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ACCELERATED PUBLICATION

Male infertility-linked point mutation disrupts the Ca^{2+} oscillation-inducing and PIP_2 hydrolysis activity of sperm PLCζ

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A male infertility-linked human PLCζ (phospholipase Cζ) mutation introduced into mouse PLCζ completely abolishes both *in vitro* PIP_2 (phosphatidylinositol 4,5-bisphosphate) hydrolysis activity and the ability to trigger *in vivo* Ca^{2+} oscillations in mouse eggs. Wild-type PLCζ initiated a normal pattern of Ca^{2+} oscillations in eggs in the presence of 10-fold higher mutant PLCζ, suggesting that infertility is not mediated by a dominant-negative mechanism.

Key words: calcium oscillation, egg activation, fertilization, infertility, phospholipase C (PLC), phospholipase Cζ (PLCζ).

INTRODUCTION

During mammalian fertilization, the fertilizing spermatozoon initiates a series of biochemical and morphological events in the egg known as 'egg activation'. In all species examined, the earliest egg activation event is an increase in the cytosolic free Ca^{2+} concentration [1,2]. In mammals, this Ca^{2+} signal is delivered in the form of long-lasting Ca^{2+} oscillations that commence after gamete fusion and persist beyond the completion of meiosis [3]. This Ca^{2+} signalling phenomenon is necessary and sufficient for the completion of all of the events of egg activation [4,5]. Much controversy existed over how the sperm induces this fundamental developmental event, but growing evidence supports the notion that, during mammalian fertilization, egg activation is triggered by a sperm-specific PLC (phospholipase C) isoform, PLCζ [6–9]. PLCζ introduced into the ooplasm is able to hydrolyse PIP_2 (phosphatidylinositol 4,5-bisphosphate) to yield IP_3 (inositol 1,4,5-trisphosphate), thus triggering Ca^{2+} oscillations within the egg via the IP_3 receptor-mediated Ca^{2+} signalling pathway [10]. PLCζ has the smallest molecular mass and most elementary domain organization among mammalian PLC isoforms [10,11]. PLCζ consists of a tandem pair of EF hand domains at the N-terminus, followed by catalytic X and Y domains, and a C-terminal C2 domain [6,10].

Further support for the importance of PLCζ in fertilization has arisen from two clinical reports demonstrating either a reduced protein level or mutated forms of PLCζ in cases of human male infertility [12,13]. One infertility case identified following failed IVF (*in vitro* fertilization) treatment was associated with a point mutation in the PLCζ catalytic Y domain [13], where replacement of histidine with a proline residue (H398P) correlated with the absence of Ca^{2+} oscillation-inducing activity of human PLCζ [13]. His^{398} is conserved in PLCζ from various mammalian species as well as in PLCδ1, the most closely related isoform to PLCζ [14]. In the present study, we have introduced the infertility-linked human PLCζ H398P mutation into the equivalent His^{435} residue of mouse PLCζ to give $PLC\zeta^{H435P}$ (Figure 1A) and analysed the effect of this mutation on *in vivo* Ca^{2+} oscillation-inducing and *in vitro* PIP_2 hydrolysis activity. For comparative analysis, we also replaced His^{435} with a neutral non helix-destabilizing residue, alanine, to produce $PLC\zeta^{H435A}$. An additional charge-reversal mutant, $PLC\zeta^{D210R}$, which produces an inactive enzyme [6], served as a negative control. We also examined the effect on PIP_2 hydrolysis activity of replacing in PLCδ1 the equivalent conserved His^{542} to yield $PLC\delta1^{H542P}$. Furthermore, we investigated potential dominant-negative inhibitory effects of $PLC\zeta^{H435P}$ on the Ca^{2+} oscillation-inducing activity of WT (wild-type) mouse PLCζ ($PLC\zeta^{WT}$) and mouse sperm.

