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Novel regulation of PLCζ activity via its XY-linker

Michail NOMIKOS*†, Khalil ELGMATI‡, Maria THEODORIDOU*†, Athena GEORGILIS*, J. Raul GONZALEZ-GARCIA*, George NOUNESIS†, Karl SWANN‡ and F. Anthony LAI*†

*Cell Signalling Laboratory, Wales Heart Research Institute & ‡Department of Obstetrics and Gynaecology, Cardiff University School of Medicine, Cardiff, UK
†Biomolecular Physics Laboratory, IRRP, National Center for Scientific Research ‘Demokritos’, 15310 Aghia Paraskevi, Greece

Short title: Distinctive role for the XY-linker region of sperm PLC-zeta

1 To whom correspondence should be addressed (email: mixosn@yahoo.com (MN), lait@cf.ac.uk (FAL)^).^Author for editorial correspondence prior to publication

Abbreviations: phospholipase C, PLC; phospholipase C-zeta, PLCζ; XY-linker, XYl; phosphatidylinositol 4,5-bisphosphate, PIP2; inositol 1,4,5-trisphosphate, IP3; open reading frame, ORF; charge-coupled device, CCD; human chorionic gonadotrophin, hCG; glutathione-S-transferase; GST

SYNOPSIS
The XY-linker region of somatic cell PLC-β, -γ, -δ and -ε isoforms confers potent catalytic inhibition suggesting a common auto-regulatory role. Surprisingly, the sperm PLCζ XY-linker does not mediate auto-inhibition. Unlike for somatic PLCs, absence of the PLCζ XY-linker significantly diminishes both in vitro PIP2 hydrolysis and in vivo Ca2+ oscillation-inducing activity, revealing evidence for a novel PLCζ enzymatic mechanism.

Keywords: phospholipase C-zeta, enzyme regulation, calcium oscillations, egg activation, fertilization

INTRODUCTION
The activation of a mammalian egg by a fertilizing sperm is effected by a characteristic series of cytoplasmic Ca2+ oscillations following sperm-egg fusion. This fundamental activation event provides the stimulus for the initiation of embryo development [1,2]. A sperm-specific phospholipase C isoform, PLC-zeta (PLCζ), is widely considered to be the physiological stimulus that triggers these intracellular Ca2+ oscillations at fertilization [3-7]. Sperm-delivered PLCζ is responsible for catalysing phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis within the fertilized egg to stimulate the inositol 1,4,5-trisphosphate (IP3) signaling pathway leading to Ca2+ oscillations [8,9]. The phosphosomitate-specific PLC family comprises thirteen isozymes grouped into six different subfamilies (β, γ, δ, ε, ζ, η), each activated by different stimuli to catalyze PIP2 hydrolysis yielding IP3, which in turn mediates intracellular Ca2+ release. All known mammalian PLCs possess homologous X and Y catalytic domains separated by a charged XY-linker (XYl) region. Likewise,
all isoforms have four tandem EF hand domains and a single C2 domain that flank the core X and Y domains, respectively [10].

Notably, the sperm-specific PLCζ is unique in displaying a positively-charged XYI region whereas in the somatic cell PLCβ, δ and ε isoforms, this region is negatively-charged. Recently, the XYI within PLCβ, δ and ε was shown to specifically mediate auto-inhibition of PIP2 hydrolytic activity, suggesting that the negatively-charged residues of the XYI directly prevent access of PIP2 to the enzyme active site via steric exclusion and electrostatic repulsion of the negatively-charged PIP2 substrate [11]. The PLCγ XYI, which comprises additional regulatory domains including two SH2 domains and an SH3 domain, regulates PLCγ via tyrosine phosphorylation [12,13]. Identification of the critical determinant for PLCγ inhibition at one of the SH2 domains has led to a proposed general mechanism of PLC auto-inhibition mediated by the XYI region [14].

