 \pm 0.8) \times 10^{-8}$ | 8.0 |
| R59A             | $(1.1 \pm 0.2) \times 10^2$ | $(4.7 \pm 0.4) \times 10^{-3}$ | $(4.3 \pm 0.9) \times 10^{-8}$ | 8.6 |
| R57/K58A         | $(1.2 \pm 0.3) \times 10^2$ | $(5.0 \pm 0.5) \times 10^{-3}$ | $(4.2 \pm 1.1) \times 10^{-8}$ | 8.4 |
| R57/59A          | $(9.8 \pm 0.6) \times 10^{-1}$ | $(4.8 \pm 0.5) \times 10^{-3}$ | $(4.9 \pm 0.6) \times 10^{-8}$ | 9.8 |
| K58/R59A         | $(9.9 \pm 0.7) \times 10^{-1}$ | $(5.0 \pm 0.4) \times 10^{-3}$ | $(5.1 \pm 0.5) \times 10^{-8}$ | 10.0 |
| R57/K58/R59A     | $(1.1 \pm 0.3) \times 10^2$ | $(5.4 \pm 0.5) \times 10^{-3}$ | $(4.9 \pm 1.4) \times 10^{-8}$ | 9.8 |
| K541/S43/S544A   | $(1.1 \pm 0.3) \times 10^2$ | $(5.0 \pm 0.5) \times 10^{-3}$ | $(4.5 \pm 1.3) \times 10^{-8}$ | 9.0 |
| POPC/C1P (97:3)  |                         |                  |            |                        |
| cPLA$_2$         | $(2.2 \pm 0.2) \times 10^2$ | $(1.1 \pm 0.1) \times 10^{-3}$ | $(5.0 \pm 0.6) \times 10^{-9}$ | 1.0 |
| R75A             | $(2.0 \pm 0.3) \times 10^2$ | $(2.4 \pm 0.3) \times 10^{-3}$ | $(1.2 \pm 0.2) \times 10^{-8}$ | 2.4 |
| K58A             | $(1.8 \pm 0.2) \times 10^2$ | $(2.3 \pm 0.4) \times 10^{-3}$ | $(1.3 \pm 0.3) \times 10^{-8}$ | 2.6 |
| R59A             | $(1.7 \pm 0.3) \times 10^2$ | $(2.0 \pm 0.3) \times 10^{-3}$ | $(1.8 \pm 0.4) \times 10^{-8}$ | 3.6 |
| R57/K58A         | $(1.8 \pm 0.4) \times 10^2$ | $(3.5 \pm 0.4) \times 10^{-3}$ | $(1.9 \pm 0.5) \times 10^{-8}$ | 3.8 |
| R57/59A          | $(1.5 \pm 0.3) \times 10^2$ | $(3.8 \pm 0.5) \times 10^{-3}$ | $(2.5 \pm 0.6) \times 10^{-8}$ | 5.0 |
| K58/R59A         | $(1.3 \pm 0.2) \times 10^2$ | $(3.7 \pm 0.4) \times 10^{-3}$ | $(2.8 \pm 0.5) \times 10^{-8}$ | 5.6 |
| R57/K58/R59A     | $(1.2 \pm 0.3) \times 10^2$ | $(4.2 \pm 0.6) \times 10^{-3}$ | $(3.5 \pm 1.0) \times 10^{-8}$ | 6.0 |
| K541/S43/S544A   | $(2.0 \pm 0.2) \times 10^2$ | $(1.2 \pm 0.1) \times 10^{-3}$ | $(6.0 \pm 0.8) \times 10^{-9}$ | 1.2 |
| POPC/PtdIns(4,5)P$_2$ (97:3) |                         |                  |            |                        |
| cPLA$_2$         | $(1.8 \pm 0.3) \times 10^2$ | $(2.9 \pm 0.2) \times 10^{-3}$ | $(1.6 \pm 0.3) \times 10^{-8}$ | 3.2 |
| K58A             | $(1.9 \pm 0.2) \times 10^2$ | $(3.2 \pm 0.4) \times 10^{-3}$ | $(1.7 \pm 0.3) \times 10^{-8}$ | 3.4 |
| R59A             | $(1.4 \pm 0.3) \times 10^2$ | $(2.8 \pm 0.5) \times 10^{-3}$ | $(2.0 \pm 0.6) \times 10^{-8}$ | 4.0 |
| R57/K58A         | $(1.6 \pm 0.4) \times 10^2$ | $(3.0 \pm 0.3) \times 10^{-3}$ | $(1.9 \pm 0.5) \times 10^{-8}$ | 3.8 |
| R57/59A          | $(1.6 \pm 0.3) \times 10^2$ | $(2.7 \pm 0.3) \times 10^{-3}$ | $(1.7 \pm 0.4) \times 10^{-8}$ | 3.4 |
| R57/K58/R59A     | $(1.5 \pm 0.2) \times 10^2$ | $(3.0 \pm 0.4) \times 10^{-3}$ | $(2.0 \pm 0.4) \times 10^{-8}$ | 4.0 |
| K541/S43/S544A   | $(1.2 \pm 0.2) \times 10^2$ | $(4.8 \pm 0.4) \times 10^{-3}$ | $(4.0 \pm 0.7) \times 10^{-8}$ | 8.0 |

* Fold increase in $K_d$ relative to the binding cPLA$_2$ to POPC/C1P (97:3) vesicles.

