Essential Role of the EF-hand Domain in Targeting Sperm Phospholipase Cζ to Membrane Phosphatidylinositol 4,5-Bisphosphate (PIP2)*

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

Results: EF-hand mutations introduced into PLCζ reduce in vivo Ca2⁺ oscillation inducing activity and in vitro interaction with PIP2.

Conclusion: EF-hand domain is essential for targeting PLCζ to PIP2-containing membranes.

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

Sperm-specific phospholipase C-ζ (PLCζ) is widely considered to be the physiological stimulus that triggers intracellular Ca2⁺ oscillations and egg activation during mammalian fertilization. Although PLCζ is structurally similar to PLCβ1, it lacks a pleckstrin homology domain, and it remains unclear how PLCζ targets its phosphatidylinositol 4,5-bisphosphate (PIP2) membrane substrate. Recently, the PLCβ1 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 PLCζ are notably conserved in PLCζ. We investigated the potential role of these conserved cationic residues in PLCζ by generating a series of mutants that sequentially neutralized three positively charged residues (Lys-49, Lys-53, and Arg-57) within the mouse PLCζ EF-hand domain. Microinjection of the PLCζ EF-hand mutants into mouse eggs enabled their Ca2⁺ oscillation inducing activities to be compared with wild-type PLCζ. Furthermore, the mutant proteins were purified, and the in vitro PIP2 hydrolysis and binding properties were monitored. Our analysis suggests that PLCζ binds significantly to PIP2, but not to phosphatidic acid or phosphatidylserine, and that sequential reduction of the net positive charge within the first EF-hand domain of PLCζ significantly alters in vivo Ca2⁺ oscillation inducing activity and in vitro interaction with PIP2 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 PLCζ mutants to PIP2. Moreover, a PLCζ mutant with mutations in the cationic residues within the first EF-hand domain and the XY linker region dramatically reduces the binding of PLCζ to PIP2, 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 (PLCζ), which is introduced into the egg upon sperm-egg fusion and leads to cycles of inositol 1,4,5-trisphosphate (IP3) production following PIP2 hydrolysis, thus activating IP3 receptor-mediated Ca2⁺ release from intracellular stores in the egg (3–12). The closest PLC homologue of sperm PLCζ is PLCβ1 (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 PLCζ over somatic PLCs has not yet been fully explained.

PLCζ is the smallest PLC with the simplest domain organization among all the mammalian isoforms. PLCζ 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 PLCζ and the other somatic PLC isoforms is that PLCζ lacks a pleckstrin homology (PH) domain at the N terminus (2, 3, 13). The membrane binding of somatic PLCs appears to be...
mediated by the PH domain, a well defined structural module of ~120-amino acid residues identified in numerous proteins (14). The PH domain of PLCδ1 is essential for interaction with its phospholipid substrate PIP2 in the plasma membrane (15). The absence of a PH domain from PLCζ sequence raises questions about how PLCζ can bind to membranes.

We have previously proposed that the PLCζ 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 PLCζ to its membrane-bound substrate PIP2 (16, 17). The XY-linker region of PLCζ is extended in length and consists of more basic residues relative to its PLCδ1 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 in the anchoring of PLCζ 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 PLCζ to PIP2-containing membranes. We have demonstrated that the absence of the XY-linker from PLCζ 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 PLCζ to its target membrane.

A recent study reported that the N-terminal lobe of the EF-hand domain of PLCδ1 binds anionic phospholipids, and this binding is due to interactions with cationic and hydrophobic residues in the first EF-hand sequence of PLCδ1 (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 PLCδ1 that contribute to anionic lipid vesicle binding are all conserved in PLCζ.

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 PLCζ in association with anionic lipids and its substrate PIP2. A series of full-length mouse PLCζ 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 PLCζ by microinjection of cRNA into unfertilized mouse eggs. The various PLCζ 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 PLCζ to phosphatidylyserine (PS), phosphatidic acid (PA), and PIP2. Furthermore, the binding properties of mutant EF-hand PLCζ proteins to PIP2 were examined. Our results suggest that PLCζ 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 PLCζ with PIP2. Moreover, we show that a PLCζ mutant where three cationic residues within the first EF-hand domain and three cationic residues within the XY-linker region of PLCζ were substituted by alanine is unable to trigger Ca2+ oscillations in mouse eggs. In vitro biochemical characterization suggests that this PLCζ mutant displays dramatically reduced binding to PIP2-containing liposomes compared with the wild-type PLCζ. Thus, we propose a novel mechanism for the sperm PLCζ 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 PLCζ, which are rich in cationic residues.

