Role of Phospholipase C-ζ Domains in Ca\(^{2+}\)-dependent Phosphatidylinositol 4,5-Bisphosphate Hydrolysis and Cytoplasmic Ca\(^{2+}\) Oscillations*

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From the ‡Cell Signalling Laboratory, Wales Heart Research Institute, School of Medicine, Cardiff University, Cardiff CF14 4XN, the ¶Department of Anatomy and Developmental Biology, University College London, London WC1E 6BT, and the §Department of Obstetrics and Gynaecology, School of Medicine, Cardiff University, Cardiff CF14 4XN, United Kingdom

The sperm-specific phospholipase C-ζ (PLCζ) elicits fertilization-like Ca\(^{2+}\) oscillations and activation of embryo development when microinjected into mammalian eggs (Saunders, C. M., Larman, M. G., Arrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K., and Lai, F. A. (2002) Development (Camb.) 129, 3533–3544; Cox, L. J., Larman, M. G., Saunders, C. M., Hashimoto, K., Swann, K., and Lai, F. A. (2002) Reproduction 124, 611–623). PLCζ may represent the physiological stimulus for egg activation and development at mammalian fertilization. PLCζ is the smallest known mammalian PLC isozyme, comprising two EF hand domains, a C2 domain, and the catalytic X and Y core domains. To gain insight into PLCζ’s structure-function, we assessed the ability of PLCζ and a series of domain-deletion constructs to cause phosphatidylinositol 4,5-bisphosphate hydrolysis in vitro and also to generate cytoplasmic Ca\(^{2+}\) changes in intact mouse eggs. PLCζ and the closely related PLCζ6 had similar \(K_m\) values for phosphatidylinositol 4,5-bisphosphate, but PLCζ was around 100 times more sensitive to Ca\(^{2+}\) than was PLCζ6. Notably, specific phosphatidylinositol 4,5-bisphosphate hydrolysis activity was retained in PLCζ constructs that had either EF hand domains or the C2 domain removed, or both. In contrast, Ca\(^{2+}\) sensitivity was greatly reduced when either one, or both, of the EF hand domains were absent, and the Hill coefficient was reduced upon deletion of the C2 domain. Microinjection into intact mouse eggs revealed that all domain-deletion constructs were ineffective at initiating Ca\(^{2+}\) oscillations. These data suggest that the exquisite Ca\(^{2+}\)-dependent features of PLCζ regulation are essential for it to generate inositol 1,4,5-trisphosphate and Ca\(^{2+}\) oscillations in intact mouse eggs.

A series of pre-programmed biochemical events are triggered during fertilization. The earliest signaling event in the activation of an egg by a sperm is a large, transient increase in intracellular free calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) (1, 2). In many nonmammalian species, such as sea urchin, the observed Ca\(^{2+}\) increase in the egg comprises a single transient, but in mammals and some marine invertebrates there is a series of repetitive Ca\(^{2+}\) oscillations (3, 4). The frequency and duration of these Ca\(^{2+}\) oscillations vary between species (1). In response to this Ca\(^{2+}\) signal, the fertilized egg completes meiosis and initiates the process of embryonic development (5).

Several lines of evidence implicate the inositol 1,4,5-trisphosphate (IP\(_3\)) signaling pathway (6) as the origin of the Ca\(^{2+}\) signals in mammalian eggs. IP\(_3\) is produced by hydrolysis of phosphatididylinositol 4,5-bisphosphate in a reaction that is catalyzed by phosphoinositide-specific phospholipase (PI-PLC) (6). Liberated IP\(_3\) then causes Ca\(^{2+}\) release by binding to IP\(_3\) receptors located on the endoplasmic reticulum of eggs and oocytes (7, 8). The essential role of IP\(_3\) and the IP\(_3\) receptor in fertilization is illustrated by studies in mouse and hamster eggs, where Ca\(^{2+}\) oscillations at fertilization can be inhibited by microinjection of antibodies that inhibit the IP\(_3\) receptor (9) or by down-regulation of IP\(_3\) receptors (10, 11). In addition, it has been shown that sustained injection of IP\(_3\), the repeated photorelease of caged IP\(_3\), or the microinjection of the IP\(_3\) analogue adenosphostin can all lead to a series of Ca\(^{2+}\) oscillations in eggs (8, 12, 13). Hence, in mammalian eggs, IP\(_3\) is both necessary and potentially sufficient to explain the Ca\(^{2+}\) oscillations observed at fertilization. However, the precise mechanism employed by a sperm to generate an IP\(_3\) increase in the egg is not established.

