Phospholipase Cζ binding to PtdIns(4,5)P2 requires the XY-linker region

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Summary
Phospholipase C-zeta (PLCζ) is a strong candidate for the mammalian sperm-derived factor that triggers the Ca2+ oscillations required for egg activation at fertilization. PLCζ lacks a PH domain, which targets PLCδ1 to the phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) substrate in the plasma membrane. Previous studies failed to detect PLCζ in the plasma membrane, hence the means of PLCζ binding to PtdIns(4,5)P2 is unclear. We find that the PLCζ XY linker, but not the C2 domain, exhibits robust binding to PtdIns(4,5)P2 or to liposomes containing near-physiological levels of PtdIns(4,5)P2. The role of positively charged residues within the XY linker was addressed by sequentially substituting alanines for three lysine residues, K374, K375 and K377. Microinjection of these mutants into mouse eggs enabled their Ca2+ oscillation-inducing activities to be compared with wild-type PLCζ. The XY-linker mutant proteins were purified and the in vitro PtdIns(4,5)P2 hydrolysis and binding properties were measured. Successive reduction of net positive charge within the PLCζ XY linker significantly affects both in vivo Ca2+-oscillation-inducing activity and in vitro PtdIns(4,5)P2 interaction of mouse PLCζ. Our data suggest that positively charged residues within the XY linker play an important role in the PLCζ interaction with PtdIns(4,5)P2, a crucial step in generating the Ca2+ activation signal that is essential for fertilization in mammals.

Key words: Phospholipase C, Phosphoinositide signalling, Calcium oscillations, Egg activation, Fertilization

Introduction
In mammalian oocytes, the fertilizing sperm evokes a striking series of intracellular Ca2+ oscillations that are essential for the initiation of egg activation and embryonic development (Swann, 1994; Stricker, 1999; Malcuit et al., 2006). Although the detailed mechanism remains unclear, accumulating evidence suggests that sperm-specific phospholipase C-zeta (PLCζ) is delivered from the fertilizing sperm into the ooplasm, triggering cytoplasmic Ca2+ oscillations via the inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] pathway (Saunders et al., 2002; Cox et al., 2002; Kouchi et al., 2004; Knott et al., 2005). Further evidence for the importance of PLCζ in mammalian fertilization has been provided by two recent clinical reports that have linked reduced expression levels and abnormal forms of PLCζ with male infertility (Yoon et al., 2008; Heytens et al., 2009). PLCζ is the smallest known mammalian PLC isozyme, possessing a similar domain organization to PLCδ1 with the notable exception that it lacks an N-terminal pleckstrin homology (PH) domain. Thus, PLCζ consists of four EF hands, the catalytic X and Y domains and a C2 domain, all of which are common to the other PLC isoforms (β, γ, δ, ε and η) (Saunders et al., 2002; Swann et al., 2006; Suh et al., 2008).

PLCs are cytosolic enzymes that require membrane association to access their phospholipid substrate. PLCδ1 binds strongly to membranes when its phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] substrate is present (Pawelczyk and Lowenstein, 1993; Lomasney et al., 1996). PLCβ2 binds strongly and non-specifically to lipid membranes (Singh and Murray, 2003), whereas PLCγ1 targets membranes by its specific, high-affinity binding to PtdIns(3,4,5)P3 (Bae et al., 1998; Falasca et al., 1998). Membrane binding of these isoforms appears to be mediated by the PH domain. PH domains are well-defined structural modules of ~120 amino acid residues identified in numerous proteins, including all isoforms of protein kinase C, phospholipase A, synaptotagmin and PLC. First identified in protein kinase C, the C2 domain was functionally implicated in Ca2+-dependent phospholipid interactions (Nalefski and Falke,
The C2 domain has since been characterized as a membrane-associating and intermolecular interaction domain in a variety of proteins (Medкова and Cho, 1999). Most C2 domains bind to Ca\(^{2+}\), a crucial determinant for the associated enzyme activity (Zheng et al., 2000). The PLC\(\varepsilon\) C2 domain interacts with phosphatidylserine (PtdSer) to form a 2-Ca\(^{2+}\)-PtdSer ternary complex, which enhances enzyme activity (Lomasney et al., 1999). The PLC\(\zeta\) C2 domain appears to have an essential role in cellular function because deletion of this domain leads to inability of the truncated PLC\(\zeta\) to cause Ca\(^{2+}\) oscillations in intact eggs, although enzyme activity is retained (Nomikos et al., 2005; Kouchi et al., 2005). There is currently no evidence for PLC\(\zeta\) C2 domain binding to membrane phospholipids through Ca\(^{2+}\)-dependent or -independent mechanisms, although screening phosphoinositides for interaction with the C2 domain of PLC\(\zeta\) revealed that it can bind to both phosphatidylinositol 3-phosphate (PtdIns3P) and phosphatidylinositol 5-phosphate (PtdIns5P) (Kouchi et al., 2005).

