

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <http://orca.cf.ac.uk/106290/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Nomikos, Michail, Kashir, Junaid and Lai, Francis Antony 2017. The role and mechanism of action of sperm PLC-zeta in mammalian fertilisation. *Biochemical Journal* 474 (21) , pp. 3659-3673. 10.1042/BCJ20160521 file

Publishers page: <http://dx.doi.org/10.1042/BCJ20160521> <<http://dx.doi.org/10.1042/BCJ20160521>>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



The role and mechanism of action of sperm PLC-zeta in mammalian fertilisation

Michail NOMIKOS*¹, Junaid KASHIR†‡[#], F. Anthony LAI†[^]

*College of Medicine, Qatar University, PO BOX 2713, Doha, Qatar

†College of Biomedical and Life Sciences, School of Biosciences, Cardiff University, Cardiff, UK

‡Alfaisal University, College of Medicine, Riyadh, Saudi Arabia

#King Faisal Specialist Hospital & Research Center, Department of Comparative Medicine, Riyadh, Saudi Arabia

¹ To whom correspondence should be addressed: Email: mixosn@yahoo.com (MN)

[^]*Author for editorial correspondence prior to publication: lait@cf.ac.uk (FAL)*

Short title: Structure and function of sperm-specific PLC ζ

Abbreviations: phospholipase C-zeta, PLC ζ ; calcium, Ca²⁺; phosphatidylinositol 4,5-bisphosphate, PIP₂; inositol 1,4,5-trisphosphate, InsP₃; pleckstrin homology domain, PH domain; phosphatidylinositol 3-phosphate, PI(3)P; phosphatidylinositol 5-phosphate, PI(5P); intracytoplasmic sperm injection, ICSI; assisted reproductive technology, ART

Key words: Phospholipase C zeta (PLC ζ), Fertilisation, Sperm, calcium oscillations, Male infertility

ABSTRACT

At mammalian fertilisation, the fundamental stimulus that triggers oocyte (egg) activation and initiation of early embryonic development is an acute rise of the intracellular free calcium (Ca^{2+}) concentration inside the egg cytoplasm. This essential Ca^{2+} increase comprises a characteristic series of repetitive Ca^{2+} oscillations, starting soon after sperm-egg fusion. Over the last fifteen years, accumulating scientific and clinical evidence supports the notion that the physiological stimulus that precedes the cytosolic Ca^{2+} oscillations is a novel, testis-specific phospholipase C (PLC) isoform, known as PLC-zeta (PLC ζ). Sperm PLC ζ catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) triggering cytosolic Ca^{2+} oscillations through the inositol 1,4,5-trisphosphate (InsP_3) signaling pathway. PLC ζ is the smallest known mammalian PLC isoform with the most elementary domain organisation. However, relative to somatic PLCs, the PLC ζ isoform possesses a unique potency in stimulating Ca^{2+} oscillations in eggs that is attributed to its novel biochemical characteristics. In this review, we discuss the latest developments that have begun to unravel the vital role of PLC ζ at mammalian fertilisation and decipher its unique mechanism of action within the fertilising egg. We also postulate the significant potential diagnostic and therapeutic capacity of PLC ζ in alleviating certain types of male infertility.

1. Introduction

Oocyte (egg) activation is the first and most critical step that initiates embryo development after fertilisation. The term 'egg activation' describes a series of biochemical and morphological events that mammalian eggs go through to prepare the egg for early embryo development after sperm-egg fusion [1]. In all species examined up-to-date, the earliest signaling event in the activation of an egg by a sperm is a large transient increase in the level of intracellular free calcium (Ca^{2+}) concentration [1-3]. In many non-mammalian species (such as frogs and sea urchins), the Ca^{2+} increase entails a single rise (one spike). Contrastingly, in mammals and ascidians the Ca^{2+} signal is delivered as a prolonged sequence of repetitive Ca^{2+} transients, known as Ca^{2+} oscillations, that start soon after gamete fusion, persisting for several hours and beyond the completion of meiosis [1, 2, 4-6]. The frequency and duration of Ca^{2+} oscillations appears to be relatively species-specific, ranging from every two minutes, to every hour [1, 7, 8]. This striking phenomenon consequently orchestrates a series of further events of egg activation, such as cortical granule exocytosis (CGE), which blocks polyspermy, meiotic resumption, and pronuclear development [1, 9, 10]. Mammalian eggs are very sensitive to the precise pattern of Ca^{2+} oscillations [11, 12]. The frequency and amplitude of Ca^{2+} oscillations are directly responsible for cell cycle progression, with varying Ca^{2+} transients resulting in different rates of cell cycle progression [13, 14]. Considering that the rate of cell cycle progression of human oocytes following fertilization has been postulated as an indicator of normal embryogenesis [15], the profile of Ca^{2+} oscillations during mammalian fertilisation may not only be necessary and sufficient for egg activation to occur but also equally important for subsequent embryogenic events [16].

Several lines of evidence suggest that the generation of Ca^{2+} oscillations in mammalian eggs is a direct consequence of inositol 1,4,5-trisphosphate (InsP_3)-mediated Ca^{2+} release [1, 4, 17]. The involvement of the universal Ca^{2+} -mobilizing messenger, InsP_3 , and its InsP_3 receptor (InsP_3R) was demonstrated by experiments in mouse and hamster eggs, where blocking or down-regulating the expression of InsP_3Rs led to the inhibition of Ca^{2+} oscillations and egg activation [18-20]. Furthermore, microinjection of InsP_3 or adenophostin A, an InsP_3 analogue, lead to Ca^{2+} oscillations in all mammalian eggs examined, demonstrating the necessity of this signaling pathway in progression of the Ca^{2+} release and egg activation process at mammalian fertilisation [1, 21, 22].

2. Sperm factor hypothesis and a novel PLC as the trigger of Ca^{2+} oscillations at fertilisation

The vigorously-contested scientific debate over the precise mechanism of how a single sperm elicits the generation of Ca^{2+} oscillations in the unfertilized egg dates over several decades. Many theories had been proposed to explain the genesis of the Ca^{2+} transients that successfully trigger egg activation during mammalian fertilisation (for review see [1]). Over the years, the weight of evidence has steadily shifted significantly in support of the 'sperm factor' hypothesis as the most appropriate model for egg activation in mammalian, and a number of marine invertebrate, species. This theory proposed that the sperm contains a soluble factor that, upon sperm-egg fusion, readily diffuses into the egg, and is functionally capable of triggering Ca^{2+} release from intracellular stores [5]. Experimental support for this hypothesis came from studies demonstrating that injection of sperm cytosolic extracts into mammalian eggs triggered a prolonged series of Ca^{2+} oscillations, indistinguishable to those seen at fertilisation, and this physiological stimulus initiated all the necessary events for successful egg activation [5, 23]. Moreover, the development of the clinical, *in vitro* fertilisation (IVF) technique, intracytoplasmic sperm injection (ICSI), a procedure that injects intact sperm directly into the egg cytoplasm, provided further support for the sperm factor hypothesis [24]. ICSI-injection of whole sperm in mammalian eggs results in egg activation and embryo development to term [25, 26].

Over the years, many candidates have been proposed as potential sperm factors. Initially, small molecules such as InsP_3 [27], nitric oxide (NO) [28] or nicotinic acid adenine dinucleotide phosphate (NAADP) [29] were considered as sperm factors but, although these molecules can induce some form of Ca^{2+} release from intracellular stores in some non-mammalian eggs, none of these substances could fully mimic the characteristic series of Ca^{2+} oscillations observed during IVF of mammalian eggs [1, 30]. *In vitro* PLC activity assays on mammalian cytosolic sperm extracts revealed that these extracts possess many fold higher PLC enzymatic activity compared with other somatic tissues that express several PLC isoforms [31]. More interestingly, although PLCs are enzymes that are generally characterized for their low Ca^{2+} sensitivity, the sperm extracts showed remarkable PLC activity even at $0.1\mu\text{M}$ Ca^{2+} concentration, the basal cytoplasmic Ca^{2+} levels found in the egg at the time of fertilisation [31]. Chromatographic fractionation studies suggested that the sperm factor should be a protein of $\sim 30\text{-}100$ kDa in size [1, 31, 32]. Microinjection of recombinant proteins corresponding to all the known and most characterized PLC isoforms at

the time, failed to trigger the pattern of Ca^{2+} oscillations observed during fertilisation [32, 33]. All these observations were consistent with the hypothetical existence of a novel, unidentified PLC isoform as the soluble sperm factor that triggers Ca^{2+} oscillations and activation of mammalian eggs, particularly since during this period the sequence of the human genome had not yet been reported.

3. PLC ζ ; the only native substance that can induce Ca^{2+} release and activation of mammalian eggs

In 2002, our laboratory in the then University of Wales College of Medicine (now Cardiff University School of Medicine) identified a novel testis-specific PLC isoform, which was termed PLC ζ [34]. Microinjection of *in vitro*-transcribed cRNA encoding PLC ζ into mouse eggs resulted in the observation of fertilisation-like Ca^{2+} release events, and subsequent early embryonic development up to the multicellular blastocyst stage [34]. Interestingly, the estimated expression levels of PLC ζ required for the initiation of Ca^{2+} oscillations was very closely comparable to the estimated amount of this enzyme contained in a single mouse sperm [34]. Moreover, the Ca^{2+} oscillation-inducing activity of sperm extracts was completely abolished following PLC ζ immunodepletion from these extracts by specific anti-PLC ζ antibodies [34]. Microinjection of recombinant mouse PLC ζ protein into mouse eggs also triggered Ca^{2+} oscillations similar to those observed after sperm extract injection [35]. Furthermore, RNA interference (RNAi) experiments producing severe disruption of PLC ζ expression in transgenic mice testes yielded sperm with otherwise normal properties that most notably induced prematurely terminating Ca^{2+} oscillations with a significantly reduced litter size [36]. Subsequent studies have also identified further mammalian orthologues of PLC ζ in human, hamster, monkey and horse sperm [4, 37-40], while non-mammalian testis-specific PLC ζ homologues have been identified in the chicken [41] and fish [42, 43]. Additional support for the fundamental role of PLC ζ at mammalian fertilisation came from a number of genetic and clinical reports that directly linked certain types of human male infertility with PLC ζ deficiencies (abnormally low expression levels or mutated forms of PLC ζ) in the sperm of IVF patient couples presenting with oocyte activation deficiencies (OAD) and thus total fertilisation failure i.e. infertility [44-49].