The molecular mechanisms involved in physiological regulation of sperm PLCζ activity, which plays a crucial role in mammalian fertilization, remain unknown. To examine whether the XYI-mediated auto-inhibition observed in somatic cell PLC isoforms also applies to PLCζ activity regulation, we specifically removed this unique PLCζ XYI region and monitored consequent changes in the in vivo Ca2+ oscillation-inducing and in vitro PIP2 hydrolysis activity relative to the wild-type sperm PLCζ. For comparative analysis, we also generated the corresponding XYI deletion within PLCδ1, as well as a chimaeric PLCζ construct, in which the last 12 amino acids from the XYI region (residues 374-385) were replaced with those of PLCδ1 (residues 480-491). Our studies show that in contrast to somatic cell PLCs, the XYI of PLCζ does not confer enzymatic auto-inhibition, indicating a disparate regulatory mechanism may apply to this distinctive, gamete-specific PLC isozyme.

MATERIALS AND METHODS
Plasmid construction and cRNA synthesis
To prepare the PLCζ XYI-deletion construct, mouse PLCζ1-307 (GenBank #AF435950) was amplified by PCR, using Phusion polymerase (Finnzymes) using appropriate primers to incorporate a 5′-KpnI and 3′-EcoRI site to generate pCR3-PLCζ1-307. Similarly, PLCζ386-647 with a 5′ EcoRI site and a 3′ primer that ablated the stop codon to create a NotI site, was cloned into the pCR3-PLCζ1-307 to generate pCR3-PLCζ1-307/386-647. The luciferase ORF amplified from pGL2 (Promega) to incorporate flanking NotI sites was then cloned into the NotI site of pCR3-PLCζ1-307/386-647 to generate PLCζ1-307/386-647-luciferase. The PLCζ1-647 was further amplified from pCR3-PLCζ1-307/386-647 to incorporate a 5′-SalI and 3′-NotI site and subcloned into a modified pET vector (pETMM30) to enable bacterial expression.

Rat PLCδ1 (GenBank #M20637) with a 5′-SalI site and a 3′-NotI site was cloned into pGEX-5X2. To generate pCR3-PLCδ1-luciferase, PLCδ1 amplified from pGEX-5X-2-PLCδ1 to incorporate a 5′-EcoRV and 3′-NotI site and cloned into pCR3, was ligated in-frame with luciferase containing 5′-NotI and 3′-NotI sites. To prepare the PLCδ1 XYI-deletion construct, pCR3-PLCδ1-440/491-756-luciferase, PLCδ1-440 with a 5′-EcoRI and 3′-EcoRV site cloned into pCR3 was ligated in-frame to PLCδ1-491-756 with a 5′-EcoRV site and a 3′-NotI site. Luciferase was then inserted via the NotI site of pCR3-PLCδ1-440/491-756. The PLCδ1-440/491-756, via the 5′-SalI and 3′-NotI sites was further subcloned into pETMM30 for bacterial expression.

The PLCζ/XYIδ1 chimaeric construct was prepared using a long primer strategy that utilised primers comprising nucleotides corresponding to XYI residues 480-491 of PLCδ1. These primers also contained a short sequence from the XYI region of PLCζ. Amplification of the two halves of PLCζ with these long primers enabled replacement of the PLCζ XYI residues 374-385 (KRRKKRKKMIAK) with the corresponding PLCδ1 XYI residues 480-491 (KPKEDKLKLVPE) to be achieved. Four silent mutations in the PLCδ1 XYI sequence were introduced to circumvent non-specific annealing of the primers. The PLCζ/XYIδ1 chimaera thus generated was cloned into
pCR XL TOPO and then subcloned into pCR3. The luciferase ORF amplified from pGL2 as above was then ligated in-frame into the NotI site of pCR3-PLCζ/XYlδ1480-491 to generate PLCζ/XYlδ1480-491-luciferase. The PLCζ/XYlδ1480-491 was further amplified from pCR3-PLCζ/XYlδ1480-491 to incorporate a 5'-Sall and 3'-NotI site and subcloned into a modified pET vector (pETMM30) to enable bacterial expression.

