

# PLC $\zeta$ causes Ca<sup>2+</sup> oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P<sub>2</sub>

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**ABSTRACT** Sperm-specific phospholipase C  $\zeta$  (PLC $\zeta$ ) activates embryo development by triggering intracellular Ca<sup>2+</sup> oscillations in mammalian eggs indistinguishable from those at fertilization. Somatic PLC isozymes generate inositol 1,4,5-trisphosphate-mediated Ca<sup>2+</sup> release by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) in the plasma membrane. Here we examine the subcellular source of PI(4,5)P<sub>2</sub> targeted by sperm PLC $\zeta$  in mouse eggs. By monitoring egg plasma membrane PI(4,5)P<sub>2</sub> with a green fluorescent protein-tagged PH domain, we show that PLC $\zeta$  effects minimal loss of PI(4,5)P<sub>2</sub> from the oolemma in contrast to control PLC $\delta$ 1, despite the much higher potency of PLC $\zeta$  in eliciting Ca<sup>2+</sup> oscillations. Specific depletion of this PI(4,5)P<sub>2</sub> pool by plasma membrane targeting of an inositol polyphosphate-5-phosphatase (Inp54p) blocked PLC $\delta$ 1-mediated Ca<sup>2+</sup> oscillations but not those stimulated by PLC $\zeta$  or sperm. Immunolocalization of PI(4,5)P<sub>2</sub>, PLC $\zeta$ , and catalytically inactive PLC $\zeta$  (ciPLC $\zeta$ ) revealed their colocalization to distinct vesicular structures inside the egg cortex. These vesicles displayed decreased PI(4,5)P<sub>2</sub> after PLC $\zeta$  injection. Targeted depletion of vesicular PI(4,5)P<sub>2</sub> by expression of ciPLC $\zeta$ -fused Inp54p inhibited the Ca<sup>2+</sup> oscillations triggered by PLC $\zeta$  or sperm but failed to affect those mediated by PLC $\delta$ 1. In contrast to somatic PLCs, our data indicate that sperm PLC $\zeta$  induces Ca<sup>2+</sup> mobilization by hydrolyzing internal PI(4,5)P<sub>2</sub> stores, suggesting that the mechanism of mammalian fertilization comprises a novel phosphoinositide signaling pathway.

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## INTRODUCTION

Mammalian embryo development is initiated by a series of intracellular Ca<sup>2+</sup> oscillations that start after sperm–egg fusion (Kline and Kline, 1992; Ozil and Swann, 1995; Swann and Yu, 2008). These Ca<sup>2+</sup> oscillations appear to be caused by a sperm-specific protein, phospholipase C  $\zeta$  (PLC $\zeta$ ), that is introduced into the egg upon sperm–egg fusion and leads to cycles of inositol 1,4,5-trisphosphate

(InsP<sub>3</sub>) production and Ca<sup>2+</sup> release (Saunders *et al.*, 2002; Swann and Yu, 2008). PLC $\zeta$  is a 70- to 75-kDa PLC, and its expression or microinjection into mammalian eggs triggers Ca<sup>2+</sup> oscillations indistinguishable from those seen at fertilization (Saunders *et al.*, 2002; Yu *et al.*, 2008). Knockdown of PLC $\zeta$  levels in mouse sperm also leads to a reduced number of Ca<sup>2+</sup> oscillations at fertilization (Knott *et al.*, 2005). PLC $\zeta$  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 PLC $\zeta$  compared with other phosphatidylinositol (PI)-specific PLCs is its ability to hydrolyze phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) and cause InsP<sub>3</sub> production at nanomolar levels of intracellular Ca<sup>2+</sup>; PLC $\zeta$  is half-maximally active at resting Ca<sup>2+</sup> levels (Kouchi *et al.*, 2004; Nomikos *et al.*, 2005). The intrinsic ability of sperm PLC $\zeta$  to cause Ca<sup>2+</sup> oscillations in eggs is significant because most other PI-specific PLCs do not trigger Ca<sup>2+</sup> oscillations in eggs. The closest and best-characterized homologue of PLC $\zeta$  is PLC $\delta$ 1, which can cause Ca<sup>2+</sup> oscillations in mouse eggs, but it has over 50 times lower potency (Kouchi *et al.*, 2004; Nomikos *et al.*, 2011c).

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Abbreviations used: ciPLC $\zeta$ , catalytically inactive PLC $\zeta$ ; cMyc-ciPLC $\zeta$ , cMyc-tagged ciPLC $\zeta$ ; Inp54p, phosphatidylinositol phosphate 5 phosphatase; InsP<sub>3</sub>, inositol 1,4,5-trisphosphate; LynPs, Lyn-GFP-tagged Inp54p; PI(4,5)P<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PLC $\zeta$ , phospholipase C  $\zeta$ .

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The domain structure of PLC $\zeta$  is similar to that of PLC $\delta$ 1, including four EF hand domains, an XY catalytic domain, an XY linker region, and a C2 domain (Katan, 1998; Rebecchi and Pentylala, 2000; Saunders *et al.*, 2002). The EF hand domains play a key role in the nanomolar Ca<sup>2+</sup> sensitivity of PLC $\zeta$  (Nomikos *et al.*, 2005). The catalytic XY domain of PLC $\zeta$  is well conserved and closely homologous to PLC $\delta$ 1. The conserved active-site residues within this catalytic domain have been identified, and a mutation has been made (D210R) leading to a catalytically inactive PLC $\zeta$  that does not trigger any Ca<sup>2+</sup> oscillations in eggs (Saunders *et al.*, 2002; Nomikos *et al.*, 2011a, 2011b). A mutation in the catalytic domain of PLC $\zeta$  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 PLC $\zeta$  from PLC $\delta$ 1 and all other vertebrate PLCs is the absence of a PH domain. This is interesting, since the PH of PLC $\delta$ 1 in particular is known to specifically bind PI(4,5)P<sub>2</sub> in the plasma membrane (Katan, 1998; Rebecchi and Pentylala, 2000). This raises questions about whether and how PLC $\zeta$  can bind to the plasma membrane. The C2 domain of PLC $\zeta$  could potentially interact with phosphoinositides in eggs, and in vitro studies of the C2 domain have suggested that it can bind to PI(3)P (Kouchi *et al.*, 2005), but it has not been shown to interact with PI(4,5)P<sub>2</sub> (Kouchi *et al.*, 2005; Nomikos *et al.*, 2011b). Of note, the XY linker of PLC $\zeta$ —the segment between the X and Y catalytic domains—has been shown to have a high affinity for PI(4,5)P<sub>2</sub> (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 PI(4,5)P<sub>2</sub> resides in the plasma membrane (Watt *et al.*, 2002; Gamper and Shapiro 2007). Furthermore, in response to agonist stimulation, it is the plasma membrane PI(4,5)P<sub>2</sub> that undergoes rapid hydrolysis to generate the InsP<sub>3</sub> required for Ca<sup>2+</sup> release. It has also been shown that PI-specific PLCs such as PLC $\beta$ , PLC $\gamma$ , PLC $\delta$ , PLC $\epsilon$ , and PLC $\eta$  all translocate to the plasma membrane upon stimulation (Katan, 1998; Rebecchi and Pentylala, 2000; Song *et al.*, 2001; Suh *et al.*, 2008). The localization of plasma membrane PI(4,5)P<sub>2</sub> has been studied using the green fluorescent protein (GFP)-tagged PH domain of PLC $\delta$ 1, since it is highly specific for PI(4,5)P<sub>2</sub> 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 PI(4,5)P<sub>2</sub> and an increase in InsP<sub>3</sub>, which competes for binding to the PH domain (Szentpetery *et al.*, 2009). The localization of PI(4,5)P<sub>2</sub> 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 PI(4,5)P<sub>2</sub> 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 PI(4,5)P<sub>2</sub> synthesis (Terada *et al.*, 2000; Wenk *et al.*, 2001; Abbott and Ducibella, 2001). Given that we expect the sperm to hydrolyze PI(4,5)P<sub>2</sub>, it is unclear why a decrease in GFP-PH staining is not seen (Halet *et al.* 2002). Of interest, studies that have tagged PLC $\zeta$  from various species with Venus GFP have failed to detect any clear sign of plasma membrane staining when PLC $\zeta$  is expressed in either eggs or cell lines (Yoda *et al.* 2004; Ito *et al.* 2008; Phillips *et al.* 2011). The only localization of PLC $\zeta$  that has been demonstrated is for the mouse PLC $\zeta$ , 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 PLC $\zeta$ -induced Ca<sup>2+</sup> oscillations have started, and this nuclear localization correlates with a loss of PLC $\zeta$  function. It is unknown precisely where within the egg the sperm PLC $\zeta$  is localized while it is actually stimulating Ca<sup>2+</sup> release via PI(4,5)P<sub>2</sub> hydrolysis.

