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Review

Sperm PLCζ: From structure to Ca^{2+} oscillations, egg activation and therapeutic potential

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ABSTRACT

Significant evidence now supports the assertion that cytosolic calcium oscillations during fertilization in mammalian eggs are mediated by a testis-specific phospholipase C (PLC), termed PLC-zeta (PLCζ) that is released into the egg following gamete fusion. Herein, we describe the current paradigm of PLCζ in this fundamental biological process, summarizing recent important advances in our knowledge of the biochemical and physiological properties of this enzyme. We describe the data suggesting that PLCζ has distinct features amongst PLCs enabling the hydrolysis of its substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2) at low Ca^{2+} levels. PLCζ appears to be unique in its ability to target PIP_2 that is present on intracellular vesicles. We also discuss evidence that PLCζ may be a significant factor in human fertility with potential therapeutic capacity.

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1. Introduction

Before fertilization, mature oocytes (henceforth referred to as eggs) of many vertebrates remain arrested at the metaphase stage of the second meiotic division (MII). Release of this arrest is vital for embryogenesis to proceed and occurs through a series of events, collectively termed '*egg activation*', acting in concert to permit formation of a totipotent zygote [1,2]. Characterized by the formation of the second polar body (2PB) and the male and female pronuclei, egg activation in vertebrates additionally involves cortical granule exocytosis, progression of the cell cycle, and maternal mRNA recruitment [1,3–5]. A number of recent reviews have summarized this fundamental biological process. Herein, we summarize recent important advances in our understanding of the current biochemical paradigms of egg activation and the sperm factor, and how these may relate to clinical application.

It is now well established in all mammalian species that oocyte/egg activation involves marked increases in the concentration of egg cytosolic calcium (Ca^{2+}), which are both necessary and sufficient for activation [1,6,7]. The importance of this Ca^{2+} signaling phenomenon extends beyond mammals since egg activation is accompanied by an increase in the level of intracellular [Ca^{2+}] in many non-mammalian species such as sea urchins and frogs. In these species the Ca^{2+} increase is a single rise, in contrast with other species such as mammals and ascidians where the Ca^{2+} signal is delivered as a train of long-lasting repetitive Ca^{2+} transients, known as Ca^{2+} oscillations, which occur after sperm–egg fusion [1,6,8,9]. The frequency and duration of Ca^{2+} oscillations varies between species and can last from a Ca^{2+} increase every 2 min, to a Ca^{2+} increase every 1 h [1,9].

Four predominant hypotheses have been proposed to explain the nature of the Ca^{2+} transients in mammalian eggs: (1) the 'Ca^{2+} bomb' hypothesis [10], (2) the 'conduit' hypothesis [11], (3) the 'contact' hypothesis [12,13], and (4) the 'sperm factor' hypothesis [8] (for more details on the proposed hypotheses explaining Ca^{2+} oscillations at fertilization, see review [1]). Numerous experimental studies now provide convincing evidence that the 'sperm factor' hypothesis of egg activation is the most appropriate model for mammals and a number of invertebrate species [8,14–17]. The 'sperm factor' hypothesis proposes that upon sperm–egg fusion, a soluble factor is delivered from sperm cytosol into the ooplasm, capable of activating the 1,4,5-trisphosphate (IP_3) signaling pathway and the subsequent Ca^{2+} oscillations in fertilized eggs [8]. Ca^{2+} oscillations in mammalian eggs are generally acknowledged to occur as a result of IP_3-mediated Ca^{2+} release from internal stores such as the endoplasmic reticulum (ER). Blocking, downregulating, or reducing levels of IP_3 receptors (IP_3Rs) in mouse and hamster eggs inhibited Ca^{2+} oscillations, preventing egg activation [18–21]. Furthermore, microinjection of IP_3 or adenophostin (an IP_3 analogue) can also lead to Ca^{2+} oscillations in mouse eggs [22–24], demonstrating the importance of this signaling pathway.

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2. From the sperm factor hypothesis to a novel sperm PLC

The above cited evidence suggest that Ca^{2+} oscillations at mammalian fertilization arise as a result of the stimulation of the phosphoinositide signalling pathway, where intracellular IP_3 is generated along with diacylglycerol (DAG) by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). DAG can stimulate the activation of protein kinase C (PKC) and IP_3 binds to the IP_3Rs on the ER, resulting in a conformational change and opening of the intrinsic IP_3R channel to allow Ca^{2+} release [25].

