Catalysis by Phospholipase C δ_1 Requires That Ca^{2+} Bind to the Catalytic Domain, but Not the C2 Domain

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ABSTRACT: The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphoinositide-specific phospholipase C (PLC) is absolutely dependent on Ca^{2+}. The PH domain truncated catalytic core of rat phospholipase C δ_1 (PLC-δ_1) has Ca^{2+} binding sites in its catalytic and C2 domains, and potential Ca^{2+} binding sites in two EF-hands. A catalytically inactive PLC-δ_1 catalytic core bound with low affinity to PIP_2-containing vesicles in the presence of Ca^{2+}. A mutant PLC-δ_1 has been engineered which lacks the C2 domain Ca^{2+} binding site and the surrounding loops known as the jaws. Isothermal calorimetric titration showed four Ca^{2+} ions bind to the wild-type PLC-δ_1 catalytic core in solution but only one binds to the C2 domain jaws deletion mutant. The activity and Ca^{2+} dependence of wild-type and mutant phospholipase Cs were determined using substrate incorporated in detergent micelles and in large unilamellar vesicles. The activities of wild-type and mutant were identical to each other in both assay systems. Wild-type and the C2 jaws deletion mutant of PLC have Hill coefficients of 1.12−1.16 with respect to [Ca^{2+}]. We conclude that a single Ca^{2+} bound to the catalytic domain is entirely responsible for the Ca^{2+} dependence of the basal activity of PLC-δ_1.

Phosphoinositide-specific phospholipase C (PLC)[1] catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), a key early step in many mammalian cell surface receptor stimulated intracellular signaling pathways. The activity of mammalian PLC depends absolutely on Ca^{2+} ions (*1*−*6*). The products of the reaction, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), are second messengers that cause an influx of calcium into the cytosol and the activation of protein kinase Cs, respectively. Three classes of PLC, β, γ, and δ, are all expressed within a given cell where each is activated by a different major signaling pathway. PLC-β is activated by guanine nucleotide binding proteins (G-proteins) (*7*, *8*) and PLC-γ by receptor tyrosine kinases (*9*). There is emerging evidence that PLC-δ is activated by transglutaminase II (G_h) (*10*), as well as a proposal that it is activated by the small GTPase rho proteins (*11*).

Members of all classes have in common four domains (*5*, *6*, *12*, *13*): an N-terminal pleckstrin homology (PH) domain, an EF-hand domain, an α/β barrel similar to that of triosephosphate isomerase, and a C2 domain. PH domains are found in over 90 different proteins where they bind phosphoinositides, proteins, or both (*14*, *15*). The PH domain of PLC-δ_1 anchors the enzyme to bilayer membranes by binding PIP_2 with high affinity in the absence of Ca^{2+} (*16*−*18*); hence, it is thought that the PH domain is solely responsible for anchoring PLC-δ_1 to membranes in the absence of Ca^{2+}.

EF-hands are ubiquitous Ca^{2+} binding motifs that normally bind one Ca^{2+} per motif, although there are naturally occurring variants that do not bind Ca^{2+} ions (*19*). The first pair of EF-hands of PLC-δ_1 conform to the consensus Ca^{2+} binding motif at the two most-conserved positions, Asp-1 and Glu-12. EF-hands 1 and 2 differ from the motif at the third most conserved, but nonessential, position, Gly-6 (*19*, *20*). The second pair of EF-hands do not conform to the Ca^{2+} binding motif, and were not recognized as EF-hands until the three-dimensional structure was determined (*12*). The PLC-δ_1 EF-hands are distributed in two lobes. The first lobe is disordered in three out of four crystallographically independent molecules analyzed (*12*, *21*). The second lobe, comprising EF-hands 3 and 4, is well-ordered and tightly associated with the α/β barrel. Ca^{2+} binding to the EF-hands is not observed crystallographically (*21*, *22*).

The active site is located in the cleft of the α/β barrel. A single Ca^{2+} ion binds in the active site. This domain is structurally similar to bacterial PI-PLC (*23*), although the bacterial PI-PLC has no Ca^{2+} binding site and its activity does not depend on Ca^{2+}.

C2 domains are ∼120 residue Ig-fold β sandwiches (*24*) found in roughly 70 membrane associated proteins where they interact with phospholipids, proteins, or both (*13*, *25*−*28*). Many, but not all, C2 domains bind Ca^{2+}. C2 domains

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[1] Abbreviations: PI-PLC or PLC, phosphoinositide-specific phospholipase C; PLC-$\delta_{\Delta 1-134}$, rat PLC-δ_1 lacking residues 1 through 134; PLC-$\delta_{\Delta 1-134,\Delta Jaws}$, rat PLC-$\delta_1$ lacking residues 1−134 and in which residues 644 through 651 have been replaced by a triglycine linker and residues 707 through 713 have been replaced by a diglycine linker, and contains the mutations D653A and D706S; PLC-$\delta_{\Delta 1-134,H311A}$, PLC-$\delta_{\Delta 1-134}$ variant resulting from replacement of H311A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfoninc acid; EDTA, ethylenediaminetetraacetic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; POPC, palmitoyloleoylphosphatidylcholine.

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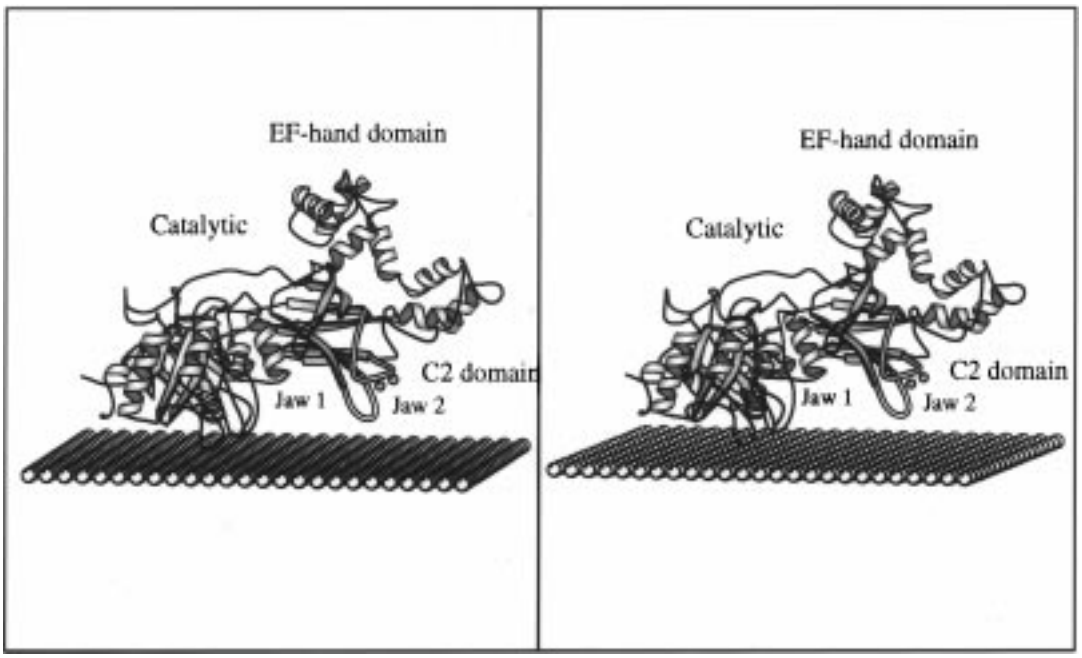