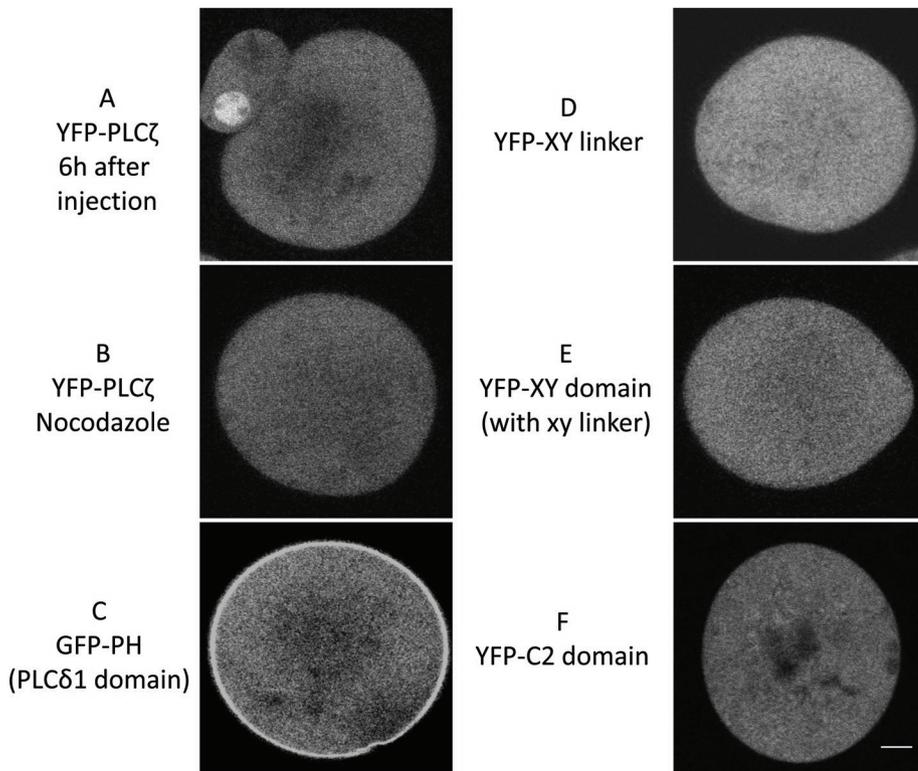
It is possible that only a small fraction of PLC $\zeta$  is present at the plasma membrane and that this is sufficient to cause InsP<sub>3</sub> production and Ca<sup>2+</sup> release in eggs. Alternatively, PLC $\zeta$  could act upon a distinct PI(4,5)P<sub>2</sub> pool that is not resident in the plasma membrane. There is evidence from studies using either the GFP-PH domain or anti-PI(4,5)P<sub>2</sub> antibodies for the presence of PI(4,5)P<sub>2</sub> on internal membranes and the nucleus (Watt *et al.*, 2002; Hammond *et al.*, 2009). Injecting fluorescently labeled PIPs also suggests that PI(4,5)P<sub>2</sub> can exist in intracellular membranes (Golebiewska *et al.*, 2008). However, in somatic cells, PI(4,5)P<sub>2</sub> 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 PI(4,5)P<sub>2</sub> 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 PLC $\zeta$  and its catalytically inactive mutant ciPLC $\zeta$  in mouse eggs and also monitored the subcellular distribution and hydrolysis of PI(4,5)P<sub>2</sub> using the GFP-PH domain or anti-PI(4,5)P<sub>2</sub> antibodies. We depleted PI(4,5)P<sub>2</sub> in specific subcellular compartments using targeted phosphatidylinositol phosphate 5 phosphatase (Inp54p) and examined the effect of these alterations upon Ca<sup>2+</sup> oscillations stimulated by PLC $\zeta$  and PLC $\delta$ 1. Our results suggest that both PLC $\zeta$ - and sperm-mediated Ca<sup>2+</sup> release preferentially use an intracellular membranous source of PI(4,5)P<sub>2</sub>, in contrast to PLC $\delta$ 1, which targets PI(4,5)P<sub>2</sub> in the plasma membrane. The data may help explain the distinctive features of PLC $\zeta$  and further suggest that fertilization in mammals involves a novel mode of phosphoinositide-induced Ca<sup>2+</sup> signaling.

## RESULTS

### PLC $\zeta$ and plasma membrane PI(4,5)P<sub>2</sub>

Previous studies failed to detect any plasma membrane targeting of PLC $\zeta$  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 PLC $\zeta$ , the catalytically inactive mutant (D210R) of PLC $\zeta$  (ciPLC $\zeta$ ), 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 PLC $\zeta$  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 PLC $\zeta$  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 PLC $\delta$ 1, 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 PLC $\zeta$  binds to and hydrolyzes PI(4,5)P<sub>2</sub> 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 PI(4,5)P<sub>2</sub> in the plasma membrane of fertilizing mouse eggs (Halet *et al.*, 2002), so we used this probe to examine dynamic PI(4,5)P<sub>2</sub> changes in the



**FIGURE 1:** Distribution of YFP-tagged PLC $\zeta$  (YFP-PLC $\zeta$ ) and PLC $\zeta$  domains in mouse eggs. YFP-PLC $\zeta$  evenly distributed in the PN stage (6 h after injection, A) or in MII stage (treated with nocodazole, B) mouse eggs, and no accumulation on specific compartments can be detected. GFP-PH (PLC $\delta$ 1) showed strong ring-like signal on the plasma membrane (C). Potential membrane association domains of PLC $\zeta$ ; XY linker (D), XY domain (E), or C2 domain (F) showed even cytoplasmic distribution similar to full-length PLC $\zeta$ . Bar, 10  $\mu$ m.

