PLCζ causes Ca²⁺ oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P₂

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ABSTRACT Sperm-specific phospholipase C ζ (PLCζ) activates embryo development by triggering intracellular Ca²⁺ oscillations in mammalian eggs indistinguishable from those at fertilization. Somatic PLC isoforms generate inositol 1,4,5-trisphosphate–mediated Ca²⁺ release by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) in the plasma membrane. Here we examine the subcellular source of PI(4,5)P₂ targeted by sperm PLCζ in mouse eggs. By monitoring egg plasma membrane PI(4,5)P₂ with a green fluorescent protein–tagged PH domain, we show that PLCζ causes Ca²⁺ oscillations in eliciting Ca²⁺ oscillations. Specific depletion of this PI(4,5)P₂ pool by plasma membrane targeting of an inositol polyphosphate-5-phosphatase (Inp54p) blocked PLCζ-mediated Ca²⁺ oscillations but not those stimulated by PLCζ or sperm. Immunolocalization of PI(4,5)P₂, PLCζ, and catalytically inactive PLCζ (ciPLCζ) revealed their colocalization to distinct vesicular structures inside the egg cortex. These vesicles displayed decreased PI(4,5)P₂ after PLCζ injection. Targeted depletion of vesicular PI(4,5)P₂ by expression of ciPLCζ-fused Inp54p inhibited the Ca²⁺ oscillations triggered by PLCζ or sperm but failed to affect those mediated by PLCζ1. In contrast to somatic PLCs, our data indicate that sperm PLCζ induces Ca²⁺ mobilization by hydrolyzing internal PI(4,5)P₂ stores, suggesting that the mechanism of mammalian fertilization comprises a novel phosphoinositide signaling pathway.

INTRODUCTION

Mammalian embryo development is initiated by a series of intracellular Ca²⁺ oscillations that start after sperm–egg fusion (Kline and Kline, 1992; Ozil and Swann, 1995; Swann and Yu, 2008). These Ca²⁺ oscillations appear to be caused by a sperm-specific protein, phospholipase C ζ (PLCζ), that is introduced into the egg upon sperm–egg fusion and leads to cycles of inositol 1,4,5-trisphosphate (InsP₃) production and Ca²⁺ release (Saunders et al., 2002; Swann and Yu, 2008). PLCζ is a 70- to 75-kDa PLC, and its expression or microinjection into mammalian eggs triggers Ca²⁺ oscillations indistinguishable from those seen at fertilization (Saunders et al., 2002; Yu et al., 2008). Knockdown of PLCζ levels in mouse sperm also leads to a reduced number of Ca²⁺ oscillations at fertilization (Knott et al., 2005). PLCζ has been found in mammals and in some other vertebrate species and could represent the essential “sperm factor” that initiates development. One unusual feature of PLCζ compared with other phosphatidylinositol (PI)-specific PLCs is its ability to hydrolyze phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and cause Ca²⁺ oscillations in eggs is significant because most other PI-specific PLCs do not trigger Ca²⁺ oscillations in eggs. The closest and best-characterized homologue of PLCζ is PLCζ1, which can cause Ca²⁺ oscillations in mouse eggs, but it has over 50 times lower potency (Kouchi et al., 2004; Nomikos et al., 2005). The intrinsic ability of sperm PLCζ to cause Ca²⁺ oscillations in eggs is significant because most other PI-specific PLCs do not trigger Ca²⁺ oscillations in eggs. The closest and best-characterized homologue of PLCζ is PLCζ1, which can cause Ca²⁺ oscillations in mouse eggs, but it has over 50 times lower potency (Kouchi et al., 2004; Nomikos et al., 2011c).

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The domain structure of PLCζ is similar to that of PLCβ1, including four EF hand domains, an XY catalytic domain, an XY linker region, and a C2 domain (Katan, 1998; Rebecchi and Pentyala, 2000; Saunders et al., 2002). The EF hand domains play a key role in the nanomolar Ca²⁺ sensitivity of PLCζ (Nomikos et al., 2005). The catalytic XY domain of PLCζ is well conserved and closely homologous to PLCβ1. The conserved active-site residues within this catalytic domain have been identified, and a mutation has been made (D210R) leading to a catalytically inactive PLCζ that does not trigger any Ca²⁺ oscillations in eggs (Saunders et al., 2002; Nomikos et al., 2011a, 2011b). A mutation in the catalytic domain of PLCζ has also been associated with loss of function in human sperm from a patient with male factor infertility (Heytens et al., 2009; Nomikos et al., 2011a). However, one major difference that distinguishes PLCζ from PLCβ1 and all other vertebrate PLCs is the absence of a PH domain. This is interesting, since the PH of PLCβ1 in particular is known to specifically bind P(4,5)P₂ in the plasma membrane (Katan, 1998; Rebecchi and Pentyala, 2000). This raises questions about whether and how PLCζ can bind to the plasma membrane. The C2 domain of PLCζ could potentially interact with phosphoinositides in eggs, and in vitro studies of the C2 domain have suggested that it can bind to PIP2 (Kouchi et al., 2005), but it has not been shown to interact with P(4,5)P₂ (Kouchi et al., 2005; Nomikos et al., 2011b). Of note, the XY linker of PLCζ—the segment between the X and Y catalytic domains—has been shown to have a high affinity for P(4,5)P₂ (Nomikos et al., 2011b). The affinity of the XY linker appears to be based on a polybasic charged region that is found in a number of other membrane-associated proteins (McLaughlin and Murray, 2005; Nomikos et al., 2007, 2011c).