FIGURE 1: Structure of the catalytic core of rat PLC δ_1. Stereoview (*58*) showing the catalytic core of PLC-δ_1 (*12*) oriented sagitally to the membrane surface (*21, 22*). The locations of crystallographically identified Ca^{2+}-binding sites in the α/β barrel and C2 domain are shown by spheres. The jaws are shown by thickened unshaded lines.

are directly responsible for the Ca^{2+}-dependent phospholipid binding of cytosolic phospholipase A_2 (*29*), synaptotagmin I (*30*), and protein kinase C (*31*). Because PLCs are membrane-associated enzymes that are dependent on Ca^{2+}, it was anticipated that C2 domains might also play a role in Ca^{2+}-stimulated membrane anchoring of PLCs. The C2 domain has been reported to be essential for activity on the basis of deletion analysis (*32*), but previous studies did not distinguish between direct effects on activity and effects on protein stability. The Ca^{2+} binding sites occur at one tip of the domain, near a set of highly variable loops that have been referred to as "the jaws" (*25*), ligand binding loops (LBLs; *33*), or Ca^{2+} binding regions (CBRs; *22*). Based on its orientation in PLC, the C2 domain Ca^{2+} binding sites, and the adjacent jaws, are predicted to be proximal to the membrane (Figure 1) and to form a Ca^{2+} bridge to phospholipid headgroups (*13*, *21*−*23*).The predicted C2 domain Ca^{2+} and phospholipid binding site is about 40 Å from the catalytic site. The putative C2 domain−membrane contact was therefore predicted to play a role in catalysis with respect to planar bilayers, but not with respect to substrate incorporated into small detergent micelles commonly used in PLC assays.

While the interaction of PLC-δ_1 with PIP_2-containing membranes has been studied extensively in the absence of Ca^{2+} , no data have been reported in the presence of Ca^{2+} . Such measurements have been difficult because in the presence of Ca^{2+} , PIP_2 is consumed by enzymatic hydrolysis in a shorter time than required for the binding measurement. Nonhydrolyzable analogues of PIP_2 are not suitable for extensive binding studies because they are laborious to synthesize in quantity. To determine whether Ca^{2+} is able to stabilize the interaction of PLC-δ_1 with bilayer membranes in the absence of the PH domain, binding of the catalytically inactive mutant H311A (*34*) to PC and PIP_2:PC vesicles was measured in the presence and absence of Ca^{2+}.

To elucidate the roles of the C2 domain Ca^{2+} binding sites in PLC-δ_1 function, we have engineered a mutant in which the C2 domain jaws have been deleted, eliminating the Ca^{2+} binding sites on this domain. The stoichiometry of Ca^{2+} binding to wild-type and mutant PLC-δ_1 was determined by isothermal titration calorimetry, and the Ca^{2+}-dependent hydrolysis of PIP_2 was characterized for both micellar and vesicular substrates.

MATERIALS AND METHODS

The template for all mutagenesis procedures was the PLC-δ_1 cDNA fragment coding for residues 135−756 of rat PLC-δ_1 subcloned into the *Nco*I and *Bam*HI sites of pET 15b (Novagen, Inc.). This vector was engineered to express PLC-$\delta_{\Delta 1-134}$, a variant of PLC-δ_1 in which the N-terminal PH domain has been deleted (*35*). Primers for mutagenesis were synthesized on a Millipore DNA synthesizer. Primers were purified by two rounds of ethanol precipitation before use. Expand DNA polymerase and deoxynucleotides were from Boehringer Mannheim. PCR was carried out using a Perkin-Elmer 9600 GeneAmp. SeaKem ME agarose, which was used for all gel separations of DNA, was from FMC. QIAEX II gel extraction kits and DNA purification kits were from Qiagen, Inc. All restriction endonucleases were from New England Biolabs. Ready-to-go DNA ligase was from Pharmacia. POROS HS and HQ ion-exchange resins were from Perseptive Biosystems. A prepacked HiLoad Superdex 200 gel filtration column (2.6 cm × 60 cm) and a prepacked Superdex 200 HR column (1.0 cm × 30 cm) were from Pharmacia. Palmitoyloleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). PIP_2 and ultrol grade *n*-dodecyl-β-D-maltoside were from Calbiochem (La Jolla, CA). [^{3}H]PIP_2 was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Other reagents, from various sources, were of the highest grade available.

Engineering of PLC Mutants. To generate the Jaw 2 deleted construct, the coding region of PLC was amplified in two reactions using first the sense primer ACGCTTCATGGTAGAGTCTGGCGGCGACTTTATTGGCCAG and the antisense T7 terminator primer and, separately, the antisense primer CGCCGCCAGACTCTACCATGAAGCG and sense primer CTGCCAGATTTTCAACTCACGTGC (Mut5′ primer). Amplification products were gel purified and included in a second reaction with additional Mut5′ and T7 terminator primers. The final amplification product was gel purified, digested with *Pml*I and *Kpn*I, and then ligated into similarly digested and phosphatase-treated vector. Deletion of Jaw 1 was accomplished essentially the same way. The first set of amplifications utilized primers AAGGGAGGAGGAGTGGCCCCCAAGGTG and the T7 terminator primer in one reaction and CACTCCTCCTCCCTTTGGCAGCTGC and the Mut 5′ primer in the other. Amplification products were gel purified and included in a second reaction with additional Mut5′ and T7 terminator primers. Gel purified amplification products were digested with *Pml*I and *Kpn*I and ligated into vector as above. The sequences of all amplification products were confirmed by DNA sequencing.

