Activation of Phospholipase C δ1 through C2 Domain by a Ca^{2+}-Enzyme-Phosphatidyserine Ternary Complex*

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The concentration of free Ca^{2+} and the composition of nonsubstrate phospholipids profoundly affect the activity of phospholipase C δ1 (PLCδ1). The rate of PLCδ1 hydrolysis of phosphatidylinositol 4,5-bisphosphate was stimulated 20-fold by phosphatidyserine (PS), 4-fold by phosphatidatic acid (PA), and not at all by phosphatidylethanolamine or phosphatidylcholine (PC). PS reduced the Ca^{2+} concentration required for half-maximal activation of PLCδ1 from 5.4 to 0.5 μM. In the presence of Ca^{2+}, PLCδ1 specifically bound to PS/PC but not to PA/PC vesicles in a dose-dependent and saturable manner. Ca^{2+} also bound to PLCδ1 and required the presence of PS/PC vesicles but not PA/PC vesicles. The free Ca^{2+} concentration required for half-maximal Ca^{2+} binding was estimated to be 8 μM. Surface dilution kinetic analysis revealed that the K_m was reduced 20-fold by the presence of 25 mol% PS, whereas V_{max} and K_d were unaffected. Deletion of amino acid residues 646–654 from the C2 domain of PLCδ1 impaired Ca^{2+} binding and reduced its stimulation and binding by PS. Taken together, the results suggest that the formation of an enzyme-Ca^{2+}-PS ternary complex through the C2 domain increases the affinity for substrate and consequently leads to enzyme activation.

Approximately 12 distinct isoforms of phospholipase C catalyze the Ca^{2+}-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield the second messengers inositol trisphosphate (IP_3) and diacylglycerol (1, 2). This constitutes one of the major pathways for receptor-coupled signaling at the plasma membrane of most eucaryotic cells. Three families of PLC isoforms have been described in mammals: PLCα, PLCβ, and PLCδ (2). The members of each family are highly homologous to one another at the amino acid sequence level, but little identity exists between members of different families (2). Three exceptions to this divergence in structure are the catalytic domain, the C2 domain, and the N-terminal pleckstrin homology (PH) domain (1, 3).

Among the initial steps in activation of PLC is a translocation to the plasma membrane. The enzyme binds to the lipid-water interface via the noncatalytic lipid binding PH domain, which is located near the N terminus of PLCδ1 and binds multiple phosphoinositides such as PIP_2 and IP_3 (4–9). This domain allows the enzyme to catalyze the hydrolysis of many substrate molecules without falling off the interface, a process referred to as processive catalysis (5, 9). Because PH domain binds IP_3 tightly, it also could function as a feedback regulator of catalysis.

Although much is known about the function of the PH domain in PLCδ1, very little is known about another lipid binding motif, the C2 domain. The C2 domain comprises approximately 130 amino acid residues and has been found in nearly 100 signaling molecules (10). The C2 domain was first identified in protein kinase C, and its function was implicated in Ca^{2+}-dependent phospholipid interactions (11). Structural studies estimate three to four divalent metal binding sites in the C2 domain of PLCδ1 (12). The C2 domain in the C terminus of PLCδ1 is essential for catalysis, because partial deletion of the C2 domain results in an inactive enzyme (13). However, the molecular mechanism by which C2 domain functions in PLCδ1 catalysis still awaits further investigation.

PLCδ1 is a prototype for enzymes that operate at an interface. As for other membrane-associated enzymes, the ability of PLCδ1 to catalyze the hydrolysis of PI or PIP_2 is influenced remarkably by the presence of nonsubstrate phospholipids in the membrane (14–19). The molecular mechanisms by which nonsubstrate phospholipid affects PLC activity is largely unknown. Nonsubstrate phospholipids could alter the structure of the PLC-membrane interface or the net charge of the interface or could promote a specific interaction between enzyme and interface.

This report examines the effect of nonsubstrate phospholipids on the activity of PLCδ1 and examines the role of Ca^{2+} in this response. These experiments revealed that PLCδ1 was specifically stimulated by PS. PS stimulates the substrate affinity of the enzyme by virtue of its ability to bind the PLCδ1 via C2 domain in a Ca^{2+}-dependent manner. We propose that Ca^{2+} regulates PLCδ1 activity by promoting the formation of an enzyme-PS-Ca^{2+} ternary complex, which leads to activation via a 20-fold reduction in the K_m for substrate.

