

Essential Role of the EF-hand Domain in Targeting Sperm Phospholipase C ζ to Membrane Phosphatidylinositol 4,5-Bisphosphate (PIP $_2$)*

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Background: The mechanism underlying sperm PLC ζ interaction with its target membrane is unresolved.

Results: EF-hand mutations introduced into PLC ζ reduce *in vivo* Ca²⁺ oscillation inducing activity and *in vitro* interaction with PIP $_2$.

Conclusion: EF-hand domain is essential for targeting PLC ζ to PIP $_2$ -containing membranes.

Significance: We propose a novel mechanism by which sperm PLC ζ is anchored to its physiological membrane substrate.

Sperm-specific phospholipase C- ζ (PLC ζ) is widely considered to be the physiological stimulus that triggers intracellular Ca²⁺ oscillations and egg activation during mammalian fertilization. Although PLC ζ is structurally similar to PLC δ 1, it lacks a pleckstrin homology domain, and it remains unclear how PLC ζ targets its phosphatidylinositol 4,5-bisphosphate (PIP $_2$) membrane substrate. Recently, the PLC δ 1 EF-hand domain was shown to bind to anionic phospholipids through a number of cationic residues, suggesting a potential mechanism for how PLCs might interact with their target membranes. Those critical cationic EF-hand residues in PLC δ 1 are notably conserved in PLC ζ . We investigated the potential role of these conserved cationic residues in PLC ζ by generating a series of mutants that sequentially neutralized three positively charged residues (Lys-49, Lys-53, and Arg-57) within the mouse PLC ζ EF-hand domain. Microinjection of the PLC ζ EF-hand mutants into mouse eggs enabled their Ca²⁺ oscillation inducing activities to be compared with wild-type PLC ζ . Furthermore, the mutant proteins were purified, and the *in vitro* PIP $_2$ hydrolysis and binding properties were monitored. Our analysis suggests that PLC ζ binds significantly to PIP $_2$, but not to phosphatidic acid or phosphatidylserine, and that sequential reduction of the net positive charge within the first EF-hand domain of PLC ζ significantly alters *in vivo* Ca²⁺ oscillation inducing activity and *in vitro* interaction with PIP $_2$ without affecting its Ca²⁺ sensitivity. Our findings are consistent with theoretical predictions provided by a mathematical model that links oocyte Ca²⁺ frequency and the binding ability of different PLC ζ mutants to PIP $_2$. Moreover, a PLC ζ mutant with mutations in the cationic residues within the first EF-hand domain

and the XY linker region dramatically reduces the binding of PLC ζ to PIP $_2$, leading to complete abolishment of its Ca²⁺ oscillation inducing activity.

During fertilization, the spermatozoon initiates activation of egg development by triggering an acute rise in cytosolic free Ca²⁺ concentration (1). In mammals, this manifests as a series of distinctive cytosolic Ca²⁺ oscillations, beginning soon after sperm-egg fusion and persisting for several hours (2). The weight of evidence now suggests that Ca²⁺ oscillations appear to be caused by a sperm-specific protein, phospholipase C- ζ (PLC ζ),³ which is introduced into the egg upon sperm-egg fusion and leads to cycles of inositol 1,4,5-trisphosphate (IP $_3$) production following PIP $_2$ hydrolysis, thus activating IP $_3$ receptor-mediated Ca²⁺ release from intracellular stores in the egg (3–12). The closest PLC homologue of sperm PLC ζ is PLC δ 1 (47% similarity, 33% identity), which is only able to cause Ca²⁺ oscillations in mouse eggs at non-physiological concentrations, because it has a >50-fold lower potency (2, 3, 12). The superior fertilization potency of the sperm PLC ζ over somatic PLCs has not yet been fully explained.

PLC ζ is the smallest PLC with the simplest domain organization among all the mammalian isoforms. PLC ζ consists of four tandem EF-hand domains, the characteristic X and Y catalytic domains in the center of the molecule, and a C-terminal C2 domain. All these domains are common to the other PLC isoforms (β , γ , δ , ϵ , and η), but they appear to individually have an essential role in the unique mode of regulation of this distinctive PLC isozyme (2). A notable structural difference between PLC ζ and the other somatic PLC isoforms is that PLC ζ lacks a pleckstrin homology (PH) domain at the N terminus (2, 3, 13). The membrane binding of somatic PLCs appears to be

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³ The abbreviations used are: PLC ζ , phospholipase C- ζ ; PIP $_2$, phosphatidylinositol 4,5-bisphosphate; IP $_3$, inositol 1,4,5-trisphosphate; PH, pleckstrin homology; PtdCho, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; CHOL, cholesterol; PtdEtn, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; PS, phosphatidylserine; PA, phosphatidic acid.

PIP₂ Binding by PLC- ζ Involves EF-hand Domain

mediated by the PH domain, a well defined structural module of ~120-amino acid residues identified in numerous proteins (14). The PH domain of PLC δ 1 is essential for interaction with its phospholipid substrate PIP₂ in the plasma membrane (15). The absence of a PH domain from PLC ζ sequence raises questions about how PLC ζ can bind to membranes.

We have previously proposed that the PLC ζ XY-linker, a segment between the X and Y catalytic domains that is notably different from the corresponding XY-linker region of somatic PLCs, is involved in the targeting of PLC ζ to its membrane-bound substrate PIP₂ (16, 17). The XY-linker region of PLC ζ is extended in length and consists of more basic residues relative to its PLC δ 1 counterpart. The affinity of the XY-linker for PIP₂ appears to involve a polybasic charged region that is found in a number of other membrane-associated proteins (16, 18). These positively charged amino acids in the XY-linker appear to assist the anchoring of PLC ζ to membranes by enhancing the local PIP₂ concentration adjacent to the XY catalytic domain via electrostatic interactions with the negatively charged PIP₂ (16, 17). However, the XY-linker might not be the only domain that mediates the binding of PLC ζ to PIP₂-containing membranes. We have demonstrated that the absence of the XY-linker from PLC ζ significantly diminishes, but does not completely abolish, the *in vivo* Ca²⁺ oscillation inducing activity (19). This suggests that other domain(s) may also be involved in anchoring PLC ζ to its target membrane.

A recent study reported that the N-terminal lobe of the EF-hand domain of PLC δ 1 binds anionic phospholipids, and this binding is due to interactions with cationic and hydrophobic residues in the first EF-hand sequence of PLC δ 1 (20). The authors propose a general mechanism that may apply to other PLC isoforms by suggesting that EF-hand domain interactions with anionic phospholipids in the target membrane provides a tether that facilitates proper substrate access and binding in the active site (20). Importantly, the cationic residues in the first EF-hand domain of PLC δ 1 that contribute to anionic lipid vesicle binding are all conserved in PLC ζ .

The aim of this study is to investigate the potential importance of a conserved cluster of cationic residues at the N-terminal lobe of the EF-hand domain of PLC ζ in association with anionic lipids and its substrate PIP₂. A series of full-length mouse PLC ζ mutants were prepared that sequentially neutralized two positively charged lysine and one arginine residues within the first EF-hand domain. The Ca²⁺ oscillation-inducing properties of these mutants were experimentally tested relative to wild-type PLC ζ by microinjection of cRNA into unfertilized mouse eggs. The various PLC ζ mutants' enzymatic properties were analyzed using an *in vitro* PIP₂ hydrolysis assay. A protein-lipid overlay and a liposome binding/enzyme assay were employed to assess the binding properties of wild-type PLC ζ to phosphatidylserine (PS), phosphatidic acid (PA), and PIP₂. Furthermore, the binding properties of mutant EF-hand PLC ζ proteins to PIP₂ were examined. Our results suggest that PLC ζ possesses significant affinity only for PIP₂ but not for PA or PS. We also find that sequential reduction of the net positive charge within the first EF-hand domain significantly reduces both *in vivo* Ca²⁺ oscillation inducing activity and the *in vitro* interaction of PLC ζ with PIP₂. Moreover, we show that

a PLC ζ mutant where three cationic residues within the first EF-hand domain and three cationic residues within the XY-linker region of PLC ζ were substituted by alanine is unable to trigger Ca²⁺ oscillations in mouse eggs. *In vitro* biochemical characterization suggests that this PLC ζ mutant displays dramatically reduced binding to PIP₂-containing liposomes compared with the wild-type PLC ζ . Thus, we propose a novel mechanism for the sperm PLC ζ interaction with PIP₂-containing membranes mediated by electrostatic interactions between the anionic PIP₂ with both the first EF-hand domain and the XY-linker region of PLC ζ , which are rich in cationic residues.

Experimental Procedures

Plasmid Construction—A pCR3-mouse PLC ζ -luciferase (PLC ζ -luc) construct (21) was subjected to site-directed mutagenesis (QuikChange II, Stratagene) to sequentially generate the three single, one double, and one triple substitutions at Lys-49, Lys-53, and Arg-57, thus producing the PLC ζ ^{K49A}, PLC ζ ^{K53A}, PLC ζ ^{R57A}, PLC ζ ^{K49A,R57A}, and PLC ζ ^{K49A,K53A,R57A} mutants.

pCR3-PLC ζ ^{K49A,K53A,R57A,K374A,K375A,K377A}-luc construct was generated by a three-step cloning strategy. PLC ζ EF^{K49A,K53A,R57A} (1–149 amino acids) was amplified from the PLC ζ ^{K49A,K53A,R57A}-luc plasmid by PCR with primers to incorporate a 5-KpnI site and a 3-EcoRI site and then cloned into the pCR3 vector. PLC ζ Δ EF^{K374A,K375A,K377A} (150–647 amino acids) was then amplified from the PLC ζ ^{K374A,K375A,K377A}-luc plasmid (17) with primers to incorporate a 5-EcoRI site and a 3-NotI site in which the stop codon had been removed and cloned into the pCR3-PLC ζ EF^{K49A,K53A,R57A} plasmid. Finally, luciferase was amplified from pGL2 with primers incorporating NotI sites, and the product was cloned into the NotI site of the pCR3-PLC ζ ^{K49A,K53A,R57A,K374A,K375A,K377A} plasmid.