Studies on somatic cells have clearly identified that the majority of cellular P(4,5)P₂ resides in the plasma membrane (Watt et al., 2002; Gampfer and Shapiro 2007). Furthermore, in response to agonist stimulation, it is the plasma membrane P(4,5)P₂ that undergoes rapid hydrolysis to generate the InsP₃ required for Ca²⁺ release. It has also been shown that PI-specific PLCs such as PLCβ1, PLCγ, PLCα, PLCε, and PLCζ all translocate to the plasma membrane upon stimulation (Katan, 1998; Rebecchi and Pentyala, 2000; Song et al., 2001; Suh et al., 2008). The localization of plasma membrane P(4,5)P₂ has been studied using the green fluorescent protein (GFP)-tagged PH domain of PLCβ1, since it is highly specific for P(4,5)P₂ binding (Lemmon et al., 1995; Holz et al., 2000). In somatic cells, the GFP-PH domain shows distinct plasma membrane staining. This localized staining is reduced in response to agonist stimulation due to a loss of P(4,5)P₂ and an increase in InsP₃, which competes for binding to the PH domain (Szentpetery et al., 2009). The localization of P(4,5)P₂ in eggs has also been studied using the GFP-PH domain (Halet et al., 2002; Chun et al., 2010) and shows distinct plasma membrane (oolemma) staining. However, this GFP-PH oolemmal P(4,5)P₂ staining in mouse eggs shows an increase in intensity at fertilization, not a decrease (Halet et al. 2002). The increase is transient and due to exocytosis, leading to increased availability of either phosphatidylinositol phosphate (PIP) or PIP kinases for P(4,5)P₂ synthesis (Terada et al., 2000; Wenk et al., 2001; Abbott and Ducibella, 2001). Given that we expect the sperm to hydrolyze P(4,5)P₂, it is unclear why a decrease in GFP-PH staining is not seen (Halet et al. 2002). Of interest, studies that have tagged PLCζ from various species with Venus GFP have failed to detect any clear sign of plasma membrane staining when PLCζ is expressed in either eggs or cell lines (Yoda et al. 2004; Ito et al. 2008; Phillips et al. 2011). The only localization of PLCζ that has been demonstrated is for the mouse PLCζ, which enters the pronuclei of activated mouse eggs (Larman et al., 2004; Yoda et al., 2004; Sone et al., 2005). However, the pronuclei do not form until many hours after fertilization or after PLCζ-induced Ca²⁺ oscillations have started, and this nuclear localization correlates with a loss of PLCζ function. It is unknown precisely where within the egg the sperm PLCζ is localized while it is actually stimulating Ca²⁺ release via P(4,5)P₂ hydrolysis. It is possible that only a small fraction of PLCζ is present at the plasma membrane and that this is sufficient to cause InsP₃ production and Ca²⁺ release in eggs. Alternatively, PLCζ could act upon a distinct P(4,5)P₂ pool that is not resident in the plasma membrane. There is evidence from studies using either the GFP-PH domain or anti-P(4,5)P₂ antibodies for the presence of P(4,5)P₂ on internal membranes and the nucleus (Watt et al., 2002; Hammond et al., 2009). Injecting fluorescently labeled PIPs also suggests that P(4,5)P₂ can exist in intracellular membranes (Golebiewska et al., 2008). However, in somatic cells, P(4,5)P₂ is kept low in internal membranes by the expression of an inositol polyphosphate-5-phosphatase (Stolz et al., 1998; Stefan et al., 2002; Yin and Janmey, 2003). In sea urchin and frog eggs, previous studies suggested that P(4,5)P₂ may be present in substantial amounts in yolk vesicles (Snow et al., 1996; Rice et al., 2000). However, mouse eggs do not contain yolk vesicles. In this study, we examined the distribution of PLCζ and its catalytically inactive mutant ciPLCζ in mouse eggs and also monitored the subcellular distribution and hydrolysis of P(4,5)P₂ using the GFP-PH domain or anti-P(4,5)P₂ antibodies. We depleted P(4,5)P₂ in specific subcellular compartments using targeted phosphatidylinositol phosphate 5 phosphatase (Inp54p) and examined the effect of these alterations upon Ca²⁺ oscillations stimulated by PLCζ and PLCβ1. Our results suggest that both PLCζ- and sperm-mediated Ca²⁺ release preferentially use an intracellular membranous source of P(4,5)P₂, in contrast to PLCβ1, which targets P(4,5)P₂ in the plasma membrane. The data may help explain the distinctive features of PLCζ and further suggest that fertilization in mammals involves a novel mode of phosphoinositide-induced Ca²⁺ signaling.

