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## Novel regulation of PLC $\zeta$ activity via its XY-linker

Michail NOMIKOS\* $\dagger^1$ , Khalil ELGMATI $\ddagger$ , Maria THEODORIDOU\* $\dagger$ , Athena GEORGILIS\*, J. Raul GONZALEZ-GARCIA\*, George NOUNESIS $\dagger$ , Karl SWANN $\ddagger$  and F. Anthony LAI\* $^1$

\*Cell Signalling Laboratory, Wales Heart Research Institute &  $\ddagger$ Department of Obstetrics and Gynaecology, Cardiff University School of Medicine, Cardiff, UK

$\dagger$ 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)^).

$^{\wedge}$ Author for editorial correspondence prior to publication

**Abbreviations:** phospholipase C, PLC; phospholipase C-zeta, PLC $\zeta$ ; XY-linker, XYI, phosphatidylinositol 4,5-bisphosphate, PIP $_2$ ; inositol 1,4,5-trisphosphate, IP $_3$ ; 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- $\beta$ , - $\gamma$ , - $\delta$  and - $\epsilon$  isoforms confers potent catalytic inhibition suggesting a common auto-regulatory role. Surprisingly, the sperm PLC $\zeta$  XY-linker does not mediate auto-inhibition. Unlike for somatic PLCs, absence of the PLC $\zeta$  XY-linker significantly diminishes both *in vitro* PIP $_2$  hydrolysis and *in vivo* Ca $^{2+}$  oscillation-inducing activity, revealing evidence for a novel PLC $\zeta$  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 Ca $^{2+}$  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 (PLC $\zeta$ ), is widely considered to be the physiological stimulus that triggers these intracellular Ca $^{2+}$  oscillations at fertilization [3-7]. Sperm-delivered PLC $\zeta$  is responsible for catalysing phosphatidylinositol 4,5-bisphosphate (PIP $_2$ ) hydrolysis within the fertilized egg to stimulate the inositol 1,4,5-trisphosphate (IP $_3$ ) signaling pathway leading to Ca $^{2+}$  oscillations [8,9]. The phosphoinositide-specific PLC family comprises thirteen isozymes grouped into six different subfamilies ( $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ), each activated by different stimuli to catalyze PIP $_2$  hydrolysis yielding IP $_3$ , which in turn mediates intracellular Ca $^{2+}$  release. All known mammalian PLCs possess homologous X and Y catalytic domains separated by a charged XY-linker (XYI) 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 PLC $\zeta$  is unique in displaying a positively-charged XYI region whereas in the somatic cell PLC $\beta$ ,  $\delta$  and  $\epsilon$  isoforms, this region is negatively-charged. Recently, the XYI within PLC $\beta$ ,  $\delta$  and  $\epsilon$  was shown to specifically mediate auto-inhibition of PIP $_2$  hydrolytic activity, suggesting that the negatively-charged residues of the XYI directly prevent access of PIP $_2$  to the enzyme active site via steric exclusion and electrostatic repulsion of the negatively-charged PIP $_2$  substrate [11]. The PLC $\gamma$  XYI, which comprises additional regulatory domains including two SH2 domains and an SH3 domain, regulates PLC $\gamma$  via tyrosine phosphorylation [12,13]. Identification of the critical determinant for PLC $\gamma$  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 PLC $\zeta$  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 PLC $\zeta$  activity regulation, we specifically removed this unique PLC $\zeta$  XYI region and monitored consequent changes in the *in vivo* Ca $^{2+}$  oscillation-inducing and *in vitro* PIP $_2$  hydrolysis activity relative to the wild-type sperm PLC $\zeta$ . For comparative analysis, we also generated the corresponding XYI deletion within PLC $\delta$ 1, as well as a chimaeric PLC $\zeta$  construct, in which the last 12 amino acids from the XYI region (residues 374-385) were replaced with those of PLC $\delta$ 1 (residues 480-491). Our studies show that in contrast to somatic cell PLCs, the XYI of PLC $\zeta$  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 PLC $\zeta$  XYI-deletion construct, mouse PLC $\zeta$  $^{1-307}$  (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-PLC $\zeta$  $^{1-307}$ . Similarly, PLC $\zeta$  $^{386-647}$  with a 5' EcoRI site and a 3' primer that ablated the stop codon to create a NotI site, was cloned into the pCR3-PLC $\zeta$  $^{1-307}$  to generate pCR3-PLC $\zeta$  $^{1-307/386-647}$ . The luciferase ORF amplified from pGL2 (Promega) to incorporate flanking NotI sites was then cloned into the NotI site of pCR3-PLC $\zeta$  $^{1-307/386-647}$  to generate PLC $\zeta$  $^{1-307/386-647}$ -luciferase. The PLC $\zeta$  $^{1-307/386-647}$  was further amplified from pCR3-PLC $\zeta$  $^{1-307/386-647}$  to incorporate a 5'-SalI and 3'-NotI site and subcloned into a modified pET vector (pETM30) to enable bacterial expression.