MATERIALS AND METHODS

Plasmid construction and cRNA synthesis

Mouse PLCζ-luciferase in pCR3 [15] was subjected to site-directed mutagenesis (QuikChange II; Stratagene) to generate the $PLC\zeta^{H435P}$, $PLC\zeta^{H435A}$ and $PLC\zeta^{D210R}$ mutants. $PLC\zeta^{WT}$ and mutants were amplified by PCR from the corresponding pCR3 plasmid using Phusion polymerase (Finnzymes) to incorporate a 5′ EcoRI site and a 3′ SalI site and were cloned into pGEX-6P1 (GE Healthcare). The primers used for amplification of WT and mutant PLCζ were: 5′-ACATGAATTCATGGAAAGCCAACTTCATGA-3′ (forward) and 5′-TAACGTCGACTCACTCTCTGAAGTACCAAAC-3′ (reverse). Similarly, rat PLCδ1 in pGEX-5X2 [15] was subjected to site-directed mutagenesis to generate $PLC\delta1^{H542P}$. Following linearization of WT and mutant PLCζs, cRNA was synthesized using the mMessage Machine T7 kit (Ambion) and a poly(A) tailing kit (Ambion), as per the manufacturer's instructions.

Abbreviations used: BAPTA, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; CCD camera, charge-coupled-device camera; GST, glutathione transferase; hCG, human chorionic gonadotropin; H-KSOM, Hepes-buffered potassium simplex optimized medium; ICSI, intracytoplasmic sperm injection; IP_3, inositol 1,4,5-trisphosphate; IPTG, isopropyl β-D-thiogalactopyranoside; IVF, *in vitro* fertilization; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; WT, wild-type.

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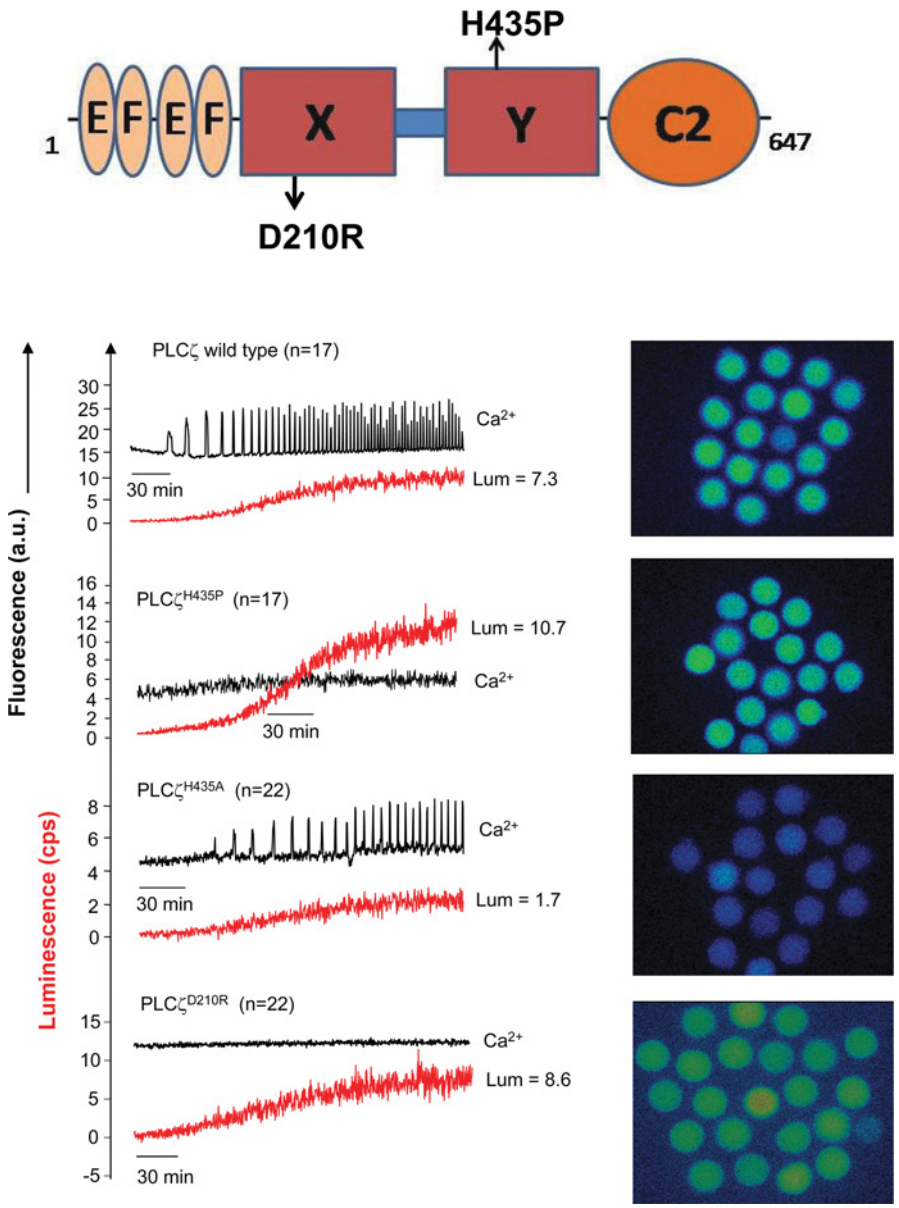

