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Activation of Phospholipase C δ1 through C2 Domain by a Ca^{2+}-Enzyme-Phosphatidylserine 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 phosphatidylserine (PS), 4-fold by phosphatidic 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)[1] 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: PLCβ, PLCδ, and PLCγ (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.

PLC is a prototype for enzymes that operate at an interface. As for other membrane-associated enzymes, the ability of PLC 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

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[1] The abbreviations used are: PIP_2, phosphatidylinositol 4,5-bisphosphate; PH, pleckstrin homology; IP_3, inositol 1,4,5-triphosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PLC, phosphoinositide-specific phospholipase C.

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 0.18 M sucrose for 18 h at 4 °C followed by mixing with an equal volume of distilled H_2O. 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 EGTA (binding buffer) and resuspended in 0.5 ml of the same buffer.

Centrifugation Binding Assay—The binding of PLCδ1 to phospholipid vesicles was estimated by a centrifugation assay (20, 23). The free Ca^{2+} 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, 2 mM EGTA, 150 μM phospholipid vesicle, and various concentrations of $CaCl_2$ 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 (25) and Kawasaki *et al.* (26). 150 μM 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 μM EGTA containing 5–120 μM of $^{45}CaCl_2$ (total cpm, 1.5 × 10^7) to yield the indicated concentration of free Ca^{2+}. Millipore polyvinylidene difluoride Immobilon-P transfer membrane was immersed in methanol and washed three times with 10 ml of 50 mM HEPES, pH 7.0, 100 mM KCl, and 10 mM EGTA. The membrane was mounted on a 96-well Bio-Dot filtration apparatus according to the manufacturer's instructions (Bio-Rad). After incubating at 30 °C for 30 min, the reaction was filtered through the membrane at a constant flow rate of 0.6–1 ml/min. Each well was washed six times with 100 μl of ice-cold 50 mM HEPES, pH 7.0, 100 mM KCl, 10 mM EGTA. The membrane was cut into sections corresponding to each sample, and the retention of $^{45}Ca^{2+}$ on the membrane was determined by liquid scintillation counting. The amount of $^{45}Ca^{2+}$ bound to the enzyme was determined in the presence or absence of vesicles containing the indicated composition of phospholipids. Calcium binding to phospholipid vesicles in the absence of enzyme was determined under the same experimental conditions. The amount of $^{45}Ca^{2+}$ binding directly to phospholipid vesicles was relatively low but consistent with published apparent K_d values for Ca^{2+}-PS interactions (27, 28). The calcium bound to membranes or to phospholipid vesicles was considered nonspecific binding and was subtracted from the amount of Ca^{2+} bound by samples containing protein plus phospholipid.

PIP_2 Hydrolysis Assay—PIP_2 hydrolysis in dodecyl maltoside/PIP_2 mixed micelles was performed in a manner similar to that described by Cifuentes *et al.* (9) with slight modifications. In brief, the indicated amount of PIP_2/[^{3}H]PIP_2 (4 × 10^5 cpm) in chloroform/methanol (19:1) in the presence or absence of the indicated phospholipids was dried under a stream of N_2 and lyophilized for 30 min. Lipids were solubilized by probe sonication in 0.95 ml of 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA plus the indicated concentration of dodecyl maltoside. Bovine serum albumin in the same buffer was added to 500 μg/ml. PLC activity was determined as a function of the concentration of substrate by keeping the total concentration of nonsubstrate phospholipid plus dodecyl maltoside at 500 μM and varying the mole fraction of PIP_2. The reaction at 30 °C was initiated by adding various concentration of $CaCl_2$ to yield the indicated concentration of free calcium. The reaction was continued for 1–5 min and was stopped by adding 0.34 ml of 10% ice-cold trichloroacetic acid and 0.17 ml of bovine serum albumin (10 mg/ml). After incubation on ice for 15 min, the unhydrolyzed [^{3}H]PIP_2 (pellet) was separated from [^{3}H]IP_3 (supernatant) by centrifugation at 2000 × *g* for 10 min at 4 °C.