Rat PLC $\delta$ 1 (GenBank #M20637) with a 5'-SalI site and a 3'-NotI site was cloned into pGEX-5X2. To generate pCR3-PLC $\delta$ 1-luciferase, PLC $\delta$ 1 amplified from pGEX-5X-2-PLC $\delta$ 1 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 PLC $\delta$ 1 XYI-deletion construct, pCR3-PLC $\delta$ 1 $^{1-440/491-756}$ -luciferase, PLC $\delta$ 1 $^{1-440}$  with a 5'-EcoRI and 3'-EcoRV site cloned into pCR3 was ligated in-frame to PLC $\delta$ 1 $^{491-756}$  with a 5'-EcoRV site and a 3'-NotI site. Luciferase was then inserted via the NotI site of pCR3-PLC $\delta$ 1 $^{1-440/491-756}$ . The PLC $\delta$ 1 $^{1-440/491-756}$ , via the 5'-SalI and 3'-NotI sites was further subcloned into pETM30 for bacterial expression.

The PLC $\zeta$ /XYI $\delta$ 1 $^{480-491}$  chimaeric construct was prepared using a long primer strategy that utilised primers comprising nucleotides corresponding to XYI residues 480-491 of PLC $\delta$ 1. These primers also contained a short sequence from the XYI region of PLC $\zeta$ . Amplification of the two halves of PLC $\zeta$  with these long primers enabled replacement of the PLC $\zeta$  XYI residues 374-385 (KKRKRKMKIAMA) with the corresponding PLC $\delta$ 1 XYI residues 480-491 (KPKEDKLLVPE) to be achieved. Four silent mutations in the PLC $\delta$ 1 XYI sequence were introduced to circumvent non-specific annealing of the primers. The PLC $\zeta$ /XYI $\delta$ 1 $^{480-491}$  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-PLC $\zeta$ /XYI $\delta$ 1<sup>480-491</sup> to generate PLC $\zeta$ /XYI $\delta$ 1<sup>480-491</sup>-luciferase. The PLC $\zeta$ /XYI $\delta$ 1<sup>480-491</sup> was further amplified from pCR3-PLC $\zeta$ /XYI $\delta$ 1<sup>480-491</sup> 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, XYI-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  $\mu$ l 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 Ca<sup>2+</sup> 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  $\mu$ M luciferin and imaged on a Nikon TE2000 microscope equipped with a cooled intensified-CCD camera (Photek Ltd, UK). Ca<sup>2+</sup> 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 A<sub>600</sub> of 0.6 then protein expression induced for 18 h, 16°C with 0.1 mM isopropyl  $\beta$ -D-thiogalactopyranoside (Promega). Cells were centrifuged at 6000  $\times$  g for 10 min, resuspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 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  $\times$  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

PIP<sub>2</sub> hydrolytic activity of PLC constructs was assayed as described previously [17]. The assay mixture final volume was 50  $\mu$ l containing 100 mM NaCl, 0.4% sodium cholate (w/v), 2 mM CaCl<sub>2</sub>, 4 mM EGTA, 20  $\mu$ g of bovine serum albumin, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl buffer, pH 6.8. The PIP<sub>2</sub> concentration in the reaction mixture was 220  $\mu$ M, containing 0.05  $\mu$ Ci of [<sup>3</sup>H]PIP<sub>2</sub>. 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 XYI of both PLC $\zeta$  (308-385aa) and PLC $\delta$ 1 (441-490aa) were excised from the wild-

