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Review

PLCζ(zeta): A sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals

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Abstract

At fertilization in mammals, the sperm activates development by causing a prolonged series of intracellular Ca^{2+} oscillations that are generated by increased production of inositol trisphosphate ($InsP_3$). It appears that the sperm initiates $InsP_3$ generation via the introduction of a sperm factor into the egg after gamete membrane fusion. We recently identified a sperm-specific form of phospholipase C (PLC), referred to as PLCζ(zeta). We review the evidence that PLCζ represents the sperm factor that activates development of the egg and discuss the characteristics of PLCζ that distinguish it from the somatic forms of PLC.

Keywords: Fertilization; Calcium; Phospholipase; Egg; Sperm

Contents

1. Introduction

One of the central questions of fertilization is how the sperm activates development of the egg. It has been well established in many different species that egg activation at fertilization is caused by a Ca^{2+} increase in the egg cytosol [1–3]. The elevated Ca^{2+} concentration has been shown to be both necessary and sufficient for egg activation in a variety of species such as sea urchin, frog, and mouse eggs [1–3]. This means that understanding how a sperm triggers egg activation requires resolution of the question of how a sperm generates the Ca^{2+} change in the egg. This is a classic signal transduction problem and has parallels with other Ca^{2+} signalling transduction problems, such as the triggering of muscle contraction or hormone secretion. For this review, we focus our attention on the problem of Ca^{2+} signalling during egg activation in mammals. We suggest that a plausible

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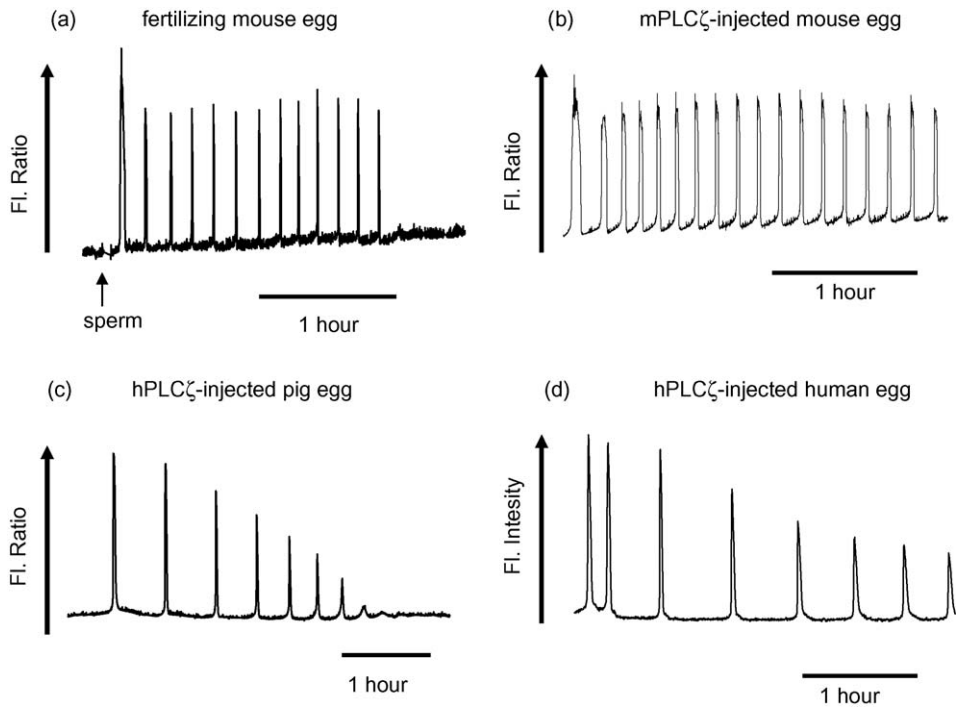

Fig. 1. Ca^{2+} oscillations in mammalian eggs. (a) Ca^{2+} oscillations in a fertilizing metaphase II mouse egg, (b) Ca^{2+} oscillations in a mouse egg that was previously injected with 0.2 mg/ml of mouse PLCζ cRNA, (c) an in vitro matured pig egg injected with 2 mg/ml of human PLCζ cRNA, and (d) an aged human egg was injected with 1 mg/ml human PLCζ cRNA. Injections volumes were ∼5% of the egg volume. Each *y*-axis is in arbitrary units and gives a relative measure of intracellular Ca^{2+} using fura 2 dextran (a), fura red (b and c) or Oregon green BAPTA dextran (d). General methods are described in references [37,44].

solution to this problem in mammals is the discovery of a novel, sperm-specific phospholipase C (PLC).