Figure 1 Ca^{2+} oscillation-inducing activity of PLCζ-luciferase and mutants in mouse eggs

(**A**) Schematic representation of mouse PLCζ domain structure identifying the location of the H435P mutation within the catalytic Y domain, as well as the D210R control mutation in the X domain. (**B**) The left-hand panels show representative fluorescence (a.u.; arbitrary units) and luminescence (c.p.s.) recordings reporting the Ca^{2+} concentration changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum) respectively in a mouse egg following microinjection of the indicated PLCζ-luciferase cRNA (encoding either PLCζ^{WT}, PLCζ^{H435P}, PLCζ^{H435A} or PLCζ^{D210R}). Right-hand panels show integrated images of luciferase luminescence from eggs microinjected with the corresponding PLCζ-luciferase cRNA. The peak luminescence (Lum) recorded is shown in c.p.s.

Preparation and handling of gametes

Experiments were carried out with mouse eggs in Hepes-buffered saline [H-KSOM (Hepes-buffered potassium simplex optimized medium)] as described previously [15,16]. Female mice were superovulated by injection of hCG (human chorionic gonadotropin; Intervet). Eggs were collected 13.5–14.5 h later and 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 [16]. IVF experiments were carried out as described previously [6].

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

Table 1 Properties of PLCζ-luciferase and mutants expressed in mouse eggs

Ca^{2+} oscillation-inducing activity (Ca^{2+} spike number in 2 h; time to first Ca^{2+} spike) and luciferase luminescence levels (peak luminescence; luminescence at first spike) are summarized for mouse eggs microinjected with each PLC-luciferase construct (see Figure 1B). Each egg was microinjected with a pipette cRNA concentration of 1.6 g/l. Values are means ± S.E.M.

PLCζ-luciferase	Ca^{2+} spikes in the first 2 h (*n*)	Peak luminescence (c.p.s.)	Time of first spike (min)	Luminescence at first spike (c.p.s.)	Number of eggs
$PLC\zeta^{WT}$	19 ± 0.14	7.33 ± 0.38	∼30	0.35 ± 0.037	17
$PLC\zeta^{H435P}$	0	10.7 ± 0.62	–	–	17
$PLC\zeta^{H435A}$	10 ± 0.65	1.74 ± 0.14	∼55	0.73 ± 0.038	22
$PLC\zeta^{D210R}$	0	8.6 ± 0.55	–	–	22

Green BAPTA–dextran fluorescence with low-level excitation light from a halogen lamp. After Ca^{2+} measurements, eggs were monitored for luminescence by integrating light emission (in the absence of fluorescence excitation) for 20 min using the same intensified CCD camera. Fluorescence signals were 10–100 times that for luminescence. Ca^{2+} measurements were considered valid only if the egg was also luminescent. To estimate expressed protein levels from luminescence values, luminescent eggs collected in a tube containing 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) with 1 mM MgATP+100 μM luciferin were lysed with 0.5 % Triton X-100 and the steady-state light emitted (in c.p.s.) was calibrated with recombinant firefly luciferase (Sigma) to give the mean protein expression of each PLCζ-luciferase construct [15]. Lower levels of protein at the start of experiments were estimated by linear extrapolation of the luminescence calibration curve.

Protein expression and purification

For GST (glutathione transferase)-fusion protein expression, *Escherichia coli* [Rosetta (DE3); Novagen], transformed with the appropriate pGEX plasmid, was cultured at 37 °C until a D_{600} of 0.6, and 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 containing 2 mM DTT (dithiothreitol) and protease inhibitor mixture (Roche) and were then sonicated four times for 15 s on ice. After centrifugation at 15000 ***g*** for 15 min at 4 °C, soluble GST-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 $PLC\zeta^{WT}$, $PLC\delta1^{WT}$ and mutant constructs was assayed as described previously [15]. The final volume of the assay mixture 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 [15]. Proteins were probed with a polyclonal anti-GST antibody (1:10000 dilution).