An alternative PLC\(\zeta\) region that could be involved in association with biological membranes is the XY-linker segment, separating the X and Y catalytic domains. In contrast to PLC\(\varepsilon\), PLC\(\zeta\) contains a more extended XY linker that is notably rich in positively charged residues (Saunders et al., 2002; Cox et al., 2002). The study reported here investigated the potential importance of the C2 domain and XY linker in PLC\(\zeta\) association with PtdIns(4,5)\(P_2\). A protein–lipid overlay and a liposome-binding assay were employed to examine the interaction of the PLC\(\zeta\) XY linker and C2 domain with PtdIns(4,5)\(P_2\). In addition, a series of full-length PLC\(\zeta\) mutants was prepared that sequentially neutralized several positively charged lysines that are clustered within the XY linker. The Ca\(^{2+}\) oscillation-inducing properties of these lysine mutants and wild-type PLC\(\zeta\) were compared by microinjecting them into unfertilized mouse eggs and analysing their enzymatic activity using an in vitro PtdIns(4,5)\(P_2\) hydrolysis assay. Furthermore, the binding properties of wild-type and mutant PLC\(\zeta\) to PtdIns(4,5)\(P_2\) was examined using a liposome binding assay, in parallel with a number of control PLC constructs. Our studies suggest that the PLC\(\zeta\) XY linker, but not the C2 domain, possesses significant affinity for PtdIns(4,5)\(P_2\) and that sequential reduction of the XY-linker net positive charge significantly affects both in vivo Ca\(^{2+}\)-oscillation-inducing activity and also the in vitro interaction of PLC\(\zeta\) with PtdIns(4,5)\(P_2\). Thus, we propose that the XY-linker region plays a major role in the binding of PLC\(\zeta\) to PtdIns(4,5)\(P_2\)-enriched membranes.

**Results**

**Binding of the XY linker and C2 domain of PLC\(\zeta\) to PtdIns(4,5)\(P_2\)**

To examine the ability of the PLC\(\zeta\) XY linker and C2 domain to bind PtdIns(4,5)\(P_2\), we employed a protein–lipid overlay and a liposome-binding assay to assess distinct GST fusion proteins of both the XY linker or C2 domain of PLC\(\zeta\), with the PH domain of PLC\(\varepsilon\) as a positive control. Fig. 1A schematically illustrates the three GST fusion proteins: the PLC\(\varepsilon\) PH domain, the PLC\(\zeta\) C2 domain and XY linker, and their corresponding sequence coordinates. These fusion proteins were expressed in *Escherichia coli* Rosetta (DE3) cells and purified by glutathione affinity chromatography. Optimal recombinant protein expression conditions required maintaining cultures at 37°C until absorbance at 600 nm (A\(_{600}\)) reached 0.5, followed by induction of expression with 0.1 mM isopropyl \(\beta\)-D-thiogalactopyranoside (IPTG) for 18 hours at 16°C. Fig. 1B shows the glutathione affinity-purified GST fusion proteins analyzed by SDS-PAGE. The predicted molecular mass, including the GST protein (26 kDa), for GST–XYlink\(\zeta\), GST–C2\(\zeta\) and GST–PH\(\varepsilon\) was 34, 39 and 40 kDa, respectively.

For each expression plasmid the corresponding protein with expected molecular mass was observed as the major band. In addition, a minor, lower band of 26 kDa was also present, consistent with the mass of GST alone, suggesting that some protein degradation had occurred during the protein purification process.

The protein–lipid overlay assay, using membrane-spotted arrays of inositol phospholipids, showed that GST–XYlink\(\zeta\) was able to bind to all the phosphoinositides though not to PtdIns (Fig. 2A). The strongest interaction occurred with the polyvalent phosphoinositides, PtdIns(4,5)\(P_2\) and PtdIns(3,4,5)\(P_3\) at the lower concentrations (e.g. at 3.13 pmol). By contrast, GST–C2\(\zeta\) bound very weakly only to PtdIns3\(P\) and PtdIns5\(P\) at the highest concentrations (50–100 pmol), whereas GST–PH\(\varepsilon\) exhibited strong binding specifically to the three monophosphates, and preferential binding to the bisphosphate, PtdIns(4,5)\(P_2\), as anticipated (Lomasney et al., 1996). The inositol phospholipid array results suggest that the PLC\(\zeta\) XY-linker region has the ability to interact with a wide range of phosphoinositides spanning mono, bis and trisphosphates, whereas the PLC\(\zeta\) C2 domain displays negligible binding to inositol lipids.

The ability of GST–XYlink\(\zeta\) and GST–C2\(\zeta\) to bind PtdIns(4,5)\(P_2\) was further monitored using unilamellar liposomes composed of phosphatidylcholine:cholesterol:phosphatidyethanolamine (PtdCho:CHOL:PtdEtn; 4:2:1) with incorporation of either 0 or 1% PtdIns(4,5)\(P_2\) (Fig. 2B). For this binding assay, GST–PH\(\varepsilon\) provided the positive control whereas the GST moiety was a negative control. Thus, GST–PH\(\varepsilon\) bound robustly to liposomes containing 1% PtdIns(4,5)\(P_2\) (Fig. 2B, top panel) and remained in the supernatant in the absence of PtdIns(4,5)\(P_2\), whereas the GST negative control did not exhibit any specific lipid binding with or without PtdIns(4,5)\(P_2\) (Fig. 2B, bottom panel). The majority (~90%) of GST–XYlink\(\zeta\) was detected in the supernatant of liposomes that did not contain PtdIns(4,5)\(P_2\). By contrast, the presence of a near-physiological concentration of 1% PtdIns(4,5)\(P_2\) in the liposomes resulted in GST–XYlink\(\zeta\) binding strongly, with...
all of this protein being detected in the liposome pellet (Fig. 2B, third panel). However, the binding of GST–C2ζ to liposomes either with or without 1% PtdIns(4,5)P2 was not detected (Fig. 2B, second panel). Thus, the results obtained using two different phosphoinositide binding interaction assays has provided congruent evidence that only the PLCζ XY linker and not the C2 domain has the ability to bind to PtdIns(4,5)P2.