Following linearization of wild-type, XY1-excised and chimaeric PLC plasmids, cRNA was synthesized using mMessage Machine T7 kit (Ambion) and 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 saline (H-KSOM) as described previously [3,4]. Female mice were superovulated by injection of human chorionic gonadotrophin (hCG; Intervet). Eggs were collected 13.5-14.5 h later and maintained in 100 μl H-KSOM under mineral oil at 37 °C. Egg microinjection was carried out 14.5-15.5 h after hCG administration [16].

**Microinjection and measurement of intracellular Ca²⁺ and luciferase expression**

Mouse eggs were microinjected with cRNA encoding the particular PLC(s) mixed with an equal volume of 1 mM Oregon Green BAPTA-dextran (Molecular Probes) in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4). All injections were 3-5 % of the egg volume. Eggs were then maintained in H-KSOM containing 100 μM luciferin and imaged on a Nikon TE2000 microscope equipped with a cooled intensified-CCD camera (Photek Ltd, UK). Ca²⁺ was monitored for 4 h after injection by measuring Oregon Green BAPTA-dextran fluorescence with low-level excitation light from a halogen lamp. Luminescence was measured with the same camera alongside fluorescence by switching, every 10 seconds, between light collection in the presence and the absence of excitation light. Fluorescence signals were 10-100 times that for luminescence. The luminescence, measured as the light emission in the absence of excitation light, was quantitatively converted into luciferase protein using a standard luminescence calibration curve generated by microinjection of eggs with known amounts of luciferase protein (Sigma) [16,17].

**Protein expression and purification**

For GST-PLC fusion protein expression, *E. coli* (Rosetta (DE3), Novagen) transformed with the appropriate plasmid, was cultured at 37 °C until A₆₀₀ of 0.6 then protein expression induced for 18 h, 16°C with 0.1 mM isopropyl β-D-thiogalactopyranoside (Promega). Cells were centrifuged at 6000 × g for 10 min, 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) then sonicated 4 x 15 s on ice. After centrifugation at 15,000 × g, 15 min, 4 °C, soluble GST-PLC fusion proteins were purified by affinity chromatography using glutathione-Sepharose™ 4B following standard procedures (GE Healthcare). Eluted proteins were dialyzed overnight (Pierce; SnakeSkin 10,000 MWCO) at 4 °C in 4 litres of PBS, and concentrated with centrifugal concentrators (Sartorius, 10,000 MWCO).

**PLC activity assay, PAGE and Western blotting**

PIP₂ hydrolytic activity of PLC constructs was assayed as described previously [17]. The assay mixture final volume 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 6.8. The PIP₂ concentration in the reaction mixture was 220 μM, containing 0.05 μCi of [³H]PIP₂. Assay conditions were optimized for linearity, requiring a 10 min, 25 °C incubation with 20 pmol protein. Recombinant proteins were separated by SDS-PAGE and immunoblot analysis performed as described previously [15]. Proteins were probed with anti-GST polyclonal antibody (1:10,000 dilution).