EXPERIMENTAL PROCEDURES

Materials—The expression vector pRSETa was from Invitrogen. To express PLCδ1 under the control of the T7 promoter, the coding sequence for PLCδ1 was cloned into pRSETa. The resulting expression construct (pRSETaPlc) was transformed into the Escherichia coli strain BL21(DE3)pLys (Novagen), and the protein was isolated and purified as described previously (20). Phosphatidylethanolamine, PA, PC, and PS were obtained from Avanti Polar Lipids Inc. PIP_2 and dodecyl maltoside was obtained from Calbiochem. The double point mutant PLCδ1 (E341G,E390G) in which the calcium-binding residues Glu-341 and Glu-390 in the cleavage center were both changed to Gly. In the C2 loop deletion mutant (Δ646–654), residues 646–654 implicated in the
divalent metal binding were deleted. These mutant forms of PLCδ1 were constructed, expressed, and purified as described previously (20, 21).

**Phospholipid Binding Assay**—Phospholipid vesicles composed of PS/PC or PA/PC mixtures were prepared as described by Mueller et al. (22) with slight modifications (20, 21). A dry phospholipid film was formed by slowly blowing 0.25 ml of chloroform/methanol (2:1 v/v) containing mixed lipids (300 nmol or the indicated concentration of each of the indicated phospholipids) under a stream of nitrogen followed by freeze-drying under vacuum for 4 h. The phospholipid film was hydrated under nitrogen with 0.5 ml of nitrogen-aerated 0.16 mm sucrose for 1.5 h, followed by mixing with an equal volume of distilled H2O. Vesicles were isolated from the pellet by centrifuging the hydrated phospholipids at 1200 × g for 20 min. The phospholipid vesicles were washed once with 1 ml of 50 mM HEPES, pH 7.0, 100 mM KCl, 2 mM phospholipids at 1200 × g for 20 min. The free and bound PLCδ1 concentration of free calcium. The reaction was carried out at 30 °C for 15 min. The free and bound PLCδ1 pellet fractions were resolved by 12% SDS-polyacrylamide gel electrophoresis, and the amount of PLCδ1 was estimated by samples containing protein plus phospholipid.

**CentrifugationBinding Assay**—The binding of PLCδ1 to phospholipid vesicles was estimated by a centrifugation assay (20, 29). The free Ca2⁺ concentration was calculated according to Fabiato and Fabiato (24). To perform the assay, 1 µg of enzyme was incubated with 200 µl of 50 mM HEPES, pH 7.0, 100 mM KCl, 10 mM EGTA (binding buffer) and resuspended in 0.5 ml of the same buffer.

**Calcium Binding Measurements**—Calcium binding was determined by a nitrocellulose membrane binding assay similar to that described by Nakamura et al. (30) with slight modifications (20, 21). 150 µg PS/PC or PA/PC phospholipid vesicles containing the indicated mole fractions of lipids were incubated with PLCδ1 (final concentration, 0.15 µM) in 100 µl of 50 mM HEPES, pH 7.0, 100 mM KCl, 150 mM phospholipid vesicle, and various concentrations of CaCl2 to yield the indicated concentration of free calcium. The reaction was carried out at 30 °C for 15 min. The free and bound PLCδ1 were separated by sedimentation at 50,000 × g for 30 min. An equal proportion of the supernatant and pellet fractions were resolved by 12% SDS-polyacrylamide gel electrophoresis, and the amount of PLCδ1 in each fraction was estimated by Western blotting analysis.

**Calcium binding measurements**—Calcium binding was determined by a nitrocellulose membrane binding assay similar to that described by Nakamura et al. (30) with slight modifications (20, 21). 150 µg PS/PC or PA/PC phospholipid vesicles containing the indicated mole fractions of lipids were incubated with PLCδ1 (final concentration, 0.15 µM) in 100 µl of 50 mM HEPES, pH 7.0, 100 mM KCl, 10 mM EGTA (binding buffer) and resuspended in 0.5 ml of the same buffer.

