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Structural Basis for Calcium and Phosphatidylserine Regulation of Phospholipase C δ1

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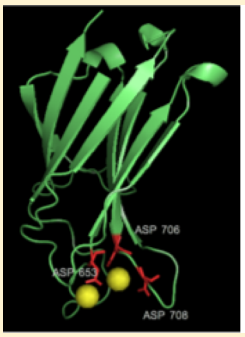
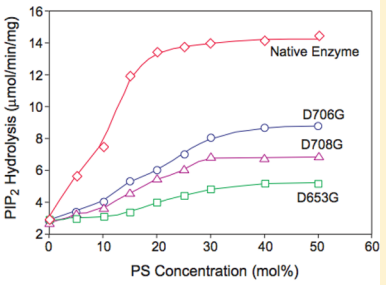
ABSTRACT: Many membrane-associated enzymes, including those of the phospholipase C (PLC) superfamily, are regulated by specific interactions with lipids. Previously, we have shown that the C2 domain of PLC δ1 is required for phosphatidylserine (PS)-dependent enzyme activation and that activation requires the presence of Ca^{2+}. To identify the site of interaction and the role of Ca^{2+} in the activation mechanism, we mutagenized three highly conserved Ca^{2+} binding residues (Asp-653, Asp-706, and Asp-708) to Gly in the C2 domain of PLC δ1. The PS-dependent Ca^{2+} binding affinities of the mutant enzymes D653G, D706G, and D708G were reduced by 1 order of magnitude, and the maximal level of Ca^{2+} binding was reduced to half of that of the native enzyme. The level of Ca^{2+}-dependent PS binding was also reduced in the mutant enzymes. Under basal conditions, the Ca^{2+} dependence and the maximal level of hydrolysis of phosphatidylinositol 4,5-bisphosphate were not altered in the mutants. However, the Ca^{2+}-dependent PS stimulation was severely defective. PS reduces the K_m of the native enzyme almost 20-fold, but far less for the mutants. Replacing Asp-653, Asp-706, and Asp-708 simultaneously with glycine in the C2 domain of PLC δ1 leads to a complete and selective loss of the stimulation and binding by PS. These results show that D653, D706, and D708 are required for Ca^{2+} binding in the C2 domain and demonstrate a mechanism by which C2 domains can mediate regulation of enzyme activity by specific lipid ligands.





The family of phospholipase C enzymes consists of six subfamilies (δ, β, γ, ε, ζ, and η) of structurally and functionally distinct isozymes. A common functional feature is the ability of all members to catalyze the hydrolysis of polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). This common phosphodiesterase activity is often the result of direct or indirect activation by receptor-coupled signaling pathways at the surface of eukaryotic cells. The mechanism by which each family is regulated is different. In general, the β isoforms are regulated by the G_q family of heterotrimeric G proteins and the γ isoforms by receptor and nonreceptor tyrosine kinases. The ε family is regulated by G12 and G13 as well as by small G proteins such as Ras and Rap2B, the ζ family by calcium, and the δ family by calcium and lipid ligands such as phosphatidylserine (PS) and PIP_2 as well as Ral and G_h,[1,2] though in recent years there have been a number of reported cases that do not fall into the traditional regulation paradigm according to each PLC subtype.[3] While there is generally little conservation of primary structure among the six families, three major motifs are found in all eukaryotic isoforms, the catalytic X and Y domains, the pleckstrin homology (PH) domain, and the C2 domain.

PLC δ1 is highly responsive to lipid stimulations. For example, the activity of PLC δ1 is enhanced by PIP_2 through the PH domain.[4,5] In cells, this PIP_2 activation is observed both in the plasma membrane and in the nucleus.[6−8] Also, PLC δ1 can be activated by other anionic lipids such as PS[9] and free fatty acids.[10,11]

The C2 domain is a conserved motif of approximately 130 amino acids that is predicted to exist in 295 distinct proteins by the human genome project.[12] The structural motif of the C2 domain is an antiparallel β sandwich consisting of eight β strands. Many of these C2 domain-containing proteins are involved in intracellular signaling or membrane trafficking. The function of the C2 domain has been examined in several of these molecules, including protein kinase C, cytoplasmic phospholipase A2, phospholipase C, and synaptotagmin. The PKC α C2 domain is known to bind both calcium and PS.[13] As for the PLC δ family, the C2 domain is also considered important in membrane targeting. The isolated C2 domains from δ1 and δ3 translocate specifically toward the plasma membrane, whereas the C2 domain of δ4 is localized to various cellular membranes, including the perinuclear membranes and plasma membrane, upon stimulation with ionomycin, a calcium-mobilizing ionophore, which presumably results from

Received: August 9, 2011
Revised: March 1, 2012
Published: March 1, 2012

a difference in their lipid specificity, especially toward PS.[14] In addition, the C2 domains of δ1 and δ3 exhibit a strong calcium-dependent membrane affinity, whereas the C2 domain of δ4 has significant calcium-independent membrane affinity. Most studies have suggested a role for the C2 domain in membrane targeting, though a few studies have also suggested a role for calcium-induced conformational changes.[15]

Previously, we demonstrated the ability of free calcium and PS to bind to the C2 domain of PLC δ1 and activate phosphodiesterase activity 10−20-fold.[9] In this work, we identify the structural determinants for calcium and PS binding. Three aspartic acid residues in the loop region of the C2 domain are identified at positions 653, 706, and 708, and each is shown to contribute to calcium and lipid binding. These studies identify the C2 domain as an allosteric modulatory domain and demonstrate the role of C2 domains in mediating the effects of calcium and phospholipid on protein function.

EXPERIMENTAL PROCEDURES

Materials. Expression vector pRSETA was from Invitrogen. To express PLC δ1 under the control of the T7 promoter, the coding sequence for PLC δ1 was cloned into pRSETA. The resulting expression construct (pRSETAplc) was transformed into *Escherichia coli* strain BL21(DE3)pLys (Novagen), and the protein was isolated and purified as described previously.[16] Phosphatidylethanolamine, PA, PC, and PS were obtained from Avanti Polar Lipids Inc. PIP_2 and dodecyl maltoside were obtained from Calbiochem.

Phospholipid Binding Assay. Phospholipid vesicles composed of PS/PC or PA/PC mixtures were prepared as described by Mueller et al.[17] with slight modifications.[16,18] A dry phospholipid film was formed by slowly blowing 0.25 mL of a chloroform/methanol mixture [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, and 2 mM EGTA (binding buffer) and resuspended in 0.5 mL of the same buffer.

Centrifugation Binding Assay. The binding of PLC δ1 to phospholipid vesicles was estimated by a centrifugation assay.[16,19] The free Ca^{2+} concentration was calculated according to the work of Fabiato and Fabiato.[20] 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 conducted at 30 °C for 15 min. Free PLC δ1 and bound PLC δ1 were separated by sedimentation at 50000*g* for 30 min. Equal proportions of the supernatant and pellet fractions were resolved by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis, and the amount of PLC δ1 in each fraction was estimated by Western blotting analysis.

Calcium Binding Measurements. Calcium binding was assessed by a nitrocellulose membrane binding assay similar to that described by Nakamura[21] and Kawasaki et al.[22] Phospholipid vesicles (150 μM PS/PC or PA/PC) containing the indicated mole fractions of lipids were incubated with PLC δ1 (final concentration of 0.15 μM) in 100 μL of 50 mM HEPES (pH 7.0), 100 mM KCl, and 10 μM EGTA containing 5−120 μM $^{45}CaCl_2$ (total counts per minute of 1.5×10^7) to yield the indicated concentration of free Ca^{2+}. A 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 being incubated at 30 °C for 30 min, the reaction mixture 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, and 10 mM EGTA. The membrane was cut into sections corresponding to each sample, and the retention of $^{45}Ca^{2+}$ on the membrane was assessed 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. The binding of calcium to phospholipid vesicles in the absence of enzyme was assessed 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.[23,24] 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 and phospholipid.