RESULTS

To assess the Ca^{2+} oscillation-inducing activity of the $PLC\zeta^{WT}$, $PLC\zeta^{H435P}$, $PLC\zeta^{H435A}$ and $PLC\zeta^{D210R}$ mutants and to verify that these constructs were faithfully expressed as functional proteins in cRNA-microinjected mouse eggs, we prepared PLCζ-luciferase fusion constructs to enable quantification of relative protein expression, as described previously [15]. $PLC\zeta^{WT}$-injected eggs displayed prominent Ca^{2+} oscillations, with the first Ca^{2+} spike occurring at a luminescence of 0.35 c.p.s., which we estimate is equivalent to a protein expression level of 35 fg/egg, similar to that reported previously [6]. In contrast, microinjecting cRNA encoding either $PLC\zeta^{H435P}$ or $PLC\zeta^{D210R}$ failed to cause any Ca^{2+} oscillations (Figure 1B), even after relatively high levels of protein expression (Table 1). However, microinjecting the $PLC\zeta^{H435A}$ mutant caused Ca^{2+} oscillations in all injected eggs (Figure 1B), exhibiting a broadly similar potency to $PLC\zeta^{WT}$, with the first Ca^{2+} spike detected after protein expression of 0.73 c.p.s. (Table 1). These results show that the mouse PLCζ H435P mutation is inactive in mouse eggs, whereas the H435A mutation retains Ca^{2+} oscillation-inducing activity.

$PLC\zeta^{H435P}$ and $PLC\zeta^{D210R}$ were subcloned into pGEX-6P1 and purified as GST-fusion proteins. We also prepared the corresponding substitution H542P in PLCδ1 and expressed $PLC\delta1^{WT}$ and $PLC\delta1^{H542P}$ to enable comparative isoform analysis of *in vitro* PIP_2 hydrolysis activity. The optimal protein yield for $PLC\zeta^{WT}$, $PLC\delta1^{WT}$ and their mutants required maintaining cultures at 37 °C until a D_{600} of 0.5, followed by induction of expression with 0.1 mM IPTG for 18 h at 16 °C. Figure 2 shows glutathione affinity-purified recombinant proteins analysed by SDS/PAGE (left-hand panels) and anti-GST immunoblot analysis (right-hand panels). The predicted molecular mass for GST–PLCζ and its corresponding mutants was 100 kDa (Figure 2A), whereas for GST–$PLC\delta1^{WT}$ and the H542P mutant it was 111 kDa (Figure 2B). The corresponding proteins with appropriate molecular masses were observed as the highest band in both the gel and immunoblot. The band at 26 kDa is consistent with cleaved GST, which, together with several other intermediate molecular-mass bands detected by the GST antibody, are the probable result of some degradation occurring through protein expression and purification.

The enzyme activity of GST-fusion proteins for $PLC\zeta^{WT}$, $PLC\delta1^{WT}$ and corresponding mutants were determined using the [^{3}H]PIP_2 hydrolysis assay at 1 μM and 1 mM Ca^{2+} (Figure 2C), as described previously [15]. $PLC\zeta^{H435P}$ and $PLC\zeta^{D210R}$ were enzymatically inactive at both low and high Ca^{2+}, in contrast with $PLC\zeta^{WT}$ which showed a specific activity of 449 ± 36 nmol/min per mg of protein at 1 μM Ca^{2+} and 382 ± 47 nmol/min per mg of protein at 1 mM Ca^{2+}. $PLC\delta1^{WT}$ displayed high activity at 1 mM Ca^{2+} with PIP_2 hydrolysis of 1385 ± 42 nmol/min per mg

