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ACCELERATED PUBLICATION

Novel regulation of PLCζ activity via its XY-linker

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The XY-linker region of somatic cell PLC (phospholipase)-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, the absence of the PLCζ XY-linker significantly diminishes both *in vitro* PIP_2 (phosphatidylinositol 4,5-bisphosphate) hydrolysis and *in vivo* Ca^{2+}-oscillation-inducing activity, revealing evidence for a novel PLCζ enzymatic mechanism.

Key words: calcium oscillation, egg activation, enzyme regulation, fertilization, phospholipase Cζ (PLCζ), XY-linker.

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 PLC (phospholipase C) isoform, PLCζ, is widely considered to be the physiological stimulus that triggers these intracellular Ca^{2+} oscillations at fertilization [3–7]. Sperm-delivered PLCζ is responsible for catalysing PIP_2 (phosphatidylinositol 4,5-bisphosphate) hydrolysis within the fertilized egg to stimulate the IP_3 (inositol 1,4,5-trisphosphate) signalling pathway leading to Ca^{2+} oscillations [8,9]. The phosphoinositide-specific PLC family comprises 13 isoenzymes grouped into six different subfamilies (β, γ, δ, ε, ζ and η), each activated by different stimuli to catalyse 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 XYl (XY-linker) 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 XYl region, whereas, in the somatic cell PLCβ, δ and ε isoforms, this region is negatively charged. The XYl within PLCβ, δ and ε has been shown to specifically mediate auto-inhibition of PIP_2 hydrolytic activity, suggesting that the negatively charged residues of the XYl 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 PLCγ XYl, which comprises additional regulatory domains including two SH2 (Src homology 2) domains and an SH3 (Src homology 3) 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 XYl 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 XYl-mediated auto-inhibition observed in somatic cell PLC isoforms also applies to PLCζ activity regulation, we specifically removed this unique PLCζ XYl 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 PLCζ. For comparative analysis, we also generated the corresponding XYl deletion within PLCδ1, as well as a chimaeric PLCζ construct, in which the last 12 amino acids from the XYl region (residues 374–385) were replaced with those of PLCδ1 (residues 480–491). Our studies show that, in contrast with somatic cell PLCs, the XYl of PLCζ does not confer enzymatic auto-inhibition, indicating that a disparate regulatory mechanism may apply to this distinctive gamete-specific PLC isoenzyme.

MATERIALS AND METHODS

Plasmid construction and cRNA synthesis

To prepare the PLCζ XYl-deletion construct ($PLC\zeta^{\Delta XYl}$), mouse $PLC\zeta^{1-307}$ (GenBank® accession number AF435950) was amplified by PCR with 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 (open reading frame) amplified from pGL2 (Promega) to incorporate the 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 amplified further from pCR3-$PLC\zeta^{1-307/386-647}$ to incorporate a 5′ SalI and 3′ NotI site, and subcloned into a modified pET vector (pETMM30) to enable bacterial expression.

Rat PLCδ1 (GenBank® accesion number 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-5X2-PLCδ1 to incorporate a 5′ EcoRV and 3′ NotI site and cloned

Abbreviations used: BAPTA 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; GST, glutathione transferase; hCG, human chorionic gonadotrophin; H-KSOM, Hepes-buffered potassium simplex optimized medium; IP_3, inositol 1,4,5-trisphosphate; ORF, open reading frame; PH, pleckstrin homology; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SH2, Src homology 2; XYl, XY-linker.

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into pCR3 produced pCR3-PLCδ1, which was ligated in-frame with luciferase containing 5′ NotI and 3′ NotI sites. To prepare the luciferase-tagged PLCδ1 XYl-deletion construct (PLC$\delta1^{\Delta XYl}$), i.e. pCR3-PLC$\delta1^{1-440/491-756}$–luciferase, PLC$\delta1^{1-440}$ with a 5′ EcoRI and 3′ EcoRV site cloned into pCR3 was ligated in-frame to PLC$\delta1^{491-756}$ with a 5′ EcoRV site and a 3′ NotI site. Luciferase was then inserted via the NotI site of pCR3-PLC$\delta1^{1-440/491-756}$. The PLC$\delta1^{1-440/491-756}$, via the 5′ SalI and 3′ NotI sites, was subcloned further into pETMM30 for bacterial expression.