A number of candidates have been proposed as the mammalian sperm factor, with early candidates suggested to be small molecules such as nitric oxide (NO) [26], or nicotinic acid adenine dinucleotide phosphate (NAADP) [27]. While such molecules generate Ca^{2+} release from intracellular stores in non-mammalian species, they do not cause Ca^{2+} release in mammalian eggs [1]. Another suggestion was that IP_3 itself was the sperm factor [28], but injection of IP_3 into mouse eggs leads to a damped series of Ca^{2+} oscillations that do not resemble those seen at fertilization [4]. Additionally, fractionation studies suggested that the sperm factor was a protein [14,29,30] ∼30–100 kDa in size [1,31,32]. Various proteins have also been proposed to be the sperm factor, including a 33 kDa protein [33], a truncated form of the kit receptor, tr-kit [34] and a post-acrosomal sheath WW domain-binding protein, termed PAWP [35]. However, like many of the small molecules proposed, none of these candidate proteins have been demonstrated to elicit repetitive Ca^{2+} release in mammalian eggs [1,36]. Furthermore, the underlying mechanisms of action of such proteins remain unclear, resulting in significant doubt over the identity of such proteins as the mammalian sperm factor [2,5].

In the end, clues that led to the identification of the sperm factor came from studies using sea urchin egg homogenates that can take up Ca^{2+} and then release Ca^{2+} in response to a range of agents including IP_3, cADPR and NAADP [37]. It was shown that the same sperm extracts that caused Ca^{2+} oscillations in mouse eggs could also generate Ca^{2+} release in the sea urchin egg homogenate [38]. The mechanism of Ca^{2+} release was shown to involve IP_3 production, and not cADPR or NAADP [24]. Further, the sperm extracts themselves were shown to contain a highly active phosphoinositide-specific phospholipase C (PLC) activity. This suggested that the sperm factor might itself be a PLC enzyme. However, the specific PLC isoform key to eliciting the repeated Ca^{2+} release at egg activation eluded investigators for some time.

An extremely promising PLC candidate was revealed following the investigation of mouse expressed sequence tag (EST) databases, which identified a set of novel PLC sequences, all derived specifically from the testis. This led to the identification of a novel, and testis-specific PLC in mouse sperm, termed PLC-zeta (PLCζ), a ∼74 kDa protein which was subsequently proven to play a fundamental role in egg activation [39]. Our laboratory also first reported the identification of human PLCζ, a 70 kDa protein [40].

Numerous studies now support the view that PLCζ is the mammalian sperm factor. The most significant evidence is that microinjection of in vitro-transcribed PLCζ RNA and PLCζ protein into mouse eggs resulted in the initiation of Ca^{2+} oscillations with the characteristic pattern of those specifically observed at fertilization. Remarkably, these injections of PLCζ in vitro transcripts also supported mouse early embryonic development up to the blastocyst stage [39,41]. Immunodepleting PLCζ from sperm extracts suppressed their ability to release Ca^{2+} [39], while protein fractionation studies correlated the presence of PLCζ in sperm extracts to their ability to induce Ca^{2+} oscillations [42,43]. Notably, sperm extracts and PLCζ transcripts from one vertebrate species (e.g. human) are able to elicit Ca^{2+} release upon microinjection into eggs from another vertebrate species (e.g. mouse) [40,44]. Furthermore, transgenic mice exhibiting disrupted PLCζ expression in the testis through RNA interference (RNAi) experiments, yielded sperm which induced prematurely ending Ca^{2+} oscillations, and while not infertile, exhibited a dramatically reduced litter size [45].

The most significant data, however, is the mounting clinical evidence that indicates the involvement of abnormal forms or aberrant function of PLCζ in cases of male factor infertility and egg activation failure in humans. In many fertility clinics, sperm is now injected directly into the eggs in a procedure known as intra-cytoplasmic sperm injection (ICSI). Sperm of infertile men that fail to activate eggs after ICSI produced either no Ca^{2+} oscillations upon injection into mouse eggs, or else produced significantly diminished Ca^{2+} oscillation profiles. [46,47]. Moreover, immunofluorescence and immunoblot analysis revealed that infertile patients whose sperm had failed in ICSI, exhibited reduced or absent levels of PLCζ within the sperm head [46,47]. The data suggest that a relative lack of PLCζ may explain some cases of male factor infertility, and they also support the strong connection between PLCζ and the ability of a sperm to generate Ca^{2+} oscillations.

Importantly, two recent studies have provided the first genetic links between PLCζ defects and infertility by identifying two heterozygous substitution mutations in the protein coding sequence of PLCζ in an infertile male [47,48]. The first mutation occurring at position 398 within the Y domain results in histidine being changed to proline (H398P) [47]. The second in the X domain at position 233 changes a histidine to leucine (H233L) [48]. Characterization studies of these mutations in mouse and human PLCζ revealed that both mutations disrupt local folding in the PLCζ active site. This results in a dramatic reduction of PLCζ in vitro enzymatic and in vivo Ca^{2+}oscillation-inducing activity suggesting that these mutations may underlie the patient's infertility [47,49,50]. Further analysis of these mutants suggested that similar loss-of-activity mutations in PLCζ may contribute not only towards male infertility, but also male sub-fertility [47,48,51].