plasma membrane induced by PLC $\zeta$  and compared this to PLC $\delta$ 1. Both PLCs were introduced into eggs by microinjection of the corresponding cRNAs, although the amount of PLC $\delta$ 1 was greater than that for PLC $\zeta$  to compensate for its much-reduced Ca $^{2+}$ -releasing potency in eggs (Kouchi *et al.*, 2004; Nomikos *et al.*, 2011c). When GFP-PH-expressing eggs were injected with PLC $\zeta$  or PLC $\delta$ 1, a set of transient increases in plasma membrane GFP-PH domain localization were detected that were coincident with Ca $^{2+}$  spikes (Figure 2, A and D). This result is consistent with previous studies of fertilization, in which elevations in PI(4,5)P $_2$  were associated with exocytosis (Halet *et al.* 2002). When eggs were injected with PLC $\zeta$  or PLC $\delta$ 1 in the presence of cytochalasin B to inhibit exocytosis, a clear decrease in plasma membrane staining was observed only after PLC $\delta$ 1 injection but not after injection of PLC $\zeta$  (Figure 2, B and E). The rate of decrease in plasma membrane GFP-PH was enhanced after Ca $^{2+}$  oscillations started. However, a decrease in plasma membrane GFP-PH staining was also seen under conditions where PLC $\delta$ 1 was expressed at levels below those that cause Ca $^{2+}$  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)P $_2$  after PLC $\delta$ 1 injection over a range of effective concentrations. However, no change in plasma membrane PI(4,5)P $_2$  was ever seen after injecting PLC $\zeta$  even though it is far more potent at causing Ca $^{2+}$  oscillations.

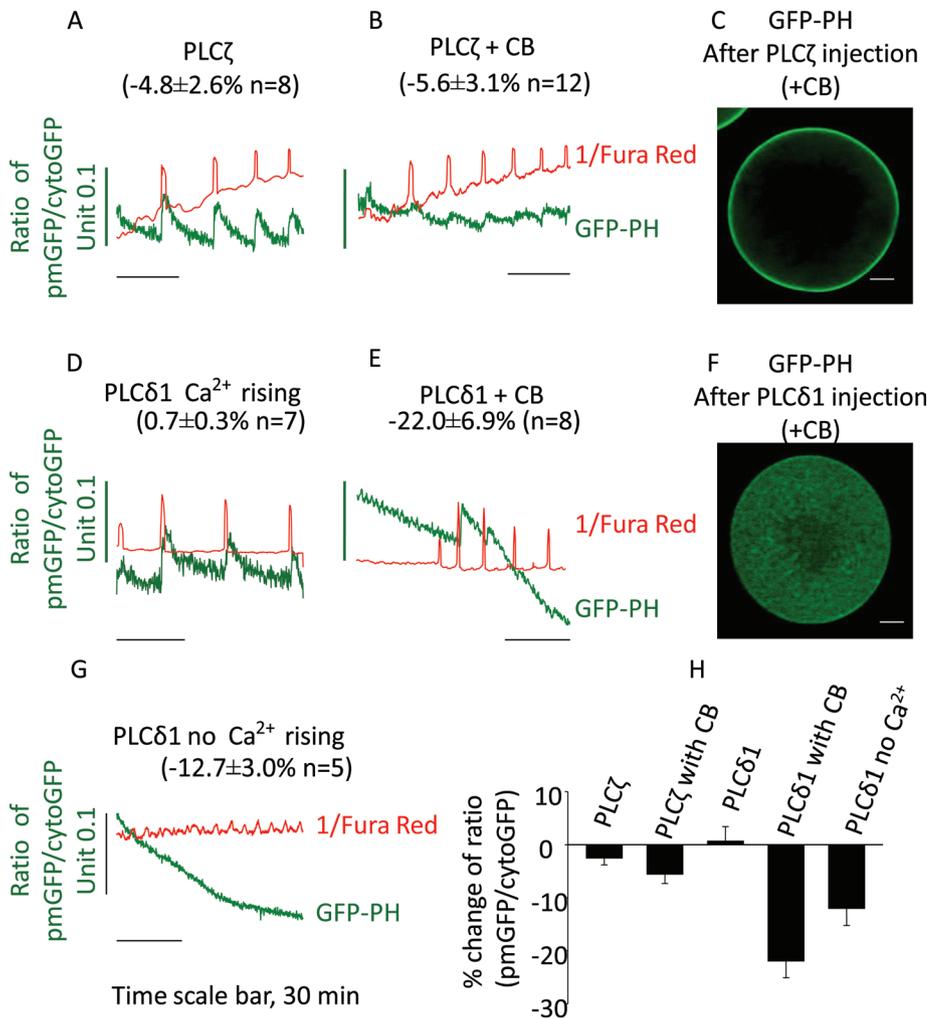
The requirement for plasma membrane PI(4,5)P $_2$  in causing Ca $^{2+}$  oscillations was then tested directly by expressing a GFP- and Lyn-tagged inositol polyphosphate-5-phosphatase (Inp54p) in eggs. The 5-phosphatase selectively removes the 5' phosphate from PI(4,5)P $_2$ , and the Lyn-tagged version was previously used to de-

plete plasma membrane PI(4,5)P $_2$  (Suh *et al.*, 2006; Johnson *et al.*, 2008; Lacramiora *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 PLC $\delta$ 1 completely failed to cause Ca $^{2+}$  oscillations in eggs, even though all similarly aged control eggs injected with PLC $\delta$ 1 displayed robust Ca $^{2+}$  oscillations (Figure 3B and Table 1). However, expressing LynPs for ~7 h had no effect on Ca $^{2+}$  oscillations triggered by PLC $\zeta$  injection, nor did it have any effect on Ca $^{2+}$  oscillations following in vitro fertilization with sperm (Figure 3, C and D, and Table 1). These data show that PLC $\delta$ 1 causes Ca $^{2+}$  oscillations in mouse eggs by hydrolyzing plasma membrane PI(4,5)P $_2$ . In contrast, neither PLC $\zeta$  nor sperm hydrolyzes any significant amount of plasma membrane PI(4,5)P $_2$ , yet both are able to generate a normal pattern of Ca $^{2+}$  oscillations in the egg.

### PLC $\zeta$ and intracellular PI(4,5)P $_2$

Confocal imaging of YFP-PLC $\zeta$  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 PLC $\zeta$  is active at ~1–10 nM in mouse eggs, the high YFP-PLC $\zeta$  expression level required is such that it may mask any pre-

cise 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 PLC $\zeta$  by injecting cMyc-tagged cPLC $\zeta$  (cMyc-PLC $\zeta$ ) or cMyc-tagged ciPLC $\zeta$  (cMyc-ciPLC $\zeta$ ) and then fixed and stained eggs with anti-cMyc antibodies (Fili *et al.*, 2006; Hammond *et al.*, 2009). The amount of cMyc-PLC $\zeta$  introduced into the egg was in the precise range that caused a physiological pattern of Ca $^{2+}$  oscillations (data not shown). Figure 4 shows that eggs injected with either cMyc-PLC $\zeta$  or cMyc-ciPLC $\zeta$  displayed an intracellular staining pattern of patches decorated with bright vesicular structures. There was also some staining of cMyc-ciPLC $\zeta$  in the microvilli (Figure 4A, iv). Because of the large size of mouse eggs (~75  $\mu$ m), 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-PLC $\zeta$  expression that fully activates eggs after injection (Figure 4B, i and ii) and with the catalytically inactive cMyc-ciPLC $\zeta$ , 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-ciPLC $\zeta$  around the metaphase II spindle (Figure 4B, iii). In contrast, cMyc-PLC $\zeta$ -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-PLC $\zeta$  constructs (Figure 4C). Consequently, these data suggest that PLC $\zeta$  has the specific ability to bind directly to internal vesicular membranes in mouse eggs.