The PLCζ/XYl$\delta1^{480-491}$ chimaeric construct was prepared using a long primer strategy that utilized primers comprising nucleotides corresponding to XYl residues 480–491 of PLCδ1. These primers also contained a short sequence from the XYl region of PLCζ. Amplification of the two halves of PLCζ with these long primers enabled replacement of the PLCζ XYl residues 374–385 (KKRKRKMKIAMA) with the corresponding PLCδ1 XYl residues 480–491 (KPKEDKLKLVPE) to be achieved. Four silent mutations in the PLCδ1 XYl sequence were introduced to circumvent non-specific annealing of the primers. The PLCζ/XYl$\delta1^{480-491}$ 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-PLCζ/XYl$\delta1^{480-491}$ to generate PLCζ/XYl$\delta1^{480-491}$–luciferase. The PLCζ/XYl$\delta1^{480-491}$ was amplified further from pCR3-PLCζ/XYl$\delta1^{480-491}$ to incorporate a 5′ SalI and 3′ NotI site and subcloned into a modified pET vector (pETMM30) to enable bacterial expression.

Following linearization of wild-type, XYl-excised and chimaeric PLC plasmids, cRNA was synthesized using the 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 H-KSOM (Hepes-buffered potassium simplex optimized medium) as described previously [3,4]. Female mice were superovulated by injection of hCG (human chorionic gonadotrophin; Intervet). Eggs were collected 13.5–14.5 h later and were maintained in 100 μl of H-KSOM under mineral oil at 37 °C. Egg microinjection was carried out 14.5–15.5 h after hCG administration [15].

All procedures were in accordance with the UK Home Office Animals Procedures Act and approved by the Cardiff University Animals Ethics Committee.

Microinjection and measurement of intracellular Ca^{2+} 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 [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid]–dextran (Molecular Probes) in injection buffer (120 mM KCl and 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 (charge-coupled-device) camera (Photek). Ca^{2+} 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 as for fluorescence by switching, every 10 s, between light collection in the presence or in the absence of excitation light. Fluorescence signals were 10–100 times that for luminescence. The luminescence, defined as the light emission recorded in the absence of excitation light, was quantitatively converted into luciferase protein using a standard luminescence calibration curve prepared by microinjection of eggs with known amounts of luciferase protein (Sigma) [15,16].

Protein expression and purification

For GST (glutathione transferase)–PLC-fusion protein expression, *Escherichia coli* [Rosetta (DE3); Novagen], transformed with the appropriate plasmid, was cultured at 37 °C until a D_{600} of 0.6, then protein expression was induced for 18 h at 16 °C with 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside) (Promega). Cells were centrifuged at 6000 ***g*** for 10 min, resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$ and 1.4 mM KH_2PO_4, pH 7.4) containing 2 mM dithiothreitol and protease inhibitor mixture (Roche) and then sonicated 4×15 s on ice. After centrifugation at 15000 ***g*** for 15 min at 4 °C, soluble GST–PLC-fusion proteins were purified by affinity chromatography using glutathione–Sepharose™ 4B following standard procedures (GE Healthcare). Eluted proteins were dialysed overnight (SnakeSkin 10000 molecular mass cut-off; Pierce) at 4 °C in 4 litres of PBS and concentrated with centrifugal concentrators (10000 molecular mass cut-off; Sartorius).

PLC activity assay, PAGE and Western blotting

PIP_2 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, 2 mM $CaCl_2$, 4 mM EGTA, 20 μg of BSA, 5 mM 2-mercaptoethanol and 20 mM Tris/HCl buffer, pH 6.8. The PIP_2 concentration in the reaction mixture was 220 μM, containing 0.05 μCi of [^{3}H]PIP_2. Assay conditions were optimized for linearity, requiring incubation for 10 min at 25 °C with 20 pmol of protein. Recombinant proteins were separated by SDS/PAGE and immunoblot analysis was performed as described previously [17]. Proteins were probed with a polyclonal anti-GST antibody (1:10000 dilution).