**RESULTS**

To understand the regulatory role of the short linker region separating the catalytic X and Y domains, the XY1 of both PLCζ (308-385aa) and PLCδ1 (441-490aa) were excised from the wild-
type PLCs to create the XYI-deletion constructs, PLCζ^{XYI} and PLCδ^{XYI}, respectively (Fig. 1). The PLCζ XYI notably contains a unique cluster of basic residues that may be involved in enzyme function [8,9]. To further examine the potential role of this short, positively-charged XYI segment in regulation of PLCζ activity, a chimerae PLCζ construct was prepared in which these 12 amino acids of PLCζ (aa374-385, KKKRRKMKIAMA; +7 charged residues) were replaced with the corresponding stretch from PLCδ1 (aa480-491, PKPEDKLLVLPE; +4/-3 charged residues), generating PLCζ/XYI61480-491 (Fig. 1). The XYI-deletion and chimerae constructs, along with the corresponding wild-type PLCs, were each tagged at the C-terminus with luciferase to enable real-time monitoring of relative protein expression by luminescence quantitation [16]. Consistent with previous reports [17,20], prominent Ca^{2+} oscillations (25 spikes/2h) are observed in unfertilized mouse eggs microinjected with PLCζ cRNA (Fig. 2), with the first Ca^{2+} spike appearing at a luminescence of 0.52 counts per second (cps) for the expressed PLC-luciferase fusion protein (Table 1). In contrast, microinjecting cRNA encoding the XYI-deletion construct, PLCζ^{XYI}, produces Ca^{2+} oscillations in mouse eggs with a significantly lower frequency (3.4 spikes/2h) relative to PLCζ, and with the first Ca^{2+} spike only appearing after luminescence had reached 3.6 cps. Similarly, microinjection of cRNA corresponding to the XYI chimera, PLCζ/XYI61480-491, also triggered relatively low frequency Ca^{2+} oscillations (5.3 spikes/2h) with the first Ca^{2+} spike appearing at a luminescence of 4.0 cps (Fig. 2; Table 1).

Microinjection of PLCδ1 cRNA into mouse eggs caused very low frequency Ca^{2+} oscillations (1.8 spikes/2h) that commenced only when the PLCδ1-luciferase protein expression produced a luminescence value of 20.4 cps. However, the PLCδ1^{XYI} deletion construct cRNA effected a ~2 fold increase in Ca^{2+} oscillation frequency (3.3 spikes/2h) compared to PLCδ1, with the first Ca^{2+} spike manifest at a luminescence of 17.2 cps. These mouse egg microinjection results show that absence of the PLCζ XYI region dramatically attenuates Ca^{2+} oscillation-inducing activity (Fig. 2), yielding a 7-fold reduction in spike frequency (25 versus 3.4 spikes/2h) and requiring a 7-fold increased level of PLCζ^{XYI} expression (3.6 versus 0.52 cps) to initiate the first Ca^{2+} spike (Table 1). In addition, replacing the cluster of basic residues in the PLCζ XYI (7 of 12 residues are positively-charged, overall +7) with the corresponding amino acids from the XYI of PLCδ1 (4 +ve, 3 -ve residues; overall +1), also dramatically reduces by 5-fold the Ca^{2+} oscillation-inducing activity of PLCζ with requirement for an 8-fold increased level of PLCζ/XYIδ1480-491 expression to initiate the first spike compared to wild type PLCζ (4.0 versus 0.52 cps). Conversely, the XYI deletion from PLCδ1 increased its Ca^{2+} oscillation-inducing activity in mouse eggs with a doubling of the Ca^{2+} spike frequency (3.3 versus 1.8 spikes/2h).

