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Structural Basis for Calcium and Phosphatidyl Serine Regulation of PLC $\delta 1$

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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 $\delta 1$ is required for phosphatidyl serine (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 $\delta 1$. The PS-dependent Ca^{2+} binding affinities of the mutant enzymes D653G, D706G and D708G were reduced by an order of magnitude and the maximal Ca^{2+} binding was reduced to half of that of the native enzyme. The Ca^{2+} dependent PS binding was also reduced in the mutant enzymes. Under basal conditions, the Ca^{2+} dependence and maximal hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) was 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 to glycine in the C2 domain of PLC $\delta 1$, leads to a complete and selective loss of the stimulation and binding by PS. These results show that D653, 706 and 708 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.

INTRODUCTION

The family of phospholipase C enzymes consist 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 eucaryotic cells. The mechanisms by which each family is regulated is different. In general, the β isoforms are regulated by the G_q family of heterotrimeric G proteins, the γ isoforms by receptor and non-receptor tyrosine kinases, the ϵ family 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

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in all eukaryotic isoforms, the catalytic X and Y domains, the pleckstrin homology (PH) domain, and the C2 domain.

PLC $\delta 1$ is highly responsive to lipid stimulations. For example, the activity of PLC $\delta 1$ is enhanced by PIP₂ through the PH domain (4, 5). In cells this PIP₂ activation is observed both in the plasma membrane and in the nucleus (6–8). Also PLC $\delta 1$ 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 anti-parallel 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. PKC α C2 is known to bind both calcium and PS (13). As for PLC δ family, the C2 domain is also considered important in membrane targeting. The isolated C2 domains from $\delta 1$ and $\delta 3$ translocate specifically towards the plasma membrane whereas the C2 domain of $\delta 4$ is localized to various cellular membranes including the perinuclear membranes and plasma membrane upon stimulation with ionomycin, a calcium-mobilizing ionophore, which is presumably resulted from a difference in their lipid specificity, especially towards PS (14). In addition, the C2 domains of $\delta 1$ and $\delta 3$ exhibit a strong calcium-dependent membrane affinity, whereas the C2 domain of $\delta 4$ 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 $\delta 1$ 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 position 653, 706 and 708 to each 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

The expression vector pRSETA was from Invitrogen. To express PLC $\delta 1$ under the control of the T7 promoter, the coding sequence for PLC $\delta 1$ was cloned into pRSETA. The resulting expression construct (pRSETA_{plc}) was transformed into the 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. PIP₂ and dodecyl maltoside was 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 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 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 EGTA (binding buffer) and resuspended in 0.5 ml of the same buffer.

Centrifugation Binding Assay

The binding of PLC $\delta 1$ to phospholipid vesicles was estimated by a centrifugation assay (16, 19). The free Ca^{2+} concentration was calculated according to 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 carried out at 30 °C for 15 min. The free and bound PLC $\delta 1$ were separated by sedimentation at 50,000 $\times g$ for 30 min. An equal proportion of the supernatant and pellet fractions were resolved by 12% SDS-polyacrylamide gel electrophoresis, and the amount of PLC $\delta 1$ 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 (21) and Kawasaki et al. (22). 150 μM PS/PC or PA/PC phospholipid vesicles containing the indicated mole fractions of lipids were incubated with PLC $\delta 1$ (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 $^{45}\text{CaCl}_2$ (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 $^{45}\text{Ca}^{2+}$ on the membrane was determined by liquid scintillation counting. The amount of $^{45}\text{Ca}^{2+}$ 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 $^{45}\text{Ca}^{2+}$ 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 plus phospholipid.

PIP₂ Hydrolysis Assay

PIP₂ hydrolysis in dodecyl maltoside/PIP₂ 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 PIP₂/[³H]PIP₂ (4×10^5 cpm) in chloroform/methanol (19:1) in the presence or absence of the indicated phospholipids was dried under a stream of N₂ 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 $\mu\text{g}/\text{ml}$. 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 PIP₂. 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 [^3H]PIP₂ (pellet) was separated from [^3H]IP₃ (supernatant) by centrifugation at $2000 \times g$ for 10 min at 4 °C.

Analysis of Kinetic Data

Surface dilution kinetics were employed to study PIP₂ hydrolysis catalyzed by PLC δ 1. Case III conditions previously described for phospholipase A2 (25) and PLC (26) 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 was used to analyze the kinetic data as previously described (9).

RESULTS

Asp653, 706 and 708 are essential for PS stimulation of PLC δ 1

Three highly conserved negatively charged amino acid residues (Asp-653, Asp-706, and Asp-708) were identified in the δ isoforms of PLC by aligning the primary sequences of the C2 domains from PLA2, PKC α and all eucaryotic PI-PLCs (Figure 1A & 1B). Crystallographic studies have implicated these three residues in coordinating calcium binding (27). To investigate the role of these residues in Ca²⁺ binding and enzyme function, all three Asp were individually mutated by site-directed mutagenesis to Gly. These residues do not participate in the Ca²⁺-dependent basal activity of PLC δ 1, but are required for the Ca²⁺-dependent PS stimulation of phosphodiesterase activity. As shown in Fig. 2, the rate of hydrolysis of PIP₂ was very similar for the native and mutant enzymes in PS-free dodecyl maltoside mixed micelles at the two free Ca²⁺ concentrations tested (the hydrolysis rates were ~0.5 and ~3.3 $\mu\text{mol PIP}_2$ hydrolyzed/min/mg at 1 μM and 10 μM free Ca²⁺, 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 Ca²⁺ concentrations (Fig. 2). At 10 μM free Ca²⁺, PS stimulated the activities of the native, D653G, D706G and D708G PLC δ 1 to 12.7, 5.5, 9.24 and 7.6 $\mu\text{mol PIP}_2$ hydrolyzed/min/mg, respectively. The fold increase in activity for the native enzyme, D653G, D706G and D708G was 3.5, 1.7, 2.8 and 2.3, respectively. More remarkable stimulation by PS on the native enzyme was seen at lower free Ca²⁺ (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 Ca²⁺ used.

These results demonstrate that D653, D706 and D708 are not required for basal phosphodiesterase activity of PLC δ 1, but are essential for PS mediated stimulation of activity. This is more clearly illustrated in Fig. 3. The PS dose response curves show that the rate of PIP₂ hydrolysis by native and mutant enzymes is dependent on the mole fraction of PS. The potency and maximal effect of PS is markedly reduced for the mutant enzymes.

Asp653, 706 and 708 mediate Ca²⁺ regulation of PLC δ 1

We have previously shown that PS stimulates PLC δ 1 by virtue of its ability to form a Ca²⁺/PS/PLC δ 1 complex via the C2 domain. PS and Ca²⁺ are interdependent; neither one can interact with nor regulate PLC δ 1 without the other. Therefore, replacing aspartate 653, 706 and 708 with glycine may reduce the ability of PS to increase the potency of Ca²⁺ for stimulating catalysis. This is clearly illustrated in Fig. 4. In the absence of PS, the calcium dose response curves for the mutant and native enzymes were identical to each other (Fig. 4A). The maximal catalytic activity of the native enzyme, D653G, D706G and D708G were estimated to be 3.3 $\mu\text{mol PIP}_2$ hydrolyzed/min/mg, and the free Ca²⁺ concentration required

for 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 (Fig. 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 $\mu\text{mol}/\text{min}/\text{mg}$ (Fig. 4A and 4B). 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 to 70% that of the native PLC $\delta 1$ in the presence of PS. The maximal PS stimulated activity of D653G, D706G and D708G PLC $\delta 1$ was 4.9, 8.2 and 6.9 μmol of $\text{PIP}_2/\text{min}/\text{mg}$, respectively, while that of the native enzyme was 13 $\mu\text{mol}/\text{min}/\text{mg}$. With 35% PS, Ca^{2+} is at least 5 to 10 fold less potent in stimulating PIP_2 hydrolysis by D653G, D706G and D708G PLC $\delta 1$ than for the native enzyme. As shown in Fig. 4B, the free Ca^{2+} concentration required for half-maximal hydrolysis of PIP_2 in mixed micelles containing 35 mol % PS by D653G, D706G and D708G PLC $\delta 1$ was estimated to be 2.5, 2.0 and 1.8 μM , respectively. This reduction in 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 carried out 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 reduced maximal effect on the mutant enzymes as compared to the PS effect on the native enzyme. The mutant enzymes have a selective deficiency in PS activation.