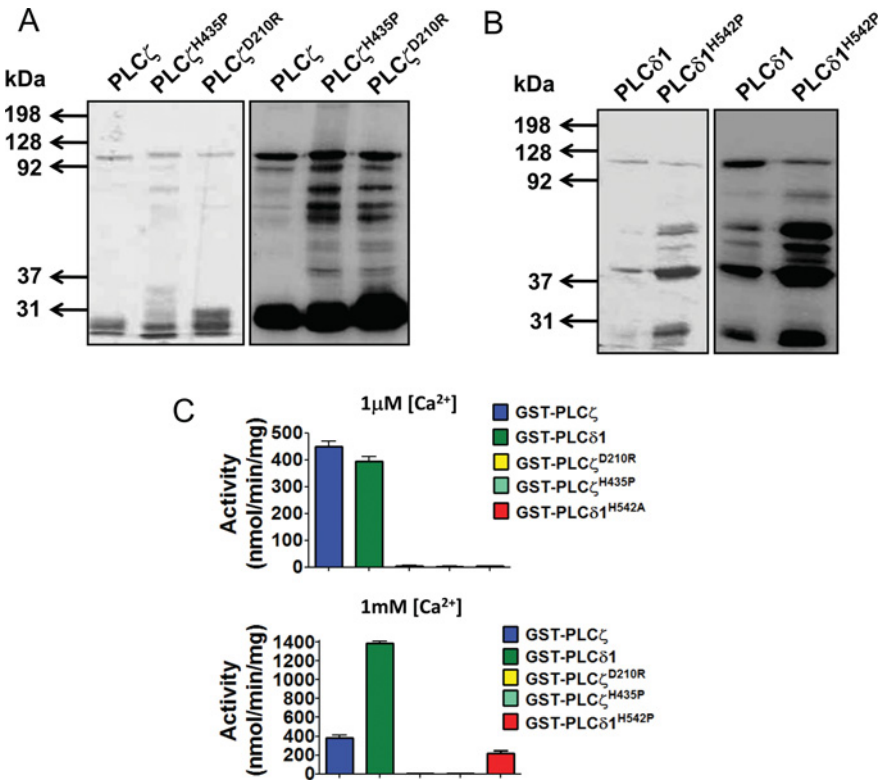

Figure 2 Expression, purification and enzyme activity of GST-fusion proteins for PLCζ, PLCδ1 and mutants

(**A**) Affinity-purified GST-fusion proteins for PLCζ^{WT}, PLCζ^{H435P} and PLCζ^{D210R} (2 μg) analysed by SDS/PAGE (8 % gels) (left-hand panel) or immunoblot analysis using a polyclonal anti-GST antibody (right-hand panel). (**B**) Affinity-purified GST-fusion proteins for PLC$\delta1^{WT}$ and PLC$\delta1^{H542P}$ (2 μg) analysed by SDS/PAGE (8 % gels) (left-hand panel) or immunoblot analysis using a polyclonal anti-GST antibody (right-hand panel). (**C**) [^{3}H]PIP$_2$ hydrolysis activity of the purified GST–PLC fusion proteins. Values are means $\pm$ S.E.M., $n = 3$.

of protein compared with 395 $\pm$ 27 nmol/min per mg of protein at 1 μM Ca^{2+}. In contrast, PLC$\delta1^{H542P}$ was completely inactive at 1 μM Ca^{2+} and retained only 16 % of the PLC$\delta1^{WT}$ activity at 1 mM Ca^{2+} (222 $\pm$ 36 nmol/min per mg of protein). These results show that the above His→Pro mutation completely inactivates the PIP_2 hydrolysis activity of both PLCζ and PLCδ1, whereas the His→Ala substitution in PLCζ retains *in vivo* function.

To investigate whether PLCζ^{H435P} can alter the Ca^{2+} oscillation-inducing activity of PLCζ^{WT}, we co-microinjected into mouse eggs an equal mixture of cRNA encoding luciferase-tagged PLCζ^{H435P} and PLCζ^{WT} (Figure 3, top panel). The co-injected cRNA produced Ca^{2+} oscillations with comparable properties with those observed with PLCζ^{WT} alone (Figure 1B and Table 1), showing a time to the first peak of ~30 min (Table 2). This suggests that the expression of PLCζ^{H435P} at similar levels to PLCζ^{WT} does not interfere with Ca^{2+} oscillations. To determine whether an excess of PLCζ^{H435P} was required to block PLCζ^{WT}-induced Ca^{2+} oscillations, we performed sequential cRNA microinjections; PLCζ^{H435P} was introduced first, followed after a period of 1 or 2 h by a second injection of PLCζ^{WT} (Figure 3, middle and lower panels). This protocol employing prior expression of PLCζ^{H435P} for 1–2 h followed by PLCζ^{WT} expression did not interfere with the induction of normal Ca^{2+} oscillations ~30 min after PLCζ^{WT} cRNA injection (Table 2). Table 2 shows that, in the double cRNA injection experiments, the cumulative luminescence for both PLCζ^{H435P} and PLCζ^{WT} at the time of first spike (7.06 and 18.68 c.p.s. for 1 and 2 h respectively) was much higher than for control PLCζ^{WT} alone (0.35 c.p.s.; Table 1). This suggests that an excess of PLCζ^{H435P}protein does not interfere with PLCζ^{WT}-induced Ca^{2+} oscillations. The estimated PLCζ^{H435P} protein level of 400 fg in the egg after 2.5 h (Figure 3 and Table 2), when the first spike is observed ~30 min after injection of PLCζ^{WT}, is well above the ~35 fg estimated to be required for Ca^{2+} oscillations with PLCζ^{WT} alone (Figure 1 and Table 1) [6,9]. This result therefore suggests that the PLCζ^{H435P} protein even when expressed at a 10-fold excess remains unable to block Ca^{2+} oscillation-inducing activity of PLCζ^{WT}. To examine whether normal sperm-induced Ca^{2+} oscillations are affected by the presence of PLCζ^{H435P}, IVF experiments with mouse sperm were performed. Even after high protein expression levels of PLCζ^{H435P} were achieved in the mouse eggs (> 40 c.p.s.), there was no discernable effect on sperm-induced Ca^{2+} oscillations (Figure 4).