The effect of removing or replacing part of the XYI on the in vitro PIP_2 hydrolysis activity of PLCζ, PLCδ1, PLCζ^{XYI}, PLCδ1^{XYI} and PLCζ/XYI61480-491 constructs was examined following their expression in bacteria and purification as GST-fusion proteins. Figure 3A shows the affinity-purified fusion proteins displayed the predicted molecular masses for GST-PLCζ, GST-PLCζ^{XYI}, GST-PLCδ1, GST-PLCδ1^{XYI} and PLCζ/XYI61480-491 recombinant proteins of 100, 94, 111, 107 and 102 kDa, respectively, as also confirmed by immunoblot analysis with anti-GST antibody. The specific PIP_2 hydrolytic enzyme activity values obtained for each protein (Fig. 3B) reveals a 30% reduction in PLCζ^{XYI} enzyme activity relative to PLCζ (302±58 vs 425±51 nmol/min/mg), and a 20% reduction in the chimera PLCζ/XYIδ1480-491 enzyme activity (342±38 vs 425±51 nmol/min/mg), indicating the presence of the XYI region and the highly positively-charged cluster are required for maximal PLCζ activity. In contrast, PLCδ1^{XYI} displayed a ~3.2-fold increase in enzymatic activity compared to PLCδ1 (2865±54 vs 1249±40 nmol/min/mg). These differential results for XYI-deleted PLCs suggest that there are disparate regulatory roles for the XYI of PLCδ1 and PLCζ with respect to enzyme hydrolytic activity. Calculation of the Michaelis-Menten constant, Km, for these proteins yielded comparable values for PLCδ1 (93 μM) and PLCδ1^{XYI} (63 μM). However, for PLCζ^{XYI} (3936 μM) the Km was 36-fold higher than that of PLCζ (110 μM) (Table...
2), indicating that deletion of the XYI has a major effect by dramatically reducing the in vitro affinity of PLCζ for the PIP2 substrate. Similarly, the Km value for the XYI chimaeric protein (1909 μM) was 17-fold higher than that of PLCζ (Table 2), highlighting the importance of the cluster of basic residues in the XYI region of PLCζ for the in vitro affinity of this enzyme for PIP2.

Further, the impact of XYI deletion or replacement on the relative Ca2+ sensitivity of PLCζ and PLCδ1 enzyme activity [5,17,18] was determined at Ca2+ concentrations ranging from 0.1 nM to 0.1 mM. The resulting EC50 value obtained for PLCζ was near identical to the corresponding XYI-truncated protein (91 nM vs 84 nM) and the XYI-chimaeric protein (91 nM vs 76 nM) (Fig. 3C, Table 2). Likewise, removing the XYI from PLCδ1 marginally altered the EC50 value from 6.3 to 7.0 μM. These results suggest that loss of the XYI or replacement of the cluster of basic residues in this region does not significantly alter the Ca2+ sensitivity of PIP2 hydrolysis for both PLCζ and PLCδ1.

**DISCUSSION**

Although the precise regulatory mechanism remains unclear, PLCζ has become established as the primary sperm-derived candidate factor that activates the egg at mammalian fertilization. Upon sperm-egg fusion, PLCζ is proposed to be delivered by the sperm into the ooplasm and catalyses PIP2 hydrolysis to generate IP3, which induces the cytoplasmic Ca2+ oscillations that initiate embryo development. Sperm-specific PLCζ is the smallest mammalian PLC isoform with the most elementary domain organization and it is the only one not found in somatic cells [3]. PLCζ is most structurally similar to PLCδ1 with the notable exception that it lacks a PH domain at the N-terminus (Fig.1). One further important and unique functional feature of PLCζ is the relatively low Ca2+ concentration (nanomolar) required for enzymatic activity, exhibiting ~100-fold higher Ca2+ sensitivity than PLCδ1 which requires micromolar Ca2+ concentration for optimal PIP2 hydrolysis. Thus, at the basal cytosolic Ca2+ concentration of 50-80 nM likely present within eggs, the PLCζ isoform but not PLCδ1 would be strongly activated. The molecular determinants that confer PLCζ’s high Ca2+ sensitivity are unknown, although previous studies suggest both EF hand and C2 domains are required for a functional PLCζ in the egg [17,18].

Another important question that remains unresolved is how PLCζ activity is intrinsically regulated. Recent structural and biochemical studies have convincingly demonstrated that the XYI region of the PLCβ, γ, δ and ε isozymes can mediate potent auto-inhibition of enzyme function [11,14]. This is consistent with the negatively-charged XYI of these isoforms conferring electrostatic repulsion of the negatively-charged PIP2 substrate, as well as providing steric hindrance by occluding the enzyme catalytic active site. However, the sperm PLCζ in this regard is very distinct from somatic PLCs in possessing a positively-charged XYI region. It was therefore important to investigate whether this putative general mechanism of XYI auto-inhibition observed in various somatic PLC isoforms also applies to the sperm-derived PLCζ.