D653G, D706G and D708G have lower affinity for 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, the effect of PS on the substrate concentration dependence of PLC $\delta 1$ catalysis was examined. When the micellar concentration of PIP_2 was increased from 0.1 to 9%, the hydrolysis of PIP_2 by the native enzyme in mixed micelles containing 35 mol % PS sharply increased from 3.1 to 19 $\mu\text{mol}/\text{min}/\text{mg}$ and was saturated as the PIP_2 concentration approached 4 mol%. 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 Fig. 5A, when the PIP_2 concentration was less than 2 mol %, 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 carried out 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 native enzyme if the reactions were carried out in the presence of 35 mol% PS. Specifically, the stimulatory effect of PS on the mutant enzymes was much less 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 the 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. K_m of the native enzyme was reduced by a factor 20 in the presence of 35% of PS, while those of D653G, D706G and D708G was reduced by a factor 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 Ca²⁺ binding

PS activation of PLC $\delta 1$ requires the simultaneous binding of Ca²⁺ and PS to the C2 domain of the enzyme. To test whether D653, D706 and D708 are involved in the lipid dependent Ca²⁺ binding to the C2 domain, we measured the binding of ⁴⁵Ca²⁺ to PLC $\delta 1$ as a function of the PS concentration and Ca²⁺ concentration. As illustrated in Fig. 6A, the binding of Ca²⁺ by the native enzyme is highly dependent on the concentration of PS; the binding was enhanced by as low as 5 mol % PS and plateaued as PS reached 30 mol%. Replacing D653, D706 or D708 to glycine reduced the total Ca²⁺ binding to PLC $\delta 1$ at saturating concentrations of PS and also required higher concentrations of PS to reach saturation (Fig. 6A).

Since little Ca²⁺ would bind to PLC $\delta 1$ in the absence of PS/PC vesicles, the Ca²⁺ saturation binding isotherm was carried out in the presence of PS/PC vesicles containing 40 mol% PS. In the presence of PS/PC vesicles, ⁴⁵Ca²⁺ bound to the native and the mutant PLC $\delta 1$ in a dose dependent and saturable manner Fig. 6B. Ca²⁺ bound to the native enzyme when the free Ca²⁺ was as low as 2 μ M and the binding increased sharply until the free concentration reached 100 μ M. The binding of Ca²⁺ to mutant enzymes was much lower than that of the native PLC $\delta 1$, and increased slowly as the free Ca²⁺ concentration increased from 10 to 1000 μ M. Saturable binding was still not obtained as the free Ca²⁺ concentration reached 1 μ M. As shown in Fig. 6B, the maximal binding of Ca²⁺ to 0.15 μ M protein was estimated to be 35 pmole, corresponding to approximately 2.3 pmole Ca²⁺/pmole protein. The binding of Ca²⁺ to D653G, D706G and D708G mutant enzymes at 1 mM free Ca²⁺ was 12.8, 16.5 and 15 pmole corresponding to 0.84, 1.08 and 0.98 pmole Ca²⁺/pmole protein, respectively. The concentration of free Ca²⁺ required for half-maximal binding to the native enzyme was estimated to be 7 μ M, while that for D653G, D706G and D708G mutants it was 128, 70 and 90 μ M, respectively. The Ca²⁺ binding isotherm revealed that the affinity for Ca²⁺ binding to PLC $\delta 1$ was reduced by a factor of 10 in D653G, D706G and D708G (Fig. 6B). Therefore, D653, D706 and D708 are required for high affinity PS-dependent Ca²⁺ binding and mutation of these residues leads to a 10-fold decrease in affinity for Ca²⁺ and a decrease in maximal Ca²⁺ binding.

D653, D706 and D708 are required for PS binding to PLC $\delta 1$

Since D653G, D706G and D708G are defective in Ca²⁺ binding and PS stimulation, these mutants may be also defective in PS binding. To test this possibility, the binding of native and mutant PLC $\delta 1$ to sucrose loaded PS/PC vesicles was determined in the presence of increasing concentrations of free Ca²⁺. The native enzyme bound to PS in a Ca²⁺-dependent manner and saturable binding was obtained when the free Ca²⁺ reached 30 μ M. As shown in Fig. 7, increasing amounts of PLC $\delta 1$ accumulated in the pellet fraction as the Ca²⁺ increased from 0 to 300 μ M, a consequence of direct binding of PLC $\delta 1$ to the sucrose-loaded PS/PC vesicles. More than 90% of the native enzyme was bound to PS/PC vesicles at 30 μ M Ca²⁺. The Ca²⁺-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 μ M free Ca²⁺, and binding is not saturated even if the free Ca²⁺ was increased to 300 μ M (Fig. 7).

The results thus far show that residues D653, D706 and D708 are essential for Ca²⁺-dependent PS stimulation of PLC $\delta 1$. However, each single residue mutant D653G, D706G and D708G still displayed some residual binding and regulation by both PS and Ca²⁺. This indicates that D653, 706 and 708 may independently contribute to the interaction of PS and Ca²⁺ with PLC $\delta 1$. To test this hypothesis, we mutated D653, 706, and 708 to glycine simultaneously. The ability of PS to stimulate the triple residue mutant enzyme and its dependence on free Ca²⁺ was examined. Analysis of the PS dose response for the D653, 706, 708G enzyme reveals that this mutant enzyme is barely stimulated by PS even at very

high concentrations of PS and Ca^{2+} ($10 \mu\text{M}$ Ca^{2+} and 50 mol % PS Fig. 8A). When the reactions were carried out at $10 \mu\text{M}$ free Ca^{2+} , the activity of the D653/D706/D708G mutant slightly increased from 2.9 to $3.9 \mu\text{mol PIP}_2$ hydrolyzed/min/mg when the PS in the mixed micelles increased from 0 to 50 mol%.

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

The D653/D706/D708G PLC $\delta 1$ triple residue mutant does not bind PS nor Ca^{2+} , nor does PS effect the affinity for substrate

The physical binding of Ca^{2+} and PS to PLC $\delta 1$ was eliminated by simultaneous mutation of D653, D706 and D708. As shown in Fig. 9A, Ca^{2+} barely binds to the triple mutant enzyme even in the presence of 35 mol% of 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 $300 \mu\text{M}$ (Fig. 9B). Although the triple mutant displays Ca^{2+} -dependence during basal catalysis similar to that of native enzyme, the mutant enzyme is severely defective in Ca^{2+} and PS binding. 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 (Fig. 10). The substrate affinity for the triple mutant was not affected by PS. These results demonstrate that each of the three aspartate residues at position 653, 706, and 708 mediate the effects of PS and Ca^{2+} on the activation of PLC $\delta 1$.

DISCUSSION

This extensive structure-function analysis is a follow-up to our previous paper that described the ability of free calcium and phosphatidylserine (PS) to potentiate the phosphodiesterase activity of PLC $\delta 1$ 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 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 nor are 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 and 50).