RESULTS

To understand the regulatory role of the short linker region separating the catalytic X and Y domains, the XYl of both PLCζ (amino acids 308–385) and PLCδ1 (amino acids 441–490) were excised from the wild-type PLCs to create the XYl-deletion constructs PLC$\zeta^{\Delta XYl}$ and PLC$\delta1^{\Delta XYl}$ respectively (Figure 1). The PLCζ XYl notably contains a unique cluster of basic residues that may be involved in enzyme function [8,9]. To examine further the potential role of this short positively charged XYl segment in the regulation of PLCζ activity, a chimaeric PLCζ construct was prepared in which these 12 amino acids of PLCζ (amino acids 374–385, KKRKRKMKIAMA; +7 charged residues) were replaced with the corresponding stretch from PLCδ1 (amino acids 480–491, KPKEDKLKLVPE; +4/−3 charged residues), generating PLCζ/XYl$\delta1^{480-491}$ (Figure 1). The XYl-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 quantification [15]. Consistent with previous reports [16,20], prominent Ca^{2+} oscillations (25 spikes/2 h) were observed in unfertilized mouse eggs microinjected with PLCζ cRNA (Figure 2), with the first Ca^{2+} spike appearing at a luminescence of 0.52 c.p.s. for the expressed PLC–luciferase-fusion protein (Table 1). In contrast, microinjecting cRNA

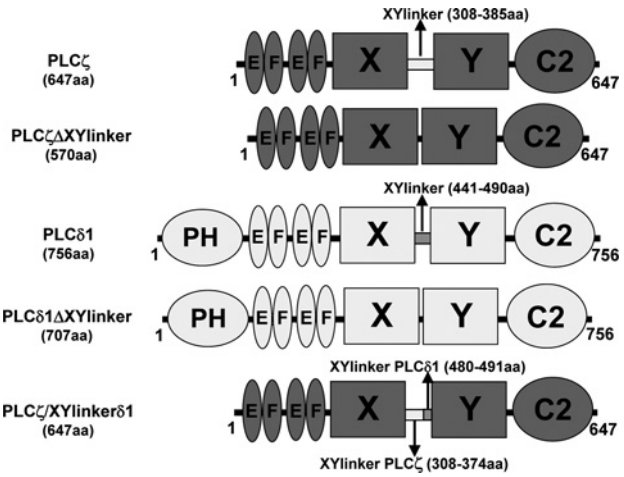

Figure 1 Domain organization of PLCζ, PLCδ1 and the deletion/chimaera constructs

Schematic representation of the domain organization of wild-type PLCζ and PLCδ1, their corresponding XYl deletions PLCζ$^{\Delta XYl}$ (PLCζ ΔXYlinker) and PLCδ1$^{\Delta XY}$ (PLCδ1ΔXYlinker), and the PLCζ/XYlδ1$^{480–491}$ (PLCζ/XYlinkerδ1) chimaera. Note the similar presence in PLCζ and PLCδ1 of the EF, X, XYl, Y and C2 domains, but the absence of the PH domain in PLCζ. The various amino acid (aa) lengths and respective XYl co-ordinates are also indicated for each construct.

encoding the XYl-deletion construct PLCζ$^{\Delta XYl}$ produced Ca^{2+} oscillations in mouse eggs with a significantly lower frequency (3.4 spikes/2 h) relative to wild-type PLCζ, and with the first Ca^{2+} spike only appearing after luminescence had reached 3.6 c.p.s. Similarly, microinjection of cRNA corresponding to the XYl chimaera PLCζ/XYlδ1$^{480–491}$ also triggered relatively low-frequency Ca^{2+} oscillations (5.3 spikes/2 h), with the first Ca^{2+} spike appearing at a luminescence of 4.0 c.p.s. (Figure 2 and Table 1).