Further biochemical studies have now also identified mammalian PLCζ orthologues in hamster, porcine, monkey, and horse sperm [40,52–54]), while non-mammalian testis-specific PLCζ homologues have been identified in the chicken [55] and fish (testis-specific in Medaka, but ovarian and brain expression in pufferfish) [56,57]. A further interesting point is that mutations in the PLCζ promoters have also been linked to semen quality (and thus fertility) in Chinese Holstein Bulls, with specific haplotypes of PLCζ promoter sequences linked to varying degrees of semen quality [58]. This suggests that the sperm PLCζ may constitute a universal biochemical mechanism of egg activation at fertilization within vertebrates, and that dysfunction may contribute to varying degrees of infertility [2,5]. All this mounting evidence highlights the central importance of sperm-specific PLCζ in mammalian fertilization and suggests that it is a critical factor in reproductive biology.

A recent outlier study has suggested that PLCζ may not be the egg-activating sperm factor due to apparent discrepancies in the immunocytochemical localisation pattern observed in mouse, bull, and human sperm [59]. This report by Aarabi et al. was primarily based upon using one antibody of limited specificity (see Fig. 1A of reference [59]). Their experiments suggested that PLCζ is secreted by epididymal cells and is not detectable in sperm after the acrosome reaction [59]. However, there are several other studies, using a variety of different antibodies, that show corroborative evidence for PLCζ localization in the perinuclear or postacrosomal regions inside the head of mouse [42,60] human [50], or bull sperm [60]. Moreover, analysis of protein extracts derived from the perinuclear theca of mouse or pig sperm were shown to contain PLCζ [42,43]. The perinuclear theca is the first region of sperm to

Fig. 1. Schematic linear representation of the domain structure of PLCζ. PLCζ exhibits a typical mammalian PLC domain structure consisting of four tandem EF hand domains, the characteristic X and Y catalytic domains which are separated by an unstructured linker region, and a single C-terminal C2 domain, all of which are common to the other PLC isoforms (β, γ, δ, ε and η).

disperse into the egg cytoplasm after gamete fusion. Hence this collective immunolocalization evidence from disparate laboratories strongly suggests that PLCζ is indeed present in a region of sperm head which is consistent with its proposed role in egg activation during fertilization and after ICSI.

3. Structure and function of PLCζ

Currently, there are 13 known mammalian PLC isozymes, categorized according to structure and regulatory activation mechanisms: PLC-beta (PLCβ 1–4), PLC-gamma (PLCγ 1 and 2), PLC-delta (PLCδ 1, 3, and 4), PLC-epsilon (PLCε), PLC-zeta (PLCζ), and PLC-eta (PLCη 1 and 2) [25,61,62]. These isozymes contain the catalytic X and Y domains as well as various regulatory domains, including a pleckstrin homology (PH) domain, EF hand motifs, and C2 domain in various conformations, depending on the isozyme, with each domain performing specific functions. Some isozymes also consist of subtype-specific domains, thought to contribute to towards specific regulatory mechanisms, including the Src homology (SH) domain in PLCγ [61] and the Ras-associating and Ras-GTPase exchange factor-like domains in PLCε [63,64].

PLCζ demonstrates a typical PLC domain structure [39] with characteristic X and Y catalytic domains which form the active site in all PLC isoforms [25,61,62] a single C2 domain and four tandem EF hand domains (Fig. 1). While PLCζ exhibits 33% homology with PLCδ [39], a major difference to other PLCs is the absence of pleckstrin homology (PH) and Src homology (SH) domains, making PLCζ the smallest known mammalian PLC with a molecular mass of ~70 kDa in humans and ~74 kDa in mice [39,40].

Importantly, PLCζ has been demonstrated to be extremely effective at triggering Ca^{2+} release within eggs, despite its small size and lack of a PH domain. Indeed, microinjection of PLCζ results in an extremely rapid down-regulation of IP_3Rs in mouse eggs [65]. In fact, microinjection of either c-myc-tagged or luciferase-tagged PLCζ fusion proteins indicated that PLCζ is effective at around 40 fg per egg [39,66]. In contrast, PLCδ1, the closest homologue to PLCζ, induces Ca^{2+} oscillations only at concentrations exceeding 1 pg per egg [41]. Furthermore, injection of 1–5 pg of PLCβ1 or PLCγ failed to elicit Ca^{2+} oscillations in mouse eggs [67]. Collectively, such data suggest that PLCζ is far more potent than other PLCs in activating IP_3 production and eliciting Ca^{2+} oscillations in mouse eggs.