**Experimental Procedures**

**Plasmid Construction**—A pCR3-mouse PLCζ-luciferase (PLCζ-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 PLCζK49A, PLCζK53A, PLCζR57A, PLCζK49A,R57A, and PLCζK49A,K53A,R57A mutants.

The pCR3-PLCζK49A,K53A,R57A (1–149 amino acids) was then amplified from the PLCζK49A,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. PLCζEFK374A,K375A,K377A (150–647 amino acids) was then amplified from the PLCζK374A,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-PLCζEFK49A,K53A,R57A plasmid. Finally, luciferase was amplified from pGL2 with primers incorporating NotI site, and the product was cloned into the NotI site of the pCR3-PLCζK49A,K53A,R57A vector with the appropriate primers to incorporate a 5-SalI site and a 3-NotI site, and the products were confirmed by dideoxynucleotide sequencing (Applied Biosystems Big-Dye Version 3.1 chemistry and model 3730 automated capillary DNA sequencer by DNA Sequencing & Services).

**cRNA Synthesis**—Following linearization of wild-type and mutated PLCζ plasmids, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and then 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 Ca\(^{2+}\) 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\(_2\)N\(_6\)N\(_8\)N\(_{10}\)-tetraacetic acid-dextran (Life Technologies, Inc.). Eggs were then maintained in H-KSOM containing 100 μl/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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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-His\(_6\)-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 A\(_{600}\) 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 × g for 10 min), resuspended in PBS containing a protease inhibitor mixture (EDTA-free; Roche Biosciences), and sonicated four times for 15 s on ice. Soluble NusA-His\(_6\)-tagged fusion protein was purified on nickel-nitrilotriacetic acid resin following standard procedures (Qia-gen) 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—PIP\(_2\) hydrolytic activity of recombinant PLC\(_{ζ}\) proteins was assayed as described previously (17, 21). The final concentration of PIP\(_2\) in the reaction mixture was 220 μM, containing 0.05 μCi of \[^{3}H\]PIP\(_2\). 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 PIP\(_2\) concentration, 0.05 μCi of \[^{3}H\]PIP\(_2\) was mixed with cold PIP\(_2\) to give the appropriate final concentration. In assays examining Ca\(^{2+}\) sensitivity, Ca\(^{2+}\) buffers were prepared by EGTA/CaCl\(_2\) 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).

**PIP\(_2\) Binding by PLC-ζ Involves EF-hand Domain**

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).
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### Results

**Effect of EF-hand Mutations on PLCζ-mediated Ca^{2+} 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 PLCζ 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 PLCζ. Thus, the residues Lys-49, Lys-53, and Arg-57 were sequentially substituted by the neutral amino acid, alanine, to create three single (PLCζ\(^{K49A},\) PLCζ\(^{K53A},\) and PLCζ\(^{R57A}\)) mutants, as well as one double (PLCζ\(^{K49A,K53A}\)) and one triple (PLCζ\(^{K49A,K53A,R57A}\)) PLCζ mutant. To test the Ca^{2+} oscillation inducing activity of PLCζ\(^{K49A},\) PLCζ\(^{K53A},\) PLCζ\(^{R57A},\) PLCζ\(^{K49A,K53A,R57A}\), and PLCζ\(^{K49A,K53A,R57A}\) 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 quantification of relative protein expression by luminescence detection of the expressed PLCζ-luciferase fusion protein, as described previously (17, 21). Prominent Ca^{2+} oscillations were observed in PLCζ\(^{K49A}\)-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 PLCζ mutants (PLCζ\(^{K49A},\) PLCζ\(^{K53A},\) and PLCζ\(^{R57A}\)) also triggered Ca^{2+} oscillations (Fig. 2), but these exhibited a lower frequency relative to PLCζ\(^{WT}\) (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 PLCζ\(^{K49A,K53A,R57A}\) or the triple PLCζ\(^{K49A,K53A,R57A}\) mutant resulted in a significant reduction in the frequency of Ca^{2+} oscillations compared with PLCζ\(^{WT}\), 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 PLCζ EF-hand domain can significantly alter their Ca^{2+} oscillation inducing activity in mouse eggs by reducing the frequency of Ca^{2+} spikes.

**Overexpression of PLCζ\(^{K49A,K53A,R57A}\) in Mouse Eggs Rescues Its Defective Ca^{2+} Oscillation-inducing Phenotype**—Judging by the number of Ca^{2+} spikes observed within the 1st h of oscillations per unit of recombinant fusion protein expression (cps), PLCζ\(^{WT}\) can be seen to be about ~3.5 times more effective at causing Ca^{2+} oscillations than the PLCζ\(^{K49A,K53A,R57A}\) triple mutant. To investigate whether we could rescue the low frequency of Ca^{2+} oscillations induced by PLCζ\(^{K49A,K53A,R57A}\), we overexpressed this PLCζ mutant in mouse eggs. As shown in Fig. 3 and Table 1, the overexpression of PLCζ\(^{K49A,K53A,R57A}\) (7.65 cps) indeed led to 8.6 spikes in the 1st h of oscillations, comparable with that for PLCζ\(^{WT}\), suggesting that loading the egg with large amounts of this PLCζ mutant can rescue its defective Ca^{2+} oscillation-inducing phenotype.