Analysis of Kinetic Data—Surface dilution kinetics were employed to study PIP_2 hydrolysis catalyzed by PLCδ1. Case III conditions previously described for phospholipase A_2 (29) and PLCβ (30) were employed. In Case III conditions, the total concentration of diluent detergent (dodecyl maltoside plus nonsubstrate phospholipid) was fixed, and the PLC activity was measured with increasing concentrations of substrate. A dual phospholipid binding model of catalysis (Equations 1 and 2) (29) was used to analyze the kinetic data.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \qquad \text{(Eq. 1)}$$

$$ES + S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} ESS \xrightarrow{k_3} ES + P \qquad \text{(Eq. 2)}$$

This model takes into account the fact that the reaction catalyzed by PLCδ1 occurs at the water-lipid interface of the phosphoinositide/dodecyl maltoside mixed micelle. Initial binding of the enzyme to the water-lipid interface of the micelle is described by the micellar dissociation constant, $K_s = k_{-1}/k_{+1}$ (molar unit). This constant is dependent on both the total enzyme concentration and the total substrate. The binding of the second lipid molecule and the subsequent catalysis by PLCδ1 is described by the interfacial Michaelis constant, $K_m = (k_{-2} + k_3)/k_{+2}$ (mole fraction, unitless). Initial rates of catalysis (v) as a function of total concentration of PIP_2 in the vesicle with a fixed concentration of diluent nonsubstrate phospholipids and dodecylmaltoside (T_o) were fitted using Equation 3 (29) to obtain the values of V_{max}, K_s, and K_m.

$$v = \frac{V_{max} S_o^2}{K_m K_s T_o + (T_o + K_s) K_m S_o + (K_m + 1) S_o^2} \qquad \text{(Eq. 3)}$$

where the absolute rate (V_{max}) occurs at an infinite substrate concentration and the saturated substrate mole fraction (S_o) is the total substrate concentration.

RESULTS

Effect of Nonsubstrate Phospholipids on the Ca^{2+} Dependence of PLCδ1 Catalysis—The concentration of free Ca^{2+} and nonsubstrate phospholipids can greatly influence the activity of PLCδ1. As shown in Fig. 1*A*, anionic phospholipids such as PS and PA stimulated PLCδ1 hydrolysis of PIP_2 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. 1*B*, 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 is 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 Ca^{2+} participates directly in the PLCδ1 catalyzed hydrolysis of PIP_2, we also examined the effect of PS concentration on the Ca^{2+} dependence of PLCδ1 activity. As illustrated in Fig. 2, the concentration of Ca^{2+} required for half-maximal stimulation in 25 mol % PS mixed micelles was 0.45 μM, whereas it was greater than 5 μM in PS-free micelles. This result demonstrated that PS significantly increases the potency for Ca^{2+} activation of PLCδ1.

Ca^{2+} Regulates Phospholipid 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 Ca^{2+}. In the absence of free Ca^{2+}, very little PLCδ1 bound to the PS/PC vesicles. PLCδ1 binding to PS/PC vesicles increased as the concentration of free Ca^{2+} increased, reaching saturation (100% of PLCδ1 in the pellet) at 50 μM free Ca^{2+}.