Expression and Purification of Mutants. Induction of protein expression in BL21(DE3) cells, harvesting, and cell lysis were as described previously (*35*). Following centrifugation, the lysate was adjusted to pH 6.0 by the addition of solid MES while stirring. Protein was applied to a POROS HS column (1.6 cm × 12 cm) attached to a BioCAD (Perseptive Biosystems) which was equilibrated in 25 mM MES, pH 6.0, 1 mM EDTA, 1 mM DTT. The column was washed with the same buffer until the A_{280} of the eluate was less than 0.1. PLC-$\delta_{\Delta 1-134}$ and its mutants were eluted using a gradient of 0−500 mM NaCl over 15 column volumes. Fractions containing enzyme were pooled and concentrated to less than 10 mL using a stirred Amicon concentrator. Concentrated protein was finally purified by gel filtration on a Superdex S-200 column (2.6 cm × 60 cm) in 25 mM MOPS, pH 6.8, 100 mM NaCl, 5 mM β-mercaptoethanol.

Molecular weights of purified proteins were measured by MALDI-TOF mass spectrometry on a Perseptive Biosystems Voyager. Samples were prepared for mass spectrometry by extensive dialysis of 1 mg/mL protein against 5% acetic acid, and then mixed with an equal volume of saturated sinapinic acid in 1% TFA in acetonitrile. Concentrations of purified protein were determined spectrophotometrically using a molar extinction coefficient at 280 nm for native PLC-$\delta_{\Delta 1-134}$ of 94 908 M^{-1} cm^{-1} obtained by the method of Gill and von Hippel (*36*). Because we noted a time-dependent loss of specific activity during storage at −80 °C, all enzyme samples were assayed within 7 days of purification.

Assay of PLC Activity. Assay mixtures contained 20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 0.4 mg/mL bovine serum albumin, 5 mM β-mercaptoethanol. Concentrations of phospholipid and EGTA-buffered Ca^{2+} were varied depending on the particular experiment. Lipids were mixed in organic solutions and then dried under vacuum in a Savant Speed Vac SC100 for at least 3 h. Large unilamellar vesicles (LUVs) were prepared by suspending lipids in assay buffer and extruding at least 15 times through a 100 nm filter. Small unilamellar vesicles (SUVs) were prepared by sonication of resuspended lipids for 5 min in 5 s bursts separated by intervals of 1 s. Micelles were prepared by resuspension of dried lipids in *N*-dodecyl β-D-maltoside followed by sonication. Assays on wild-type and mutant enzymes were carried out concurrently on the same vesicle preparations in order to avoid artifacts due to a small degree of irreproducibility in the vesicle preparations. Reactions were started by the addition of protein to the reaction mixture which had been preequilibrated to 37 °C. Mixtures were incubated at 37 °C for 5 min and then stopped by adding 1 mL of chloroform/methanol/HCl (100:100:0.6). The water soluble product, IP_3, was extracted with 0.3 mL of 1 N HCl. Product formation was measured by the level of radioactivity in the aqueous phase and was measured for 5 min in an LKB liquid scintillation counter.

Analysis of Kinetic Data. Activity was analyzed in terms of the two-step binding model (*37*) in which binding to membranes or micelles depends on the bulk concentration of lipid, and subsequent substrate binding depends on its mole fraction. The equation describing this model is

$$v = V_{max} X_s [S_0] / (K_m K_s + K_m [S_0] + X_s [S_0]) \qquad (1)$$

where V_{max} is the maximum rate, X_s is the mole fraction of substrate, $[S_0]$ is the bulk concentration of substrate, K_s is the binding constant of PLC to vesicles, and K_m is the binding constant of substrate once PLC is associated with the membrane. A complete characterization of surface dilution kinetics requires three sets of assays are performed to determine all the kinetic parameters: (i) vary $[S_0]$ while keeping X_s constant; vary X_s while keeping $[S_0]$ constant; and (iii) vary both $[S_0]$ and X_s. Because we were unable to saturate activity at bulk lipid and Ca^{2+} concentrations at which LUVs were stable, we carried out partial characterizations with assays i and ii. Experiments were all done at 10 μM free Ca^{2+}. All parameters were determined by nonlinear fitting using the program Origin.

In experiments performed to determine the effect of Ca^{2+} on the activity of PLC, lipid concentrations were fixed and $[Ca^{2+}]_{free}$ was varied. In all reactions, the concentration of EGTA was kept fixed at 5 mM, and the appropriate concentration of free Ca^{2+} was attained by varying the total concentration of Ca^{2+} present in the reaction mixture. $[Ca^{2+}]_{free}$ was calculated based on the composition of the buffer (*38*). Under these conditions:

$$v = V_{max}' [Ca^{2+}]^n / (K_{Act} + [Ca^{2+}]^n) \qquad (2)$$

where V_{max}' is the maximum rate at the $[S_0]$ and X_s used, K_{Act} is the binding constant of PLC for Ca^{2+}, and n is the stoichiometry of Ca^{2+} binding to PLC necessary for activity.

Titration Calorimetry. Calorimetric titrations were performed in an isothermal titration calorimeter from MCS (*39*). Titrations were performed at either 25 °C or 37 °C. Following thermal equilibration for 1 h, injections of 4 μL of titrant were made automatically using a precision stepper motor. An initial delay of 120 s preceded the first injection, and subsequent injections were made at interval of 300 s. Sample buffer consisted of 100 mM NaCl and 5 mM β-mercaptoethanol buffered by 25 mM MOPS−NaOH. All buffers were treated with Chelex 100 to remove any metals before use. The reference cell contained water. Samples were prepared by gel filtration on S-200 Sepharose equili-