The aspartic acid residues at positions 653, 706 and 708 of the loop regions of the C2 domain of PLC $\delta 1$ 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 $\delta 1$ complexed with the calcium analog lanthanum revealed that the

negatively charged carboxyl groups of residues Asp 653, 706 and 708 participated in coordinating the binding of three lanthanum ions to the C2 domain of PLC δ 1 (27). To investigate the functional role of calcium binding to the C2 domain of PLC δ 1, we eliminated the negative charges in the holo protein 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 δ 1 to bind Ca^{2+} in the absence of substrate and also ablates PS binding. Since substrate is necessary for Ca^{2+} binding to the catalytic domain but not to the C2 domain, non-catalytic Ca^{2+} binding sites are assessed by excluding substrate from the assay mixture. Ca^{2+} can still bind to the catalytic site of the triple mutant, since Ca^{2+} binding 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 δ 1 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 δ 1 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 δ 1 is required for the formation of the ternary Ca^{2+} -PS-enzyme activation complex(9). Because D653/D706/D708G PLC δ 1 cannot bind PS, the enzyme is not regulated at all by PS, unlike the native enzyme which is stimulated 20 fold by μM concentrations of PS and calcium. The lack of PS stimulation and binding to D653/D706/D708G PLC δ 1 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 from Ananthanarayanan *et al.* support this model. They have demonstrated Ca^{2+} -dependent translocation of the C2 domain of PLC δ 1 to the inner plasma membrane which is considered rich in PS content (14). It is concluded that Ca^{2+} binding to the C2 domain is essential for the enzyme to interact with and be regulated by PS. Work by Corbin *et al.* also demonstrate that PKC alpha and cPLA2-alpha C2 associate to the membrane via calcium drive interactions with specific lipids (49).

We have determined the stoichiometry of Ca^{2+} binding to PLC δ 1 to be 2.3, a number in agreement with the co-crystal structures (27). From analysis of the single point mutants, D653G, D706G, and D708G, it is concluded that all three residues play a role in Ca^{2+} and PS binding. Each of the point mutants has an intermediate phenotype between wild type and triple mutant D653/D706/D708G PLC δ 1 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 was 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 δ 1, 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 δ 1 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 δ 1 (4). It may be possible that the method we employed here was able to detect only strong interactions between the metal cation and the protein, however, the obtained number of bound Ca^{2+} is in good agreement with the co-crystal structures.

Ca^{2+} can have multiple roles on 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 promote protein-protein interactions with adaptor proteins (30, 32). Several reports suggest that the role of the C2

domain of PLC $\delta 1$ is to correctly orient the catalytic domain 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 $\delta 1$ by virtue of its ability to increase substrate affinity. The native enzyme's affinity for PIP₂ was increased 20-fold in the presence of PS, while the affinity between PIP₂ and the triple mutant enzyme remained unchanged. It appears that the C2 domain of PLC $\delta 1$ acts primarily as an allosteric regulator, since the K_m for substrate is reduced dramatically after Ca²⁺ and PS binding. Kinetic analyses comparing wildtype 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 is also 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 $\delta 1$ as part of the holo protein, since 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 $\delta 1$ reveals that there is an extensive and rigid interface between C2 domain and core structure of 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 $\beta 3$ comprising the most of the enzyme including the C2 domain, was co-crystallized with G α_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 $\beta 3$, whose mutations markedly reduced the PLC activity in the presence of G α_q . It is possible that PS/Ca²⁺-binding to the C2 domain of PLC $\delta 1$ 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 $\beta 3$ complexed with G α_q , which ultimately affects the enzymatic activity.

C terminal deletion mutagenesis of PLC $\delta 1$ resulted in production of an inactive protein (37, 38), furthermore, the Ca²⁺ binding loops in the C2 domain are located in the same face as the cleavage site. These findings led to the proposal that membrane binding to the C2 domain of PLC $\delta 1$ 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 $\delta 1$ 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 $\delta 1$ is interdependent. The present biochemical studies of mutated PLC $\delta 1$ demonstrate that Ca²⁺ binding 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 PIP₂ at the active site is increased by 20-fold as the enzyme binds to Ca²⁺ and 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 Ca²⁺ binding residues in the C2 domain lead to a reduction in the PS mediated increase in affinity for substrate? The simplest explanation is that the mutant enzymes are not able to interact with PS, since this interaction requires the binding of Ca²⁺ to the C2 domain. Disruption of the Ca²⁺ binding leads to defective PS-PLC $\delta 1$ interaction thus PS is not able to stimulate PLC $\delta 1$. Because PS binding to the C2 domain is highly sensitive to the free Ca²⁺ concentration, we postulate that Ca²⁺ is the primary regulator of

PLC $\delta 1$, since Ca^{2+} concentrations clearly can vary several magnitudes while the concentration of PS likely varies little in the cell (Figure 11). This mechanism may be particularly important for the regulation of PLC $\delta 1$ in the cell. The affinity of the enzyme for PIP_2 has been determined by kinetic analysis from several laboratories. The K_m values ranged from 2 to 20 mol% of membrane lipids. (4) Since the concentration of PIP_2 in the plasma membrane is below 1%, it is highly unlikely that PLC $\delta 1$ can catalyze the hydrolysis of PIP_2 in the cell unless there is a mechanism to increase its affinity for substrate. The present results provide a mechanism whereby Ca^{2+} may function as an activator of PLC $\delta 1$ in the cell. Actually, several observations reveal that activation of the PLC $\delta 1$ 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 participating in the catalytic reaction, the present results shows that Ca^{2+} may increase the affinity for substrate by promoting PS binding via the C2 domain.

Although D653, D706, and D708 are conserved in PLC $\delta 1$, 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. Asp708 is replaced with a 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). Since elimination of any one of these three conserved aspartates in PLC $\delta 1$ leads to a substantial reduction in 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 of the Ca^{2+} dependence of PLC isoforms (39, 42). PLC $\delta 1$ but not PLC $\beta 1$ nor PLC $\gamma 1$ is stimulated by physiologic concentrations of Ca^{2+} to hydrolyze cellular inositol lipids (40).

Many studies in excitable tissues show that a rise in intracellular Ca^{2+} activates PLC activity (40, 43–47). Indeed, it has been demonstrated that PLC $\delta 1$ 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 $\delta 1$ and demonstrate a potential mechanism for Ca^{2+} to regulate PLC $\delta 1$ through the C2 domain. Thus activation of a receptor coupled to PLC β leads to increased intracellular free Ca^{2+} , which in turn can further activate a Ca^{2+} -sensitive form of PLC (like PLC $\delta 1$) 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 $\delta 1$ 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. Since many other molecules contain C2 domains, these studies suggest a mechanism by which these other molecules could be regulated.