RESULTS

PLCζ and plasma membrane P(4,5)P₂
Previous studies failed to detect any plasma membrane targeting of PLCζ using fluorescent fusion protein tags (Larman et al., 2004; Yoda et al., 2004; Sone et al., 2005; Phillips et al., 2011). However, its subcellular localization in MII eggs and that of any of its discrete domains was not specifically investigated. We made yellow fluorescent protein (YFP)-tagged constructs of wild-type PLCζ, the catalytically inactive mutant (D210R) of PLCζ (ciPLCζ), the X-Y linker, the XY domain, and the C2 domain. Expression of these constructs all showed uniform fluorescence in the cytoplasm (Figure 1, A–D) regardless of whether eggs were held in metaphase, such as with nocodazole. The only localization of PLCζ noted was in the nucleus of the second polar body (Figure 1A). This nuclear localization was evidently similar to that seen in previous studies, since PLCζ also accumulated in the germinal vesicle, which is the large nucleus of the immature oocyte (Supplemental Figure S1). This lack of specific localization was in sharp contrast to that seen with GFP-tagged PH domain of PLCβ1, which showed distinctive plasma membrane localization (Figure 1C), consistent with a previous study (Halet et al., 2002).

It is possible that only a small fraction of PLCζ binds to and hydrolyzes P(4,5)P₂ in the plasma membrane and that this fraction is too low to detect with fluorescent fusion protein tags. GFP-PH localization was used to record the relative changes of P(4,5)P₂ in the plasma membrane of fertilizing mouse eggs (Halet et al., 2002), so we used this probe to examine dynamic P(4,5)P₂ changes in the
plasma membrane induced by PLCζ and compared this to PLCδ1. Both PLCs were introduced into eggs by microinjection of the corresponding cRNAs, although the amount of PLCδ1 was greater than that for PLCζ to compensate for its much-reduced Ca2+-releasing potency in eggs (Kouchi et al., 2004; Nomikos et al., 2011c). When GFP-PH–expressing eggs were injected with PLCζ or PLCδ1, a set of transient increases in plasma membrane GFP-PH domain localization were detected that were coincident with Ca2+ spikes (Figure 2, A and D). This result is consistent with previous studies of fertilization, in which elevations in PI(4,5)P2 were associated with exocytosis (Halet et al. 2002). When eggs were injected with PLCζ or PLCδ1 in the presence of cytochalasin B to inhibit exocytosis, a clear decrease in plasma membrane staining was observed only after PLCδ1 injection but not after injection of PLCζ (Figure 2, B and E). The rate of decrease in plasma membrane GFP-PH was enhanced after Ca2+ oscillations started. However, a decrease in plasma membrane GFP-PH staining was also seen under conditions where PLCδ1 was expressed at levels below those that cause Ca2+ oscillations (Figure 2G). These data are summarized for many different eggs in Figure 2H, which indicates that the GFP-PH domain readily detects decreases in plasma membrane PI(4,5)P2 after PLCδ1 injection over a range of effective concentrations. However, no change in plasma membrane PI(4,5)P2 was ever seen after injecting PLCζ even though it is far more potent at causing Ca2+ oscillations.

The requirement for plasma membrane PI(4,5)P2 in causing Ca2+ oscillations was then tested directly by expressing a GFP- and Lyn-tagged inositol polyphosphate-5-phosphatase (Inp54p) in eggs. The 5-phosphatase selectively removes the 5’ phosphate from PI(4,5)P2, and the Lyn-tagged version was previously used to deplete plasma membrane PI(4,5)P2 (Suh et al., 2006; Johnson et al., 2008; Lacramioara et al. 2010). Figure 3A shows that Lyn-GFP-Inp54p (LynPs) localized mostly to the plasma membrane in eggs. Following ~7 h of expression of LynPs, subsequent expression of PLCδ1 completely failed to cause Ca2+ oscillations in eggs, even though all similarly aged control eggs injected with PLCδ1 displayed robust Ca2+ oscillations (Figure 3B and Table 1). However, expressing LynPs for ~7 h had no effect on Ca2+ oscillations triggered by PLCζ injection, nor did it have any effect on Ca2+ oscillations following in vitro fertilization with sperm (Figure 3, C and D, and Table 1). These data show that PLCδ1 causes Ca2+ oscillations in mouse eggs by hydrolyzing plasma membrane PI(4,5)P2. In contrast, neither PLCζ nor sperm hydrolyzes any significant amount of plasma membrane PI(4,5)P2, yet both are able to generate a normal pattern of Ca2+ oscillations in the egg.