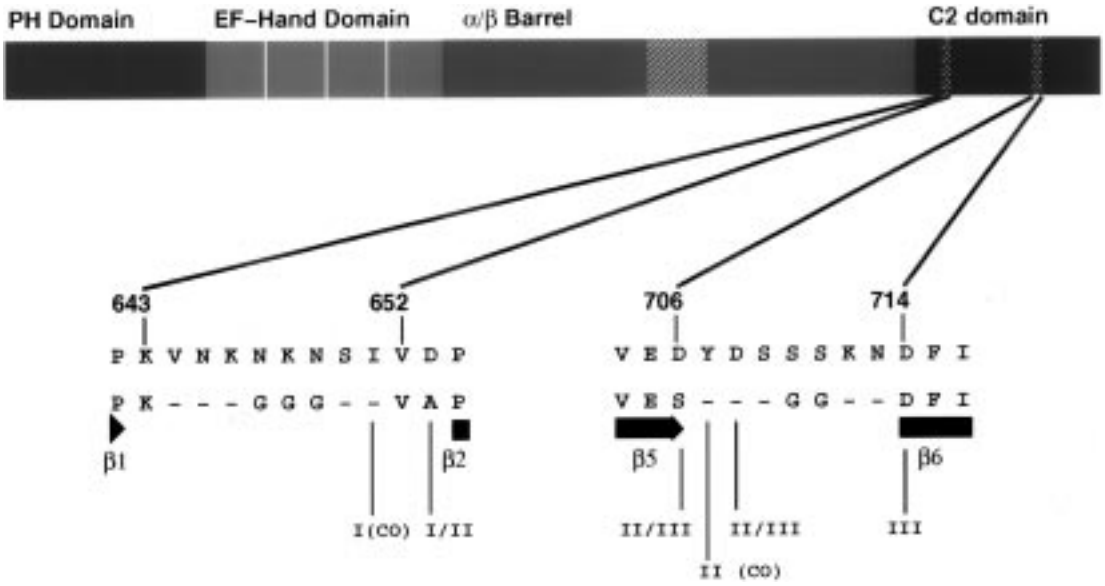

FIGURE 2: Design of C2 domain jaws-deleted mutant and domain structure of PLC-δ_1. Roman numerals indicate ligands for Ca^{2+} or lanthanide sites I, II, and III (*21*, *22*).

brated in buffer. The absence of residual Ca^{2+} in protein samples used for calorimetry was confirmed by atomic absorption spectroscopy using a Perkin-Elmer Model 3110 atomic absorption spectrometer. In all cases, the presence of Ca^{2+} was lower than the experimental limits of detection of 1 Ca^{2+} atom per 20 protein molecules. Atomic absorption was used to determine the Ca^{2+} concentrations in the stock solution used to prepare titrants. ITC data were analyzed using the Origin data analysis package. Data were fit using the single set of sites model. All parameters were allowed to float freely during fitting.

Gel Filtration PLC Lipid Binding Studies. Gel filtration experiments were carried out either in MSE (50 mM MOPS, pH 6.8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT) or in MSC (50 mM MOPS, pH 6.8, 100 mM NaCl, 0.5 mM $CaCl_2$, 1 mM DTT). For all experiments, a prepacked Pharmacia Superdex 200 HR column was equilibrated with either buffer. The Superdex 200 HR column was calibrated using gel filtration standards. Samples were loaded in volumes of 250 μL and consisted of 530 μg of PLC (7.6 nmol) and either 200 nmol of PC or 200 nmol of PC and 50 nmol of PIP_2. The column was developed at a rate of 0.5 mL/min using the same buffer in which the column was equilibrated. Fractions of 0.5 mL were collected.

RESULTS

Design and Properties of PLC Mutants. PLC-$\delta_{\Delta1-134,H311A}$ was made to study binding of PLC-δ_1 to phospholipids in the presence of Ca^{2+}. H311A inactivates PLC-δ_1 by removing one of the catalytic residues in the hydrolysis reaction, without altering any active site Ca^{2+} ligands (*34*). PLC-$\delta_{\Delta1-134,\Delta Jaws}$ was designed to remove the Ca^{2+} binding residues in the C2 domain jaws. The jaws comprise the two longest of three loops, also known as CBR-1 and -3 (*22*) or LBL-1 and -3 (*33*), involved in Ca^{2+} and probably phospholipid binding (*21*). There are naturally occurring C2 domains, such as those of the Ca^{2+}-independent protein kinase Cs, that lack acidic residues in the jaws (*13*, *25*−*27*). These C2 domains are not known to bind Ca^{2+}, but some of them bind phospholipids in a Ca^{2+}-independent manner (*25*−*27*). Phospholipid binding may occur at the distal portions of the jaws in some Ca^{2+}-independent C2 domains (*25*, *33*). To remove the potential phospholipid binding site, and to avoid unintentionally creating a Ca^{2+}-independent phospholipid binding site, the entire jaws were removed. Residues 644 through 652 were replaced by three glycines and residues 707 through 713 were replaced by two glycines (Figure 2). To minimize disruption to the C2 domain β sandwich scaffold, Asp 653 and Asp 706 were mutated to alanine and serine, respectively, rather than deleted. The Ca^{2+} ligand Asn 677 and the proposed Ca^{2+} ligand Asp 714 were not altered in PLC-$\delta_{\Delta1-134,\Delta Jaws}$, but we were able nevertheless to confirm that Ca^{2+} binding to the C2 domain had been eliminated. The lengths of the polyglycine linkers used to replace the jaws were the shortest possible needed to connect the β strands without disrupting their Cα conformations and were determined using molecular graphics (*40*).

Both mutants were expressed in *E. coli* as soluble proteins and purified at high yield by the same procedure as wild-type. PLC-$\delta_{\Delta1-134}$, PLC-$\delta_{\Delta1-134,\Delta Jaws}$, and PLC-$\delta_{\Delta1-134,H311A}$ were all therefore presumed to be normally folded. All proteins eluted as single sharp peaks from a Superdex 200 HR gel filtration column at an elution volume corresponding to 70 kDa, consistent with monomeric protein. In SDS−PAGE, all deletion mutants ran at their expected molecular weights and appeared to be homogeneous. Analysis by mass spectrometry demonstrated that both PLC-$\delta_{\Delta1-134}$ and PLC-$\delta_{\Delta1-134,\Delta Jaws}$ were within 0.2% of their calculated molecular weights of 70 345 and 68 877, respectively.

Gel Filtration of PLC. In the absence of vesicles, PLC-$\delta_{\Delta1-134,H311A}$ eluted at a volume corresponding to a molecular mass of approximately 70 kDa, consistent with previous gel filtration (*41*) and dynamic light scattering results (*35*). The elution of phospholipid vesicles at the void volume was apparent by a small UV absorption peak and confirmed by assay for phosphate. Chromatograms of gel filtration of mixtures of PLC-$\delta_{\Delta1-134,H311A}$ and vesicles consisting of only PC or PC containing 20% mole fraction PIP_2 in the presence