Microinjection of wild-type PLCδ1 cRNA into mouse eggs caused very low-frequency Ca^{2+} oscillations (1.8 spikes/2 h) that commenced only when the PLCδ1–luciferase protein expression produced a relatively large luminescence value of 20.4 c.p.s. However, the PLCδ1$^{\Delta XYl}$ deletion construct cRNA effected a ~2-fold increase in Ca^{2+} oscillation frequency (3.3 spikes/2 h) compared with PLCδ1, with the first Ca^{2+} spike manifested at a reduced luminescence of 17.2 c.p.s.

These mouse egg microinjection results show that the absence of the PLCζ XYl region dramatically attenuated the Ca^{2+}-oscillation-inducing activity (Figure 2), yielding a 7-fold reduction in spike frequency (25 compared with 3.4 spikes/2 h) and requiring a 7-fold increased level of PLCζ$^{\Delta XYl}$ expression (3.6 compared with 0.52 c.p.s.) to initiate the first Ca^{2+} spike (Table 1). In addition, replacing the cluster of basic residues in the PLCζ XYl (seven out of 12 residues are positively charged; overall +7) with the corresponding amino acids from the XYl of PLCδ1 (four positively charged residues and three negatively charged residues; overall charge +1), also dramatically reduced by 5-fold the Ca^{2+}-oscillation-inducing activity of PLCζ with a requirement for an 8-fold increased level of PLCζ/XYlδ1$^{480–491}$ expression to initiate the first spike compared with wild-type PLCζ (4.0 compared with 0.52 c.p.s.). Conversely, the XYl deletion from PLCδ1 increased its Ca^{2+}-oscillation-inducing activity in mouse eggs with a doubling of the Ca^{2+} spike frequency (3.3 compared with 1.8 spikes/2 h).

The effect of removing or replacing part of the XYl on the *in vitro* PIP_2 hydrolysis activity of PLCζ or PLCδ1, i.e. PLCζ$^{\Delta XYl}$, PLCδ1$^{\Delta XYl}$ and PLCζ/XYlδ1$^{480–491}$ constructs, was