**FIGURE 2. SPR binding analysis of cPLA$_2$α.** A, sensorgrams from kinetic measurements of cPLA$_2$α to POPC/C1P (97:3) vesicles. cPLA$_2$α was injected at 30 µl/min at varying concentrations (4, 8, 16, 32, and 64 nM). Solid lines represent the best-fit theoretical curves. RU, resonance unit. B, equilibrium SPR measurements of cPLA$_2$α to POPC/C1P (97:3) vesicles. cPLA$_2$α was injected at 2 µl/min at varying concentrations (1, 2, 4, 12, 25, 50, 100, and 200 nM), and $R_{eq}$ values were measured. A binding isotherm (shown) was generated from the $R_{eq}$ versus the concentration of cPLA$_2$α. A solid line represents a theoretical curve constructed from $R_{max}$ (215 ± 5) and $K_d$ (5.2 ± 0.4 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 10 µM Ca$^{2+}$ was used for both sets of measurements.

The addition of 3 mol % C1P ($K_d = 4.1 \pm 0.4$ nM) was similar to the $K_d$ (5.2 ± 0.4 nM) value determined from kinetic analysis.

Mutants of cPLA$_2$α were first monitored for affinity to POPC vesicles to demonstrate that none of the mutants played a significant role in binding of cPLA$_2$α to zwitterionic vesicles. Indeed, all mutants, including a triple cationic mutant (R57A/K58A/R59A), displayed little change in POPC vesicle affinity ($K_d$), with rate constant ($k_a$ and $k_d$) values within respective error bar ranges (Table 1). To quantitatively assess the effects of the cationic mutants on C1P binding, we monitored their bind-
ing to POPC/C1P (97:3) vesicles in 10 μM Ca2+. Single mutants (R57A, K58A, and R59A) reduced binding 2.4–4.6-fold to POPC/C1P vesicles, whereas having little effect on POPC bind-
ing, suggesting their involvement in specific C1P binding. In support of this C1P-specific binding hypothesis, these mutations increased $k_d$ without significantly decreasing $k_a$. Next, we monitored the binding of double and triple cationic mutants (R57A/R59A, K58A/R59A, and R57A/K58A/R59A) to POPC/C1P vesicles. All mutations reduced binding to POPC/C1P vesicles 4–6-fold, without effecting POPC vesicle binding. Furthermore, all three mutations primarily influenced $k_d$ (faster $k_d$), supporting the specific nature of the interaction between these residues and C1P.

Recent reports have demonstrated that PtdIns(4,5)P2 is able to increase cPLA$_2$α affinity for the membrane as well as enhance cPLA$_2$α activation (25–28). Unlike the C1P binding site that is located in the C2 domain, this binding site resides in the catalytic domain (25, 26). Therefore, it was expected that the above cationic site mutations would not affect the binding of cPLA$_2$α to PtdIns(4,5)P2-containing vesicles. In fact, cationic mutants of full-length cPLA$_2$α (Table 1) displayed analogous affinity to wild type for 3 mol % in POPC/PtdIns(4,5)P2 (97:3) vesicles. This again underscores the specific role of the C2 domain cationic β-groove residues in C1P coordination. To demonstrate that a reduction in cationic charge by abolishing one to three cationic residues was not solely responsible for the reduction in C1P binding (i.e. a nonspecific electrostatic effect), we measured the binding of another triple cationic mutant (K541/543/544α) to the panel of lipids. This mutant displayed similar affinity to wild type for both POPC and POPC/C1P vesicles; however, the 3-fold increase with 3 mol % PtdIns(4,5)P$_2$ was abolished for this mutation, which is in line with the proposed role of these amino acids in PtdIns(4,5)P$_2$ binding (22).