All the above PLC ζ mutants were amplified from their corresponding pCR3 plasmid with the appropriate primers to incorporate a 5-SalI site and a 3-NotI site, and the products were cloned into the pETMM60 vector to enable bacterial protein expression. The primers used for the amplifications were as follows: 5'-GAACGTCGACATGGAAAGCCAACTTCATGAGCTCGC-3' (forward) and 5'-GGAAGCGGCCGCTCACTCTCTGAAGTACCAAAC-3' (reverse). Successful mutagenesis and cloning of the above expression vector constructs were confirmed by dideoxynucleotide sequencing (Applied Biosystems Big-Dye Version 3.1 chemistry and model 3730 automated capillary DNA sequencer by DNA Sequencing & ServicesTM).

cRNA Synthesis—Following linearization of wild-type and mutated PLC ζ plasmids, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and then was polyadenylated using the poly(A) tailing kit (Ambion), as per the manufacturer's instructions.

Preparation and Handling of Gametes—Female mice were super-ovulated and mature MII eggs were collected from excised oviducts 13.5–14.5 h after injection of human chorionic gonadotrophin and maintained in droplets of M2 media (Sigma) under mineral oil at 37 °C. Experimental recordings of Ca²⁺ release or luciferase expression were carried out with

mouse eggs in Hepes-buffered media (H-KSOM), as described previously (22). All compounds were from Sigma unless stated otherwise. All procedures using animals were performed in accordance with the United Kingdom Home Office Animals Procedures Act and were approved by the Cardiff University Animals Ethics Committee.

Microinjection and Measurement of Intracellular Ca²⁺ and Luciferase Expression—Mouse eggs were washed in M2 and microinjected with cRNA diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4). The volume injected was estimated from the diameter of cytoplasmic displacement caused by the bolus injection. All injections were 3–5% of the oocyte volume. Eggs were microinjected with the appropriate cRNA in the injection buffer, mixed with an equal volume of 1 mM Oregon Green 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-dextran (Life Technologies, Inc.). Eggs were then maintained in H-KSOM containing 100 μ M luciferin and imaged on a Nikon TE2000 microscope equipped with a cooled intensified CCD camera (Photek Ltd., UK). The luminescence (luciferase expression) and fluorescence (for Ca²⁺ measurements) from eggs were collected by switching back and forth between the two modes on a 10-s cycle (23, 24). These two signals were then displayed as two separate signals over the same time period for each egg. The fluorescent light used to measure Ca²⁺ is shown in relative units. Luminescence was recorded as photon counts/s and plotted as a running average over 5 min. All live imaging experiments on eggs were made during a 1-month period.

Protein Expression and Purification—For NusA-His₆-fusion protein expression, *Escherichia coli* (BL21-CodonPlus(DE3)-RILP; Stratagene) cells were transformed with the appropriate pETMM60 plasmid and cultured at 37 °C until the A₆₀₀ reached 0.6, and protein expression was induced for 18 h at 16 °C with 0.1 mM isopropyl 1-thio- β -D-galactopyranoside (ForMedium). Cells were harvested (6000 \times g for 10 min), resuspended in PBS containing a protease inhibitor mixture (EDTA-free; Roche Applied Science), and sonicated four times for 15 s on ice. Soluble NusA-His₆-tagged fusion protein was purified on nickel-nitrilotriacetic acid resin following standard procedures (Qiagen) and eluted with 250 mM imidazole. Eluted proteins were dialyzed overnight (10,000 molecular weight cutoff; Pierce) at 4 °C against 4 liters of PBS and concentrated with centrifugal concentrators (Sartorius; 10,000 molecular weight cutoff).

Assay of PLC Activity—PIP₂ hydrolytic activity of recombinant PLC ζ proteins was assayed as described previously (17, 21). The final concentration of PIP₂ in the reaction mixture was 220 μ M, containing 0.05 μ Ci of [³H]PIP₂. The assay conditions were optimized for linearity, requiring a 1-min incubation of 200 pmol of PLC ζ protein sample at 25 °C. In assays to determine dependence on PIP₂ concentration, 0.05 μ Ci of [³H]PIP₂ was mixed with cold PIP₂ to give the appropriate final concentration. In assays examining Ca²⁺ sensitivity, Ca²⁺ buffers were prepared by EGTA/CaCl₂ admixture, as described previously (17, 21).

Protein Lipid Overlay Assay—PIP array membranes (Echelon Biosciences) were blocked for 2 h with binding buffer (TBS-T: 20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.4) containing 3% bovine serum albumin (lipid-free) and incubated with 25

pmol of each NusA-PLC ζ fusion protein for 1 h at room temperature. After washing three times in TBS-T, NusA-PLC ζ fusion protein interaction with the inositol phosphate lipids was detected by first incubating the PIP array membranes with penta-His monoclonal antibody (Qiagen, 1:5000 dilution in 5 ml of binding buffer) overnight at 4 °C, followed by three 15-min washes. This was followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody in the same binding buffer for 1 h at room temperature, followed by three 15-min washes with TBS-T. Detection of horseradish peroxidase-coupled secondary antibody was achieved using enhanced chemiluminescence detection (ECL; Amersham Biosciences).

Liposome Preparation and Binding Assay—Unilamellar liposomes were prepared as described previously (17, 25) by the extrusion method using a laboratory extruder (LiposoFast-Pneumatic, Avestin Inc., Ottawa, Ontario, Canada) with lipids purchased from Avanti Polar Lipids Inc. (Alabaster, AL). In a typical experiment for preparing a 2-ml dispersion of liposomes, 0.038 mmol (19 \times 10⁻³ M) of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PtdCho), 0.019 mmol (9.5 \times 10⁻³ M) of cholesterol (CHOL; molar ratio of PtdCho/CHOL, 2:1), 0.0095 mmol (4.8 \times 10⁻³ M) of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (PtdEtn; molar ratio of PtdCho/PtdEtn, 4:1), and 5% 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (PS) or 1–5% 1,2-diacyl-*sn*-glycero-3-phosphate sodium salt (PA) or 1% of 1,2-diacyl-*sn*-glycero-3-phospho-(1-D-myoinositol 4,5-bisphosphate) sodium salt (PIP₂) were dissolved in a chloroform/methanol solution (2:1 v/v) for the formation of lipid films. The film was hydrated with 2 ml of PBS, and the resultant suspension was extruded through two stacked polycarbonate filters of 100-nm pore size. Twenty five cycles of extrusion were applied at 50 °C. Dynamic light scattering was employed to determine the size of the liposomes, which used a light scattering apparatus (AXIOS-150/EX, Triton Hellas, Thessaloniki, Greece) with a 30-milliwatt laser source and an Avalanche photodiode detector set at a 90° angle. Dynamic light scattering measurements of the extruded lipid preparation showed a narrow monomodal size distribution with average liposome diameter of 100 nm and a polydispersity index of 0.20–0.25. For protein binding studies, liposomes (100 μ g) were incubated with 1 μ g of recombinant protein for 30 min at room temperature and centrifuged for 5 h at 4 °C. The supernatant and pellet were then analyzed either by SDS-PAGE and Coomassie Blue staining or by the [³H]PIP₂ hydrolysis assay described above.

SDS-PAGE and Western Blotting—Recombinant proteins were separated by SDS-PAGE as described previously (25, 26). Separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) using a semi-dry transfer system (Trans-Blot S.D.; Bio-Rad) in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% v/v methanol) at 20 V for 1 h. Membranes were incubated overnight at 4 °C in Tris-buffered saline, 0.1% Tween 20 (TBS-T) containing 5% nonfat milk powder, and probed with a penta-His mouse monoclonal antibody (Novagen, 1:100,000 dilution). Detection of horseradish peroxidase-coupled secondary antibody was achieved using enhanced chemiluminescence detection (ECL; Amersham Biosciences).

PIP₂ Binding by PLC- ζ Involves EF-hand Domain

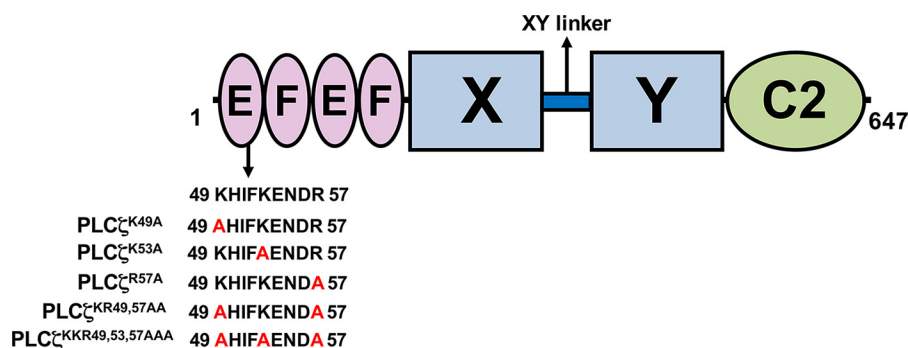


FIGURE 1. **Generation of PLC ζ EF-hand mutants.** Schematic representation of the domain structure of mouse PLC ζ identifying the location of the successive Lys or Arg residue substitutions to Ala, between residues 49 and 57 within the first EF-hand domain that are prepared by site-directed mutagenesis for this study.

