Novel regulation of PLCζ activity via its XY-linker

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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 PLC (phospholipase C) isoform, 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 PIP2 (phosphatidylinositol 4,5-bisphosphate) hydrolysis within the fertilized egg to stimulate the IP3 (inositol 1,4,5-trisphosphate) signalling pathway leading to Ca2+ 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 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 (XY-linker). Notably, the sperm-specific PLCζ is unique in displaying a XY-linker deletion within PLCζ1 deletion construct [10]. Notably, the sperm-specific PLCζ is uniquely in displaying a positively charged XY1 region, whereas, in the somatic cell PLCβ, δ and ε isoforms, this region is negatively charged. The XY1 within PLCζ, δ and ε has been shown to specifically mediate auto-inhibition of PIP2 hydrolytic activity, suggesting that the negatively charged residues of the XY1 directly prevent access of PIP2 to the enzyme active site via steric exclusion and electrostatic repulsion of the negatively charged PIP2 substrate [11]. The PLCζ γ, γ1, which comprises additional regulatory domains including two SH2 (Src homology 2) domains and an SH3 (Src homology 3) domain, regulates PLCζ via tyrosine phosphorylation [12,13]. Identification of the critical determinant for PLCζ inhibition at one of the SH2 domains has led to a proposed general mechanism of PLC auto-inhibition mediated by the XY1 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 XY1-mediated auto-inhibition observed in somatic cell PLC isoforms also applies to PLCζ activity regulation, we specifically removed this unique PLCζ XY1 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 XY1 deletion within PLCζ1, as well as a chimaeric PLCζ construct, in which the last 12 amino acids from the XY1 region (residues 374–385) were replaced with those of PLCδ1 (residues 480–491). Our studies show that, in contrast with somatic cell PLCs, the XY1 of PLCζ does not confer enzymatic auto-inhibition, indicating that a disparate regulatory mechanism may apply to this distinctive gamete-specific PLC isoenzyme.

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

MATERIALS AND METHODS

Plasmid construction and cRNA synthesis

To prepare the PLCζ-XY1-deletion construct (PLCζΔXY1), mouse PLCζ1 (GenBank accession number AF435950) was amplified by PCR with Phusion polymerase (Finnzymes) using appropriate primers to incorporate a 5′ EcoRI and 3′NotI site and a 5′XhoI and a 3′BglII site to generate pCR3-PLCζ1. Similarly, PLCζ1-luciferase construct 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-luciferase. To prepare the PLCζδ-deletion construct (PLCζΔδ), 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-PLCζ1-luciferase. The PLCζζ-luciferase construct with a 5′Sall restriction site and a modified pET vector (pETMM30) to enable bacterial expression.

Rat PLCζ1 (GenBank accession number M20637) with a 5′Sall site and a 3′ NotI site was cloned into pGEX-5X2. To generate pCR3-PLCζ-luciferase, PLCζ1 amplified from pGEX-5X2-PLCζ1 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; IP3, inositol 1,4,5-trisphosphate; ORF, open reading frame; PH, pleckstrin homology; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SH2, Src homology 2; XY1, 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 (PLCδ1ΔXYL), i.e. pCR3-PLCδ1ΔXYL–luciferase, PLCδ1ΔXYL with a 5’ EcoRI and 3’ EcoRV site cloned into pCR3 was ligated in-frame to PLCδ1ΔXYL–luciferase and a 3’ NotI site. Luciferase was then inserted via the NotI site of pCR3-PLCδ1ΔXYL–luciferase to enable real-time monitoring of relative protein expression by luminescence quantification [15]. Consistent with previous reports [16,20], prominent Ca²⁺ oscillations (25 spikes/2 h) were observed in unfertilized mouse eggs microinjected with PLCδ1 cRNA (Figure 2), with the first Ca²⁺ spike appearing at a latency 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 Na₂HPO₄·7H₂O and 1.4 mM KH₂PO₄, 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**

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, 2 mM CaCl₂, 4 mM EGTA, 20 μg of BSA, 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 [³²P]PIP₂. 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 PLC[ζ] amino acids 308–385 and PLCδ1 (amino acids 441–490) were excised from the wild-type PLCs to create the XYL-deletion and XYL-rescued constructs PLC[ζ]ΔXYL and PLCδ1ΔXYL respectively (Figure 1). The PLC[ζ] 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 PLC[ζ] activity, a chimaeric PLCδζ construct was prepared in which these 12 amino acids of PLCδ (amino acids 374–385, KRRKKRMKIA; +7 charged residues) were replaced with the corresponding stretch from PLCζ1 (amino acids 480–491, KPKEDKLKLVE; +4/–3 charged residues), generating PLCζ/XYLζΔ480–491 (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 Ca²⁺ oscillations (25 spikes/2 h) were observed in unfertilized mouse eggs microinjected with PLCζ cRNA (Figure 2), with the first Ca²⁺ spike appearing at a latency of 0.52 c.p.s. for the expressed PLC–luciferase fusion protein (Table 1). In contrast, microinjecting cRNA