**PLCζ and intracellular PI(4,5)P2**

Confocal imaging of YFP-PLCζ failed to show any specific localization other than in the nucleus. However, the detection limit for fluorescent protein probes in cells is >100 nM (Niswender et al. 1995), and since PLCζ is active at ~1–10 nM in mouse eggs, the high YFP-PLCζ expression level required is such that it may mask any precise localization in the cell (Saunders et al., 2002). Selective immunostaining of expressed proteins in the cell offers the advantage of sensitivity. So we investigated the subcellular distribution of PLCζ by injecting cMyc-tagged cPLCζ (cMyc-PLCζ) or cMyc-tagged ciPLCζ (cMyc-ciPLCζ) and then fixed and stained eggs with anti-cMyc antibodies (Fili et al., 2006; Hammond et al., 2009). The amount of cMyc-PLCζ introduced into the egg was in the precise range that caused a physiological pattern of Ca2+ oscillations (data not shown). Figure 4 shows that eggs injected with either cMyc-PLCζ or cMyc-ciPLCζ displayed an intracellular staining pattern of patches decorated with bright vesicular structures. There was also some staining of cMyc-ciPLCζ in the microvolta (Figure 4A, iv). Because of the large size of mouse eggs (~75 μm), a higher-resolution view was obtained by scanning the top cortical section that most clearly illustrates the labeled vesicles. The overall pattern of staining was similar for both cMyc-PLCζ and cMyc-ciPLCζ expression that fully activates eggs after injection (Figure 4B, i and ii) and with the catalytically inactive cMyc-ciPLCζ, where eggs remain arrested at metaphase II of meiosis. This difference in enzymatic activity between the two constructs might, however, explain why we were able to observe some cMyc-ciPLCζ around the metaphase II spindle (Figure 4B, iii). In contrast, cMyc-PLCζ–activated eggs were able to form a pronucleus 4 h after injection, and some translocation into the nucleus was evident; this became very marked in eggs after 7 h (Figure 4B, i and ii). No antibody staining was observed in control eggs that were not injected with cMyc-PLCζ constructs (Figure 4C). Consequently, these data suggest that PLCζ has the specific ability to bind directly to internal vesicular membranes in mouse eggs.
For the binding of PLCζ to internal vesicular structures to be physiologically significant, there should be an intracellular source of PI(4,5)P2. We investigated this by using immunocytochemistry with anti-PI(4,5)P2 antibodies. Eggs were fixed and permeabilized with formaldehyde and Triton X-100 (see Materials and Methods). Figure 5 shows that eggs stained positively for PI(4,5)P2 in regions near the plasma membrane, as well as near the cortex, several microns from the plasma membrane. The cortical sections illustrate more clearly that PI(4,5)P2 staining was present in discrete vesicles within the egg cytoplasm. As shown in the cortical scan and magnification, these bright vesicles were similar to the distribution pattern of PLCζ. Much of the staining in the plasma membrane region showed many bright areas near the base of microvilli. It was of note that such PI(4,5)P2 immunostaining increased in intensity during oocyte maturation (see Supplemental Figure S2, A and B), suggesting that the temporal-specific augmentation of PI(4,5)P2 inside the egg might correlate physiologically with preparation for fertilization. We also noted that, in contrast to mature eggs, there were very few PI(4,5)P2-containing vesicles evident in the cytoplasm of CHO cells even though PI(4,5)P2 could be readily detected in the nucleus of CHO cells (Figure 5C).

To understand its potential physiological relevance in eggs, we investigated whether PI(4,5)P2 was depleted from these internal vesicular stores after PLCζ injection. Previous studies and our preliminary experiments showed that fixation conditions affect the relative preservation of the phospholipids in the plasma membrane versus the internal membranes (Sharma et al., 2008; Hammond et al., 2009). So in the subsequent experiments we examined the amount of PI(4,5)P2 staining in the plasma membrane and in the internal membranes, using two different fixation conditions. Figure 6 shows PI(4,5)P2 immunostaining of eggs fixed with formaldehyde (4%) and glutaraldehyde (0.05%), which preserves the plasma membrane. Immunodetection of PI(4,5)P2 was recorded in the plasma membrane, but this was not significantly affected by injection of PLCζ, either with or without the inhibition of exocytosis by cytochalasin B (Figure 6, A and B). After injection of PLCζ there was also no effect on PI(4,5)P2 staining in the plasma membrane of normal eggs, but in the presence of cytochalasin B there was a marked reduction in plasma membrane PI(4,5)P2 labeling. These data closely mimic those in Figure 2 and show that the intensity of PI(4,5)P2 levels in membrane compartments. We then fixed eggs in formaldehyde alone and Triton X-100, which we observed to better preserve the internal membranes over the plasma membrane, and, as shown in Figure 5, we found distinct labeling of PI(4,5)P2 in internal vesicular membranes. When PLCζ or PLCζ1 was injected into eggs, we found some loss of PI(4,5)P2 in internal vesicles, but the reduction of PI(4,5)P2 staining with PLCζ was much larger than with PLCζ1 (Figure 6, C and D). The diminution of PI(4,5)P2 staining was unaffected by cytochalasin B. These data suggest that PLCζ1 predominantly hydrolyzes PI(4,5)P2 in the plasma membrane, whereas PLCζ predominantly hydrolyzes an intracellular vesicle membrane source of PI(4,5)P2.