Mathematical Modeling of Oocyte Ca²⁺ Dynamics—Theoretical predictions of the oscillatory Ca²⁺ activity associated with the various PLC ζ constructs were provided by a mathematical model of oocyte IP₃/Ca²⁺ dynamics. The mathematical model, which has previously been presented in detail (25), employs three inter-dependent variables, namely free cytosolic Ca²⁺, Ca²⁺ sequestered in the endoplasmic reticulum, and intracellular concentrations of IP₃. To account for the specific binding activity of each PLC ζ variant, the effective activity of a PLC ζ concentration is defined as: $V_{\text{PLC}}^e = V_{\text{PLC}} \cdot b$, where V_{PLC} is the nominal PLC ζ concentration, and b is the binding activity estimated experimentally for each construct. Coefficient b assumes a value between 0 and 1, whereas V_{PLC} can assume values beyond the physiological range when the protein is over-expressed. The mathematical model was coded and numerically integrated on both C++ and a MATLAB platform (MathWorks).

Results

Effect of EF-hand Mutations on PLC ζ -mediated Ca²⁺ Oscillations in Mouse Eggs—To investigate the potential importance of a cluster of cationic residues within the first EF-hand unit of the first pair of PLC ζ EF-hand domains (Fig. 1), we performed site-directed mutagenesis to produce a panel of cumulative mutations within this positively charged region of the full-length mouse PLC ζ . Thus, the residues Lys-49, Lys-53, and Arg-57 were sequentially substituted by the neutral amino acid, alanine, to create three single (PLC ζ ^{K49A}, PLC ζ ^{K53A}, and PLC ζ ^{R57A}) mutants, as well as one double (PLC ζ ^{K53A,K57A}) and one triple (PLC ζ ^{K49A,K53A,R57A}) PLC ζ mutant. To test the Ca²⁺ oscillation inducing activity of PLC ζ ^{K49A}, PLC ζ ^{K53A}, PLC ζ ^{R57A}, PLC ζ ^{K49A,R57A}, and PLC ζ ^{K49A,K53A,R57A} mutants and to verify that these constructs were faithfully expressed as proteins in cRNA-microinjected mouse eggs, we generated C-terminal luciferase-tagged versions of these constructs to enable quantitation of relative protein expression by luminescence detection of the expressed PLC ζ -luciferase fusion protein, as described previously (17, 21). Prominent Ca²⁺ oscillations were observed in PLC ζ ^{WT}-luciferase cRNA-injected mouse eggs (9.7 spikes in the 1st h of oscillations) following successful protein expression to a level indicated by a luminescence reading of 0.47 counts/s (Fig. 2 and Table 1), in accord with previous reports (17, 21). Microinjection of cRNA encoding the three single PLC ζ

mutants (PLC ζ ^{K49A}, PLC ζ ^{K53A}, and PLC ζ ^{R57A}) also triggered Ca²⁺ oscillations (Fig. 2), but these exhibited a lower frequency relative to PLC ζ ^{WT} (3.6, 4.4, and 4.3 spikes in the 1st h, respectively), although the proteins were expressed at comparable expression levels (Table 1). Similarly, egg microinjection with cRNA encoding either the double PLC ζ ^{K49A,R57A} or the triple PLC ζ ^{K49A,K53A,R57A} mutant resulted in a significant reduction in the frequency of Ca²⁺ oscillations compared with PLC ζ ^{WT}, causing 3.7 and 2.8 spikes/1 h, respectively, again when protein was expressed at comparable levels (Fig. 2 and Table 1). These data indicate that the substitution of even one Lys or Arg residue for a neutral Ala within the positively charged cluster of the PLC ζ EF-hand domain can significantly alter their Ca²⁺ oscillation inducing activity in mouse eggs by reducing the frequency of Ca²⁺ spikes.

Overexpression of PLC ζ ^{K49A,K53A,R57A} in Mouse Eggs Rescues Its Defective Ca²⁺ Oscillation-inducing Phenotype—Judging by the number of Ca²⁺ spikes observed within the 1st h of oscillations per unit of recombinant fusion protein expression (cps), PLC ζ ^{WT} can be seen to be about ~3.5 times more effective at causing Ca²⁺ oscillations than the PLC ζ ^{K49A,K53A,R57A} triple mutant. To investigate whether we could rescue the low frequency of Ca²⁺ oscillations induced by PLC ζ ^{K49A,K53A,R57A}, we overexpressed this PLC ζ mutant in mouse eggs. As shown in Fig. 3 and Table 1, the overexpression of PLC ζ ^{K49A,K53A,R57A} (7.65 cps) indeed led to 8.6 spikes in the 1st h of oscillations, comparable with that for PLC ζ ^{WT}, suggesting that loading the egg with large amounts of this PLC ζ mutant can rescue its defective Ca²⁺ oscillation-inducing phenotype.

Expression and Enzymatic Characterization of PLC ζ EF-hand Mutants—Each of the PLC ζ ^{K49A}, PLC ζ ^{K53A}, PLC ζ ^{R57A}, PLC ζ ^{K49A,R57A}, and PLC ζ ^{K49A,K53A,R57A} mutants was subcloned into the pETMM60 vector and purified as NusA-His₆ fusion proteins by affinity chromatography. We have recently demonstrated that NusA is an effective fusion protein partner for PLC ζ , significantly increasing soluble expression of PLC ζ protein in *E. coli*, as well as enhancing the enzymatic stability of the purified protein over time (11). Following expression of NusA-PLC ζ fusion proteins in *E. coli* and purification by nickel-nitrilotriacetic acid affinity chromatography, samples of each protein were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining and immunoblotting using an

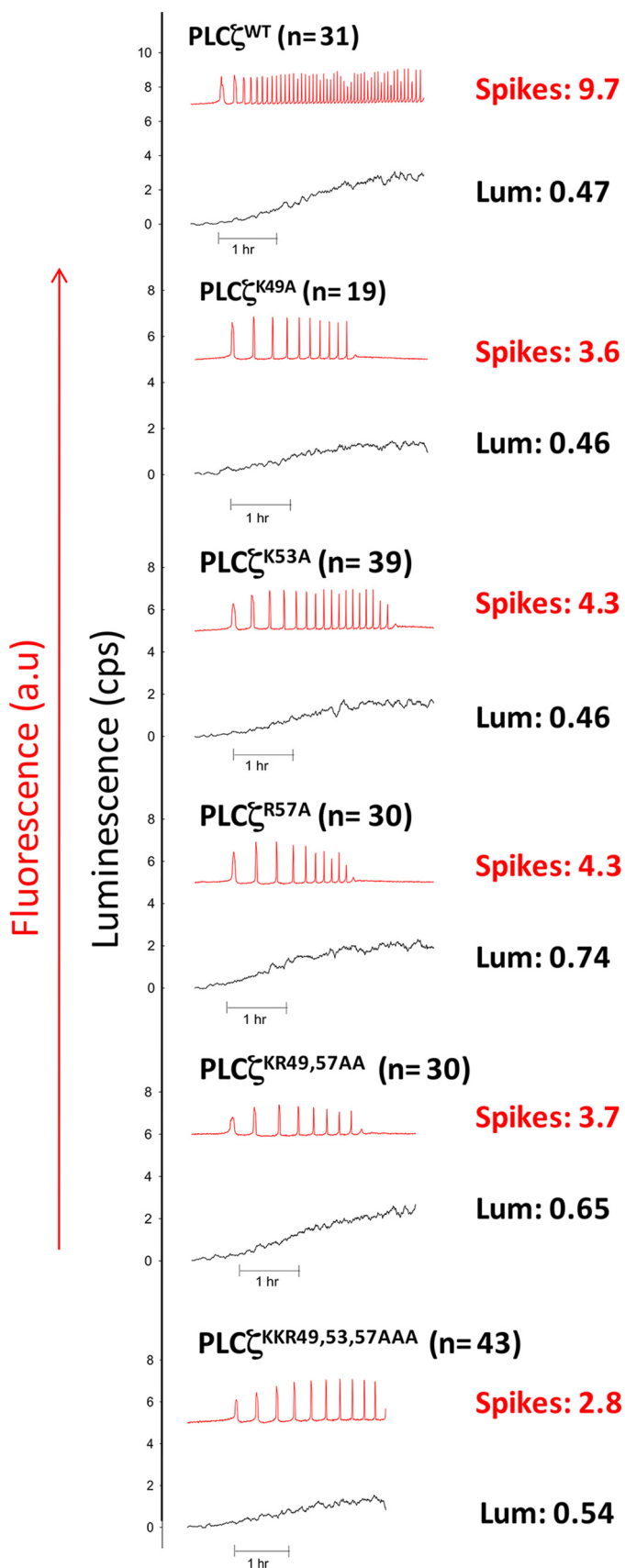


FIGURE 2. Expression of wild-type and mutant PLC ζ constructs (PLC ζ^{K49A} , PLC ζ^{K53A} , PLC ζ^{R57A} , PLC $\zeta^{\text{K53A,K57A}}$, and PLC $\zeta^{\text{K49A,K53A,R57A}}$) in unfertilized mouse eggs. Fluorescence and luminescence (*Lum*) recordings reported the Ca²⁺ changes (red traces; Ca²⁺) and luciferase expression ((black traces; lumi-