In mammalian eggs there is evidence for the existence of different types of PI-PLCs. The stimulation of egg-derived PLC isoforms of the \(\beta\) or \(\gamma\) class by receptor tyrosine kinases (14) or by guanine nucleotide-binding proteins (G-proteins) (15) can lead to Ca\(^{2+}\) release in different species of eggs. However, there is evidence that neither the tyrosine kinase nor the G-protein pathways are necessary for Ca\(^{2+}\) release at fertilization in mouse eggs (2). In contrast to classical transmembrane receptor signaling, there is evidence that mammalian eggs can be activated by a sperm protein component that is introduced into the egg after gamete membrane fusion (1, 4). Egg microinjection of sperm extracts, or whole sperm, triggers Ca\(^{2+}\) oscillations similar to fertilization in a range of different species (17–23). The injection of such a sperm factor also activates the development of eggs and hence could represent the physiological agent that triggers egg activation and embryonic development at fertilization (24).

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The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; PI-PLC, phosphatidylinositol 4,5-bisphosphatase; PLC, phospholipase C; PI-PLC, phosphoinositide-specific phospholipase; BAPTA, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N’-tetraacetic acid; GST, glutathione S-transferase; PH, pleckstrin homology.
We demonstrated that the ability of soluble mammalian sperm extracts to cause Ca\(^{2+}\) oscillations can be explained by the presence of a sperm-specific PLC activity (25, 26). This activity was shown to be due to a novel mammalian PI-PLC, phospholipase C\(_{\gamma}\) (PLC\(_{\gamma}\)), that we isolated from a spermatid cDNA library (27). Microinjection of cRNA encoding the mouse (27), human, and human sperm PLC\(_{\gamma}\) isozymes have in common a C2 domain, which binds to lipids in the membrane containing PIP2, and this association is Ca\(^{2+}\) dependent. The specific reasons why sperm PLC\(_{\gamma}\) is much more effective than other PLC isoforms at causing Ca\(^{2+}\) oscillations in eggs are currently unknown.

The unique functional features of PLC\(_{\gamma}\) may be attributable to its distinct domain structure. There are five subfamilies of PI-PLCs (\(\beta\), \(\gamma\), \(\delta\), \(\epsilon\), and \(\zeta\)) classified on the basis of their sequence homology (27, 31). The regions of greatest sequence identity are the X and Y domains, which form the active site for enzymatic cleavage of PIP2 in all PI-PLCs. In addition, all isoforms have in common a C2 domain, which binds to lipids such as phosphatidylserine. In studies with PI-PLC extracts to cause Ca\(^{2+}\) oscillations in eggs at very low concentrations (e.g. 10 fg/egg) (24, 27, 29). In contrast, other studies have shown that PLC isoforms of the \(\beta\), \(\gamma\), \(\delta\) class are either ineffective (25) or at least much less effective than PLC\(_{\gamma}\) at causing Ca\(^{2+}\) release when microinjected into eggs (2, 29, 30).

The specific reasons why sperm PLC\(_{\gamma}\) is much more effective than other PLC isoforms at causing Ca\(^{2+}\) oscillations in eggs are currently unknown.

PLC\(_{\gamma}\) Domain Regulation of Calcium Oscillations

The aim of this study is to examine the importance of the EF hands and Ca\(^{2+}\) binding domains in PLC isozymes is not clear. These domains usually bind one Ca\(^{2+}\) ion each, although vari-
stability sites. The following primers were used for each PLC\(_{\gamma}\) isoform: for the forward primer was 5'-ATATCAATGGACTCGGGTAGGGACTT-3' (forward) and 5'-CATGTTTTCATCGAATTCTGGGTT-3' (reverse); and for the \(\gamma\)Y, the primers were ACCGGATCCATATGGACTCGGGTAGGGACTT-3' (forward) and 5'-CATGTTTTCATCGAATTCTGGGTT-3' (reverse). The rat PLC\(_{\gamma}\) clone (GenBank\textsuperscript{TM} accession number M20637) was kindly provided by M. Katian (Cancer Research UK, Centre for Cell and Molecular Biology, London, UK). Appropriate primers were designed to incorporate a 5'-SalI site and a 3'-NotI site at the ends of PLC\(_{\gamma}\). PLC\(_{\gamma}\) was amplified by PCR and cloned into pGEM-5X2 by using these restriction sites. The following primers were used for PLC\(_{\gamma}\): 5'-CTTGGCAATCATGAGGTCAGAGTGC-3' (forward) and 5'-CACGGACCCCTTATCTGGATTCAGAG-3' (reverse). These domains usually bind one Ca\(^{2+}\) ion each, although vari-

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PLC\(_{\gamma}\) Domain Regulation of Calcium Oscillations