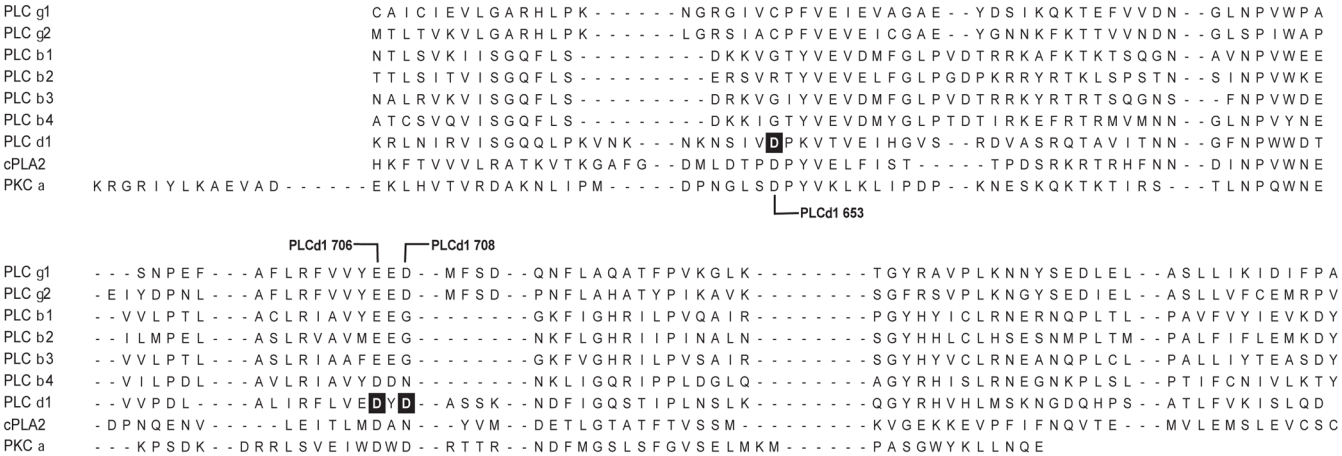
PIP_2 Hydrolysis Assay. PIP_2 hydrolysis in dodecyl maltoside/PIP_2 mixed micelles was performed in a manner similar to that described by Cifuentes et al.[4] with slight modifications. In brief, the indicated amount of PIP_2/[^{3}H]PIP_2 (4×10^5 cpm) in a chloroform/methanol mixture (19:1) in the presence 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, and the indicated concentration of dodecyl maltoside. Bovine serum albumin in the same buffer was added to a final concentration of 500 μg/mL. PLC activity was determined as a function of the concentration of substrate by keeping the total concentration of nonsubstrate phospholipid and 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 PLC δ1. Case III conditions previously described for phospholipase A2[25] and PLC[26] were employed. Under case III conditions, the total concentration of diluent detergent (dodecyl maltoside and nonsubstrate phospholipid) was fixed, and the PLC activity was measured with increasing concentrations of substrate. A dual phospholipid binding model of catalysis was used to analyze the kinetic data as previously described.[9]

RESULTS

Asp-653, Asp-706, and Asp-708 Are Essential for PS Stimulation of PLC δ1. Three highly conserved negatively charged amino acid residues (Asp-653, Asp-706, and Asp-708)

A



B

Figure 1. (A) Amino acid alignment of C2 domains. Asp residues mutated in PLC δ1 are highlighted. (B) Crystal structure of the C2 domain of PLC δ1 (Protein Data Bank entry 1DJI). D653, D706, and D708 are colored red.

were identified in the δ isoforms of PLC by aligning the primary sequences of the C2 domains from PLA2, PKCα, and all eukaryotic PI-PLCs (Figure 1A,B). Crystallographic studies have implicated these three residues in coordinating calcium binding.[27] To investigate the role of these residues in Ca^{2+} binding and enzyme function, all three Asp residues were individually mutated by site-directed mutagenesis to Gly. These residues do not participate in the Ca^{2+}-dependent basal activity of PLC δ1 but are required for the Ca^{2+}-dependent PS stimulation of phosphodiesterase activity. As shown in Figure 2, the rate of hydrolysis of PIP_2 was very similar for the native and mutant enzymes in PS-free dodecyl maltoside mixed micelles at the two free Ca^{2+} concentrations tested (the hydrolysis rates were ~0.5 and ~3.3 μmol of PIP_2 hydrolyzed min^{-1} mg^{-1} at 1 and 10 μM free Ca^{2+}, respectively). However, when 35% PS was included in the mixed micelles, significantly higher activity was seen for the native enzyme than for the mutant enzymes at both of the two free Ca^{2+} concentrations (Figure 2). At 10 μM free Ca^{2+}, PS stimulated the activities of native, D653G, D706G, and D708G PLC δ1 to 12.7, 5.5, 9.24, and 7.6 μmol of PIP_2 hydrolyzed min^{-1} mg^{-1}, respectively. The increases in activity for the native enzyme, D653G, D706G, and D708G were 3.5-, 1.7-, 2.8-, and 2.3-fold, respectively. More remarkable stimulation by PS on the native enzyme was seen at a lower free Ca^{2+} concentration (1 μM); the native enzyme was stimulated by a factor of 20, while the activities of all three mutant enzymes remained unchanged. The activities of the mutant enzymes in the absence of PS were comparable to those of the native enzyme, irrespective of the concentration of free Ca^{2+} used.

These results demonstrate that D653, D706, and D708 are not required for the basal phosphodiesterase activity of PLC δ1 but are essential for PS-mediated stimulation of activity. This is more clearly illustrated in Figure 3. The PS dose–response curves show that the rate of PIP_2 hydrolysis by native and mutant enzymes is dependent on the mole fraction of PS. The potency and maximal effect of PS are markedly reduced for the mutant enzymes.

Asp-653, Asp-706, and Asp-708 Mediate the Ca^{2+} Regulation of PLC δ1. We have previously shown that PS stimulates PLC δ1 by virtue of its ability to form a Ca^{2+}–PS–PLC δ1 complex via the C2 domain. PS and Ca^{2+} are interdependent; neither can interact with or regulate PLC δ1 without the other. Therefore, replacing Asp-653, Asp-706, and Asp-708 with glycine may weaken the ability of PS to increase the potency of Ca^{2+} for stimulating catalysis. This is clearly illustrated in Figure 4. In the absence of PS, the calcium dose–response curves for the mutant and native enzymes were

dx.doi.org/10.1021/bi201252f | *Biochemistry* 2012, 51, 2246–2257

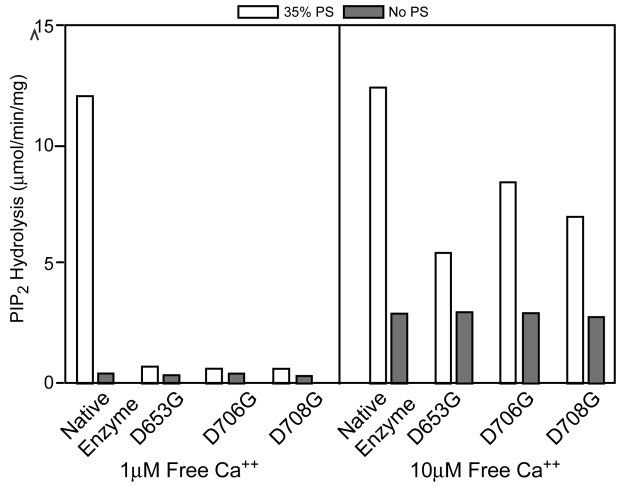


Figure 2. Phosphatidylserine stimulation of native, D653G, D706G, and D708G PLC δ1. Hydrolysis of 5 μM (1 mol %) PIP_2 in PIP_2/dodecyl maltoside mixed micelles in the presence (white bars) or absence of 35% PS (black bars). The catalytic reaction was conducted in either 1 or 10 μM free Ca^{2+}. The total concentration of dodecyl maltoside and nonsubstrate phospholipid was constant at 495 μM. The catalytic reaction was conducted in 50 μL of 50 mM HEPES (pH 7.0), 100 mM NaCl, 2 mM EGTA, 500 μg/mL BSA, and $CaCl_2$ to yield 1 or 10 μM free Ca^{2+}. The free Ca^{2+} concentration in an EGTA/$CaCl_2$ buffer was calculated according to the method of Fabiato and Fabiato.[20] The reaction was conducted at 30 °C for 1−5 min, stopped, and quantitated as described in Experimental Procedures.