anti-NusA monoclonal antibody. Fig. 4A shows that the major protein band following affinity isolation, with mobility corresponding to the predicted molecular mass of ~134 kDa for each construct, was present for all fusion proteins analyzed (*left panel*), and these major bands were also recognized in the corresponding anti-NusA immunoblot (*right panel*), confirming the appropriate expression of all PLC ζ mutants. Some intermediate molecular mass bands detected by the anti-NusA antibody are the probable result of some degradation occurring through the various protein expression and purification procedures. Similarity of protein expression profile, including degradation products, for the various PLC ζ constructs being examined suggests that experimental comparison of relative enzymatic data may be appropriate. Hence, the specific PIP₂ hydrolytic enzyme activity for PLC ζ^{WT} and each recombinant mutant protein was determined by the standard micellar [³H]PIP₂ hydrolysis assay. The histogram of Fig. 4B and Table 2 summarize the enzyme specific activity values obtained for each recombinant protein. The enzymatic activities of all recombinant proteins was very similar, suggesting that mutating the basic residues of the first pair of EF-hands to a neutral residue has no effect on the ability of PLC ζ to hydrolyze PIP₂ *in vitro*. Moreover, to investigate the impact of the EF-hand mutations on Ca²⁺ sensitivity of PLC ζ enzyme activity, we assessed the ability of these PLC ζ recombinant proteins to hydrolyze [³H]PIP₂ at different Ca²⁺ concentrations ranging from 0.1 nM to 0.1 mM. These experiments indicated that there was no significant difference in the Ca²⁺ sensitivity of PIP₂ hydrolysis for the wild type, and the five EF-hand mutants (Fig. 4C) with a very similar EC₅₀ value (67–85 nM) displayed by all recombinant PLC ζ proteins (Table 2). To compare the enzyme kinetics of wild-type and mutant PLC ζ s, the Michaelis-Menten constant, *K_m*, was calculated for each construct (Table 2). The *K_m* values obtained were similar for human PLC ζ^{WT} (84 μ M), PLC ζ^{K49A} (121 μ M), and PLC ζ^{R57A} (115 μ M), whereas the *K_m* value for PLC ζ^{K53A} (169 μ M) and PLC $\zeta^{\text{K49A,R57A}}$ (219 μ M) mutants was ~2- and ~2.6-fold higher compared with that of PLC ζ^{WT} . Interestingly, the *K_m* value for PLC $\zeta^{\text{K49A,K53A,R57A}}$ (432 μ M) was ~5.1-fold higher compared with PLC ζ^{WT} (84 μ M), suggesting that replacement of these three positively charged residues within the first EF-hand domain affects the *in vitro* affinity of PLC ζ for PIP₂ without affecting the Ca²⁺ sensitivity of this enzyme.

Binding of PLC ζ to PS, PA, and PIP₂—To examine the ability of PLC ζ to bind the membrane lipids, PS, PA, and PIP₂, we employed three different approaches. First, we used a protein-lipid overlay assay to assess the binding of PLC ζ to membrane-spotted arrays of inositol phospholipids containing PS, PA, or PIP₂. As shown in Fig. 5A, no binding to PS or PA was evident, although PLC ζ was able to bind to membrane arrays containing PIP₂. This result is consistent with our liposome binding assays (Fig. 5B). For these binding assays, we made unilamellar liposomes composed of phosphatidylcholine/CHOL/phos-

nescence, in counts/s), respectively, in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged PLC ζ constructs. Mean number of Ca²⁺ oscillations in the 1st h of oscillating (*spikes*) and mean luminescence (*cps*) in the 1st h of oscillating (*Lum*) are shown. *a.u.*, arbitrary units.

PIP₂ Binding by PLC-ζ Involves EF-hand Domain

TABLE 1

Properties of PLC-ζ-luciferase EF-hand mutants expressed in unfertilized mouse eggs

Ca²⁺ oscillation inducing activity (number of Ca²⁺ spikes in the 1st h of oscillations) and luciferase luminescence levels (counts/s of luminescence in 1st h of oscillating) are summarized for mouse eggs microinjected with each of the PLC-ζ-luciferase mutants as follows: PLC-ζ^{K49A}, PLC-ζ^{K53A}, PLC-ζ^{R57A}, PLC-ζ^{K53A,R57A}, PLC-ζ^{K49A,K53A,R57A}, PLC-ζ^{DMM}, and wild type PLC-ζ-luciferase (see Figs. 2, 3, and 8B). The data are expressed to two significant figures, with means ± S.E. Ratios of total luminescence (counts) per spike in the 1st h of oscillating are also shown expressed to two significant figures with means ± S.E. This is not shown where the number of spikes is zero or where the expression of PLC-ζ is sufficiently high that the relationship between number of spikes and expression is no longer linear. The results of Mann-Whitney tests for significant differences between the number of spikes for each of the wild-type and mutant PLC-ζ-luciferase constructs are indicated with *p* values. The results of this same test for a significant difference between the PLC-ζ mutant ratios (counts/spike) and the wild-type PLC-ζ-luciferase ratio are also denoted in this way. NA, not applicable.

PLC-ζ cRNA	No. of eggs	Mean no. of oscillations in 1st h of spiking	Mean expression in 1st h of spiking	No. of spikes significantly different from wild type?	Mean total counts/spike in 1st h of oscillating (counts/spike)	Counts/spike significantly different from wild type?
PLC-ζ ^{WT}	31	9.7 ± 0.63	0.47 ± 0.038	NA	14.61 ± 2.05	NA
PLC-ζ ^{K49A}	19	3.6 ± 0.16	0.48 ± 0.024	Yes (<i>p</i> = <0.001)	25.92 ± 1.17	Yes (<i>p</i> = <0.001)
PLC-ζ ^{K53A}	39	4.4 ± 0.13	0.46 ± 0.014	Yes (<i>p</i> = <0.001)	20.78 ± 0.85	Yes (<i>p</i> = <0.001)
PLC-ζ ^{R57A}	30	4.3 ± 0.13	0.74 ± 0.032	Yes (<i>p</i> = <0.001)	38.21 ± 2.1	Yes (<i>p</i> = <0.001)
PLC-ζ ^{K49A,R57A}	30	3.7 ± 0.14	0.65 ± 0.030	Yes (<i>p</i> = <0.001)	34.96 ± 1.94	Yes (<i>p</i> = <0.001)
PLC-ζ ^{K49A,K53A,R57A}	43	2.8 ± 0.074	0.54 ± 0.031	Yes (<i>p</i> = <0.001)	37.35 ± 2.24	Yes (<i>p</i> = <0.001)
PLC-ζ ^{K49A,K53A,R57A}	11	8.6 ± 1.7	7.65 ± 0.92	No (<i>p</i> = 0.15)	NA	NA
PLC-ζ ^{DMM}	25	0 ± 0	0.53 ± 0.046	Yes (<i>p</i> = <0.001)	NA	NA
PLC-ζ ^{DMM}	20	0 ± 0	14.43 ± 0.80	Yes (<i>p</i> = <0.001)	NA	NA

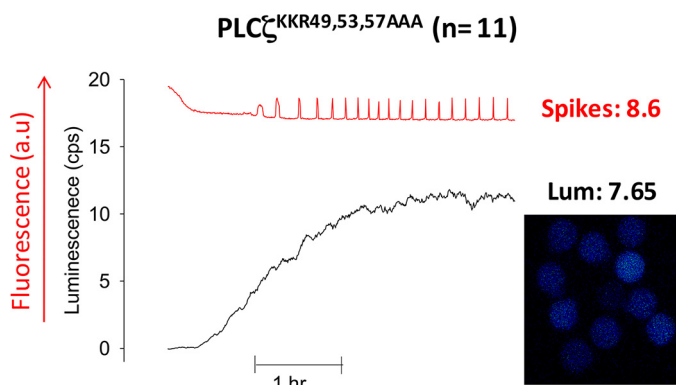


FIGURE 3. Overexpression of PLC-ζ^{K49A,K53A,R57A} in unfertilized mouse eggs. The left panel shows representative fluorescence (a.u., arbitrary units) and luminescence (cps) recordings reporting the Ca²⁺ concentration changes (red traces; Ca²⁺) and luciferase expression (black traces; Lum), respectively, in a mouse egg following microinjection of PLC-ζ^{K49A,K53A,R57A} luciferase cRNA. The right panel shows an integrated image of luciferase luminescence from eggs microinjected with the corresponding PLC-ζ^{K49A,K53A,R57A} luciferase cRNA for the 1st h of recording. The mean luminescence in the 1st h of oscillating (Lum) and mean number of Ca²⁺ spikes in the 1st h of oscillating (spikes) are shown. a.u., arbitrary units.

phatidylethanolamine (4:2:1) with incorporation of either 5% PS, 1 or 5% PA, and 1% PIP₂. To diminish any nonspecific protein binding to highly charged lipids, the liposome binding assays were performed in the presence of a near-physiological concentration of MgCl₂ (0.5 mM). PLC-ζ displayed robust binding only to liposomes containing 1% PIP₂, whereas the protein was only detected in the supernatant of liposomes containing 5% PS and 1 or 5% PA (Fig. 5B). Finally, we incubated 1 μg of PLC-ζ recombinant protein with the liposomes composed of the different phospholipids, and after centrifugation, the supernatants were separated and assayed for their ability to hydrolyze PIP₂ *in vitro*, using the standard [³H]PIP₂ hydrolysis assay. As shown in Fig. 5C, only the supernatant obtained after the interaction of recombinant PLC-ζ protein with the liposomes containing 1% PIP₂ showed a dramatic ~94% reduction in its PIP₂ hydrolytic activity. All these data suggest that the PLC-ζ binds specifically to PIP₂, not generically to any anionic phospholipid.