**Expression and Enzymatic Characterization of PLCζ EF-hand Mutants**—Each of the PLCζ\(^{K49A},\) PLCζ\(^{K53A},\) PLCζ\(^{R57A},\) PLCζ\(^{K49A,K53A,R57A},\) and PLCζ\(^{K49A,K53A,R57A}\) mutants was subcloned into the pETMM60 vector and purified as NusA-His\(_6\) fusion proteins by affinity chromatography. We have recently demonstrated that NusA is an effective fusion protein partner for PLCζ, significantly increasing soluble expression of PLCζ protein in *E. coli*, as well as enhancing the enzymatic stability of the purified protein over time (11). Following expression of NusA-PLCζ 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
**PIP<sub>2</sub> Binding by PLC-ζ Involves EF-hand Domain**

![Diagram of PLCζ constructs](image)

**Fig. 2. Expression of wild-type and mutant PLCζ constructs (PLCζ<sup>K49A</sup>, PLCζ<sup>K53A</sup>, PLCζ<sup>R57A</sup>, PLCζ<sup>KR49,57AA</sup>, and PLCζ<sup>KKR49,53,57AAA</sup>) in unfertilized mouse eggs.**

Fluorescence and luminescence (Lum) recordings reported the Ca<sup>2+</sup> changes (red traces; Ca<sup>2+</sup>) and luciferase expression (black traces; luminescence). The constructs were incubated in the presence of cRNA encoding luciferase-tagged PLCζ constructs, and their expression was monitored in unfertilized mouse eggs. The constructs are denoted as follows:

- **PLCζ<sup>WT</sup> (n=31)**: Spikes: 9.7, Lum: 0.47
- **PLCζ<sup>K49A</sup> (n=19)**: Spikes: 4.3, Lum: 0.46
- **PLCζ<sup>K53A</sup> (n=39)**: Spikes: 3.6, Lum: 0.46
- **PLCζ<sup>R57A</sup> (n=30)**: Spikes: 4.3, Lum: 0.74
- **PLCζ<sup>KR49,57AA</sup> (n=30)**: Spikes: 3.7, Lum: 0.65
- **PLCζ<sup>KKR49,53,57AAA</sup> (n=43)**: Spikes: 2.8, Lum: 0.54

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 PLCζ constructs. 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 PLCζ constructs being examined suggests that experimental comparison of relative enzymatic data may be appropriate. Hence, the specific PIP<sub>2</sub> hydrolytic enzyme activity for PLCζ<sup>WT</sup> and each recombinant mutant protein was determined by the standard micellar [H]PIP<sub>2</sub> hydrolysis assay. The histograms 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 PLCζ to hydrolyze PIP<sub>2</sub> in vitro. Moreover, to investigate the impact of the EF-hand mutations on Ca<sup>2+</sup> sensitivity of PLCζ enzyme activity, we assessed the ability of these PLCζ recombinant proteins to hydrolyze [H]PIP<sub>2</sub> at different Ca<sup>2+</sup> concentrations ranging from 0.1 mM to 0.1 mM. These experiments indicated that there was no significant difference in the Ca<sup>2+</sup> sensitivity of PIP<sub>2</sub> hydrolysis for the wild type, and the five EF-hand mutants (Fig. 4C) with a very similar EC<sub>50</sub> value (67–85 nM) displayed by all recombinant PLCζ proteins (Table 2). To compare the enzyme kinetics of wild-type and mutant PLCζ, the Michaelis-Menten constant, K<sub>m</sub>, was calculated for each construct (Table 2). The K<sub>m</sub> values obtained were similar for human PLCζ<sup>WT</sup> (84 μM), PLCζ<sup>K49A</sup> (121 μM), and PLCζ<sup>R57A</sup> (115 μM), whereas the K<sub>m</sub> value for PLCζ<sup>K53A</sup> (169 μM) and PLCζ<sup>KRR49,57AA</sup> (219 μM) mutants was ~2- and ~2.6-fold higher compared with that of PLCζ<sup>WT</sup>. Interestingly, the K<sub>m</sub> value for PLCζ<sup>KRR49,53,57AAA</sup> (432 μM) was ~5.1-fold higher compared with PLCζ<sup>WT</sup> (84 μM), suggesting that replacement of these three positively charged residues within the first EF-hand domain affects the in vitro affinity of PLCζ for PIP<sub>2</sub> without affecting the Ca<sup>2+</sup> sensitivity of this enzyme.