Analysis of XY-linker mutations on PLCζ-mediated Ca2+-oscillations in mouse eggs

To investigate the potential importance of the cluster of basic residues within the PLCζ XY linker (Fig. 3A), we performed site-directed mutagenesis to produce a panel of three cumulative mutations within this positively charged region of mouse PLCζ. Thus, the three lysines, K374, K375 and K377, were sequentially replaced with the neutral amino acid, alanine, to create single (PLCζK374A), double (PLCζK374,5AA) and triple (PLCζK374,5,7AAA) substituted PLCζ mutants. In order to test the ability of PLCζK374A, PLCζK374,5AA and PLCζK374,5,7AAA to trigger Ca2+ oscillations and to verify their expression upon microinjection of the corresponding cRNA into eggs, we generated luciferase fusion constructs of each of these mutants. This approach enables the accurate quantification of

Fig. 2. In vitro binding of PLCζ XY linker and C2 domain to PtdIns(4,5)P2. (A) PLC domain protein–lipid overlay assays. Recombinant protein binding to spotted phosphoinositides on the PIP Arrays was detected using a polyclonal anti-GST antibody. PI, phosphoinositide (e.g. PI(4,5)P2 is PtdIns(4,5)P2. (B) Liposome ‘pull-down’ assay of PLC domains. Unilamellar liposomes comprising PtdCho:CHOL:PtdEtn (4:2:1) with or without 1% PtdIns(4,5)P2 were incubated with recombinant protein. Following centrifugation, both the supernatant (s) and liposome pellet (p) were subjected to SDS-PAGE and Coomassie Brilliant Blue staining. Previous control experiments showed that GST alone does not bind to phosphoinositides (Nomikos et al., 2007).

Fig. 3. Effect of PLCζ XY-linker mutations on Ca2+-oscillation-inducing activity in mouse eggs. (A) Schematic representation of mouse PLCζ domain structure (comprising the N-terminal EF hands, X and Y catalytic domains and C-terminal C2 domain) identifying the successive K-to-A mutations between residues 374 and 379 in the XY-linker region, as well as the position of the D210R mutation in the X catalytic domain (arrows). (B) Fluorescence and luminescence recordings reporting the Ca2+ changes (black traces; Ca2+) and luciferase expression (red traces; Lum, in counts per second; cps) in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged, wild-type PLCζ or the indicated single, double, triple K-to-A and D210R mutants of PLCζ (left panels). The average luminescence level (cps) achieved in mouse eggs (n is number of eggs) is indicated for each microinjected cRNA, e.g. PLCζwt Lum=7.33 (top trace). Panels on the right are the integrated luminescence images of individual mouse eggs following cRNA microinjection of either wild-type or mutant PLCζ (Table 1). Two eggs at the bottom left of the PLCζwt panel were poorly fluorescing and not included in the analysis of Ca2+ oscillations. All eggs were microinjected with 1.5 g/l cRNA.
relative protein expression by luminescence detection for each of the luciferase-tagged PLCζ proteins (Nomikos et al., 2005). A catalytically inactive PLCζ mutant (PLCζD210R) served as a control in these experiments in order to establish the degree of inhibition effected by the K-to-A mutations. We have previously reported that point mutation of Asp210 (D210R) in the XY catalytic domain of PLCζ results in loss of PLCζ Ca2+-oscillation inducing activity in the oocytes (Saunders et al., 2002; Nomikos et al., 2011). Fig. 3B and Table 1 summarize the results of the wild-type and mutant PLCζ-luciferase cRNA microinjection experiments. Prominent Ca2+ oscillations (19 spikes in the first 2 hours) were observed in the wild-type PLCζ cRNA-injected eggs, with the first Ca2+ spike occurring ~30 minutes after microinjection at a luminescence corresponding to PLCζ protein expression of ~35 fg/egg. Microinjection of cRNA encoding the PLCζK374A single mutant also caused Ca2+ oscillations in mouse eggs, exhibiting a similar potency to wild-type PLCζ (17 spikes in 2 hours) with the first Ca2+ spike detected at a protein expression level of ~33 fg/egg. By contrast, egg microinjection with cRNA of either PLCζK374,5AA or PLCζK374,5,7AAA resulted in a significant reduction in the frequency of Ca2+ oscillations compared with wild-type PLCζ, causing 8 and 2.6 spikes in 2 hours, respectively. Moreover, there was also a significant increase in time required for initiation of Ca2+ oscillations for the double and triple PLCζ mutants, with the first Ca2+ spike appearing at ~65 minutes (Fig. 3B; Table 1). Furthermore, the luminescence level required to produce the first Ca2+ spike was equivalent to protein expression of ~55 and ~68 fg/egg for the double and triple mutant, respectively. Finally, microinjection of cRNA encoding the PLCζD210R mutant failed to cause any Ca2+ oscillations even though it was expressed at much higher levels than wild-type PLCζ and PLCζ K-to-A mutants (Table 1). These data indicate that the substitution of two or more alanines for lysines within the positively charged cluster of the PLCζ XY linker dramatically alters their Ca2+-oscillation-inducing activity in mouse eggs by reducing the positively charged cluster of the PLCζ XY linker by two or more alanines for lysines within the positively charged cluster of the PLCζ XY linker dramatically alters their Ca2+-oscillation-inducing activity in mouse eggs by reducing the significantly.