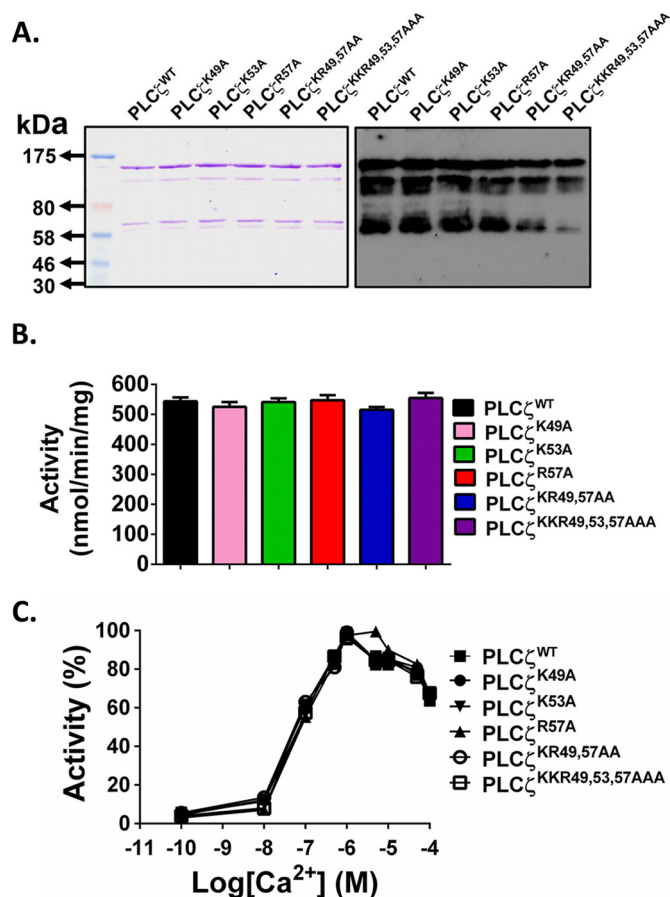


FIGURE 4. Expression and enzymatic characterization of recombinant NusA-His₆-PLC-ζ EF-hand mutants. A, expression of recombinant NusA-His₆-PLC-ζ^{WT} and the various mutant PLC-ζ proteins. Affinity-purified PLC-ζ proteins (1 μg) were analyzed by SDS-PAGE followed by either Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using the anti-NusA monoclonal antibody at 1:100,000 dilution (right panel). B, enzyme activity of the various PLC-ζ mutants. PIP₂ hydrolysis enzyme activities of the purified NusA-His₆-PLC-ζ fusion proteins were determined with the standard [³H]PIP₂ cleavage assay, *n* = 4 ± S.E., using two different preparations of each recombinant protein. An unpaired Student's *t* test showed no significant statistical difference between the enzymatic activities of PLC-ζ and PLC-ζ EF-hand mutants. In all cases, *p* > 0.1. C, effect of varying [Ca²⁺] on the normalized activity of NusA-His₆-tagged wild-type and mutant PLC-ζ fusion proteins. For these assays, *n* = 4 ± S.E., using two different preparations of each recombinant protein.

TABLE 2

In vitro enzymatic properties of NusA-His₆-PLCζ EF-hand mutants

Summary of specific enzyme activity and *K_m* and *EC*₅₀ values of Ca²⁺ dependence for PIP₂ hydrolysis determined by non-linear regression analysis (GraphPad Prism 5) for the NusA-His₆ fusion proteins (see Figs. 4 and 9) is shown.

PLC protein	PIP ₂ hydrolysis enzyme activity	Ca ²⁺ dependence <i>EC</i> ₅₀	<i>K_m</i>
	nmol/min/mg	nM	μM
PLCζ ^{WT}	544 ± 23	72	84
PLCζ ^{K49A}	525 ± 28	68	121
PLCζ ^{K53A}	541 ± 22	75	169
PLCζ ^{R57A}	547 ± 30	85	115
PLCζ ^{K49A,R57A}	515 ± 17	67	219
PLCζ ^{K49A,K53A,R57A}	555 ± 30	79	432
PLCζ ^{DMM}	434 ± 28	108	4975

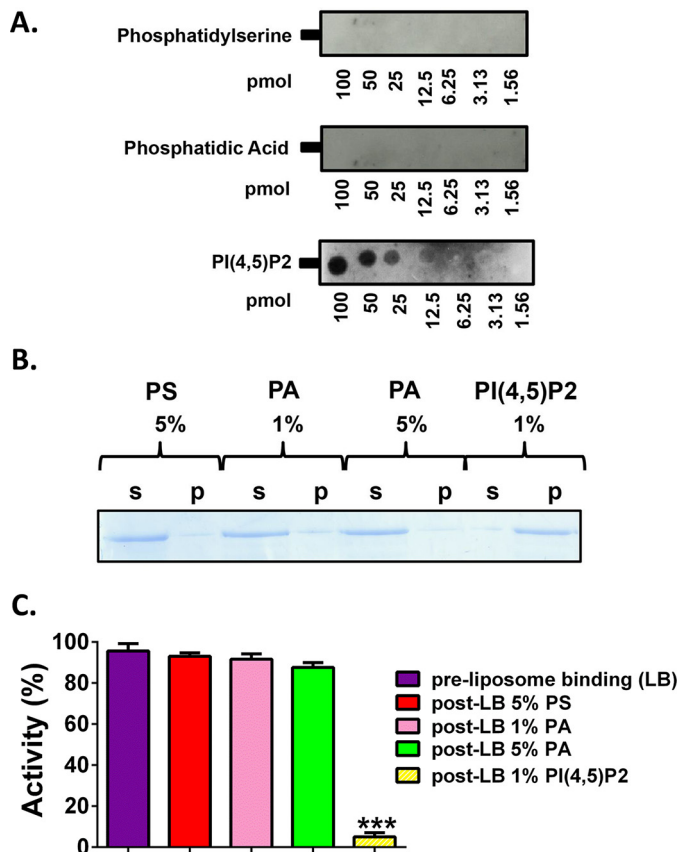


FIGURE 5. In vitro binding of wild-type PLCζ to PS, PA and PIP₂. A, PLCζ protein-lipid overlay assays. Recombinant protein binding to spotted phospholipids on the PIP arrays was detected using the monoclonal penta-His antibody. B, liposome “pull-down” assay of PLCζ. Unilamellar liposomes containing either PS (5%), or PA (1 or 5%), or PIP₂ (1%) were incubated with PLCζ recombinant protein. Following liposome centrifugation, both the supernatant (s) and liposome pellet (p) were subjected either to SDS-PAGE and Coomassie Brilliant Blue staining. C, supernatants were assayed for their ability to hydrolyze PIP₂ *in vitro*, using the standard [³H]PIP₂ hydrolysis assay, *n* = 4 ± S.E., using two different preparations of recombinant protein. Significant statistical differences (asterisks) were calculated by an unpaired Student’s *t* test; ***, *p* < 0.0005 (GraphPad, Prism 5).

Binding of PLCζ EF-hand Mutants to PIP₂-containing Liposomes—To investigate the effect of cumulative EF-hand mutations on the PIP₂-binding properties of wild-type PLCζ, we employed the liposome/activity binding assay as described above (see Fig. 5C). Thus, 1 μg of recombinant protein corresponding to PLCζ^{WT} and the five EF-hand mutants were each incubated with liposomes containing 1% PIP₂. After centrifugation, the supernatants were separated, and the PIP₂ hydro-

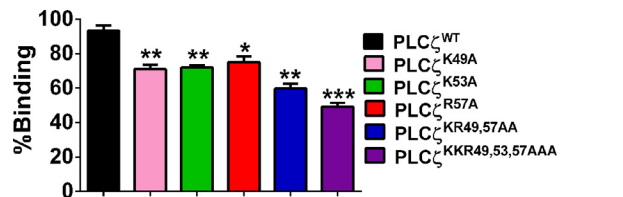


FIGURE 6. Binding of PLCζ mutants to PIP₂-containing liposomes. Normalized binding of PLCζ^{WT}, PLCζ^{K49A}, PLCζ^{K53A}, PLCζ^{R57A}, PLCζ^{K53A,K57A}, and PLCζ^{K49A,K53A,R57A} to unilamellar liposomes containing 1% PIP₂ is shown. Following centrifugation, the supernatants were assayed for their ability to hydrolyze PIP₂ *in vitro*, using the standard [³H]PIP₂ hydrolysis assay (*n* = 4 ± S.E., using two different preparations of recombinant protein). Based on the percentage of the PIP₂ hydrolytic activity pre- and post- liposome binding, the relative binding of each PLCζ protein to the PIP₂-containing liposomes was determined. Significant statistical differences (asterisks) were calculated by an unpaired Student’s *t* test; *, *p* < 0.05; **, *p* < 0.005; and ***, *p* < 0.0005, (GraphPad, Prism 5).

lytic activity was assayed using the standard [³H]PIP₂ hydrolysis assay. Based on the percentage of the PIP₂ hydrolytic activity pre- and post-liposome binding, we estimated the relative binding of each PLCζ protein to the PIP₂-containing liposomes. As shown in Fig. 6, although 94% of PLCζ^{WT} bound to the liposomes, the three single EF-hand mutants (PLCζ^{K49A}, PLCζ^{K53A}, and PLCζ^{R57A}) showed ~71–75% liposome binding. The effect of the double and the triple mutation was even more notable, as PLCζ^{K53A,K57A} displayed ~59% and PLCζ^{K49A,K53A,R57A} ~49% relative liposome binding. These data indicate that sequential neutralization of the basic residues within the EF-hand region substantially reduces the PIP₂-binding ability of PLCζ.