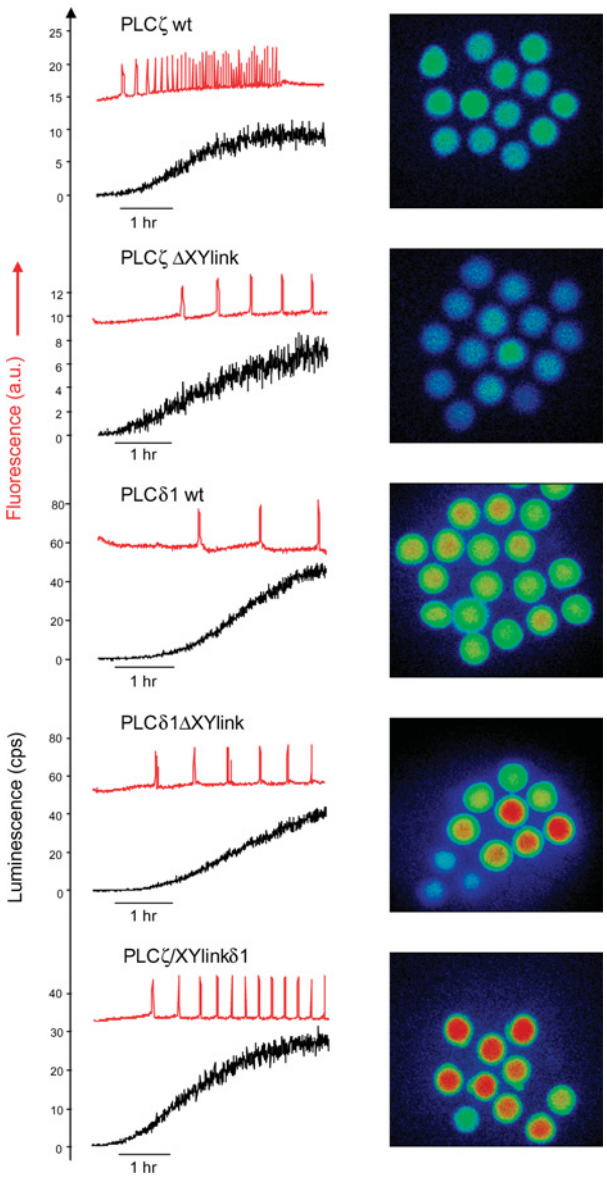

Figure 2 Ca^{2+}-oscillation-inducing activity of the PLC and XYl deletion/chimaera expressed in mouse eggs

Fluorescence and luminescence recordings reporting the Ca^{2+} changes [fluorescence (red traces), in arbitrary units (a.u.), and luciferase expression (black traces; luminescence) in c.p.s. respectively] in unfertilized mouse eggs following microinjection of cRNA encoding luciferase-tagged PLCζ, PLCδ1, their corresponding XYl deletions [PLCζ$^{\Delta XYl}$ (PLCζ ΔXYlink) and PLCδ1$^{\Delta XY}$ (PLCδ1ΔXYlink] and chimaera [PLCζ/XYlδ1$^{480–491}$; (PLCζ/XYlinkδ1)] (left-hand panels). Right-hand panels show the integrated luminescence image of a field of mouse eggs following cRNA microinjection of each PLC construct (see Table 1). wt, wild-type.

examined following their expression in bacteria and purification as GST-fusion proteins. Figure 3(A) shows that the affinity-purified fusion proteins displayed the predicted molecular masses for the GST–PLCζ, GST–PLCζ$^{\Delta XYl}$, GST–PLCδ1, GST–PLCδ1$^{\Delta XYl}$ and PLCζ/XYlδ1$^{480–491}$ recombinant proteins of 100, 94, 111, 107 and 102 kDa respectively, as also confirmed by immunoblot analysis with the anti-GST antibody. The specific PIP_2 hydrolytic enzyme activity values obtained for each protein (Figure 3B) revealed a 30% reduction in PLCζ$^{\Delta XYl}$ enzyme activity relative to PLCζ (302 ± 58 compared with 425 ± 51 nmol/min per mg of protein), and a 20% reduction in the chimaera PLCζ/XYlδ1$^{480–491}$ enzyme activity (342 ± 38 compared with 425 ± 51 nmol/min per mg of

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 h) and luciferase luminescence levels (peak luminescence and luminescence at first spike) are summarized for mouse eggs microinjected with each of the PLC–luciferase constructs, PLCζ, PLC$\zeta^{\Delta XYl}$, PLCδ1, PLC$\delta1^{\Delta XYl}$ and PLC$\zeta^{XYl\delta1}$ (see Figure 2). Each egg was microinjected with a pipette cRNA concentration of 1.6 g/l. Results are expressed are means ± S.E.M.

PLC–luciferase injected	Ca^{2+} oscillations (spikes/2 h)	Peak luminescence (c.p.s.)	Luminescence at first spike (c.p.s.)	Number of eggs
PLCζ	24.5 ± 0.88	8.7 ± 1.16	0.5 ± 0.06	13
PLC$\zeta^{\Delta XYl}$	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
PLC$\delta1^{\Delta XYl}$	3.3 ± 0.20	40.2 ± 1.7	17.2 ± 0.35	19
PLC$\zeta^{XYl\delta1}$	5.3 ± 0.16	30.5 ± 2.0	4.0 ± 0.39	9

Table 2 Ca^{2+}-dependent [^{3}H]PIP_2 hydrolysis activity and K_m of purified GST–PLC-fusion proteins

Specific enzyme activity values obtained for the various GST–PLC-fusion proteins showing the EC_{50} value of Ca^{2+}-dependent enzyme activity and the Michaelis–Menten constant, K_m, for PIP_2 determined by non-linear regression analysis (GraphPad, Prism 5) for the GST–fusion proteins PLCζ, PLC$\zeta^{\Delta XYl}$, PLCδ1, PLC$\delta1^{\Delta XYl}$ and PLC$\zeta^{XYl\delta1}$ (see Figure 3C).