**Binding of PLCζ to PS, PA, and PIP<sub>2</sub>**—To examine the ability of PLCζ to bind the membrane lipids, PS, PA, and PIP<sub>2</sub>, we employed three different approaches. First, we used a protein-lipid overlay assay to assess the binding of PLCζ to membrane-spotted arrays of inositol phospholipids containing PS, PA, or PIP<sub>2</sub>. As shown in Fig. 5A, no binding to PS or PA was evident, although PLCζ was able to bind to membrane arrays containing PIP<sub>2</sub>. This result is consistent with our lipid binding assays (Fig. 5B). For these binding assays, we made unilamellar liposomes composed of phosphatidylcholine/CHOL/phosphatidylethanolamine, in counts/s, respectively, in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged PLCζ constructs. Mean number of Ca<sup>2+</sup> 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.
**PIP\(_2\) Binding by PLC-\(\zeta\) Involves EF-hand Domain**

**TABLE 1**

Properties of PLC\(_{\zeta}\)-luciferase EF-hand mutants expressed in unfertilized mouse eggs

<table>
<thead>
<tr>
<th>PLC(_{\zeta}) crRNA</th>
<th>No. of eggs</th>
<th>Mean no. of oscillations in 1st h of spiking</th>
<th>Mean expression in 1st h of spiking</th>
<th>No. of spikes significantly different from wild type?</th>
<th>Mean total counts/spike in 1st h of oscillating (counts/spike)</th>
<th>Counts/spike significantly different from wild type?</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC(_{\zeta}) WT</td>
<td>31</td>
<td>9.7 ± 0.63</td>
<td>0.47 ± 0.038</td>
<td>NA</td>
<td>14.6 ± 2.05</td>
<td>NA</td>
</tr>
<tr>
<td>PLC(_{\zeta}) K49A</td>
<td>19</td>
<td>3.6 ± 0.16</td>
<td>0.48 ± 0.024</td>
<td>Yes ((p &lt; 0.001))</td>
<td>25.9 ± 1.17</td>
<td>Yes ((p &lt; 0.001))</td>
</tr>
<tr>
<td>PLC(_{\zeta}) K53A</td>
<td>39</td>
<td>4.4 ± 0.13</td>
<td>0.46 ± 0.014</td>
<td>Yes ((p &lt; 0.001))</td>
<td>20.7 ± 0.85</td>
<td>Yes ((p &lt; 0.001))</td>
</tr>
<tr>
<td>PLC(_{\zeta}) R57A</td>
<td>30</td>
<td>4.3 ± 0.13</td>
<td>0.74 ± 0.032</td>
<td>Yes ((p &lt; 0.001))</td>
<td>38.2 ± 2.1</td>
<td>Yes ((p &lt; 0.001))</td>
</tr>
<tr>
<td>PLC(_{\zeta}) K49A+K53A+R57A</td>
<td>30</td>
<td>3.7 ± 0.14</td>
<td>0.65 ± 0.030</td>
<td>Yes ((p &lt; 0.001))</td>
<td>34.9 ± 1.94</td>
<td>Yes ((p &lt; 0.001))</td>
</tr>
<tr>
<td>PLC(_{\zeta}) K49A+K53A+R57A+PIP2</td>
<td>43</td>
<td>2.8 ± 0.074</td>
<td>0.54 ± 0.031</td>
<td>Yes ((p &lt; 0.001))</td>
<td>37.35 ± 2.24</td>
<td>Yes ((p &lt; 0.001))</td>
</tr>
<tr>
<td>PLC(_{\zeta}) K49A+K53A+R57A+PI(3,4,5)P3</td>
<td>11</td>
<td>8.6 ± 1.7</td>
<td>7.65 ± 0.92</td>
<td>No ((p = 0.15))</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PLC(_{\zeta}) K53A+R57A</td>
<td>25</td>
<td>0 ± 0</td>
<td>0.53 ± 0.046</td>
<td>Yes ((p &lt; 0.001))</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PLC(_{\zeta}) K53A</td>
<td>20</td>
<td>0 ± 0</td>
<td>14.43 ± 0.80</td>
<td>No ((p = 0.01))</td>
<td>NA</td>
<td>NA</td>
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Phatidylinositol (4:2) with incorporation of either 5% PS, 1% PS, or 5% PA, and 1% PIP\(_2\). 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 MgCl\(_2\) (0.5 mM). PLC\(_\zeta\) displayed robust binding only to liposomes containing 1% PIP\(_2\), whereas the protein was only detected in the supernatant of liposomes containing 5% PS or 1% PA (Fig. 5B). Finally, we incubated 1 μg of PLC\(_\zeta\) recombinant protein with the liposomes composed of the different phospholipids, and after centrifugation, the supernatants were separated and assayed for their ability to hydrolyze PIP\(_2\) in vitro, using the standard \(^{3}H\)PI(4,5)P2 hydrolysis assay. As shown in Fig. 5C, only the supernatant obtained after the interaction of recombinant PLC\(_\zeta\) protein with the liposomes containing 1% PIP\(_2\) showed a dramatic ~94% reduction in its PIP\(_2\) hydrolytic activity. All these data suggest that the PLC\(_\zeta\) binds specifically to PIP\(_2\) not generally to any anionic phospholipid.