Modeling of Ca²⁺ Oscillations Induced by PLCζ EF-hand Mutants—The Ca²⁺ oscillatory activity associated with each of the PLCζ mutants constructed was simulated by using the parameters calculated in Fig. 6 and Tables 1 and 2. The most marked differentiation between constructs is the binding activity of each protein (Fig. 6), which is in agreement with a progressive destabilization of the EF-hand binding regime. By contrast, the Ca²⁺ dependence of IP₃ production (plotted in Fig. 4 and quantified in Table 2 as Ca²⁺-dependent *EC*₅₀ value) is very similar for each of the PLCζ constructs. Ca²⁺ oscillations simulated with this set of parametric values (Fig. 7, top panel) closely match those observed experimentally for each construct (Fig. 2) in terms of frequency. The theoretical relationship between Ca²⁺ oscillatory frequency and binding activity was produced by the mathematical model for *EC*₅₀ = 65, 75, and 85 nM (Fig. 7, bottom panel, lines left to right). The experimentally computed operating points of PLCζ wild-type and its various constructs (Fig. 7, bottom panel, circles) are located very close to the theoretical curves, confirming that the variability in Ca²⁺ oscillatory frequency can be accounted for almost exclusively by the gradual reduction in binding activity. When PLCζ^{K49A,K53A,R57A} was highly overexpressed, the oscillatory activity was largely restored, as indicated by the operating point of this scenario (Fig. 7, bottom panel, solid circle at the right of the panel). The fact that the circle lies below the theoretical frequency curve (Fig. 7, bottom panel, dashed line) may be due to the sub-optimal binding of the protein to PIP₂ at non-physiologically elevated concentrations.

Ca²⁺ Oscillation Inducing Activity of PLCζ Double Motif Mutant Expressed in Mouse Eggs—To investigate whether there is synergy between the cationic residues of the first EF-hand

PIP₂ Binding by PLC-ζ Involves EF-hand Domain

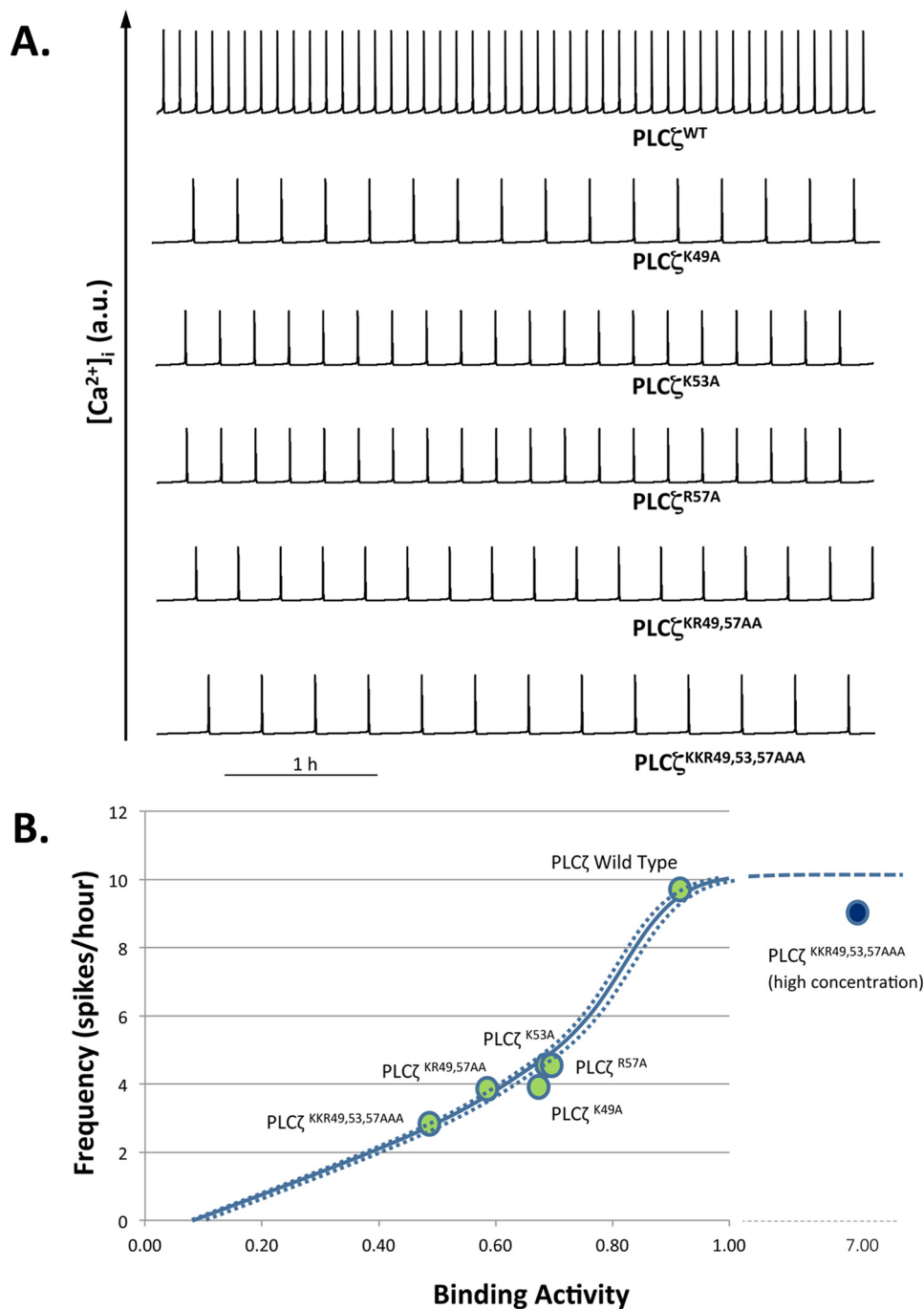


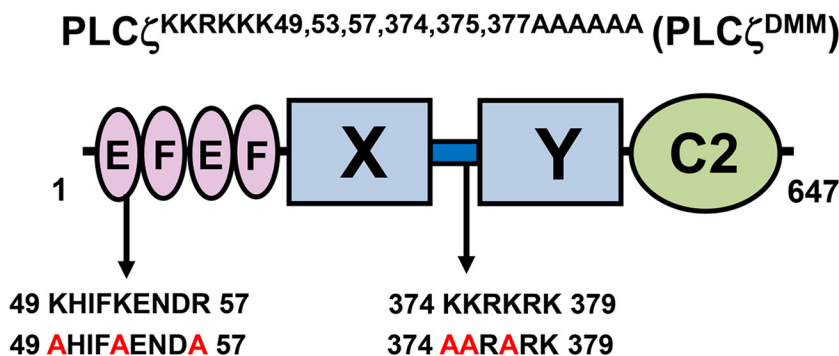
FIGURE 7. **Simulated time series of Ca²⁺ oscillations within an egg for wild-type and the various PLC ζ mutants.** Top panel, physiological parameters were taken from experimental measurements summarized in Fig. 6 and Tables 1 and 2. The theoretical relationship between PIP₂ binding activity of the PLC ζ constructs and the Ca²⁺ oscillatory frequency is plotted in the bottom panel as a solid line for $EC_{50} = 75$ nM. The solid curve is framed by two dotted lines corresponding to $EC_{50} = 65$ and 85 nM (left and right panels, respectively) to account for the small variability in EC_{50} estimated for the various constructs (indicated by circles). The curve is plotted against a normalized range of 0 to 1 to account for the binding activity estimated as a percentile in Fig. 6. Oscillatory activity associated with overexpressed PLC $\zeta^{K49A,K53A,R57A}$ is indicated by the solid circle (top right). This point lies below the theoretical binding activity versus frequency curve (dashed line).

domain and the XY-linker region of PLC ζ and whether these residues are necessary and sufficient to anchor this sperm protein to its PIP₂-containing membranes, we generated a PLC ζ mutant, in which charge-neutralization mutations were introduced within these two PLC ζ motifs. Thus, the residues Lys-49, Lys-53, and Arg-57 within the first EF-hand domain and the residues Lys-374, Lys-375, and Lys-377 within the XY-linker of PLC ζ were substituted by the neutral Ala residue giving rise to a PLC ζ double motif mutant

(PLC $\zeta^{K49A,K53A,R57A,K374A,K375A,K377A}$; PLC ζ^{DMM1}) containing six neutralization mutations (Fig. 8A). Interestingly, microinjection of cRNA encoding a luciferase-tagged version of PLC ζ^{DMM1} failed to cause any Ca²⁺ release, even after relatively high levels of protein expression in unfertilized mouse eggs (Fig. 8B and Table 1).

To investigate whether the luciferase-tagged PLC ζ^{WT} and PLC ζ^{DMM1} fusion constructs were expressed as structurally intact proteins in mouse eggs, we performed immunoblot anal-

A.



B.