type PLCs to create the XYI-deletion constructs, PLC $\zeta^{\Delta XYI}$  and PLC $\delta 1^{\Delta XYI}$ , respectively (Fig. 1). The PLC $\zeta$  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 PLC $\zeta$  activity, a chimaeric PLC $\zeta$  construct was prepared in which these 12 amino acids of PLC $\zeta$  (aa374-385, KKRKRKMKIAMA; +7 charged residues) were replaced with the corresponding stretch from PLC $\delta 1$  (aa480-491, KPKEDKLVPE; +4/-3 charged residues), generating PLC $\zeta$ /XYI $\delta 1^{480-491}$  (Fig. 1). The XYI-deletion and chimaeric 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<sup>2+</sup> oscillations (25 spikes/2h) are observed in unfertilized mouse eggs microinjected with PLC $\zeta$  cRNA (Fig. 2), with the first Ca<sup>2+</sup> 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, PLC $\zeta^{\Delta XYI}$ , produces Ca<sup>2+</sup> oscillations in mouse eggs with a significantly lower frequency (3.4 spikes/2h) relative to PLC $\zeta$ , and with the first Ca<sup>2+</sup> spike only appearing after luminescence had reached 3.6 cps. Similarly, microinjection of cRNA corresponding to the XYI chimaera, PLC $\zeta$ /XYI $\delta 1^{480-491}$ , also triggered relatively low frequency Ca<sup>2+</sup> oscillations (5.3 spikes/2h) with the first Ca<sup>2+</sup> spike appearing at a luminescence of 4.0 cps (Fig. 2; Table 1).

Microinjection of PLC $\delta 1$  cRNA into mouse eggs caused very low frequency Ca<sup>2+</sup> oscillations (1.8 spikes/2h) that commenced only when the PLC $\delta 1$ -luciferase protein expression produced a luminescence value of 20.4 cps. However, the PLC $\delta 1^{\Delta XYI}$  deletion construct cRNA effected a ~2 fold increase in Ca<sup>2+</sup> oscillation frequency (3.3 spikes/2h) compared to PLC $\delta 1$ , with the first Ca<sup>2+</sup> spike manifest at a luminescence of 17.2 cps. These mouse egg microinjection results show that absence of the PLC $\zeta$  XYI region dramatically attenuates Ca<sup>2+</sup> 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 PLC $\zeta^{\Delta XYI}$  expression (3.6 versus 0.52 cps) to initiate the first Ca<sup>2+</sup> spike (Table 1). In addition, replacing the cluster of basic residues in the PLC $\zeta$  XYI (7 of 12 residues are positively-charged, overall +7) with the corresponding amino acids from the XYI of PLC $\delta 1$  (4 +ve, 3 -ve residues; overall +1), also dramatically reduces by 5-fold the Ca<sup>2+</sup> oscillation-inducing activity of PLC $\zeta$  with requirement for an 8-fold increased level of PLC $\zeta$ /XYI $\delta 1^{480-491}$  expression to initiate the first spike compared to wild type PLC $\zeta$  (4.0 versus 0.52 cps). Conversely, the XYI deletion from PLC $\delta 1$  increased its Ca<sup>2+</sup> oscillation-inducing activity in mouse eggs with a doubling of the Ca<sup>2+</sup> spike frequency (3.3 versus 1.8 spikes/2h).