The aim of this study is to examine the importance of the EF hands and Ca\(^{2+}\) domains in PLC\(_{\gamma}\) domain-deletion constructs were prepared, together with the well-characterized PLC\(_{\gamma}\) (34, 35) for comparison, and were tested by using an in vitro PLC activity assay (hydrolysis of PIP2) and also in situ assay by microinjection into eggs of the corresponding cRNA constructs (Ca\(^{2+}\) oscillations). Our studies show that although the XY catalytic domain is minimally sufficient for \(\omega\) in vitro enzymatic hydrolysis of PIP2, all of the disparate domains, i.e. EF hands and C2 domains, are required for PLC\(_{\gamma}\) to initiate the trademark Ca\(^{2+}\) oscillations observed in mammalian eggs upon fertilization by sperm.
plied by PCR from the original clone cDNA using appropriate primers and Pfu polymerase, as described above. PCR products were cloned into pCR-Blunt-TOPO (Invitrogen), sequence-verified, and then subcloned into the pTarget vector (Promega). cRNAs encoding the wild type PLC and deletion constructs were produced from the linearized pTarget constructs using the Ribomax RNA synthesis kit (Promega) in the presence of 3 mM mGTP 5'ppp5'G (37 °C, 2 h). Synthesized cRNA products were analyzed by agarose gel electrophoresis, isopropyl alcohol-precipitated, and resuspended in diethyl pyrocarbonate-treated water containing 4 units of RNasin l−1 (Promega). Integrity of complementary RNAs was assayed by in vitro expression of proteins (Reticulocyte Lysate System, Promega) in the presence of [35S]methionine (Amer sham Biosciences) and analyzed by 8% SDS-PAGE and autoradiography, as described previously (27).

Preparation and Handling of Gametes—Experiments were carried out with mouse eggs in Hepes-buffered saline (H-KSOM) as described previously (38). All compounds were from Sigma unless stated otherwise. Female mice were superovulated by injection of human chorionic gonadotrophin (Intervet). Eggs were collected 13.5–14.5 h later (5) and maintained in 100-μl droplets of H-KSOM under mineral oil at 37 °C. Microinjection of the eggs was carried out 14.5–15.5 h after human chorionic gonadotrophin injection (27).

Microinjection and Measurement of Intracellular Ca2+ and Luciferase Expression—Mouse eggs were washed in H-KSOM and microinjected as described previously (27) 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 egg volume. In experiments with untagged PLCγ, Ca2+ changes were monitored with a CGB-based imaging system using a Zeiss Axiosvert 100 microscope, with illumination from a monochromator (Photonics) controlled by MetaFluor version 4.0 (Universal Imaging Corp.). Eggs were loaded for 10 min with 4 μM Fura Red-AM (Molecular Probes) and dissolved in Me2SO 0.5% (w/v) pluronic acid, and the loading medium was supplemented with sulfipyrazone, which helps prevent compartmentalization and extrusion of the dye (5).

For experiments with luciferase-tagged PLCγ, eggs were microinjected with the appropriate cRNA mixed with an equal volume of 1 mM Oregon Green BAPTA dextran (Molecular Probes) in KC1 Hepes buffer. Eggs were then maintained in H-KSOM with 100 μM luciferin and imaged on a Nikon TE2000 or Zeiss Axiovert 100 microscope equipped with a cooled intensified CCD camera (Photokon Ltd., UK). Ca2+ was monitored in these eggs for 4 h after injection by measuring the Oregon Green BAPTA dextran fluorescence with low level excitation light from a halogen lamp. At the end of Ca2+ measurements, the same set of eggs was then monitored for luminescence by integrating light emission (in the absence of fluorescence excitation) for 30 min using the same intensified CCD camera. The fluorescence signals were typically 10–100 times greater than the luminescence signals. Ca2+ measurements for an egg were considered valid only if the same egg was also luminescent. Eggs verified as being luminescent were then counted and placed in a test tube containing PBS with 1 mM MgATP + 100 μM luciferin, which was held in a custom-made luminometer equipped with a cooled S20 photomultiplier tube (Electron Tubes Ltd., UK). The eggs were then lysed with 0.5% Triton X-100, and the steady state light was compared with that emitted from calibrated amounts of recombinant firefly luciferase (Sigma). The amount of luciferase activity measured for each group of eggs was then divided by the number of luminous eggs to obtain the mean value for protein expression of each type of luc-PLCγ.

SDS-PAGE and Western Blotting—Recombinant proteins were separated by SDS-PAGE as described previously (27). Separated proteins were transferred onto polyvinylidene difluoride membrane, incubated overnight at 4 °C in Tris-buffered saline, 0.1% Tween 20 (TBS-T) containing 5% nonfat milk powder, and probed with anti-GST polyclonal antibody (1:5000 dilution). Detection of horseradish peroxidase-coupled secondary antibody was achieved using SuperSignal West Dura (Pierce) and a Bio-Rad ChemiDoc gel documentation system for image capture.