It is the PS that is essential for PLCδ1 to bind to the PS/PC

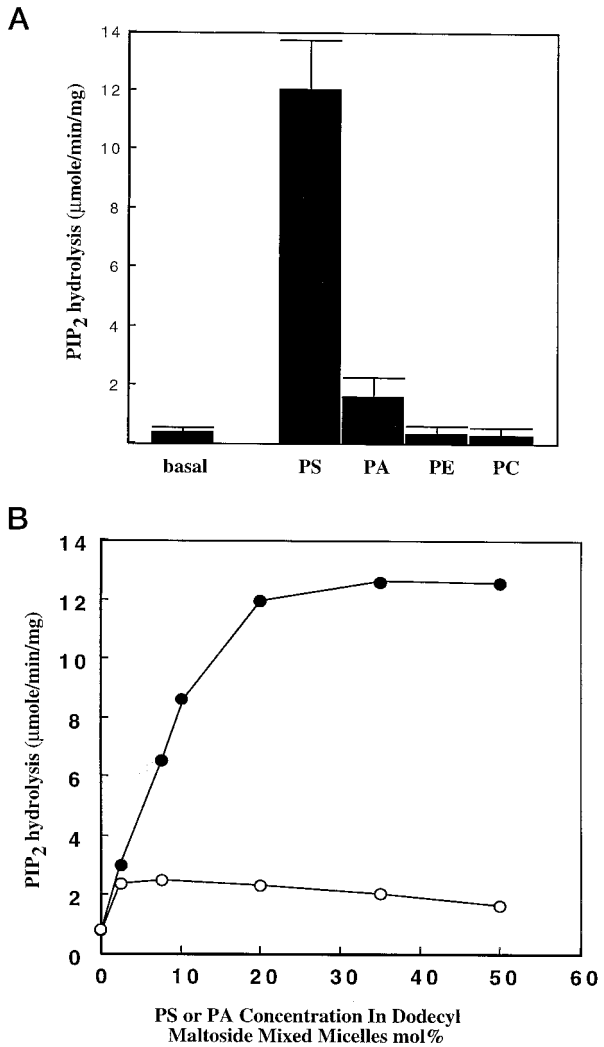

FIG. 1. **Influence of nonsubstrate phospholipid on PIP_2 hydrolysis catalyzed by PLCδ1.** Hydrolysis of 5 μM (1 mol %) PIP_2 in PIP_2/dodecyl maltoside mixed micelles (basal) and in mixed micelles containing 25 mol % PA, PS, phosphatidylethanolamine (*PE*), or PC (*A*). PLCδ1 catalyzed hydrolysis of 5 μM of PIP_2 in mixed micelles containing increasing concentrations of PS (●) or PA (○) (*B*). PIP_2 was present in mixed micelles at 1 mol %. The total concentration of dodecyl maltoside plus nonsubstrate phospholipid was constant at 495 μM. PLCδ1-catalyzed hydrolysis of PIP_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 $CaCl_2$ to yield 1 μM of free Ca^{2+}. The free Ca^{2+} concentration in an EGTA-$CaCl_2$ 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."

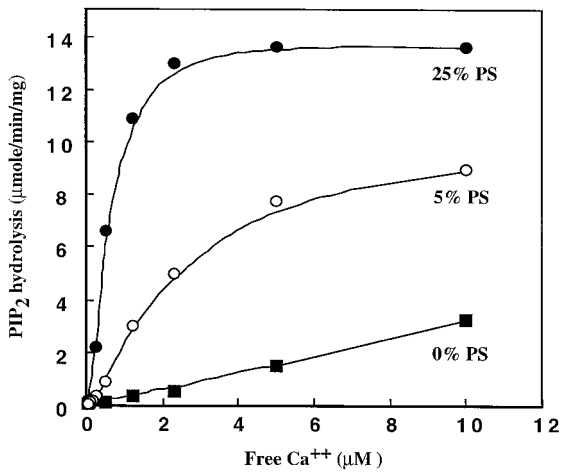

FIG. 2. **Effect of PS on the Ca^{2+} dependence of PLCδ1 catalytic activity.** Calcium concentration dependence of hydrolysis of 5 μM PIP_2 (corresponding to 1 mol % in the mixed micelles) by PLCδ1 in PIP_2/dodecyl maltoside mixed micelles containing 25 (●), 5 (○), and 0 (■) mol % PS. The reaction was carried out in a buffer of 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA plus 500 μg/ml bovine serum albumin and various concentrations of $CaCl_2$ to yield the indicated concentrations of free Ca^{2+}. PLCδ1 catalyzed hydrolysis of PIP_2 in the mixed micelles was measured as described (Fig. 1 and "Experimental Procedures").

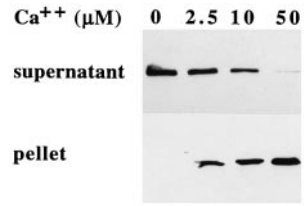

FIG. 3. **Ca^{2+}-dependent phospholipid binding to PLCδ1.** Centrifugation binding assay of PLCδ1 and sucrose-loaded PS/PC vesicles in the presence of the indicated concentration of free Ca^{2+} ion is shown. 1 μg of PLCδ1 protein was incubated with 150 μM PS/PC vesicles (molar ratio, 1:1) in 0.2 ml of 50 mM HEPES, pH 7.0, 100 mM KCl, 2 mM EGTA, and various concentrations of $CaCl_2$ to yield the indicated concentration of free Ca^{2+}. The reaction was incubated 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."

phospholipid vesicles. No binding of PLCδ1 was detected in vesicles devoid of PS. As the concentration of PS increased (Fig. 4*a*), more PLCδ1 bound to the vesicle; saturation of PLCδ1 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 PLCδ1 was remarkably specific for PS; in contrast, only a small amount of PLCδ1 bound to PA/PC vesicles (Fig. 4*b*).

Phophatidylserine Regulates Ca^{2+} Binding to PLCδ1—A nitrocellulose filter Ca^{2+} protein binding assay was used to investigate the role of Ca^{2+} in regulating the binding of phospholipid to PLCδ1. In the absence of PLCδ1, $^{45}Ca^{2+}$ was poorly retained on the nitrocellulose membrane (Fig. 5*A*). Even the binding between 0.15 μM PLCδ1 and 40 μM free $^{45}Ca^{2+}$ (1.5×10^7 cpm) was barely detectable (7,000–10,000 cpm). However, the binding of $^{45}Ca^{2+}$ to PLCδ1 was significantly increased by including PS/PC but not PA/PC vesicles in the binding mixture. As shown in Fig. 5*A*, as much as 1% (150,000 cpm) of the total $^{45}Ca^{2+}$ was bound to PLCδ1 when 150 μM of PS/PC (mole ratio, 1:1) vesicles were co-incubated with 0.15 μM PLCδ1 and 40 μM of free Ca^{2+}. Binding of Ca^{2+} to PLCδ1 specifically required PS, because the binding was minimal with PA/PC phospholipid vesicles.