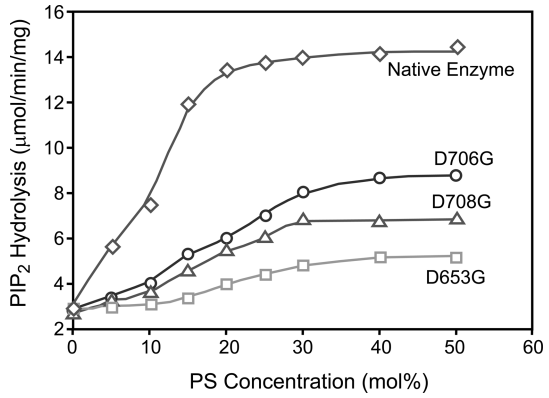


Figure 3. Dependence of PS concentration on PIP_2 hydrolysis by native and mutant PLC δ1. Catalytic hydrolysis of 5 μM PIP_2 in dodecyl maltoside mixed micelles containing increasing concentration of PS (from 0 to 50 mol %) by the native (◇), D653G (□), D706G (○), and D708G (△) enzymes. The total concentration of dodecyl maltoside and nonsubstrate phospholipid was constant at 495 μM. The catalytic reaction was conducted as described in the legend of Figure 2 and Experimental Procedures.

identical to each other (Figure 4A). The maximal catalytic activity of the native enzyme, D653G, D706G, and D708G was estimated to be 3.3 μmol of PIP_2 hydrolyzed min^{-1} mg^{-1}, and the free Ca^{2+} concentration required for the half-maximal activity of each enzyme (EC_{50}) was approximately 6 μM. Including 35 mol % PS in the mixed micelles shifted the Ca^{2+} dose−response curves up and to the left for native and mutant enzymes, though the shift was much more pronounced for the native enzyme (Figure 4B). PS increased both the potency of Ca^{2+} and the maximal activity. The maximal activity of the native enzyme was increased from 3.2 to 13 μmol min^{-1} mg^{-1} (Figure 4A,B). The free Ca^{2+} concentration for half-maximal activity (EC_{50}) of the native enzyme was reduced by a factor of at least 20 (from 6 to 0.25 μM). The maximal activity of the mutants was 40−70% of that of native PLC δ1 in the presence of PS. The maximal PS-stimulated activities of D653G, D706G, and D708G PLC δ1 were 4.9, 8.2, and 6.9 μmol of PIP_2 min^{-1} mg^{-1}, respectively, while that of the native enzyme was 13 μmol min^{-1} mg^{-1}. With 35% PS, Ca^{2+} is at least 5−10-fold less potent in stimulating PIP_2 hydrolysis by D653G, D706G, and D708G PLC δ1 than for the native enzyme. As shown in Figure 4B, the free Ca^{2+} concentrations required for half-maximal hydrolysis of PIP_2 in mixed micelles containing 35 mol % PS by D653G, D706G, and D708G PLC δ1 were estimated to be 2.5, 2.0, and 1.8 μM, respectively. This reduction in the potency of Ca^{2+} for activation of the mutant enzymes may explain why we were not able to observe the stimulatory effect of PS on the mutants when assays were conducted at 1 μM free Ca^{2+}. In the absence of PS, the calcium dose−response curves for native and mutant enzymes are identical. However, in the presence of PS, calcium has a reduced potency and a reduced maximal effect on the mutant enzymes as compared to the effect of PS on the native enzyme. The mutant enzymes have a selective deficiency in PS activation.

D653G, D706G, and D708G Have Lower Affinities for the Substrate Than the Native Enzyme. We have previously shown that the stimulation of PIP_2 hydrolysis by PS was primarily due to an increase in the affinity for the substrate at the catalytic site.[9] To understand the enzymatic mechanism underlying the impaired phenotypes of the D653G, D706G, and D708G mutant enzymes, we examined the effect of PS on the substrate concentration dependence of PLC δ1 catalysis. When the micellar concentration of PIP_2 was increased from 0.1 to 9%, the extent of hydrolysis of PIP_2 by the native enzyme in mixed micelles containing 35 mol % PS sharply increased from 3.1 to 19 μmol min^{-1} mg^{-1} and was saturated as the PIP_2 concentration approached 4 mol %. The level of PIP_2 hydrolysis catalyzed by the native enzyme in PS-free mixed micelles increased slowly and did not reach a maximum even at 9 mol % PIP_2. Comparing the substrate dependence of PIP_2 hydrolysis in the presence and absence of PS demonstrated that the most dramatic stimulatory effect of PS on the hydrolysis of PIP_2 was at low substrate concentrations. As shown in Figure 5A, when the PIP_2 concentration was less than 2 mol %, the level of PIP_2 hydrolysis in mixed micelles containing 35 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. The substrate dependence for the D653G, D706G, and D708G mutant enzymes was very comparable to that of the native enzyme, when the hydrolysis of PIP_2 was conducted in PS-free mixed micelles. The rates of PIP_2 hydrolysis by the D653G, D706G, and D708G mutant enzymes were much lower than that of the native enzyme if the reactions were conducted in the presence of 35 mol % PS. Specifically, the stimulatory effect of PS on the mutant enzymes was much weaker than that of the native enzyme at low substrate concentrations. This observation reflects a decrease in substrate affinity for the mutant enzymes. The kinetic parameters of the native and mutant enzymes revealed that the mutant enzymes were partially impaired in PS-stimulated reduction in the interfacial Michaelis constant (K_m), which reflects the affinity for the substrate (Table 1). The K_m of the native enzyme was reduced by a factor of 20 in the presence of 35% PS, while

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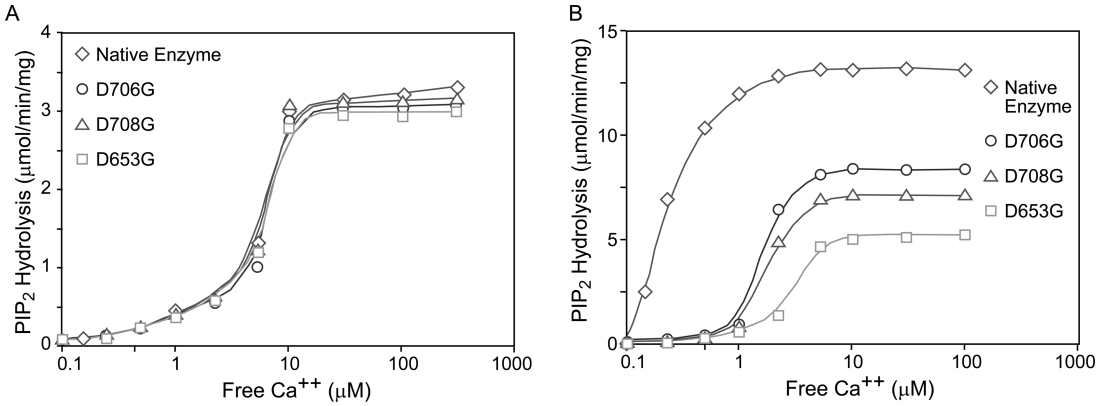