RESULTS
Expression and Enzymatic Characterization of Recombinant PLCγ—PLCγ1 and PLCγ2 were expressed in E. coli BL21 cells and purified by GST affinity chromatography, as described under “Experimental Procedures.” Empirically determined, optimal protein production required maintaining bacterial cultures at 37 °C until A600 of 0.5–0.6, followed by induction of protein expression upon addition of 0.5 mM isopropyl 1-thio-β-d-galactopyranoside and 4 h of vigorous shaking at 25 °C. Fig. 1A schematically depicts the distinct domains of PLCγ1 and PLCγ2, which illustrate their close similarity because of common structural features, specifically the two EF hands, catalytic X and Y domains, and C2 domain, with the notable exception of an N-terminal PH domain absent from PLCγ2 (27). Fig. 1B shows the GST affinity-purified recombinant proteins analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining and immunoblot detection using an anti-GST antibody. The predicted molecular mass, including the GST tag (26 kDa), for GST-PLCγ1 and GST-PLCγ2 (1 μg) were analyzed by 8% SDS-PAGE and then either Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using anti-GST polyclonal antibody (right panel).

![Fig. 1. Expression of recombinant PLCγ1 and PLCγ2](image-url)

The corresponding proteins with appropriate molecular mass were observed as the top band in both Coomassie Brilliant Blue staining and on the immunoblot (Fig. 1B). The lowest band of 26 kDa is consistent with cleaved GST, which along with the much fainter intermediate molecular mass bands detected by the anti-GST antibody are probably the result of protein degradation occurring through the bacterial expression and purification processes. Hydrolysis of [3H]PPIP2 to [3H]IP3 was monitored to the enzymatic activities of recombinant PLCγ1 and PLCγ2 (36) was optimized for PLC activity by varying a series of parameters, including incubation time, reaction temperature, and protein concentrations. Linearity of [3H]PIP2 cleavage was obtained with 20 pmol of recombinant protein incubated with 220 μM [3H]PIP2, for 10 min at 25 °C (data not shown).

Fig. 2A shows the effect of total PIP2 concentration on PLCγ1 and PLCγ2 enzyme activity. For both PLCγ1 and PLCγ2, the maximum hydrolytic enzyme activity was obtained at 660 μM PIP2 with specific activity values of 1884 and 770 nmol/min/mg measured, respectively. The Michaelis-Menten constant, Km, was calculated by a Lineweaver-Burk reciprocal plot for both recombinant proteins. The Kms were similar in the range of 87–90 μM.
Although PLCδ1 and PLCζ had common enzymatic properties with regard to PIP2, the Ca2+ dependence of their activities was markedly different (Fig. 3A). PLCδ1 was activated between 0.01 and 0.1 μM Ca2+, whereas the threshold for PLCζ was 0.1 μM, with maximum activity at about 100 μM. The EC50 was 82 nM (Hill constant, 4.3) for PLCδ1 and 6 μM (Hill constant, 1.5) for PLCζ (calculated from Fig. 3A). PLCδ1 showed maximal activity over a broad pH range, varying between 5.2 and 6.0, in contrast with PLCζ, which displayed an optimum pH at 6.0 (Fig. 3B).

**Enzymatic Analysis of Domain-truncated PLCζ in Vitro**—To examine the role of the distinct structural domains on enzymatic activity, four domain-deletion constructs of PLCζ were expressed and purified as GST fusion proteins. Fig. 4A schematically illustrates the full-length PLCζ and the various domain-truncated PLCζ versions that have one or both EF hands removed (ζEF1 and ζEF1,2, respectively), the C2 domain deleted (ζC2) or all the above domains absent (ζXY), and their specific amino acid sequence coordinates. Following expression in E. coli and purification by affinity chromatography, samples of each protein were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using anti-GST polyclonal antibody (right panel). Lanes 1–4 show ζEF1, ζEF1,2, ζC2, and ζXY, respectively. C. PIP2 hydrolysis activity of the PLCζ domain-deletion constructs (20 pmol) using the standard [3H]PIP2 cleavage assay as described under “Experimental Procedures,” n = 3 ± S.E., using three different batches of recombinant protein, and each experiment was performed in duplicate.

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**Fig. 2.** Enzymatic characterization of recombinant PLCδ1 and PLCζ. A, [3H]PIP2 hydrolysis assay (see “Experimental Procedures”) of PLCδ1 and PLCζ activities as a function of PIP2 concentration. B, Lineweaver-Burk reciprocal plots for determination of the Km value for PIP2, yielding values of 75 and 87 μM for PLCδ1 and PLCζ, respectively. A, n = 2 ± S.E., using two different batches of recombinant proteins, and each experiment was performed in duplicate.