**FIGURE 2:** Plasma membrane PI(4,5)P<sub>2</sub> changes monitored with GFP fused to the PLC $\delta$ 1 PH domain (GFP-PH). Calcium and plasma membrane PI(4,5)P<sub>2</sub> changes (ratio of pmGFP/cytoGFP) in eggs caused by PLC $\zeta$  or PLC $\delta$ 1 were recorded (A–G) starting ~20 min after injection of RNA. Injection of PLC $\zeta$  cRNA (0.002  $\mu$ g/ $\mu$ l) caused Ca $^{2+}$  oscillations and plasma membrane PI(4,5)P<sub>2</sub> increases following each Ca $^{2+}$  spike (A). A slight decrease (4.8  $\pm$  2.6%) in resting level of PI(4,5)P<sub>2</sub> was detected (A, H). Keeping eggs in medium containing 10  $\mu$ g/ml cytochalasin B had little effect on changes in plasma membrane PI(4,5)P<sub>2</sub> in PLC $\zeta$ -injected eggs (B, H). PLC $\delta$ 1 (cRNA, >10  $\mu$ g/ $\mu$ l) triggered Ca $^{2+}$  oscillations and plasma membrane PI(4,5)P<sub>2</sub> increases (D). During 2 h of imaging the resting level of PI(4,5)P<sub>2</sub> showed little change in eggs with Ca $^{2+}$  oscillations caused by PLC $\delta$ 1 (C, H). In contrast, when eggs were treated with cytochalasin B, a dramatic consumption of plasma membrane PI(4,5)P<sub>2</sub> was detected (22.0  $\pm$  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 consumption of plasma membrane PI(4,5)P<sub>2</sub> was also detected (12.7  $\pm$  3.0%) in eggs injected with reduced amounts of PLC $\delta$ 1 (5  $\mu$ g/ $\mu$ l), which was insufficient to cause Ca $^{2+}$  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 PLC $\zeta$  or PLC $\delta$ 1 and kept in cytochalasin B for 4 h are shown in C and F. Bar, 10  $\mu$ m.

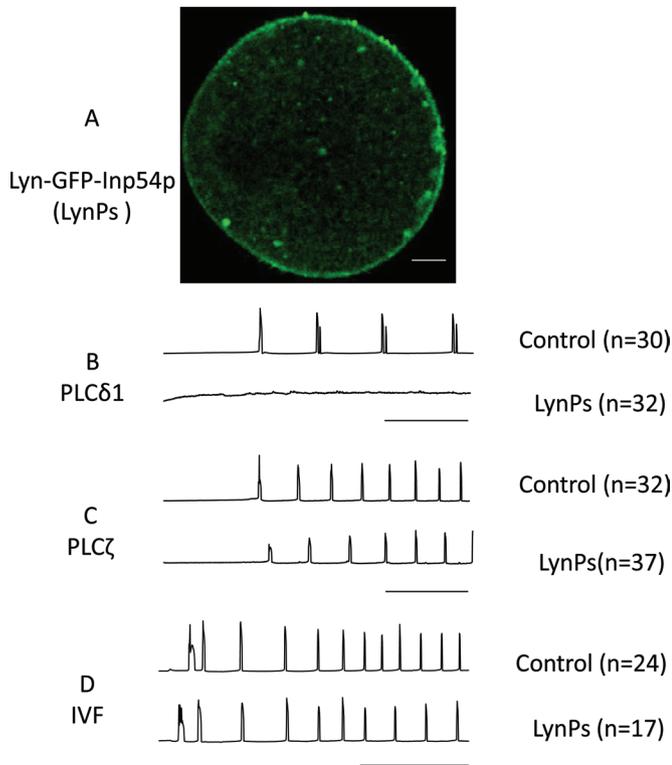
For the binding of PLC $\zeta$  to internal vesicular structures to be physiologically significant, there should be an intracellular source of PI(4,5)P<sub>2</sub>. We investigated this by using immunocytochemistry with anti-PI(4,5)P<sub>2</sub> antibodies. Eggs were fixed and permeabilized with formaldehyde and Triton X-100 (see *Materials and Methods*). Figure 5 shows that eggs stained positively for PI(4,5)P<sub>2</sub> 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)P<sub>2</sub> 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 PLC $\zeta$ . 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)P<sub>2</sub> immunostaining increased in intensity during oocyte maturation (see Supplemental Figure S2, A and B), suggesting that the temporal-specific augmentation of PI(4,5)P<sub>2</sub> 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)P<sub>2</sub>-containing vesicles evident in the cytoplasm of CHO cells even though PI(4,5)P<sub>2</sub> 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)P<sub>2</sub> was depleted from these internal vesicular stores after PLC $\zeta$  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)P<sub>2</sub> staining in the plasma membrane and in the internal membranes, using two different fixation conditions. Figure 6 shows PI(4,5)P<sub>2</sub> immunostaining of eggs fixed with formaldehyde (4%) and glutaraldehyde (0.05%), which preserves the plasma membrane. Immunodetection of PI(4,5)P<sub>2</sub> was recorded in the plasma membrane, but this was not significantly affected by injection of PLC $\zeta$ , either with or without the inhibition of exocytosis by cytochalasin B (Figure 6, A and B). After injection of PLC $\delta$ 1 there was also no effect on PI(4,5)P<sub>2</sub> 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)P<sub>2</sub> labeling. These data closely mimic those in Figure 2 and show that the intensity of PI(4,5)P<sub>2</sub> immunostaining can be used to estimate relative PI(4,5)P<sub>2</sub> 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)P<sub>2</sub> in internal membrane vesicles. When PLC $\zeta$  or PLC $\delta$ 1 was injected into eggs, we found some loss of PI(4,5)P<sub>2</sub> in internal vesicles, but the reduction of PI(4,5)P<sub>2</sub> staining with PLC $\zeta$  was much larger than with PLC $\delta$ 1 (Figure 6, C and D). The diminution of PI(4,5)P<sub>2</sub> staining was unaffected by cytochalasin B. These data suggest that PLC $\delta$ 1 predominantly hydrolyzes PI(4,5)P<sub>2</sub> in the plasma membrane, whereas PLC $\zeta$  predominantly hydrolyzes an intracellular membrane vesicular source of PI(4,5)P<sub>2</sub>.