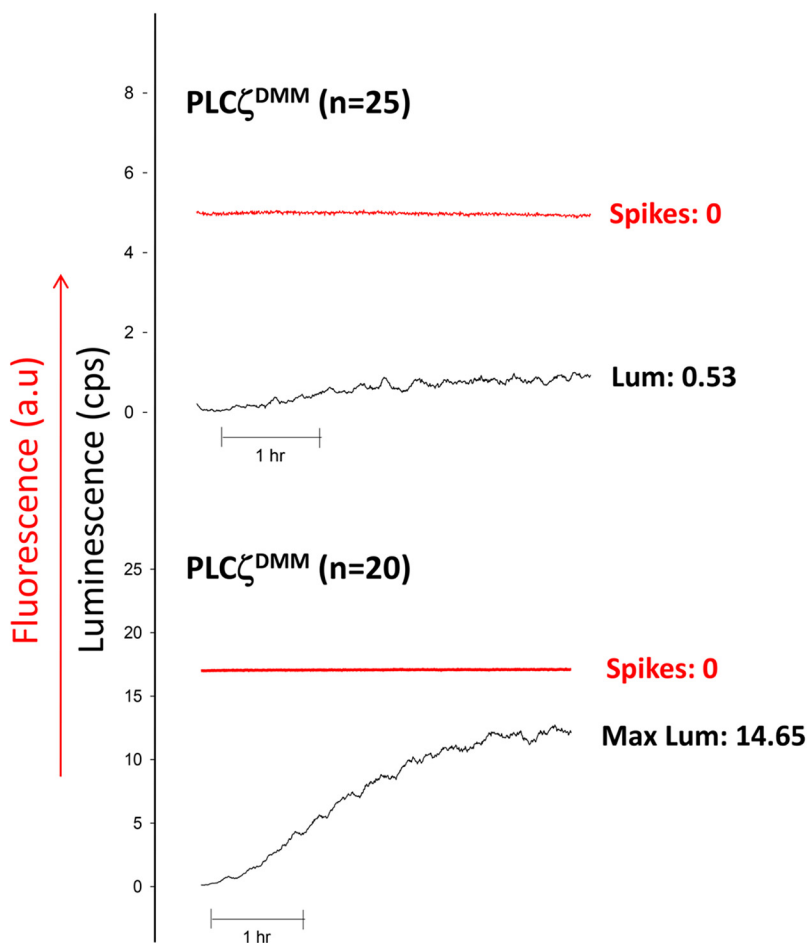


FIGURE 8. **Generation and expression of PLC_ζ^{K49A,K53A,R57A,K374A,K375A,K377A} (PLC_ζ^{DMM}) in unfertilized mouse eggs.** A, schematic representation of mouse PLC_ζ domain structure identifying the location of the successive Lys or Arg residue substitutions to Ala, between residues 49 and 57 within the first EF-hand domain, as well as the location of the successive Lys substitutions to Ala between residues 374 and 379 in the XY-linker region. B, traces showing the changes in fluorescence (*a.u.*, arbitrary units) and luminescence (cps) denoting alterations in Ca²⁺ concentrations (*red trace*) and luciferase expression (*black trace*), respectively, following the microinjection of high and low concentrations of PLC_ζ^{DMM}-luciferase cRNA into unfertilized mouse eggs. The mean values for the number of Ca²⁺ oscillations (*spikes*) and luminescence (*Lum*) during the 1st h of oscillating are shown.

ysis of two groups of mouse eggs microinjected with 0.5 μg/μl cRNA encoding either PLC_ζ^{WT}-LUC or the PLC_ζ^{DMM}-LUC mutant. Expression was followed for ~3 h and then the two groups of eggs were analyzed by SDS-PAGE and immunoblot detection using an anti-luciferase antibody. A single protein band was observed with mobility corresponding to the predicted molecular mass (~129 kDa) for both PLC_ζ^{WT}-LUC and PLC_ζ^{DMM}-LUC fusion proteins (Fig. 9), suggesting that each of the two cRNAs was faithfully expressed as full-length PLC-lu-

ciferase proteins and at similar expression levels in the cRNA-injected mouse eggs.

Expression, Enzymatic Characterization, and in Vitro Binding of PLC_ζ Double Motif Mutant to PIP₂-containing Liposomes—PLC_ζ^{DMM} was then subcloned into the pETMM60 vector and bacterially expressed and purified as a NusA-His₆-tagged fusion protein. Fig. 10A shows NusA-His₆-PLC_ζ^{DMM} recombinant protein analyzed by SDS-PAGE (*left panel*) and immunoblot detection with the anti-NusA monoclonal anti-

PIP₂ Binding by PLC- ζ Involves EF-hand Domain

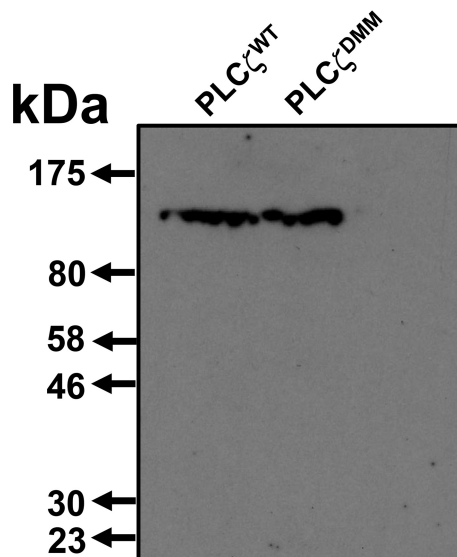


FIGURE 9. Confirmation of expression of PLC ζ^{WT} - and PLC ζ^{DMM} -LUC fusion proteins in mouse eggs. Two sets of mouse eggs (50 eggs each) were microinjected with 0.5 μ g/ μ l cRNA corresponding to either PLC ζ^{WT} or PLC ζ^{DMM} . Expression was allowed for \sim 3 h, and then the two sets of eggs were analyzed by SDS-PAGE and Western blotting using an anti-firefly luciferase antibody (1:10,000; Pierce).

body (right panel). The corresponding protein with the appropriate molecular mass (\sim 134 kDa) was observed as the top band in both Coomassie Brilliant Blue staining and on the immunoblot (Fig. 10A). Some low molecular weight bands were also detected by the anti-NusA antibody, and these are probably the result of protein degradation occurring through the bacterial expression and purification processes. Enzymatic analysis using the [³H]PIP₂ hydrolysis assay showed that PLC ζ^{DMM} retained \sim 80% of the enzymatic activity of PLC ζ^{WT} (434 ± 28 versus 544 ± 23 nmol/min/mg) (Fig. 10B) and that there was no significant difference in the Ca²⁺ sensitivity of PIP₂ hydrolysis for PLC ζ^{WT} and PLC ζ^{DMM} , with a very similar EC₅₀ value (72 versus 108 nM) (Fig. 10C and Table 2). However, the K_m value for PLC ζ^{DMM} (4975 μ M) was \sim 59-fold higher compared with PLC ζ^{WT} (84 μ M). More interestingly, when we performed the liposome/activity binding assay for PLC ζ^{DMM} , we found that this mutant displayed only \sim 15% relative liposome binding compared with PLC ζ^{WT} (Fig. 10D). These data indicate that neutralization of the positively charged residues within the first EF-hand and the XY-linker region dramatically reduces the binding of PLC ζ to PIP₂, leading to complete loss of its *in vivo* Ca²⁺ oscillation inducing activity.

Discussion

A significant body of scientific and clinical evidence suggests that the sperm-specific PLC ζ protein is the physiological molecule that, following sperm-egg fusion, stimulates cytoplasmic Ca²⁺ oscillations, egg activation, and early embryonic development to effect mammalian fertilization (3, 5, 7, 8, 11, 21, 27). The most compelling observation is that solely introducing PLC ζ mimics all of the signaling processes initiated by the sperm, triggering the same pattern of Ca²⁺ release as seen at normal fertilization and leading to the successful development of a blastocyst embryo. Thus, the current model of egg activa-

tion at fertilization is that the PLC ζ of a fertilizing spermatozoon is introduced into the egg cytoplasm where it catalyzes PIP₂ hydrolysis, stimulating the IP₃ signaling pathway, and leading to Ca²⁺ oscillations (5, 13).

The sperm PLC ζ is the smallest, with the most elementary domain organization, of all the mammalian PLC isoforms (3). Hence, the intrinsic ability of sperm PLC ζ to cause robust Ca²⁺ oscillations in eggs is significant because all the other PI-specific PLCs are unable to trigger Ca²⁺ oscillations in eggs at physiological protein expression levels. It therefore appears most plausible that PLC ζ employs a novel mechanism to potentially induce Ca²⁺ release in eggs and each of its individual domains appears to play an important role in the distinct molecular and biochemical characteristics, as well as in the unique regulatory mechanism of this sperm-derived PLC isozyme (2, 12). PLC ζ shares the greatest homology with PLC δ 1, but one major structural difference that distinguishes PLC ζ from PLC δ 1 is the lack of an N-terminal PH domain (2, 13). This is mechanistically interesting because the PH domain of PLC δ 1 in particular is known to specifically bind PIP₂ in the plasma membrane (15, 28). In contrast, we have recently shown that PLC ζ does not localize to the plasma membrane-bound PIP₂, but instead it targets distinct vesicular structures inside the egg cortex (29). Interestingly, the chimeric addition of a PH domain at the N terminus of the PLC ζ sequence does not alter the ability of PLC ζ to trigger Ca²⁺ oscillations in mouse eggs, and the PH-PLC ζ chimera is unable to target PLC ζ to the plasma membrane PIP₂ (25). The precise mechanism employed by PLC ζ to enable interaction with the PIP₂-containing vesicular membranes inside the egg cytosol is not understood.