**Fig. 3.** Calcium and pH dependence of recombinant PLCδ1 and PLCζ. A, effect of [Ca2+] on the PIP2 hydrolysis activity of PLCδ1 and PLCζ. Enzyme assays were performed in different free [Ca2+] ranging from 0.1 mM to 0.1 μM, as outlined under “Experimental Procedures.” B, effect of pH on enzyme activity of PLCδ1 and PLCζ. The pH of the reaction was varied between pH 5 and pH 9, as outlined under “Experimental Procedures.” For all assays, n = 2 ± S.E., using two different batches of recombinant proteins, and each experiment was performed in duplicate.

**Fig. 4.** Effect of PLCζ domain deletion on PIP2 hydrolysis. A, schematic representation of the domain-deletion constructs of PLCζ, expressed as GST fusion proteins. B, affinity-purified, truncated PLCζ proteins (1 μg) analyzed by 8% SDS-PAGE and then either Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using anti-GST polyclonal antibody (right panel). Lanes 1–4 show ζEF1, ζEF1,2, ζC2, and ζXY, respectively. C. PIP2 hydrolysis activity of the PLCζ domain-deletion constructs (20 pmol) using the standard [3H]PIP2 cleavage assay as described under “Experimental Procedures,” n = 3 ± S.E., using three different batches of recombinant protein, and each experiment was performed in duplicate.
truncated PLCζ proteins. Enzyme activity assays performed for each of the recombinant proteins, determined by using the standard [3H]PIP$_2$ hydrolysis assay, showed that every domain-deletion construct retained some of the enzymatic activity present with the full-length PLCζ. The histogram of Fig. 4C plots the enzyme specific activity values obtained for each protein and reveals that the PLCζ proteins lacking either one or both EF hand domains or the C2 domain retained about 70% of the activity of the full-length PLCζ protein. Even the XY catalytic site alone exhibited well over half of the activity of the full-length PLCζ. These data suggest that the PLCζ catalytic site alone, comprising the X and Y domains, is capable of binding and hydrolyzing PIP$_2$ and that the C2 and EF hand domains are not essential for enzymatic activity in vitro.

Activity Analysis of PLCζ Domain Deletions Expressed in Mouse Eggs—Because the recombinant deletion constructs of PLCζ displayed robust enzymatic activity in vitro, further experiments were conducted to determine whether this is matched by their ability to trigger Ca$^{2+}$ oscillations when the corresponding cRNA is injected into mouse eggs (Fig. 5A). Injection of 0.2 mg/ml cRNA encoding the full-length PLCζ (WT in Fig. 5) was used as the positive control, and this caused a robust series of Ca$^{2+}$ oscillations in all eggs, as reported previously (27, 28). The Ca$^{2+}$ oscillations started about 20 min after injection, and spiking behavior persisted at an interval of about 3 min for over 2 h. In contrast, when the cRNAs for ΔEF1-PLCζ, ΔEF1,2-PLCζ, or ΔC2-PLCζ were microinjected into eggs under the same conditions, there were no Ca$^{2+}$ oscillations observed in any of the injected eggs (Fig. 5B). All the cRNAs used in these experiments were synthesized in parallel with batches of control (wild type PLCζ) cRNA, and as demonstrated previously (27, 28), the cRNA used for these microinjection experiments was able to generate proteins of the correct size when expressed in an in vitro reticulocyte lysate expression system (data not shown). Consequently, these data suggest that the absence of either of the EF hand domains or the C2 domain of PLCζ prevents it from being able to trigger Ca$^{2+}$ oscillations in intact eggs.

Quantitation of PLCζ Expression in Mouse Eggs—To verify that the domain-deleted versions of PLCζ were faithfully expressed as proteins in cRNA-microinjected eggs, we generated luciferase-tagged versions of these constructs to enable quantitation of relative protein expression. Fig. 6A shows that eggs injected with cRNA encoding the wild type PLCζ-luciferase (PLCζ-luc) fusion construct caused a series of Ca$^{2+}$ oscillations in eggs. This indicates that a fusion tag at the C terminus of PLCζ can also retain the ability to generate Ca$^{2+}$ oscillations, as has been previously shown for N-terminal tags (27). At the end of 4 h of monitoring the changes in Ca$^{2+}$, we measured the light emitted from the same set of eggs (in the absence of fluorescence excitation), and we found that they were luminescent (Fig. 6B). Every mouse egg injected with PLCζ-luc cRNA that showed clear expression of luciferase activity after 4 h ($n$ = 19) had also exhibited prior robust Ca$^{2+}$ oscillations. When
eggs were injected with ΔEF1-PLCζ-luc cRNA, however, none of the eggs showed any Ca2+ increase (n = 26), although they were visibly expressing luciferase activity, as confirmed by the intense luminescence detected at the end of the experiment (4 h), which was similar to that of the full-length PLCζ protein (Fig. 6, C and D). Similarly, upon injection of ΔC2-PLCζ-luc cRNA, all 25 eggs failed to show any Ca2+ oscillations, although they exhibited strong luciferase luminescence (Fig. 6, E and F). The exact level of luminescence in cells can depend upon the amount of luciferase protein, the intracellular pH, and the ATP concentration (39). Consequently, we quantified the relative expression of the luciferase fusion protein by lysing the mouse eggs in a buffer with a fixed concentration of ATP. Upon lysing the eggs in a luminometer, we determined that at the end of the experiment (4 h), a mean value of 0.19 pg of wild type PLCζ-luc protein was expressed per egg (n = 19). With ΔEF1-PLCζ-luc, a mean of 0.98 pg of protein was expressed per egg (n = 26), and with ΔC2-PLCζ-luc a mean of 2.7 pg of protein was expressed per egg (n = 25). These data show that the two PLCζ-luc domain-deletion constructs did not cause any Ca2+ oscillations but were demonstrably being expressed in the eggs that were injected with cRNA. The ΔEF1-PLCζ-luc and ΔC2-PLCζ-luc were expressed at levels that were 5- and 14-fold that of PLCζ-luc, respectively. This high level of expression of domain-deletion constructs more than compensates for the reduced specific activity of these proteins (70%) relative to full-length PLCζ. The threshold for PLCζ to cause a Ca2+ oscillation is around 50 fg (27), the two domain-deletion constructs were expressed at levels that are 20-50 times the amount required to cause Ca2+ oscillations with PLCζ.