Figure 4. Effect of PS on the Ca^{2+} dependence of the native and the mutant PLC δ1 catalytic activities. Calcium concentration dependence of hydrolysis of 5 μM PIP_2 (corresponding to 1 mol % in the mixed micelles) by native (◇), D653G (□), D706G (○), and D708G (△) PLC δ1 in PIP_2/dodecyl maltoside mixed micelles containing 0 (A) or 35 mol % PS (B). The reaction was conducted in a buffer that consisted of 50 mM HEPES (pH 7.0), 100 mM NaCl, 2 mM EGTA, 500 μg/mL BSA, and various concentrations of $CaCl_2$ to yield the indicated concentration of free Ca^{2+}. PLC δ1-catalyzed hydrolysis of PIP_2 in the mixed micelles was assessed as described in the legend of Figure 2 and Experimental Procedures.

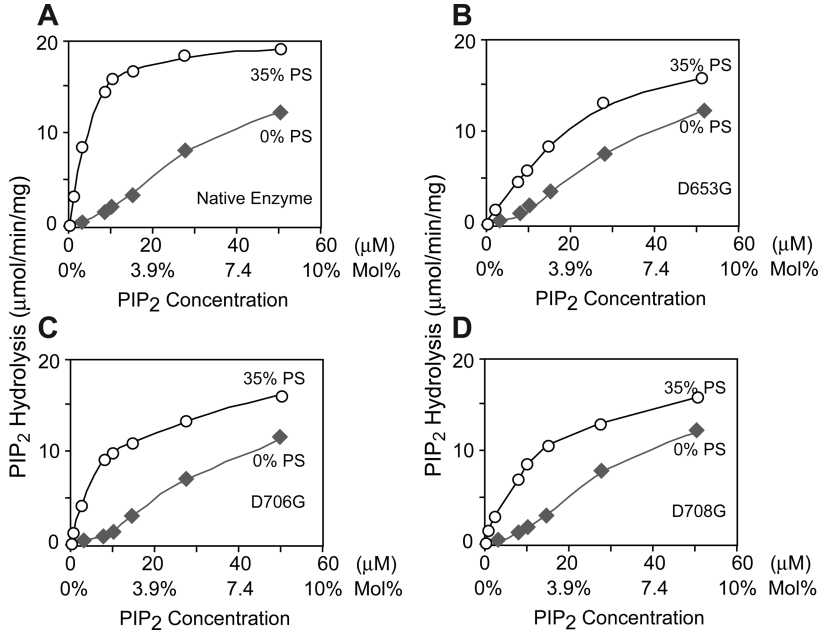

Figure 5. Influence of PS on the substrate dependence of catalytic hydrolysis of PIP_2 by native and mutant PLC δ1. Hydrolysis of increasing concentrations of PIP_2 by native (A), D653G (B), D706G (C), and D708G (D) PLC δ1 in PIP_2/dodecyl maltoside mixed micelles containing 0 (◆) and 35 mol % PS (○). The mole fraction of PIP_2 was increased while the concentration of PS was fixed at 0 and 35 mol %, and the combined total concentration of PS and dodecyl maltoside was maintained at 495 μM (see Experimental Procedures). The reaction was conducted in the presence of 5 μM free Ca^{2+} and stopped, and the $[^3H]IP_3$ was separated and quantitated as described in Experimental Procedures.

Table 1. Effect of PS on the Kinetic Properties of Native and Mutant PLC δ1[a]

	concentration of PLC δ1 and PS (mol %)	V_{max} (μmol min^{-1} mg^{-1})	K_m (mole fraction)	K_s (μM)
native PLC δ1	0	17 ± 3	0.045 ± 0.001	70 ± 2
	35	19 ± 4	0.0025 ± 0.0002	45 ± 3
D653G PLC δ1	0	14 ± 2	0.045 ± 0.001	71 ± 3
	35	17 ± 3	0.01 ± 0.01	47 ± 2
D706G PLC δ1	0	15 ± 2	0.051 ± 0.003	69 ± 2
	35	18 ± 3	0.008 ± 0.002	44 ± 2
D708G PLC δ1	0	14 ± 3	0.048 ± 0.004	73 ± 4
	35	18 ± 4	0.009 ± 0.001	46 ± 2

[a]Hydrolysis of increasing concentrations of PIP_2 at 5 μM free Ca^{2+} by PLC δ1 was assessed in PIP_2/dodecyl maltoside mixed micelles containing 35 and 0 mol % PS (see Experimental Procedures). V_{max}, K_m, and K_s correspond to the constants defined in the surface dilution model; values of V_{max}, K_m, and K_s were calculated by fitting the data to eq 1 of ref 26.

dx.doi.org/10.1021/bi201252f | *Biochemistry* 2012, 51, 2246−2257

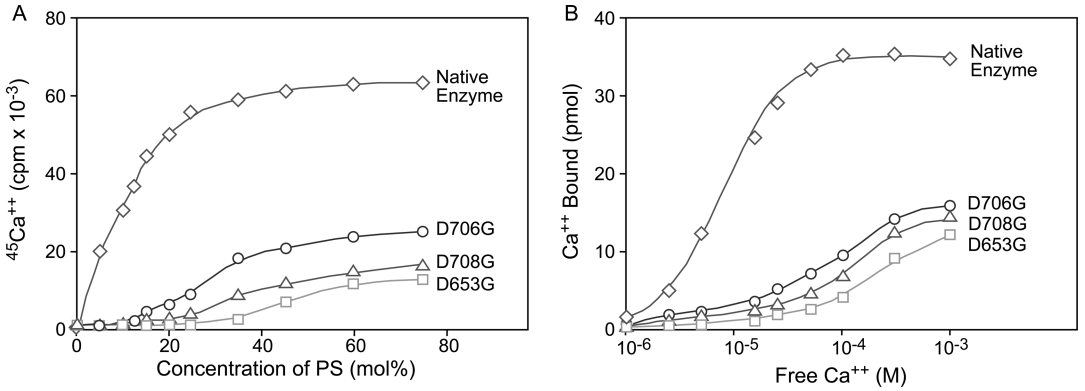

Figure 6. Dependence of PS and free Ca^{2+} on the binding of Ca^{2+} to native and mutant PLC δ1. PS dependence of the binding of Ca^{2+} to native (◇), D653G (□), D706G (○), and D708G (△) PLC δ1. For the binding of 0.15 μM enzyme with 100 μM free $^{45}Ca^{2+}$ (total of 1.5×10^7 cpm) in 0.1 mL of buffer containing 50 mM Hepes (pH 7.0), 100 mM KCl, and 10 μM EGTA and 150 μM PS/PC vesicles containg 0−75 mol % PS, the determination of the amount of bound Ca^{2+} was conducted as described in Experimental Procedures (A). Binding of 0.15 μM native (◇), D653G (□), D706G (○), and D708G (△) PLC δ1 to increasing concentrations of free $^{45}Ca^{2+}$ (total of 1.5×10^7 cpm) in the presence of 150 μM PS/PC vesicles composed of 50 mol % PS was conducted as described in Experimental Procedures (B).