**Effect of Nonsubstrate Phospholipids on the Ca²⁺ Dependence of PLCδ1 Catalysis**—The concentration of free Ca²⁺ and nonsubstrate phospholipids can greatly influence the activity of PLCδ1. As shown in Fig. 1A, anionic phospholipids such as PS and PA stimulated PLCδ1 hydrolysis of PIP2 by a factor of 20 and 4, respectively, whereas cationic phospholipids such as phosphatidylethanolamine and PC had no effect or were inhibitory. Although PS and PA are both anionic phospholipids, PS stimulation was much greater than that of PA. As shown in Fig. 1B, the maximal stimulation of PLCδ1 activity by PS (12 µmol/min/mg) was at least 5-fold higher than that by PA (2.3 µmol/min/mg). The concentration (mol %) of PA for half-maximal stimulation of PLCδ1 was lower than that of PS. This analysis revealed that the affinity of PLCδ1 appears to be greater for PA than for PS, whereas the maximal stimulation of PLCδ1 activity by PS is much greater than that by PA.

Because Ca²⁺ participates directly in the PLCδ1 catalyzed hydrolysis of PIP2, we also examined the effect of PS concentration on the Ca²⁺ dependence of PLCδ1 activity. As illustrated in Fig. 2, the concentration of Ca²⁺ required for half-maximal stimulation in 25 mol % PS mixed micelles was 0.45 mM, whereas it was greater than 5 µM in PS-free micelles. This result demonstrated that PS significantly increases the potency for Ca²⁺ activation of PLCδ1.

Ca²⁺ RegulatesPhospholipid Binding to PLCδ1—PS could activate PLCδ1 by interacting with the enzyme directly or through nonspecific mechanisms. To distinguish between these possibilities, centrifugation binding experiments were performed with vesicles to examine the interaction between PLCδ1 and PS. Fig. 3 shows that PLCδ1 accumulates in the pellet fraction, a consequence of direct binding of PLCδ1 to the sucrose-loaded PS/PC vesicles. Furthermore, this binding was dependent on Ca²⁺. In the absence of free Ca²⁺, very little PLCδ1 bound to the PS/PC vesicles. PLCδ1 binding to PS/PC vesicles increased as the concentration of free Ca²⁺ increased, reaching saturation (100% of PLCδ1 in the pellet) at 50 µM free Ca²⁺.

It is the PS that is essential for PLCδ1 to bind to the PS/PC vesicles.
Phosphatidylserine Regulates Ca\textsuperscript{2+} Binding to PLC\textsubscript{61}-PLC\textsubscript{61} catalyzed hydrolysis of PIP\textsubscript{2} in dodecyl maltoside mixed micelles containing 25 mol % PA, PS, phosphatidylethanolamine (PE), or PC (A). PLC\textsubscript{61} catalyzed hydrolysis of PIP\textsubscript{2} in mixed micelles containing increasing concentrations of PS (●) or PA (○) (B). PIP\textsubscript{2} was present in mixed micelles at 1 mol %. The total concentration of dodecyl maltoside plus nonsubstrate phospholipid was constant at 495 μM. PLC\textsubscript{61} catalyzed hydrolysis of PIP\textsubscript{2} in the mixed micelles was measured in a 50-μl reaction buffer (50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA) plus 500 μg/ml bovine serum albumin and CaCl\textsubscript{2} to yield 1 μM of free Ca\textsuperscript{2+}. The free Ca\textsuperscript{2+} concentration in an EGTA-CaCl\textsubscript{2} buffer was calculated according to Fabiato and Fabiato (24). The reaction was carried at 30 °C for 1–5 min, stopped, and quantitated as described under "Experimental Procedures."

phospholipid vesicles. No binding of PLC\textsubscript{61} was detected in vesicles devoid of PS. As the concentration of PS increased (Fig. 4a), more PLC\textsubscript{61} bound to the vesicle; saturation of PLC\textsubscript{61} binding occurred at a concentration of 35 mol % PS. The concentration of PS required in the mixed micelles for half-maximal binding was estimated to be 15 mol %. The binding of phospholipid vesicles by PLC\textsubscript{61} was remarkably specific for PS; in contrast, only a small amount of PLC\textsubscript{61} bound to PA/PC vesicles (Fig. 4b).