PLCζ identified from all species to date are similar in size, ranging between 70–75 kDa. However, all PLCζ isoforms seem to display a significant degree of variance in their calculated isoelectric points (p*I*), ranging from 5.29 in rat PLCζ, to 9.14 in human PLCζ [1,9], perhaps indicating a wide solubility range of the enzyme across species. Furthermore, the biochemical factors underlying the high levels of PLCζ activity comparative to other PLC isoforms currently remains unanswered [36]. However, each individual domain of PLCζ exhibits an essential role.

3.1. EF hand domains contribute to the high Ca^{2+}-sensitivity of PLCζ

PLCζ contains two pairs of EF hand domains at the N-terminal end of the protein, each of which consists of four helix–loop–helix motifs divided in two pair-wise lobes. In PLCδ1, the EF hands form a flexible link between the catalytic and PH domains, and possess residues capable of binding Ca^{2+} [68]. Compared to PLCδ1, PLCζ exhibits a 100-fold higher Ca^{2+}-sensitivity. At the resting cytosolic Ca^{2+} levels in oocytes, PLCζ is predicted to be at least half-maximally active and any increase in basal Ca^{2+} will lead to a significant rise in activity. This suggests there may be a positive feedback loop of Ca^{2+} and IP_3 increases that could underlie the oscillation mechanism. Deletion of one or both EF hand domains of PLCζ led to a complete loss of its oscillatory ability in mouse eggs [69,70]. Intriguingly, these PLCζ deletion constructs retained their ability to hydrolyze PIP_2 in vitro. However, even the deletion of the first EF hand domain raised the EC_{50} of PLCζ for Ca^{2+} by ~ninefold [70]. Deletion of both EF hands dramatically altered the EC_{50} of PLCζ for Ca^{2+} from ~80 nM to ~30 μM [70]. This suggests that truncation of EF hands ablates the enzyme's ability to generate IP_3 in an intact egg cell since it has a probable basal Ca^{2+} concentration of ~100 nM [1,70].

Notably, an additional role for the EF hand domains of PLCζ has been proposed based on the findings that point mutations within the EF hands disrupt the nuclear translocation of mouse PLCζ [71]. However, this contrasts with studies reporting that the XY-linker region in mouse PLCζ comprises a nuclear localization signal (discussed in 'XY-linker region' section below). Regardless, the significantly increased Ca^{2+} sensitivity conferred by the EF hands makes PLCζ one of the most Ca^{2+}-sensitive PLC isoforms in mammals, enabling PLCζ to elicit Ca^{2+} release at relatively low Ca^{2+} levels within eggs.

3.2. XY-linker region: critical role in the regulation of enzymatic activity and substrate targeting of PLCζ

PLCζ possesses characteristic X and Y catalytic domains which form the active site common to all PLCs [1,9]. Indeed, all PLCζ active site residues are conserved, or conservatively replaced, mutagenesis of which leads to the loss of Ca^{2+} induction ability, confirming that as with PLCδ1, the active site of PLCζ is responsible for hydrolyzing PIP_2 to cause IP_3-mediated Ca^{2+} release [9]. The other region of PLCζ that plays an important role in regulating its enzymatic activity and its substrate targeting is the segment that joins the X and Y catalytic domains, termed the XY-linker [1,72–74]. Found in all PLCs, the XY-linker region remains the only part of PLC whose structure as of yet remains unresolved, with its specific role in PLC enzymatic activity unclear. Notably the XY-linker region of PLCζ is extended in length and consisting of more basic residues relative to its PLCδ1 counterpart [39,40]. Structural and biochemical evidence suggests that the XY-linker region of PLCβ, γ, δ, and η, mediates potent auto-inhibition of their enzymatic activity [75,76]. Such data are consistent with the negatively-charged XY-linker region of these PLC isoforms, which may confer electrostatic repulsion alongside steric hindrance to occlude PIP_2 from the active site [75,76].

In contrast with somatic PLCs, recent evidence suggests that PLCζ operates via a novel enzymatic mechanism, because deletion of the XY-linker of PLCζ significantly diminishes PIP_2 hydrolysis in vitro, and Ca^{2+} oscillation-inducing ability in vivo [73]. It has been proposed that the PLCζ XY-linker, a region which is notably

different from the corresponding region of other PLCs, may be involved in the targeting of PLCζ to its membrane-bound PIP_2 via electrostatic interactions, assisting in anchoring PLCζ to membranes, while enhancing local PIP_2 concentrations [72]. Indeed, substitution mutation of three lysines to alanines (K374A, K375A and K377A) within the XY-linker region of mouse PLCζ provided a successive reduction of net positive charge within the XY-linker region, which in turn significantly reduced both in vivo Ca^{2+} oscillation-inducing activity, and the efficacy of PIP_2 interaction in vitro [74]. Interestingly, porcine PLCζ remains functionally active following cleavage within the XY-linker region, suggesting that an intact polypeptide is not essential for PIP_2 substrate hydrolysis [77]. PLCζ contains in its XY-linker region a predicted nuclear localization signal (NLS) sequence located close to the start of the Y domain, which may play a role in the mode of regulation of at least the mouse PLCζ [56,78–80], (discussed in detail later).