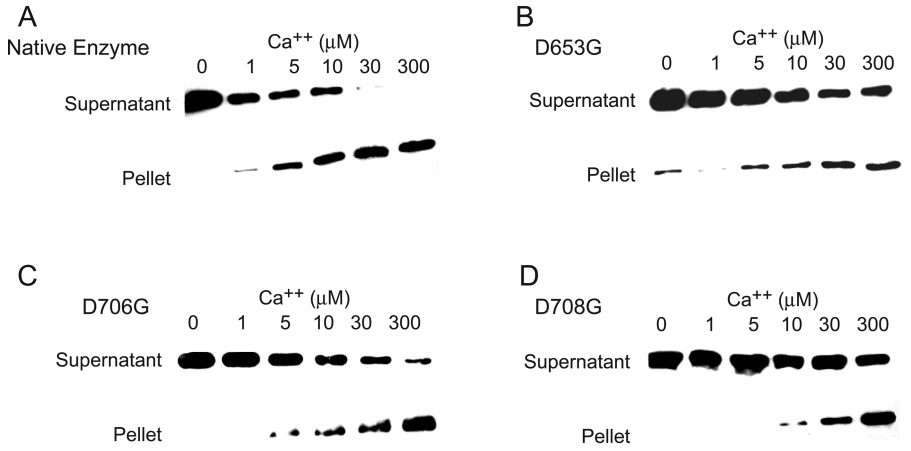

Figure 7. Ca^{2+}-dependent phospholipid binding to native and mutant PLC δ1. Centrifugation binding assay of PLC δ1 and sucrose-loaded PS/PC vesicles in the presence of the indicated concentration of free Ca^{2+} ion; 1 μg of native (A), D653G (B), D706G (C), and D708G (D) protein was incubated with 150 μM PS/PC vesicles (molar ratio of 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 mixture 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 in Experimental Procedures.

those of D653G, D706G, and D708G were reduced by factors of 3, 6, and 4.5, respectively. PS had little effect on the maximal rate of catalysis (V_{max}) or on the affinity for the interface (K_s) for all enzymes.

D653G, D706G, and D708G Are Defective in Ca^{2+} Binding. PS activation of PLC δ1 requires the simultaneous binding of Ca^{2+} and PS to the C2 domain of the enzyme. To test whether D653, D706, and D708 are involved in the lipid-dependent binding of Ca^{2+} to the C2 domain, we measured the binding of $^{45}Ca^{2+}$ to PLC δ1 as a function of the PS concentration and Ca^{2+} concentration. As illustrated in Figure 6A, the binding of Ca^{2+} by the native enzyme is highly dependent on the concentration of PS; the binding was enhanced by as little as 5 mol % PS and plateaued as the level of PS reached 30 mol %. Replacing D653, D706, or D708 with glycine reduced the total level of binding of Ca^{2+} to PLC δ1 at saturating concentrations of PS and also required higher concentrations of PS to reach saturation (Figure 6A).

Because little Ca^{2+} would bind to PLC δ1 in the absence of PS/PC vesicles, the Ca^{2+} saturation binding isotherm was determined in the presence of PS/PC vesicles containing 40 mol % PS. In the presence of PS/PC vesicles, $^{45}Ca^{2+}$ bound to native and mutant PLC δ1 in a dose-dependent and saturable manner (Figure 6B). Ca^{2+} bound to the native enzyme when the free Ca^{2+} concentration was as low as 2 μM, and the level of binding increased sharply until the free concentration reached 100 μM. The binding of Ca^{2+} to mutant enzymes was much weaker than that of native PLC δ1, and the level increased slowly as the free Ca^{2+} concentration increased from 10 to 1000 μM. Saturable binding was still not obtained as the free Ca^{2+} concentration reached 1 μM. As shown in Figure 6B, the maximal amount of Ca^{2+} binding to 0.15 μM protein was estimated to be 35 pmol, corresponding to approximately 2.3 pmol of Ca^{2+}/pmol of protein. The amounts of Ca^{2+} binding to D653G, D706G, and D708G mutant enzymes at 1 mM free Ca^{2+} were 12.8, 16.5, and 15 pmol, respectively, corresponding to 0.84, 1.08, and 0.98 pmol of Ca^{2+}/pmol of protein, respectively. The concentration of free Ca^{2+} required for half-maximal binding to the native enzyme was estimated to be 7 μM, while those for D653G, D706G, and D708G mutants were 128, 70, and 90 μM, respectively. The Ca^{2+} binding isotherm revealed that the affinity of Ca^{2+} for PLC δ1 was reduced by a

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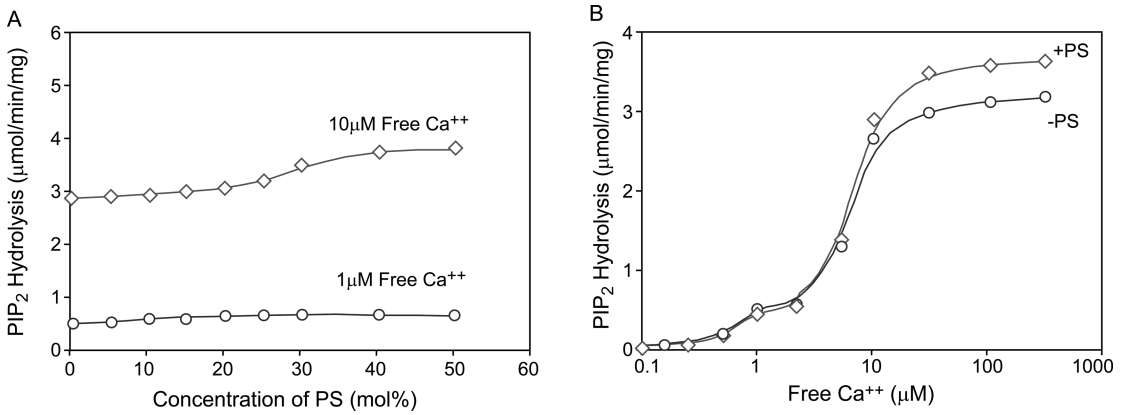

Figure 8. Ca^{2+} and PS dependence of PIP_2 hydrolysis by the D653/D706/D708G mutant PLC δ1. (A) PS concentration dependence of the D653/D706/D708G mutant PLC δ1-catalyzed hydrolysis of 5 μM PIP_2 (corresponding to 1 mol % in the mixed micelles) in the presence of 1 (○) or 10 μM free Ca^{2+} (◇). (B) Ca^{2+} dependence of hydrolysis of 5 μM PIP_2 (corresponding to 1 mol % in the mixed micelles) by the D653/D706/D708G mutant PLC δ1 in mixed micelles containing 0 (○) or 50 mol % PS (◇). The catalytic reaction was conducted as described in the legend of Figures 2 and 3 and Experimental Procedures.