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The abbreviations used are

PIP₂	phosphatidylinositol 4,5-bisphosphate
IP₃	inositol 1,4,5-trisphosphate
PI	phosphatidylinositol
PC	phosphatidylcholine
PA	phosphatidic acid
PS	phosphatidyl serine
PLC	phosphoinositide-specific phospholipase C

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A

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PLC 1      CA I C I E V L G A R H L P K - - - - - N G R G I V C P F V E I E V A G A E - - Y D S I K Q K T E F V V D N - - G L N P V W P A
PLC 2      M T L T V K V L G A R H L P K - - - - - L G R S I A C P F V E V E I C G A E - - Y G N N K F K T T V V N D N - - G L S P I W A P
PLC 1      N T L S V K I I S G Q F L S - - - - - D K K V G T Y V E V D M F G L P V D T R R K A F K T K T S Q G N - - A V N P V W E E
PLC 2      T T L S I T V I S G Q F L S - - - - - E R S V R T Y V E V E L F G L P G D P K R R Y R T K L S P S T N - - S I N P V W K E
PLC 3      N A L R V K V I S G Q F L S - - - - - D R K V G I Y V E V D M F G L P V D T R R K Y R T R T S Q G N S - - - F N P V W D E
PLC 4      A T C S V Q V I S G Q F L S - - - - - D K K I G T Y V E V D M Y G L P T D T I R K E F R T R M V M N N - - G L N P V Y N E
PLC 1      K R L N I R V I S G Q Q L P K V N K - - - N K N S I V D P K V T V E I H G V S - - R D V A S R Q T A V I T N N - - G F N P W W D T
cPLA2     H K F T V V V L R A T K V T K G A F G - - D M L D T P D P Y V E L F I S T - - - - - T P D S R K R T R H F N N - - D I N P V W N E
PKC       K R G R I Y L K A E V A D - - - - - E K L H V T V R D A K N L I P M - - - - - D P N G L S D P Y V K L K L I P D P - - K N E S K Q K T K T I R S - - T L N P Q W N E

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PLCδ1 706
PLCδ1 708
PLCδ1 653

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PLC 1      - - - S N P E F - - - A F L R F V V Y E E D - - M F S D - - Q N F L A Q A T F P V K G L K - - - - - T G Y R A V P L K N N Y S E D L E L - - A S L L I K I D I F P A
PLC 2      - E I Y D P N L - - - A F L R F V V Y E E D - - M F S D - - P N F L A H A T Y P I K A V K - - - - - S G F R S V P L K N G Y S E D I E L - - A S L L V F C E M R P V
PLC 1      - - V V L P T L - - - A C L R I A V Y E E G - - - - - G K F I G H R I L P V Q A I R - - - - - P G Y H Y I C L R N E R N Q P L T L - - P A V F Y Y I E V K D Y
PLC 2      - - I L M P E L - - - A S L R V A V M E E G - - - - - N K F L G H R I I P I N A L N - - - - - S G Y H H L C L H S E S N M P L T M - - P A L F I F L E M K D Y
PLC 3      - - V V L P T L - - - A S L R I A A F E E G - - - - - G K F V G H R I L P V S A I R - - - - - S G Y H Y V C L R N E A N Q P L C L - - P A L L I Y T E A S D Y
PLC 4      - - V I L P D L - - - A V L R I A V Y D D N - - - - - N K L I G Q R I P P L D G L Q - - - - - A G Y R H I S L R N E G N K P L S L - - P T I F C N I V L K T Y
PLC 1      - - V V P D L - - - A L I R F L V E D Y D - - A S S K - - N D F I G Q S T I P L N S L K - - - - - Q Q Y R H V H L M S K N G D Q H P S - - A T L F V K I S L Q D
cPLA2     - D P N Q E N V - - - - - L E I T L M D A N - - - Y V M - - D E T L G T A T F T V S S M - - - - - K V G E K K E V P F I F N Q V T E - - - M V L E M S L E V C S C
PKC       - - - K P S D K - - D R R L S V E I W D W D - - R T T R - - N D F M G S L S F G V S E L M K M - - - - - P A S G W Y K L L N Q E

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B



Fig. 1.
 (A) Amino acid alignment of C2 domains. Asp residues mutated in PLC δ1 are highlighted.
 (B) The crystal structure of the C2 domain of PLC δ1 (PDB: 1DJI). The residues colored in red are D653, D706 and D708.

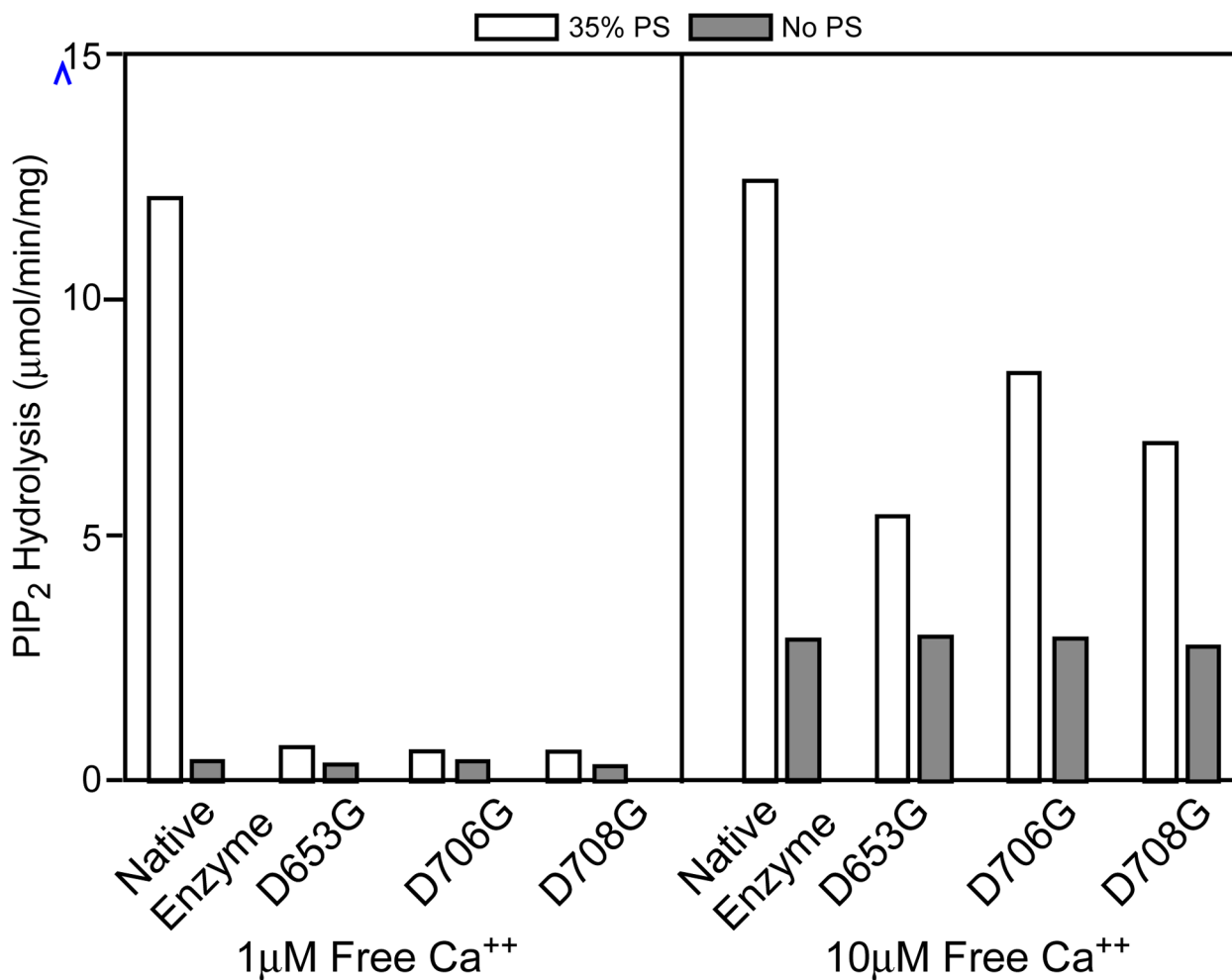


Fig. 2. Phosphatidylserine stimulation of the native, D653G, D706G and D708G mutant PLC $\delta 1$. Hydrolysis of 5 μM (1 mol%) PIP₂ in PIP₂/dodecyl maltoside mixed micelles in the presence (open bar) or absence of 35% PS (solid bar). The catalytic reaction was carried out in either 1 or 10 μM of free Ca²⁺. The total concentration of dodecyl maltoside plus nonsubstrate phospholipid was constant at 495 μM . The catalytic reaction was carried out in 50 μl of 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EGTA plus 500 $\mu\text{g/ml}$ BSA and CaCl₂ to yield 1 or 10 μM of free Ca²⁺. The free Ca²⁺ concentration in an EGTA-CaCl₂ buffer was calculated according to Fabiato and Fabiato (20). The reaction was carried at 30°C for 1–5 min, stopped and quantitated as described in Experimental Procedures.