We further sought to establish the functional consequences of PI(4,5)P2 depletion in intracellular vesicles. To this, we again targeted the phosphatase Inp54p by fusing it to ciPLCζ, which was

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**FIGURE 2:** Plasma membrane PI(4,5)P2 changes monitored with GFP fused to the PLCζ1 PH domain (GFP-PH). Calcium and plasma membrane PI(4,5)P2 changes (ratio of pmGFP/cytoGFP) in eggs caused by PLCζ or PLCζ1 were recorded (A–G) starting 20 min after injection of RNA. Injection of PLCζ cRNA (0.002 μg/μl) caused Ca2+ oscillations and plasma membrane PI(4,5)P2 increases following each Ca2+ spike (A). A slight decrease (4.8 ± 2.6%) in resting level of PI(4,5)P2 was detected (A, H). Keeping eggs in medium containing 10 μg/ml cytochalasin B had little effect on changes in plasma membrane PI(4,5)P2 in PLCζ1-injected eggs (B, H). PLCζ1 (cRNA, >10 μg/μl) triggered Ca2+ oscillations and plasma membrane PI(4,5)P2 increases (D). During 2 h of imaging the resting level of PI(4,5)P2 showed little change in eggs with Ca2+ oscillations caused by PLCζ1 (C, H). In contrast, when eggs were treated with cytochalasin B, a dramatic consumption of plasma membrane PI(4,5)P2 was detected (22.0 ± 6.9%, E and H) as judged by the percentage change in signal over the 2 h from the start to end of the recording. A clear diminution of PI(4,5)P2 was also detected (12.7 ± 3.0%) in eggs injected with reduced amounts of PLCζ1 (5 μg/μl), which was insufficient to cause Ca2+ oscillations. A summary of the data is shown in H, with egg numbers indicated for each condition above the traces in A–G. Images of GFP-PH distribution in eggs injected with PLCζ1 or PLCζ1 and kept in cytoplasmic B for 4 h are shown in C and F, Bar, 10 μm.

For the binding of PLCζ to internal vesicular structures to be physiologically significant, there should be an intracellular source of PI(4,5)P2. We investigated this by using immunocytochemistry with anti-PI(4,5)P2 antibodies. Eggs were fixed and permeabilized with formaldehyde and Triton X-100 (see Materials and Methods). Figure 5 shows that eggs stained positively for PI(4,5)P2 in regions near the plasma membrane, as well as near the cortex, several microns from the plasma membrane. The cortical sections illustrate more clearly that PI(4,5)P2 staining was present in discrete vesicles within the egg cytoplasm. As shown in the cortical scan and magnification, these bright vesicles were similar to the distribution pattern of PLCζ. Much of the staining in the plasma membrane region showed many bright areas near the base of microvilli. It was of note that such PI(4,5)P2 immunostaining increased in intensity during oocyte maturation (see Supplemental Figure S2, A and B), suggesting that the temporal-specific augmentation of PI(4,5)P2 inside the egg might correlate physiologically with preparation for fertilization. We also noted that, in contrast to mature eggs, there were very few PI(4,5)P2-containing vesicles evident in the cytoplasm of CHO cells even though PI(4,5)P2 could be readily detected in the nucleus of CHO cells (Figure 5C).
blocked Ca\(^{2+}\) oscillations caused by PLC\(\delta\) accumulated at plasma membrane (A). Expression of LynPs expression either totally blocked or greatly inhibited the Ca\(^{2+}\) oscillations induced by injection of PLC\(\delta\) (Figure 2). This decrease in PI(4,5)P\(_2\) could not be due to InsP\(_3\) generation displacing the membrane PI(4,5)P\(_2\). It was notable that PLC\(\delta\) injection, even though PLC\(\delta\) is over an order of magnitude more potent than PLC\(\delta\) in causing Ca\(^{2+}\) oscillations. It is significant that we identified both PLC\(\delta\) and PI(4,5)P\(_2\) localization to intracellular vesicles, with PI(4,5)P\(_2\) staining in these vesicles being reduced greatly after PLC\(\delta\) injection than with PLC\(\delta\). For the first time in any type of egg, PI(4,5)P\(_2\)-specific phosphatases were used to confirm the requirement for PI(4,5)P\(_2\) to generate Ca\(^{2+}\) oscillations (Figures 3 and 7). However, different sources of PI(4,5)P\(_2\) are apparent, with PLC\(\delta\) having a requirement for plasma membrane PI(4,5)P\(_2\), whereas the sperm and PLC\(\delta\) require intracellular vesicular PI(4,5)P\(_2\).