Enzymatic properties of PLCζ XY-linker mutants

The PLCζK374A, PLCζK374,5AA and PLCζK374,5,7AAA mutants were expressed as GST fusion proteins and purified by glutathione affinity chromatography. The optimal protein production for the full-length PLCζ constructs, PLCζ C2 and XY linker, required induction with 0.1 mM IPTG for 18 hours at 16°C. Fig. 4A shows the four glutathione affinity-purified PLCζ fusion proteins analyzed by SDS-PAGE and immunoblotting with anti-GST antibody (right panel). The predicted molecular mass for the GST–PLCζ and mutants, including the GST tag is 100 kDa. A protein of ~100 kDa was observed as the major band in both the SDS gels and the immunoblots (Fig. 4A). A protein migrating at 26 kDa in both the gels and immunoblots is consistent with cleaved GST, which, along with several fainter intermediate molecular mass proteins detected by the GST antibody, is probably the result of protease degradation occurring during protein purification.

To examine the impact of the XY-linker mutations on Ca2+ sensitivity of PLCζ enzyme activity, we assessed the ability of these GST–PLCζ fusion proteins to hydrolyze [3H]PtdIns(4,5)P2 at different Ca2+ concentrations, ranging from 0.1 nM to 0.1 mM.

![Fig. 4. Ca2+-dependent enzyme activity of PLCζ XY-linker mutations.](image)

(A) Glutathione affinity-purified, wild-type and each of the K-to-A mutant GST–PLCζ fusion proteins (1 μg) were analyzed by 8% SDS-PAGE followed by either Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using anti-GST antibody (right panel). Lanes 1-4 (left to right) show wild-type PLCζ and single, double and triple K-to-A PLCζ XY-linker mutants, respectively. (B) PtdIns(4,5)P2 hydrolysis activity of the purified wild-type and XY-linker mutant PLCζ proteins were determined in vitro with [3H]PtdIns(4,5)P2 at different Ca2+ concentrations. For these enzyme assays, using two different batches of recombinant proteins and each experiment was performed in duplicate; values are means. In control experiments with GST, there was no specific PtdIns(4,5)P2 hydrolysis activity observed (data not shown).

| K-to-A Mutations | Ca2+ Oscillations (spikes/2 hours) | Peak Luminescence (cps) | Time to First Spike (minutes) | Luminescence at First Spike (cps) | No. of Eggs |
|------------------|-----------------------------------|------------------------|-----------------------------|----------------------------------|------------|
| PLCζ             | 19±0.14                           | 7.3±0.38               | ~30                         | 0.35±0.037                       | 17         |
| PLCζK374A        | 17±1.0                            | 3.1±0.23               | ~30                         | 0.28±0.03                       | 12         |
| PLCζK374,5AA     | 8.0±0.30                          | 3.0±0.10               | ~65                         | 0.93±0.07                       | 16         |
| PLCζK374,5,7AAA  | 2.6±0.50                          | 5.8±1.5                | ~65                         | 1.43±0.30                       | 29         |
| PLCζD210R        | 0                                 | 14.5±0.87              | 0                           | 0                                | 17         |

The data are expressed to two significant figures (means ± s.e.m.). All eggs were microinjected with 1.5 g/l cRNA.

cps, counts per second.
These experiments indicated that there was no significant difference in the Ca\(^{2+}\) sensitivity of PtdIns(4,5)\(P_2\) hydrolysis for the wild-type and the three XY-linker mutants (Fig. 4B), with a very similar EC\(_{50}\) value (81–90 nM) for all four recombinant PLC\(_{\zeta}\) proteins (Table 2). To compare the enzyme kinetics of the wild-type and mutant PLC\(_{\zeta}\), the Michaelis–Menten constant, \(K_m\), was calculated from a Lineweaver–Burk reciprocal plot of the PtdIns(4,5)\(P_2\) hydrolysis activity of these proteins (Fig. 5; Table 2). The \(K_m\) values obtained for wild-type PLC\(_{\zeta}\) (80 \(\mu\)M) and PLC\(_{\zeta}K374A\) (88 \(\mu\)M) were very similar. By contrast, the \(K_m\) value for PLC\(_{\zeta}K374,5AA\) and PLC\(_{\zeta}K374,5,7AAA\) was approximately 9-fold (752 \(\mu\)M) and 61-fold (4919 \(\mu\)M) higher than that of wild-type PLC\(_{\zeta}\), respectively. These results suggest that sequential neutralization of these three positively-charged residues within the XY-linker region reduces the in vitro affinity of PLC\(_{\zeta}\) for PtdIns(4,5)\(P_2\) without affecting the Ca\(^{2+}\) sensitivity of this enzyme.