Phosphatidylserine Regulates Ca\textsuperscript{2+} Binding to PLC\textsubscript{61}-A nitrocellulose filter Ca\textsuperscript{2+} protein binding assay was used to investigate the role of Ca\textsuperscript{2+} in regulating the binding of phospholipid to PLC\textsubscript{61}. In the absence of PLC\textsubscript{61}, 45Ca\textsuperscript{2+} was poorly retained on the nitrocellulose membrane (Fig. 5A). Even the binding between 0.15 μM PLC\textsubscript{61} and 40 μM free 45Ca\textsuperscript{2+} (1.5 × 10\textsuperscript{7} cpm) was barely detectable (7,000–10,000 cpm). However, the binding of 45Ca\textsuperscript{2+} to PLC\textsubscript{61} was significantly increased by including PS/PC but not PA/PC vesicles in the binding mixture. As shown in Fig. 5A, as much as 1% (150,000 cpm) of the total 45Ca\textsuperscript{2+} was bound to PLC\textsubscript{61} when 150 μM of PS/PC (molar ratio, 1:1) was co-incubated with 0.15 μM PLC\textsubscript{61} and 40 μM of free Ca\textsuperscript{2+}. Binding of Ca\textsuperscript{2+} to PLC\textsubscript{61} specifically required PS, because the binding was minimal with PA/PC phospholipid vesicles.

PS stimulated PLC\textsubscript{61}-Ca\textsuperscript{2+} binding in a dose-dependent and saturable manner. As shown in Fig. 5B, Ca\textsuperscript{2+} binding by PLC\textsubscript{61} was significantly stimulated by as low as 5 mol % PS, and the binding plateaued at 40 mol % PS. The concentration of PS required for half-maximal Ca\textsuperscript{2+} binding under these conditions was estimated to be 10 mol %. In contrast, stimulation of Ca\textsuperscript{2+}-PLC\textsubscript{61} binding by PA/PC vesicles was minimal. The maximal PA-dependent Ca\textsuperscript{2+} binding by PLC\textsubscript{61} was lower than that of PS and occurred at 10 mol % PA; no further stimulation of binding was observed even if the concentration of PA was increased to 75 mol %. This result demonstrates the specificity for the head group of PS required for PLC\textsubscript{61} to bind Ca\textsuperscript{2+}.

To understand how PS facilitates Ca\textsuperscript{2+} binding to PLC\textsubscript{61}, the effect of PS on the dose dependence of Ca\textsuperscript{2+} binding to PLC\textsubscript{61} was examined. 45Ca\textsuperscript{2+} bound to PLC\textsubscript{61} in a dose-dependent and saturable manner when the binding was carried out in the presence of the indicated concentration of free Ca\textsuperscript{2+} ion is shown. 45Ca\textsuperscript{2+} bound to PLC\textsubscript{61} in the presence of the indicated concentration of free Ca\textsuperscript{2+} was 10 mol % PS. The reaction was carried at 30 °C for 15 min. The bound enzyme (pellet fraction) and the free enzyme (supernatant fraction) were separated and quantitated as described under "Experimental Procedures."
presence of phospholipid vesicles containing PS. As shown in Fig. 6, in the presence of PS/PC vesicles containing 35 mol % PS the maximal binding of Ca\(^{2+}\) to 0.15 \(\mu\)M PLC\(_{\delta1}\) was 35 pmol, which corresponds to approximately 2.3 pmol Ca\(^{2+}\)/pmol protein. The concentration of Ca\(^{2+}\) required for half-maximal binding was estimated to be 7 \(\mu\)M. In the presence of vesicles containing 10% PS, the total binding at 1 mM Ca\(^{2+}\) was reduced to 23 pmol, corresponding to 1.4 pmol Ca\(^{2+}\)/pmol protein. With vesicles containing 2.5 mol % PS, saturation of Ca\(^{2+}\) binding was not reached, even at a free Ca\(^{2+}\) concentration of 1 mM. These results demonstrate that PS specifically increases the affinity of Ca\(^{2+}\) for PLC\(_{\delta1}\).