**FIGURE 3.** Overexpression of PLC\(_{\zeta}\)K49A,K53A,R57A in unfertilized mouse eggs. The left panel shows representative fluorescence (a.u., arbitrary units) and luminescence (cps) recordings reporting the Ca\(^{2+}\) concentration changes (red traces; Ca\(^{2+}\)) and luciferase expression (black traces; Lum), respectively, in a mouse egg following microinjection of PLC\(_{\zeta}\)K49A,K53A,R57A-luciferase cRNA. The right panel shows an integrated image of luciferase luminescence from eggs microinjected with the corresponding PLC\(_{\zeta}\)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 Ca\(^{2+}\) spikes in the 1st h of oscillating (spikes) are shown. a.u., arbitrary units.

**FIGURE 4.** Expressions and enzymatic characterization of recombinant NusA-His\(_{6}\)-PLC\(_{\zeta}\) EF-hand mutants. A, expression of recombinant NusA-His\(_{6}\)-PLC\(_{\zeta}\) and the various mutant PLC\(_{\zeta}\) proteins. Affinity-purified PLC\(_{\zeta}\) proteins (1 μg) were analyzed by SDS-PAGE followed by either Coomassie Blue staining (left panel) or immunoblot analysis using the anti-NusA-PLC\(_{\zeta}\) protein. B, enzyme activity of the various PLC\(_{\zeta}\) mutants. PIP\(_2\) hydrolysis enzyme activities of the purified NusA-His\(_{6}\)-PLC\(_{\zeta}\) fusion proteins were determined with the standard \(^{3}H\)PI(4,5)P2 cleavage assay. C, effect of varying [Ca\(^{2+}\)] on the normalized activity of NusA-His\(_{6}\) tagged wild-type and mutant PLC\(_{\zeta}\) 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-His<sub>6</sub>-PLC<sub>ζ</sub> EF-hand mutants
Summary of specific enzyme activity and \( K_m \) and \( EC_{50} \) values of Ca<sup>2+</sup> dependence for PI<sub>3</sub> hydrolysis determined by non-linear regression analysis (GraphPad Prism 5) for the NusA-His<sub>6</sub> fusion proteins (see Figs. 4 and 9) is shown.

<table>
<thead>
<tr>
<th>PLC protein</th>
<th>PI&lt;sub&gt;3&lt;/sub&gt; hydrolysis enzyme activity</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt; dependence ( EC_{50} )</th>
<th>( K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC&lt;sub&gt;C&lt;/sub&gt;WT</td>
<td>544 ± 23</td>
<td>72</td>
<td>84</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;C&lt;/sub&gt;K49A</td>
<td>525 ± 28</td>
<td>68</td>
<td>121</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;C&lt;/sub&gt;K53A</td>
<td>541 ± 22</td>
<td>75</td>
<td>169</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;C&lt;/sub&gt;K57A</td>
<td>547 ± 30</td>
<td>85</td>
<td>115</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;C&lt;/sub&gt;K49A,K53A</td>
<td>515 ± 17</td>
<td>67</td>
<td>219</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;C&lt;/sub&gt;K49A,K53A,R57A</td>
<td>555 ± 30</td>
<td>79</td>
<td>432</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;C&lt;/sub&gt;R57M</td>
<td>434 ± 28</td>
<td>118</td>
<td>4975</td>
</tr>
</tbody>
</table>

**FIGURE 6. Binding of PLC<sub>ζ</sub> mutants to PI<sub>3</sub>-containing liposomes.** Normalized binding of PLC<sub>C</sub>WT, PLC<sub>C</sub> K49A, PLC<sub>C</sub> K53A, PLC<sub>C</sub> K57A, PLC<sub>C</sub> K49A,K53A, PLC<sub>C</sub> K49A,K53A,R57A, and PLC<sub>C</sub> K49A,K53A,K57A to unilamellar liposomes containing 1% PI<sub>3</sub> is shown. Following centrifugation, the supernatants were assayed for their ability to hydrolyze PI<sub>3</sub>, in vitro, using the standard \([^3]H\)PI<sub>3</sub> hydrolysis assay (\( n = 4 \) ± S.E., using two different preparations of recombinant protein). Based on the percentage of the PI<sub>3</sub> hydrolytic activity pre- and post-liposome binding, the relative binding of each PLC<sub>ζ</sub> protein to the PI<sub>3</sub>-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).