### Binding of wild-type PLC\(_{\zeta}\) and the XY-linker K-to-A mutants to PtdIns(4,5)\(P_2\)

To examine the PtdIns(4,5)\(P_2\) binding properties of wild-type PLC\(_{\zeta}\) and the three XY-linker K-to-A mutants, we employed the liposome binding assay as described above (see Fig. 2B). In order to diminish any non-specific binding to highly charged lipids by this series of full-length PLC proteins, the liposome binding assays were performed in the presence of a near-physiological concentration of MgCl\(_2\) (0.5 mM). In addition to the wild-type PLC\(_{\zeta}\) and PLC\(_{\zeta}\) K-to-A mutants, the PtdIns(4,5)\(P_2\) binding of three other control PLC constructs (PLC\(_{\zeta}1\), PLC\(_{\zeta}AC2\) and PLC\(_{\zeta}D210R\)) was also assessed. The PLC\(_{\zeta}1\) served as positive control for these PtdIns(4,5)\(P_2\) binding assays, whereas PLC\(_{\zeta}AC2\), a deletion construct lacking the PLC\(_{\zeta}\) C2 domain (Nomikos et al., 2005) was used to examine whether the C2 domain has any role in binding to PtdIns(4,5)\(P_2\). We also included PLC\(_{\zeta}D210R\) to investigate whether PtdIns(4,5)\(P_2\) binding requires the catalytically competent enzyme. Fig. 6 shows that PLC\(_{\zeta}\), PLC\(_{\zeta}D210R\), PLC\(_{\zeta}AC2\), PLC\(_{\zeta}K374A\) and PLC\(_{\zeta}K374,5AA\) bind robustly only to the liposomes containing 1% PtdIns(4,5)\(P_2\), similar to that observed for the positive control, PLC\(_{\zeta}1\). There was no binding of proteins to liposomes without PtdIns(4,5)\(P_2\). By contrast, the majority (~60%) of the triple K-to-A mutant, PLC\(_{\zeta}K374,5,7AAA\), was detected in the supernatant of liposomes containing 1% PtdIns(4,5)\(P_2\), indicating that although PLC\(_{\zeta}\) lacks a PH domain, it remains capable of binding to PtdIns(4,5)\(P_2\). The binding observed with PLC\(_{\zeta}AC2\) and PLC\(_{\zeta}D210R\) reveals that PLC\(_{\zeta}\) interaction with PtdIns(4,5)\(P_2\) in liposomes does not require the C2 domain, and there is no requirement for catalytic enzyme function, respectively. However, the replacement of three positively-charged residues within the PLC\(_{\zeta}\) XY-linker region does dramatically diminish the interaction with PtdIns(4,5)\(P_2\).

### Discussion

During mammalian fertilization, egg activation leading to embryo development is believed to be triggered by entry of the sperm-specific PLC\(_{\zeta}\) (Saunders et al., 2002; Cox et al., 2002; Kouchi et...
PLCζ interaction with PtdIns(4,5)P2

al., 2004). PLCζ stimulates repetitive Ca2+ oscillations in the egg upon fusion of the sperm and egg membranes by initiating Ca2+ release from intracellular stores via the activation of the Ins(1,4,5)P3 receptor signalling pathway. Recent clinical reports providing further evidence for the importance of PLCζ in mammalian fertilization have directly linked reduced expression levels and abnormal forms of PLCζ with documented cases of human male infertility (Yoon et al., 2008; Heytens et al., 2009). Despite the crucial significance of PLCζ in intracellular Ca2+ signalling at fertilization, the precise mechanism by which PLCζ locates and targets the substrate in eggs is still unclear. The potential role of positively charged PLCζ residues in targeting this enzyme to biological membranes via electrostatic interactions with the negatively charged PtdIns(4,5)P2 has been previously suggested (Nomikos et al., 2007). Fluorescence resonance energy transfer (FRET) experiments with a synthetic peptide corresponding to a portion of the XY linker have suggested that basic amino acids could help anchor PLCζ to the membrane and enhance local PtdIns(4,5)P2 concentration adjacent to the catalytic site.

In this report, we have examined the ability of the mouse PLCζ XY-linker and C2 domain to interact with PtdIns(4,5)P2 by preparing recombinant protein constructs to enable expression and purification of two structurally distinct PLCζ regions (Fig. 1). The protein-lipid overlay and liposome-binding assay results suggest that only the XY-linker, and not the C2 domain, binds strongly to PtdIns(4,5)P2 spotted onto nitrocellulose membrane (Fig. 2A) or to liposomes containing a near-physiological (1%) concentration of PtdIns(4,5)P2 (Fig. 2B). The lipid array analysis of interactions between the PLCζ C2 domain and other phosphoinositides detected binding only to the monophosphates, PtdIns3P and PtdIns5P (Fig. 2A). This result is consistent with previous observations using a similar protein-lipid overlay assay that showed the PLCζ C2 domain binds to PtdIns3P and with lower affinity to PtdIns5P (Kouchi et al., 2005). There is presently no evidence that C2 domain interaction with PtdIns3P or PtdIns5P plays a significant role in the subcellular targeting of PLCζ to biological membranes. However, a C2–PtdIns3P interaction might be involved in the regulation of PLCζ enzymatic activity, because it has been reported that PtdIns3P reduces the PtdIns(4,5)P2 hydrolytic activity of PLCζ (Kouchi et al., 2005).

In contrast to the C2 domain, the PLCζ XY-linker showed strong binding to all phosphoinositides, with highest preference for PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (Fig. 2A). In agreement with the protein-lipid overlay approach, strong binding of the PLCζ XY-linker but not the C2 domain was also observed to liposomes containing PtdIns(4,5)P2 (Fig. 2B). Given that PLCζ lacks a PH domain, our results suggest that electrostatic interactions of the positively charged PLCζ XY-linker might provide the major contribution to the physiological interaction of PLCζ with PtdIns(4,5)P2. However, without further detailed kinetic analysis and high-resolution protein structure the role of additional PLCζ domains cannot be ruled out.