In the present study, a truncated PLCζ lacking the XYI region, as well as a chimaeric PLCζ in which the cluster of basic residues at the C-terminal end of the XYI was replaced by the homologous region of PLCδ1 were prepared. These two novel PLCζ constructs enabled the specific examination of how these targeted XYI changes might alter the in vivo Ca2+ oscillation-inducing and in vitro PIP2 hydrolysis activity relative to wild-type PLCζ. Parallel studies were simultaneously performed using the corresponding construct derived from the most closely-related PLC isoform, PLCδ1. Notably, PLCδ1 is absent from differentiated spermatids and is not believed to play a role in mammalian fertilization [19], but it provides a useful comparative PLC isoform control. The bacterially-expressed and purified PLCδ1 exhibited a much higher in vitro PIP2 hydrolytic activity than PLCζ (Fig. 3B), although the in vivo Ca2+ oscillation-inducing activity observed for PLCδ1 in mouse eggs was much lower than that of PLCζ (Fig. 2). This is consistent with a previous study showing PLCδ1 was capable of inducing only low frequency Ca2+ oscillations in mouse eggs, even at a 20-fold higher concentration than PLCζ [5]. Interestingly, deletion of the XYI from PLCδ1
resulted in a 2-fold increase in Ca\(^{2+}\) oscillation-inducing activity in eggs (Fig. 2), which correlates with the \textit{in vitro} PIP\(_2\) hydrolysis assays showing a ~2.3-fold increased enzymatic activity relative to wild-type PLC\(\delta1\) (Fig. 3B, Table 1). In contrast, the deletion of XYI from PLC\(\zeta\) decreased both the \textit{in vitro} enzymatic activity (Fig.3B) and the PIP\(_2\) substrate affinity (Table 2), which was consistent with the observed 7-fold reduction in Ca\(^{2+}\) oscillation-inducing activity in eggs (Table 1). The XYI appears not to be directly involved in Ca\(^{2+}\)-dependent regulation of enzyme activity, as the Ca\(^{2+}\) sensitivity of in vitro PIP\(_2\) hydrolysis was essentially unchanged between the wild-type and XYI-deleted PLC constructs (Fig. 3C, Table 2). Significantly, replacement of only the PLC\(\zeta\) XYI cluster of basic residues (overall charge +7) by the homologous twelve amino acids of the XYI region of PLC\(\delta1\) (overall charge +1) also resulted in decrease of both the \textit{in vitro} enzymatic activity (Fig. 3B) and the PIP\(_2\) substrate affinity (Table 2). These \textit{in vitro} results are consistent with the observed 5-fold reduction in Ca\(^{2+}\) oscillation-inducing activity in eggs with this chimaeric PLC\(\zeta\) (Table 1), whereas the Ca\(^{2+}\) sensitivity remained comparable to the wild-type enzyme (Fig. 3C, Table 2).

Our findings suggest that the XYI of PLC\(\zeta\) serves a different regulatory role to that of the XYI in PLC\(\delta1\). A critical determinant for this disparity may be the high density of basic amino acids in the XYI of PLC\(\zeta\) that is absent from PLC\(\delta1\) and other somatic PLC isoforms. Previously, we have proposed that this unstructured cluster of positively-charged residues at the C-terminal end of the PLC\(\zeta\) XYI may play a role in facilitating interaction with biological membranes, particularly the negatively-charged substrate, PIP\(_2\) [20,21]. Direct involvement of the XYI positively-charged residues in PIP\(_2\) interaction was recently examined by sequentially substituting three XYI lysine residues, K374, K375 and K377, for alanine to produce single (K374A), double (K374,5AA) and triple (K374,5,7AAA) substitutions [21]. The Ca\(^{2+}\) oscillation-inducing activity in mouse eggs, PIP\(_2\) binding and enzymatic hydrolysis measurements of these K-A mutants revealed that the cumulative reduction of the PLC\(\zeta\) XYI net positive charge progressively abated both the \textit{in vitro} Ca\(^{2+}\) oscillations and \textit{in vitro} PIP\(_2\) interaction/enzyme function of mouse PLC\(\zeta\) [21]. These results indicate that the XYI cluster of positively-charged residues may perform a central role in the interaction of PLC\(\zeta\) with the substrate, PIP\(_2\) [20,21]. Such a proposed role for the XYI of PLC\(\zeta\) in PIP\(_2\) binding is entirely consistent with the present study in which excision of the complete XYI or exchanging a discrete XYI segment, and thereby removing the entire cluster of basic residues, causes significant diminution of both PLC\(\zeta\) functional properties and PIP\(_2\) interaction without altering Ca\(^{2+}\) sensitivity.