Although the precise identity of the intracellular PIP₂-containing vesicles is currently unknown, we have proposed that PLC ζ associates with vesicular PIP₂ via electrostatic interactions mediated by the positively charged XY-linker region, assisting in anchoring PLC ζ to membranes, while enhancing local concentrations of the negatively charged PIP₂ (16, 17). In PLC ζ , the XY-linker region is more extended compared with that of PLC δ 1, and the proximal part to the Y catalytic domain contains a distinctive cluster of basic amino acid residues not found in the homologous region of any of the other somatic PLC isoforms (3). It is also notable that the XY-linker of somatic PLCs confers potent inhibition of their enzymatic activity (30, 31). In contrast, the XY-linker of PLC ζ does not confer enzymatic auto-inhibition but conversely appears to be required for maximal enzymatic activity (19). We have recently shown that deletion of PLC ζ XY-linker significantly diminishes its *in vivo* Ca²⁺ oscillation inducing activity but does not completely abolish it (19). This suggests that the XY-linker is essential for the association of PLC ζ with PIP₂-containing vesicular membranes, but it is not the sole region of PLC ζ responsible for this association.

Another candidate region that might be involved in the sequestration of PLC ζ to membranes containing its substrate PIP₂ is the C2 domain. The current data indicate that the C2 domain of PLC ζ may interact, albeit with low affinity, with membrane phospholipids (17, 32). Indeed, such interactions were observed *in vitro* with phosphatidylinositol 3-phosphate and phosphatidylinositol 5-phosphate. It is possible that the association of the C2 domain with phosphatidylinositol 3-phosphate may

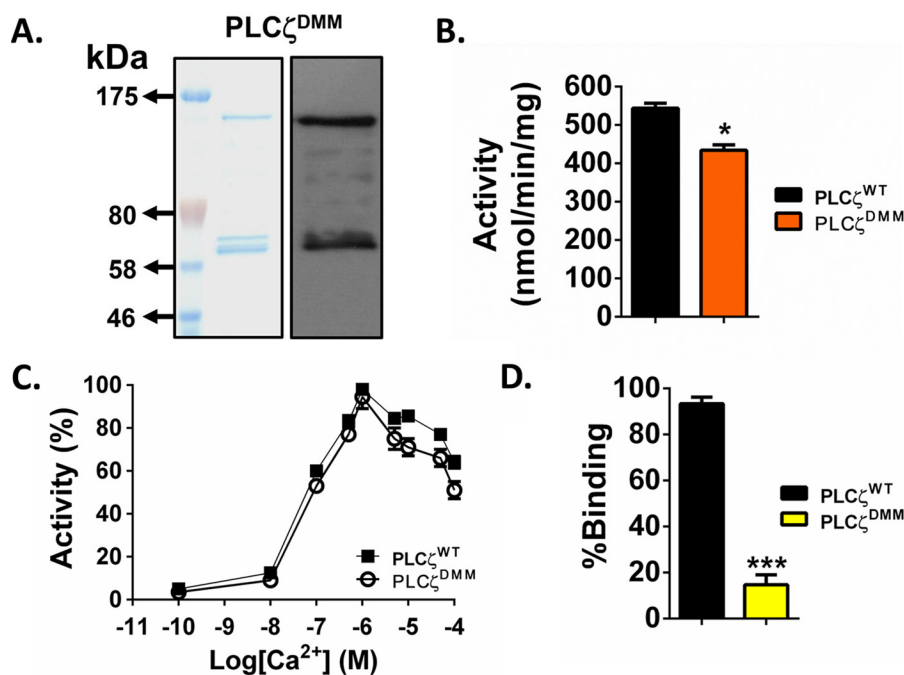


FIGURE 10. **Expression, enzymatic characterization and *in vitro* binding of NusA-His₆-PLC ζ^{DMM} to PIP₂-containing liposomes.** *A*, expression of recombinant PLC ζ^{DMM} protein. Affinity-purified NusA-His₆-tagged PLC ζ^{DMM} protein (1 μg) was analyzed by SDS-PAGE followed by either Coomassie Brilliant Blue staining (*left panel*) or immunoblot analysis using the anti-NusA monoclonal antibody at 1:100,000 dilution (*right panel*). *B*, enzyme activity of PLC ζ^{DMM} . PIP₂ hydrolysis enzyme activities of the purified recombinant proteins were determined with the standard [³H]PIP₂ cleavage assay, $n = 4 \pm \text{S.E.}$, using two different preparations of each recombinant protein. Significant statistical differences (*asterisks*) were calculated by an unpaired Student's *t* test; *, $p < 0.05$ (GraphPad, Prism 5). *C*, effect of varying [Ca²⁺] on the normalized activity of NusA-His₆-tagged PLC ζ^{DMM} fusion protein. For these assays $n = 4 \pm \text{S.E.}$, using two different preparations of each recombinant protein. *D*, normalized binding of PLC ζ^{DMM} to unilamellar liposomes containing 1% PIP₂ ($n = 4 \pm \text{S.E.}$, using two different preparations of recombinant protein). Significant statistical differences (*asterisks*) were calculated by an unpaired Student's *t* test; ***, $p < 0.0005$, (GraphPad, Prism 5).

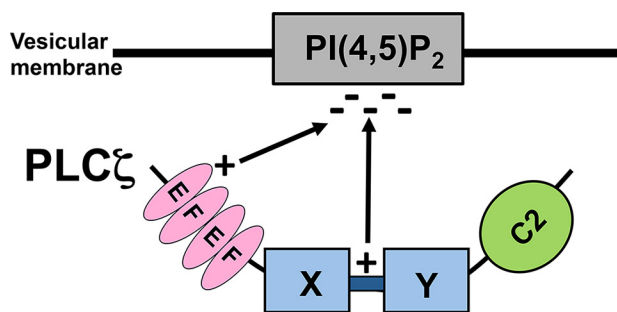


FIGURE 11. **Schematic illustration of the proposed mechanism that PLC ζ utilizes to target intracellular vesicular PIP₂-containing membranes.** Association of PLC ζ with the negatively charged PIP₂ involves electrostatic interactions with the positively charged first EF-hand domain and the XY-linker region. The catalytic XY domain subsequently proceeds with the enzymatic cleavage of PIP₂.

play a role in PLC ζ localization, or even perhaps regulation of enzymatic activity, as the presence of phosphatidylinositol 3-phosphate reduced PIP₂ hydrolysis by PLC ζ *in vitro* (32).

A recent study demonstrated that the N-terminal lobe of the EF-hand domain of PLC δ 1 binds to anionic phospholipid-containing vesicles, suggesting that the EF-hand domain aids substrate binding in the active site when the protein is membrane-anchored (20). The binding of the PLC δ 1 EF-hand domain to anionic phospholipid is mediated by a number of cationic residues within the first EF-hand motif of PLC δ 1. Interestingly, the positively charged residues that have been shown to contribute to the binding of PLC δ 1 (Arg-182, Lys-183, and Arg-186) by vesicles containing anionic lipids are specifically conserved in PLC ζ . We have shown that PLC ζ EF-hand domains play an important role in the high Ca²⁺ sensitivity relative to the other

PLC isoforms, especially in comparison with PLC δ 1 (21). PLC ζ appears to be 100-fold more sensitive to Ca²⁺ than PLC δ 1, which would enable the enzyme to be active at the resting nanomolar Ca²⁺ levels within the egg cytosol (21). Deletion of one or both pairs of EF-hand domains of PLC ζ completely abolishes its Ca²⁺ oscillation inducing activity in mouse eggs (21). Our current data suggest that this might be the result of both altered Ca²⁺ sensitivity and loss of ability to associate with PIP₂-containing membranes, as these PLC ζ EF-hand deletion constructs were unable to trigger Ca²⁺ release even when overexpressed in mouse eggs (21). Our mutagenesis analysis indicates that the substitution of even one Lys or Arg residue to Ala within the positively charged cluster of the PLC ζ EF-hand domain diminishes the Ca²⁺ oscillation inducing activity of PLC ζ (Fig. 2) without affecting its ability to hydrolyze PIP₂ *in vitro* or the Ca²⁺ sensitivity of its enzymatic activity (Fig. 4). Interestingly, the K_m value for the triple mutant PLC $\zeta^{\text{K49A,K53A,R57A}}$ (432 μM) was ~ 5.1 -fold higher compared with PLC ζ^{WT} (84 μM), suggesting that replacement of these three positively charged residues within the first EF-hand domain has an effect on the *in vitro* binding ability of PLC ζ to PIP₂ (Table 2). Moreover, we used a variety of approaches and demonstrated that PLC ζ binds only to PIP₂-containing liposomes, and sequential neutralization of these basic residues within the first EF-hand region of PLC ζ can significantly diminish the PIP₂-binding ability of PLC ζ (Figs. 5 and 6). As shown in our proposed mechanism in Fig. 11, which is supported by our studies on the PLC $\zeta^{\text{K49A,K53A,R57A,K374A,K375A,K377A}}$ mutant (PLC ζ^{DMM}), it is plausible that PLC ζ is attracted to the anionic PIP₂-containing

PIP₂ Binding by PLC- ζ Involves EF-hand Domain

component of the intracellular vesicular membranes through electrostatic interactions with both the first EF-hand domain and the XY-linker regions, which are rich in basic residues.

Our study provides an important advance in understanding the complex regulatory mechanism of PLC ζ and suggests that the N-terminal lobe of the EF-hand domain of PLC ζ has an essential role in the interaction of this enzyme with its target membrane, which together with the XY-linker may combine to provide a tether that facilitates proper PIP₂ substrate access and binding in the PLC ζ active site.

Author Contributions—M. N., K. S., and F. A. L. designed the study; J. R. S. conducted the oocyte experiments; D. P. generated the simulation data; M. N., L. B., B. L. C., P. S., A. S., and M. C. performed the molecular cloning, protein expression, purification, and characterization experiments; Z. S. prepared the liposomes, and all authors contributed to manuscript preparation. M. N. compiled the figures and together with F. A. L. prepared the final draft.

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