The importance of obtaining in vivo evidence led us to employ an additional approach to demonstrate the potential involvement of the PLCζ XY-linker in Ca2+-oscillation-inducing activity. Using site-directed mutagenesis to generate three cumulative mutations within the positively charged region of the mouse PLCζ XY linker (Fig. 3A), a cluster of three lysine residues (K374, K375 and K377) were sequentially replaced with the neutral amino acid alanine, to produce single (K374A), double (K374A, K375A) and triple (K374,375,377AAA) mutant constructs of PLCζ. The lysine to alanine change was chosen as it is likely to bring about relatively small changes compared with charge-reversed amino acid substitution effects on PLCζ structure. Microinjection of the cRNA for luciferase-tagged PLCζ, PLCζK374A, PLCζK374,5AA or PLCζK374,5,7AAA into mouse eggs uniformly resulted in robust recombinant protein expression followed by the initiation of a series of Ca2+ oscillations that persisted for several hours (Fig. 3B). There was a significant reduction in the frequency of Ca2+ oscillations observed with the double and triple K-to-A mutants, PLCζK374,5AA (40% vs wild type) and PLCζK374,5,7AAA (14% vs wild type; Fig. 3B; Table 1). By contrast, oscillation frequency of the single PLCζK374AA mutant was only slightly affected (90% vs wild type), suggesting that significantly altered in vivo PLCζ function was mediated by the loss of two or more positive charges in the XY-linker. Notably, for the double and triple K-to-A mutants there was an extended delay (~65 minutes) before the first Ca2+ spike appeared, compared with wild-type PLCζ (~30 minutes). In addition, a higher protein concentration was required of these two mutants (266% and 409% vs wild type) to initiate the first Ca2+ spike (Fig. 3B; Table 1). The requirement for substantially increased expression level of the double and triple K-to-A mutant proteins to generate the first Ca2+ spike, together with the lower Ca2+ oscillation frequency they produced, suggests that reduction of the net positive charge by two or more in the XY linker diminishes the affinity of PLCζ for its negatively charged, membrane-bound substrate, PtdIns(4,5)P2.

To determine the effect of the single, double and triple K-to-A mutations on the enzymatic properties of PLCζ, we purified each of them as GST fusion proteins (Fig. 4A) to enable in vitro analysis of their ability to hydrolyze PtdIns(4,5)P2. Previous characterization of PLCζ activity has shown a steep Ca2+ dependence, with a nanomolar EC50 (82 nM) for the hydrolysis of PtdIns(4,5)P2, in contrast to a micromolar EC50 (6 μM) for PLCβ1 (Nomikos et al., 2005). We found no evidence of a role for these XY-linker lysines in Ca2+ regulation of PLCζ activity because all three K-to-A mutants exhibited an EC50 similar to that of the wild-type enzyme (81–90 nM vs 88 nM; Table 2). By contrast, the Km values for PLCζK374,5AA and PLCζK374,5,7AAA mutants were approximately 9-fold and 61-fold higher than wild-type PLCζ (Fig. 5; Table 2). This suggests that the sequential cumulative neutralization of these basic residues within the XY-linker region substantially reduces the in vitro affinity of PLCζ for PtdIns(4,5)P2 without affecting the Ca2+ sensitivity of this enzyme (Fig. 4B; Table 2).

Further evidence of a specific role for the polybasic region within the PLCζ XY-linker in interacting with PtdIns(4,5)P2-enriched biological membranes was obtained from liposome binding assays performed with expressed wild-type and mutant PLCζs in the presence of near-physiological Mg2+ (Fig. 6). We observed strong PtdIns(4,5)P2 binding of both the wild-type PLCζ and the catalytically inactive mutant PLCζD210R, indicating that substrate interaction and hydrolytic activity are distinct, separable steps in the mechanism of enzyme action. Interestingly, both single and double K-to-A mutants also displayed robust PtdIns(4,5)P2 binding, whereas binding of PLCζK374,5,7AAA was dramatically reduced, by over 60%, suggesting that the cumulative reduction of XY-linker positive residues in the PLCζ triple mutant had crossed below a crucial charge-density threshold resulting in diminution of PtdIns(4,5)P2 binding (Fig. 6). Furthermore, the PLCζ deletion construct lacking the C2 domain showed binding to liposomes.
containing PtdIns(4,5)P₂ equivalent to that of wild-type PLCζ, suggesting that the C2 domain does not play a direct role in the interaction with PtdIns(4,5)P₂.

Notably, ClustalW sequence alignment analysis of PLCζ from various species shows that the XY-linker region, which connects the two highly conserved halves of the catalytic barrel (the X and Y domains) is the most non-conserved region of PLCζ (Saunders et al., 2007). However, although the primary structure is poorly conserved, the XY-linker region of all mammalian PLCζ species thus far sequenced retains a net positive charge. The significance of this XY-linker structure diversity is unclear, but it might explain the different rates of PtdIns(4,5)P₂ hydrolysis and relative potency in inducing Ca²⁺ oscillations between PLCζ isoforms of different species. For example, the deduced isoelectric points of human (9.1), monkey (7.2) and mouse (5.4), appears to correlate with the ability of the PLCζ of these different species to trigger Ca²⁺ oscillations in mouse eggs; increased potency is observed with higher isoelectric point (Saunders et al., 2002; Cox et al., 2002; Saunders et al., 2007).