GST–PLC protein	Ca^{2+}-dependence EC_{50} (nM)	Michaelis–Menten K_m (μM)
PLCζ	91	110
PLC$\zeta^{\Delta XYl}$	84	3936
PLCδ1	6289	93
PLC$\delta1^{\Delta XYl}$	6973	63
PLC$\zeta^{XYl\delta1}$	76	1909

protein), indicating that the presence of the XYl region and the highly positively charged cluster are required for maximal PLCζ activity. In contrast, PLC$\delta1^{\Delta XYl}$ displayed a ~2.3-fold increase in enzymatic activity compared with PLCδ1 (2865 ± 54 compared with 1249 ± 40 nmol/min per mg of protein). These differential results for XYl-deleted PLCs suggest that there are disparate regulatory roles for the XYl of PLCδ1 and PLCζ with respect to enzyme hydrolytic activity.

Calculation of the Michaelis–Menten constant, K_m, for these proteins yielded comparable values for PLCδ1 (93 μM) and PLC$\delta1^{\Delta XYl}$ (63 μM). However, for PLC$\zeta^{\Delta XYl}$ (3936 μM), the K_m was 36-fold higher than that of PLCζ (110 μM) (Table 2), indicating that deletion of the XYl has a major effect by dramatically reducing the *in vitro* affinity of PLCζ for the PIP_2 substrate. Similarly, the K_m value for the XYl 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 XYl region of PLCζ for the *in vitro* affinity of this enzyme for PIP_2.

The impact of the XYl deletion or replacement on the relative Ca^{2+} sensitivity of PLCζ and PLCδ1 enzyme activity [5,16,18] was determined at Ca^{2+} concentrations ranging from 0.1 nM to 0.1 mM. The resulting EC_{50} value obtained for PLCζ was near identical with the corresponding XYl-truncated protein (91 compared with 84 nM) and the XYl chimaeric protein (91 compared with 76 nM) (Figure 3C and Table 2). Likewise, removing the XYl from PLCδ1 marginally altered the EC_{50} value from 6.3 to 7.0 μM. These results suggest that loss of the XYl or replacement of the cluster of basic residues in this region does not significantly alter the Ca^{2+} sensitivity of PIP_2 hydrolysis for both PLCζ and PLCδ1.