It is now well characterised and widely believed that upon sperm-egg membrane fusion, PLC ζ is delivered from the sperm head into the egg cytoplasm and this then catalyses the hydrolysis of its membrane-bound substrate, phosphatidylinositol 4,5-bisphosphate (PIP $_2$)

which is located on the membrane of an intracellular vesicle compartment [1, 34, 50]. The resulting liberation of InsP₃ stimulates opening of the InsP₃R, a Ca²⁺ release channel in the endoplasmic reticulum resulting in Ca²⁺ oscillations, causing egg activation and subsequent early embryonic development [1, 34, 50]; (Figure 1).

It is worth noting that over the previous 2 decades other proteins have been proposed as potential sperm factor candidates, including a 33 kDa protein (prematurely/imprudently termed ‘oscillin’) [51], a truncated form of the c-kit receptor (tr-kit) [52] and more recently, the postacrosomal sheath WW domain-binding protein (PAWP) [53, 54]. However, as various studies have demonstrated, none of these molecules induce the Ca²⁺ oscillations observed at fertilisation and nor fulfil all the physiological criteria for the sperm factor [1, 55-60]. Thus, all the mounting experimental and clinical evidence emanating from a number of different laboratories in the world indicate that the only identified molecule up to date, which is capable of initiating Ca²⁺ oscillations during mammalian fertilisation, is the testis-specific PLC ζ [50, 61].

4. PLC ζ structure and domain organization

Mammalian phosphoinositide-specific phospholipase C (PI-PLC) comprises a family of ubiquitous intracellular enzymes that play an essential role in activating intracellular signal transduction pathways to regulate various cellular functions [4, 62]. PI-PLCs directly participate in the phosphoinositide signalling pathway, catalysing the hydrolysis of PIP₂ and giving rise to two important second messenger molecules; InsP₃ and diacylglycerol (DAG). Fourteen distinct mammalian PLC isoforms have thus far been identified and are grouped into six distinct classes [β (1-4), γ (1,2), δ (1-4), ϵ , ζ , and η (1,2)], based on domain organisation and mode of activation. PLC isoforms also differ in tissue distribution, expression levels, Ca²⁺ sensitivity, catalytic regulation, and cellular localisation [4, 62, 63].

Despite its superior Ca²⁺ oscillation-inducing activity in eggs relative to somatic PLCs, sperm PLC ζ is the smallest PLC isoform with the most basic domain organization. PLC ζ enzymes in all the species characterized up to date, share a similar size of 70-75 kDa [4, 50, 64]. PLC ζ demonstrates a typical PLC domain structure consisting of four EF hand motifs, followed by the characteristic X&Y catalytic domains and a single C2 domain at its C-terminus. These domains are common to all PLC isoforms (β , γ , δ , ϵ , ζ and η) [50]. The catalytic X&Y domains form the active site in all PLCs and their sequence is the most highly conserved domain between the different PLC isoforms relative to the other regulatory

domains. The sequence similarity among all PLC isoforms is 60%, but much higher among the isoforms of the same class. The catalytic X&Y domains of PLC ζ shares a 64% sequence similarity with that of PLC δ 1 and by structural homology, is predicted to be organised in eight repetitive beta sheet/alpha helix sequences, forming a distorted barrel [4, 50]. Mutagenesis of conserved active site residues within the XY catalytic domain of PLC ζ resulted in loss of enzymatic activity, and thus its inability to induce Ca²⁺ release in mammalian eggs [1, 50]. In all PLC isoforms, a discrete region separates the X and Y catalytic domains, known as the XY-linker. In PLC ζ , this is an unstructured linker region with a distinctive cluster of basic amino acid residues not found in the XY-linker regions of the other PLC isoforms, and playing an important regulatory role in PLC ζ function [1, 34, 50].

A notable structural difference of sperm PLC ζ to somatic PLC isoforms is its lack of a pleckstrin homology (PH) domain [34]. PH domains are well defined structural modules of ~120 amino acid residues long, which have been identified in more than 100 different proteins [1, 4]. All PLC isoforms except PLC ζ and PLC ϵ possess a PH domain. It is believed that the PH domain facilitates the binding of PLCs to biological membranes [62, 65]. The PH domain of PLC δ 1 mediates the binding of this enzyme to the cell plasma membrane by its high-affinity and -specificity binding to its membrane-bound substrate, PIP₂ [66]. With the significant absence of a PH domain, PLC ζ is most closely related in domain structure to PLC δ 1 enzyme exhibiting a 33% identity and 47% similarity [34]. Due to the lack of a PH domain, PLC ζ appears to employ a unique mechanism to target its biological membrane substrate, involving synergy of the positively-charged N-terminal lobe EF-hand domain with the basic amino acids at the C-terminal end of its XY-linker region, that coalesce through electrostatic interactions with its negatively-charged substrate, PIP₂ [67-69].

5. Essential role of the EF hand domains in the Ca²⁺ sensitivity and membrane targeting of PLC ζ

Arguably the most critical physiological feature of PLC ζ allowing it to be enzymatically very active in mammalian eggs relative to other somatic PLCs, is its uniquely high Ca²⁺ sensitivity [35, 70]. PLC ζ is ~100-fold more Ca²⁺ sensitive compared with its PLC δ 1 counterpart, displaying an EC₅₀ of ~80nM [70], well within the range of reported resting Ca²⁺ concentrations in mammalian eggs, ~120nM, explaining why PLC ζ 's biochemical activity is observed very soon after its release from the sperm into the egg cytoplasm [70].

We have previously shown that deletion of the EF-hand domains dramatically increases the EC_{50} of PLC ζ from ~ 80 nM to ~ 30 μ M [70], while replacement of PLC ζ EF-hand domains with that from PLC $\delta 1$ results in a ~ 10 -fold decrease in the Ca^{2+} sensitivity of PLC ζ , without significantly affecting its enzymatic activity [71]. In addition, in parallel with our empirical data, mathematical modelling approaches have strongly suggested that the EF-hand motifs are the major determinants of the high Ca^{2+} sensitivity of PLC ζ , which in turn leads to its unsurpassed effectiveness in triggering high frequency Ca^{2+} oscillations in mammalian eggs [71].

Recently, we investigated whether replacement of the EF-hand domains of rat PLC $\delta 1$ with mouse PLC ζ , in a PLC $\delta 1$ construct lacking the PH domain can confer to this chimeric enzyme the high Ca^{2+} sensitivity of PLC ζ . We generated the Δ PH/PLC $\delta 1$ /EF ζ construct, as shown in Figure 2A comprising the N-terminal 150 aa of PLC ζ (containing its EF-hand domains) fused to a PLC $\delta 1$ deletion construct lacking the first 284 aa (comprising both its PH & EF-hand domain regions). This Δ PH/PLC $\delta 1$ /EF ζ construct encodes a 624 aa chimaeric protein that was expressed using the pETMM60 vector and isolated by affinity chromatography as previously described [69, 71-73] enabling characterization of the purified ~ 129 kDa NusA-tagged PLC chimaera (Fig. 2B). The PIP $_2$ hydrolytic enzyme activity of PLC ζ , PLC $\delta 1$ and Δ PH/PLC $\delta 1$ /EF ζ was determined (Fig. 3A & Table 1) and indicates that chimaeric protein displayed a $\sim 31\%$ reduced enzymatic activity (2015 ± 51 nmol/min/mg) compared to PLC $\delta 1$. To investigate the effect of replacing EF-hand domains of PLC $\delta 1$ with that of PLC ζ on the Ca^{2+} sensitivity of PLC $\delta 1$ (lacking the PH domain), we assessed the [3 H]PIP $_2$ hydrolysis at different Ca^{2+} concentrations ranging from 0.1nM to 0.1mM [69, 71-73]; (Figure 3B, Table 1). The resulting EC_{50} values for PLC ζ (73 nM) and PLC $\delta 1$ (5.96 μ M) were in good agreement with those obtained at previous studies [70, 72, 73], while the EC_{50} for Δ PH/PLC $\delta 1$ /EF ζ (4.71 μ M) was slightly lower compared to PLC $\delta 1$, but ~ 64 -fold higher compared to the EC_{50} of PLC ζ . Our experiments suggest that the EF-domains of PLC ζ on their own are not sufficient to confer the high Ca^{2+} sensitivity of PLC ζ to a PLC $\delta 1$ -like protein lacking the PH domain, indicating that the EF-domains play a vital role in conferring the high Ca^{2+} sensitivity of PLC ζ but the overall PLC ζ protein tertiary structure is an additional essential requirement.

Interestingly, the N-terminal lobe of the EF-hand domains of PLC ζ contains a cluster of basic amino acid residues. We recently demonstrated that sequential neutralisation of these

basic residues within the first EF-hand domain of PLC ζ significantly diminishes the PIP₂-binding properties of PLC ζ [69]. We surmised that PIP₂ might be attracted to the negatively-charged PIP₂-containing component of the intracellular vesicular membrane through electrostatic interactions and thus, both the EF-hand and the XY-linker regions which are rich in basic residues, are essential for this interaction. This molecular interaction provides a tether that facilitates proper PIP₂ substrate access to and binding with the PLC ζ active site [69].

