Anionic lipids activate group IVA cytosolic phospholipase A\(_2\) via distinct and separate mechanisms

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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\(_2\)\(_{\alpha}\) with its membrane substrate phosphatidylcholine. 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.

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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 of cPLA\(_2\)\(_{\alpha}\).

Abbreviations: AA, arachidonic acid; cPLA\(_2\)\(_{\alpha}\), group IVA cytosolic phospholipase A\(_2\); CIP, ceramide-1-phosphate; K\(_{d}\), dissociation constant; OPPC, 1-oleoyl-2-palmitoyl-phosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine; PC, phosphatidylcholine; PI(4,5)P\(_2\), phosphatidylinositol-4,5-bisphosphate; SPR, surface plasmon resonance.

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of cPLA₂α. Mosior, Six, and Dennis (12) also showed that cPLA₂α binds with increased affinity to surfaces with PI(4,5)P₂ 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)P₂ binding. In the current study, we identified the mechanistic difference in the activation of cPLA₂ by anionic lipids C1P and PI(4,5)P₂. 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)P₂ 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-α-ω-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and used without further purification. [³⁵S]POPC was purchased from American Radiolabeled Chemicals. 1,2-Dipalmitoyl derivatives of PI(4,5)P₂ 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 the presence of cPLA₂α (17, 18). Each lipid layer was stabilized with 10 μM Ca²⁺ and 100 μM PMSF (9, 18, 20). 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 cPLA₂α (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 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 the dissociation constant (Kd), 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 BIAlavuation 3.0 software (Biacore) as described previously (9, 18, 20). The Kd was then calculated from the equation Kd = kₐ/kₐ, kₐ is the membrane dissociation rate constant, and kₐ is the membrane association constant. Kd 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

**Recombinant expression of cPLA₂α**

Recombinant human cPLA₂α 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-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 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, 10% glycerol) 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**

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### Table 1. Binding parameters for group IVA cytosolic phospholipase A₂ determined from surface plasmon resonance analysis

| Lipid          | kₐ  | kₐ  | Kd   |
|----------------|-----|-----|------|
| POPC           | 1.2 | 0.2 | 10^5 |
| POPC/CIP (97:3)| 2.4 | 3.4 | 10^5 |
| POPC/CIP (95:5)| 2.8 | 0.6 | 10^5 |
| POPC/PI(4,5)P₂ | 1.7 | 0.2 | 10^5 |
| POPC/PI(4,5)P₂ | 1.9 | 0.3 | 10^5 |

C1P, ceramide-1-phosphate; kₐ membrane association constant; kₐ membrane dissociation rate constant; Kd dissociation constant; PI(4,5)P₂, 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 Ca²⁺, and 5% glycerol.

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of 5 μl/min to allow sufficient time for the \( R \) values of the association phase to reach saturating response values (\( R_{\text{sat}} \)). \( R_{\text{sat}} \) values were then plotted versus protein concentrations (\( C \)), and the \( K_a \) value was determined by a nonlinear least-squares analysis of the binding isotherm using the equation \( R_{\text{eq}} = R_{\text{sat}}/(1 + K_a/C) \). 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\(_2\) into the phospholipid monolayer of different lipid compositions was measured in terms of the change in surface pressure (\( \pi \)) 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 (\( \pi_0 \)) 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 (\( \Delta \pi \)) was monitored for 45 min. The \( \Delta \pi \) value reached a maximum after 25 min in all experiments.

**Mixed-micelle assay for cPLA\(_2\)**

cPLA\(_2\) 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\(_2\)SO\(_4\), 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\(_2\) 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 cPLA\(_2\). Data are representative of six separate determinations on three separate occasions.

**Mixed-vesicle assay for cPLA\(_2\)**

cPLA\(_2\) activity was measured in vesicles composed of 30 μM PAPC alone or 10 μM PAPC and 100 μM 1-oleoyl-2-palmitoyl-phosphatidylcholine (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 lipids 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\(_2\)SO\(_4\), 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.
cPLA2α and ≥2 molecules of C1P per molecule of cPLA2α. Thus, both C1P and PI(4,5)P2 induce dramatic increases in the activity of the enzyme but different stoichiometries.

Effects of PI(4,5)P2 and C1P on the membrane dissociation rate of cPLA2α

To demonstrate the effects of PI(4,5)P2 and C1P on the dissociation rate constant or membrane residence time, we measured the kinetics and affinity of cPLA2α binding with lipid vesicles using SPR. Both C1P and PI(4,5)P2 increased the affinity of cPLA2α 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)P2 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 cPLA2α for PC-rich membranes.