DISCUSSION

Accumulating evidence suggests that sperm-specific PLCζ is the probable agent that stimulates Ca^{2+} oscillations and consequent egg activation after sperm–egg membrane fusion. Identification of PLCζ in several different mammalian species indicates that PLCζ should play a pivotal role at fertilization in all mammalian kingdoms. The importance of PLCζ in mammalian fertilization has been supported by two clinical studies which linked PLCζ with some cases of human male infertility [12,13]. ICSI (intracytoplasmic sperm injection) is a powerful IVF technique that has been extensively employed to overcome many male infertility conditions such as severe oligospermia, asthenospermia

Table 2 Properties of PLCζ^{WT} and PLCζ^{H435P} co-expressed in mouse eggs

Ca^{2+} oscillation-inducing activity (Ca^{2+} spike number in 2 h; time to first Ca^{2+} spike) and luciferase luminescence levels (peak luminescence; luminescence at first spike) are summarized for mouse eggs microinjected with each PLC-luciferase construct (see Figure 3). Each egg was microinjected with a pipette cRNA concentration of 1.6 g/l. The asterisk denotes that this value is the time taken after the second injection of cRNA for PLCζ^{WT}. Values are means ± S.E.M.

PLCζ-luciferase injected	Ca^{2+} spikes in the first 2 h (*n*)	Peak luminescence (c.p.s.)	Time of first spike (min)	Luminescence at first spike (c.p.s.)	Number of eggs
PLCζ^{H435P} and PLCζ^{WT} mixture	21 ± 1.7	3.4 ± 0.14	~30	0.68 ± 0.045	19
PLCζ^{H435P} +1 h, then PLCζ^{WT}	17.2 ± 1.0	15.8 ± 0.47	~30*	7.06 ± 0.72	21
PLCζ^{H435P} +2 h, then PLCζ^{WT}	15.5 ± 1.1	28.1 ± 1.6	~30*	18.68 ± 1.2	13