6. The regulatory role of the non-conserved XY-linker region

The XY-linker region of PLC ζ is extended in length and contains more positively-charged amino acid residues relative to its PLC δ 1 counterpart [34, 50]. Notably, the XY-linker is the most non-conserved region of the PLC ζ domain sequences determined from different species, but in all cases this linker region contains a number of positively-charged residues in close proximity to the Y catalytic domain [4, 50]. The specific physiological rationale for this XY-linker diversity is still unclear. Perhaps the diversity in this sequence may explain the species-specific differences in specific patterns of Ca²⁺ oscillations observed for various mammalian PLC ζ enzymes, as well as the divergent relative potencies of these enzymes in triggering cytosolic Ca²⁺ oscillations when expressed in mouse eggs [4, 6]. Notably, we have demonstrated that the XY-linker plays a key role in the functional regulation of PLC ζ enzyme activity and also in its important ability to contribute to the interaction with its membrane-resident lipid substrate, PIP₂ [67, 68, 74]. Finally, the XY-linker region contains a predicted nuclear localization signal (NLS) sequence that may play a role in the regulation of at least mouse PLC ζ [75].

7. The vital role of PLC ζ C2 domain is highlighted by male infertility-linked point mutation

All PLC isoforms possess a C2 domain, following the XY catalytic domain. These domains comprise ~120 amino acid residues and play an important role in the Ca²⁺-dependent subcellular membrane targeting of several lipid-metabolizing enzymes, such as PLC δ 1 and cPLCA2 [76]. In PLC δ 1, the structurally closest protein to PLC ζ , the C2 domain interacts with the membrane phospholipid, phosphatidylserine (PS) to form a C2-Ca²⁺-phosphatidylserine quaternary complex, which enhances its enzymatic activity [77].

The exact physiological role of PLC ζ C2 domain is still unresolved. There is experimental evidence for low-affinity binding of this domain to membrane phospholipids; phosphatidylinositol 3-phosphate [PI(3)P] and phosphatidylinositol 5-phosphate [PI(5P)] [68, 78]. We have previously demonstrated that deletion or replacement of the C2 domain of PLC ζ with the corresponding domain of PLC δ 1 abolishes the Ca²⁺ oscillation-inducing activity in intact eggs, without affecting the in vitro PIP₂ hydrolytic properties of this enzyme [70, 71]. These observations suggest a disparate, vital (but apparently non-hydrolytic) role of the C2 domain in PLC ζ function. Further support for this suggestion came from the identification of a male infertility-linked point mutation located in the C2 domain of PLC ζ [49]. This is the first PLC ζ infertility-linked mutation to be reported in a domain other than the XY catalytic domain. In this mutant, an isoleucine (I) residue is substituted with a phenylalanine (F) residue in 489 position (I489F) of human PLC ζ sequence [49]. Analysis of this mutation revealed that at physiological concentrations this PLC ζ mutant is unable to trigger Ca²⁺ release in mouse eggs [79]. However, microinjection of non-physiological levels could rescue the infertile phenotype, leading to Ca²⁺ oscillations and egg activation. Further biochemical characterization suggested that the I489F had no effect on the enzymatic properties of PLC ζ , but dramatically reduced its in vitro binding to PI(3)P- and PI(5)P-containing liposomes [79]. These findings highlight the critical role of this domain in PLC ζ function due to its novel membrane binding/localisation characteristics (Figure 4). Further investigation is required to delineate the physiological significance of PLC ζ -PI(3)P and -PI(5)P interactions and to identify other potential egg-derived binding partners of this domain that might regulate PLC ζ function within the egg.

8. Localisation of PLC ζ in the sperm

PLC ζ has been identified in sperm of numerous species and generally appears localised at distinct subcellular regions of the sperm head, with potentially differential functional roles for each specific population [64]. PLC ζ has been identified in acrosomal and post-acrosomal regions of mouse and porcine sperm, with a tail population also identified in porcine sperm [80-82]. In equine sperm, PLC ζ was reported in the acrosome, equatorial segment, head mid-piece, as well as the principal piece of the flagellum [40]. In humans, three distinct populations of PLC ζ have been identified in the acrosomal, equatorial and post-acrosomal regions of the sperm head, with a potentially additional tail localisation [44, 47, 83-86]. However, it remains unclear whether such populations are physiologically valid.

Specific PLC ζ localisation remains a concern, with numerous studies identifying variable patterns within the same mammalian species, often using the same antibody probe.

A previous study suggested that PLC ζ was localised within the acrosome of mouse and human sperm, with further PLC ζ populations present on the sperm surface [87]. However, using the same antibodies, multiple studies indicated PLC ζ localisation to the post-acrosomal region of the mouse sperm [80, 88], as well as to the acrosomal, equatorial, and post-acrosomal regions of human sperm [44, 84-86, 89]. Indeed, there only seems to be consensus regarding PLC ζ localisation in mouse sperm. This variance in PLC ζ localisation is not only limited to observations between different species, with a study reporting significant variance in localisation patterns observed in sperm between human subjects [86].

Perhaps such variance is attributable to differences in immunolocalisation protocols used by different studies, or due to limited specificity of the polyclonal antibodies that have been used in all such studies to date, particularly in relation to human sperm. Recently developed epitope-specific PLC ζ polyclonal antibodies to human, mouse and porcine PLC ζ , have exhibited reliable consistency in results throughout multiple studies for both recombinant and native PLC ζ [90-92]. Further to such progress, it has recently been demonstrated that specific antigen unmasking/retrieval protocols are required to enhance the visualisation efficacy of PLC ζ in mammalian sperm, perhaps due to strong intra- or inter-molecular conformation(s)/interaction(s) between discrete domains of monomeric and/or oligomeric PLC ζ [92]. Following the application of these specific antibodies and enhanced protocols, PLC ζ has been identified in the acrosomal and post-acrosomal, acrosomal and equatorial, as well as, post-acrosomal and equatorial compartments of mouse, human and porcine sperm, respectively. Furthermore, use of these antibodies has consistently observed potential tail localisation in all species [92]. These recent observations from application of new PLC ζ -specific probes and protocols suggest that previously published results regarding PLC ζ subcellular localisation in sperm of mammalian species may require further detailed evaluation. For example, Grasa *et al.*, [89] and Young *et al.*, [88] demonstrated in human and mouse sperm, respectively, that prior to capacitation acrosomal populations of PLC ζ were prominent, shifting to a predominantly post-acrosomal localisation following capacitation. While such results remain supportive of PLC ζ 's proposed identity as the mammalian sperm factor, it is important that further high-resolution localisation analysis enables a consensus view to emerge.

9. Localisation of PLC ζ in the egg and the search for a putative ‘egg factor’

Another unique biochemical feature of sperm PLC ζ is its localisation in mammalian eggs. Somatic PLC isoforms are known to localise to the plasma membrane where they hydrolyse their membrane-bound substrate PIP₂ [50]. However, there is no detectable PLC ζ localisation in the plasma membrane of eggs [93]. Moreover, the depletion of plasma membrane PIP₂ using a targeted inositol lipid phosphatase has no effect upon PLC ζ - or sperm-induced Ca²⁺ oscillations, even though such a PIP₂-depletion strategy effectively abolishes PLC δ 1-induced Ca²⁺ oscillations [93]. Further, immunocytochemical approaches suggest that the introduced sperm PLC ζ becomes localised to small vesicles throughout the egg cytoplasm [93]. Interestingly, PIP₂ is also detected in such cytoplasmic vesicles. This putative intracellular PIP₂ source is very significant because the expression of an inositol lipid phosphatase fused with catalytically-inactive PLC ζ in mouse eggs (to specifically target and deplete this vesicular PIP₂), potently inhibited both sperm- and PLC ζ -induced Ca²⁺ oscillations [93]. This indicates that the egg cytosolic Ca²⁺ oscillations induced by PLC ζ or sperm rely upon hydrolysis of PIP₂ from intracellular vesicles primarily, and not the plasma membrane, which in itself represents an entirely new aspect of PI-mediated Ca²⁺ signalling.

Immediately after PLC ζ is injected into eggs it is capable of triggering Ca²⁺ oscillations, hence PLC ζ appears to be autonomously active. However, relative to the egg, PLC ζ is present at >1000 times higher concentration in sperm where it appears to be either enzymatically inactive or compartmentalised. Transfection studies have shown that PLC ζ can be stably expressed in CHO cells at concentrations 100-1000 times that found in an egg during fertilization, and yet this does not cause any change in Ca²⁺ homeostasis [91]. However, when these PLC ζ -expressing CHO cells, or extracts from these cells, are injected into mouse eggs, Ca²⁺ oscillations are induced [91]. These data suggest that PLC ζ may only be active in eggs, and this further implies that eggs possess an essential factor(s) that is specifically required for PLC ζ to hydrolyse PIP₂. The target molecule or ‘receptor’ for PLC ζ is likely to be on the egg vesicles that contain PIP₂ and if this target interacts very specifically with PLC ζ then this hypothesis may also explain why other mammalian PLC isoforms e.g. PLC δ 1, are orders of magnitude less effective in triggering Ca²⁺ oscillations when injected into mouse eggs. The identification of an egg receptor for PLC ζ will have

major implications for understanding wider cases of infertility and for animal reproductive technologies.

10. PLC ζ , ICSI failure and male infertility

ICSI, a powerful IVF technique whereby a single sperm is injected into oocytes, is an effective method of assisted reproductive technology (ART) for men presenting with suboptimal parameters, such as abnormal sperm concentration, motility, or morphology. Such methodology is generally applied in cases when couples experience low fertilisation success or complete fertilisation failure following conventional IVF. However, despite relatively high rates of ICSI success, total fertilisation failure occurs in ~1–5% of ICSI cycles, usually recurring in subsequent ART cycles [94, 95]. Potential causative factors include failed sperm head decondensation, premature sperm chromatin condensation, oocyte spindle defects and sperm aster defects [46], while issues such as incorrect sperm injection/expulsion, or low gamete quality [96, 97] may also contribute. However, a failure of the oocyte activation mechanism is frequently considered to be the main contributory factor [46, 95, 98-101].