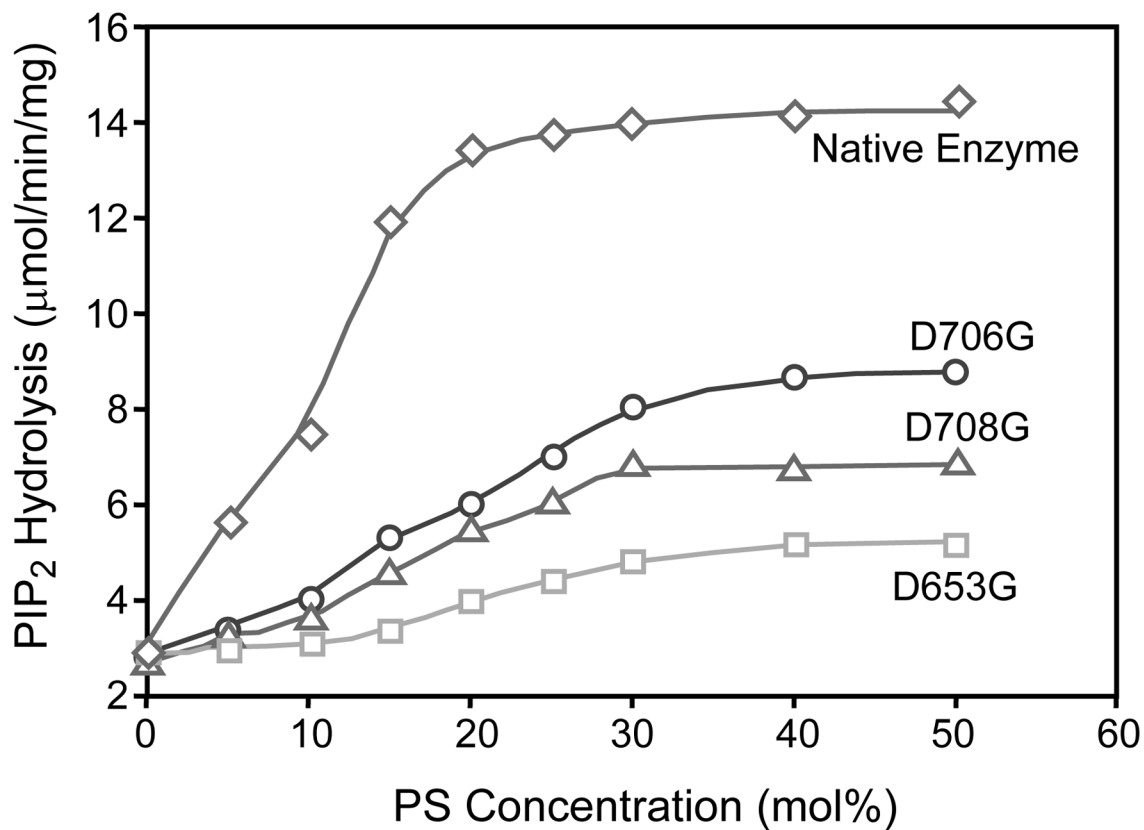
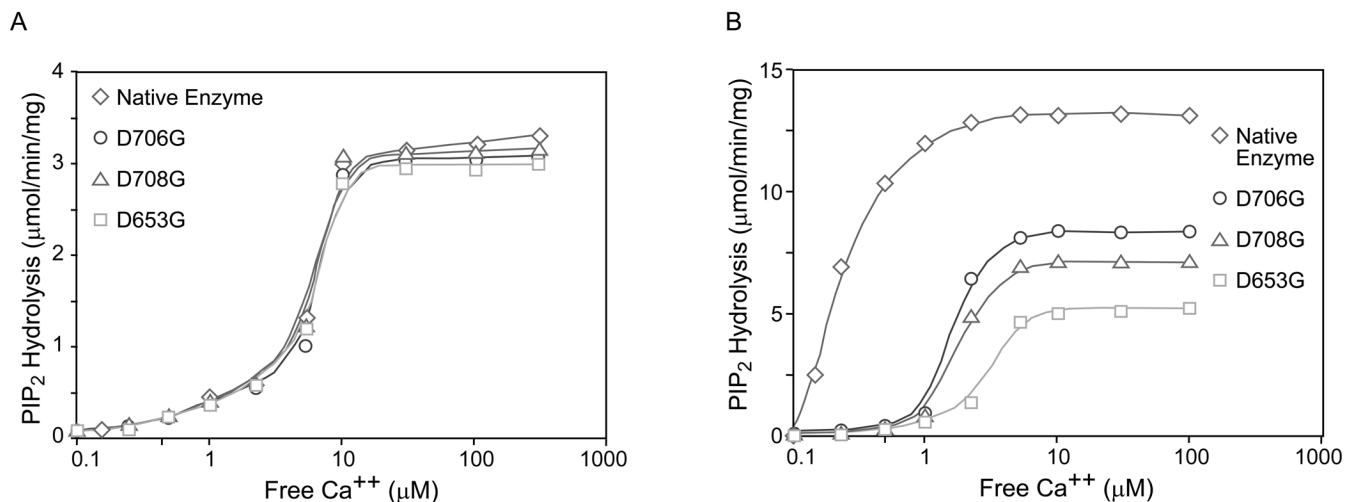


Fig. 3. Dependence of PS concentration on PIP₂ hydrolysis by the native and mutant PLC $\delta 1$. Catalytic hydrolysis of 5 μM of PIP₂ in dodecyl maltoside mixed micelles containing increasing concentration of PS (from 0 to 50 mol%) by the native (diamond), D653G (square), D706G (circle) and D708G (triangle) mutant enzyme. The total concentration of dodecyl maltoside plus nonsubstrate phospholipid was constant at 495 μM . The catalytic reaction was carried out as described in Fig. 1 and Experimental Procedures.

**Fig. 4.**

Effect of PS on the Ca²⁺ dependence of the native and the mutant PLC δ1 catalytic activities. Calcium concentration dependence of hydrolysis of 5 μM PIP₂ (corresponding to 1 mol% in the mixed micelles) by native (diamond), D653G (square), D706G (circle) and D708G (triangle) mutant PLC δ1 in PIP₂/dodecyl maltoside mixed micelles containing 0 (A) or 35 (B) 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 BSA and various CaCl₂ to yield indicated concentration of free Ca²⁺. PLC δ1 catalyzed hydrolysis of PIP₂ in the mixed micelles was measured as described (Fig. 1 and Experimental Procedures).