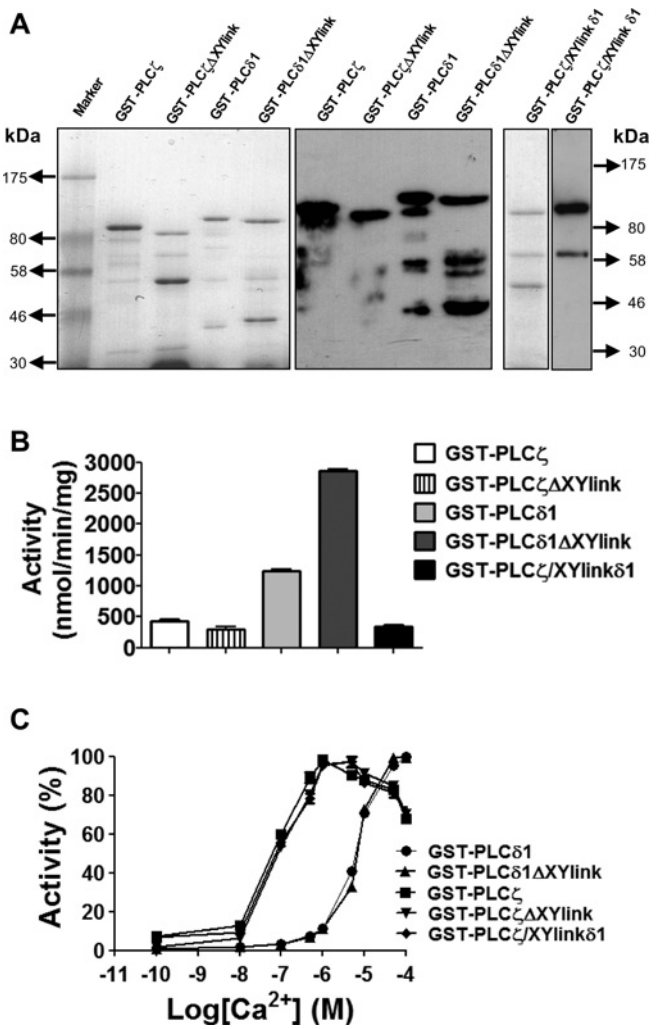

Figure 3 Expression, purification and enzyme activity of PLC and XYl/chimaera proteins

(**A**) Glutathione affinity-purified GST–PLC-fusion proteins (1 μg) were analysed by SDS/PAGE (8 % gels), followed by either Coomassie Brilliant Blue staining (left-hand panel) or immunoblot analysis using an anti-GST antibody (middle panel). Lanes 1–4 show PLCζ, PLC$\zeta^{\Delta XYl}$, PLCδ1 and PLC$\delta1^{\Delta XYl}$ respectively. The panel containing the pair of single lanes on the right shows Coomassie gel and immunoblot analysis of PLCζ/XYl$\delta1^{480–491}$, the PLCζ/XYlδ1 chimaera. (**B**) PIP_2 hydrolysis enzyme activity of PLCζ, PLCδ1 and their XYl deletions and chimaera (20 pmol) obtained with the standard [^{3}H]PIP_2 cleavage assay. Values are means ± S.E.M. (n = 3), using two different preparations of recombinant protein and with each experiment performed in duplicate. (**C**) Effect of various [Ca^{2+}] on the normalized activity of PLCζ, PLCδ1 and their XYl deletions and chimaera. For these assays, values are means ± S.E.M. (n = 2), using two different batches of recombinant proteins and with each experiment performed in duplicate (see Table 2).

DISCUSSION

Although the precise regulatory mechanism remains unclear, PLCζ has become established as the primary sperm factor candidate 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 PIP_2 hydrolysis to generate IP_3, which induces the cytoplasmic Ca^{2+} 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 structurally most similar to PLCδ1 with the notable exception that it lacks a PH (pleckstrin homology) domain at the N-terminus (Figure 1). One further important and unique functional feature of PLCζ is the relatively low Ca^{2+} concentration (nanomolar) required for enzymatic activity, exhibiting ∼100-fold higher Ca^{2+} sensitivity than PLCδ1, which requires micromolar Ca^{2+} concentrations for optimal PIP_2 hydrolysis. Thus, at the basal cytosolic Ca^{2+} concentration of 50–80 nM likely to be present within eggs, the PLCζ isoform but not PLCδ1 would be strongly activated. The molecular determinants that confer the high Ca^{2+} sensitivity of PLCζ are unknown, although previous studies suggest that both EF hand and C2 domains are required for a functional PLCζ in the egg [16,18].

Another important question that remains unresolved is how PLCζ activity is intrinsically regulated. Structural and biochemical studies have convincingly demonstrated that the XYl region of the PLCβ, γ, δ and ε isoenzymes can mediate potent auto-inhibition of enzyme function [11,14]. This is consistent with the negatively charged XYl of these isoforms conferring electrostatic repulsion of the negatively charged PIP_2 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 XYl region. It was therefore important to investigate whether this putative general mechanism of XYl auto-inhibition observed in various somatic PLC isoforms also applies to the sperm-derived PLCζ.