Cationic Mutants of cPLA$_2$α Fail to Respond to C1P without Effects on PtdIns(4,5)P$_2$ Activation—Based on the SPR studies above, we predicted that the mutants of the cationic β-groove of cPLA$_2$α would demonstrate decreased response to C1P in vitro as compared with the wild-type cPLA$_2$α. To determine whether our prediction was correct, we examined all of these cPLA$_2$α mutants for activation with increasing mol % of C1P using a mixed-micelle assay. As shown in Fig. 3A (see also Table 2), C1P increased the $V_{max}$ value of wild-type cPLA$_2$α by about 10-fold. For single mutants, R57A and K58A, however, C1P caused a smaller increase in the $V_{max}$ (Table 2). In accord with the SPR analysis, the triple mutant (R57A/K58A/R59A), the double mutants (R57A/K58A and R57A/R59A), and the single mutant (R59A) of cPLA$_2$α had even smaller $V_{max}$ values in the presence of C1P (Fig. 3A and Table 2). Both the basal activity

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**TABLE 2**

| cPLA$_2$α Wild type/mutants | App. $V_{max}$ | $K_a^*$ |
|-----------------------------|----------------|--------|
|                             | $-$C1P   | $+$C1P | $-$C1P  | $+$C1P   |
| Wild type                   | 24.05 ± 2.9 | 240.5 ± 11.13 | 249.89 ± 73.45 | 106.301 ± 17.3 |
| R57A                       | 25.57 ± 3.6 | 198.53 ± 13.3 | 228.34 ± 81.6 | 106.1 ± 25.13 |
| K58A                       | 26.9 ± 4.8 | 216.73 ± 11 | 242.01 ± 96.02 | 137.37 ± 21.8 |
| R59A                       | 19.8 ± 0.9 | 112.19 ± 11.7 | 227.45 ± 23.19 | 178.9 ± 52.5 |
| R57A/R59A                  | 22.3 ± 3.6 | 149 ± 14.5 | 221.26 ± 92 | 158.68 ± 45.5 |
| K58A/R59A                  | 25.7 ± 2.9 | 158.26 ± 12.3 | 253.91 ± 30 | 168.8 ± 37.5 |
| R57A/K58A                  | 21.38 ± 1.3 | 88 ± 12.8 | 201.91 ± 32.9 | 164.97 ± 27 |
| R57A/K58A/R59A             | 22.8 ± 4.6 | 94.5 ± 4.2 | 265.23 ± 26.5 | 228.58 ± 25.37 |

$K_a^*$ is the dissociation constant which is expressed in bulk concentration terms. $V_{max}$ is the true $V_{max}$ at an infinite bulk concentration of lipid substrate. App., apparent
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and the activation of cPLA₂α by PtdIns(4,5)P₂ were not significantly affected by cationic β-groove mutations (Fig. 3B). These data again demonstrate that mutation of one or more basic amino acids (Arg⁵⁷, Lys⁵⁸, and Arg⁵⁹) in this cationic β-groove inhibits the response of cPLA₂α to C1P without affecting basal activity or the response to PtdIns(4,5)P₂. These data also support the specific nature of this interaction and a lack of structural defects due to mutagenesis of these amino acid residues.

Cationic Mutants of cPLA₂α Fail to Decrease the Dissociation Constant (Kₛ) in Response to C1P—In our previous studies, we have examined the kinetic interaction of C1P-cPLA₂α using the surface dilution model (19). This model takes into account both two-dimensional surface interaction and the three-dimensional bulk interaction between an interfacial enzyme and lipid substrates (29–33). We previously demonstrated that C1P activates cPLA₂α activity by lowering the apparent dissociation constant (Kₛ) of the enzyme, thereby decreasing its dissociation from its membrane substrate (19). Thus, we examined whether mutation of these basic amino acids showed any effect on the ability of C1P to lower the dissociation constant (Kₛ). As shown in Fig. 4, supplemental Fig. 2, and Table 2, C1P lowered the Kₛ by 2.4-fold but had smaller effects on the triple mutants (R57A/K58A/R59A), the double mutants (R57A/K58A, R57A/R59A, and K58A/R59A), and the single mutant (R59A). These results corroborate the notion that the specific binding of C1P to the cationic β-groove (Arg⁵⁷/Lys⁵⁸/Arg⁵⁹) activates cPLA₂α by lowering its membrane dissociation.