We further sought to establish the functional consequences of PI(4,5)P<sub>2</sub> depletion in intracellular vesicles. To do this, we again targeted the phosphatase Inp54p by fusing it to cPLC $\zeta$ , which was



Time scale bar, 1h

**FIGURE 3:** Inhibition of  $\text{Ca}^{2+}$  oscillations following plasma membrane  $\text{PI}(4,5)\text{P}_2$  depletion using Lyn-GFP-Inp54p (LynPs). The majority of LynPs accumulated at plasma membrane (A). Expression of LynPs blocked  $\text{Ca}^{2+}$  oscillations caused by PLC $\delta 1$  (B, 100%). However, LynPs did not affect  $\text{Ca}^{2+}$  oscillations in PLC $\zeta$ -injected eggs (C) or in IVF eggs (D). Bar, 10  $\mu\text{m}$ .

shown earlier to bind to intracellular vesicles (Figure 4). Expressed ciPLC $\zeta$ -GFP-Inp54p (ciPPs) was verified to be confined to the cytoplasm without any accumulation at the plasma membrane (Figure 7A). Expression of ciPPs had no effect on the  $\text{Ca}^{2+}$  oscillations induced by injection of PLC $\delta 1$  (Figure 7 and Table 2). However, ciPPs expression either totally blocked or greatly inhibited the  $\text{Ca}^{2+}$  oscillations induced by PLC $\zeta$  or those induced by mouse sperm in fertilizing eggs (Figure 7, C and D, and Table 2). We could classify such ciPPs-expressing eggs into two distinct groups: those with no  $\text{Ca}^{2+}$  oscillations, and those with slower and less frequent  $\text{Ca}^{2+}$  oscillations.

Group		Blocked eggs	First spike timing (min)	Interval (min)
PLC $\delta 1$	Control	0/30	96.7 $\pm$ 12.7	52.6 $\pm$ 2.6
	LynPs	32/32	—	—
PLC $\zeta$	Control	0/32	90.6 $\pm$ 7.9	26.5 $\pm$ 4.6
	LynPs	0/37	85.7 $\pm$ 4.1	28.6 $\pm$ 3.2
IVF	Control	0/24	3.7 $\pm$ 0.4 <sup>a</sup>	10.1 $\pm$ 1.3
	LynPs	0/17	3.7 $\pm$ 0.4 <sup>a</sup>	10.1 $\pm$ 1.6

<sup>a</sup>First spike duration.

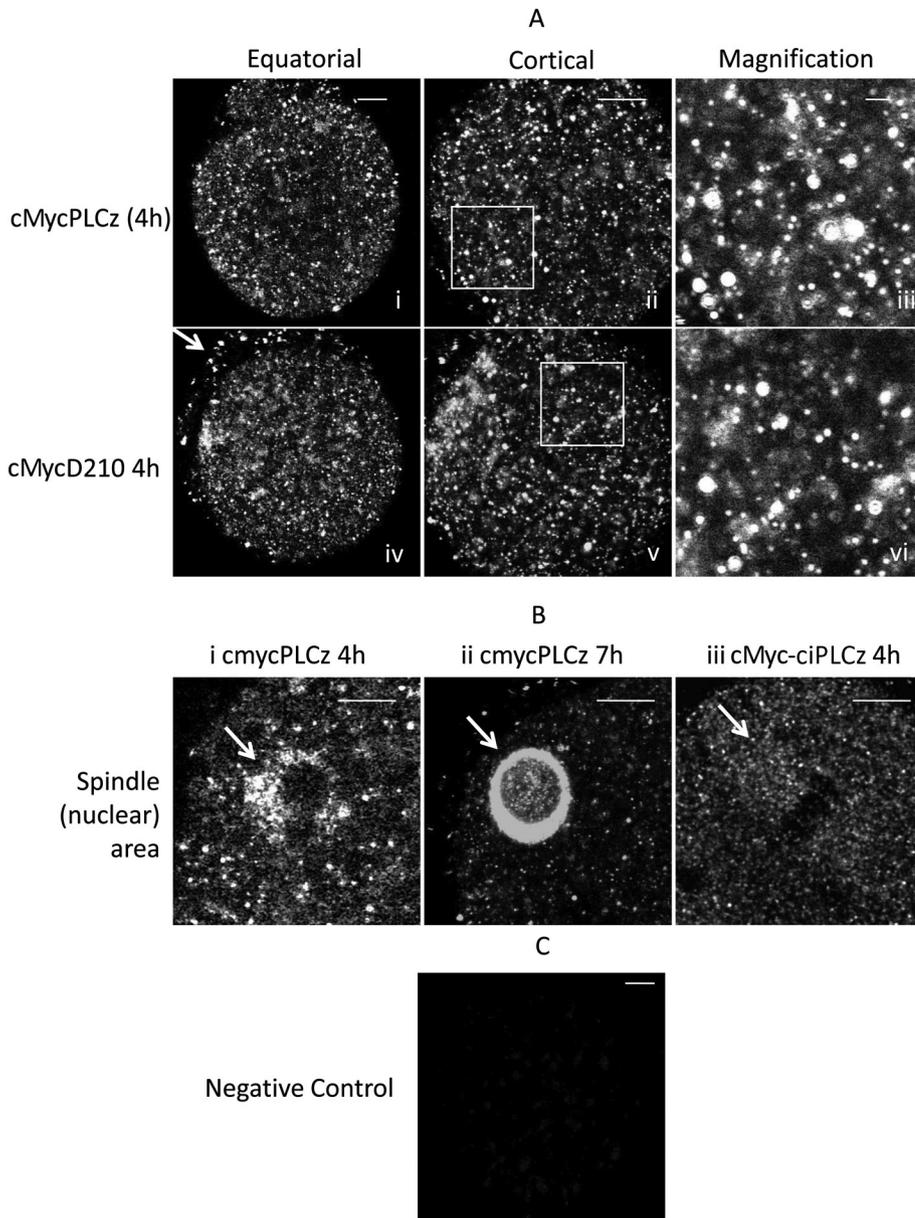
**TABLE 1:** Block of calcium oscillation by  $\text{PI}(4,5)\text{P}_2$  depletion with overdose of LynPs.

tions. In either group there was a clear indication that  $\text{Ca}^{2+}$  release had been perturbed; this result is in complete contrast to the lack of ciPPs effect on PLC $\delta 1$ -induced  $\text{Ca}^{2+}$  release. It was notable that these effects depended entirely on the specific subcellular targeting of Inp54p, since expression of untargeted GFP-Inp54p, which remained mostly in the cytosol, had little or no effect on  $\text{Ca}^{2+}$  oscillations triggered by PLC $\delta 1$ , PLC $\zeta$ , or sperm (see Supplemental Figure S3). These data are consistent with PLC $\zeta$  and sperm hydrolyzing intracellular sources of  $\text{PI}(4,5)\text{P}_2$  in mouse eggs, indicating that this  $\text{PI}(4,5)\text{P}_2$  source is essential for them to be able to cause  $\text{Ca}^{2+}$  oscillations. This is very different from the mechanism of PLC $\delta 1$ -mediated hydrolysis, which acts like other somatic PLCs by targeting the plasma membrane  $\text{PI}(4,5)\text{P}_2$  pool to generate  $\text{InsP}_3$ -induced  $\text{Ca}^{2+}$  release.