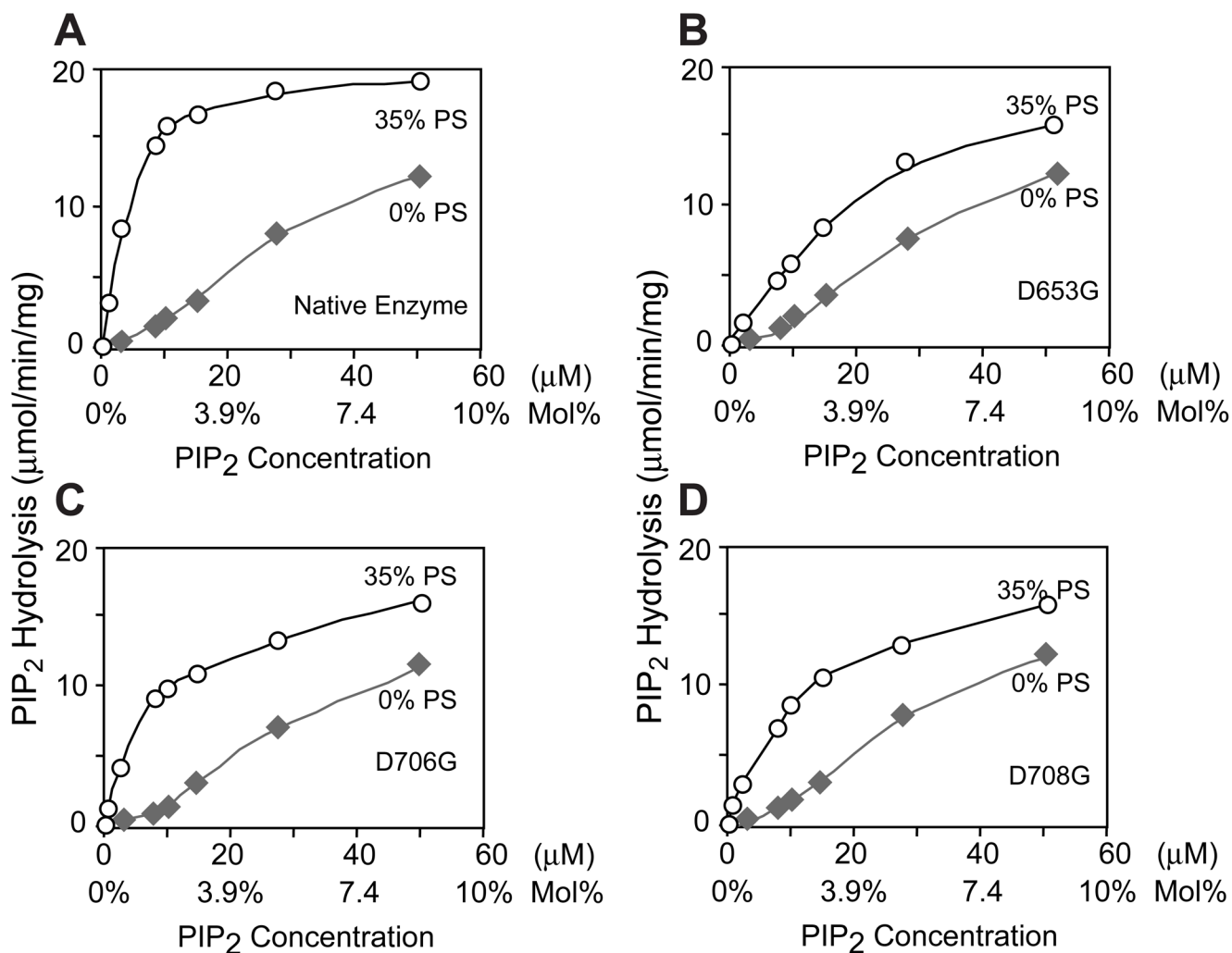


Fig. 5. Influence of PS on the substrate dependence of catalytic hydrolysis of PIP₂ by the native and the mutant PLC $\delta 1$. Hydrolysis of increasing concentrations of PIP₂ by the native (A), D653G (B), D706G (C) and D708G (D) PLC $\delta 1$ in PIP₂/dodecyl maltoside mixed micelles containing 0 (diamond) and 35 (circle) mol% PS. The mol% of PIP₂ was increased while the concentration of PS was fixed at 0 and 35 mol%, and the combined total concentration of PS plus dodecyl maltoside was maintained at 495 μM , see Experimental Procedures. The reaction was carried out in the presence of 5 μM free Ca^{2+} , stopped and the [³H]IP₃ was separated and quantitated as described in Experimental Procedures.

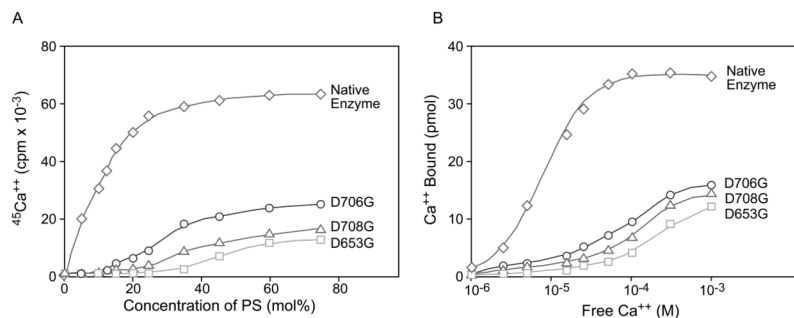


Fig. 6. Dependence of PS and free Ca^{2+} on the binding of Ca^{2+} to the native and the mutant PLC $\delta 1$. PS dependence of the binding of Ca^{2+} to the native (diamond), D653G (square), D706G (circle) and D708G (triangle) mutant PLC $\delta 1$. Binding of $0.15 \mu\text{M}$ enzyme with $100 \mu\text{M}$ free $^{45}\text{Ca}^{2+}$ (total 1.5×10^7 cpm) in 0.1 ml buffer containing 50 mM HEPES, pH 7.0, 100 mM KCl, and $10 \mu\text{M}$ EGTA and $150 \mu\text{M}$ PS/PC vesicles containing 0–75 mol% of PS, the determination of bound Ca^{2+} were carried as described in Experimental Procedures (A). Binding of $0.15 \mu\text{M}$ native (diamond), D653G (square), D706G (circle) and D708G (triangle) mutant PLC $\delta 1$ to increasing concentrations of free $^{45}\text{Ca}^{2+}$ (total 1.5×10^7 cpm) in the presence of $150 \mu\text{M}$ PS/PC vesicles composed of 50 mol% of PS was carried out as described in Experimental Procedures (B).