PS stimulated PLCδ1-Ca^{2+} binding in a dose-dependent and saturable manner. As shown in Fig. 5*B*, Ca^{2+} binding by PLCδ1 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^{2+} binding under these conditions was estimated to be 10 mol %. In contrast, stimulation of Ca^{2+}-PLCδ1 binding by PA/PC vesicles was minimal. The maximal PA-dependent Ca^{2+} binding by PLCδ1 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 PLCδ to bind Ca^{2+}.

To understand how PS facilitates Ca^{2+} binding to PLCδ1, the effect of PS on the dose dependence of Ca^{2+} binding to PLCδ1 was examined. $^{45}Ca^{2+}$ bound to PLCδ1 in a dose-dependent and saturable manner when the binding was carried out in the

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 μM PLCδ1 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 μM. 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 PLCδ1.

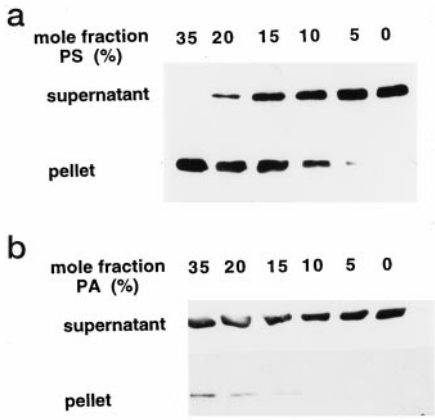

FIG. 4. **Dose dependence of PLCδ1 binding to phospholipid vesicles.** The binding of 1 μg of PLCδ1 to 150 μM PS/PC (*a*) or PA/PC (*b*) vesicles containing the indicated mole fraction of PS or PA was carried out in 0.2 ml of 50 mM HEPES, pH 7.0, 100 mM KCl, 2 mM EGTA, and 100 μM free Ca^{2+}. The mol % of PS or PA was varied by adjusting the concentration of PC such that the total concentration of PA or PS plus that of PC was kept at 150 μM. After incubation 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."

PS-dependent Ca^{2+} Binding to the Cleavage Center Mutant (E341G,E390G) and C2 Domain Deletion Mutant (Δ646–654)—Two structure features in PLCδ1 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 (Δ646–654). 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 Δ646–654 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 PLCδ1, although defective in cleavage activity, was able to bind Ca^{2+} in a PS-dependent manner indistinguishable from the native enzyme (Fig. 7*A*), and the binding also displayed Ca^{2+} dependence similar to that of the native enzyme (Fig. 7*B*). Although the Δ646–654 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 Δ646–654 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 Δ646–654 Deletion Mutant Is Defective in PS Stimulation and PS Binding—To study whether the Δ646–654 mutant is also defective in PS mediated stimulation, we examined the effect of PS on the Ca^{2+}-dependent catalysis of substrate. In