Although the specific amino acid sequence of the XYI in PLC\(\zeta\) is poorly conserved across species, the presence of positively-charged residues is a common feature of the PLC\(\zeta\) sequences currently available [8,9]. The significance of this sequence diversity, albeit with charge conservation, might explain the different rates of PIP\(_2\) hydrolysis observed for PLC\(\zeta\) isoforms from different species and thus the species-specific frequency of sperm-induced Ca\(^{2+}\) oscillations observed in the eggs of different mammals [9]. Interestingly, a study of bovine PLC\(\zeta\) has found that it remains functionally active even after proteolytic cleavage occurs specifically within the XYI region [22]. Further investigation is required to delineate the precise molecular mechanism of action of the various PLC\(\zeta\) domains and this may lead to important implications in the therapeutic approach to PLC\(\zeta\)-mediated male infertility [15].

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AUTHOR CONTRIBUTIONS
Michail Nomikos, J. Raul Gonzalez-Garcia, George Nounesis, Karl Swann and F. Anthony Lai devised the project strategy, MN and FAL designed the experiments, which were performed by MN,
Khalil Elgmati, Maria Theodoridou, Athena Georgilis and JRG. MN, KS and FAL prepared the manuscript.

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FIGURE LEGENDS

Figure 1
Domain organisation of PLCζ, PLCδ1 and deletion/chimaera constructs
Schematic representation of the domain organisation of wild-type PLCζ and PLCδ1, their corresponding XY-linker deletions PLCζΔXYlinker and PLCδ1ΔXYlinker, and the PLCζ/XYlinkerδ1 chimaera. Note the similar presence in PLCζ and PLCδ1 of the EF, X, XY-linker, Y and C2 domains, but absence of the PH domain in PLCζ. The various amino acid lengths and respective XY-linker coordinates are also indicated for each construct.

Figure 2
Ca2+ oscillation-inducing activity of PLC and XY-linker deletion/chimaera expressed in mouse eggs
Fluorescence and luminescence recordings reporting the Ca2+ changes (red traces; Fluorescence, arbitrary units (a.u.)) and luciferase expression (black traces; Luminescence, counts per second (cps)), respectively, in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged PLCζ, PLCδ1, their corresponding XY-linker deletions and the PLCζ/XYlinkerδ1 chimaera (left panels). Panels on the right show the integrated luminescence image of a field of mouse eggs following cRNA microinjection of each PLC construct (see Table 1).

Figure 3
Expression, purification and enzyme activity of PLC and XY-linker/chimaera proteins
(A) Glutathione affinity-purified GST-PLC fusion protein (1 μg) was analyzed by 8% SDS-PAGE followed by either Coomassie Brilliant Blue staining (left panel) or immunoblot analysis using anti-GST antibody (middle panel). Lanes 1-4 show PLCζ, PLCζΔXY1, PLCδ1 and PLCδ1ΔXY1, respectively. The pair of single lane panels on the right shows Coomassie gel and immunoblot analysis of the PLCζ/XYlinkerδ1 chimaera. (B) PIP2 hydrolysis enzyme activity assay of PLCζ, PLCδ1 their XY-linker deletions and chimaera (20 pmol) obtained with the standard [3H]PIP2 cleavage assay, n = 3 ± S.E., using two different preparations of recombinant protein and with each experiment performed in duplicate. (C) Effect of varying [Ca2+] on the normalized activity of PLCζ, PLCδ1 and their XY-linker deletions and chimaera. For these assays n = 2 ± S.E., using two different batches of recombinant proteins and with each experiment performed in duplicate (see Table 2).
TABLE LEGENDS