Importantly, morphologically normal sperm from several human patients have been found to fail to activate mouse eggs [45, 101], with sperm either unable to elicit Ca²⁺ release following injection into mouse eggs, or else producing significantly diminished and abnormal Ca²⁺ oscillation profiles, being severely reduced in frequency and amplitude [44, 45]. Furthermore, immunofluorescence and immunoblot analysis of such sperm exhibited reduced or absent levels of PLC ζ within the sperm head, while the Ca²⁺ oscillation-inducing activity of such sperm was ‘rescued’ in mouse oocytes by the co-injection of PLC ζ cRNA [44, 45]. Subsequent to these studies, numerous investigations performing PLC ζ assessment in human sperm undergoing fertility treatment indicate that sperm with defective oocyte activation capacity are associated with reduced/absent levels of PLC ζ [44-46, 86, 102, 103], while PLC ζ deficiencies are also associated with sperm conditions, such as globozoospermia [45, 46, 102, 104, 105] and recurrent partial hydatidiform moles (abnormal pregnancies) [106].

Importantly, abrogation of PLC ζ gene function in patients diagnosed with oocyte activation deficiency has also now been reported. Two PLC ζ mutations were identified from an infertile man, whose sperm lacked the capacity to elicit Ca²⁺ oscillations that led to disrupted PLC ζ activity and an infertility phenotype [45, 47]. Biochemical characterisation indicated that the mutations, both occurring in the active site domains of PLC ζ , led to disruption of local protein folding, while injection of correspondingly mutated cRNA and

recombinant protein into mouse eggs resulted in highly abnormal Ca^{2+} transients that were unable to initiate oocyte activation [48, 90, 107]. Both mutations were reported to be heterozygous, with one inherited from the patient's father, with the other inherited from the patient's mother, indicating for the first time that such maternally-inherited loss-of-activity mutations contribute towards male infertility [47, 107].

Subsequently, a homozygous mutation was identified by Escoffier *et al.*, (2016) from a similarly infertile patient, occurring within the C2 domain of PLC ζ [49]. As discussed above, this PLC ζ C2 domain mutant displays similar enzymatic activity compared to wild-type PLC ζ , however exhibiting dramatically reduced binding to PI(3)P and PI(5)P-containing liposomes [79]. Single nucleotide polymorphisms (SNPs) have also been reported either within the coding sequence of PLC ζ or its associated bi-directional promoter in human patients [44, 108]. Further polymorphisms have also been identified in the PLC ζ gene of bulls and stallions, at promoter regions and exonic loci, which were shown to positively correlate to semen parameters and the fertility status of such sperm [109, 110]. However, as these polymorphisms were not physiologically or biochemically characterised, any potential contributory effects exerted by SNPs upon sperm PLC ζ function are currently unclear.

Recent data suggest a significant level of variance in terms of total PLC ζ levels and localisation patterns in human sperm from normal, fertile men [44, 45, 84, 85]. Such variance in sperm PLC ζ presents an interesting problem, particularly when considering that absent/reduced levels of PLC ζ have previously been implicated in cases of infertility, and that levels of PLC ζ directly impact upon the frequency and amplitude of the resulting Ca^{2+} oscillations. Injection of increasing PLC ζ cRNA levels in human oocytes results in increasing frequencies and amplitudes of Ca^{2+} oscillations [111], which may also exert effects upon gene expression profiles in a Ca^{2+} -dependent manner during early embryogenesis [13, 14]. Indeed, preimplantation development of oocytes activated by differential levels of human PLC ζ suggests that too little, or too much, PLC ζ leads to poor development to the blastocyst stage despite apparently normal oocyte activation. Thus, abnormalities in sperm PLC ζ levels may underlie not only infertility through fertilisation failure, but also cases of male sub-fertility, whereby adequate PLC ζ may be delivered to oocytes to cause activation only, but which may be insufficient for embryonic competence.

11. Clinical applications of PLC ζ

The success of ART is reflected by estimates that such methods now account for ~7% of total birth rates in some developing countries [46]. However, several conditions such as severe male infertility (19-57% of infertility cases) remain untreatable, even after ICSI treatment [112]. It seems clear that sperm from males that fail to activate human eggs following ICSI exhibit abrogated, reduced, or aberrant forms of PLC ζ . Thus, alongside a potential diagnostic parameter, PLC ζ may represent an endogenous method to clinically treat cases of egg activation failure.

Currently, oocyte activation failure is clinically treated by assisted oocyte activation (AOA) methods, predominantly application of Ca²⁺ ionophores, resulting in improved rates of fertilization and successful pregnancy. However, concerns currently exist that such synthetic non-native chemicals may be potentially detrimental to embryo viability [113], considering that such methods elicit a single large Ca²⁺ increase unlike the repetitive pattern of Ca²⁺ oscillations observed at fertilisation. Indeed, computational modelling and experimental studies suggest that the temporal pattern of Ca²⁺ changes may exert specific effects upon rates of cell cycle progression, and thus, subsequent embryogenesis [114]. Thus, it is important that an endogenous, potentially safer method of assisted egg activation is established, for which PLC ζ is an extremely attractive candidate. Rogers *et al.* [115] obtained parthenogenetically-generated human blastocysts by injection of PLC ζ cRNA into eggs, while Yoon *et al.* [44] demonstrated that abnormalities in sperm PLC ζ could be counteracted by co-injection with mouse PLC ζ cRNA. Moreover, a very recent study reported the determination of the optimal concentration of human PLC ζ cRNA to activate human oocytes [111].

However, while representing a significant research tool, clinical use of cRNA injections may prove problematic due to uncontrolled expression and potential reverse transcription activity inherent to mammalian oocytes [46]. Thus, the production of purified, enzymatically-active, recombinant human PLC ζ protein has been a goal for many laboratories around the world, to enable dose-controlled delivery of functionally-viable PLC ζ protein. To this degree, recent efforts have been able to produce such versions of human PLC ζ , which is able to effectively rescue activation in mouse and human oocytes, representing a significant milestone in potential clinical applicability to reproductive medicine [90]. However, while this recombinant protein may be significantly advantageous to patients suffering from recurrent ICSI failure, it remains unclear whether a PLC ζ ‘therapeutic’ may be able to aid further types of patients where normal activation at

fertilisation occurs, but subsequent initiation of early embryogenesis is poor, a causative factor for recurrent implantation failure.

12. Concluding remarks

The 2002 discovery of PLC ζ , the putative physiological ‘sperm factor’ that plays a vital role at mammalian fertilisation represented a major breakthrough in the field of reproductive biology. It is now widely accepted that PLC ζ is the sole physiological stimulus that is delivered from the fertilising sperm into the egg cytoplasm, soon after sperm-egg membrane fusion, triggering the Ca²⁺ oscillations required for successful egg activation and early embryogenesis. Despite all the recent advances that have improved our knowledge about the unique biochemical properties of this gamete-specific enzyme, the precise biological mechanism of PLC ζ action and regulation within the fertilising sperm and egg has not yet been fully characterised. All the recent genetic and clinical reports that have directly linked male infertility cases with reduced expression levels and mutated forms of this sperm-specific protein necessitate elucidation of the molecular mechanism that PLC ζ employs to ‘kick start’ a new life. For example, the participation of PLC ζ in a standard biochemical pathway [PI signalling pathway] that is known to be present in all types of cells in the body, but uniquely, PLC ζ appears to only be fully active within eggs. It is still currently unclear the mechanism by which PLC ζ is kept inactive within the sperm head but soon after its release within the egg cytosol, it is immediately able to hydrolyse its membrane-bound substrate PIP₂, inducing Ca²⁺ release and subsequent egg activation. Moreover, while the biochemical properties of PLC ζ domains have been characterised, a full understanding of how all these distinct domains work together in synchrony within the egg remains incomplete. The recent genetic report that identified the infertility-linked point mutation in the C2 domain of PLC ζ , clearly suggests that this domain plays a fundamental role in PLC ζ function, although it is not directly involved in the hydrolysis of PIP₂. This necessitates further investigation of the exact role of this domain in PLC ζ function. There is a possibility that PLC ζ interacts through this domain with an unidentified egg protein or receptor that mediates PLC ζ action in the egg. Unravelling and understanding the full mechanism of PLC ζ action and mode of regulation, can provide the basis for creating new advances not only in clinical medicine but also in animal breeding technologies.

AUTHOR CONTRIBUTIONS

M.N. prepared the first draft of the manuscript, which was revised and approved by J.K. and F.A.L.

FUNDING

This work was supported by an EU-FP7 Marie-Curie Intra-European Fellowship 628634 and an internal College of Medicine (Qatar University) grant to M.N.; and a Health Fellowship award from the National Institute for Social Care and Health Research (NISCHR) to J.K.

DECLARATIONS OF INTEREST: The authors declare no financial interests.