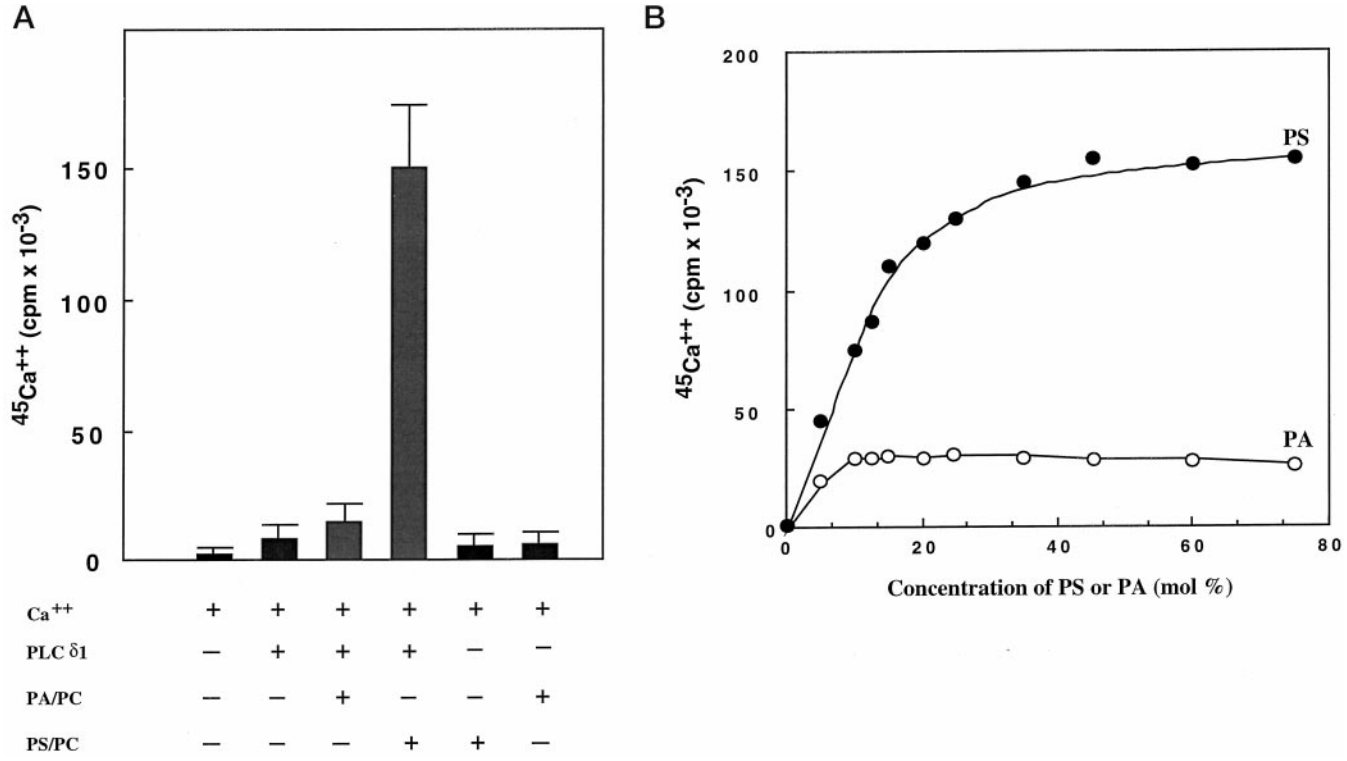

FIG. 5. **Phospholipid stimulates Ca^{2+} binding to PLCδ1.** Binding of 0.15 μM enzyme with 40 μM free $^{45}Ca^{2+}$ (total 1.5×10^7 cpm) in 0.1 ml of buffer containing 50 mM HEPES, pH 7.0, 100 mM KCl, 10 μM EGTA, and 150 μM of the indicated phospholipid vesicles (PA/PC or PS/PC molar ratio, 1:1) (*A*) or in the same buffer containing 150 μM of PA/PC or PS/PC vesicles composed of 0–75 mol % of PA (○) or PS (●), respectively (*B*). After incubation at 30 °C for 30 min, the reaction mixture was filtered through a nitrocellulose membrane and washed extensively with ice-cold 50 mM HEPES, pH 7.0, 5 mM EGTA. The amount of Ca^{2+} bound to the enzyme retained on the filter membrane was determined by scintillation counting.

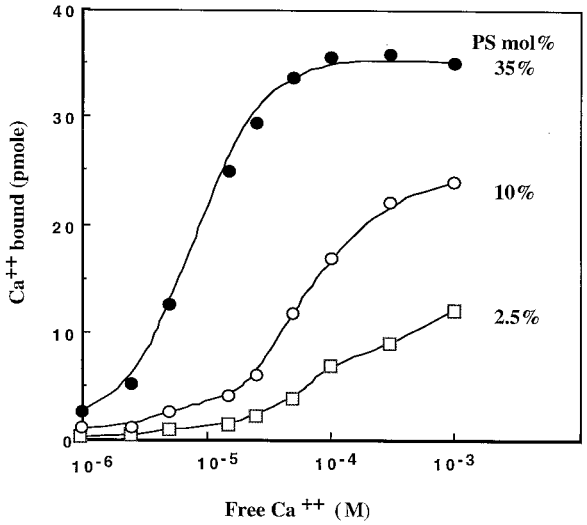

FIG. 6. **Effect of PS on the Ca^{2+} dependence of PLCδ1-Ca^{2+} binding.** Binding of 0.15 μM PLCδ1 to $^{45}Ca^{2+}$ (total 1.5×10^7 cpm) in the presence of 150 μM PS/PC vesicles composed of 35 (●), 10 (○), and 2.5 mol % (□) of PS was determined at variable concentrations of free $^{45}Ca^{2+}$. Reactions were carried out and the determination of Ca^{2+} binding to PLCδ1 was performed as described under "Experimental Procedures."