During in vitro fertilization in mammals there are a distinctive and long-lasting series of Ca^{2+} oscillations observed in the egg (Fig. 1a) [1–3]. The frequency of Ca^{2+} oscillations varies from one species to another and can range from a Ca^{2+} increase every 2 min, to a Ca^{2+} increase every 1 h [4,5]. The Ca^{2+} oscillations persist for several hours, and in mouse eggs they have been found to stop around the time of pronuclear formation [6]. There is considerable evidence that the fertilization-induced Ca^{2+} oscillations in mammalian eggs are caused by increased $InsP_3$ production (see reviews by Miyazaki and Fissore, this volume). The signal transduction problem is, therefore, reduced to one of understanding how the sperm can initiate increased $InsP_3$ production in eggs. Since increased $InsP_3$ production and Ca^{2+} oscillations occurs so widely during hormonal stimulation of somatic cells, it was considered that the sperm acts like a 'giant hormone molecule' and stimulates receptors on the membrane surface that couple to transmembrane signalling systems [3,7,8]. It has been specifically suggested that either GTP binding proteins, or tyrosine kinsases, may link the hypothetical sperm receptors to phospholipase C, of the β or γ class, within the egg [3,7]. The case for GTP binding proteins has been made because the injection of non-hydrolysable analogues of GTP stimulate Ca^{2+} oscillations in hamster and mouse eggs [9]. However, the Ca^{2+} oscillations stimulated by GTP analogues tend to 'run down' in a way that is not seen at fertilization [2]. In addition, there is now evidence to show that G-protein inhibitors and functionally inhibitory antibodies do not block egg activation at fertilization in mammals [10]. The proposed role of tyrosine kinases was largely based upon the finding that the expression and subsequent stimulation of EGF receptors, via tyrosine kinase activation, also leads to Ca^{2+} oscillations in mouse eggs [3]. However, subsequent studies have again demonstrated that tyrosine kinases are unlikely to be involved in triggering Ca^{2+} release during fertilization [11].

2. The sperm factor hypothesis

There are now several lines of evidence supporting the idea that the mammalian sperm delivers a soluble factor into the egg to initiate Ca^{2+} release. In mammals, the sperm and egg membranes have been shown to fuse 1–3 min before the onset of the first Ca^{2+} increase at fertilization [12,13]. This means that factors from the sperm would be able to enter the egg cytoplasm before $InsP_3$ production commences. The existence of the relevant sperm factors is suggested by the finding that injection of soluble, cytosolic sperm extracts triggers the pattern of Ca^{2+} oscillations that occurs at fertilization in eggs of a variety of mammalian species [14–18]. The active factor in these experiments was shown to be protein-based and sperm-specific [14–18]. As well as the injection of soluble sperm extracts, the injection of whole sperm, a procedure termed intracytoplasmic sperm injection (ICSI), has also been shown to activate mouse and human eggs by generating a series of Ca^{2+} oscillations [19–21]. The active factor in ICSI also appears to be a protein [22]. However, experiments using mouse sperm suggest that the protein is predominantly insoluble and can remain bound to the perinuclear theca around the sperm

head [22,23]. This factor was referred to as the Sperm Oocyte Activating Factor, or SOAF [23]. SOAF is an 'insoluble' or perinuclear sperm factor and was suggested to be different from the soluble sperm factor that was being studied by other groups.

A number of different proteins have been suggested as 'sperm factor' candidates. Our initial attempts to identify the soluble sperm factor protein involved the purification of hamster soluble sperm extracts and resulted in a candidate protein of 33 kDa [24]. This protein has since been shown to be unrelated to the ability of sperm extracts to cause Ca^{2+} oscillations in eggs [25,26]. Another protein that was suggested to represent an egg activating 'sperm factor' is a truncated form of the c-kit receptor, referred to as tr-kit. This protein is a truncated and intracellular part of the c-kit protein which is a transmembrane tyrosine kinase receptor [27]. Microinjection of the tr-kit protein, or cRNA, into mouse eggs has been shown to cause egg activation [27]. However, there is as yet no evidence that tr-kit can cause Ca^{2+} oscillations in eggs. In addition, there are no reported studies that have repeated the experiments using tr-kit to activate mammalian eggs. The relevance of tr-kit to fertilization is also questionable since the activation of mouse eggs by tr-kit is blocked by injection of SH3 containing domains, however the Ca^{2+} oscillations at fertilization are apparently unaffected by injection of PLCγ1 SH2 and SH3 containing domains proteins [28,11]. Other proteins, such as components of the perinuclear matrix, have been proposed to represent a sperm factor but again no evidence exists that these proteins cause Ca^{2+} release in eggs [29].