REFERENCES

- 1 Nomikos, M., Swann, K. and Lai, F. A. (2012) Starting a new life: sperm PLC-zeta mobilizes the Ca²⁺ signal that induces egg activation and embryo development: an essential phospholipase C with implications for male infertility. *Bioessays*. **34**, 126-134
- 2 Stricker, S. A. (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol*. **211**, 157-176
- 3 Runft, L. L., Jaffe, L. A. and Mehlmann, L. M. (2002) Egg activation at fertilization: where it all begins. *Dev Biol*. **245**, 237-254
- 4 Nomikos, M., Kashir, J., Swann, K. and Lai, F. A. (2013) Sperm PLCzeta: from structure to Ca²⁺ oscillations, egg activation and therapeutic potential. *FEBS Lett*. **587**, 3609-3616
- 5 Swann, K. (1990) A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development*. **110**, 1295-1302
- 6 Swann, K., Saunders, C. M., Rogers, N. T. and Lai, F. A. (2006) PLCzeta(zeta): a sperm protein that triggers Ca²⁺ oscillations and egg activation in mammals. *Semin Cell Dev Biol*. **17**, 264-273
- 7 Kline, D. and Kline, J. T. (1992) Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol*. **149**, 80-89
- 8 Fissore, R. A., Dobrinsky, J. R., Balise, J. J., Duby, R. T. and Robl, J. M. (1992) Patterns of intracellular Ca²⁺ concentrations in fertilized bovine eggs. *Biol Reprod*. **47**, 960-969
- 9 Schultz, R. M. and Kopf, G. S. (1995) Molecular basis of mammalian egg activation. *Curr Top Dev Biol*. **30**, 21-62
- 10 Jones, K. T. (1998) Ca²⁺ oscillations in the activation of the egg and development of the embryo in mammals. *Int J Dev Biol*. **42**, 1-10
- 11 Malcuit, C., Kurokawa, M. and Fissore, R. A. (2006) Calcium oscillations and mammalian egg activation. *J Cell Physiol*. **206**, 565-573
- 12 Stitzel, M. L. and Seydoux, G. (2007) Regulation of the oocyte-to-zygote transition. *Science*. **316**, 407-408
- 13 Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R. M., Kopf, G. S., Fissore, R., Madoux, S. and Ozil, J. P. (2002) Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Dev Biol*. **250**, 280-291
- 14 Ducibella, T., Schultz, R. M. and Ozil, J. P. (2006) Role of calcium signals in early development. *Semin Cell Dev Biol*. **17**, 324-332
- 15 Wong, C. C., Loewke, K. E., Bossert, N. L., Behr, B., De Jonge, C. J., Baer, T. M. and Reijo Pera, R. A. (2010) Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol*. **28**, 1115-1121
- 16 Yu, Y., Saunders, C. M., Lai, F. A. and Swann, K. (2008) Preimplantation development of mouse oocytes activated by different levels of human phospholipase C zeta. *Hum Reprod*. **23**, 365-373
- 17 Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y. (1993) Essential role of the inositol 1,4,5-trisphosphate receptor/Ca²⁺ release channel in Ca²⁺ waves and Ca²⁺ oscillations at fertilization of mammalian eggs. *Dev Biol*. **158**, 62-78
- 18 Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S. and Mikoshiba, K. (1992) Block of Ca²⁺ wave and Ca²⁺ oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science*. **257**, 251-255
- 19 Brind, S., Swann, K. and Carroll, J. (2000) Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm or adenophostin A but not to increases in intracellular Ca(2+) or egg activation. *Dev Biol*. **223**, 251-265
- 20 Jellerette, T., He, C. L., Wu, H., Parys, J. B. and Fissore, R. A. (2000) Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilization or parthenogenetic activation. *Dev Biol*. **223**, 238-250

- 21 Wu, H., Smyth, J., Luzzi, V., Fukami, K., Takenawa, T., Black, S. L., Allbritton, N. L. and Fissore, R. A. (2001) Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol Reprod.* **64**, 1338-1349
- 22 Jones, K. T. and Nixon, V. L. (2000) Sperm-induced Ca²⁺ oscillations in mouse oocytes and eggs can be mimicked by photolysis of caged inositol 1,4,5-trisphosphate: evidence to support a continuous low level production of inositol 1, 4,5-trisphosphate during mammalian fertilization. *Dev Biol.* **225**, 1-12
- 23 Stricker, S. A. (1997) Intracellular injections of a soluble sperm factor trigger calcium oscillations and meiotic maturation in unfertilized oocytes of a marine worm. *Dev Biol.* **186**, 185-201
- 24 Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A. C. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet.* **340**, 17-18
- 25 Tesarik, J., Sousa, M. and Testart, J. (1994) Human oocyte activation after intracytoplasmic sperm injection. *Hum Reprod.* **9**, 511-518
- 26 Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y. and Miyazaki, S. (1997) Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Mol Hum Reprod.* **3**, 1087-1093
- 27 Tosti, E., Palumbo, A. and Dale, B. (1993) Inositol tri-phosphate in human and ascidian spermatozoa. *Mol Reprod Dev.* **35**, 52-56
- 28 Kuo, R. C., Baxter, G. T., Thompson, S. H., Stricker, S. A., Patton, C., Bonaventura, J. and Epel, D. (2000) NO is necessary and sufficient for egg activation at fertilization. *Nature.* **406**, 633-636
- 29 Lim, D., Kyojuka, K., Gragnaniello, G., Carafoli, E. and Santella, L. (2001) NAADP⁺ initiates the Ca²⁺ response during fertilization of starfish oocytes. *Faseb J.* **15**, 2257-2267
- 30 Swann, K. (1994) Ca²⁺ oscillations and sensitization of Ca²⁺ release in unfertilized mouse eggs injected with a sperm factor. *Cell Calcium.* **15**, 331-339
- 31 Rice, A., Parrington, J., Jones, K. T. and Swann, K. (2000) Mammalian sperm contain a Ca²⁺-sensitive phospholipase C activity that can generate InsP(3) from PIP(2) associated with intracellular organelles. *Dev Biol.* **228**, 125-135
- 32 Parrington, J., Jones, M. L., Tunwell, R., Devader, C., Katan, M. and Swann, K. (2002) Phospholipase C isoforms in mammalian spermatozoa: potential components of the sperm factor that causes Ca²⁺ release in eggs. *Reproduction.* **123**, 31-39
- 33 Jones, K. T., Matsuda, M., Parrington, J., Katan, M. and Swann, K. (2000) Different Ca²⁺-releasing abilities of sperm extracts compared with tissue extracts and phospholipase C isoforms in sea urchin egg homogenate and mouse eggs. *Biochem J.* **346 Pt 3**, 743-749
- 34 Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K. and Lai, F. A. (2002) PLC zeta: a sperm-specific trigger of Ca²⁺ oscillations in eggs and embryo development. *Development.* **129**, 3533-3544
- 35 Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T. and Miyazaki, S. (2004) Recombinant phospholipase C zeta has high Ca²⁺ sensitivity and induces Ca²⁺ oscillations in mouse eggs. *J Biol Chem.* **279**, 10408-10412
- 36 Knott, J. G., Kurokawa, M., Fissore, R. A., Schultz, R. M. and Williams, C. J. (2005) Transgenic RNA interference reveals role for mouse sperm phospholipase C zeta in triggering Ca²⁺ oscillations during fertilization. *Biol Reprod.* **72**, 992-996
- 37 Cox, L. J., Larman, M. G., Saunders, C. M., Hashimoto, K., Swann, K. and Lai, F. A. (2002) Sperm phospholipase C zeta from humans and cynomolgus monkeys triggers Ca²⁺ oscillations, activation and development of mouse oocytes. *Reproduction.* **124**, 611-623
- 38 Yoneda, A., Kashima, M., Yoshida, S., Terada, K., Nakagawa, S., Sakamoto, A., Hayakawa, K., Suzuki, K., Ueda, J. and Watanabe, T. (2006) Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase C zeta. *Reproduction.* **132**, 393-401
- 39 Cooney, M. A., Malcuit, C., Cheon, B., Holland, M. K., Fissore, R. A. and D'Cruz, N. T. (2010) Species-specific differences in the activity and nuclear localization of murine and bovine phospholipase C zeta 1. *Biol Reprod.* **83**, 92-101

- 40 Bedford-Guaus, S. J., McPartlin, L. A., Xie, J., Westmiller, S. L., Buffone, M. G. and Roberson, M. S. (2011) Molecular cloning and characterization of phospholipase C zeta in equine sperm and testis reveals species-specific differences in expression of catalytically active protein. *Biol Reprod.* **85**, 78-88
- 41 Coward, K., Ponting, C. P., Chang, H. Y., Hibbitt, O., Savolainen, P., Jones, K. T. and Parrington, J. (2005) Phospholipase C zeta, the trigger of egg activation in mammals, is present in a non-mammalian species. *Reproduction.* **130**, 157-163
- 42 Coward, K., Ponting, C. P., Zhang, N., Young, C., Huang, C. J., Chou, C. M., Kashir, J., Fissore, R. A. and Parrington, J. (2011) Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C zeta (PLCzeta) in pufferfish. *Mol Reprod Dev.* **78**, 48-56
- 43 Ito, M., Shikano, T., Oda, S., Horiguchi, T., Tanimoto, S., Awaji, T., Mitani, H. and Miyazaki, S. (2008) Difference in Ca²⁺ oscillation-inducing activity and nuclear translocation ability of PLCZ1, an egg-activating sperm factor candidate, between mouse, rat, human, and medaka fish. *Biol Reprod.* **78**, 1081-1090
- 44 Yoon, S. Y., Jellerette, T., Salicioni, A. M., Lee, H. C., Yoo, M. S., Coward, K., Parrington, J., Grow, D., Cibelli, J. B., Visconti, P. E., Mager, J. and Fissore, R. A. (2008) Human sperm devoid of PLC, zeta 1 fail to induce Ca(2+) release and are unable to initiate the first step of embryo development. *J Clin Invest.* **118**, 3671-3681
- 45 Heytens, E., Parrington, J., Coward, K., Young, C., Lambrecht, S., Yoon, S. Y., Fissore, R. A., Hamer, R., Deane, C. M., Ruas, M., Grasa, P., Soleimani, R., Cuvelier, C. A., Gerris, J., Dhont, M., Deforce, D., Leybaert, L. and De Sutter, P. (2009) Reduced amounts and abnormal forms of phospholipase C zeta (PLCzeta) in spermatozoa from infertile men. *Hum Reprod.* **24**, 2417-2428
- 46 Kashir, J., Heindryckx, B., Jones, C., De Sutter, P., Parrington, J. and Coward, K. (2010) Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update.* **16**, 690-703
- 47 Kashir, J., Konstantinidis, M., Jones, C., Lemmon, B., Lee, H. C., Hamer, R., Heindryckx, B., Deane, C. M., De Sutter, P., Fissore, R. A., Parrington, J., Wells, D. and Coward, K. (2012) A maternally inherited autosomal point mutation in human phospholipase C zeta (PLCzeta) leads to male infertility. *Hum Reprod.* **27**, 222-231
- 48 Nomikos, M., Elgmati, K., Theodoridou, M., Calver, B. L., Cumbes, B., Nounesis, G., Swann, K. and Lai, F. A. (2011) Male infertility-linked point mutation disrupts the Ca²⁺ oscillation-inducing and PIP(2) hydrolysis activity of sperm PLCzeta. *Biochem J.* **434**, 211-217
- 49 Escoffier, J., Lee, H. C., Yassine, S., Zouari, R., Martinez, G., Karaouzene, T., Coutton, C., Kherraf, Z. E., Halouani, L., Triki, C., Nef, S., Thierry-Mieg, N., Savinov, S. N., Fissore, R., Ray, P. F. and Arnoult, C. (2016) Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. *Hum Mol Genet.* **25**, 878-891
- 50 Nomikos, M. (2015) Novel signalling mechanism and clinical applications of sperm-specific PLCzeta. *Biochem Soc Trans.* **43**, 371-376
- 51 Parrington, J., Swann, K., Shevchenko, V. I., Sesay, A. K. and Lai, F. A. (1996) Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature.* **379**, 364-368
- 52 Sette, C., Bevilacqua, A., Bianchini, A., Mangia, F., Geremia, R. and Rossi, P. (1997) Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. *Development.* **124**, 2267-2274
- 53 Wu, A. T., Sutovsky, P., Manandhar, G., Xu, W., Katayama, M., Day, B. N., Park, K. W., Yi, Y. J., Xi, Y. W., Prather, R. S. and Oko, R. (2007) PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem.* **282**, 12164-12175
- 54 Aarabi, M., Balakier, H., Bashar, S., Moskovtsev, S. I., Sutovsky, P., Librach, C. L. and Oko, R. (2014) Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *Faseb J.* **28**, 4434-4440