**Effect of [Ca2+[^2]] on the Activity of PLCζ Domain Deletions**—The activity assays described above suggest that the X and Y catalytic domains alone are sufficient for in vitro enzymatic activity (Fig. 4), although all domains appear to be essential for Ca2+ oscillations activity in intact eggs (Figs. 5 and 6). To examine the role of selected domains on the Ca2+ sensitivity of PLCζ activity, we tested the ability of the domain-deletion constructs of PLCζ to hydrolyze [3H]PIP2 at different Ca2+ concentrations ranging from 0.1 mM to 0.1 nM (Fig. 7). Fig. 7A illustrates the Ca2+ dependence of specific PIP2 hydrolytic activity for the full-length PLCζ and each of the truncated proteins, and these are shown normalized to the maximum specific activity in Fig. 7B. Table I summarizes the EC50 and Hill coefficients of PLCζ, PLCζ, and domain-deletion constructs. Deletion of EF1 increased the EC50 by 10-fold and reduced the Hill coefficient from 4.135 to 2.204. Deletion of both EF hands led to a dramatic increase of the EC50 of PLCζ from 82 to 30 µM and a decrease of the Hill coefficient from 4.315 to 0.642. Deletion of the C2 domain did not change the EC50 of PLCζ but reduced the Hill coefficient from 4.315 to 1.139. Finally, deletion of both EF hands and C2 domain (PLCζ-XY) drastically changed the EC50 and Hill coefficient (62 and 0.327 µM, respectively). These results suggest the EF hands may play a direct modulatory role in the Ca2+ regulation of PLCζ activity.

**DISCUSSION**

PLCζ was identified as a sperm-specific PLC that is highly effective in causing Ca2+ oscillations and activation in mouse eggs (27). It appears to be responsible for the previously described PLC activity and Ca2+-releasing activity present in sperm extracts (27). PLCζ has also been identified as the protein factor that is responsible for causing Ca2+ oscillations after injection of whole sperm into mouse eggs (24). This "sperm factor" is proposed to enter the egg upon fusion with the sperm at fertilization and to be responsible for activating embryonic development in mammals (27, 28). Despite its significance in cell signaling at fertilization, the regulation of PLCζ activity and the mechanism by which PLCζ locates and targets the substrate in eggs are currently unknown (40). The aims of this study were to compare the enzymatic properties of the bacterially expressed, full-length PLCζ and PLCζ in vitro. We then determined which structural domains within the PLCζ sequence are essential for its enzymatic function and whether this correlates with its unique ability to cause sustained Ca2+ oscillations in mouse eggs.

The dependence of PLCζ and PLCζ activity on PIP2 concentration indicated that the K0.5 values for these closely related isoforms were very similar (Fig. 2) and in reasonable agreement with the K0.5 value obtained for recombinant PLCζ3 (142 µM) in another study (41). This suggests that the enzymes have similar affinity for their substrate PIP2, which is consistent with the presence of highly conserved X and Y active site domains found throughout the PI-PLC family.