Effects of PI(4,5)P2 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)P2 interactions with cPLA2α, 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 ($K_d^A$) of the enzyme by 80% and increasing the $V_{\max}$ of the reaction by ~8- to 10-fold (9). The dissociation constant, $K_d^A$, 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)P2 on the $K_d^A$ of the reaction was examined using the same methodology. PI(4,5)P2 induced a dramatic 15-fold increase in the $V_{\max}$ of the reaction but had only a small effect on the $K_d^A$ of the reaction (Fig. 2A). This suggests that PI(4,5)P2 does not have as great a role as C1P in mediating the membrane residence time of cPLA2α, 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)P2 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)P2 on the kinetic behavior of cPLA2α. A: Effect of PI(4,5)P2 on the dissociation constant of cPLA2α. cPLA2α 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)P2 for 45 min at 37°C. The PC mole fraction was held constant at 0.137. Data are presented as cPLA2α activity measured as nmol arachidonic acid (AA) produced/min/mg recombinant cPLA2α ± 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)P2 on the Michaelis-Menten constant of cPLA2α. cPLA2α activity was measured as a function of the mole fraction of PC ([PC]/[PC + Triton X-100 + PI(4,5)P2]) in the absence (closed squares) and presence (open squares) of 2 mol% PI(4,5)P2 for 45 min at 37°C. Data are presented as cPLA2α activity measured as nmol AA produced/min/mg recombinant cPLA2α ± SEM. Data are representative of six separate determinations on three separate occasions.](image-url)
Thus, the two anionic lipids activate cPLA2α via different mechanisms: C1P having an effect on $K^A$, and PI(4,5)P2 having an effect on the $K^B$ of the reaction.

**Effects of C1P and PI(4,5)P2 on the membrane penetration of cPLA2α**

A number of studies have demonstrated the ability of cPLA2α 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 cPLA2α might also contribute to the penetration of the enzyme. Our finding that C1P and PI(4,5)P2 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 cPLA2α. 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 $\pi_c$ value should be >30 mN/m.

As shown in Fig. 3A, 3 mol% of C1P did not significantly influence the monolayer penetration of cPLA2α to PC monolayers, as both with and without C1P the $\pi_c$ value (x-intercept) was ~28 mN/m. However, when 3 mol% of PI(4,5)P2 was added to the monolayer, cPLA2α penetration increased significantly to a $\pi_c$ of ~34 mN/m. PI(4,5)P2 could increase the penetration of cPLA2α by enhancing the penetration of the C2 domain, the catalytic domain, or both. To assess these possibilities, we monitored the penetration of hydrophobic 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)P2 (Fig. 3C). This demonstrates that PI(4,5)P2 binding to the catalytic domain enhances/increases the penetration of hydrophobic residues around the rim of the active site to enhance the activity of cPLA2α.

**Comparison of the effects of C1P versus PI(4,5)P2 on surface dilution in a vesicle-based assay**

Traditionally, cPLA2α activity has been measured using a vesicle-based assay (5). This assay requires 1,2-dioleoyl glycerol or ceramide for cPLA2α 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)P2 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)P2 interaction.

**C1P competes with PI(4,5)P2 for activation of cPLA2α**

We measured the activity of cPLA2α in response to both C1P and PI(4,5)P2 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)P2 showed a 16-fold increase in the activity. However, upon the addition of both C1P and PI(4,5)P2 to the micelle, there was no synergistic or additive increase in the activity of the enzyme. Furthermore, addition of PI(4,5)P2 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)P2 for cPLA2α 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 cPLA2α by the anionic lipids C1P and PI(4,5)P2. 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)P2 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 cPLA2α.

Our first indicator of two distinct mechanisms for C1P and PI(4,5)P2 was the stoichiometry of the activation of cPLA2α by PI(4,5)P2. We have previously demonstrated that C1P increases 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 cPLA2α.

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**C1P competes with PI(4,5)P2 for activation of cPLA2α**

We measured the activity of cPLA2α in response to both C1P and PI(4,5)P2 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)P2 showed a 16-fold increase in the activity. However, upon the addition of both C1P and PI(4,5)P2 to the micelle, there was no synergistic or additive increase in the activity of the enzyme. Furthermore, addition of PI(4,5)P2 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)P2 for cPLA2α activation as a result of the higher affinity for C1P.
The efficiency of cPLA₂α, the enzyme demonstrated increased membrane penetration in the presence of PIP₂, whereas C1P was ineffective. Thus, PIP₂ activates cPLA₂α 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 cPLA₂α 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)P₂ on the activation of cPLA₂α 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 cPLA₂α 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...
substrate PAPC with OPPC, the activity of cPLA₂α was increased by 5-fold in the presence of C1P versus 80% increase observed using vesicles composed of only PAPC. PI(4,5)P₂ 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)P₂ 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)P₂ activates cPLA₂α 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)P₂ but not C1P. Instead, C1P increases the residence time through electrostatic interactions with cationic residues in the C2 domain (29).

In the last part of this study, we explored the possibility that PI(4,5)P₂ and C1P act synergistically or additively on cPLA₂α 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)P₂ do not coordinate activate cPLA₂α in vitro. These data are also interesting given the observation that the addition of C1P negated the PI(4,5)P₂ activation of the enzyme. Therefore, C1P, having higher affinity for cPLA₂α, recruited the enzyme to the micelle, not allowing any interaction with PI(4,5)P₂. 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)P₂ may be localized in lipid subdomains and require colocalization at a specific distance to activate cPLA₂α 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)P₂ and C1P activate cPLA₂α via separate mechanisms; 2) PI(4,5)P₂ activates cPLA₂α by increasing the enzyme’s catalytic efficiency and membrane penetration once bound to the membrane; and 3) C1P and PI(4,5)P₂ do not coordinate activate cPLA₂α in a coordinated manner in vitro. These studies open an entirely new avenue to investigate the nature and orientation of cPLA₂α at the C1P and PI(4,5)P₂ 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 cPLA₂α in cells in a coordinated manner? How? Can we simulate this effect in vitro, or are these lipids important for entirely different signaling mechanisms?
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