The XY-linker region of PLCζ is the least conserved region between species, being longest in the monkey *Macaca fascicularis*, and shortest in humans [9]. The role played by such diversity is currently unclear, with such variance perhaps accounting for the different rates of enzymatic activity and relative potency between PLCζ species [81]. Indeed, the proximity of this apparently unstructured cluster of residues to the active site indicates potential involvement in regulating catalytic activity, or PIP_2 binding [1,9].

3.3. C2 domain: critical for the Ca^{2+} oscillation-inducing activity of PLCζ

C2 domains in some proteins can bind Ca^{2+} to varying degrees. However, there is no predicted Ca^{2+} binding site in the PLCζ C2 domain [36]. Furthermore, deletion of the PLCζ C2 domain led to some loss of enzymatic activity, but no change in the Ca^{2+} sensitivity of the enzyme in vitro [66]. However, removal of the C2 domain from PLCζ led to inability of PLCζ to elicit Ca^{2+} release in mouse eggs, as indicated by microinjection of PLCζ in vitro transcript lacking the C2 domain [66]. Such data indicate that the C2 domain plays an essential role for in vivo PLCζ activity, and is essential for egg activation. The specific role played by this domain, however, remains unclear.

A recent line of enquiry suggests that the C2 domain may play a role in the localization of PLCζ within the egg, possibly aiding PLCζ sequestration to the membrane containing its substrate PIP_2. Indeed, C2 domains are able to bind to phospholipid-containing membranes, as is the case with PLCδ1 binding to phosphatidylserine (PS) or PLA_2 binding to phosphatidylcholine (PC) [82]. Furthermore, most C2 domains can bind to Ca^{2+}, which in turn exerts a significant effect upon enzymatic activity [83]. However, the C2 domains of some enzymes such as ApIII PKC and PI3K-C2β do not bind Ca^{2+}, leading to phospholipid binding with relatively low affinity and specificity [1,84]. The data indicate that the C2 domain of PLCζ may interact, albeit with low-affinity, with membrane phospholipids. Indeed, such interactions were observed in vitro with phosphatidylinositol-3-phosphate (PI3P) and phosphatidylinositol-5-phosphate (PI5P) [69,74]. It has been suggested that the association of the C2 domain with PI3P may play a role in PLCζ localization, or even perhaps regulation of enzymatic activity, as the presence of PI3P reduced PIP_2 hydrolysis by PLCζ in vitro [69].

4. Localization of PLCζ

4.1. PLCζ localization in the sperm

PLCζ has been identified in numerous mammalian sperm and generally appears localized to distinct regions within the sperm head, with suggestions of differential functional roles for each population [50,85–87]. Indeed, this general localization pattern is consistent with the proposal that PLCζ causes Ca^{2+} release in eggs, as localization to such regions would facilitate rapid diffusion of the enzyme into the ooplasm to initiate Ca^{2+} oscillations either at, or within a few minutes following, gamete fusion [1].

In mouse, hamster, and boar sperm, two PLCζ populations have been identified, acrosomal and post-acrosomal [42,86,88]. In equine sperm, PLCζ was reported to be localized to the acrosome, equatorial segment and head mid-piece, as well as to the principal piece of the flagellum [53]. In porcine sperm, PLCζ was identified in the post-acrosomal region and the tail [89]. Furthermore, three distinct populations of PLCζ have been identified in the human sperm head: acrosomal, equatorial and post-acrosomal [47,50,85,87]. A recent study reported a dynamic shift in PLCζ localization within mouse sperm following capacitation and the acrosome reaction [86], findings that were echoed in capacitated and non-capacitated fertile human sperm [85]. Furthermore, identification of an isoform of PLCζ, termed NYD-SP27, was reported in the acrosome of human and mouse sperm, and suggested that this protein was necessary for capacitation and the acrosome reaction, functioning as an 'intrinsic decapacitation factor' [90]. However, a particular conundrum was presented by a recent study that did not identify a consistent motif of PLCζ localization in sperm from either fertile men or sperm from ICSI-failed men, with significant variance in predominant pattern exhibited [91].