**PS-dependent Ca\(^{2+}\) Binding to the Cleavage Center Mutant (E341G,E390G) and C2 Domain Deletion Mutant (D646–654)—**

Two structure features in PLC\(_{\delta1}\) have been shown to be involved in divalent metal ion binding, the cleavage center and the C2 domain near the C terminus of the enzyme. To determine whether PS-dependent Ca\(^{2+}\) binding is mediated by these structural determinants, we examined the Ca\(^{2+}\) binding activity of a cleavage center double point mutant (E341G,E390G) and a C2 domain loop deletion mutant (D646–654). The double mutant enzyme E341G,E390G is completely defective in catalysis (Table I), presumably because of the loss of Ca\(^{2+}\) binding at the cleavage center. In contrast, C2 loop deletion D646–654 mutant catalyzes the hydrolysis of PI and PIP\(_2\) in a manner comparable with that of the native enzyme (Table I).

The E341G,E390G mutant PLC\(_{\delta1}\), although defective in cleavage activity, was able to bind Ca\(^{2+}\) in a PS-dependent manner indistinguishable from the native enzyme (Fig. 7A), and the binding also displayed Ca\(^{2+}\) dependence similar to that of the native enzyme (Fig. 7B). Although the D646–654 deletion mutant is as active as the native enzyme in catalyzing the hydrolysis of PI or PIP\(_2\), this mutant was severely defective in PS-dependent Ca\(^{2+}\) binding. The D646–654 deletion mutant enzyme bound Ca\(^{2+}\) poorly, even at saturating concentrations of either PS or free Ca\(^{2+}\) (Fig. 7, A and B). These results identify the C2 domain as the structural motif responsible for mediating PS-dependent Ca\(^{2+}\) binding.

**The D646–654 Deletion Mutant Is Defective in PS Stimulation and PS Binding—** To study whether the D646–654 mutant is also defective in PS mediated stimulation, we examined the effect of PS on the Ca\(^{2+}\) dependent catalysis of substrate. In
expressed as release of PIP2 in mixed micelles containing 25 mol % PS was compared as a function of the total PIP2 concentration (from 0.5 to 50 mol %). When the concentration of PIP2 was increased from 0.1 to 9 mol %, the hydrolysis of PIP2 in mixed micelles containing 25 mol % PS sharply increased from 3 to 18 μmol/min/mg and was saturated as the PIP2 concentration approached 4 mol % (Fig. 10). PIP2 hydrolysis in PS-free mixed micelles increased slowly and did not reach a maximum even at a 9 mol % PIP2 (Fig. 10). The most dramatic stimulatory effect of PS on the hydrolysis of PIP2 was found at a low substrate concentration. As shown in Fig. 10, when PIP2 concentration was less than 2 mol %, PIP2 hydrolysis in mixed micelles containing 25 mol % PS was at least 10-fold higher than in PS-free mixed micelles. The stimulatory effect of PS diminished as the concentration of PIP2 increased (Fig. 10).

The kinetic parameters of PLCδ1 (Table II) demonstrate that the stimulation of PIP2 hydrolysis by PS was primarily because of a reduction in the interfacial Michaelis constant (K_m), which governs the affinity between the catalytic site and the substrate. K_m was reduced by a factor of 20, from 0.065 to 0.003, as PS in the mixed micelles was increased from 0 to 25 mol %. In contrast, PS had little effect on the affinity of the enzyme for the membrane (K_a) and its maximal rate of catalysis (V_max).
In this report we examine in detail the effects of nonsubstrate phospholipids on PLCδ1 activity and the molecular mechanism of their interaction. Although both PA and PS stimulate PLCδ1, the mechanism of stimulation may be different for PA and PS. They differ in affinity and maximal stimulation of PLCδ1. A direct interaction between PLCδ1 and PS but not PA was demonstrated by the binding assay. Furthermore, Ca²⁺ binding to PLCδ1 requires PS not PA. These observations suggested that there is a specific interaction between PS and PLCδ1. The similar effect of PS on enzyme stimulation has also been demonstrated for protein kinase C (31, 32).