The effect of removing or replacing part of the XYI on the *in vitro* PIP<sub>2</sub> hydrolysis activity of PLC $\zeta$ , PLC $\delta 1$ , PLC $\zeta^{\Delta XYI}$ , PLC $\delta 1^{\Delta XYI}$  and PLC $\zeta$ /XYI $\delta 1^{480-491}$  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-PLC $\zeta$ , GST-PLC $\zeta^{\Delta XYI}$ , GST-PLC $\delta 1$ , GST-PLC $\delta 1^{\Delta XYI}$  and PLC $\zeta$ /XYI $\delta 1^{480-491}$  recombinant proteins of 100, 94, 111, 107 and 102 kDa, respectively, as also confirmed by immunoblot analysis with anti-GST antibody. The specific PIP<sub>2</sub> hydrolytic enzyme activity values obtained for each protein (Fig. 3B) reveals a 30% reduction in PLC $\zeta^{\Delta XYI}$  enzyme activity relative to PLC $\zeta$  (302±58 vs 425±51 nmol/min/mg), and a 20% reduction in the chimaera PLC $\zeta$ /XYI $\delta 1^{480-491}$  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 PLC $\zeta$  activity. In contrast, PLC $\delta 1^{\Delta XYI}$  displayed a ~2.3-fold increase in enzymatic activity compared to PLC $\delta 1$  (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 PLC $\delta 1$  and PLC $\zeta$  with respect to enzyme hydrolytic activity. Calculation of the Michaelis-Menten constant, *K<sub>m</sub>*, for these proteins yielded comparable values for PLC $\delta 1$  (93 μM) and PLC $\delta 1^{\Delta XYI}$  (63 μM). However, for PLC $\zeta^{\Delta XYI}$  (3936 μM) the *K<sub>m</sub>* was 36-fold higher than that of PLC $\zeta$  (110 μM) (Table

2), indicating that deletion of the XYI has a major effect by dramatically reducing the *in vitro* affinity of PLC $\zeta$  for the PIP<sub>2</sub> substrate. Similarly, the *K<sub>m</sub>* value for the XYI chimaeric protein (1909  $\mu$ M) was 17-fold higher than that of PLC $\zeta$  (Table 2), highlighting the importance of the cluster of basic residues in the XYI region of PLC $\zeta$  for the *in vitro* affinity of this enzyme for PIP<sub>2</sub>.

Further, the impact of XYI deletion or replacement on the relative Ca<sup>2+</sup> sensitivity of PLC $\zeta$  and PLC $\delta$ 1 enzyme activity [5,17,18] was determined at Ca<sup>2+</sup> concentrations ranging from 0.1 nM to 0.1 mM. The resulting EC<sub>50</sub> value obtained for PLC $\zeta$  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 PLC $\delta$ 1 marginally altered the EC<sub>50</sub> value from 6.3 to 7.0  $\mu$ M. These results suggest that loss of the XYI or replacement of the cluster of basic residues in this region does not significantly alter the Ca<sup>2+</sup> sensitivity of PIP<sub>2</sub> hydrolysis for both PLC $\zeta$  and PLC $\delta$ 1.

## DISCUSSION

Although the precise regulatory mechanism remains unclear, PLC $\zeta$  has become established as the primary sperm-derived candidate factor that activates the egg at mammalian fertilization. Upon sperm-egg fusion, PLC $\zeta$  is proposed to be delivered by the sperm into the ooplasm and catalyses PIP<sub>2</sub> hydrolysis to generate IP<sub>3</sub>, which induces the cytoplasmic Ca<sup>2+</sup> oscillations that initiate embryo development. Sperm-specific PLC $\zeta$  is the smallest mammalian PLC isoform with the most elementary domain organization and it is the only one not found in somatic cells [3]. PLC $\zeta$  is most structurally similar to PLC $\delta$ 1 with the notable exception that it lacks a PH domain at the N-terminus (Fig.1). One further important and unique functional feature of PLC $\zeta$  is the relatively low Ca<sup>2+</sup> concentration (nanomolar) required for enzymatic activity, exhibiting ~100-fold higher Ca<sup>2+</sup> sensitivity than PLC $\delta$ 1 which requires micromolar Ca<sup>2+</sup> concentration for optimal PIP<sub>2</sub> hydrolysis. Thus, at the basal cytosolic Ca<sup>2+</sup> concentration of 50-80 nM likely present within eggs, the PLC $\zeta$  isoform but not PLC $\delta$ 1 would be strongly activated. The molecular determinants that confer PLC $\zeta$ 's high Ca<sup>2+</sup> sensitivity are unknown, although previous studies suggest both EF hand and C2 domains are required for a functional PLC $\zeta$  in the egg [17,18].

Another important question that remains unresolved is how PLC $\zeta$  activity is intrinsically regulated. Recent structural and biochemical studies have convincingly demonstrated that the XYI region of the PLC $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  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 PIP<sub>2</sub> substrate, as well as providing steric hindrance by occluding the enzyme catalytic active site. However, the sperm PLC $\zeta$  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 PLC $\zeta$ .