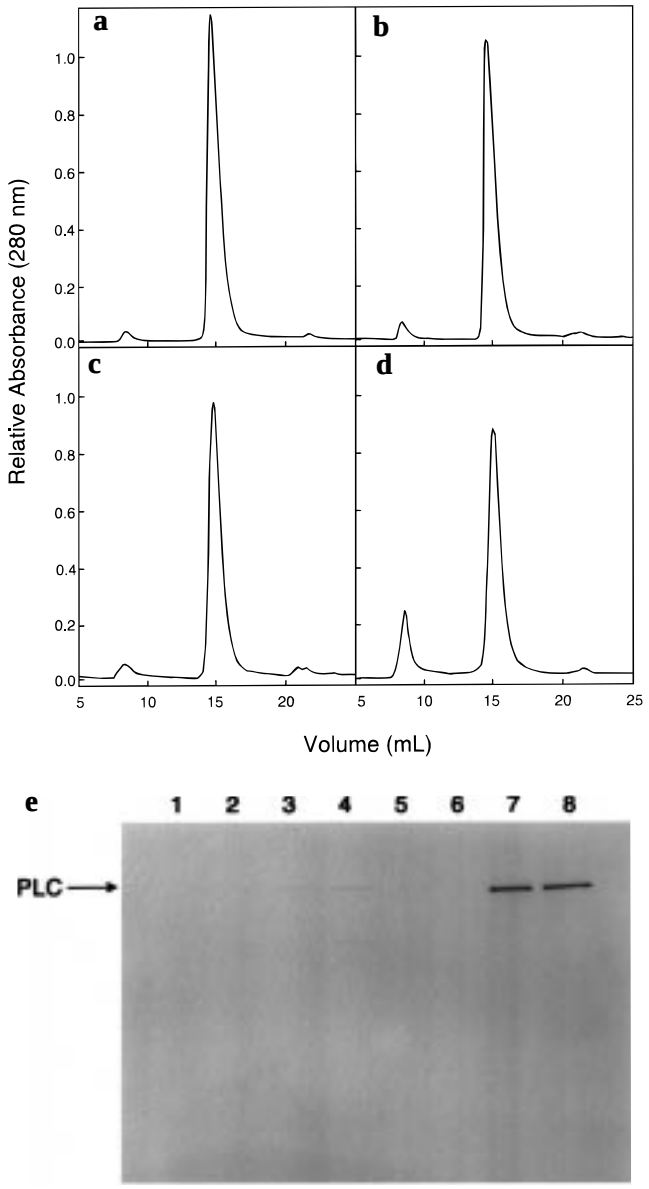

FIGURE 3: PLC binding to phospholipid vesicles. Chromatograms of gel filtration of PLC-$\delta_{\Delta1-134,H311A}$ mixed with PC vesicles (200 nmol of PC) or PC/PIP_2 vesicles (200 nmol of PC/50 nmol of PIP_2) in 50 mM MOPS, pH 6.8, 100 mM NaCl, and either 1.0 mM EDTA (MSE) or 0.5 mM $CaCl_2$ (MSC). Shown are results for gel filtration with (a) PC vesicles in MSE, (b) PC vesicles in MSC, (c) PC/PIP_2 vesicles in MSE, and (d) PC/PIP_2 vesicles in MSC. SDS−PAGE of the void volume fractions from each experiment is shown in (e). Shown are void volume fractions from gel filtration of PLC-$\delta_{\Delta1-134,H311A}$ with PC vesicles in MSE in lanes 1 and 2, with PC, vesicles in MSC in lanes 3 and 4, with PC/PIP_2 vesicles in MSE in lanes 5 and 6, and with PC/PIP_2 vesicles in MSC in lanes 7 and 8.

of either 1.0 mM EDTA or 0.5 mM Ca^{2+} are shown in Figure 3a-d. PLC-$\delta_{\Delta1-134,H311A}$ did not bind to vesicles consisting only of PC either in the absence or in the presence of 1 mM Ca^{2+}. The protein did bind to vesicles containing PIP_2 at a mole fraction of 20% and an initial bulk concentration of 0.2 mM in the presence of 0.5 mM Ca^{2+}, but not in the presence of 1.0 mM EDTA. Protein which eluted at the void volume and which was presumed to be associated with phospholipid vesicles is shown in Figure 3e. The elution of the protein at the void volume represents Ca^{2+}-dependent association with the vesicles and not Ca^{2+}-induced protein aggregation, as essentially no protein eluted at the void volume when PLC-$\delta_{\Delta1-134,H311A}$ was run in the presence of Ca^{2+} but in the absence of vesicles.

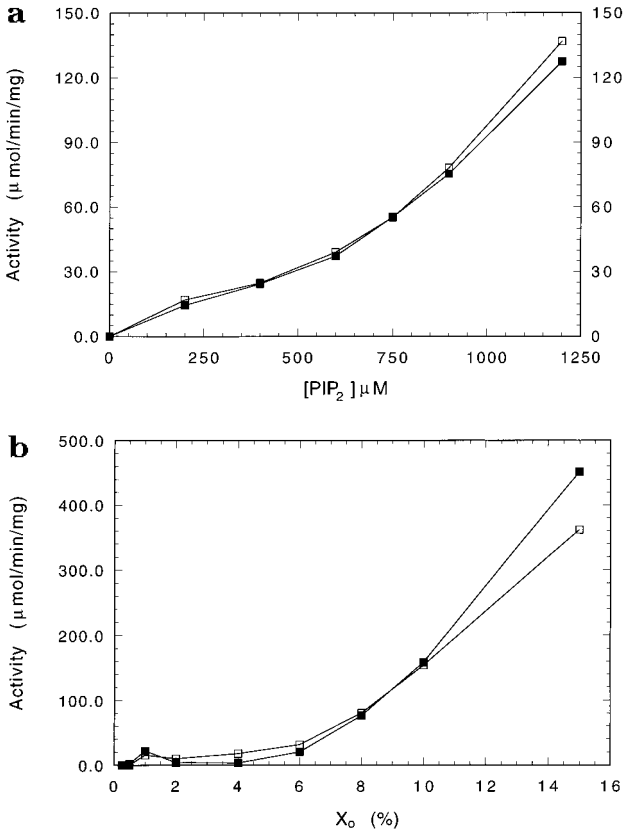

FIGURE 4: Activities of PLC-$\delta_{\Delta1-134}$ and PLC-$\delta_{\Delta1-134,\Delta Jaws}$. Wild-type (filled boxes) and mutant (open boxes) activities (a) as a function of PIP_2 bulk concentration with a mole fraction of 0.05 in a background of POPC; and (b) as a function of the mole fraction (X_o) of PIP_2 with the bulk concentration of PIP_2 fixed at 200 μM.

Kinetics of PIP_2 Hydrolysis. Neither PLC-$\delta_{\Delta1-134}$ nor PLC-$\delta_{\Delta1-134,\Delta Jaws}$ showed significant cooperativity as a function of PIP_2 mole fraction in micelles (not shown), as found previously (*35*, *41*). Their activities with respect to hydrolysis of micellar substrates were essentially identical. PLC-$\delta_{\Delta1-134,\Delta Jaws}$ was essentially identical in activity with wild-type when assayed as a function of bulk PIP_2 concentration at a 5% mole fraction in PC/PIP_2 large unilamellar vesicles (Figure 4a). We found that it was not possible to saturate the activity of PLC $\delta_{\Delta1-134}$ with increasing bulk concentration of PIP_2 at concentrations under which the LUVs were stable, and we were therefore unable to calculate the three kinetic parameters of the surface dilution model. Comparisons between mutant and wild-type are based on direct comparison of the raw data. PLC-$\delta_{\Delta1-134,\Delta Jaws}$ was also indistinguishable from wild-type when the mole fraction of PIP_2 was varied in a background of POPC (Figure 4b) at constant [PIP_2].