To determine the role of different parts of PLCζ in PIP2 hydrolyzing activity, we generated a series of truncated proteins with deletions of selected structural domains (Fig. 4). Our data showed that the recombinant X + Y catalytic domains alone are able to retain considerable enzyme activity (70%) compared with that of the full-length PLCζ. Furthermore, the proteins with deletions of either the EF hands or the C2 domains also showed a similar PLC activity in vitro compared with that of full-length PLCζ. Thus, the absence of both EF...
hands and the C2 domain is unable to ablate PLC\(_\gamma\) enzyme activity when the X + Y catalytic domains are presented with

In order to assess the relevance of PLC\(_\gamma\) structural domains in triggering Ca\(^{2+}\) oscillations in intact cells, various truncated forms of PLC\(_\gamma\) were prepared, and individual mouse eggs were microinjected with cRNA corresponding to each domain-deletion construct (Fig. 5). Microinjection of PLC\(_\gamma\) cRNA into eggs is used in preference to recombinant PLC\(_\gamma\) protein because it avoids the problems of maintaining protein activity and solubility and to negate potential cross-contamination from bacterial proteins during the purification process. In previous work with recombinant PLCs, we have found that “purified” preparations can be also contaminated with IP\(_3\), and this can give the misleading impression that microinjection of a purified PLC directly triggers Ca\(^{2+}\) oscillations in intact eggs (25). In contrast, the use of microinjected cRNA ensures the sample delivered into the egg is free from such contamination; moreover, it is a robust and reliable method because we have demonstrated previously that the mouse egg is capable of efficiently translating the cRNA encoding the active sperm PLC\(_\gamma\) protein (27, 28, 42, 43). By using cRNA injection, we found that expression of the PLC\(_\gamma\) domain-deletion constructs in mouse eggs did not lead to the generation of any Ca\(^{2+}\) oscillations (Fig. 5). Because the "in vitro" assays demonstrated that these shortened versions possess PLC enzyme activity (Fig. 4), we can infer that the absence of either of the EF hands or the C2 domain of PLC\(_\gamma\) causes it to be unable to hydrolyze PIP\(_2\) under the conditions found in intact cells.

One issue that remains to be addressed with the use of microinjected cRNA samples into eggs is the level of expression achieved with each of the various PLC\(_\gamma\) truncations. For example, if the full-length PLC\(_\gamma\) is expressed with much greater efficiency than the PLC\(_\gamma\) domain deletions, then microinjection of equivalent amounts of cRNA would not be directly comparable. Thus, to examine whether expression of specific domain-deletion constructs was selectively impaired relative to full-length PLC\(_\gamma\), a series of luciferase-tagged fusion constructs for the domain deletions was microinjected into eggs. Although at levels of luciferase expression for the two domain deletions that were significantly higher than that of the full-length PLC\(_\gamma\)-luc (Fig. 6), no Ca\(^{2+}\) oscillations were produced by any of the domain-deleted PLC\(_\gamma\)-luc constructs. It was notable that the Ca\(^{2+}\) oscillations observed upon injection of the full-length PLC\(_\gamma\)-luc cRNA occurred about 1 h after injection, and at the end of the experiment (4 h) the level of expressed PLC\(_\gamma\)-luc protein was determined to be 190 fg/egg. If we assume a linear increase in PLC\(_\gamma\)-luc protein during the 4 h of recording, then we can estimate that the amount of PLC\(_\gamma\)-luc required to initiate Ca\(^{2+}\) oscillations is around 50 fg. This value is similar to our previous estimate that 20–50 fg of PLC\(_\gamma\) is required to initiate Ca\(^{2+}\) oscillations in eggs (27), as well as the estimate that 10–40 fg of VenusGFP-PLC\(_\gamma\) is required to initiate Ca\(^{2+}\) release (44). These data therefore suggest that the PLC\(_\gamma\)-luc has a similar efficiency in generating IP\(_3\) in eggs to other N-terminally tagged PLC\(_\gamma\) fusion proteins.

One potential reason for the striking difference between the activity observed in vitro and in intact eggs could be the way in which Ca\(^{2+}\) regulates PLCs. Most mammalian PLCs show some stimulation of activity with increasing Ca\(^{2+}\) concentration, but PLCs are often only stimulated by Ca\(^{2+}\) concentrations that are much higher than those found in resting cells. This is even true for PLC\(_{61}\) which has been reported previously to be one of the most Ca\(^{2+}\)-sensitive of the PLC isozymes, with marked stimulation by Ca\(^{2+}\) concentrations in the micro-
molar range (45). Our assays of PLC\(_{61}\) are consistent with this earlier work, because we found the EC\(_{50}\) value for Ca\(^{2+}\) stimulation of PLC\(_{61}\) to be about 6 \(\mu\)M (Fig. 3). In contrast to PLC\(_{61}\), PLC\(_\gamma\) is about 100 times more sensitive to Ca\(^{2+}\) with an EC\(_{50}\) of 50–80 nm, which is well within the range of reported resting Ca\(^{2+}\) concentration in eggs (3, 4). The difference between these isoforms means that PLC\(_\gamma\) is not only likely to show significant activity at resting Ca\(^{2+}\) levels, but it will be maximally active at 1 \(\mu\)M Ca\(^{2+}\), although PLC\(_{61}\) will not be fully activated until Ca\(^{2+}\) reaches 30 \(\mu\)M. As well as the high Ca\(^{2+}\) sensitivity, we found that the dependence of PLC\(_\gamma\) activity on Ca\(^{2+}\) had a Hill coefficient of 4.3, suggesting the binding of 4 Ca\(^{2+}\) molecules/protein. This is greater than the previous value of 0.9 observed by Kouchi et al. (29) in their study of PLC\(_\gamma\) expressed in insect cells. The reason for the difference is unclear, but it is not likely to be due to systematic differences in the assay, because the calculated EC\(_{50}\) values and the Hill coefficients for the Ca\(^{2+}\) dependence of PLC\(_{61}\) were very similar between the present data and those of Kouchi et al. (1.5 and 1.7, respectively). The difference in Ca\(^{2+}\) sensitivity and Hill coefficient between PLC\(_{61}\) and PLC\(_\gamma\) is of interest because it could be one source of explanation for why PLC\(_\gamma\) is so effective at causing Ca\(^{2+}\) oscillations in mouse eggs, and yet PLC\(_{61}\) is reported to be either ineffective (25) or at least much less effective (29). This idea is supported by our current observations of the behavior of the PLC\(_\gamma\) domain-deletion constructs in eggs.