Although both C2 domain and cleavage center could be the Ca²⁺ binding site, the present data show that PS-dependent Ca²⁺ binding occurs at the C2 domain of PLCδ1. In the presence of saturation level of PS but no substrate, the maximal binding of Ca²⁺ to PLCδ1 was measured to be 2.1 pmol Ca²⁺/pmol protein. The concentration of Ca²⁺ required for half-maximal binding was estimated to be 7 μM. This stoichiometry is in good agreement with other studies indicating that a total of three to five metal ions bind to a single molecule of PLCδ1 (3). One site exists in the catalytic domain where a single calcium ion binds and is required for catalysis (3). Substrate facilitates calcium binding at this site. A second site was maintained at 495 nM in PIP₂/dodecyl maltoside mixed micelles containing 25, 10, and 0 mol % PS plus dodecyl maltoside was maintained at 495 μM (see “Experimental Procedures”). The reaction was carried out and stopped, and the [³²P]IP₃ was separated and quantitated as described under “Experimental Procedures.”

**FIG. 8. Effect of PS concentration on the Ca²⁺ dependence of Δ646–654 deletion mutant PLCδ1 catalytic activity.** Hydrolysis of 5 μM PIP₂ (corresponding to 1 mol % in the mixed micelles) by the native (closed symbols) or Δ646–654 deletion mutant (open symbols) PLCδ1 in PIP₂/dodecyl maltoside mixed micelles containing 25 (circles) and 0 (squares) mol % PS is shown. PLCδ1 catalyzed hydrolysis of PIP₂ in the mixed micelles was carried out as described (Fig. 2 and “Experimental Procedures”) using various concentrations of CaCl₂ to yield the indicated concentration of free Ca²⁺.

**FIG. 9. Ca²⁺-dependent PS binding to the E341G,E390G and the Δ646–654 deletion mutant PLCδ1.** Centrifugation binding assay of the E341G,E390G (a) and the Δ646–654 deletion (b) PLCδ1 to sucrose-loaded PS/PC vesicles in the presence of the indicated concentration of free Ca²⁺ ion. 1 μg of PLCδ1 was incubated with 150 μM PS/PC vesicles (molar ratio, 1:1) in a 0.2-ml reaction containing 50 mM HEPES, pH 7.0, 100 mM KCl, 2 mM EGTA, and various concentrations of CaCl₂ to yield the indicated concentration of free Ca²⁺. The reaction was carried out and binding was determined as described under “Experimental Procedures.”

**FIG. 10. Effect of PS concentration on the substrate dependence of PLCδ1 catalysis of PIP₂.** Hydrolysis of increasing concentrations of PIP₂ by PLCδ1 in PIP₂/dodecyl maltoside mixed micelles containing 0 (●), 10 (□), and 25 (▲) mol % PS is shown. The mol % of PIP₂ was increased, while the concentration of PS was fixed at 0, 10, and 25 mol %, and the combined total concentration of PS plus dodecyl maltoside was maintained at 495 μM (see “Experimental Procedures”). The reaction was carried out and stopped, and the [³²P]IP₃ was separated and quantitated as described under “Experimental Procedures.”

**TABLE II**

| PS concentration (mol %) | Vmax (μmol/min/mg) | Kn (mol fraction) | Ks (μM) |
|--------------------------|--------------------|-------------------|---------|
| 0                        | 16 ± 3             | 0.065 ± 0.01      | 71 ± 8  |
| 10                       | 18 ± 3             | 0.012 ± 0.03      | 61 ± 8  |
| 25                       | 19 ± 4             | 0.003 ± 0.001     | 43 ± 7  |

**DISCUSSION**

In this report we examine in detail the effects of nonsubstrate phospholipids on PLCδ1 activity and the molecular mechanism of their interaction. Although both PA and PS stimulate PLCδ1, the mechanism of stimulation may be different for PA and PS. They differ in affinity and maximal stimulation of PLCδ1. A direct interaction between PLCδ1 and PS but not PA was demonstrated by the binding assay. Furthermore, Ca²⁺ binding to PLCδ1 requires PS not PA. These observations suggested that there is a specific interaction between PS and PLCδ1. The similar effect of PS on enzyme stimulation has also been demonstrated for protein kinase C (31, 32).