- 55 Wolosker, H., Kline, D., Bian, Y., Blackshaw, S., Cameron, A. M., Fralich, T. J., Schnaar, R. L. and Snyder, S. H. (1998) Molecularly cloned mammalian glucosamine-6-phosphate deaminase localizes to transporting epithelium and lacks oscillin activity. *Faseb J.* **12**, 91-99
- 56 Wolny, Y. M., Fissore, R. A., Wu, H., Reis, M. M., Colombero, L. T., Ergun, B., Rosenwaks, Z. and Palermo, G. D. (1999) Human glucosamine-6-phosphate isomerase, a homologue of hamster oscillin, does not appear to be involved in Ca²⁺ release in mammalian oocytes. *Mol Reprod Dev.* **52**, 277-287
- 57 Nomikos, M., Sanders, J. R., Theodoridou, M., Kashir, J., Matthews, E., Nounesis, G., Lai, F. A. and Swann, K. (2014) Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not cause Ca²⁺ release in mouse oocytes. *Mol Hum Reprod.* **20**, 938-947
- 58 Nomikos, M., Swann, K. and Lai, F. A. (2015) Is PAWP the "real" sperm factor? *Asian J Androl.* **17**, 444-446
- 59 Kashir, J., Nomikos, M., Swann, K. and Lai, F. A. (2015) PLCzeta or PAWP: revisiting the putative mammalian sperm factor that triggers egg activation and embryogenesis. *Mol Hum Reprod.* **21**, 383-388
- 60 Nomikos, M., Sanders, J. R., Kashir, J., Sanusi, R., Buntwal, L., Love, D., Ashley, P., Sanders, D., Knaggs, P., Bunkheila, A., Swann, K. and Lai, F. A. (2015) Functional disparity between human PAWP and PLCzeta in the generation of Ca²⁺ oscillations for oocyte activation. *Mol Hum Reprod.* **21**, 702-710
- 61 Swann, K. and Lai, F. A. (2016) Egg Activation at Fertilization by a Soluble Sperm Protein. *Physiol Rev.* **96**, 127-149
- 62 Suh, P. G., Park, J. I., Manzoli, L., Cocco, L., Peak, J. C., Katan, M., Fukami, K., Kataoka, T., Yun, S. and Ryu, S. H. (2008) Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep.* **41**, 415-434
- 63 Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem.* **70**, 281-312
- 64 Kashir, J., Nomikos, M., Lai, F. A. and Swann, K. (2014) Sperm-induced Ca²⁺ release during egg activation in mammals. *Biochem Biophys Res Commun.* **450**, 1204-1211
- 65 Rebecchi, M. J. and Pentylala, S. N. (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev.* **80**, 1291-1335
- 66 Lomasney, J. W., Cheng, H. F., Wang, L. P., Kuan, Y., Liu, S., Fesik, S. W. and King, K. (1996) Phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology domain of phospholipase C-delta1 enhances enzyme activity. *J Biol Chem.* **271**, 25316-25326
- 67 Nomikos, M., Mulgrew-Nesbitt, A., Pallavi, P., Mihalyne, G., Zaitseva, I., Swann, K., Lai, F. A., Murray, D. and McLaughlin, S. (2007) Binding of phosphoinositide-specific phospholipase C-zeta (PLC-zeta) to phospholipid membranes: potential role of an unstructured cluster of basic residues. *J Biol Chem.* **282**, 16644-16653
- 68 Nomikos, M., Elgmami, K., Theodoridou, M., Calver, B. L., Nounesis, G., Swann, K. and Lai, F. A. (2011) Phospholipase C-zeta binding to PtdIns(4,5)P₂ requires the XY-linker region. *J Cell Sci.* **124**, 2582-2590
- 69 Nomikos, M., Sanders, J. R., Parthimos, D., Buntwal, L., Calver, B. L., Stamatiadis, P., Smith, A., Clue, M., Sideratou, Z., Swann, K. and Lai, F. A. (2015) Essential Role of the EF-hand Domain in Targeting Sperm Phospholipase C-zeta to Membrane Phosphatidylinositol 4,5-Bisphosphate (PIP₂). *J Biol Chem.* **290**, 29519-29530
- 70 Nomikos, M., Blayney, L. M., Larman, M. G., Campbell, K., Rossbach, A., Saunders, C. M., Swann, K. and Lai, F. A. (2005) Role of phospholipase C-zeta domains in Ca²⁺-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca²⁺ oscillations. *J Biol Chem.* **280**, 31011-31018
- 71 Theodoridou, M., Nomikos, M., Parthimos, D., Gonzalez-Garcia, J. R., Elgmami, K., Calver, B. L., Sideratou, Z., Nounesis, G., Swann, K. and Lai, F. A. (2013) Chimeras of sperm PLCzeta reveal

disparate protein domain functions in the generation of intracellular Ca²⁺ oscillations in mammalian eggs at fertilization. *Mol Hum Reprod.* **19**, 852-864

72 Nomikos, M., Theodoridou, M., Elgmati, K., Parthimos, D., Calver, B. L., Buntwal, L., Nounesis, G., Swann, K. and Lai, F. A. (2014) Human PLCzeta exhibits superior fertilization potency over mouse PLCzeta in triggering the Ca²⁺ oscillations required for mammalian oocyte activation. *Mol Hum Reprod.* **20**, 489-498

73 Nomikos, M., Thanassoulas, A., Beck, K., Theodoridou, M., Kew, J., Kashir, J., Calver, B. L., Matthews, E., Rizkallah, P., Sideratou, Z., Nounesis, G. and Lai, F. A. (2016) Mutations in PLCdelta1 associated with hereditary leukonychia display divergent PIP₂ hydrolytic function. *Febs J.* **283**, 4502-4514

74 Nomikos, M., Elgmati, K., Theodoridou, M., Georgilis, A., Gonzalez-Garcia, J. R., Nounesis, G., Swann, K. and Lai, F. A. (2011) Novel regulation of PLCzeta activity via its XY-linker. *Biochem J.* **438**, 427-432

75 Larman, M. G., Saunders, C. M., Carroll, J., Lai, F. A. and Swann, K. (2004) Cell cycle-dependent Ca²⁺ oscillations in mouse embryos are regulated by nuclear targeting of PLCzeta. *J Cell Sci.* **117**, 2513-2521

76 Nalefski, E. A. and Falke, J. J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci.* **5**, 2375-2390

77 Lomasney, J. W., Cheng, H. F., Roffler, S. R. and King, K. (1999) Activation of phospholipase C delta1 through C2 domain by a Ca²⁺-enzyme-phosphatidylserine ternary complex. *J Biol Chem.* **274**, 21995-22001

78 Kouchi, Z., Shikano, T., Nakamura, Y., Shirakawa, H., Fukami, K. and Miyazaki, S. (2005) The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Czeta. *J Biol Chem.* **280**, 21015-21021

79 Nomikos, M., Stamatiadis, P., Sanders, J. R., Beck, K., Calver, B. L., Buntwal, L., Lofty, M., Sideratou, Z., Swann, K. and Lai, F. A. (2017) Male infertility-linked point mutation reveals a vital binding role for the C2 domain of sperm PLCzeta. *Biochem J.* **474**, 1003-1016

80 Fujimoto, S., Yoshida, N., Fukui, T., Amanai, M., Isobe, T., Itagaki, C., Izumi, T. and Perry, A. C. (2004) Mammalian phospholipase Czeta induces oocyte activation from the sperm perinuclear matrix. *Dev Biol.* **274**, 370-383

81 Nakai, M., Ito, J., Sato, K., Noguchi, J., Kaneko, H., Kashiwazaki, N. and Kikuchi, K. (2011) Pre-treatment of sperm reduces success of ICSI in the pig. *Reproduction.* **142**, 285-293

82 Kaewmala, K., Uddin, M. J., Cinar, M. U., Grosse-Brinkhaus, C., Jonas, E., Tesfaye, D., Phatsara, C., Tholen, E., Looft, C. and Schellander, K. (2012) Investigation into association and expression of PLCz and COX-2 as candidate genes for boar sperm quality and fertility. *Reprod Domest Anim.* **47**, 213-223

83 Heytens, E., Soleimani, R., Lierman, S., De Meester, S., Gerris, J., Dhont, M., Van der Elst, J. and De Sutter, P. (2008) Effect of ionomycin on oocyte activation and embryo development in mouse. *Reprod Biomed Online.* **17**, 764-771

84 Kashir, J., Jones, C., Lee, H. C., Rietdorf, K., Nikiforaki, D., Durrans, C., Ruas, M., Tee, S. T., Heindryckx, B., Galione, A., De Sutter, P., Fissore, R. A., Parrington, J. and Coward, K. (2011) Loss of activity mutations in phospholipase C zeta (PLCzeta) abolishes calcium oscillatory ability of human recombinant protein in mouse oocytes. *Hum Reprod.* **26**, 3372-3387