The activity of PLC-$\delta_{\Delta1-134,\Delta Jaws}$ was determined at a fixed bulk concentration and mole fraction of PIP_2 but in the presence of varying amounts of buffered Ca^{2+}. The activity of PLC-$\delta_{\Delta1-134,\Delta Jaws}$ was nearly identical to that of PLC-$\delta_{\Delta1-134}$ over the entire range of Ca^{2+} concentrations assessed (Figure 5). Data were fitted to eq 2, and Hill coefficients of Ca^{2+} binding to PLC-$\delta_{\Delta1-134}$ and to PLC-$\delta_{\Delta1-134,\Delta Jaws}$ were 1.12 and 1.16, respectively. Activities of both PLC-$\delta_{\Delta1-134}$ and PLC-$\delta_{\Delta1-134,\Delta Jaws}$ were saturated at 10 μM Ca^{2+} and at

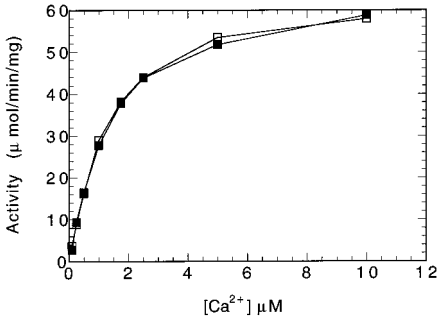

FIGURE 5: Ca^{2+} requirements of PLC-$\delta_{\Delta1-134}$ and PLC-$\delta_{\Delta1-134,\Delta Jaws}$. Activities were measured with $[PIP_2]$ fixed at 100 μM and a fixed mole fraction of 5% in a background of PC. $[Ca^{2+}]_{free}$ was varied by adjusting $[Ca^{2+}]_{total}$ in the reaction mix which contained 5 mM EDTA (*38*). Activity of PLC-$\delta_{\Delta1-134}$ is shown in filled squares and PLC-$\delta_{\Delta1-134,\Delta Jaws}$ in open squares.

Table 1: Activation of PLC-$\delta_{\Delta1-134}$ and PLC-$\delta_{\Delta1-134,\Delta Jaws}$ by Ca^{2+} [a]

protein	n	K_{Act} (μM)	V_{max}' (μmol min^{-1} mg^{-1})
PLC $\delta_{\Delta1-134}$	1.12 ± 0.04	1.31 ± 0.06	64.0 ± 0.9
PLC $\delta_{\Delta1-134,\Delta Jaws}$	1.16 ± 0.03	1.22 ± 0.04	63.0 ± 0.7
PLC $\delta_{\Delta1-134}$ [b]		1.45 ± 0.08	67.6 ± 0.1
PLC $\delta_{\Delta1-134,\Delta Jaws}$ [b]		1.40 ± 0.09	67.4 ± 1.2

[a] Kinetic parameters were calculated by fitting to eq 2 (Materials and Methods). [b] Fixed $n = 1$ during fitting.

100 μM PIP_2 had a K_{Act} of 1.4 μM (Table 1) when $n = 1$ is assumed.

Isothermal Titration Calorimetry. We used ITC to investigate the stoichiometry of Ca^{2+} binding to PLC-$\delta_{\Delta1-134,H311A}$ and to PLC-$\delta_{\Delta1-134,\Delta Jaws}$. ITC experiments were performed by injecting Ca^{2+} into a sample chamber which contained the protein solution. To avoid overfitting, data were fit to a model in which Ca^{2+} binding sites are equivalent and noninteracting. A typical titration of Ca^{2+} binding to PLC-$\delta_{\Delta1-134,H311A}$ at 25° C is shown in Figure 6. Titration of PLC-$\delta_{\Delta1-134,H311A}$ demonstrated three binding sites for Ca^{2+}, with an average of −1.5 kcal/mol released per site (Table 2). The calculated average free energy of Ca^{2+} binding was −5.6 kcal/mol per site. Repeating the experiment at 37 °C did not significantly change the thermodynamic parameters; hence, there is little change in the heat capacity of the system. PLC-$\delta_{\Delta1-134,\Delta Jaws}$, in contrast, bound only a single Ca^{2+}. Because only a single site remained in PLC-$\delta_{\Delta1-134,\Delta Jaws}$, heat released per injection was approximately one-third of that obtained with PLC-$\delta_{\Delta1-134,H311A}$, and reliable data were only obtained at higher concentrations of protein. We were unable to detect by ITC any Ca^{2+}-dependent binding of PLC to phospholipids either by injection of protein into 10 μM PIP_2/190 μM dodecyl maltoside micelles in the presence of 350 μM Ca^{2+} or by injection of Ca^{2+} into a mixture of 47 μM protein and 910 μM PIP_2/PC SUVs (X_o = 10%).

DISCUSSION

We used ITC to confirm that all but one of the Ca^{2+} binding sites had been removed from PLC-$\delta_{1\Delta1-134,\Delta Jaws}$. Mutation of the Ca^{2+} ligand Glu341 to Gly in human PLC-δ is inactivating (*42*). PLC-$\delta_{\Delta1-134,\Delta Jaws}$ has activity with respect to substrate incorporated in micelles or LUVs that is indistinguishable from wild type in the equivalent assay

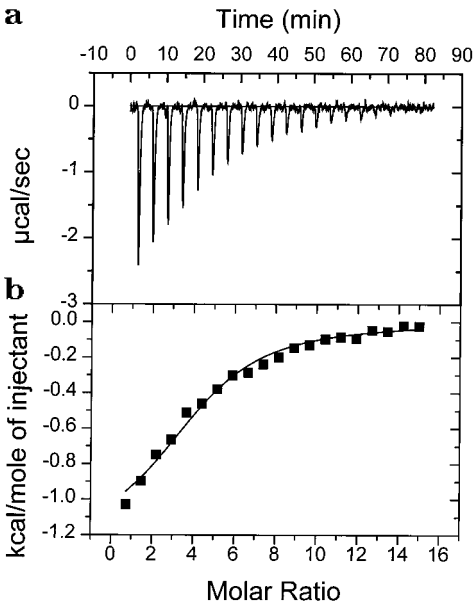

FIGURE 6: Isothermal calorimetric titration of Ca^{2+} binding to PLC-$\delta_{\Delta1-134,H311A}$. (a) Raw ITC data; (b) binding isotherm obtained for the binding of Ca^{2+} to PLC-$\delta_{\Delta1-134,H311A}$. The curve through the data is the best nonlinear fit to the data calculated with Origin (Microcal) using the equivalent noninteracting sites model.