While it is not yet clear whether a particular pattern of PLCζ localization is required, or whether a combination of different populations is required for functional ability, the equatorial and post-acrosomal populations would indeed permit rapid access to the ooplasm following sperm–oocyte fusion. However, further evidence is urgently required to test the veracity and validity of these apparent multiple locations of PLCζ in sperm, and whether discrete populations perform functions other than oocyte activation. It remains to be determined whether different populations identified in these studies are due to species specificity, or rather simply a reflection of antibody specificity and laboratory protocol. Thus, more specific tools would also be required for investigations regarding the precise location and potential role of multiple PLCζ isoforms.

4.2. PLCζ localization in the egg

The subcellular localization of PLCζ has been extensively examined using injection of in vitro transcripts encoding Venus- or YFP-tagged PLCζ fusion proteins, or indirect immunofluorescence. Both datasets indicate that PLCζ does not localize to the plasma membrane, but instead appears to be distributed uniformly within the egg cytoplasm [79,92]. In mouse eggs, this even distribution of PLCζ has been identified as localization to small intracellular vesicles distributed throughout the egg cytoplasm, with similar small vesicles also identified as containing PIP_2 [36,92]. This suggests that the PIP_2 hydrolysed by PLCζ to produce IP_3 may be widely distributed within the egg. Indeed, such assertions gain support from modeling studies showing that in fertilizing ascidian eggs, a fertilization-like profile of Ca^{2+} release is only possible if one assumes the presence of a uniformly-distributed cytoplasmic source of PIP_2, alongside widely-dispersed cytoplasmic PLC activity [36]. However, the precise subcellular targeting mechanisms underlying such postulated vesicular/organelle localization of PLCζ is currently unknown (Fig. 2).

5. Mechanism of PLCζ regulation in mammalian eggs

The exact molecular mechanism of PLCζ regulation in mammalian eggs is still unclear. As previously mentioned, PLCζ contains in its XY-linker region a predicted nuclear localization signal (NLS)

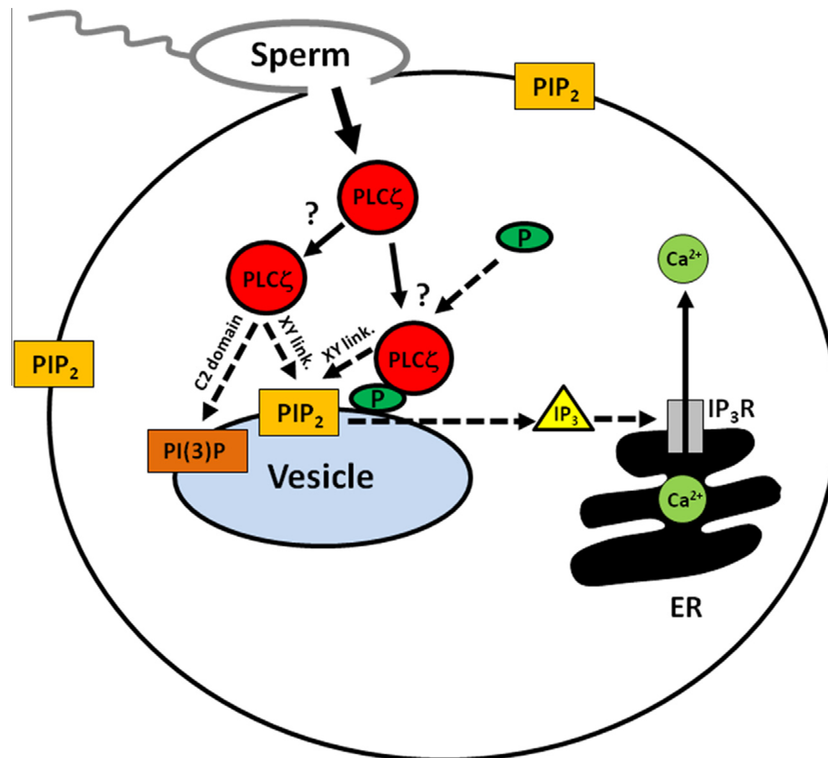

Fig. 2. Hypothetical mechanism of PLCζ domain regulation of function based on studies on sperm PLCζ. After sperm–egg fusion, PLCζ diffuses from the sperm head into the egg cytosol and targets a distinct intracellular vesicular PIP_2-containing membrane. The EF hand domains of PLCζ confer its high Ca^{2+} sensitivity, enabling the enzyme to be active at resting cytosolic Ca^{2+} levels (nM) in the egg, and results in further increases in activity as Ca^{2+} levels rise. Association of PLCζ with its specific membrane target may be mediated by interaction of the C2 domain with either PI(3)P and/or an unidentified egg membrane-targeting protein (P). The positively-charged amino acids in the XY-linker region (XY link) further assists the anchoring of PLCζ to the membrane, thus enhancing the local PIP_2 concentration adjacent to the catalytic domain via electrostatic interactions with the negatively-charged PIP_2. Once PLCζ is associated with the membrane PIP_2 the catalytic X/Y barrel binds and hydrolyzes its substrate to produce IP_3 that in turn induces Ca^{2+} release from the endoplasmic reticulum (ER).