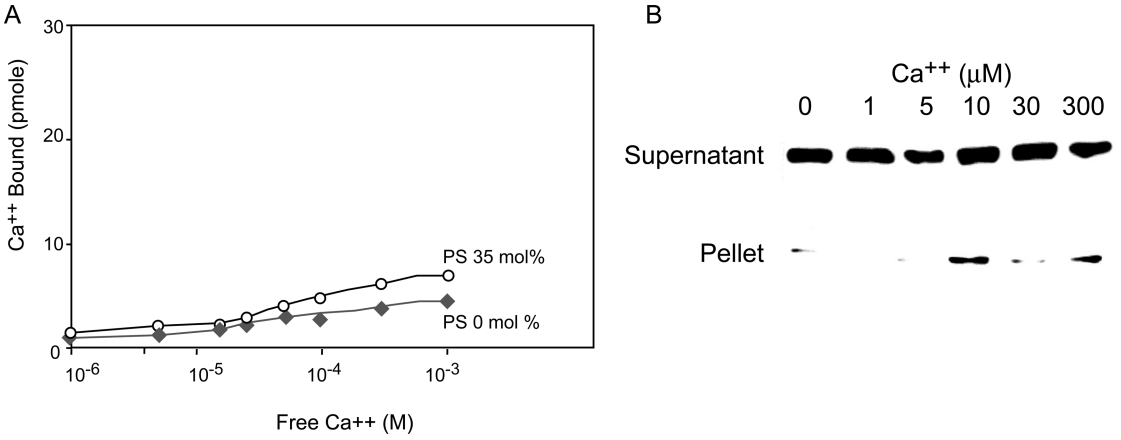

Figure 9. Binding of Ca^{2+} and PS to the D653/D706/D708G mutant of PLC δ1. (A) For binding of 0.15 μM D653/D706/D708G mutant enzyme with indicated concentrations of free $^{45}Ca^{2+}$ (total of 1.5×10^7 cpm) in 0.1 mL of buffer containing 50 mM Hepes (pH 7.0), 100 mM KCl, and 10 μM EGTA and 150 μM PS/PC vesicles containing 0 (◇) and 35 mol % PS (○), the determination of the amount of bound Ca^{2+} was conducted as described in Experimental Procedures. (B) Centrifugation binding assay of the D653/D706/D708G mutant enzyme and sucrose-loaded PS/PC vesicles in the presence of the indicated concentration of free Ca^{2+} ion. Binding of 1 μg of protein to 150 μM PS/PC vesicles (molar ratio of 1:1) in the presence of the indicated concentration of free Ca^{2+} was conducted as described in the legend of Figure 6 and Experimental Procedures.

factor of 10 in D653G, D706G, and D708G (Figure 6B). Therefore, D653, D706, and D708 are required for high-affinity PS-dependent Ca^{2+} binding, and mutation of these residues leads to a 10-fold decrease in the affinity for Ca^{2+} and a decrease in the maximal level of Ca^{2+} binding.

D653, D706, and D708 Are Required for Binding of PS to PLC δ1. Because D653G, D706G, and D708G are defective in Ca^{2+} binding and PS stimulation, these mutants may be also defective in PS binding. To test this possibility, we assessed the binding of native and mutant PLC δ1 to sucrose-loaded PS/PC vesicles in the presence of increasing concentrations of free Ca^{2+}. The native enzyme bound to PS in a Ca^{2+}-dependent manner, and saturable binding was obtained when the free Ca^{2+} concentration reached 30 μM. As shown in Figure 7, increasing amounts of PLC δ1 accumulated in the pellet fraction as the Ca^{2+} concentration increased from 0 to 300 μM, a consequence of direct binding of PLC δ1 to the sucrose-loaded PS/PC vesicles. More than 90% of the native enzyme was bound to PS/PC vesicles at 30 μM Ca^{2+}. The Ca^{2+}-dependent binding of PS to D653G, D706G, and D708G mutant enzymes was impaired. Less than 50% of the mutant protein was bound to PS at 30 μM free Ca^{2+}, and binding is not saturated even if the free Ca^{2+} concentration is increased to 300 μM (Figure 7).

The results thus far show that residues D653, D706, and D708 are essential for Ca^{2+}-dependent PS stimulation of PLC δ1. However, each single-residue mutant (D653G, D706G, and D708G) still displayed some residual binding and regulation by both PS and Ca^{2+}. This indicates that D653, D706, and D708 may independently contribute to the interaction of PS and Ca^{2+} with PLC δ1. To test this hypothesis, we mutated D653, D706, and D708 to glycine simultaneously. The ability of PS to stimulate the triple-mutant enzyme and its dependence on free Ca^{2+} were examined. Analysis of the PS dose–response curves for the D653/D706/D708G enzyme reveals that this mutant enzyme is barely stimulated by PS even at very high concentrations of PS and Ca^{2+} [10 μM Ca^{2+} and 50 mol % PS (Figure 8A)]. When the reactions were conducted at 10 μM free Ca^{2+}, the activity of the D653/D706/D708G mutant slightly increased from 2.9 to 3.9 μmol of PIP_2 hydrolyzed min^{-1} mg^{-1} when the level of PS in the mixed micelles increased from 0 to 50 mol %.

dx.doi.org/10.1021/bi201252f | Biochemistry 2012, 51, 2246–2257

As shown in Figure 8B, PIP_2 hydrolysis by D653/D706/D708G PLC δ1 in the presence of increasing concentrations of free Ca^{2+} revealed that the activity of D653/D706/D708G mutant PLC δ1 was stimulated as the free Ca^{2+} concentration increased from 1 μM, and the activity reached a plateau when the free Ca^{2+} concentration approached 10 μM. This pattern of Ca^{2+} dependence is very similar to that of the native enzyme under basal conditions. In contrast to that of the native enzyme, the activity in the presence of PS was only slightly higher than that in the absence of PS. The estimated free Ca^{2+} concentration (5.5 μM) required for half-maximal stimulation in the absence of PS was comparable to that (6 μM) in the presence of PS. These results indicate that the affinity of Ca^{2+} for the D653/D706/D708G mutant PLC δ1 was not affected by the presence of PS. This observation is quite in contrast to the observation with the native enzyme and the single mutants D653G, D706G, and D708G, where the Ca^{2+} affinity was increased 20-, 2.3-, 3.3-, and 3-fold, respectively, in the presence of PS.

The D653/D706/D708G PLC δ1 Triple Mutant Does Not Bind PS or Ca^{2+}, and PS Does Not Affect the Affinity for the Substrate. The physical binding of Ca^{2+} and PS to PLC δ1 was eliminated by simultaneous mutation of D653, D706, and D708. As shown in Figure 9A, Ca^{2+} barely binds to the triple-mutant enzyme even in the presence of 35 mol % PS. Centrifugation phospholipid vesicle binding assays also revealed that the triple mutant binds very little to PS/PC vesicles when the free Ca^{2+} concentration ranged from 0 to 300 μM (Figure 9B). Although the triple mutant displays Ca^{2+} dependence during basal catalysis similar to that of the native enzyme, the mutant enzyme is severely defective in Ca^{2+} and PS binding. The lack of this physical interaction with the mutant enzyme leads to a severe loss of activation by PS. Analysis of the substrate concentration dependence of the triple mutant revealed that the mutant enzyme is barely stimulated by PS (Figure 10). The substrate affinity for the triple mutant was not affected by PS. These results demonstrate that each of the three aspartate residues at positions 653, 706, and 708 mediates the effects of PS and Ca^{2+} on the activation of PLC δ1.

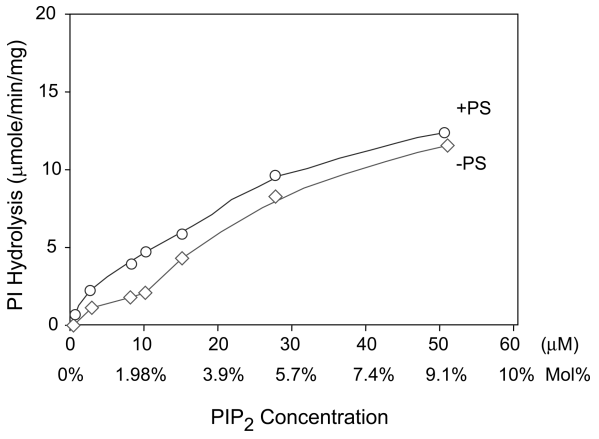

Figure 10. Effect of PS on the substrate dependence of the PIP_2 hydrolysis catalyzed by the D653/D706/D708G mutant of PLC δ1. Hydrolysis of increasing concentrations of PIP_2 by the D653/D706/D708G mutant of PLC δ1 in PIP_2/dodecyl maltoside mixed micelles containing 0 (◇) and 50 mol % PS (○). The reaction was conducted in the presence of 20 μM free Ca^{2+} and stopped, and the [^{3}H]IP_3 was separated and quantitated as described in the legend of Figure 4 and Experimental Procedures.