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encoding the XYL-deletion construct PLCζ\textsuperscript{ΔXYL} produced Ca\textsuperscript{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\textsuperscript{2+} spike only appearing after luminescence had reached 3.6 c.p.s. Similarly, microinjection of cRNA corresponding to the XYL chimaera PLCζ/XYL\textsuperscript{1480–491} also triggered relatively low-frequency Ca\textsuperscript{2+} oscillations (5.3 spikes/2 h), with the first Ca\textsuperscript{2+} spike appearing at a luminescence of 4.0 c.p.s. (Figure 2 and Table 1).

Microinjection of wild-type PLC\δ1 cRNA into mouse eggs caused very low-frequency Ca\textsuperscript{2+} oscillations (1.8 spikes/2 h) that commenced only when the PLC\δ1-luciferase protein expression produced a relatively large luminescence value of 20.4 c.p.s. However, the PLC\δ1\textsuperscript{ΔXYL} deletion construct cRNA effected a ∼2-fold increase in Ca\textsuperscript{2+} oscillation frequency (3.3 spikes/2 h) compared with PLC\δ1, with the first Ca\textsuperscript{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 XYL1 region dramatically attenuated the Ca\textsuperscript{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\textsuperscript{ΔXYL} expression (3.6 compared with 0.52 c.p.s.) to initiate the first Ca\textsuperscript{2+} spike (Table 1). In addition, replacing the cluster of basic residues in the PLCζ XYL1 (seven out of 12 residues are positively charged; overall +7) with the corresponding amino acids from the XYL1 of PLC\δ1 (four positively charged residues and three negatively charged residues; overall charge +1), also dramatically reduced by 5-fold the Ca\textsuperscript{2+}-oscillation-inducing activity of PLCζ with a requirement for an 8-fold increased level of PLCζ/XYL1\textsuperscript{1480–491} expression to initiate the first spike compared with wild-type PLCζ (4.0 compared with 0.52 c.p.s.). Conversely, the XYL1 deletion from PLC\δ1 increased its Ca\textsuperscript{2+}-oscillation-inducing activity in mouse eggs with a doubling of the Ca\textsuperscript{2+} spike frequency (3.3 compared with 1.8 spikes/2 h).

The effect of removing or replacing part of the XYL1 on the in vitro PIP\textsubscript{2} hydrolysis activity of PLCζ or PLC\δ1, i.e. PLCζ\textsuperscript{ΔXYL}, PLC\δ1\textsuperscript{ΔXYL} and PLCζ/XYL1\textsuperscript{1480–491} constructs, was 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\textsuperscript{ΔXYL}, GST–PLC\δ1, GST–PLC\δ1\textsuperscript{ΔXYL} and PLCζ/XYL1\textsuperscript{1480–491} 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\textsubscript{2} hydrolytic enzyme activity values obtained for each protein (Figure 3B) revealed a 30% reduction in PLCζ\textsuperscript{ΔXYL} enzyme activity relative to PLCζ (302 ± 58 compared with 425 ± 51 nmol/min per mg of protein), and a 20% reduction in the chimera PLCζ/XYL1\textsuperscript{1480–491} enzyme activity (342 ± 38 compared with 425 ± 51 nmol/min per mg of protein).
Table 1 Properties of PLC–luciferase and deletion/chimaera constructs expressed in mouse eggs

<table>
<thead>
<tr>
<th>PLC–luciferase injected</th>
<th>Ca2+ oscillations (spikes/2 h)</th>
<th>Peak luminescence (c.p.s.)</th>
<th>Luminescence at first spike (c.p.s.)</th>
<th>Number of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLCζ</td>
<td>24.5 ± 0.88</td>
<td>8.7 ± 1.16</td>
<td>0.5 ± 0.06</td>
<td>13</td>
</tr>
<tr>
<td>PLCζΔXYI</td>
<td>3.4 ± 0.27</td>
<td>7.1 ± 0.20</td>
<td>3.6 ± 0.20</td>
<td>20</td>
</tr>
<tr>
<td>PLCΔ1</td>
<td>1.8 ± 0.10</td>
<td>45.0 ± 1.7</td>
<td>20.4 ± 3.00</td>
<td>17</td>
</tr>
<tr>
<td>PLCΔ1ΔXYI</td>
<td>3.2 ± 0.20</td>
<td>40.2 ± 1.7</td>
<td>17.2 ± 0.35</td>
<td>19</td>
</tr>
<tr>
<td>PLCζ/ΔXYI</td>
<td>5.3 ± 0.16</td>
<td>30.5 ± 2.0</td>
<td>4.0 ± 0.39</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2 Ca2+ -dependent [3H]PIP2 hydrolysis activity and Km of purified GST–PLC-fusion proteins