Significantly, none of the other mammalian PLC isozymes possesses a highly positively charged XY-linker region. In contrast to PLCζ, the XY-linker of PLCβ, δ and ε is highly negatively charged, mostly disordered and it has been suggested that these XY linkers might mediate auto-inhibition of PLC activity (Hicks et al., 2008). It has been proposed that the negatively charged XY-linkers of these PLCs prevent PtdIns(4,5)P₂ gaining access to the active site, by a combination of steric exclusion and electrostatic repulsion of negatively charged membranes (Hicks et al., 2008). The PLCγ XY-linker possesses additional regulatory domains: a PH domain, two SH2 domains and an SH3 domain, and this enzyme is activated by tyrosine phosphorylation within the XY-linker region (Rodriguez et al., 2001; Ozdener et al., 2002; Sekiya et al., 2004). A recent study suggested that the general mechanism of PLC auto-inhibition mediated by the XY-linker region also applies to PLCζ isoforms and the crucial determinant for the auto-inhibition is the C-terminal SH2 domain (Gresset et al., 2010). Interestingly, the XY-linkers of PLCζ isoforms are not negatively charged overall but contain clusters of acidic residues near their ends. However, it is unclear whether there is a role of this region in the regulation of PLCζ activity (Fukami et al., 2010).

PLCζ has the simplest domain organization and could be considered a prototypic mammalian PLC (Saunders et al., 2002; Swann et al., 2006; Saunders et al., 2007). This is consistent with the pivotal physiological role it plays in reproduction by triggering the primary Ca²⁺ signalling event at mammalian fertilization to initiate egg activation and embryo development. However, our observations suggest that the PLCζ regulatory mechanism and the role of its XY-linker region might be distinct and not follow the pattern observed for other mammalian somatic cell PLCs. Further understanding of the potentially unique molecular mechanism of PLCζ action will be aided by additional kinetic analysis and structural definition of the mammalian PLCζ. High resolution three-dimensional structure analysis by X-ray crystallography could help to reveal the crucial ion, lipid and protein binding sites within PLCζ. This would provide a useful base for further kinetic investigation of the putative interactions of PLCζ with the ions, lipids or proteins that might play an important role in its function and mode of regulation. Unravelling the complex subcellular processes mediated by PLCζ should provide a molecular explanation for the fundamental Ca²⁺ signalling event that initiates early embryo development.

### Materials and Methods

#### Cloning of XY-linker, C2ζ and PHζ constructs

The XY-linker (amino acids (aa) 252–118) and the C2 domain (aa521–647) of mouse PLCζ were amplified by PCR from the original cDNA clone (GenBank™ accession number AF435950) (Saunders et al., 2002), using Phusion polymerase (Finnzymes) and the appropriate primers to incorporate a 5’-EcoRI site and a 3’-SalI site. PCR products were cloned into the pGEX-6P1 vector (GE Healthcare). The following primers were used for each construct for XY-linker ζ: 5’-GTCGAAATCTCAAGTGCGGAGCTCTATCTCGA-3’ (forward) and 5’-GATGTCGACTCAAGGAGCTGATTTTCACT-3’ (reverse), and for C2ζ, 5’-GCTGAAATCCCTCACAATCCGAATC-3’ (forward) and 5’-TAACGTGACTACCTGTAAGGACACACCAAC-3’ (reverse), PHζ (aa1–136) was amplified by PCR from the rat PLCζ clone (GenBank™ accession number M28637) that was kindly provided by M. Katan (Cancer Research UK, Centre for Cell and Molecular Biology, London, UK). We used the appropriate primers to incorporate a 5’-EcoRI site and a 3’-SalI site and the PCR product was cloned into the pGEX-6P1 vector. The forward primer was 5’-ACATGAATCTGACTGCCTGAGGAGCTCTC-3’ and the reverse 5’-GCCAGTGCACGTGACGCCGACGTG-3’. Each of the above expression vector constructs was confirmed by dyeodeoxysequencing (Prism Big Dye kit; ABI Prism® 3100 Genetic Analyzer, Applied Biosystems).

#### Protein expression and purification

For bacterial expression of glutathione S-transferase (GST) fusion proteins, E. coli (Roset3B DE3) cells; Novagen was transformed with the appropriate pGEX construct and cultured at 37°C until the A₆₀₀ reached 0.6, and then protein expression was induced for 18 hours at 16°C with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Promega). Cells were harvested by centrifugation at 6000 g for 10 minutes, resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.4) containing 2 mM dithiothreitol and protease inhibitor mixture (Roche), and then sonicated four times for 15 seconds on ice. After 15 minutes of centrifugation at 15,000 g at 4°C, soluble GST fusion proteins were purified by affinity chromatography using glutathione–Sepharose™ 4B beads following standard procedures (GE Healthcare). Eluted proteins were dialysed overnight (Pierce; SnakeSkin 10,000 molecular weight cut-off) at 4°C against 4 litres of PBS, and concentrated with centrifugal concentrators (Sartorius; 10,000 molecular weight cut-off).

#### Protein–lipid overlay assay

PIP Array membranes (Mammalian Proteome) were blocked for 2 hours with binding buffer [TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.4)] containing 3% bovine serum albumin (protein-free), and incubated with 25 pmoles of each GST–PLCζ fusion protein for 1 hour at room temperature. After washing three times in TBS-T, GST–PLCζ fusion protein interaction with the phosphatidylinositolinositol was detected by first incubating the PIP Array membranes with rabbit anti-GST polyclonal antibody (T103; 1:10,000 dilution in 5 ml of binding buffer) (Nomikos et al., 2005; Nomikos et al., 2007) overnight at 4°C, followed by 3× 15-minute washes. This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody in the same binding buffer for 1 hour at room temperature, followed by 3× 15-minute washes with TBS-T. Super Signal West Dura (Pierce) was used to visualise the HRP-coupled antibodies followed by a Bio-Rad Gel Doc system for image capture.