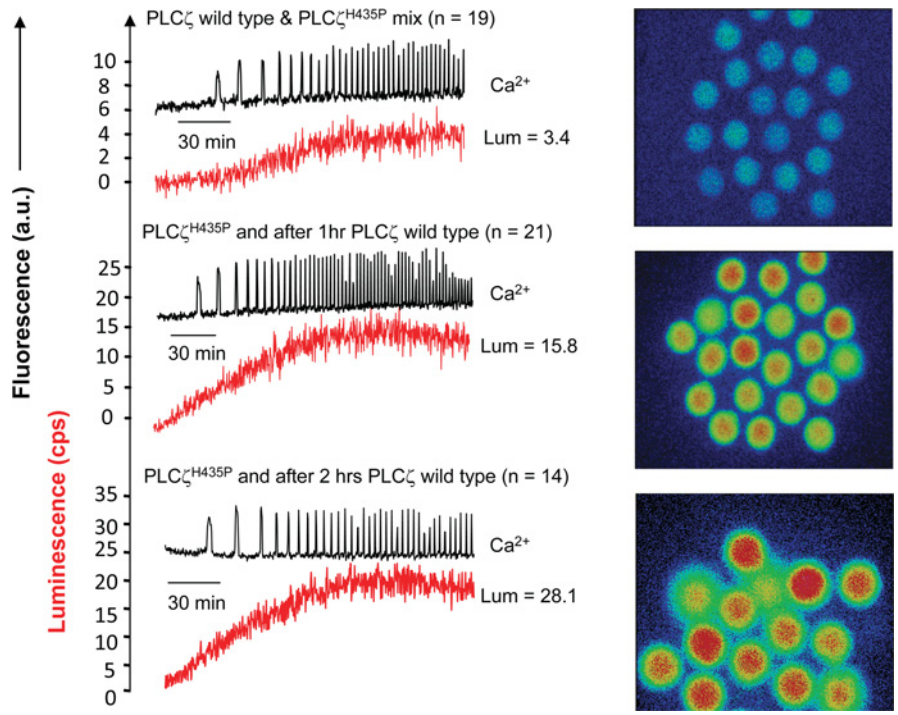

Figure 3 Co-expression of PLCζ^{H435P} and PLCζ^{WT} in mouse eggs

Left-hand panels show representative fluorescence and luminescence recordings reporting Ca^{2+} concentration changes (black traces; Ca^{2+}) and luciferase expression (red traces; Lum) respectively in a mouse egg. The egg was co-microinjected with equal amounts of PLCζ-luciferase cRNA encoding PLCζ^{WT} and PLCζ^{H435P} (top panel), or was initially microinjected with cRNA for PLCζ^{H435P} followed, after a period of 1 h (middle panel) or 2 h (bottom panel), by the microinjection of cRNA for PLCζ^{WT}. Right-hand panels show the integrated image of luciferase luminescence from eggs microinjected with PLCζ^{H435P} and PLCζ^{WT} cRNA. The peak luminescence (Lum) recorded is shown in c.p.s.

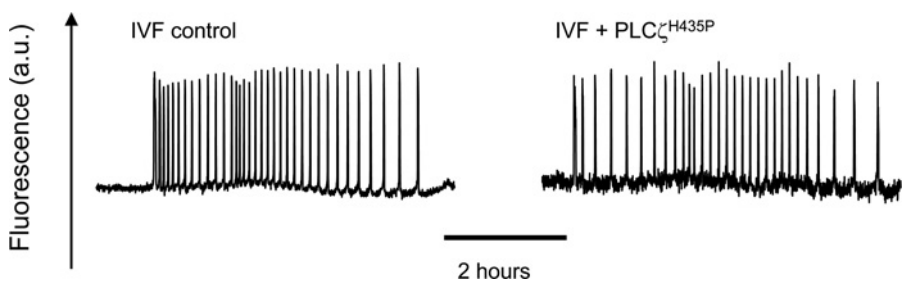

Figure 4 Effect of PLCζ^{H435P} on sperm-induced Ca^{2+} oscillations

Mouse eggs were either untreated (IVF control) or injected with PLCζ^{H435P} cRNA (IVF+PLCζ^{H435P}) 3 h prior to the start of recording. PLCζ^{H435P} expression produced luminescence of 41.7 ± 1.8 c.p.s. (value is mean ± S.E.M., *n* = 11). Fluorescence recordings [arbitrary units (a.u.)] reporting Ca^{2+} concentration changes were monitored after the addition of capacitated mouse sperm. Following IVF, both control and PLCζ^{H435P} cRNA-injected eggs exhibited robust Ca^{2+} oscillations and formed pronuclei. The number of Ca^{2+} spikes in control eggs was 33.4 ± 3.8 (mean ± S.E.M., *n* = 7), and the number of Ca^{2+} spikes in PLCζ^{H435P} cRNA-injected eggs was 32.9 ± 4.5 (mean ± S.E.M., *n* = 11).

and teratospermia [12]. A study [12] has identified a number of patients with repeatedly unsuccessful ICSI due to egg activation failure. Sperm from these patients was unable to initiate Ca^{2+} oscillations and this deficiency was associated with reduced or absent PLCζ expression in their sperm [12]. A second clinical study reported identification of a point mutation in the PLCζ gene of a patient with failed ICSI [13]. In this mutant PLCζ, His^{398} in the catalytic Y domain was replaced with a proline residue. Injection of infertile sperm or the mutant PLCζ from this patient failed to induce the typical pattern of Ca^{2+} oscillations in unfertilized mouse eggs [13]. The His^{398} residue is conserved not only in PLCζ of all mammalian species, but also in PLCδ1, the most closely related PLC isoform to PLCζ. Interestingly, His^{398} does not correlate with one of the five critical active-site residues within the catalytic domain of PLCδ1 (His^{311}, Glu^{341}, Asp^{343}, His^{356} and Glu^{390}) that were identified previously by structural studies [17] and which are conserved in PLCζ.