TABLE I
PI and PIP_2 hydrolysis activity of native and mutant PLCδ1

Type of enzymes	Enzymatic activity	
	PI[a]	PIP_2[a]
	μmol/min/mg	
Native PLCδ1	62 ± 5	35 ± 3
E341G, E390G	<0.1	<0.1
Δ646–654	61 ± 4	33 ± 4

[a] PIP_2 and PI hydrolysis activities were determined as described (20), expressed as release of μmol of IP_3 or IP/min/mg of enzyme.

the absence of PS, the Δ646–654 mutant catalyzed the hydrolysis of PIP_2 with a similar Ca^{2+} dependence as the native enzyme (Fig. 8). In contrast, in the presence of 25 mol % PS, the activity of the Δ646–654 mutant is at least lower than that of the native enzyme by a factor of 3 (Fig. 8). This result illustrates that residues 646–654 are not essential for the basal activity of PLCδ1 but are required for PS-dependent enzyme activation.

The Δ646–654 mutant is also impaired in its ability to bind PS. Although E341G,E390G was able to bind a PS vesicle in a Ca^{2+}-dependent manner (Fig. 9*a*), the Δ646–654 mutant was severely defective in Ca^{2+}-dependent PS binding (Fig. 9*b*). This finding revealed that residues 646–654 in the C2 domain of PLCδ1 are also involved in PS binding.

PS Reduces the K_m for PIP_2—PS stimulation of PIP_2 hydrolysis in the mixed micelles could be because of an increase in the maximal turnover of the enzyme, an increase in affinity for the substrate, or an increase in affinity of the enzyme for the membrane. To distinguish between these possibilities or a combination thereof, the effects of PS concentration in the mixed micelles on the substrate dependence of PLCδ1 catalysis was examined. The rate of hydrolysis of PIP_2 in mixed micelles containing either 0, 2.5, or 35 mol % PS was compared as a function of the total PIP_2 concentration (from 0.5 to 50 μM, corresponding to a molar fraction from 0.1 to 9 mol %). When the concentration of PIP_2 was increased from 0.1 to 9%, the hydrolysis of PIP_2 in mixed micelles containing 25 mol % PS sharply increased from 3 to 18 μmol/min/mg and was saturated as the PIP_2 concentration approached 4 mol % (Fig. 10). PIP_2 hydrolysis in PS-free mixed micelles increased slowly and did