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**FIG. 10. Effect of PS concentration on the substrate dependence of PLCδ1 catalysis of PIP₂.** Hydrolysis of increasing concentrations of PIP₂ by PLCδ1 in PIP₂/dodecyl maltoside mixed micelles containing 0 (●), 10 (□), and 25 (▲) mol % PS is shown. The mol % of PIP₂ was increased, while the concentration of PS was fixed at 0, 10, and 25 mol %, and the combined total concentration of PS plus dodecyl maltoside was maintained at 495 μM (see “Experimental Procedures”). The reaction was carried out and stopped, and the [³²P]IP₃ was separated and quantitated as described under “Experimental Procedures.”

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| 25                       | 19 ± 4             | 0.003 ± 0.001     | 43 ± 7  |
hydrolysis in PC vesicles. However, this loop is required for the enzyme to interact with and is stimulated by PS, because Δ646–654 mutant enzyme was much less active than the native enzyme when catalysis was performed in the presence of PS. Although the loop connecting β1 and β2 strand in the C2 domain is required for PS mediated activation and Ca2+ binding of PLCδ1, other residues in the C2 domain were mapped and found essential for PS to bind and stimulate PLCδ1.2

The relationship between calcium, PS, and PLCδ1 appears to be very complex because all three are interdependent. While calcium regulates PS binding, PS also regulates calcium binding to PLCδ1. PS increases the potency for free calcium on the activation of PLCδ1. PS facilitates the binding of calcium to PLCδ1 by increasing the affinity. In the absence of PS, very little calcium binds to PLCδ1. PA has no effect on calcium binding correlating with its weak ability to activate PLCδ1. Furthermore, deleting residues 646–654 not only abolished Ca2+ binding but also eliminated the activation and binding of PLCδ1 by PS. All these observations indicated that the simultaneous presence of Ca2+1, PLCδ1, and PS stabilizes a ternary complex formed by these three components.

The predominant effect of PS on the stimulation of PLCδ1 is to reduce the interfacial Michaelis constant (Km) (increasing the substrate affinity for the catalytic site). The Km was reduced by a factor of 20. A similar result was also found for protein kinase C; a decrease in Km results when rat brain enzyme binds PS and Ca2+ (37). PS has also been found to increase the affinity between coagulation factor IXa and VIIIa and to reduce the interfacial Michaelis constant (Km) (increasing the affinity). In the absence of PS, very little calcium binds to PLCδ1, and PS stabilizes a ternary complex through the C2 domain (41) and Ca2+1 is relatively inactive to catalyze the factor IXa catalyzed proteolysis reaction (38).

Although two distinct mechanisms have been proposed by isolated reports (39, 40), the binding of calcium and PS to the C2 domain may also be an important mechanism for regulation of this enzyme in vivo. Our data demonstrated that in the absence of PS, PLCδ1 is relatively inactive to catalyze the hydrolysis of physiologival concentrations of PIP2 (<1 mol % of total membrane phospholipids). However, PS and calcium stimulate PLCδ1 at physiologival concentration of PIP2 by increasing the affinity for the substrate. The concentration of PS (41) and Ca2+ required to stimulate PLCδ1 are within limits of their intracellular concentrations. The intracellular concentration of calcium is usually below 1 μM; however, the local calcium can rise to almost mM levels after stimulation by various calcium mobilizing agonists. We suggest that the formation of a stimulatory ternary complex is a mechanism by which calcium can regulate this isoform. If we assume that the mol % of PS is relatively constant in the plasma membranes of cells, then calcium becomes the primary modulator of PLCδ1 activity. Of course, this fits into a well established and broad paradigm in which fluxes of calcium are modulators of protein function and cellular effects.

In summary, the present results show that the formation of an enzyme-PS-calcium ternary complex through the C2 domain increases its affinity for substrate and consequently stimulates the enzyme. We postulate that the formation of this ternary complex plays a role in the in vivo activation and regulation of PLCδ1.