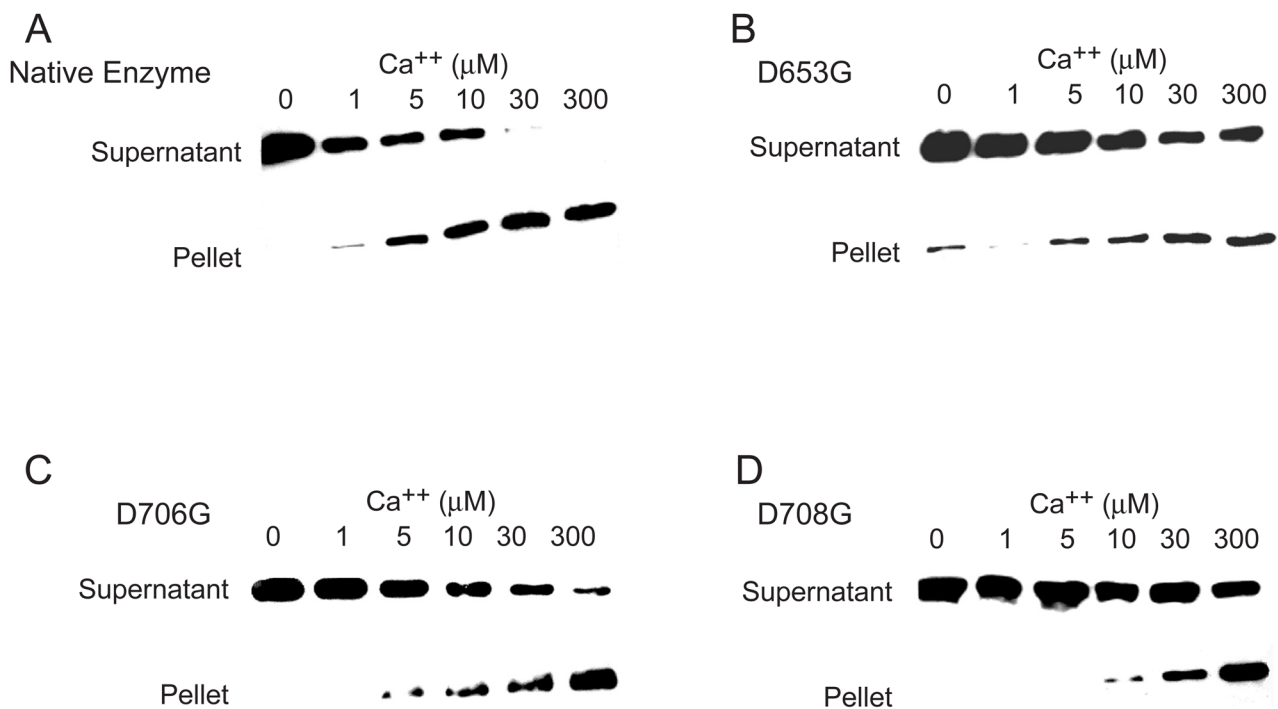


Fig. 7. Ca^{2+} -dependent phospholipid binding to the native and the mutant PLC $\delta 1$. Centrifugation binding assay of PLC $\delta 1$ and sucrose loaded PS/PC vesicles in the presence of the indicated concentration of free Ca^{2+} ion. $1\mu\text{g}$ of the native (A), D653G (B), D706G (C) and D708G (D) mutant protein was incubated with $150\mu\text{M}$ PS/PC vesicles (molar ratio = 1:1) in 0.2 ml of 50 mM Hepes, $\text{pH } 7.0$, 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.

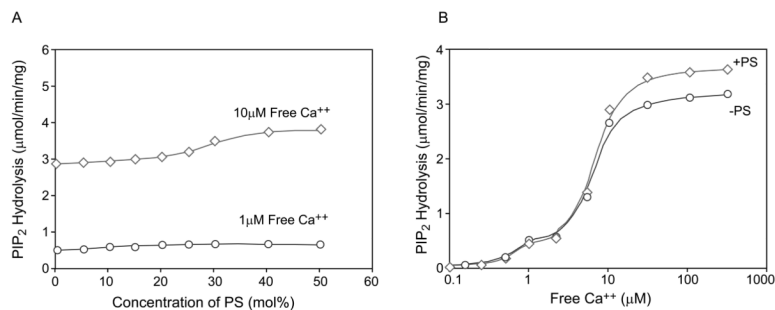


Fig. 8. Ca²⁺ and PS dependence of PIP₂ hydrolysis by the D653/D706/D708G mutant PLC δ1. PS concentration dependence of the D653/D706/D708G mutant PLC δ1 catalyzed hydrolysis of 5 μM PIP₂ (corresponding to 1 mol % in the mixed micelles) in the presence of 1 (circle) or 10 (diamond) μM free Ca²⁺ (A). Ca²⁺ dependence of hydrolysis of 5 μM of PIP₂ (corresponding to 1 mol % in the mixed micelles) by the D653/D706/D708G mutant PLC δ1 in mixed micelles containing 0 (circle) or 50 (diamond) mol % of PS is shown. The catalytic reaction was carried as described (Fig. 2 and 3, and Experimental Procedures).

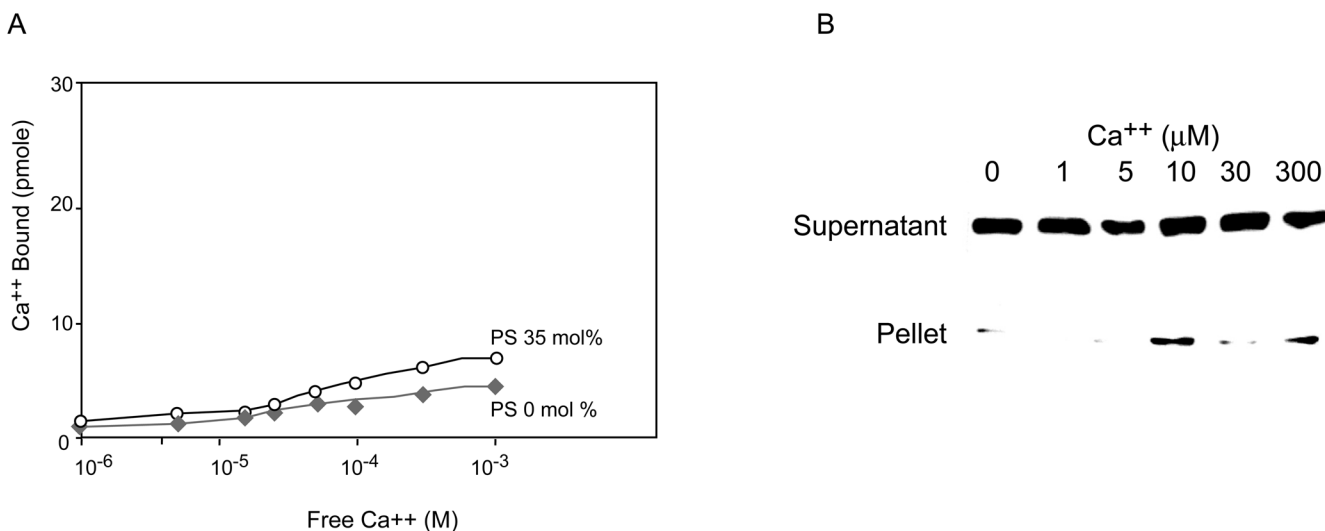


Fig. 9. Binding of Ca²⁺ and PS to the D653, 706, 708G mutant PLC δ 1. Binding of 0.15 μ M the D653/D706/D708G mutant enzyme with indicated concentration of free ⁴⁵Ca²⁺ (total 1.5×10^7 cpm) in 0.1 ml buffer containing 50 mM Hepes, pH 7.0, 100 mM KCl, and 10 μ M EGTA and 150 μ M PS/PC vesicles containing 0 (diamond) and 35 (circle) mol% of PS, the determination of bound Ca²⁺ were carried as described in Experimental Procedures (A). 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 Ca²⁺ ion. Binding of 1 μ g protein to 150 μ M PS/PC vesicles (molar ratio = 1:1) in the presence of indicated concentration of free Ca²⁺ was carried as described (Fig. 6 and Experimental Procedures) (B).