system. Because we did not alter the catalytic Ca^{2+} binding site of PLC-$\delta_{\Delta1-134\Delta Jaws}$, we conclude that the catalytic domain site must account for the remaining Ca^{2+} binding of PLC-$\delta_{\Delta1-134\Delta Jaws}$. This is consistent with the complete abolition of Ca^{2+} binding to the C2 domain. Because Ca^{2+} is absolutely required for the activity of both PLC-δ_1 and PLC-$\delta_{\Delta1-134,\Delta Jaws}$, and PLC-$\delta_{\Delta1-134,\Delta Jaws}$ has normal activity, we conclude that Ca^{2+} binding to the single site on the catalytic domain is both necessary and sufficient for catalytic activity. We also conclude that the C2 domain Ca^{2+} binding site plays no role in catalytic activity with respect to a simple, but reasonable, model membrane system consisting of PC and PIP_2.

The PH domain truncated PLC-δ_1 catalytic core displays little surface dilution kinetics with respect to micellar substrate over a wide range of substrate concentrations (*35*, *41*). The same is true with vesicular substrate at a mole fraction of PIP_2 less than 0.05. This was expected since is has been amply demonstrated that the processivity of PLC-δ_1 under physiologically reasonable concentrations (under 2%) of PIP_2 depends on the presence of the PH domain (*41*). At $X(PIP_2) > 0.05$, the catalytic velocity begins to increase with mole fraction for vesicular, but not micellar, substrate. The kinetics are essentially identical for wild-type and PLC-$\delta_{\Delta1-134,\Delta Jaws}$ for $X(PIP_2) < 0.15$. At a mole fraction of 0.15, the highest tested, PLC-$\delta_{\Delta1-134\Delta Jaws}$ is marginally less active than wild-type. The kinetics of enzymes with respect to substrates incorporated into LUVs are relatively poorly understood, in contrast to the well-characterized mixed micelle systems (*37*, *43*). It is therefore not surprising that some qualitative differences are seen between hydrolysis on micellar and vesicular substrates. For example, Scarlata et al. (*44*) showed that sphingomyelin inhibits PLC-δ_1 in LUVs, but not in micelles, consistent with a selective effect of sphingomyelin on membrane structure but not micelle structure.

Table 2: Thermodynamic Parameters of Ca^{2+} Binding to PLC

protein	temp (K)	n	K_{eq}/M ($\times 10^3$)	ΔH (cal/mol)	ΔG (cal/mol)	ΔS (cal mol^{-1} deg^{-1})
PLC-$\delta_{\Delta 1-134,H311A}$	298	4.08 ± 0.11	11.0 ± 0.8	-1272 ± 55	-5510	14.22
PLC-$\delta_{\Delta 1-134,H311A}$	298	4.05 ± 0.06	31.4 ± 1.6	-1506 ± 45	-6131	15.52
PLC-$\delta_{\Delta 1-134,H311A}$	298	3.82 ± 0.10	19.9 ± 1.2	-1516 ± 59	-5861	14.48
PLC-$\delta_{\Delta 1-134,H311A}$	310	4.24 ± 0.24	9.95 ± 1.2	-1372 ± 133	-5670	13.86
PLC-$\delta_{\Delta 1-134,\Delta Jaws}$	298	0.98 ± 0.02	22.8 ± 1.3	-1600 ± 57	-5941	14.57
PLC-$\delta_{\Delta 1-134,\Delta Jaws}$	298	1.11 ± 0.02	29.2 ± 1.6	-1371 ± 43	-6088	15.83
PLC-$\delta_{\Delta 1-134,\Delta Jaws}$	310	0.84 ± 0.03	19.6 ± 2.1	-1752 ± 116	-5852	13.23

The possibility that the phospholipid mixture in the vesicle undergoes a phase transition at high $X(PIP_2)$ cannot be excluded. Lateral domains of PIP_2 form at physiologically relevant concentrations in the presence of the basic MARCKS peptide (*45*). PLC is excluded from these domains, and its activity is inhibited. It is easy to imagine a scenario in which, at high concentrations, PIP_2 molecules cluster about the basic patches rimming the active site of PLC-δ_1 (12) to form activity-enhancing microdomains. Another possibility is that processivity is observed at high concentrations of PIP_2 due to interactions between PLC-δ_1 and the lipid surface which are formed with near-planar membranes, but not with small and highly curved micelles. These interactions are not primarily with the C2 domain jaws, however, because they are observed with nearly equal strength in both wild-type and PLC-$\delta_{\Delta 1-134,\Delta Jaws}$. We are unsure of the origin of the high $X(PIP_2)$ surface dilution kinetics of PLC-$\delta_{\Delta 1-134}$. We feel they are unlikely to contribute to enzyme processivity under physiological conditions, for which the PH domain is primarily responsible (*41*). In sum, these data clearly show that the C2 domain mutant does not significantly alter enzyme kinetics even at artificially high mole fractions of $X(PIP_2)$.

We find a Hill coefficient for activity of 1 for both the wild-type and C2 domain deletion mutants. This shows that removing Ca^{2+} binding sites from the C2 domain does not alter the Ca^{2+} dependence of PLC, and strongly suggests that binding of only a single ion is sufficient for catalytic activity. These conclusions differ from those reported by Rebecchi et al. (*46*) where approximately three ions per protein molecule were required when assaying against submicellar concentrations of 1,2-dihexanoyl-PI or dioctanoyl-PI. Cifuentes et al. (*41*) reported Hill coefficients of approximately 2 for the intact enzyme for active proteolytic fragments of PLC. To obtain the most accurate possible determination of the Hill coefficient, we measured activity with special attention to Ca^{2+} concentrations of (0.1−1.0)-K_{Act}. Only experimental observations were included in the curve fitting, and in particular the origin was omitted. The dependence of activity on Ca^{2+} concentration was linear in this regime. The data obtained here are very similar to those reported previously (*41, 46*), although the derived Hill coefficients are not. We believe the difference in Hill coefficients reflects differences in curve fitting rather than in the underlying data. The observation that the $K_{Act}(Ca^{2+})$ values for both PLC-δ_1 and PLC-$\delta_{\Delta 1-134,\Delta Jaws}$ are identical strongly suggests their Ca^{2+} activation mechanisms are identical. We conclude that the single Ca^{2+} binding site on the catalytic domain is solely responsible for the Ca^{2+} dependence of PLC-δ in vitro.