#### Liposome preparation and binding assay

Unilamellar liposomes were prepared by the extrusion method using a laboratory extractor (LipoFast-Pneumatic, Avestin Inc., Ottawa, ON, Canada) with lipids purchased from Avanti Polar Lipids Inc. (Alabaster, AL). In a typical experiment requiring a 2 ml dispersion of liposomes, 0.038 mmol (19.5 x 10⁻³ M) 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (PtdCho), 0.019 mmol (9.5 x 10⁻³ M) of cholesterol (CHOL; molar ratio of PtdCho:CHOL 2:1), 0.0095 mmol (4.8 x 10⁻³ M) 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine (PtdEtn; molar ratio of PtdCho:PtdEtn 4:1) and 1% of total lipids (16:50:38 phosphatidylcholine and phosphatidylinositol (PtdIns) were dissolved in chloroform:methanol (2:1 v/v) for the formation of lipid films. The film was hydrated with 2 ml of PBS and the resultant suspension was extruded through two stacked polycarbonate filters of 100 nm pore size. Twenty-five cycles of extrusion were applied at 30°C. For the control experiments, unilamellar liposomes without PtdIns were prepared in an analogous manner. Dynamic light scattering (DLS) was employed to determine the size of the liposomes, which used a light scattering apparatus (AXIOS–150/EX, Thriton Hellas, Thessaloniki, Greece) with a 30 mW laser source and an Avalanche photodiode detector set at a 90° angle. DLS measurements of the extruded lipid preparation showed a narrow monomodal size distribution with average liposome diameter of 100±10 nm and polydispersity index of 0.20–0.25. For system-binding index of the liposomes (100 µg of 4:2:1 PtdCho:CHOL:PtdEtn containing 0–1% PtdIns(4,5)P₂; were incubated with 1 µg of recombining protein for 20 minutes at room temperature and centrifuged for 5 hours at 4°C. The supernatant and pellet were then analyzed by SDS-PAGE and Coomassie Blue staining.

#### Mutagenesis and cRNA synthesis

A pCR3 mouse PLCζ- luciferase construct (Nomikos et al., 2005) was subjected to site-directed mutagenesis (QuickChange II, Stratagene) to sequentially generate the
three substitutions of alanine for lysine at K374, K375 and K377, thus producing the PLCζK374, K375,5AA mutant. Following lineairization of wild-type and mutated PLCζ constructs, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and then polyadenylated using the poly(A) tailing kit (Ambion), as per the manufacturer's instructions.

Preparation and handling of gametes
Experiments were carried out with mouse eggs in Hepes-buffered medium (H-KSOM) as described previously (Summers et al., 2000; Nomikos et al., 2005). All compounds were from Sigma unless stated otherwise. Female mice were superovulated and eggs were collected 13.5–14.5 hours after injection of human chorionic gonadotrophin and maintained in droplets of M2 medium (Sigma) or H-KSOM under mineral oil at 37°C. Microinjection of the eggs was carried out 14.5–15.5 hours after the hormone injection (Nomikos et al., 2005).

Microinjection and measurement of intracellular Ca2+ and luciferase expression
Mouse eggs were washed in M2 and microinjected with cRNA diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4) (Nomikos et al., 2005). 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. Eggs were microinjected with the appropriate cRNA, mixed with an equal volume of 1 mM Oregon Green BAPTA dextran (Molecular Probes) in the injection buffer. Eggs were then maintained in H-KSOM with 100 µM luciferin and imaged on a Nikon TE2000 or Zeiss Axiophot 100 microscope equipped with a cooled intensified CCD camera (Photokon Ltd., St Leonards on Sea, UK). Ca2+ was monitored in these eggs for 4 hours 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 monitored for luminescence by integrating light emission (in the absence of fluorescence excitation) for 20 minutes 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 further analysed only if the same egg was luminescence. The luminescence from eggs was converted into an arbitrary number for each egg, as these were measured using the Nikon software for the luminescence from each egg to be converted into a single number.

Assay of PLC activity
PtdIns(4,5)P2 hydrolytic activity of recombinant wild-type PLCζ and the various mutant constructs was assayed as described previously (Nomikos et al., 2005). The final volume of the assay mixture was 50 µl containing 100 mM NaCl, 0.4% sodium cholate (w/v), 2 mM CaCl2, 4 mM EGTA, 20 µg of bovine serum albumin, 5 mM 2-mercaptoethanol and 20 mM Tris-HCl buffer, pH 8.6. The final concentration of PtdIns(4,5)P2 in the reaction mixture was 220 µM, containing 0.05 µCi of [3H]PtdIns(4,5)P2. The assay conditions were optimized for linearity, requiring a 10-minute incubation of 20 pmol GST–PLCζ protein sample at 25°C. Reactions were stopped by the addition of 0.25 ml chloroform:methanol:concentrated HCl (95:7:4 v/v/v). Any unhydrolyzed PtdIns(4,5)P2 was removed by centrifugation at 10000 g for 2 minutes, and then 0.2 ml of the upper aqueous phase was removed and added to 10 ml Optiphase ‘Hisafe 3’ scintillation mixture (Wallac), and the radioactivity was determined by liquid scintillation counting.

Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate.
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