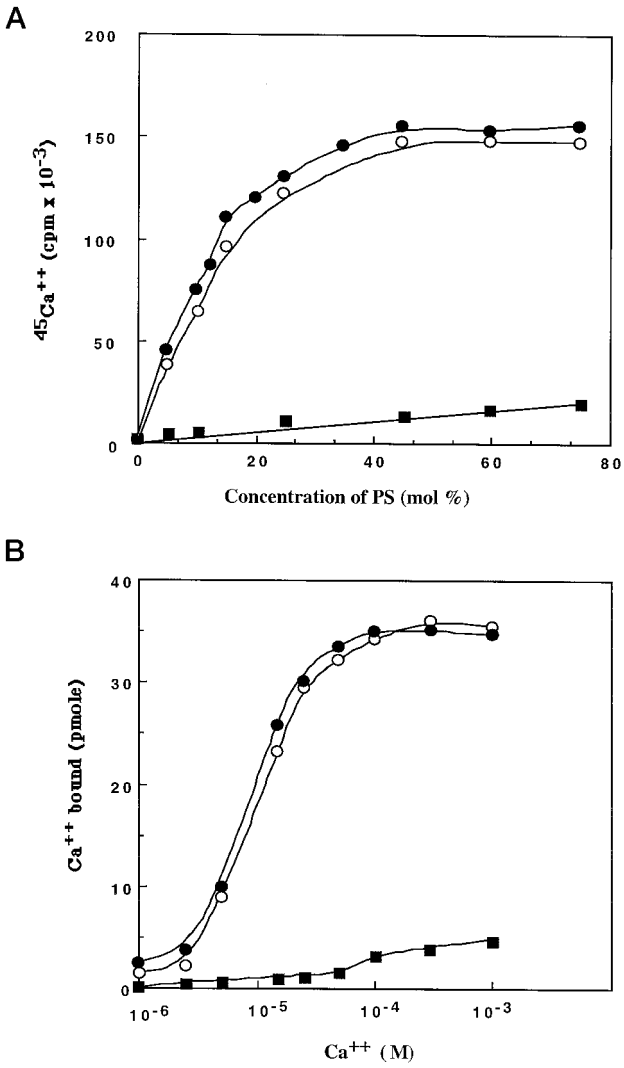

FIG. 7. **Ca^{2+} binding to the E341G,E390G mutant and the Δ646–654 deletion mutant PLCδ1.** PS dependence of the binding of Ca^{2+} to native (●), E341G,E390G mutant (○), or Δ646–654 deletion mutant (■) PLCδ1 is shown. The enzyme-Ca^{2+} binding in the presence 150 μM of PS/PC vesicles containing 0–75 mol % of PS and the determination of bound Ca^{2+} were carried as described under "Experimental Procedures" (*A*). Binding of 0.15 μM native (●), E341G,E390G mutant (○), or Δ646–654 deletion mutant (■) PLCδ1 to increasing concentrations of free $^{45}Ca^{2+}$ (total 1.5×10^7 cpm) in the presence of 150 μM PS/PC vesicles composed of 35 mol % of PS was carried out as described under "Experimental Procedures" (*B*).

not reach a maximum even at a 9 mol % PIP_2 (Fig. 10). The most dramatic stimulatory effect of PS on the hydrolysis of PIP_2 was found at a low substrate concentration. As shown in Fig. 10, when PIP_2 concentration was less than 2 mol %, PIP_2 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 PIP_2 increased (Fig. 10).

The kinetic parameters of PLCδ1 (Table II) demonstrate that the stimulation of PIP_2 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. The 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_s) and its maximal rate of catalysis (V_{max}).

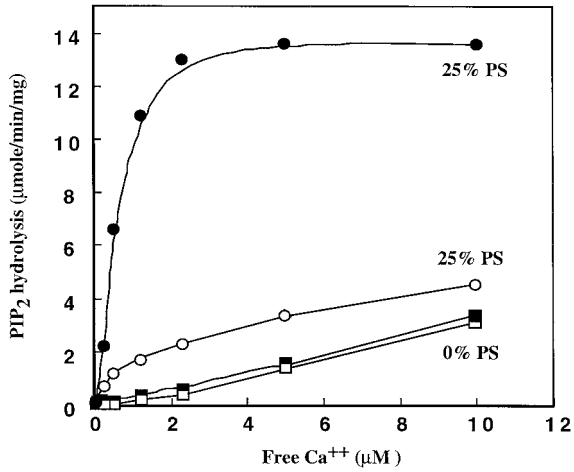

FIG. 8. **Effect of PS concentration on the Ca^{2+} dependence of Δ646–654 deletion mutant PLCδ1 catalytic activity.** Hydrolysis of 5 μM PIP_2 (corresponding to 1 mol % in the mixed micelles) by the native (*closed symbols*) or Δ646–654 deletion mutant (*open symbols*) PLCδ1 in PIP_2/dodecyl maltoside mixed micelles containing 25 (*circles*) and 0 (*squares*) mol % PS is shown. PLCδ1 catalyzed hydrolysis of PIP_2 in the mixed micelles was carried out as described (Fig. 2 and "Experimental Procedures") using various concentrations of $CaCl_2$ to yield the indicated concentration of free Ca^{2+}.

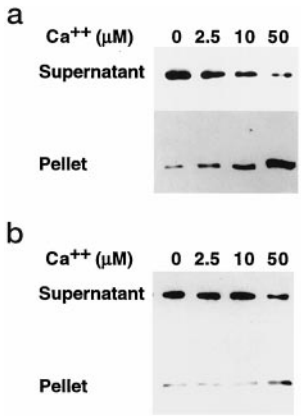

FIG. 9. **Ca^{2+}-dependent PS binding to the E341G,E390G and the Δ646–654 deletion mutant PLCδ1.** Centrifugation binding assay of the E341G,E390G (*a*) and the Δ646–654 deletion (*b*) PLCδ1 to sucrose-loaded PS/PC vesicles in the presence of the indicated concentration of free Ca^{2+} ion. 1 μg of 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 $CaCl_2$ to yield the indicated concentration of free Ca^{2+}. The reaction was carried out and binding was determined as described under "Experimental Procedures."