85 Kashir, J., Heynen, A., Jones, C., Durrans, C., Craig, J., Gadea, J., Turner, K., Parrington, J. and Coward, K. (2011) Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa. *Reprod Biomed Online.* **23**, 263-267

86 Kashir, J., Jones, C., Mounce, G., Ramadan, W. M., Lemmon, B., Heindryckx, B., de Sutter, P., Parrington, J., Turner, K., Child, T., McVeigh, E. and Coward, K. (2013) Variance in total levels of phospholipase C zeta (PLC-zeta) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertil Steril.* **99**, 107-117

- 87 Aarabi, M., Yu, Y., Xu, W., Tse, M. Y., Pang, S. C., Yi, Y. J., Sutovsky, P. and Oko, R. (2012) The testicular and epididymal expression profile of PLCzeta in mouse and human does not support its role as a sperm-borne oocyte activating factor. *PLoS One*. **7**, e33496
- 88 Young, C., Grasa, P., Coward, K., Davis, L. C. and Parrington, J. (2009) Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. *Fertil Steril*. **91**, 2230-2242
- 89 Grasa, P., Coward, K., Young, C. and Parrington, J. (2008) The pattern of localization of the putative oocyte activation factor, phospholipase C zeta, in uncapacitated, capacitated, and ionophore-treated human spermatozoa. *Hum Reprod*. **23**, 2513-2522
- 90 Nomikos, M., Yu, Y., Elgmati, K., Theodoridou, M., Campbell, K., Vassilakopoulou, V., Zikos, C., Livaniou, E., Amso, N., Nounesis, G., Swann, K. and Lai, F. A. (2013) Phospholipase C zeta rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril*. **99**, 76-85
- 91 Phillips, S. V., Yu, Y., Rossbach, A., Nomikos, M., Vassilakopoulou, V., Livaniou, E., Cumbes, B., Lai, F. A., George, C. H. and Swann, K. (2011) Divergent effect of mammalian PLCzeta in generating Ca²⁺(+) oscillations in somatic cells compared with eggs. *Biochem J*. **438**, 545-553
- 92 Kashir, J., Buntwal, L., Nomikos, M., Calver, B. L., Stamatiadis, P., Ashley, P., Vassilakopoulou, V., Sanders, D., Knaggs, P., Livaniou, E., Bunkheila, A., Swann, K. and Lai, F. A. (2017) Antigen unmasking enhances visualization efficacy of the oocyte activation factor, phospholipase C zeta, in mammalian sperm. *Mol Hum Reprod*. **23**, 54-67
- 93 Yu, Y., Nomikos, M., Theodoridou, M., Nounesis, G., Lai, F. A. and Swann, K. (2012) PLCzeta causes Ca²⁺ oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P₂. *Mol Biol Cell*. **23**, 371-380
- 94 Esfandiari, N., Javed, M. H., Gotlieb, L. and Casper, R. F. (2005) Complete failed fertilization after intracytoplasmic sperm injection--analysis of 10 years' data. *Int J Fertil Womens Med*. **50**, 187-192
- 95 Flaherty, S. P., Payne, D. and Matthews, C. D. (1998) Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod*. **13 Suppl 1**, 155-164
- 96 Dam, A. H., Feenstra, I., Westphal, J. R., Ramos, L., van Golde, R. J. and Kremer, J. A. (2007) Globozoospermia revisited. *Hum Reprod Update*. **13**, 63-75
- 97 Yanagida, K. (2004) Complete fertilization failure in ICSI. *Hum Cell*. **17**, 187-193
- 98 Sousa, M. and Tesarik, J. (1994) Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. *Hum Reprod*. **9**, 2374-2380
- 99 Liu, J., Nagy, Z., Joris, H., Tournaye, H., Devroey, P. and Van Steirteghem, A. (1995) Successful fertilization and establishment of pregnancies after intracytoplasmic sperm injection in patients with globozoospermia. *Hum Reprod*. **10**, 626-629
- 100 Rawe, V. Y., Olmedo, S. B., Nodar, F. N., Doncel, G. D., Acosta, A. A. and Vitullo, A. D. (2000) Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Mol Hum Reprod*. **6**, 510-516
- 101 Heindryckx, B., De Gheselle, S., Gerris, J., Dhont, M. and De Sutter, P. (2008) Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reprod Biomed Online*. **17**, 662-668
- 102 Kashir, J., Deguchi, R., Jones, C., Coward, K. and Stricker, S. A. (2013) Comparative biology of sperm factors and fertilization-induced calcium signals across the animal kingdom. *Mol Reprod Dev*. **80**, 787-815
- 103 Yelumalai, S., Yeste, M., Jones, C., Amdani, S. N., Kashir, J., Mounce, G., Da Silva, S. J., Barratt, C. L., McVeigh, E. and Coward, K. (2015) Total levels, localization patterns, and proportions of sperm exhibiting phospholipase C zeta are significantly correlated with fertilization rates after intracytoplasmic sperm injection. *Fertil Steril*. **104**, 561-568 e564
- 104 Escoffier, J., Yassine, S., Lee, H. C., Martinez, G., Delaroche, J., Coutton, C., Karaouzene, T., Zouari, R., Metzler-Guillemain, C., Pernet-Gallay, K., Hennebicq, S., Ray, P. F., Fissore, R. and Arnoult, C. (2015) Subcellular localization of phospholipase C zeta in human sperm and its absence in

- DPY19L2-deficient sperm are consistent with its role in oocyte activation. *Mol Hum Reprod.* **21**, 157-168
- 105 Park, J. H., Kim, S. K., Kim, J., Kim, J. H., Chang, J. H., Jee, B. C. and Kim, S. H. (2015) Relationship between phospholipase C zeta immunoreactivity and DNA fragmentation and oxidation in human sperm. *Obstet Gynecol Sci.* **58**, 232-238
- 106 Nikiforaki, D., Vanden Meerschaut, F., De Gheselle, S., Qian, C., Van den Abbeel, E., De Vos, W. H., Deroo, T., De Sutter, P. and Heindryckx, B. (2014) Sperm involved in recurrent partial hydatidiform moles cannot induce the normal pattern of calcium oscillations. *Fertil Steril.* **102**, 581-588 e581
- 107 Kashir, J., Konstantinidis, M., Jones, C., Heindryckx, B., De Sutter, P., Parrington, J., Wells, D. and Coward, K. (2012) Characterization of two heterozygous mutations of the oocyte activation factor phospholipase C zeta (PLCzeta) from an infertile man by use of minisequencing of individual sperm and expression in somatic cells. *Fertil Steril.* **98**, 423-431
- 108 Ferrer-Vaquero, A., Barragan, M., Freour, T., Vernaeve, V. and Vassena, R. (2016) PLCzeta sequence, protein levels, and distribution in human sperm do not correlate with semen characteristics and fertilization rates after ICSI. *J Assist Reprod Genet.* **33**, 747-756
- 109 Pan, Q., Ju, Z., Huang, J., Zhang, Y., Qi, C., Gao, Q., Zhou, L., Li, Q., Wang, L., Zhong, J., Liu, M. and Wang, C. (2013) PLCz functional haplotypes modulating promoter transcriptional activity are associated with semen quality traits in Chinese Holstein bulls. *PLoS One.* **8**, e58795
- 110 Schrimpf, R., Dierks, C., Martinsson, G., Sieme, H. and Distl, O. (2014) Genome-wide association study identifies phospholipase C zeta 1 (PLCz1) as a stallion fertility locus in Hanoverian warmblood horses. *PLoS One.* **9**, e109675
- 111 Yamaguchi, T., Ito, M., Kuroda, K., Takeda, S. and Tanaka, A. (2017) The establishment of appropriate methods for egg-activation by human PLCZ1 RNA injection into human oocyte. *Cell Calcium.* **65**, 22-30
- 112 Kashir, J., Jones, C. and Coward, K. (2012) Calcium oscillations, oocyte activation, and phospholipase C zeta. *Adv Exp Med Biol.* **740**, 1095-1121
- 113 Ross, P. J., Beyhan, Z., Lager, A. E., Yoon, S. Y., Malcuit, C., Schellander, K., Fissore, R. A. and Cibelli, J. B. (2008) Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. *BMC Dev Biol.* **8**, 16
- 114 Dupont, G. and Dumollard, R. (2004) Simulation of calcium waves in ascidian eggs: insights into the origin of the pacemaker sites and the possible nature of the sperm factor. *J Cell Sci.* **117**, 4313-4323
- 115 Rogers, N. T., Hobson, E., Pickering, S., Lai, F. A., Braude, P. and Swann, K. (2004) Phospholipase C zeta causes Ca²⁺ oscillations and parthenogenetic activation of human oocytes. *Reproduction.* **128**, 697-702

TABLE LEGENDS

Table 1

***In vitro* enzymatic properties of NusA-tagged Δ PH/PLC δ /EF ζ chimaera.** Summary of specific enzyme activity and EC₅₀ values of Ca²⁺-dependence for PIP₂ hydrolysis, determined by non-linear regression analysis (GraphPad Prism 5), (see Fig. 3) for PLC ζ , PLC δ 1 and Δ PH/PLC δ 1/EF ζ fusion proteins.

FIGURE LEGENDS

Figure 1

Schematic representation of egg activation triggered by sperm-specific PLC ζ . Following sperm-egg membrane fusion, PLC ζ is released from the sperm into the egg cytosol and targets a distinct intracellular vesicular membrane containing its membrane-bound substrate, phosphoinositide 4,5-bisphosphate (PIP₂). PLC ζ -mediated PIP₂ hydrolysis produces two second messengers, inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG). InsP₃ subsequently binds to the InsP₃ receptor (InsP₃R) on the endoplasmic reticulum, an interaction that triggers Ca²⁺ release from intracellular stores, which subsequently produces the characteristic pattern of Ca²⁺ oscillations that initiate egg activation (Figure modified from [1, 59]).