The stoichiometry of Ca^{2+} binding to C2 domains, and to PLC-δ_1 in particular, has been a source of confusion. Ca^{2+}, Ba^{2+}, and Sm^{3+} bind to PLC-δ_1 in crystals at one site on the α/β domain and at two sites on the C2 domain (*12, 21, 22*). In contrast to other metals, La^{3+} binds to three sites on the C2 domain (*22*), where the third site is structurally similar to the low-affinity Ca^{2+} binding site in the C2 domain of synaptotagmin I (*47*). The third La^{3+} site may be a marker for a functional Ca^{2+} site which might not have bound Ca^{2+} crystallographically due to packing constraints, the presence of salt and slightly acidic pH in the crystal, or the absence of phospholipid. On the other hand, the weak binding of La^{3+} need not represent any biological function. Calorimetric titration of PLC-$\delta_{\Delta 1-134}$ reveals binding of a total of four Ca^{2+} ions. Three of these sites are removed by the deletion of the C2 domain jaws. We used atomic absorption spectroscopy to verify that our PLC-δ_1 preparations were devoid of residual Ca^{2+} prior to ITC and to verify the Ca^{2+} concentration of solutions used to prepare titrant. This argues against the possibility that additional Ca^{2+} binding sites were unobserved because they were already occupied. The calorimetric data are most consistent with binding of three Ca^{2+} ions to the PLC-δ_1 C2 domain (*22*), one to the catalytic domain, and none to the EF-hands.

We and others (*41, 42, 46*) have observed that the K_{Act} for Ca^{2+} activation of wild-type and C2 domain mutants of PLC-δ_1 is 1.40−1.45 μM with activity being saturated at 10 μM. From ITC analysis of PLC-$\delta_{\Delta 1-134\Delta Jaws}$, the calculated dissociation constant of Ca^{2+} from the active site of PLC-δ1 in the absence of phospholipid is 30−50 μM, more than 20 times weaker than expected from the kinetics. The difference between the Ca^{2+} activation constant and the binding constant in the absence of phospholipid we observe for the PLC-δ_1 catalytic domain is analogous to observations on C2 domains of other proteins. Dissociation constants of Ca^{2+} binding to the C2 domain of synaptotagmin I of 40 and 600 μM were determined by NMR titration (*47*). The dissociation constants for binding of Ca^{2+} to the synaptotagmin C2 domain in the absence of phospholipids are significantly higher than the Ca^{2+} concentrations required for association of synaptotagmin with lipid vesicles (*30*). A similar disparity has been documented for the C2 domain of cytosolic phospholipase A_2 and rationalized in terms of a model of reciprocally synergistic binding between Ca^{2+} and phospholipid (*48*). Our observations are in good agreement with the finding the Ca^{2+} forms a bridge between the PIP_2 headgroup and the catalytic domain in the crystal structure of PLC-δ_1 (*12*).

A model in which the Ca^{2+} binding site is created at the lipid/protein interface has been proposed for the C2 domains of protein kinase C (*49*−*52*), $cPLA_2$ (*48*), and PLC-δ_1 (*21*). Ca^{2+} bridging has been directly demonstrated for the catalytic domain of PLC-δ_1 (*12*) and for the unrelated Ca^{2+} and phospholipid binding protein annexin V (*53*). To test

whether Ca^{2+} enhanced the affinity of PLC-δ_1 for PIP_2-containing membranes, it was necessary to prepare a catalytically inactive PLC-δ_1 variant to avoid hydrolyzing the membrane in the course of the binding measurement. For this reason, gel filtration and ITC measurements were carried out on PLC-$\delta_{\Delta1-134,H311A}$, which is catalytically inactive (*34*). Binding could be detected by gel filtration in the presence of 0.2 mM PIP_2 at injection and 0.5 mM Ca^{2+}, but not by ITC at a somewhat lower amount of Ca^{2+} and lipid. PLC-$\delta_{\Delta1-134,H311A}$ bound weakly to LUVs composed of PC/PIP_2 (5:1) in the presence but not the absence of Ca^{2+} as measured by the semiquantitative gel filtration assay. This line of investigation was not pursued further. The stimulation of vesicle binding by Ca^{2+} is consistent with synergistic binding of Ca^{2+} and phospholipid binding to PLC-δ_1, although it has not been measured quantitatively enough for us to account for the difference between K_d and K_{Act} for Ca^{2+}. We have not directly determined whether the Ca^{2+}-dependent vesicle binding is mediated by the catalytic domain or the C2 domain. An interaction with the catalytic domain would most readily explain why $K_d > K_{Act}$.

The data which we present here rule out a catalytically productive interaction between the PLC-δ_1 C2 domain jaws and a simple, but reasonable, model membrane composed of PC and PIP_2, at least with respect to unstimulated activity. One possibility is that phospholipids bind to a region of the C2 domain outside of the jaws. This seems unlikely, because most Ca^{2+} binding C2 domains that bind phospholipid do so in a Ca^{2+}-dependent manner, and the Ca^{2+} binding sites have been eliminated in the present study. Further, in studies of the C2 domain of the Ca^{2+}-dependent protein kinase C-βII, Newton and co-workers have ruled out the most likely alternative phospholipid binding site, a basic patch on the protein surface remote from the jaws (*54*). The same workers have demonstrated a role for the jaws in Ca^{2+}-dependent binding and kinase activation by phosphatidylserine (*31*).

Our observations are consistent with recent cross-linking studies by Tall et al. (*55*) that demonstrated PIP_2 analogue binding to the PLC-δ_1 PH domain and to the active site, but not to any other sites, including the C2 domain. Several possibilities for PLC-δ_1 C2 domain function remain. The C2 domain could interact with a phospholipid other than PC or PIP_2. The C2 domain could interact with a protein, as seen for the C2B domain of synaptotagmin (*56*). The C2 domain may be evolutionary detritus which could have anchored an ancestral PLC, perhaps one lacking a PH domain, to membranes, but serves no contemporary purpose other than that of structural stability. A peptide from Gh7 which is capable of binding to and activating PLC-δ_1 has recently been identified (*10*). The binding of this peptide is inhibited by EGTA, suggesting that it is a Ca^{2+}-dependent event. Interaction between PLC-δ and Gh7 might therefore be mediated by the C2 domain, whose ability to interact with membranes might in turn be enhanced. It remains possible that the presence of the PH domain could also potentiate C2 domain/membrane interactions. Lastly, the noncatalytic domains of PLC-δ_1 could function as adaptors in signal transduction rather than having a role in regulating phospholipase catalytic activity, as seen for protein kinase C activation of phospholipase D (*57*).

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