In the present study, a truncated PLC $\zeta$  lacking the XYI region, as well as a chimaeric PLC $\zeta$  in which the cluster of basic residues at the C-terminal end of the XYI was replaced by the homologous region of PLC $\delta$ 1 were prepared. These two novel PLC $\zeta$  constructs enabled the specific examination of how these targeted XYI changes might alter the *in vivo* Ca<sup>2+</sup> oscillation-inducing and *in vitro* PIP<sub>2</sub> hydrolysis activity relative to wild-type PLC $\zeta$ . Parallel studies were simultaneously performed using the corresponding construct derived from the most closely-related PLC isoform, PLC $\delta$ 1. Notably, PLC $\delta$ 1 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 PLC $\delta$ 1 exhibited a much higher *in vitro* PIP<sub>2</sub> hydrolytic activity than PLC $\zeta$  (Fig. 3B), although the *in vivo* Ca<sup>2+</sup> oscillation-inducing activity observed for PLC $\delta$ 1 in mouse eggs was much lower than that of PLC $\zeta$  (Fig. 2). This is consistent with a previous study showing PLC $\delta$ 1 was capable of inducing only low frequency Ca<sup>2+</sup> oscillations in mouse eggs, even at a 20-fold higher concentration than PLC $\zeta$  [5]. Interestingly, deletion of the XYI from PLC $\delta$ 1

resulted in a 2-fold increase in  $\text{Ca}^{2+}$  oscillation-inducing activity in eggs (Fig. 2), which correlates with the *in vitro*  $\text{PIP}_2$  hydrolysis assays showing a ~2.3-fold increased enzymatic activity relative to wild-type PLC $\delta$ 1 (Fig. 3B, Table 1). In contrast, the deletion of XYI from PLC $\zeta$  decreased both the *in vitro* enzymatic activity (Fig. 3B) and the  $\text{PIP}_2$  substrate affinity (Table 2), which was consistent with the observed 7-fold reduction in  $\text{Ca}^{2+}$  oscillation-inducing activity in eggs (Table 1). The XYI appears not to be directly involved in  $\text{Ca}^{2+}$ -dependent regulation of enzyme activity, as the  $\text{Ca}^{2+}$  sensitivity of *in vitro*  $\text{PIP}_2$  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 $\delta$ 1 (overall charge +1) also resulted in decrease of both the *in vitro* enzymatic activity (Fig. 3B) and the  $\text{PIP}_2$  substrate affinity (Table 2). These *in vitro* results are consistent with the observed 5-fold reduction in  $\text{Ca}^{2+}$  oscillation-inducing activity in eggs with this chimaeric PLC $\zeta$  (Table 1), whereas the  $\text{Ca}^{2+}$  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 $\delta$ 1. 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 $\delta$ 1 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,  $\text{PIP}_2$  [20,21]. Direct involvement of the XYI positively-charged residues in  $\text{PIP}_2$  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  $\text{Ca}^{2+}$  oscillation-inducing activity in mouse eggs,  $\text{PIP}_2$  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 *in vivo*  $\text{Ca}^{2+}$  oscillations and *in vitro*  $\text{PIP}_2$  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,  $\text{PIP}_2$  [20,21]. Such a proposed role for the XYI of PLC $\zeta$  in  $\text{PIP}_2$  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  $\text{PIP}_2$  interaction without altering  $\text{Ca}^{2+}$  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  $\text{PIP}_2$  hydrolysis observed for PLC $\zeta$  isoforms from different species and thus the species-specific frequency of sperm-induced  $\text{Ca}^{2+}$  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 PLC $\zeta$ , PLC $\delta$ 1 and deletion/chimaera constructs

Schematic representation of the domain organisation of wild-type PLC $\zeta$  and PLC $\delta$ 1, their corresponding XY-linker deletions PLC $\zeta$  $\Delta$ XYlinker and PLC $\delta$ 1 $\Delta$ XYlinker, and the PLC $\zeta$ /XYlinker $\delta$ 1 chimaera. Note the similar presence in PLC $\zeta$  and PLC $\delta$ 1 of the EF, X, XY-linker, Y and C2 domains, but absence of the PH domain in PLC $\zeta$ . The various amino acid lengths and respective XY-linker coordinates are also indicated for each construct.