### DISCUSSION

PLC\(\zeta\) has been identified as a sperm-specific protein that can trigger embryonic development in mammals (Saunders et al., 2002). The functional role of its domains in enzyme catalysis has been characterized (Kouchi et al., 2004; Nomikos et al., 2005, 2011). However, its mode of action in the egg has not been established. Because somatic PI-specific PLCs hydrolyze PI(4,5)P\(_2\) in the plasma membrane, a natural assumption is that PLC\(\zeta\) also targets plasma membrane PI(4,5)P\(_2\), given the strong evidence for a significant oolemmal pool of PI(4,5)P\(_2\) in mouse eggs (Halet et al., 2002). However, studies of fluorescently tagged PLC\(\zeta\) failed to show any plasma membrane localization in either eggs or somatic cell lines (Yoda et al., 2004; Sone et al., 2005; Ito et al. 2008; Phillips et al., 2011). Using PLC\(\delta\) as a comparative control, we observe a decrease in plasma membrane PI(4,5)P\(_2\) both during and after Ca\(^{2+}\) oscillations induced by PLC\(\delta\) if exocytosis is inhibited (Figure 2). This decrease in PI(4,5)P\(_2\) could not be due to InsP\(_3\) generation displacing our GFP-PH domain probe since this can only occur with high InsP\(_3\) levels, which would cause high-frequency Ca\(^{2+}\) oscillations (Halet et al., 2002). Yet we found a displacement of the GFP-PH domain from the plasma membrane with PLC\(\delta\) expression in the absence of any Ca\(^{2+}\) oscillations. These data clearly suggest that we can establish conditions to readily detect plasma membrane PI(4,5)P\(_2\) consumption after injection of PLC\(\delta\). However, under these conditions no decrease in plasma membrane PI(4,5)P\(_2\) is detected after PLC\(\delta\) injection, even though PLC\(\zeta\) is over an order of magnitude more potent than PLC\(\delta\) in causing Ca\(^{2+}\) oscillations. It is significant that we identified both PLC\(\zeta\) and PI(4,5)P\(_2\) localization to intracellular vesicles, with PI(4,5)P\(_2\) staining in these vesicles being reduced much greater after PLC\(\delta\) injection than with PLC\(\delta\). For the first time in any type of egg, PI(4,5)P\(_2\)-specific phosphatases were used to confirm the requirement for PI(4,5)P\(_2\) to generate Ca\(^{2+}\) oscillations (Figures 3 and 7).

Numerous internal membranes could be involved in making PI(4,5)P\(_2\) in cells. A previous study reported low levels of PI(4,5)P\(_2\) in the nuclear membrane, Golgi stack, endoplasmic reticulum, and various multivesicular bodies (Osborne et al., 2001; Watt et al. 2002; Stallings et al., 2005; Hammond et al., 2009). However, the nuclear membrane, Golgi, and various trafficking membranous systems

<table>
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<tr>
<th>Group</th>
<th>Blocked eggs</th>
<th>First spike timing (min)</th>
<th>Interval (min)</th>
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<tr>
<td>PLC(\delta) Control</td>
<td>0/30</td>
<td>96.7 ± 12.7</td>
<td>52.6 ± 2.6</td>
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<tr>
<td>LynPs</td>
<td>32/32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PLC(\zeta) Control</td>
<td>0/32</td>
<td>90.6 ± 7.9</td>
<td>26.5 ± 4.6</td>
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<tr>
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<td>10.1 ± 1.3</td>
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<tr>
<td>LynPs</td>
<td>0/17</td>
<td>3.7 ± 0.4*</td>
<td>10.1 ± 1.6</td>
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*First spike duration.
undergo fragmentation during the mitotic metaphase, and there are a range of vesicular membrane bodies that are dispersed throughout the cell (Altan-Bonnet et al., 2003; Xiang et al., 2007; Krauss and Haucke, 2007). Because mammalian eggs are arrested at metaphase of the second meiosis, the Golgi and other membrane-trafficking organelles can be transformed into many small vesicles (Payne and Schatten, 2003). The staining pattern we obtain for PLCζ organelles can be transformed into many small vesicles (Payne and Schatten, 2003). Because mammalian eggs are arrested at metaphase of the second meiosis, the Golgi and other membrane-trafficking organelles can be transformed into many small vesicles (Payne and Schatten, 2003).

The staining pattern we observe in mouse eggs appears more consistent with the vesicular distribution of the Golgi and other membrane-trafficking systems. It is not clear which PLCζ domains mediate interaction with such intracellular vesicles, as we did not find evidence for specific binding of the XY linker region or the C2 domain using YFP tags (Figure 1) or c-Myc tags or by immunocytochemistry (unpublished data). It is possible that precise targeting requires the combined interaction of both XY and C2 domains to bind specifically to intracellular membranes, since the type of polybasic cluster found in the PLCζ XY linker can potentially play a role in protein binding to the plasma membrane as much as any other membrane (Heo et al., 2006).

In our studies using mouse eggs, we made novel use of the Inp54p phosphatase, which specifically dephosphorylates P(4,5)P2 (Guo et al., 1999; Lacramioara et al., 2010). This yeast phosphatase was previously used to reduce P(4,5)P2 levels in the plasma membrane of somatic cells (Suh et al., 2006; Johnson et al., 2008). It had not been previously used to deplete internal membrane sources of P(4,5)P2, possibly because P(4,5)P2 in intracellular membranes is already kept very low by the action of 5-phosphatases (Stolz et al., 1998; Stefan et al., 2002; Yin and Janmey, 2003). We found that targeting Inp54p to an internal membrane with catalytically inactive PLCζ could inhibit Ca2+ oscillations in response to sperm and PLCζ but not to the intrinsically less potent PLCδ1. Although this result argues that PLCζ requires an internal membrane P(4,5)P2, we found that the targeted Inp54p did not completely block Ca2+ oscillations in all eggs. This may be because P(4,5)P2 is more difficult to deplete in the intracellular membranes of mammalian eggs (Snow et al., 1996).