DISCUSSION

This extensive structure–function analysis is a follow-up to our previous paper that described the ability of free calcium and PS to potentiate the phosphodiesterase activity of PLC δ1 through a C-terminal C2 domain.[9] C2 domains mediate the regulatory effects of the second messenger calcium for many proteins that act at an interface between the cytosol and plasma membrane, nuclear membrane, and endoplasmic reticulum.[15,28] They have a very conserved tertiary structure consisting of an eight-stranded antiparallel β sandwich. Despite this conservation of tertiary structure, C2 domains vary substantially in their primary structure, which likely explains their differential ability to bind ligands such as calcium (a minority of C2 domains do not bind and are not regulated by calcium), phospholipids, and proteins. Much of the diversity in primary structure resides in the loops that connect the β strands at the bottom and top of the β sandwich. Specific residues from these loops have been implicated in calcium and phospholipid binding.[29,30,49,50]

The aspartic acid residues at positions 653, 706, and 708 of the loop regions of the C2 domain of PLC δ1 are highly conserved in the C2 domains from a number of signaling molecules that display Ca^{2+}-dependent activation. Subsets of C2 domains that are calcium-independent are missing most if not all of these conserved residues. The solution of a crystal structure of PLC δ1 in a complex with the calcium analogue lanthanum revealed that the negatively charged carboxyl groups of residues Asp-653, Asp-706, and Asp-708 participated in coordinating the binding of three lanthanum ions to the C2 domain of PLC δ1.[27] To investigate the functional role of binding of calcium to the C2 domain of PLC δ1, we eliminated the negative charges in the holoprotein at these three positions by mutating each Asp residue to Gly, individually and all together to create a triple mutant.

Replacement of all three Asp residues with Gly (D653/D706/D708G) ablates the ability of PLC δ1 to bind Ca^{2+} in the absence of substrate and also ablates PS binding. Because a substrate is necessary for binding of Ca^{2+} to the catalytic domain but not to the C2 domain, noncatalytic Ca^{2+} binding sites are assessed by excluding the substrate from the assay mixture. Ca^{2+} can still bind to the catalytic site of the triple mutant, because binding of Ca^{2+} to the catalytic domain is absolutely necessary for hydrolysis and the basal activity of this enzyme is unchanged. In fact, the activity of D653/D706/D708G PLC δ1 is still responsive to increasing Ca^{2+} concentrations; its Ca^{2+} dose–response curve is similar to that of the native enzyme in the absence of PS. Because the binding of Ca^{2+} and PS is interdependent, D653/D706/D708G PLC δ1 does not bind PS. This finding is also in agreement with our previous results that showed the simultaneous presence of PS, Ca^{2+}, and PLC δ1 is required for the formation of the ternary Ca^{2+}–PS–enzyme activation complex.[9] Because D653/D706/D708G PLC δ1 cannot bind PS, the enzyme is not regulated at all by PS, unlike the native enzyme that is stimulated 20-fold by micromolar concentrations of PS and calcium. The lack of PS stimulation and binding to D653/D706/D708G PLC δ1 definitively identifies the C2 domain as the site of Ca^{2+} and PS interaction and defines a role for the C2 domain in enzyme activation. Data of Ananthanarayanan et al. support this model. They have demonstrated Ca^{2+}-dependent translocation of the C2 domain of PLC δ1 to the inner plasma

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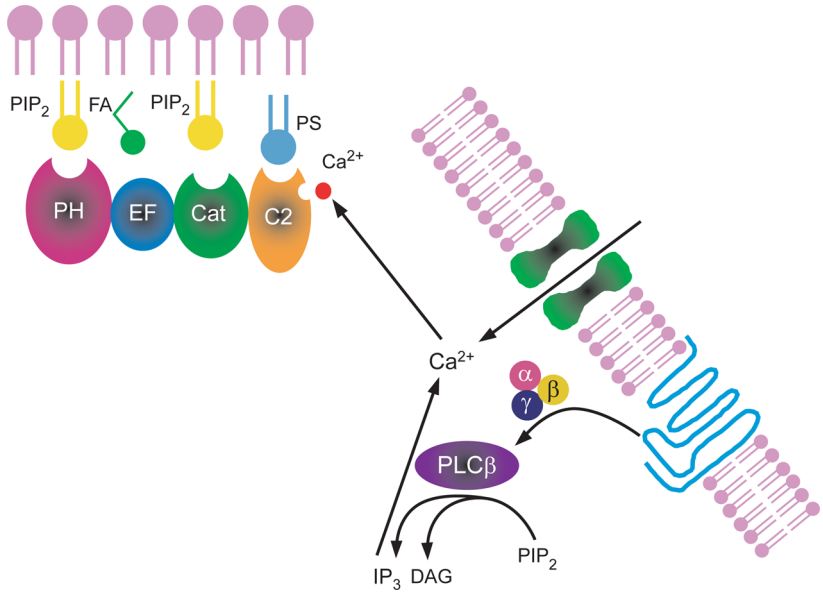

Figure 11. Schematic depicting the proposed model for PLC δ1 activation.

membrane, which is considered rich in PS content.[14] It is concluded that binding of Ca^{2+} to the C2 domain is essential for the enzyme to interact with and be regulated by PS. Work by Corbin et al. also demonstrated that PKCα and cPLA2-α C2 associate with the membrane via calcium drive interactions with specific lipids.[49]

We have determined the stochiometry of binding of Ca^{2+} to PLC δ1 to be 2.3, a number in agreement with the cocrystal structures.[27] From analysis of the single-point mutants, D653G, D706G, and D708G, we conclude that all three residues play a role in Ca^{2+} and PS binding. Each of the point mutants has an intermediate phenotype between the wild-type and triple-mutant D653/D706/D708G PLC δ1 enzyme. For example, each of the single-point mutants could be regulated by PS and Ca^{2+}; however, the potency of PS and Ca^{2+} was substantially reduced as was the maximal efficacy of these compounds. The reduced potency and maximal efficacy were a result of the reduced affinity of Ca^{2+} and PS for these mutant enzymes. While it is still not known how many Ca^{2+} ions actually bind to the C2 domain of PLC δ1, it is at least two and perhaps as many as three. It is clear, however, that all three Asp acid residues (D653, D706, and D708G) in the C2 domain of PLC δ1 play an equal role in binding to these Ca^{2+} ions, and that the binding of multiple Ca^{2+} ions is cooperative. Grobler and Hurley reported binding of a total of four Ca^{2+} ions, one to the catalytic pocket and three to the C2 domain, estimated from the Hill coefficients of calorimetric titration.[31] On the other hand, Cifuentes et al. approximated the number of bound Ca^{2+} ions to be two for both the intact and active proteolytic fragments of PLC δ1.[4] It may be possible that the method we employed here could detect only strong interactions between the metal cation and the protein; however, the obtained number of bound Ca^{2+} atoms is in good agreement with the cocrystal structures.