<table>
<thead>
<tr>
<th>GST–PLC protein</th>
<th>Ca2+ -dependence EC50 (nM)</th>
<th>Michaelis–Menten Km (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLCζ</td>
<td>91</td>
<td>110</td>
</tr>
<tr>
<td>PLCζΔXYI</td>
<td>84</td>
<td>3936</td>
</tr>
<tr>
<td>PLCΔ1</td>
<td>6289</td>
<td>93</td>
</tr>
<tr>
<td>PLCΔ1ΔXYI</td>
<td>6973</td>
<td>63</td>
</tr>
<tr>
<td>PLCζ/ΔXYI</td>
<td>76</td>
<td>1909</td>
</tr>
</tbody>
</table>

protein, indicating that the presence of the XY1 region and the highly positively charged cluster are required for maximal PLCζ activity. In contrast, PLCΔ1–XY1 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 XY1-deleted PLCs suggest that there are disparate regulatory roles for the XY1 of PLCζ 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–XY1 (63 μM). However, for PLCζ–XY1 (3936 μM), the Km was 36-fold higher than that of PLCζ (110 μM) (Table 2), indicating that deletion of the XY1 has a major effect by dramatically reducing the in vitro affinity of PLCζ for the PIP2 substrate. Similarly, the Km value for the XY1 chimaeric protein (1900 μM) was 17-fold higher than that of PLCζ (Table 2), highlighting the importance of the cluster of basic residues in the XY1 region of PLCζ for the in vitro affinity of this enzyme for PIP2.

The impact of the XY1 deletion or replacement on the relative Ca2+ sensitivity of PLCζ and PLCΔ1 enzyme activity [5,16,18] was determined at Ca2+ concentrations ranging from 0.1 nM to 0.1 μM. The resulting EC50 value obtained for PLCζ was near identical with the corresponding XY1-truncated protein (91 compared with 84 nM) and the XY1 chimaeric protein (91 compared with 76 nM) (Figure 3C and Table 2). Likewise, removing the XY1 from PLCΔ1 marginally altered the EC50 value from 6.3 to 7.0 μM. These results suggest that loss of the XY1 or replacement 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 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 PyP2 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 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 Ca2+ concentration (nanomolar) required for enzymatic activity, exhibiting ~100-fold higher Ca2+ sensitivity than PLCδ1, which requires micromolar Ca2+ concentrations for optimal PyP2 hydrolysis. Thus, at the basal cytosolic Ca2+ concentration (nanomolar) likely to be present within eggs, the PLCζ isoform but not PLCδ1 would be strongly activated. The molecular determinants that confer the high Ca2+ 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 PLCδ, 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 PyP2 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 vitro Ca2+-oscillation-inducing and in vitro PyP2 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 PLCζ [5]. Interestingly, deletion of the XYl from PLCδ1 resulted in a 2-fold increase in Ca2+-oscillation-inducing activity in eggs (Figure 2), which correlates with the in vitro PyP2 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 PyP2 substrate affinity (Table 2), which was consistent with the observed 7-fold reduction in Ca2+-oscillation-inducing activity in eggs (Table 1). The XYl appears not to be directly involved in Ca2+-dependent regulation of enzyme activity, as the Ca2+ sensitivity of in vitro PyP2 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 PyP2 substrate affinity (Table 2). These in vitro results are consistent with the observed 5-fold reduction in Ca2+-oscillation-inducing activity in eggs with this chimaeric PLCζ (Table 1), whereas the Ca2+ 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 PyP2 [20,21]. Direct involvement of the XYl positively charged residues in the PyP2 interaction was recently examined by sequentially replacing three XYl lysine residues, Lys374, Lys375 and Lys377, for alanine to produce single (K374A), double (K374,5AA) and triple (K374,5,7AAA) substitutions [21]. The Ca2+-oscillation-inducing activity in mouse eggs, PyP2 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 vitro Ca2+-oscillations and in vitro PyP2 interaction/enzyme function of 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 PyP2 [20,21]. Such a proposed role for the XYl of PLCζ in PyP2 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 PyP2 interaction without altering Ca2+ 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 PyP2 hydrolysis observed for PLCζ isoforms from different species and thus the species-specific frequency of sperm-induced Ca2+ 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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ACKNOWLEDGEMENTS

We thank Matilda Katan (Institute of Cancer Research, London, U.K.) for providing the rat PLCζ.

FUNDING

This work was supported by the Wellcome Trust [grant number 080701]. K.E. and M.T. hold research scholarships supported by the Libyan Government and NCSR Demokritos respectively.

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Received 31 May 2011/18 July 2011; accepted 18 July 2011
Published as BJ Immediate Publication 18 July 2011, doi:10.1042/BJ20110953