**FIGURE 5. In vitro binding of wild-type PLC<sub>ζ</sub> to PS, PA and PI<sub>3</sub>.** A, PLC<sub>ζ</sub> protein-lipid overlay assays. Recombinant protein binding to spotted phospholipids on the PIP arrays was detected using the monoclonal penta-His antibody. B, liposome "pulldown" assay of PLC<sub>ζ</sub>. Unilamellar liposomes containing either PS (5%), or PA (1 or 5%), or PI(4,5)P<sub>2</sub> (1%) were incubated with PLC<sub>ζ</sub> 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 PI<sub>3</sub>, in vitro, using the standard \([^3]H\)PI<sub>3</sub> 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 PLC<sub>ζ</sub> EF-hand Mutants to PI<sub>3</sub>-containing Liposomes**—To investigate the effect of cumulative EF-hand mutations on the PI<sub>3</sub>-binding properties of wild-type PLC<sub>ζ</sub>, we employed the liposome/activity binding assay as described above (see Fig. 5C). Thus, 1 μg of recombinant protein corresponding to PLC<sub>C</sub>WT and the five EF-hand mutants were each incubated with liposomes containing 1% PI<sub>3</sub>. After centrifugation, the supernatants were separated, and the PI<sub>3</sub> hydrolytic activity was assayed using the standard \([^3]H\)PI<sub>3</sub> hydrolysis assay. Based on the percentage of the PI<sub>3</sub> hydrolytic activity pre- and post-liposome binding, we estimated the relative binding of each PLC<sub>ζ</sub> protein to the PI<sub>3</sub>-containing liposomes. As shown in Fig. 6, although 94% of PLC<sub>C</sub>WT bound to the liposomes, the three single EF-hand mutants (PLC<sub>C</sub>K49A, PLC<sub>C</sub>K53A, and PLC<sub>C</sub>K57A) showed ~71–75% liposome binding. The effect of the double and the triple mutation was even more notable, as PLC<sub>C</sub>K49A,K53A,K57A displayed ~59% and PLC<sub>C</sub>K49A,K53A,K57A ~49% relative liposome binding. These data indicate that sequential neutralization of the basic residues within the EF-hand region substantially reduces the PI<sub>3</sub>-binding ability of PLC<sub>ζ</sub>.

**Modeling of Ca<sup>2+</sup> Oscillations Induced by PLC<sub>ζ</sub> EF-hand Mutants**—The Ca<sup>2+</sup> oscillatory activity associated with each of the PLC<sub>ζ</sub> 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 Ca<sup>2+</sup> dependence of IP<sub>3</sub> production (plotted in Fig. 4 and quantified in Table 2 as Ca<sup>2+</sup>-dependent EC<sub>50</sub> value) is very similar for each of the PLC<sub>ζ</sub> constructs. Ca<sup>2+</sup> 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 Ca<sup>2+</sup> oscillatory frequency and binding activity was produced by the mathematical model for EC<sub>50</sub> = 65, 75, and 85 nm (Fig. 7, bottom panel, lines left to right). The experimentally computed operating points of PLC<sub>ζ</sub> wild-type and its various constructs (Fig. 7, bottom panel, circles) are located very close to the theoretical curves, confirming that the variability in Ca<sup>2+</sup> oscillatory frequency can be accounted for almost exclusively by the gradual reduction in binding activity. When PLC<sub>C</sub>K49A,K53A,K57A 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 PI<sub>3</sub> at non-physiologically elevated concentrations.

**Ca<sup>2+</sup> Oscillation Inducing Activity of PLC<sub>ζ</sub> Double Motif Mutant Expressed in Mouse Eggs**—To investigate whether there is synergy between the cationic residues of the first EF-hand...
domain and the XY-linker region of PLCζ and whether these residues are necessary and sufficient to anchor this sperm protein to its PIP2-containing membranes, we generated a PLCζ mutant, in which charge-neutralization mutations were introduced within these two PLCζ 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 PLCζ were substituted by the neutral Ala residue giving rise to a PLCζ double motif mutant (PLCζK49A,K53A,R57A,K374A,K375A,K377A; PLCζDMM) containing six neutralization mutations (Fig. 8A). Interestingly, microinjection of cRNA encoding a luciferase-tagged version of PLCζDMM 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 PLCζWT and PLCζDMM fusion constructs were expressed as structurally intact proteins in mouse eggs, we performed immunoblot anal-