Although our studies focused on mature mouse eggs, we noted that the intracellular vesicular staining of P(4,5)P2 in mouse eggs developed during oocyte maturation. This maturation of P(4,5)P2 was coincident with the reorganization of internal organelles (Payne and Schatten, 2003; FitzHarris et al., 2003, 2007; Dumollard et al., 2007; Yu et al., 2010). Several proteins have been reported to be involved in synthesis of internal membrane P(4,5)P2, including Arf1 (Roth et al., 1999; Jones et al., 2000), PIP (Cockcroft and Carvou, 2007), and PLD (Roth et al., 1999; Freyberg et al., 2003). Because mammalian eggs acquire the ability to produce appropriate Ca2+ oscillations after maturation (Carroll et al., 1996; Machaca, 2004), it is possible that the synchronous increase in internal levels of P(4,5)P2 represents a significant feature in maturation of the oocyte cytoplasm. Of interest,
Equatorial section  Cortical section  Magnification

**FIGURE 5:** The distribution of P(4,5)P2 in MII eggs. (A) Both ring-like plasma membrane P(4,5)P2 and intracellular P(4,5)P2 were detected in MII eggs. The cortical section and magnification illustrate the presence of intracellular P(4,5)P2 in vesicular structures, which aggregate into patch-like areas (ii and iii). (B) A negative control for P(4,5)P2 staining in which P(4,5)P2 antibody showed that P(4,5)P2 was present in yolk platelets (Snow et al., 2003). Fertilization might now also provide an exemplar of an InsP3 signaling mechanism that completely bypasses the plasma membrane.

**MATERIALS AND METHODS**

**Handling of gametes and microinjection of mouse eggs**

MF1 female mice (6–8 wk) were obtained from Harlan Laboratories (Indianapolis, IN) and were primed with pregnant mare’s serum gonadotrophin and human chorionic gonadotropin (hCG) 48 h apart. MII eggs were collected from mice ~15 h after hCG injection and kept in M2 medium. All the cRNAs or Ca2+ dyes were injected into eggs with a pulled fine needle driven by an air pump. The volume of injected sample can be controlled by air pressure as described previously (Swann et al., 2009). The eggs were handled in M2 medium through the whole process. For in vitro fertilization experiments, sperm was collected from the epididymis of 10-wk-old hybrid male mice (C57/CBA) and released into T6 medium containing 1.6 g/ml bovine serum albumin (BSA). Sperm were kept in T6 for 3 h for capacitation before they were added into Heps KsOM (Saunders et al., 2002; hKSOM), where the eggs were stuck onto the glass bottom of the dish.

In all our experiments for detecting and depleting P(4,5)P2 in eggs, we found that the results obtained with PLCζ were the same as for in vitro fertilization with sperm. The data imply that fertilization in mammals is an important example of a novel mechanism for phosphoinositide signaling in cells, in which the stimulus (i.e., PLCζ) acts upon intracellular P(4,5)P2 and not plasma membrane P(4,5)P2. This mechanism would appear to be physiologically appropriate because PLCζ is a soluble protein factor that should be able to diffuse throughout the egg cytosol and access all cytoplasmic membranes. Previous work in sea urchin eggs and frog eggs showed that P(4,5)P2 was present in yolk platelets (Snow et al., 1996; Rice et al., 2000). In ascidian eggs, a mathematical model of Ca2+ oscillations found that the Ca2+ wavefront could be simulated only if one assumed that InsP3 production (and hence P(4,5)P2 hydrolysis) occurred widely throughout the cell’s cytoplasm (Dupont and Dumollard, 2004). Of note, fertilization provided some of the first and preeminent examples of Ca2+ waves, Ca2+ oscillations, and InsP3-induced Ca2+ release that were the prelude to the ubiquitous establishment of phosphoinositide signaling in generating Ca2+ release from plasma membrane signals (Berridge et al., 2003). Fertilization might now also provide an exemplar of an InsP3 signaling mechanism that completely bypasses the plasma membrane.