### Figure 2

#### Ca<sup>2+</sup> oscillation-inducing activity of PLC and XY-linker deletion/chimaera expressed in mouse eggs

Fluorescence and luminescence recordings reporting the Ca<sup>2+</sup> 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 PLC $\zeta$ , PLC $\delta$ 1, their corresponding XY-linker deletions and the PLC $\zeta$ /XYlinker $\delta$ 1 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  $\mu$ g) 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 PLC $\zeta$ , PLC $\zeta$  $\Delta$ XY1, PLC $\delta$ 1 and PLC $\delta$ 1 $\Delta$ XY1, respectively. The pair of single lane panels on the right shows Coomassie gel and immunoblot analysis of the PLC $\zeta$ /XYlinker $\delta$ 1 chimaera. (B) PIP<sub>2</sub> hydrolysis enzyme activity assay of PLC $\zeta$ , PLC $\delta$ 1 their XY-linker deletions and chimaera (20 pmol) obtained with the standard [<sup>3</sup>H]PIP<sub>2</sub> cleavage assay,  $n = 3 \pm$ S.E., using two different preparations of recombinant protein and with each experiment performed in duplicate. (C) Effect of varying [Ca<sup>2+</sup>] on the normalized activity of PLC $\zeta$ , PLC $\delta$ 1 and their XY-linker deletions and chimaera. For these assays  $n = 2 \pm$  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<sup>2+</sup> oscillation-inducing activity (Ca<sup>2+</sup> spike number in 2 hours) and luciferase luminescence levels (Peak luminescence; Luminescence at 1<sup>st</sup> spike) are summarized for mouse eggs microinjected with each of the PLC-luciferase constructs, PLC $\zeta$ , PLC $\zeta^{\Delta XY1}$ , PLC $\delta 1$ , PLC $\delta 1^{\Delta XY1}$  and PLC $\zeta^{XY\delta 1}$  (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  $\pm$  s.e.m.

**Table 2****[<sup>3</sup>H]PIP<sub>2</sub> hydrolysis activity and *K<sub>m</sub>* of purified GST-PLC fusion proteins**

Specific enzyme activity values obtained for the various GST-PLC fusion proteins showing the EC<sub>50</sub> of Ca<sup>2+</sup>-dependent enzyme activity and the Michaelis-Menten constant, *K<sub>m</sub>*, determined by non-linear regression analysis (GraphPad, Prism 5) for the GST-fusion proteins PLC $\zeta$ , PLC $\zeta^{\Delta XY1}$ , PLC $\delta 1$ , PLC $\delta 1^{\Delta XY1}$  and PLC $\zeta^{XY\delta 1}$  (see Fig. 3C).