Figure 2

Expression of recombinant NusA-tagged Δ PH/PLC δ 1/EF ζ chimaeric protein. (A) Schematic representation of the domain structure of PLC ζ (brown), PLC δ 1 (purple) and their corresponding Δ PH/PLC δ 1/EF ζ chimaera. The various amino acid lengths and respective coordinates are indicated for each construct. (B) Expression of recombinant NusA-tagged Δ PH/PLC δ 1/EF ζ chimaeric protein. 1 μ g of recombinant NusA-tagged Δ PH/PLC δ 1/EF ζ protein analysed by 7% SDS-PAGE and Coomassie Brilliant Blue staining (left panel) and immunoblot analysis using the anti-NusA antibody (1:25,000 dilution; right panel).

Figure 3

***In vitro* enzyme specific activity and Ca²⁺ sensitivity of Δ PH/PLC δ 1/EF ζ chimaera.** (A) PIP₂ hydrolysis enzyme activity of PLC ζ , PLC δ 1 and Δ PH/PLC δ 1/EF ζ proteins obtained with the standard [³H]PIP₂ hydrolysis assay [70-74]. Values are means \pm S.E.M. (n=4), using

two different preparations of recombinant protein and each experiment was performed in duplicate. (B) Effect of various $[Ca^{2+}]$ on the normalized activity of PLC ζ , PLC δ 1 and Δ PH/PLC δ 1/EF ζ recombinant proteins. For these assays, values are \pm S.E.M. (n=4), using two different batches of recombinant proteins and with each experiment performed in duplicate (see Table 1).

Figure 4

Schematic illustration of the proposed intracellular mechanism of action of PLC ζ in mammalian eggs. After its delivery from the fertilising sperm, PLC ζ associates with a specific vesicular membrane by a potential interaction of the C2 domain with PI(3)P, PI(5)P or an as yet unidentified (membrane or cytosolic) egg protein. Then, PLC ζ associates with its negatively-charged substrate PIP $_2$ via electrostatic interactions with the positively-charged 1st EF-hand domain and the C-terminal part of the XY-linker region. The catalytic XY domain subsequently proceeds with the enzymatic cleavage of PIP $_2$. The high Ca $^{2+}$ sensitivity of PLC ζ , which enables it to be active at resting nanomolar Ca $^{2+}$ levels, is conferred by its EF hand domains. It has been reported that the XY-linker of mouse PLC ζ contains a nuclear localisation signal (NLS), which targets the enzyme to pronuclei in a cell-cycle dependent manner (Figure modified from [1, 69, 79]).

TABLES

Table 1

Recombinant PLC protein	PIP ₂ hydrolysis enzyme activity (nmol/min/mg)	Ca ²⁺ dependence EC ₅₀ (nM)
PLC ζ	532 \pm 24	73
PLC δ 1	2934 \pm 36	5960
Δ PH/PLC δ 1/EF ζ	2015 \pm 51	4710

FIGURES

Figure 1

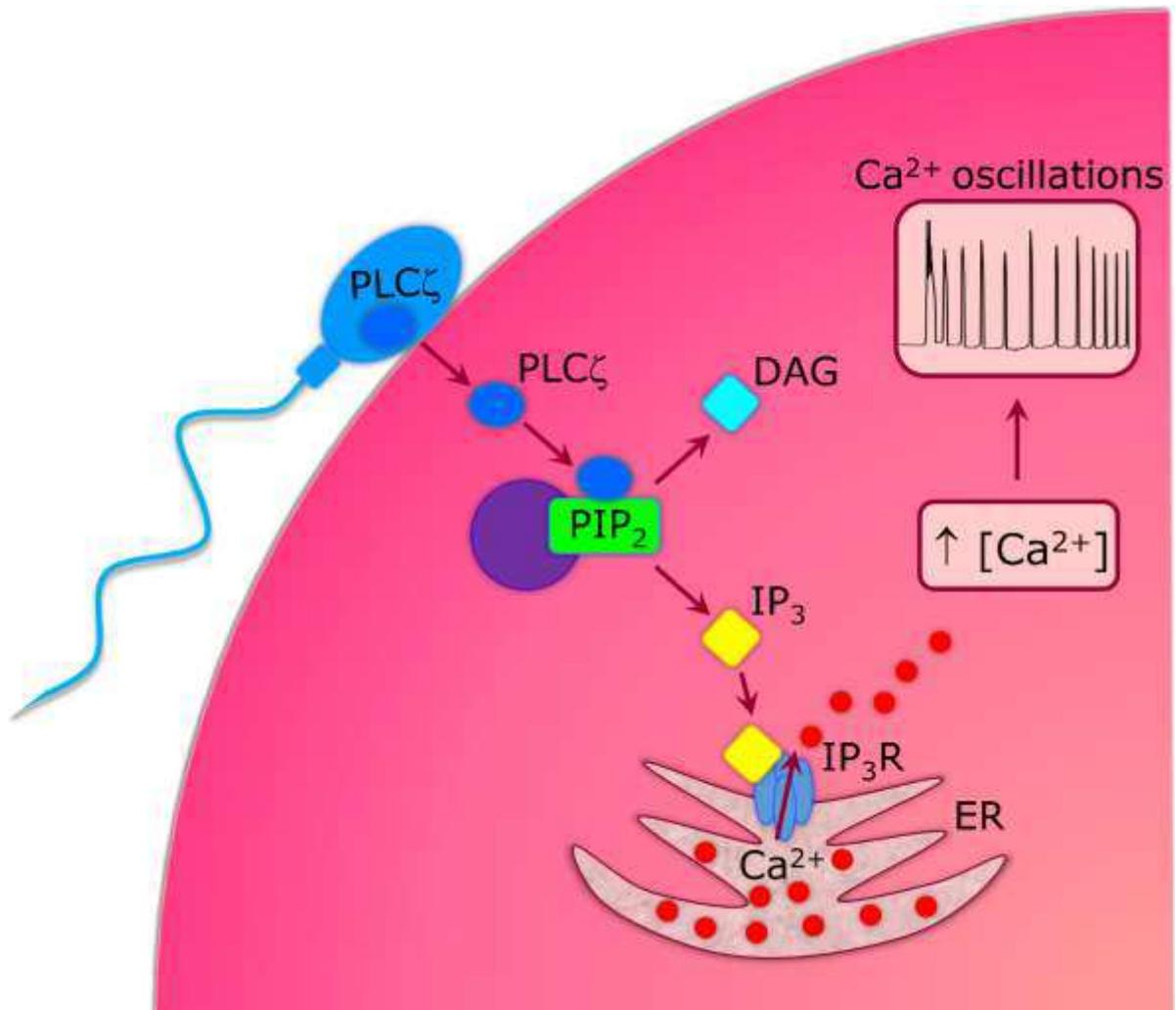
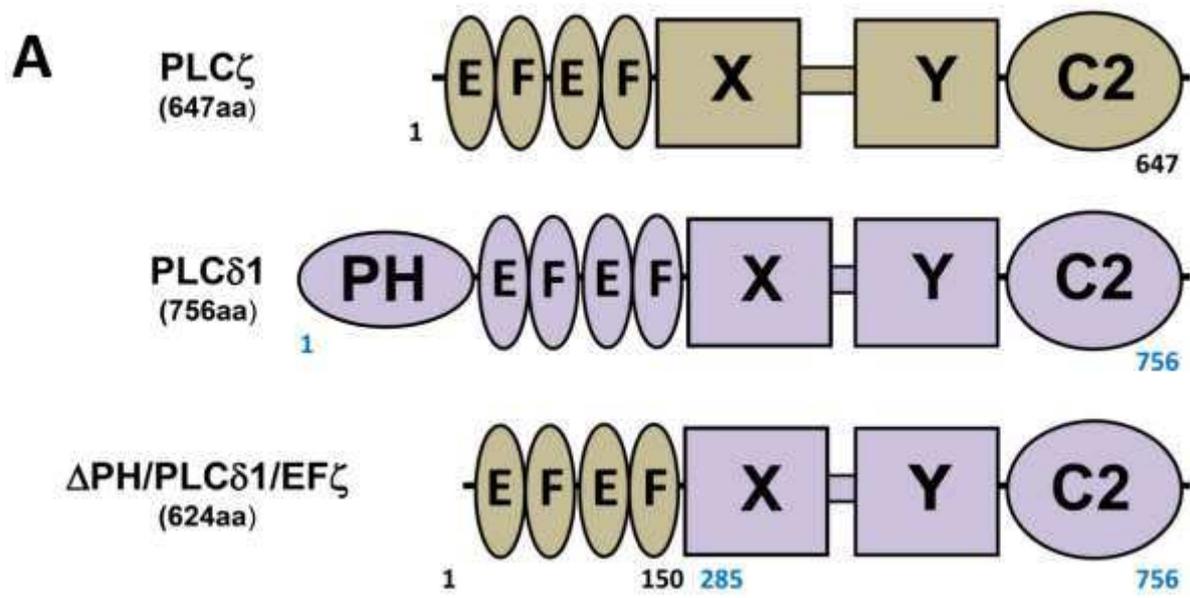


Figure 2



B

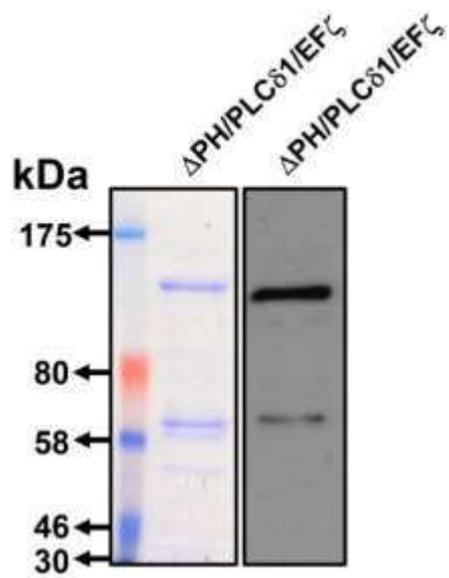


Figure 3