sequence [9,78,79], the same region that is also thought to be necessary for PIP_2 binding [72,74]. Substitution of basic for acidic residues in the mouse PLCζ NLS causes loss of PLCζ nuclear translocation ability without affecting its in vivo ability to elicit Ca^{2+} release, which concurrently enables Ca^{2+} oscillations to proceed beyond pronuclei formation [78,79]. However, a notable conundrum with such a theory is that this mechanism only appears to apply to Ca^{2+} oscillations within mouse zygotes. It currently remains unresolved how Ca^{2+} oscillations terminate in eggs of other species, particularly since bovine, rat and human PLCζ do not appear to undergo nuclear localization [56], even though a range of PLCζ species possess a putative NLS, including mouse, rat, human, monkey, cow, pig, dog and medaka fish [9,56]. Furthermore, rat PLCζ does not translocate to the pronuclei of rat zygotes, while mouse PLCζ does. This divergence in nuclear localization is all the more striking considering that the rat and mouse PLCζ NLS sequence share a sequence identity of 87% [1,56]. Thus, whilst PLCζ nuclear sequestration may play a role in the termination of Ca^{2+} oscillations at interphase in mouse embryos, disparate mechanisms may be involved in other organisms.

An alternative explanation is that PLCζ may require association or interaction with a specific egg factor(s) to achieve an active state. Indeed, a current key unanswered question regarding PLCζ activity is how the enzyme is kept inactive within sperm, where it is present at significantly higher concentrations than within eggs [36]. Studies in CHO cells, in which PLCζ was expressed to levels ~1000 times higher than that which is active in eggs, did not exhibit any significant Ca^{2+} changes following ATP-induced Ca^{2+} release, despite the fact that PLCζ-transfected CHO cell extracts exhibited high intrinsic PLC enzyme activity. Surprisingly, when such transfected cells, or extracts made from these cells, were injected into mouse eggs, Ca^{2+} oscillations were successfully triggered [93]. This suggests that PLCζ may be held in an inactive state in sperm or that PLCζ is active in the egg due to specific interaction(s) with a protein(s) that is only expressed in the egg cytoplasm [93].

An alternative suggested mechanism of PLCζ regulation involves its three-dimensional folding. Analysis of the structure of PLCδ1 (PLCζ's closest homologue) indicates folding at the XY-linker region such that the C2 domain has extensive contact with the EF hands domain and the catalytic domain. Considering the significant level of sequence identity between PLCζ and PLCδ1, the EF hands and C2 domain association may be essential in forming the active conformation of the enzyme [71]. Furthermore, hydrophobic residues in the EF hands domain of PLCζ may play an important role in EF hand-C2 domain interactions. Truncations or mutations in the EF hands resulted in a decrease in Ca^{2+} oscillation-inducing ability of PLCζ, which may be due to dissociation of the EF hands from the C2 domain [71]. A similar conformational change may disrupt the close interactions between the X and Y catalytic domains, perturbing substrate binding [71]. However, more specific studies are required, including determination of the crystal structure of PLCζ before such questions can be addressed. Indeed, such crystallization studies would elucidate the three-dimensional properties of PLCζ and its domains, shedding further insight into how local interactions, and their perturbations, may affect protein stability and activity.

6. Clinical applications of PLCζ

Human infertility affects ~1 in 7 couples [2,94–96]. While in vitro fertilization (IVF) methods represent ~7% of total birth

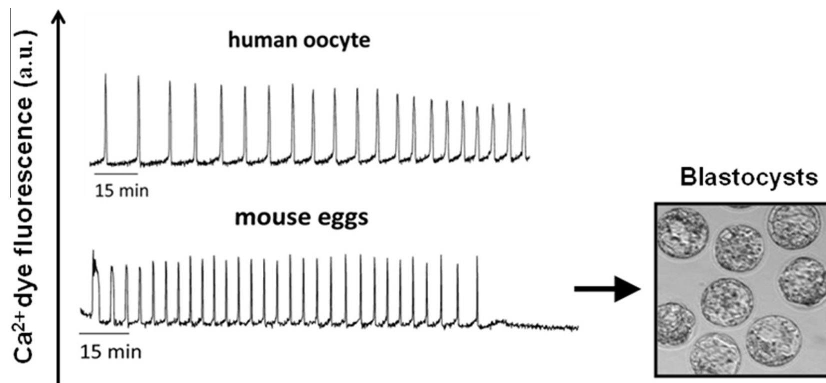

Fig. 3. Microinjection of recombinant human PLCζ protein induces Ca^{2+} oscillations in human oocyte and mouse eggs and triggers development. Representative fluorescence (a.u.; arbitrary units) recordings of the cytosolic Ca^{2+} concentration changes occurring in an unfertilized human oocyte (upper trace) and mouse egg (lower trace) following the microinjection of human PLCζ recombinant protein. Micrograph on right illustrates the mouse embryo development to the blastocyst stage after microinjection of an unfertilized mouse egg with human PLCζ recombinant protein. Modified from [53].