## 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, 2011c). However, its mode of action in the egg has not been established. Because somatic PI-specific PLCs hydrolyze  $\text{PI}(4,5)\text{P}_2$  in the plasma membrane, a natural assumption is that PLC $\zeta$  also targets plasma membrane  $\text{PI}(4,5)\text{P}_2$ , given the strong evidence for a significant oolemmal pool of  $\text{PI}(4,5)\text{P}_2$  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 $\delta 1$  as a comparative control, we observe a decrease in plasma membrane  $\text{PI}(4,5)\text{P}_2$  both during and before  $\text{Ca}^{2+}$  oscillations induced by PLC $\delta 1$  if exocytosis is inhibited (Figure 2). This decrease in  $\text{PI}(4,5)\text{P}_2$  could not be due to  $\text{InsP}_3$  generation displacing our GFH-PH domain probe since this can only occur with high  $\text{InsP}_3$  levels, which would cause high-frequency  $\text{Ca}^{2+}$  oscillations (Halet *et al.* 2002). Yet we found a displacement of the GFP-PH domain from the plasma membrane with PLC $\delta 1$  expression in the absence of any  $\text{Ca}^{2+}$  oscillations. These data clearly suggest that we can establish conditions to readily detect plasma membrane  $\text{PI}(4,5)\text{P}_2$  consumption after injection of PLC $\delta 1$ . However, under these conditions no decrease in plasma membrane  $\text{PI}(4,5)\text{P}_2$  is detected after PLC $\zeta$  injection, even though PLC $\zeta$  is over an order of magnitude more potent than PLC $\delta 1$  in causing  $\text{Ca}^{2+}$  oscillations. It is significant that we identified both PLC $\zeta$  and  $\text{PI}(4,5)\text{P}_2$  localization to intracellular vesicles, with  $\text{PI}(4,5)\text{P}_2$  staining in these vesicles being reduced much greater after PLC $\zeta$  injection than with PLC $\delta 1$ . For the first time in any type of egg,  $\text{PI}(4,5)\text{P}_2$ -specific phosphatases were used to confirm the requirement for  $\text{PI}(4,5)\text{P}_2$  to generate  $\text{Ca}^{2+}$  oscillations (Figures 3 and 7). However, disparate sources of  $\text{PI}(4,5)\text{P}_2$  are apparent, with PLC $\delta 1$  having a requirement for plasma membrane  $\text{PI}(4,5)\text{P}_2$ , whereas the sperm and PLC $\zeta$  require intracellular vesicular  $\text{PI}(4,5)\text{P}_2$ . To our knowledge, this is the first demonstration of a requirement for non-plasma membrane  $\text{PI}(4,5)\text{P}_2$  in order to elicit  $\text{Ca}^{2+}$  signaling in cellular signal transduction. These data also strongly suggest that PLC $\zeta$  is different from somatic PLCs in using intracellular  $\text{PI}(4,5)\text{P}_2$  and distinct from PLC $\delta 1$  in not interacting with plasma membrane  $\text{PI}(4,5)\text{P}_2$ .

Numerous internal membranes could be involved in making  $\text{PI}(4,5)\text{P}_2$  in cells. A previous study reported low levels of  $\text{PI}(4,5)\text{P}_2$  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



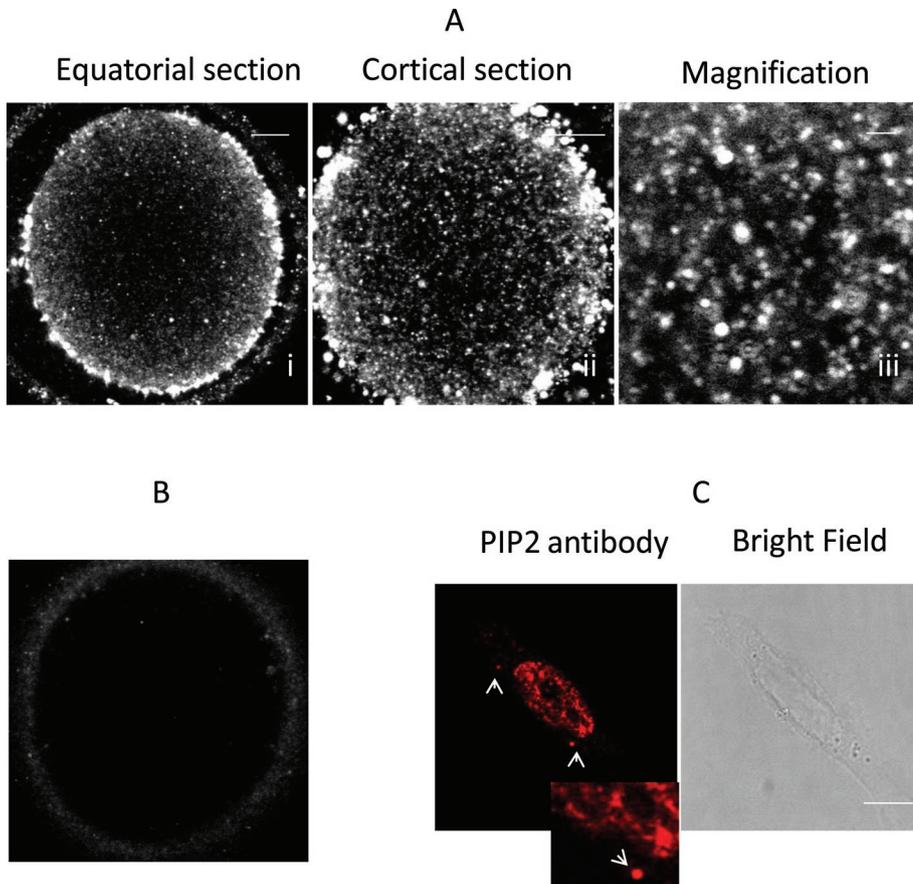
**FIGURE 4:** Distribution of PLC $\zeta$  in mouse eggs. Mouse eggs expressing cMyc-tagged PLC $\zeta$  (cMyc-PLC $\zeta$ ) or the catalytically inactive mutant (cMyc-ciPLC $\zeta$ ) were stained with cMyc antibody. (A) A pattern of distinctive vesicles for both cMyc-PLC $\zeta$ - and cMyc-ciPLC $\zeta$ -expressing eggs. The microvilli were also stained in cMyc-ciPLC $\zeta$ -injected eggs (iv, arrow point) but not in cMyc-PLC $\zeta$ -injected eggs (i). The cortical section and magnification clearly show that the appearance of vesicles in the cytoplasm. (B) Distribution in the nuclear/spindle area. PLC $\zeta$  translocated into the egg nucleus 4–7 h after PLC $\zeta$  injection (i and ii), but a spindle area distribution was seen in cMyc-ciPLC $\zeta$ -injected eggs (iii). Eggs that were not injected with cMyc-PLC $\zeta$  or cMyc-ciPLC $\zeta$  but were stained in the same way were treated as negative controls. (C) Equatorial section image. Bar, 1  $\mu$ m in magnification views; 10  $\mu$ m in all other images.

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 PLC $\zeta$  and PI(4,5)P $_2$  is not consistent with the endoplasmic reticulum since that membrane system in eggs is unusual in retaining characteristics of many somatic cells and forms clusters within the cortex (Fitzharris *et al.*,

2007). Of note, the pattern of PI(4,5)P $_2$  and PLC $\zeta$  staining that 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 PLC $\zeta$  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 PLC $\zeta$  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 PI(4,5)P $_2$  (Guo *et al.*, 1999; Lacramioara *et al.*, 2010). This yeast phosphatase was previously used to reduce PI(4,5)P $_2$  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 PI(4,5)P $_2$ , possibly because PI(4,5)P $_2$  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 PLC $\zeta$  could inhibit Ca $^{2+}$  oscillations in response to sperm and PLC $\zeta$  but not to the intrinsically less potent PLC $\delta$ 1. Although this result argues that PLC $\zeta$  requires an internal membrane PI(4,5)P $_2$ , we found that the targeted Inp54p did not completely block Ca $^{2+}$  oscillations in all eggs. This may be because PI(4,5)P $_2$  is more difficult to deplete in the intracellular vesicles. Studies in frog eggs suggest that there is substantially more PI(4,5)P $_2$  present in intracellular vesicles than in the plasma membrane (Snow *et al.*, 1996).