In the present study, a truncated PLCζ lacking the XYl region, as well as a chimaeric PLCζ in which the cluster of basic residues at the C-terminal end of the XYl was replaced by the homologous region of PLCδ1, were prepared. These two novel PLCζ constructs enabled the specific examination of how these targeted XYl changes might alter the *in vivo* Ca^{2+}-oscillation-inducing and *in vitro* PIP_2 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* PIP_2 hydrolytic activity than PLCζ (Figure 3B), although the *in vivo* Ca^{2+}-oscillation-inducing activity observed for PLCδ1 in mouse eggs was much lower than that of PLCζ (Figure 2). This is consistent with a previous study showing that PLCδ1 was capable of inducing only low-frequency Ca^{2+} oscillations in mouse eggs, even at a 20-fold higher concentration than PLCζ [5]. Interestingly, deletion of the XYl from PLCδ1 resulted in a 2-fold increase in Ca^{2+}-oscillation-inducing activity in eggs (Figure 2), which correlates with the *in vitro* PIP_2 hydrolysis assays showing an ∼2.3-fold increased enzymatic activity relative to wild-type PLCδ1 (Figure 3B and Table 1).

In contrast, the deletion of the XYl from PLCζ decreased both the *in vitro* enzymatic activity (Figure 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 XYl 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 XYl-deleted PLC constructs (Figure 3C and Table 2). Significantly, replacement of only the PLCζ XYl cluster of basic residues (overall charge +7) by the homologous 12 amino acids of the XYl region of PLCδ1 (overall charge +1) also resulted in a decrease in both the *in vitro* enzymatic activity (Figure 3B) and the PIP_2 substrate affinity (Table 2). These *in vitro* results are consistent with the observed 5-fold reduction in Ca^{2+}-oscillation-inducing activity in eggs with this chimaeric PLCζ (Table 1), whereas the Ca^{2+} sensitivity remained comparable with the wild-type enzyme (Figure 3C and Table 2).

Our findings suggest that the XYl of PLCζ serves a different regulatory role to that of the XYl in PLCδ1. An important determinant for this disparity may be the high density of basic amino acids in the XYl of PLCζ that is absent from PLCδ1 and other somatic PLC isoforms. Previously, we have proposed that this unstructured cluster of positively charged residues at the C-terminal end of the PLCζ XYl may play a role in facilitating interactions with biological membranes, particularly the negatively charged substrate PIP_2 [20,21]. Direct involvement of the XYl positively charged residues in the PIP_2 interaction was recently examined by sequentially replacing three XYl lysine residues, Lys^{374}, Lys^{375} and Lys^{377}, 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 PLCζ XYl net positive charge progressively abated both the *in vivo* Ca^{2+} oscillations and *in vitro* PIP_2 interaction/enzyme function of mouse PLCζ [21]. These results indicate that the XYl cluster of positively charged residues may perform a central role in the interaction of PLCζ with the substrate PIP_2 [20,21]. Such a proposed role for the XYl of PLCζ in PIP_2 binding is entirely consistent with the present study in which excision of the complete XYl or exchanging a discrete XYl segment, and thereby removing the entire cluster of basic residues, causes significant diminution of both PLCζ functional properties and PIP_2 interaction without altering Ca^{2+} sensitivity.

Although the specific amino acid sequence of the XYl in PLCζ is poorly conserved across species, the presence of positively charged residues is a common feature of the PLCζ sequences currently available [8,9]. The significance of this species PLCζ XYl sequence diversity, albeit with charge conservation, might explain the different rates of PIP_2 hydrolysis observed for PLCζ 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 PLCζ has found that it remains functionally active even after proteolytic cleavage occurs specifically within the XYl region [22]. Further investigation is required to delineate the precise molecular mechanism of action of the various PLCζ domains and this may lead to important implications in the therapeutic approach to PLCζ-mediated male infertility [17].

AUTHOR CONTRIBUTION

Michail Nomikos, Raul Gonzalez-Garcia, George Nounesis, Karl Swann and Anthony Lai devised the project strategy; Michail Nomikos and Anthony Lai designed the experiments, which were performed by Michail Nomikos, Khalil Elgmati, Maria Theodoridou, Athena Georgilis and Raul Gonzalez-Garcia. Michail Nomikos, Karl Swann and Anthony Lai prepared the paper.

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