## TABLES

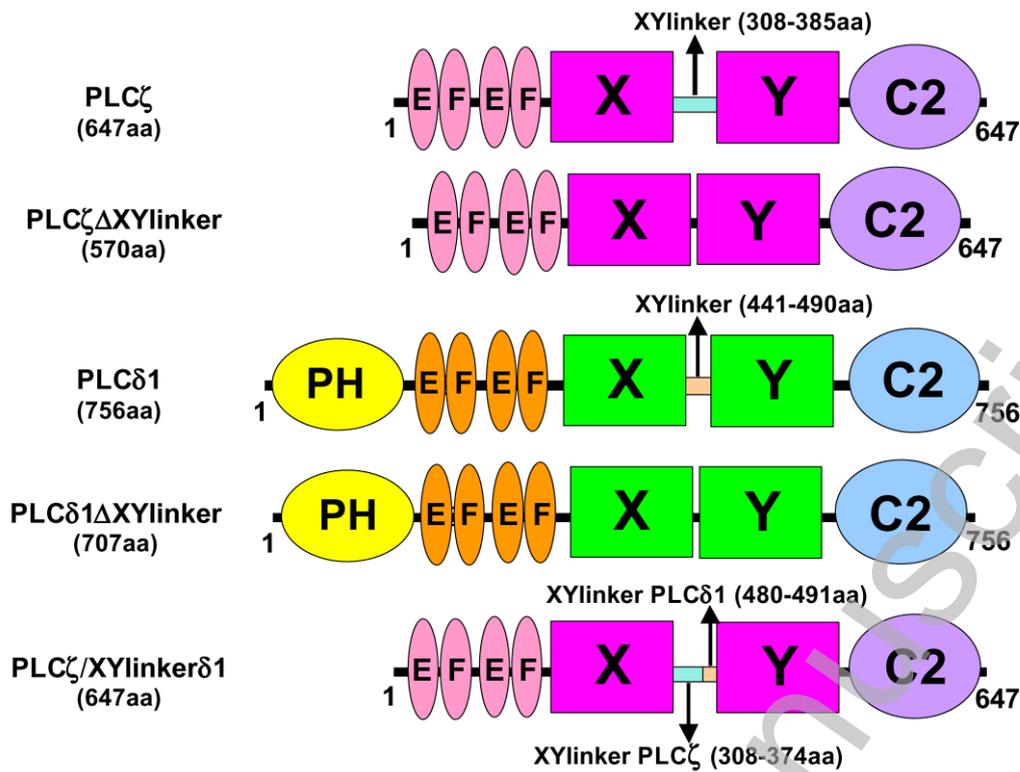
Table 1

PLC-luciferase injected	Ca <sup>2+</sup> oscillations (spikes/2 hour)	Peak luminescence (cps)	Luminescence at 1 <sup>st</sup> spike (cps)	No. of eggs
PLC $\zeta$	24.5 $\pm$ 0.88	8.7 $\pm$ 1.16	0.5 $\pm$ 0.06	13
PLC $\zeta^{\Delta XY1}$	3.4 $\pm$ 0.27	7.1 $\pm$ 0.20	3.6 $\pm$ 0.20	20
PLC $\delta 1$	1.8 $\pm$ 0.10	45.0 $\pm$ 1.7	20.4 $\pm$ 3.00	17
PLC $\delta 1^{\Delta XY1}$	3.3 $\pm$ 0.20	40.2 $\pm$ 1.7	17.2 $\pm$ 0.35	19
PLC $\zeta^{XY1\delta 1}$	5.3 $\pm$ 0.16	30.5 $\pm$ 2.0	4.0 $\pm$ 0.39	9

Table 2

GST-PLC protein	Ca <sup>2+</sup> -dependence EC <sub>50</sub> (nM)	Michaelis-Menten <i>K<sub>m</sub></i> ( $\mu$ M)
PLC $\zeta$	91	110
PLC $\zeta^{\Delta XY1}$	84	3936
PLC $\delta 1$	6289	93
PLC $\delta 1^{\Delta XY1}$	6973	63
PLC $\zeta^{XY1\delta 1}$	76	1909