In the present study, we introduced the infertility-linked human PLCζ H398P mutation into the equivalent His^{435} residue of mouse PLCζ (H435P; Figure 1A) and assessed the effects of PLCζ^{H435P} upon *in vivo* Ca^{2+} oscillation-inducing and *in vitro* PIP_2 hydrolysis activity. Our results indicate that the H435P mutation totally abolishes the ability of PLCζ to trigger Ca^{2+} oscillations in mouse eggs (Figure 1B and Table 1) and also fully abrogates enzyme activity in a PIP_2 hydrolysis assay. To examine whether the His^{435} mutation to a proline residue, which in protein structure prediction is known to have helix-destabilizing ability, specifically causes sufficient perturbation of the catalytic Y domain to annul PLCζ enzymatic activity, we employed an additional PLCζ mutant in which His^{435} was replaced with a neutral non-helix-destabilizing amino acid, alanine. Interestingly, in contrast with the inactive PLCζ^{H435P}, robust Ca^{2+} oscillations were generated by PLCζ^{H435A} cRNA injection with a similar potency to PLCζ^{WT} (Figure 1B and Table 1). This suggests that the H435P substitution may cause inactivation of PLCζ due to the introduction of major protein structural changes, consistent with that proposed previously [13]. We examined further the effect of the PLCζ^{H435P} equivalent mutation upon the *in vitro* enzymatic properties of a closely related PLC isoform PLCδ1. We observed very similar results with the equivalent PLC$\delta1^{H542P}$ mutant, which retained only 16% of the PIP_2 hydrolysis activity of the PLC$\delta1^{WT}$ at high Ca^{2+} levels, a condition where PLC$\delta1^{WT}$ is known to be fully enzymatically active [11,15].

Interestingly, Heytens et al. [13] reported that the H398P mutation identified to correlate with male infertility appeared to be heterozygous, suggesting that in humans PLCζ^{H398P} may exert a dominant-negative effect on PLCζ^{WT}. To investigate whether mouse PLCζ^{H435P} acts in a dominant-negative fashion, we performed both cRNA co-microinjection and sequential injection experiments using PLCζ^{H435P} and PLCζ^{WT}. The aim of these experiments was to determine whether an equivalent or excess level of PLCζ^{H435P} protein expressed in mouse eggs can block the Ca^{2+} oscillation-inducing activity of PLCζ^{WT}. However, when PLCζ^{H435P} was expressed at up to 10-fold higher levels relative to PLCζ^{WT}, this did not block the ability of PLCζ^{WT} to trigger Ca^{2+} oscillations, suggesting that, in the mouse, PLCζ^{H435P} does not display dominant-negative behaviour. This observation is intriguing as it leaves unexplained why the sperm of this heterozygous infertile male is unable to cause Ca^{2+} oscillations after ICSI. Further analysis is required to confirm whether there are any other defects, such as PLCζ mislocalization, in the sperm of this infertile patient. Transgenic heterozygous animal models carrying this mutant allele would also help to enable further investigation of the reason for failure of egg activation in this clinical case.

The present study extends the previous work of Heytens et al. [13] by revealing that (i) a mouse PLCζ H435P mutation, equivalent to the infertile human H398P, is functionally inactive both *in vivo* and *in vitro*, as is the equivalent PLCδ1 mutation H542P, and (ii) injection of luciferase-tagged mutant PLCs enables quantitative analysis of their *in vivo* expression in eggs and the demonstration that a dominant-negative inhibition mechanism by the mouse H435P mutant PLCζ does not appear to operate. Further advances in understanding the precise role and importance of PLCζ in mammalian fertilization may provide a major step in overcoming some cases of male infertility. It has been reported that, in some cases of egg activation failure after ICSI, treatment with a Ca^{2+} ionophore has been successful in producing assisted egg activation [18]. However, it remains to be determined whether routine ionophore treatment to assist with activation carries a risk of abnormal embryo development, or whether it is the most effective means of overcoming activation failure [19]. The application of recombinant PLCζ might represent a potential alternative physiological therapeutic agent that can overcome certain cases of failed fertilization after ICSI.

AUTHOR CONTRIBUTION

Michail Nomikos, 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, Brian Calver and Bevan Cumbes; and Michail Nomikos, Karl Swann and Anthony Lai prepared the manuscript.

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