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, Ca^{2+} 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 Ca^{2+} binding site, the present data show that PS-dependent Ca^{2+} binding occurs at the C2 domain of PLCδ1. In the presence of saturation level of PS but no substrate, the maximal

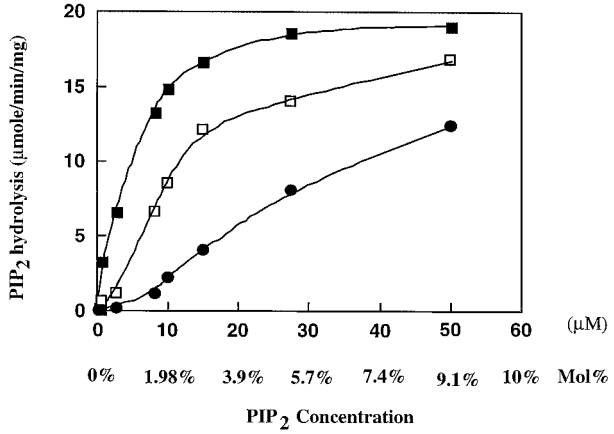

FIG. 10. **Effect of PS concentration on the substrate dependence of PLCδ1 catalysis of PIP_2.** Hydrolysis of increasing concentrations of PIP_2 by PLCδ1 in PIP_2/dodecyl maltoside mixed micelles containing 0 (●), 10 (□), and 25 (■) mol % PS is shown. The mol % of PIP_2 was increased, while the concentration 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 [^{3}H]IP_3 was separated and quantitated as described under "Experimental Procedures."

TABLE II
Effect of PS on the kinetic properties of PLCδ1

Hydrolysis of increasing concentration of PIP_2 by PLCδ1 was measured in PIP_2/dodecyl maltoside mixed micelles containing 25, 10, and 0 mol % PS (see "Experimental Procedures"). V_{max}, K_m, and K_s correspond to the constants defined in the surface dilution model (Equations 1, 2, and 3); values of V_{max}, K_m, and K_s were calculated by fitting the data to Equation 3 (29).

PS concentration (mol %)	V_{max} (μmol/min/mg)	K_m (mol fraction)	K_s (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

binding of Ca^{2+} to PLCδ1 was measured to be 2.1 pmol Ca^{2+}/pmol protein. The concentration of Ca^{2+} 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 exists in the C2 domain, where four putative metal ion binding sites are present. Two of these sites have been shown to bind calcium ions in structural studies, and three can be occupied by lanthanum (12). The actual number of calciums binding to the C2 domain of PLCδ1 is unknown. Because calcium analogs such as lanthanum may actually have greater affinity for true calcium binding sites, three calcium binding sites could well be an overestimate. The present studies indicate a minimum of two calciums binding to the C2 domain.

Residues 646–654 constitute the C terminus of the loop that connects β1 and β2 strands in the C2 domain of PLCδ1 (3). The phenotype of the Δ646–654 deletion mutant revealed that this region of C2 domain is required for PLCδ1 to interact with PS and Ca^{2+}. This finding is consistent with the role of the C2 domain in other molecules. The C2 domains of cytosolic phospholipase A2, synaptotagmin I, and protein kinase C all mediate calcium-dependent phospholipid binding (33–35). Our data indicated that abolishing Ca^{2+} binding to the C2 domain did not affect the basal activity of PLCδ1. This is consistent with the findings of Grobler and Hurley (36), which show that deleting similar loop in PLCδ1 does not affect its catalysis of PIP_2

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 Ca^{2+} 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 Ca^{2+} binding but also eliminated the activation and binding of PLCδ1 by PS. All these observations indicated that the simultaneous presence of Ca^{2+}, 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 (K_m) (increasing the substrate affinity for the catalytic site). The K_m was reduced by a factor of 20. A similar result was also found for protein kinase C; a decrease in K_m results when rat brain enzyme binds PS and Ca^{2+} (37). PS has also been found to increase the affinity between coagulation factor IXa and VIIIa and to reduce the K_m for the factor IXa catalyzed proteolysis reaction (38). Both K_s (which governs the affinity of the enzyme for membrane) and V_{max} (which reflects the velocity at infinite substrate concentrations) were not affected by the presence of PS. The present kinetic analysis may not have been sensitive enough to detect the contribution of PS on membrane anchorage (K_s), because membrane association may be primarily driven by PIP_2 binding to the PH domain. The binding of PIP_2 to the PH domain is at least 10-fold tighter than that for the Ca^{2+}-dependent binding of PS by the enzyme (5).

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 physiological concentrations of PIP_2 (<1 mol % of total membrane phospholipids). However, PS and calcium stimulate PLCδ1 at physiological concentration of PIP_2 by increasing the affinity for the substrate. The concentration of PS (41) and Ca^{2+} 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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[2] J. W. Lomasney, H.-F. Cheng, S. R. Roffler, and K. King, manuscript in preparation.

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