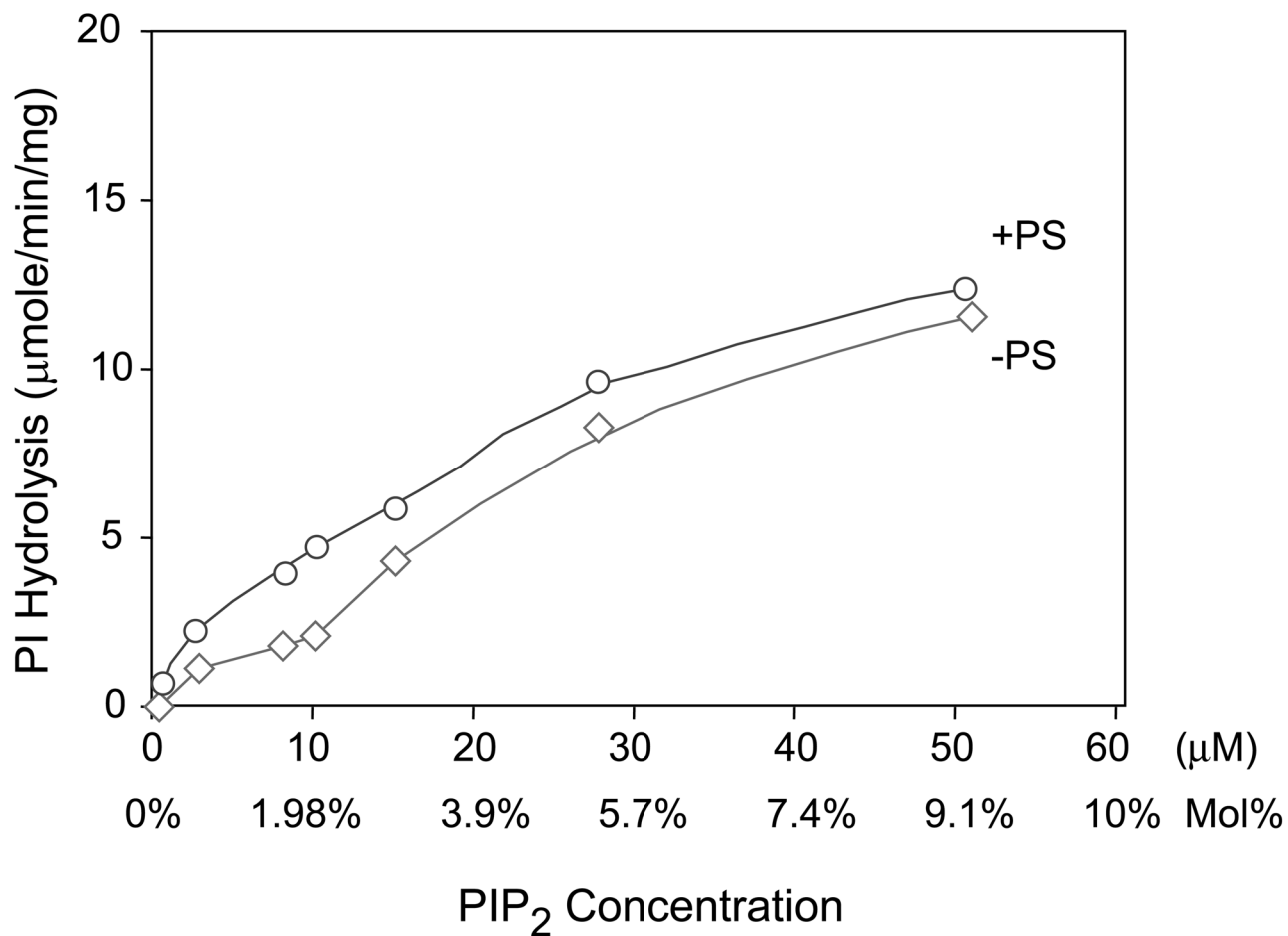


Fig. 10.

Effect of PS on the substrate dependence of the PIP₂ hydrolysis catalyzed by the D653/D706/D708G mutant PLC δ 1. Hydrolysis of increasing concentrations of PIP₂ by the D653/D706/D708G mutant PLC δ 1 in PIP₂/dodecyl maltoside mixed micelles containing 0 (diamond) and 50 (circle) mol% PS. The reaction was carried out in the presence of 20 μ M free Ca²⁺, stopped and the [³H]IP₃ was separated and quantitated as described in Fig. 4 and Experimental Procedures.

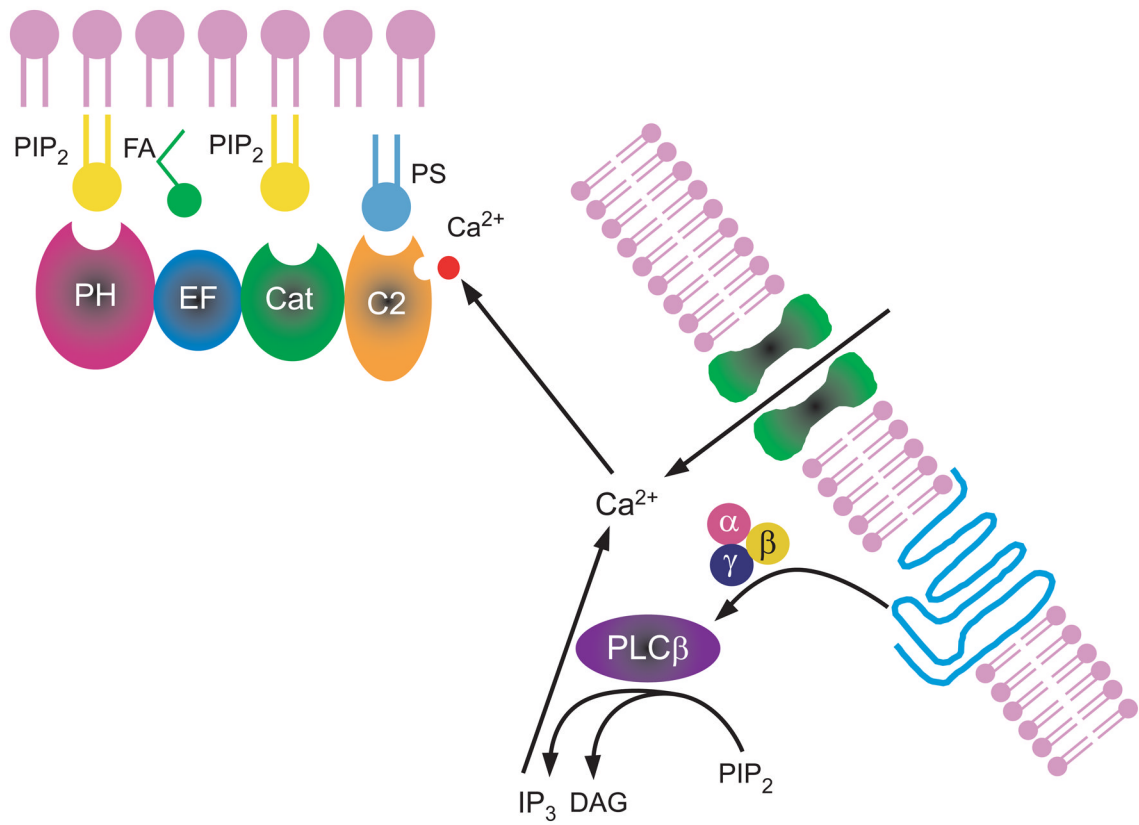


Fig 11.
Schematic depicting proposed model for PLC $\delta 1$ activation.

Table 1Effect of PS on the kinetic properties of the native and the mutant PLC $\delta 1$

	PLC $\delta 1$ PS concentration (mol %)	V _{max} ($\mu\text{mol}/\text{min}/\text{mg}$)	K _m (mol fraction)	K _s (μM)
Native PLC $\delta 1$	0	17 \pm 3	0.045 \pm 0.001	70 \pm 2
	35	19 \pm 4	0.0025 \pm 0.0002	45 \pm 3
D653G PLC $\delta 1$	0	14 \pm 2	0.045 \pm 0.001	71 \pm 3
	35	17 \pm 3	0.01 \pm 0.01	47 \pm 2
D706G PLC $\delta 1$	0	15 \pm 2	0.051 \pm 0.003	69 \pm 2
	35	18 \pm 3	0.008 \pm 0.002	44 \pm 2
D708G PLC $\delta 1$	0	14 \pm 3	0.048 \pm 0.004	73 \pm 4
	35	18 \pm 4	0.009 \pm 0.001	46 \pm 2

Hydrolysis of increasing concentration of PIP₂ at 5 μM free Ca²⁺ by PLC $\delta 1$ was measured in PIP₂/dodecyl maltoside mixed micelles containing 35 and 0 mol % PS (see Experimental Procedures). V_{max}, K_m and K_s corresponding 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 Equation 1 in ref (26).