It is clear from our data that PLC\(_\gamma\) is not effective in stimulating Ca\(^{2+}\) oscillations when it lacks one or both of its EF hand domains (Figs. 5 and 6). This result is consistent with previous observations with a short form of PLC\(_\gamma\) that lacks the first 110 amino acids at the N terminus (29), which corresponds to removal of the first EF hand and part of the second (Fig. 4A). The basic ability to hydrolyze PIP\(_2\) in vitro would be preserved for these deletion constructs, so the lack of Ca\(^{2+}\) oscillation-inducing activity in eggs injected with EF hand domain deletions of PLC\(_\gamma\) may be explained by their differential response to Ca\(^{2+}\) regulation. EF hands appear to play a vital role in the Ca\(^{2+}\) sensitivity of PLC\(_\gamma\) activity, because deletion of both EF hands dramatically changed the EC\(_{50}\) of PLC\(_\gamma\) from 82 nm to 30 \(\mu\)M. The N-terminal truncation of both EF hands would ablate the ability of this domain deletion to generate IP\(_3\) in an intact cell with a Ca\(^{2+}\) level of around 100 nm. Even deletion of the first EF hand domain raised the EC\(_{50}\) for Ca\(^{2+}\) to >700 nm, which is well above the resting Ca\(^{2+}\) level in an egg. The Hill coefficients were also decreased by deletion of one or both of the EF hand domains. The Hill coefficient describes the minimum number of interacting active sites required for enzyme function, suggesting that upon removal of the EF hands the minimum number of sites is reduced from 4 to 1. This in turn suggests that Ca\(^{2+}\) binding to the EF hands is important for the interaction of the X-Y domain with PIP\(_2\) substrate and thus for PLC\(_\gamma\) enzyme activity.

It is of interest that the loss of the C2 domain from PLC\(_\gamma\) also leads to the inability to cause Ca\(^{2+}\) oscillations in intact eggs, although the EC\(_{50}\) value for Ca\(^{2+}\) stimulation was unchanged. This result suggests two potential explanations. One possibility is linked to the significant change in the Hill coefficient, as the C2 domain removal caused a marked reduction in the Hill coefficient for Ca\(^{2+}\) stimulation from ~4 to 1. This loss of cooperativity in Ca\(^{2+}\) stimulation could be important for generating Ca\(^{2+}\) oscillations (see below). The other possibility is that the C2 domain plays an important role in targeting PLC\(_\gamma\) to the correct subcellular source of PIP\(_2\) in eggs. C2 domains display functional diversity and can be involved in binding to lipids, or proteins, in a way that can be Ca\(^{2+}\)-dependent (46).

The precise physiological mechanism by which IP\(_3\) produc-
tion leads to the exquisite pattern of Ca\textsuperscript{2+} oscillations at fertilization in mammalian eggs is unknown. By analogy with mechanisms proposed in somatic cells, the positive and delayed negative feedback effect of Ca\textsuperscript{2+} on the IP\textsubscript{3} receptor may be an important part of the mechanism for generating regular Ca\textsuperscript{2+} oscillations (6). The observation that Ca\textsuperscript{2+} oscillations similar, but not identical, to those occurring at fertilization can be supported this idea (12, 13). However, because of the high Ca\textsuperscript{2+} dependence of PLC\textgreekz activity and the properties of the IP\textsubscript{3} receptor combine to generate Ca\textsuperscript{2+} oscillations at fertilization in mammals.

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Addendum—A similar PLC\textgreekz study showing the importance of the EF and C2 domains has been reported (49) in overall agreement with our data.

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