**Plasmid construction and cRNA preparation**

pCR3-GFP-PHδ1 plasmid was kindly provided by G. Halet (University College London, London, United Kingdom). Full-length mouse PLCζ (1–647 amino acids), the EF hands (1–150 amino acids), the XY linker (308–385 amino acids), the XY domain (151–533 amino acids), and the C2 domain (521–647 amino acids) of mouse PLCζ were amplified by PCR from the original cDNA clone (GenBank accession number AF435950; Saunders et al., 2002) and Phusion polymerase enzymes (Finnzymes, Thermo Scientific, Vantaa, Finland), and the appropriate primers were used to incorporate a 5’-EcoRI site and a 3’-NotI site. The PCR product was cloned into a modified pCR3 vector containing an N’-terminal YFP tag. pcDNA3-1-cMyC-PLCζ was prepared as described previously (Saunders et al., 2002; Nomikos et al., 2005). Rat PLCζ (GenBank accession number M20637) was kindly provided by M. Katan (Cancer Research UK Centre for Cell and Molecular Biology, London, United Kingdom). We used the appropriate primers to incorporate a 5’-EcoRV site and a 3’-NotI site, and the PCR product was cloned into the pCR3 vector. The pcDNA3-Lyn-GFP-Inp54p plasmid was purchased from Addgene (Cambridge, MA). The pCR3-Lyn-GFP-Inp54p and pCR3-GFP-Inp54p plasmids were constructed by subcloning of Lyn-GFP-Inp54p or GFP-Inp54p into the pCR3 vector. For the construction of pCR3-ciPLCζ-GFP-Inp54p
ciPLCζ was amplified by PCR from a pCR3-PLCζD210R-luciferase plasmid described previously (Nomikos et al., 2011a) using Phusion polymerase and the appropriate primers to incorporate a 5′-KpnI site and a 3′-KpnI site. PCR product was cloned into the pCR3-GFP-Inp54p vector and restriction digests performed to confirm the correct orientation of the cloned insert. Each of the foregoing expression vector constructs was confirmed by dideoxynucleotide sequencing (Prism Big Dye Kit, ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Warrington, United Kingdom).

Following linearization of constructs, cRNA was synthesized using the mMessage Machine T7 kit (Applied Biosystems/Ambion, Austin, TX) and then polyadenylated using the poly(A) tailing kit (Applied Biosystems/Ambion), as per the manufacturer’s instructions.

Antibodies, immunostaining, and confocal imaging
Mouse monoclonal anti-PI(4,5)P2 antibody (clone 2C11; immunoglobulin M [IgM]) was purchased from Echelon Bioscience (Salt Lake City, UT) and used at 2.5 μg/μl. In negative controls, this antibody was preabsorbed with PI(4,5)P2 (Echelon Bioscience) for 20 min at room temperature. Rabbit polyclonal anti-cMyc (IgG) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 600-fold dilution. Fluor 488–conjugated goat anti–rabbit IgG and Fluor 594–conjugated goat anti–mouse IgG were purchased from Invitrogen (Carlsbad, CA), and both were used at 800-fold dilution. Methanol-free formaldehyde (FA), 16% (wt/vol), and 25% (vol/vol) electron microscopy-grade glutaraldehyde (GA) stock solutions were purchased from Park Scientific (Sunnyvale, CA) and Sigma-Aldrich (Poole, United Kingdom). FA was diluted to 8% (vol/vol) in phosphate-buffered saline (PBS) and stored at −20°C. Ca2+ and Mg2+-free PBS normal goat serum were purchased from Invitrogen. Mouse Ig blocking reagent (MKB-1113) was purchased from Vector Laboratories (Burlingame, CA) and used at 300-fold dilution.

In most experiments, eggs were fixed with 4% FA for 15 min. However, for plasma membrane PI(4,5)P2 staining, eggs were fixed with a combination of 4% FA and 0.05% GA (Sharma et al., 2008). We found that this method gave similar results for egg plasma membrane staining of lipids to those of Hammond et al. (2009), who used saponin permeabilization and a phosphate-free buffer. Fixed eggs from either protocol were quenched in 50 mM NH4Cl for 15 min and then permeabilized in 0.1% Triton X-100 for 15 min after rinses with PBS (Fili et al., 2006). Then these eggs were rinsed with PBS containing 0.2% BSA and blocked in 5% normal goat serum for 30 min. For mouse primary antibodies, eggs were incubated in Ig remover reagent for 1 h before blocking in serum. Eggs were incubated in primary antibody at 4°C overnight for PI(4,5)P2 staining or room temperature for 1 h for cMyc staining. Then they were washed twice by

<table>
<thead>
<tr>
<th>Group</th>
<th>Blocked eggs</th>
<th>First spike timing (min)</th>
<th>Interval (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i</td>
<td>ii</td>
<td></td>
</tr>
<tr>
<td>PLCδ1</td>
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<tr>
<td></td>
<td>ciPPs</td>
<td>12/51</td>
<td>39/51</td>
</tr>
</tbody>
</table>

aFirst spike duration.
bp < 0.05.

TABLE 2: Block of calcium oscillation by PI(4,5)P2 depletion with overdose of CiPPs.
Ca2+ oscillations were monitored by measuring Rhod Dextran fluorescence in eggs exposed to excitation light of 540–560 nm (emission measured with a 615-nm long pass filter) with a charge-coupled device camera driven by Image-Pro Plus (Media Cybernetics, Bethesda, MD). All calcium traces were plotted with SigmaPlot 6 (Systat Software, San Jose, CA) and displayed in a self-normalized ratio, \( \frac{f_t - f_0}{f_0} \). In PI(4,5)P2 depletion experiments, the Ca2+ oscillations were observed for the first calcium spike timing (in PLCζ or PLCβ1 groups) or the first spike duration (in the in vitro fertilization [IVF] groups) and the interval between spikes were calculated and the differences between groups analyzed with Student’s t test.

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