**DISCUSSION**

In this study, for the first time, a novel interaction site for C1P has been identified for a target protein, specifically cPLA₂α. C1P binds to a cationic patch (Arg⁵⁷, Lys⁵⁸, and Arg⁵⁹) on the β-groove of the C2 domain that is adjacent to but distinct from the membrane-penetrating CBRs. The interaction, with just 3 mol % C1P in the vesicles, increases cPLA₂α affinity nearly 10-fold in 10 μM Ca²⁺. The affinity increase is due to a modest 2-fold increase in kₐ and a more prominent 4.5-fold decrease in kₐ. Thus, C1P functions in increasing the membrane residence time of cPLA₂α, reminiscent of other interactions of peripheral proteins with phosphatidylinositol and/or diacylglycerol (18, 34–36), which is generally attributed to the specific nature of the binding and/or membrane penetration induced via the interaction (16). In line with this specificity, mutations of cationic residues in the C2 domain (triple, double, or single), reduced binding to C1P-containing vesicles 2–6-fold without observable effects on PC or PC/PtdIns(4,5)P₂ vesicles. Thus, mutagenesis of these cationic residues did not affect the structure of the enzyme. It is important to note that none of the C2 domain cationic mutants appreciably lowered PtdIns(4,5)P₂ or PtdIns(4,5)P₂ vesicle binding, demonstrating the unique nature of the C1P and PtdIns(4,5)P₂ binding sites. In fact, it has been suggested that the PtdIns(4,5)P₂ binding site resides in the catalytic domain (28, 31). Furthermore, furthering the validity of the C1P interaction, all mutations of the cationic groove residues increased kₐ and slightly decreased kₐ, similar to the effects of C1P on wild-type cPLA₂α binding. Thus, these studies have established a role of C1P in the activation of cPLA₂α via a novel binding site localized to the cationic β-groove of the C2 domain.

Currently, the exact mechanism of stereospecific recognition of C1P by the cPLA₂α C2 domain is unknown. Among three cationic residues investigated in this study, Arg⁵⁷ seems to be most important because its mutation consistently has a bigger effect for C1P interaction than mutations of Arg⁵⁷ and Lys⁵⁸. This is intriguing in that Arg⁵⁷ is more proximal than Arg⁵⁷ and Lys⁵⁸ to the calcium binding loops. Thus, when the Ca²⁺ binding loops interact with and partially penetrate the membrane, the cationic groove, Arg⁵⁷ in particular, seems to be well positioned to bind an anionic lipid head group (37, 38). Under these conditions, C1P may serve as a bridge between cPLA₂α and the membrane, similar to that proposed for the AP180 N-terminal
homology domain (ANTH) domain of PtdIns(4,5)P$_2$ (36, 39) interaction or the Ca$^{2+}$-bridge suggested for the C2 domain of protein kinase Ca (40). Alternatively, C1P could induce the more effective penetration of cPLA$_2$$\alpha$ through the C2 and/or catalytic domains. Our earlier study demonstrated that the effects of C1P binding are more prominent on the isolated C2 domain than full-length cPLA$_2$$\alpha$, suggesting that C1P effects are more local to the C2 domain binding of cPLA$_2$$\alpha$. The current study opens an avenue to investigate the nature and orientation of cPLA$_2$$\alpha$ as well as its isolated C2 domain at the C1P- and PtdIns(4,5)P$_2$-containing membrane interface through lipid penetration analysis (10), EPR (37, 41, 42), x-ray reflectivity studies (38), or molecular dynamics simulations (41).

The involvement of the $\beta$-groove in lipid binding was first suggested by Fukuda and coworkers who demonstrated the ability of the C2B domain of synaptotagmin II and IV to bind soluble inositol polyphosphates (43, 44). Subsequently, a number of other C2 domains have been shown to bind lipids through their $\beta$-groove in both Ca$^{2+}$-dependent and Ca$^{2+}$-independent manners (45–47). Although most C2 domains reported to bind lipids through their $\beta$-groove interact nonspecifically with phosphatidylinositides, such as PtdIns(4,5)P$_2$, the cPLA$_2$$\alpha$ C2 domain is one of the first C2 domains demonstrated to harbor such selectivity for anionic lipids, only displaying an affinity increase with C1P. Furthermore, this is the first known C2 domain to interact with a phosphorylated sphingolipid. Although this study opens an avenue to better understand the function of C1P in the recruitment of cPLA$_2$$\alpha$ to the Golgi, it also serves as a framework to systematically study the unique nature of C2 domain lipid interactions with particular emphasis on the $\beta$-groove.

In this study, for the first time, we have determined the amino acids (Arg$_{57}$/Lys$_{58}$/Arg$_{59}$) critical for the C1P-cPLA$_2$$\alpha$ interaction. The interaction site for C1P was localized to the cationic $\beta$-groove of the C2 domain of the enzyme. Cationic mutants of cPLA$_2$$\alpha$ demonstrated decreased response to C1P as shown by SPR and mixed-micelle activity assays. This effect was also shown to be specific to C1P as these mutants retained their response to PtdIns(4,5)P$_2$. Thus, this study further defines a specific role for C1P in the activation of cPLA$_2$$\alpha$. The identification of the C1P binding site will now allow for “in-depth” studies on the requirement of the C1P-cPLA$_2$$\alpha$ interaction for cPLA$_2$$\alpha$ translocation to membranes.

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