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**REFERENCES**

1. Rhee, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048
2. Rhee, S. G., Suh, P. G., Ryu, S. H., and Lee, Y. S. (1989) Science 244, 546–550
3. Essen, L. O., Perisic, O., Cheung, R., Katan, M., and Williams, R. L. (1996) Nature 380, 595–602
4. Yagiawa, H., Sakuma, K., Paterson, H. F., Cheung, R., Allen, V., Hirata, H., Watanabe, Y., Hirata, M., Williams, R. L., and Katan, M. (1998) J. Biol. Chem. 273, 417–424
5. Lomasney, J. W., Cheng, H.-F., Wang, L.-P., Kuan, Y.-S., Liu, S.-M., Fesik, S. W., and King, K. (1996) J. Biol. Chem. 271, 25316–25326
6. Lemmon, M., Ferguson, K., O’Brien, B., Sigler, P., and Schlessinger, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10472–10476
7. Hyvonen, M., Maricas, M. J., Nilges, M., Oechschnat, H., Saraste, M., and Wilmanns, M. (1995) EMBO J. 14, 4676–4685
8. Ferguson, K., Lemmon, M., Schlessinger, J., and Sigler, P. (1995) Cell 83, 1037–1046
9. Ciferriente, M. E., Honkanen, L., and Rebicchi, M. J. (1993) J. Biol. Chem. 268, 11586–11593
10. Rizo, J., and Sudhof, T. C. (1998) J. Biol. Chem. 273, 15879–15882
11. Nelsestuen, G. L., and Lim, T. K. (1977) Biochemistry 16, 780–790
12. Portis, A., Newton, C., Pangborn, W., and Papahadjopoulos, D. (1979) Biochemistry 18, 780–790
13. Hendrickson, H. S., and Dennis, E. A. (1984) J. Biol. Chem. 259, 5734–5739
14. James, S. R., Paterson, A., Harden, T. K., and Downes, C. P. (1995) J. Biol. Chem. 270, 11718–11724
15. Taylor, S. J., and Exton, J. H. (1987) Biochem. J. 248, 791–799
16. Low, M. G., Carroll, R. C., and Cox, A. C. (1986) Biochem. J. 237, 139–145
17. Wilson, D. B., Bross, T. E., Hofmann, S. L., and Magerus, P. W. (1984) J. Biol. Chem. 259, 11718–11724
18. Jackowski, S., and Rock, C. O. (1989) Arch. Biochem. Biophys. 268, 516–524
19. Cheng, H.-F., Jiang, M.-J., Chen, C.-L., Liu, S.-M., Weng, L.-P., Lomasney, S. W., and King, K. (1996) J. Biol. Chem. 271, 5495–5505
20. Wang, L.-P., Lim, C., Kuan, Y.-S., Chen, C.-L., Chen, H.-F., and King, K. (1996) J. Biol. Chem. 271, 24505–24516
21. Mueller, P., Chen, T. F., and Rudy, B. (1983) Biochim. Biophys. Acta 749, 495–501
22. Kawasaki, H., Kasai, H., and Okuyama, T. (1985) Anal. Biochem. 148, 297–302
23. Nelsestuen, G. L., and Lim, T. K. (1977) Biochemistry 16, 4164–4171
24. Portis, A., Newton, C., Pangborn, W., and Papahadjopoulos, D. (1979) Biochemistry 18, 780–790
25. Hendrickson, H. S., and Dennis, E. A. (1984) J. Biol. Chem. 259, 5734–5739
26. James, S. R., Paterson, A., Harden, T. K., and Downes, C. P. (1995) J. Biol. Chem. 270, 11718–11724
27. Lee, M. G., Carroll, R. C., and Cox, A. C. (1986) Biochem. J. 237, 139–145
28. Portis, A., Newton, C., Pangborn, W., and Papahadjopoulos, D. (1979) Biochemistry 18, 780–790
29. Edwards, A. S., and Newton, A. C. (1997) J. Biol. Chem. 272, 15045–15048
30. Rhee, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048
31. Newton, A. C., and Keranen, L. M. (1994) J. Biol. Chem. 269, 20179–20188
32. Newton, A. C., and Keranen, L. M. (1994) Biochemistry 33, 139–145
33. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., and deChamplain, J. (1994) Hypertension 23, 722–728