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**FIGURE 7.** Simulated time series of Ca2+ oscillations within an egg for wild-type and the various PLCζ 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 PLCζ constructs and the Ca2+ oscillatory frequency is plotted in the bottom panel as a solid line for EC50 = 75 nM. The solid curve is framed by two dotted lines corresponding to EC50 = 65 and 85 nM (left and right panels, respectively) to account for the small variability in EC50 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 PLCζK49A,K53A,R57A is indicated by the solid circle (top right). This point lies below the theoretical binding activity versus frequency curve (dashed line).
ysis of two groups of mouse eggs microinjected with 0.5 μg/μl cRNA encoding either PLCζWT-LUC or the PLCζDMM-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 PLCζWT-LUC and PLCζDMM-LUC fusion proteins (Fig. 9), suggesting that each of the two cRNAs was faithfully expressed as full-length PLC-luciferase proteins and at similar expression levels in the cRNA-injected mouse eggs.

Expression, Enzymatic Characterization, and in Vitro Binding of PLCζ Double Motif Mutant to PIP2-containing Liposomes—PLCζDMM was then subcloned into the pETMM60 vector and bacterially expressed and purified as a NusA-His6-tagged fusion protein. Fig. 10A shows NusA-His6-PLCζDMM recombinant protein analyzed by SDS-PAGE (left panel) and immunoblot detection with the anti-NusA monoclonal anti-
body (right panel). The corresponding protein with the appropriate molecular mass (~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 PLCζ<sup>WT</sup> retained ~80% of the enzymatic activity of PLCζ<sup>DMM</sup> (434 ± 28 versus 544 ± 23 nmol/min/mg) (Fig. 10B) and that there was no significant difference in the Ca<sup>2+</sup> sensitivity of PIP2 hydrolysis for PLCζ<sup>WT</sup> and PLCζ<sup>DMM</sup>, with a very similar EC<sub>50</sub> value (72 versus 108 nm) (Fig. 10C and Table 2). However, the K<sub>m</sub> value for PLCζ<sup>DMM</sup> (4975 μM) was ~59-fold higher compared with PLCζ<sup>WT</sup> (84 μM). More interestingly, when we performed the lipidosome/activity binding assay for PLCζ<sup>DMM</sup>, we found that this mutant displayed only ~15% relative liposome binding compared with PLCζ<sup>WT</sup> (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 PLCζ to PIP2, leading to complete loss of its in vivo Ca<sup>2+</sup> oscillation inducing activity.

**Discussion**

A significant body of scientific and clinical evidence suggests that the sperm-specific PLCζ protein is the physiological molecule that, following sperm-egg fusion, stimulates cytoplasmic Ca<sup>2+</sup> 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 PLCζ mimics all of the signaling processes initiated by the sperm, triggering the same pattern of Ca<sup>2+</sup> release as seen at normal fertilization and leading to the successful development of a blastocyst embryo. Thus, the current model of egg activation at fertilization is that the PLCζ of a fertilizing spermatozoan is introduced into the egg cytoplasm where it catalyzes PIP<sub>2</sub> hydrolysis, stimulating the IP<sub>3</sub> signaling pathway, and leading to Ca<sup>2+</sup> oscillations (5, 13).

The sperm PLCζ is the smallest, with the most elementary domain organization, of all the mammalian PLC isoforms (3). Hence, the intrinsic ability of sperm PLCζ to cause robust Ca<sup>2+</sup> oscillations in eggs is significant because all the other PI-specific PLCs are unable trigger Ca<sup>2+</sup> oscillations in eggs at physiological protein expression levels. It therefore appears most plausible that PLCζ employs a novel mechanism to potently induce Ca<sup>2+</sup> 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). PLCζ shares the greatest homology with PLCδ1, but one major structural difference that distinguishes PLCζ from PLCδ1 is the lack of an N-terminal PH domain (2, 13). This is mechanistically interesting because the PH domain of PLCδ1 in particular is known to specifically bind PIP2 in the plasma membrane (15, 28). In contrast, we have recently shown that PLCζ 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 PLCζ sequence does not alter the ability of PLCζ to trigger Ca<sup>2+</sup> oscillations in mouse eggs, and the PH-PLCζ chimera is unable to target PLCζ to the plasma membrane PIP2 (25). The precise mechanism employed by PLCζ 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 PLCζ associates with vesicular PIP2 via electrostatic interactions mediated by the positively charged XY-linker region, assisting in anchoring PLCζ to membranes, while enhancing local concentrations of the negatively charged PIP2 (16, 17). In PLCζ, the XY-linker region is more extended compared with that of PLCδ1, 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 PLCζ does not confer enzymatic autoinhibition but conversely appears to be required for maximal enzymatic activity (19). We have recently shown that deletion of PLCζ XY-linker significantly diminishes its in vivo Ca<sup>2+</sup> oscillation inducing activity but does not completely abolish it (19). This suggests that the XY-linker is essential for the association of PLCζ with PIP2-containing vesicular membranes, but it is not the sole region of PLCζ responsible for this association.