rates in some developing countries [2,97], several conditions such as severe male infertility (19–57% of cases of infertility) remain untreatable [5]. Even with intra-cytoplasmic sperm injection (ICSI), a modified IVF technique where the sperm is directly injected into the eggs, up to 5% of ICSI treatment cycles still fail, affecting at least 1000 couples per year in the UK alone [96]. Deficiencies in the egg activation process are currently regarded as the principal source of this failure [2,98,99].

A number of clinical reports have linked defects in human PLCζ with cases of egg activation deficiency. Sperm of some infertile men which fail IVF and ICSI are unable to induce Ca^{2+} oscillations upon microinjection into mouse oocytes [46,47]. Such ICSI-failure patients exhibited reduced/absent levels of PLCζ within the sperm head [46,47,87]. It appears likely that PLCζ may be contributing not only towards male infertility, but also perhaps to cases of male sub-fertility [5,47]. Egg activation failure can currently be treated by using artificial egg activation methods, such as applying Ca^{2+} ionophores [100–102]. Indeed, high fertilization rates and a successful pregnancy were achieved with patients whose sperm were deficient in PLCζ when a Ca^{2+} ionophore was used to artificially activate eggs following ICSI [102].

However, such chemicals may be detrimental to embryo viability, posing concerns regarding their future health due to potential cytotoxic, mutagenic and teratogenic effects on eggs and embryos [97]. Moreover, there is only a single large Ca^{2+} increase following ionophore treatment and this does not mimic the repetitive pattern of Ca^{2+} changes that is observed at fertilization [47]. While a recent computational approach suggested that two sequential applications of ionophore should improve successful activation rates, supporting experimental data, the same study also indicates that the temporal pattern of Ca^{2+} may exert an effect upon rates of cell cycle progression, and thus, subsequent embryogenesis [103]. Thus, a more endogenous therapeutic agent is urgently required as a replacement for current synthetic methodology and hence the use of sperm PLCζ seems the obvious physiological candidate.

It has been demonstrated that abnormalities in sperm PLCζ could be counteracted by co-injection with mouse PLCζ mRNA [46], while the parthenogenetic generation of blastocysts was achieved by injection of in vitro transcripts encoding PLCζ into human oocytes [104]. However, the clinical use of in vitro-transcribed PLCζ mRNA is not likely to be viable, since it is hard to control the precise level of PLCζ translation and any protein over-expression subsequently leads to developmental arrest defects in embryos [104,105]. Consequently, a pure, active recombinant protein form of PLCζ is likely to be more effective as a potential therapeutic in cases of male infertility and sub-fertility due to aberrant or absent PLCζ [50]. Significantly, it has recently been demonstrated that the prokaryotic production of purified recombinant human PLCζ protein in a stable state was able to induce Ca^{2+} oscillations in mammalian eggs within a physiological range (Fig. 3). In this study, the deleterious effect of mutant versions of PLCζ on Ca^{2+} oscillations and egg activation was shown to be efficiently overcome ('rescued') by microinjection of purified recombinant PLCζ protein [50].

7. Closing remarks

In 2002 the discovery of PLCζ instigated a shift in thinking about how fertilization works in mammals and other animals. The previously preferred model of fertilization, whereby a sperm acts on an egg plasma membrane receptor to trigger Ca^{2+} release is now being replaced, for many vertebrates, by the idea that a soluble sperm factor i.e. PLCζ is introduced into the egg following gamete fusion, thus triggering egg activation. Although the sperm PLCζ discovery represents an important breakthrough in the field, we currently still do not fully understand how PLCζ works. For example, although PLCζ participates in a standard biochemical pathway (hydrolysis of PIP_2) that is known to be present in all types of cells in the body, PLCζ appears to be unique amongst PLCs; it appears to be effective only in eggs. PLCζ also seems to interact with small membranous vesicles inside eggs, which is very different from the way other types of PLC proteins work, since they interact with their substrate located on the plasma membrane. There are also significant variations in the activity of PLCζ in sperm from different species, which function with varying efficacy in eggs from different animal species, observations that remain to be explained. While we know that all parts of the PLCζ protein are important for it to work, we do not fully understand how all these parts work together. Further investigation is required to fully elucidate the fundamental mechanism of egg activation by a sperm, the earliest signaling event required for a new life to begin.

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