Fig. 1



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Fig. 2

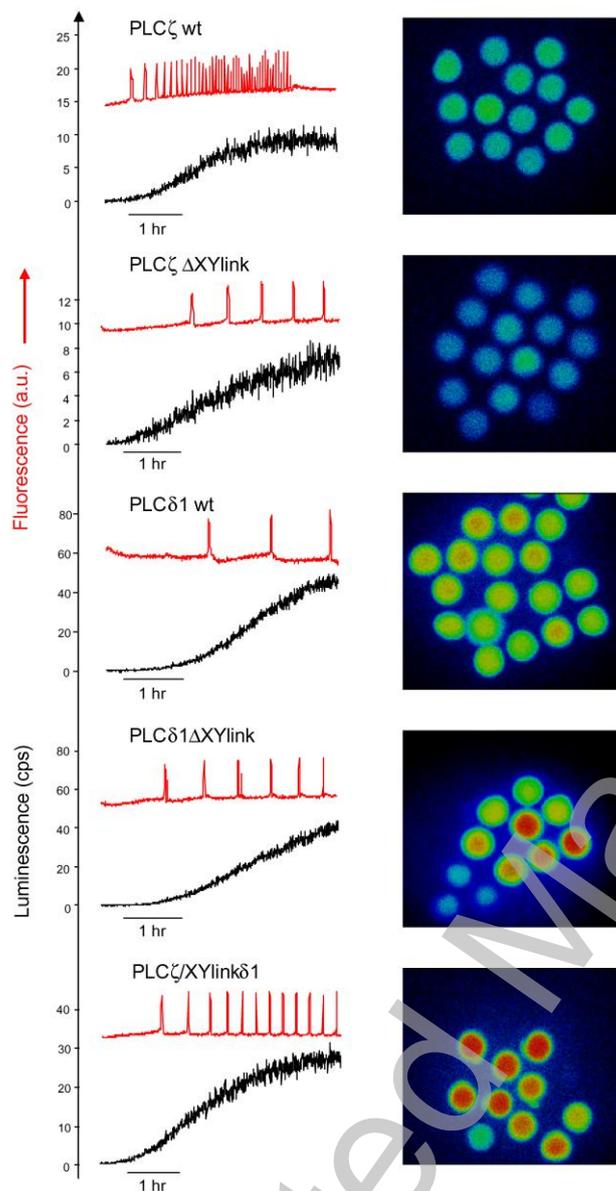
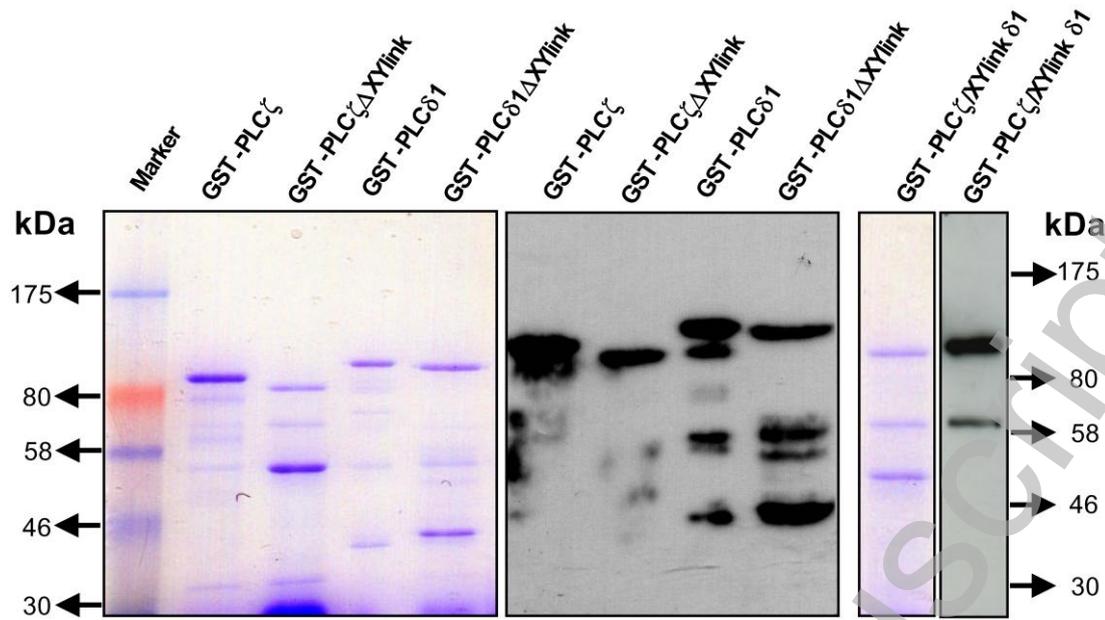


Fig. 3A



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Fig. 3B

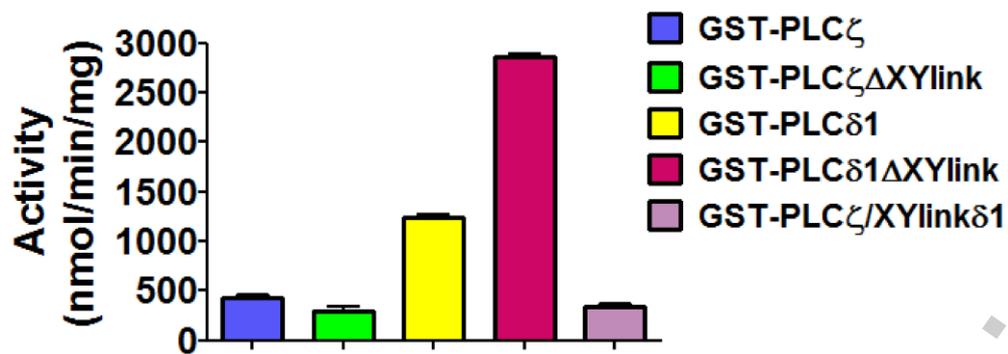


Fig. 3C