Another candidate region that might be involved in the sequestration of PLCζ to membranes containing its substrate PIP2 is the C2 domain. The current data indicate that the C2 domain of PLCζ 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...
Significant statistical differences (asterisks) were calculated by an unpaired Student’s t test; ***, p < 0.0005.

**FIGURE 10.** Expression, enzymatic characterization and in vitro binding of NusA-His6-PLC<sub>DMM</sub> to PIP<sub>2</sub>-containing liposomes. A, expression of recombinant PLC<sub>DMM</sub> protein. Affinity-purified NusA-His<sub>6</sub>-tagged PLC<sub>DMM</sub> 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<sub>DMM</sub>. PIP<sub>2</sub> hydrolysis enzyme activities of the purified recombinant proteins were determined with the standard ([<sup>3</sup>H]PIP<sub>2</sub> cleavage assay, n = 4 ± 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 [Ca<sup>2+</sup>] on the normalized activity of NusA-His<sub>6</sub>-tagged PLC<sub>DMM</sub> fusion protein. For these assays n = 4 ± S.E., using two different preparations of each recombinant protein. D, normalized binding of PLC<sub>DMM</sub> to unilamellar liposomes containing 1% PIP<sub>2</sub> (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).

**FIGURE 11.** Schematic illustration of the proposed mechanism that PLC<sub>ζ</sub> utilizes to target intracellular vesicular PIP<sub>2</sub>-containing membranes. Association of PLC<sub>ζ</sub> with the negatively charged PIP<sub>2</sub> 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 PIP<sub>2</sub>.

PLC isoforms, especially in comparison with PLC<sub>δ1</sub> (21). PLC<sub>ζ</sub> appears to be 100-fold more sensitive to Ca<sup>2+</sup> than PLC<sub>δ1</sub>, which would enable the enzyme to be active at the resting nanomolar Ca<sup>2+</sup> levels within the egg cytosol (21). Deletion of one or both pairs of EF-hand domains of PLC<sub>ζ</sub> completely abolishes its Ca<sup>2+</sup> oscillation inducing activity in mouse eggs (21). Our current data suggest that this might be the result of both altered Ca<sup>2+</sup> sensitivity and loss of ability to associate with PIP<sub>2</sub>-containing membranes, as these PLC<sub>ζ</sub> EF-hand deletion constructs were unable to trigger Ca<sup>2+</sup> 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 residues within the first EF-hand domain has an effect on the oscillation inducing activity of PLC<sub>ζ</sub> (Fig. 2) without affecting its ability to hydrolyze PIP<sub>2</sub> in vitro or the Ca<sup>2+</sup> sensitivity of its enzymatic activity (Fig. 4). Interestingly, the K<sub>m</sub> value for the triple mutant PLC<sub>ζ</sub><sup>K49A,K53A,K57A</sup> (432 μM) was ~5.1-fold higher compared with PLC<sub>ζ</sub><sup>WT</sup> (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<sub>ζ</sub> to PIP<sub>2</sub> (Table 2). Moreover, we used a variety of approaches and demonstrated that PLC<sub>ζ</sub> binds only to PIP<sub>2</sub>-containing liposomes, and sequential neutralization of these basic residues within the first EF-hand region of PLC<sub>ζ</sub> can significantly diminish the PIP<sub>2</sub>-binding ability of PLC<sub>ζ</sub> (Figs. 5 and 6). As shown in our proposed mechanism in Fig. 11, which is supported by our studies on the PLC<sub>ζ</sub><sup>K49A,K53A,K57A</sup> mutant (PLC<sub>ζ</sub><sup>DMM</sup>), it is plausible that PLC<sub>ζ</sub> is attracted to the anionic PIP<sub>2</sub>-containing

**A.** Vesicular membrane

**B.** PI(4,5)P<sub>2</sub>
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 PLCζ and suggests that the N-terminal lobe of the EF-hand domain of PLCζ 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 PLCζ 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.

References

Lipids:
Essential Role of the EF-hand Domain in Targeting Sperm Phospholipase Cζ to Membrane Phosphatidylinositol 4,5-Bisphosphate (PIP₂)

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