Ca^{2+} can have multiple roles in regulating C2 domain function. Calcium can alter the electrostatic potential of the surface of the C2 domain, can act as a bridge between the C2 domain and a ligand, can induce conformational changes, and/or can promote protein−protein interactions with adaptor proteins.[30,32] Several reports suggest that the role of the C2 domain of PLC δ1 is to correctly orient the catalytic domain with respect to the interface.[14,33] This may indeed be a role for the C2 domain; however, its dramatic effect on enzyme catalysis is likely to involve more complex mechanisms. Kinetic analysis revealed that PS stimulates PLC δ1 by virtue of its ability to increase substrate affinity. The native enzyme's affinity for PIP_2 was increased 20-fold in the presence of PS, while the affinity between PIP_2 and the triple-mutant enzyme remained unchanged. It appears that the C2 domain of PLC δ1 acts primarily as an allosteric regulator, because the K_m for the substrate is reduced dramatically after Ca^{2+} and PS binding. Kinetic analyses comparing wild-type and C2 domain mutant enzymes using a soluble substrate such as cIP should be able to discriminate true allosteric effects from those mediated by interactions with the interface.

Our approach examines the C2 domain as it exists in nature, as a part of a macromolecule. This approach not only has the potential to be more informative but also is a more conservative approach than studies that seek to examine the function of isolated C2 domains. It is especially important to determine the functional role(s) of the C2 domain of PLC δ1 as part of the holoprotein, because the C2 domain is very closely apposed to the catalytic domain in this phospholipase.[33] The extensive contact between the C2 and catalytic domain is likely necessary for proper folding of both domains and allows for the transfer of allosteric effects. The crystal structure of PLC δ1 reveals that there is an extensive and rigid interface between the C2 domain and core structure of the catalytic domain.[33] Additional evidence suggesting that the C2 domain is specifically important for enzymatic activity was provided by Kanematsu et al. In their study, the replacement of the C2 domain with that of its related molecule, p130, completely abolished the activity of the enzyme.[34] The C2 domain of the PLC family is considered to have evolved together with two other domains, the catalytic and EF-hand domains.[35] More recently, a fragment of PLC β3 comprising most of the enzyme, including the C2 domain, was cocrystallized with $G\alpha_q$, and the structure of the complex was examined.[36] The interface between the two proteins included the region between the C2 and catalytic domains of PLC β3, whose mutations markedly reduced the

dx.doi.org/10.1021/bi201252f | *Biochemistry* 2012, 51, 2246−2257

PLC activity in the presence of $G\alpha_q$. It is possible that binding of PS and Ca^{2+} to the C2 domain of PLC δ1 could subsequently cause a structural change in the C2 domain itself as well as the region between the C2 and catalytic domains similar to the one in PLC β3 complexed with $G\alpha_q$, which ultimately affects the enzymatic activity.

C-Terminal deletion mutagenesis of PLC δ1 resulted in the production of an inactive protein;[37,38] furthermore, the Ca^{2+} binding loops in the C2 domain are located in the same face as the cleavage site. These findings led to the proposal that binding of the membrane to the C2 domain of PLC δ1 was essential to correctly orient the catalytic site to the substrate-containing interface.[33] However, the proposal did not distinguish between direct effects of the C2 domain on activity, on protein stability, or both. The isolated C2 domain of PLC δ1 or of the C2 domain truncation enzyme is expressed mainly in the insoluble fraction of bacterial extracts, suggesting that correct folding of the catalytic core and of the C2 domain of PLC δ1 is interdependent. These biochemical studies of mutated PLC δ1 demonstrate that binding od Ca^{2+} to the C2 domain is not essential for catalysis of substrate but has a direct affect on the catalytic center. The affinity for the substrate PIP_2 at the active site is increased by 20-fold as the enzyme binds to Ca^{2+} and the membrane containing PS via its C2 domain. The modification of K_m is a common mechanism by which enzymes are regulated.

Why does deletion of the Ca^{2+}-binding residues in the C2 domain lead to a reduction in the PS-mediated increase in the affinity for the substrate? The simplest explanation is that the mutant enzymes are not able to interact with PS, because this interaction requires the binding of Ca^{2+} to the C2 domain. Disruption of Ca^{2+} binding leads to defective PS−PLC δ1 interaction; thus, PS is not able to stimulate PLC δ1. Because binding of PS to the C2 domain is highly sensitive to the free Ca^{2+} concentration, we postulate that Ca^{2+} is the primary regulator of PLC δ1, because Ca^{2+} concentrations clearly can vary by several orders of magnitude while the concentration of PS likely varies little in the cell (Figure 11). This mechanism may be particularly important for the regulation of PLC δ1 in the cell. The affinity of the enzyme for PIP_2 has been determined by kinetic analysis by several laboratories. The K_m values ranged from 2 to 20 mol % membrane lipids.[4] Because the concentration of PIP_2 in the plasma membrane is below 1%, it is highly unlikely that PLC δ1 can catalyze the hydrolysis of PIP_2 in the cell unless there is a mechanism for increasing its affinity for the substrate. These results provide a mechanism whereby Ca^{2+} may function as an activator of PLC δ1 in the cell. Actually, several observations reveal that activation of the PLC δ1 isozyme might occur as an event secondary to receptor-mediated activation of other PLC isozymes or Ca^{2+} channels.[39,40] In addition, to directly participate in the catalytic reaction, these results show that Ca^{2+} may increase the affinity for the substrate by promoting PS binding via the C2 domain.

Although D653, D706, and D708 are conserved in PLC δ1, primary sequence analysis shows that these Ca^{2+} binding residues are not conserved in the C2 domains of the β and γ families of PLC. Asp-653 is replaced with Gly and Cys in PLC β and γ, respectively. Asp-708 is replaced with an Asn or Gly in PLC β. The activity of the δ class is much more sensitive to the concentration of free Ca^{2+} than that of the β, γ, or ε enzymes. Only the newly identified ζ isozyme whose expression is limited to sperm appears to have similar or even greater sensitivity to free Ca^{2+}.[41] Because elimination of any one of these three conserved aspartates in PLC δ1 leads to a substantial reduction in the extent of Ca^{2+}-dependent enzyme activation, it is predicted that the β and γ isoenzymes should be much less sensitive to the concentration of free calcium. This prediction is consistent with both in vitro and in vivo findings about the Ca^{2+} dependence of PLC isoforms.[39,42] PLC δ1 but not PLC β1 or PLC γ1 is stimulated by physiologic concentrations of Ca^{2+} to hydrolyze cellular inositol lipids.[40]

Many studies in excitable tissues show that an increase in the intracellular Ca^{2+} concentration activates PLC activity.[40,43−47] Indeed, it has been demonstrated that PLC δ1 is activated by capacitative calcium entry that follows PLC β activation upon bradykinin stimulation.[39] We hypothesize that this calcium-induced PLC activity may be primarily due to activation of PLC δ1 and demonstrate a potential mechanism by which Ca^{2+} regulates PLC δ1 through the C2 domain. Thus, activation of a receptor coupled to PLC β leads to an increased intracellular free Ca^{2+} concentration, which in turn can further activate a Ca^{2+}-sensitive form of PLC (like PLC δ1) forming a positive feedback loop of Ca^{2+} and inositol phospholipid signaling (Figure 11). Because receptor-mediated PLC activation is usually transient and rapidly desensitized,[48] the positive Ca^{2+}−inositol phospholipid loop could prolong the action of PLC and may operate in slower or more prolonged synaptic or cellular responses. We propose that PLC δ1 is in fact responsible for these prolonged effects, such as those seen in hypertension and cardiac hypertrophy.

These studies identify the C2 domain as an allosteric modulatory domain and demonstrate the role of C2 domains in mediating the effects of calcium and phospholipid on protein function. Because many other molecules contain C2 domains, these studies suggest a mechanism by which these other molecules could be regulated.

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Funding

This work was supported by National Institutes of Health Grants HL 03961 and HL 55591 (to J.W.L.) and the National Sciences Council of the Republic of China, the Institute of Biomedical Sciences, and Academia Sinica Intramural Funding (to K.K.).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PLC, phosphoinositide-specific phospholipase C.

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