Although our studies focused on mature mouse eggs, we noted that the intracellular vesicular staining of PI(4,5)P $_2$  in mouse eggs developed during oocyte maturation. This maturation of PI(4,5)P $_2$  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 PI(4,5)P $_2$ , including Arf1 (Roth *et al.*, 1999; Jones *et al.*, 2000), PITP (Cockcroft and Carvou, 2007), and PLD (Roth *et al.*, 1999; Freyberg *et al.*, 2003). Because mammalian eggs acquire the ability to produce appropriate Ca $^{2+}$  oscillations after maturation (Carroll *et al.*, 1996; Machaca, 2004), it is possible that the synchronous increase in internal levels of PI(4,5)P $_2$  represents a significant feature in maturation of the oocyte cytoplasm. Of interest,



**FIGURE 5:** The distribution of PI(4,5)P<sub>2</sub> in MII eggs. (A) Both ring-like plasma membrane PI(4,5)P<sub>2</sub> and intracellular PI(4,5)P<sub>2</sub> were detected in MII eggs. The cortical section and magnification illustrate the presence of intracellular PI(4,5)P<sub>2</sub> in vesicular structures, which aggregate into patch-like areas (ii and iii). (B) A negative control for PI(4,5)P<sub>2</sub> staining in which PI(4,5)P<sub>2</sub> antibody was incubated with PI(4,5)P<sub>2</sub> for 20 min at room temperature; the only weak staining seen is in the zona pellucida. Bar, 1 μm in magnification views; 10 μm in all other images. (C) Distinctive nuclear PI(4,5)P<sub>2</sub> was detected in CHO cells, but very few intracellular vesicles were seen to contain PI(4,5)P<sub>2</sub>. The only vesicles seen are pointed to by the white arrow and one can be seen more clearly in the inset. Bar, 10 μm.

use of the same procedure that we used to stain PI(4,5)P<sub>2</sub> in eggs did not result in any detectable PI(4,5)P<sub>2</sub> in the cytoplasm of CHO cells. In accord with this observation, we recently showed that PLCζ is unable to induce Ca<sup>2+</sup> oscillations in CHO cells and appears to be inactive in CHO cell cytoplasm (Phillips *et al.*, 2011). The absence of PI(4,5)P<sub>2</sub> in the appropriate organelles in CHO cells might be one explanation for a lack of PLCζ activity in this cell line.

In all our experiments for detecting and depleting PI(4,5)P<sub>2</sub> 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 PI(4,5)P<sub>2</sub> and not plasma membrane PI(4,5)P<sub>2</sub>. 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 PI(4,5)P<sub>2</sub> was present in yolk platelets (Snow *et al.*, 1996; Rice *et al.*, 2000). In ascidian eggs, a mathematical model of Ca<sup>2+</sup> oscillations found that the Ca<sup>2+</sup> wavefront could be simulated only if one assumed that InsP<sub>3</sub> production (and hence PI(4,5)P<sub>2</sub> hydrolysis) occurred widely throughout the cell's cytoplasm

(Dupont and Dumollard, 2004). Of note, fertilization provided some of the first and preeminent examples of Ca<sup>2+</sup> waves, Ca<sup>2+</sup> oscillations, and InsP<sub>3</sub>-induced Ca<sup>2+</sup> release that were the prelude to the ubiquitous establishment of phosphoinositide signaling in generating Ca<sup>2+</sup> release from plasma membrane signals (Berridge *et al.*, 2003). Fertilization might now also provide an exemplar of an InsP<sub>3</sub> signaling mechanism that completely bypasses the plasma membrane.

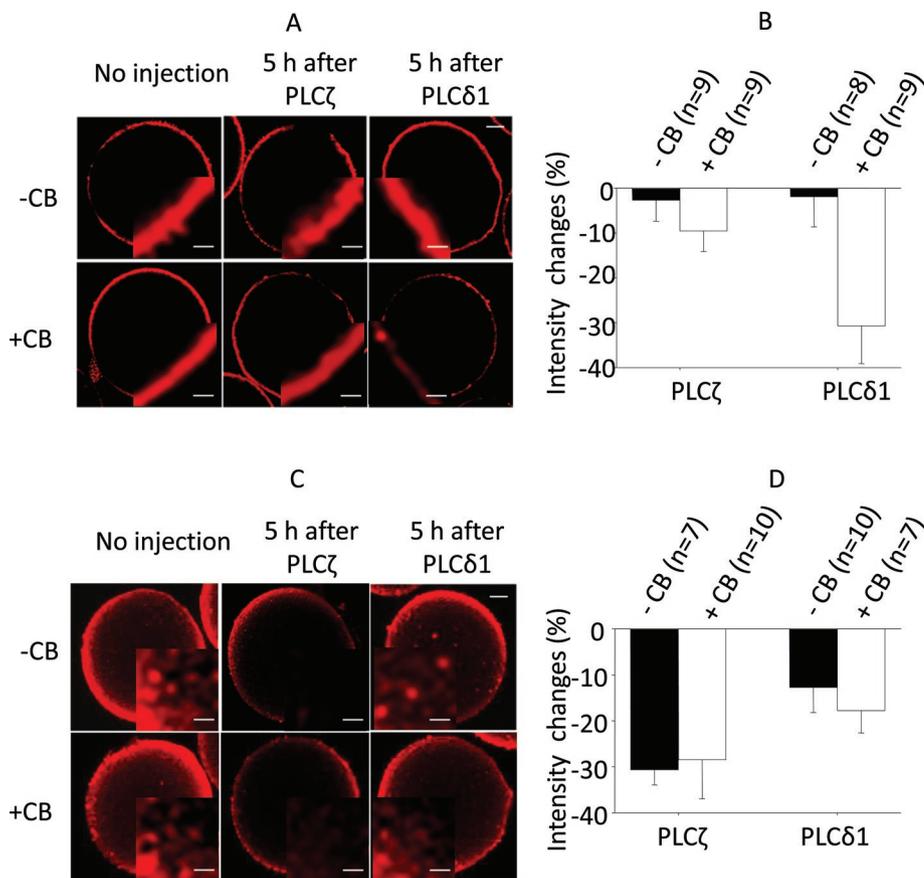
## MATERIALS AND METHODS

### 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 Ca<sup>2+</sup> 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 Hepes KSOM (Saunders *et al.*, 2002; hKSOM), where the eggs were stuck onto the glass bottom of the dish.