Table 1

Properties of PLC-luciferase and deletion/chimaera constructs expressed in mouse eggs

Ca\(^{2+}\) oscillation-inducing activity (Ca\(^{2+}\) spike number in 2 hours) and luciferase luminescence levels (Peak luminescence; Luminescence at 1\(^{st}\) spike) are summarized for mouse eggs microinjected with each of the PLC-luciferase constructs, PLC\(\zeta\), PLC\(\zeta^{\Delta XYl}\), PLC\(\delta1\), PLC\(\delta1^{\Delta XYl}\) and PLC\(\zeta^{XYl\delta1}\) (see Fig. 2). Each egg was microinjected with a pipette cRNA concentration of 1.6 g/l. The data are expressed to 2 significant figures, with means ± s.e.m.

Table 2

\(^{3}H\)PIP\(_2\) hydrolysis activity and \(Km\) of purified GST-PLC fusion proteins

Specific enzyme activity values obtained for the various GST-PLC fusion proteins showing the EC\(_{50}\) of Ca\(^{2+}\)-dependent enzyme activity and the Michaelis-Menten constant, \(Km\), determined by non-linear regression analysis (GraphPad, Prism 5) for the GST-fusion proteins PLC\(\zeta\), PLC\(\zeta^{\Delta XYl}\), PLC\(\delta1\), PLC\(\delta1^{\Delta XYl}\) and PLC\(\zeta^{XYl\delta1}\) (see Fig. 3C).
TABLES

Table 1

| PLC-luciferase injected | Ca^{2+} oscillations (spikes/2 hour) | Peak luminescence (cps) | Luminescence at 1st spike (cps) | No. of eggs |
|-------------------------|-------------------------------------|-------------------------|-------------------------------|-------------|
| PLCζ                    | 24.5 ± 0.88                         | 8.7 ± 1.16              | 0.5 ± 0.06                    | 13          |
| PLCζ^{ΔXYI}             | 3.4 ± 0.27                          | 7.1 ± 0.20              | 3.6 ± 0.20                    | 20          |
| PLCδ1                   | 1.8 ± 0.10                          | 45.0 ± 1.7              | 20.4 ± 3.00                   | 17          |
| PLCδ1^{ΔXYI}            | 3.3 ± 0.20                          | 40.2 ± 1.7              | 17.2 ± 0.35                   | 19          |
| PLCζ^{XYIδ1}            | 5.3 ± 0.16                          | 30.5 ± 2.0              | 4.0 ± 0.39                    | 9           |

Table 2

| GST-PLC protein | Ca^{2+}-dependence EC_{50} (nM) | Michaelis-Menten Km (μM) |
|-----------------|---------------------------------|--------------------------|
| PLCζ            | 91                              | 110                      |
| PLCζ^{ΔXYI}     | 84                              | 3936                     |
| PLCδ1           | 6289                            | 93                       |
| PLCδ1^{ΔXYI}    | 6973                            | 63                       |
| PLCζ^{XYIδ1}    | 76                              | 1909                     |
Fig. 1
Fig. 2

- PLCζ wt
- PLCζ ΔXYlink
- PLCζ1 wt
- PLCζ1 ΔXYlink
- PLCζ XYlinkζ1

Fluorescence (a.u.)

Luminescence (cpm)

1 hr
Fig. 3A
Fig. 3B
Fig. 3C