### 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 (Finnzymes, Thermo Scientific, Vantaa, Finland), and the appropriate primers were used to incorporate a 5'-EcoRI site and a 3'-NotI site. PCR products were 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 PLCδ1 (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,



Bar=10 μm in whole egg image and 1 μm in insertion

**FIGURE 6:** Differential changes in intracellular PI(4,5)P<sub>2</sub> and plasma membrane PI(4,5)P<sub>2</sub> upon PLCζ and PLCδ1 expression. Each egg is shown in low resolution with a higher-resolution insert. Plasma membrane PI(4,5)P<sub>2</sub> (A) or intracellular PI(4,5)P<sub>2</sub> (C) was specifically preserved by different fixation protocols as described in *Materials and Methods*. In the presence of cytochalasin B, PLCδ1 caused ~30% reduction of plasma membrane PI(4,5)P<sub>2</sub> (fluorescence intensities measured in the whole of each egg cortex; A, B), in contrast to the 10% plasma membrane PI(4,5)P<sub>2</sub> reduction caused by PLCζ (A, B;  $p < 0.01$ , measured in the whole egg). However, PLCζ caused higher reduction of intracellular PI(4,5)P<sub>2</sub> than PLCδ1 regardless of cytochalasin B treatment (C, D). Bar, 10 μm for main image; 1 μm for insets. The numbers of eggs analyzed for each treatment are indicated above the bars in B and D.

	Group	Blocked eggs		First spike timing (min)	Interval (min)
		i	ii		
PLCδ1	Control	0	22	121.7 ± 21.1	49.5 ± 9.2
	ciPPs	0/17	17/17	141.7 ± 20.9	46.4 ± 9.0
PLCζ	Control	0/33	33/33	90.7 ± 8.7	22.1 ± 2.6
	ciPPs	11/31	20/31	134.2 ± 16.9*	42.1 ± 4.4*
IVF	Control	0/46	46/46	3.4 ± 0.7 <sup>a</sup>	9.8 ± 0.5
	ciPPs	12/51	39/51	2.8 ± 0.1 <sup>a,*</sup>	13.2 ± 0.9*

<sup>a</sup>First spike duration.

\* $p < 0.05$ .

**TABLE 2:** Block of calcium oscillation by PI(4,5)P<sub>2</sub> depletion with overdose of CiPPs.

ciPLCζ was amplified by PCR from a pCR3-PLCζ<sup>D210R</sup>-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).

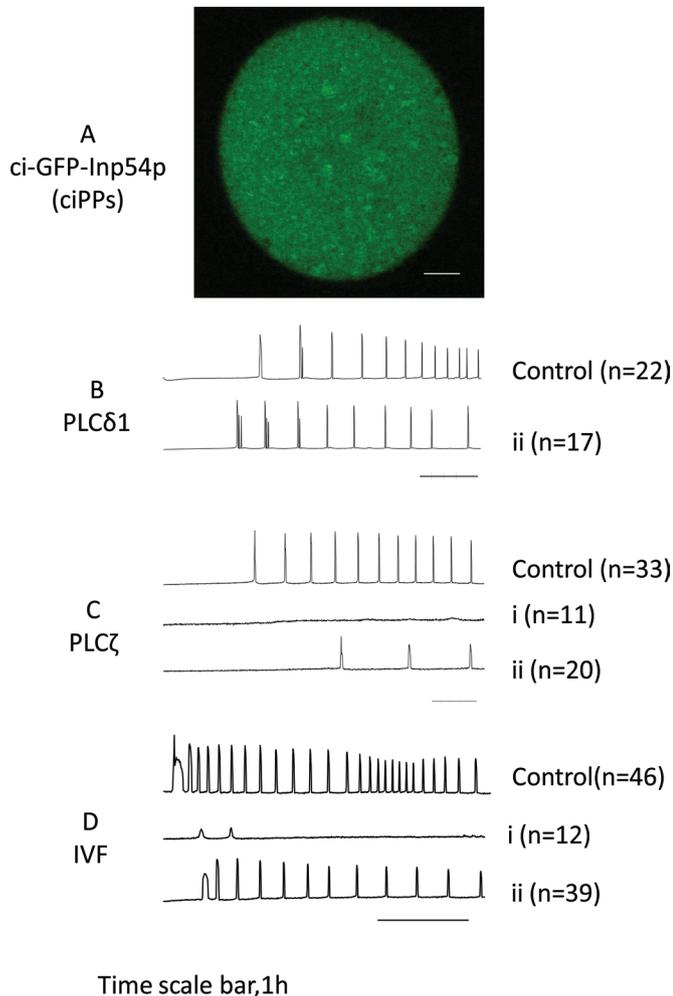
Following linearization of constructs, cRNA was synthesized using the mMessage Machine T7 kit (Applied Biosystems/Ambion, Austin, TX) and then polyadenylated using the poly(A) tailing kit (Applied Biosystems/Ambion), as per the manufacturer's instructions.

#### Antibodies, immunostaining, and confocal imaging

Mouse monoclonal anti-PI(4,5)P<sub>2</sub> 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)P<sub>2</sub> (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. Ca<sup>2+</sup> and Mg<sup>2+</sup>-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)P<sub>2</sub> 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 NH<sub>4</sub>Cl for 15 min and 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)P<sub>2</sub> staining or room temperature for 1 h for cMyc staining. Then they were washed twice by



**FIGURE 7:** Block of calcium oscillations following PI(4,5)P<sub>2</sub> depletion with ciPLC $\zeta$ -GFP-Inp54p, which distributed throughout the cytoplasm without any accumulation in the plasma membrane (A). Expression of this construct did not affect Ca<sup>2+</sup> oscillations caused by PLC $\delta$ 1 (B). However, Ca<sup>2+</sup> oscillations were completely blocked in 35% of PLC $\zeta$ -expressing eggs (C, i) and 24% of fertilized eggs (D, i). In eggs in which Ca<sup>2+</sup> oscillations were observed using PLC $\zeta$  (C) or sperm (D), they were greatly attenuated. Bar, 10  $\mu$ m.

15-min incubation in PBS before they were stained with secondary antibody diluted with PBS containing 5% goat serum for 45 min.

All the images were observed using a Leica (Wetzlar, Germany) SP5 confocal microscope with 20 $\times$  or 100 $\times$  oil objective. For the Fluor 488- and Fluor 594-conjugated secondary antibody, samples were exposed to 488- or 594-nm laser illumination, respectively, with emission ranges of 500–540 and 610–660 nm. All images were extracted to .tif files and analyzed with ImageJ (National Institutes of Health, Bethesda, MD). The significance of the hydrolysis of PI(4,5)P<sub>2</sub> from plasma membrane or internal membrane was analyzed for significance with a Student's *t* test.

#### Measurement and analysis of intracellular Ca<sup>2+</sup>

Eggs were injected with ~5  $\mu$ l of a solution containing 2 mM Rhod Dextran (Invitrogen, R34676). They were placed in M2 medium in a chamber settled on a Leica inverted fluorescent microscope with heating system. For in vitro fertilization, egg zonae were removed by a short incubation in Tyrode's acid solution (Sigma-Aldrich) and stuck onto the glass bottom of the chamber in BSA-free hKSOM medium. Ca<sup>2+</sup> oscillations were monitored by measuring Rhod Dextran fluo-

rescence 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,  $(f_t - f_0)/f_0$ . In PI(4,5)P<sub>2</sub> depletion experiments, the Ca<sup>2+</sup> oscillation features for the first calcium spike timing (in PLC $\zeta$  or PLC $\delta$ 1 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.

#### ACKNOWLEDGMENTS

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