3. Evidence that the soluble sperm factor is a PLC

A clue to the nature of the mammalian soluble sperm factor emerged from studies using sea urchin egg homogenates. The homogenates of sea urchin eggs can sequester Ca^{2+} in an ATP-dependent manner and can release Ca^{2+} in response to a range of second messengers, such as $InsP_3$, cyclic ADP ribose and NAADP [30]. Sea urchin egg homogenates also release Ca^{2+} in response to the addition of mammalian soluble sperm extracts [31]. Fractionation experiments suggested that the same factor is responsible for causing Ca^{2+} release in egg homogenates and Ca^{2+} oscillations in intact mouse eggs [26]. The soluble sperm factor-mediated Ca^{2+} release in the sea urchin egg homogenate is completely abolished by prior desensitization of $InsP_3$-induced Ca^{2+} release, but it is unaffected by desensitization of cADPR- or NAADP-induced Ca^{2+} release [31]. Furthermore, addition of the soluble sperm factor to the homogenate system leads to a large increase in $InsP_3$ that can quantitatively account for the observed Ca^{2+} release. These data demonstrated that the sperm factor works via the generation of $InsP_3$ in eggs, and have been corroborated by the demonstration that the $InsP_3$ concentration increases after injection of sperm extracts into Xenopus oocytes [32].

One explanation for how sperm extracts cause $InsP_3$ production in egg homogenates is that the extracts themselves contain a relevant phosphoinositide-specific phospholipase C (PLC), an enzyme that hydrolyzes phosphatidylinositol bisphosphate ($PtdInsP_2$) to generate $InsP_3$ [31]. This idea was supported by the finding that sperm extracts contain a soluble PLC activity that is very active at low Ca^{2+} concentrations, compared to PLCs in other tissue extracts [33]. We suggested that the soluble sperm factor may comprise a highly Ca^{2+}-sensitive PLC, and proceeded to identify the PLC isoforms present in mammalian sperm. However, we and others were unable to find a precise correlation between the sperm factor activity and the presence of known PLC isoforms of the β, γ and δ class [32–34]. Moreover, it was also apparent that most of the known PLCs were unable either to cause Ca^{2+} oscillations when injected into eggs, or to cause Ca^{2+} release from sea urchin egg homogenates [35]. PLCγ became a potential candidate when it was reported to cause Ca^{2+} oscillations in mouse eggs upon injection of around 100 pg protein/egg [36]. However, the injected protein is vastly in excess of the amount of PLCγ that is likely to be present in a single sperm. This observation lead to the suggestion that soluble sperm factor therefore could not be a PLC [3,36]. However, this did not take into account the possibility that another, novel type of sperm PLC may be involved.

4. Sperm-specific PLCζ(zeta)

Evidence for a novel type of PLC was first obtained upon examination of short EST-sequences derived from mouse and human testis and this enabled us to isolate and characterise a full-length cDNA encoding a sperm protein that is referred to as PLCζ (zeta) [37]. PLCζ is unusual in that it is appears to be a gamete-specific protein that is expressed only in spermatids [37]. PLCζ is a protein of about 70 kDa, which makes it smaller in size than any of the other known vertebrate isoforms of phosphoinositide-specific PLC. The most important experiment with PLCζ is to test whether it is effective at causing Ca^{2+} release in eggs. To do this, we injected cRNA encoding the full-length PLCζ protein and found that this leads to a series of prolonged Ca^{2+} oscillations in mouse eggs [37]. The more PLCζ cRNA that is injected, the higher the frequency of Ca^{2+} oscillations. Fig. 1b shows an example of Ca^{2+} oscillations in mouse eggs stimulated by the injection of PLCζ cRNA. These oscillations require the synthesis of PLCζ protein since they are prevented if the protein synthesis inhibitor cycloheximide is included in the media [37]. Furthermore, the tagging of PLCζ with either a Myc epitope, the Venus fluorescent protein, or with luciferase all leads to a measurable amount of PLCζ protein synthesis following injection of the cRNA [37,38]. We used the Myc-tagged PLCζ to estimate that the amount of PLCζ cRNA that leads to a frequency of Ca^{2+} oscillations similar to those seen during fertilization, corresponds to 45–75 fg of PLCζ protein. This estimate has now been supported by experiments using either the Venus- or the luciferase-tagged versions of PLCζ and these data have shown that as little as 5–10 fg/egg of PLCζ is active in causing a single Ca^{2+} increase and that around 50 fg/egg of PLCζ per egg is effective at causing a prolonged series of Ca^{2+} oscillations [38]. The injection of recombinant PLCζ protein has also been shown to generate Ca^{2+} oscillations in mouse eggs [39]. The amount of recombinant PLCζ protein required to cause a series of Ca^{2+} oscillations release was around 300 fg per egg. This is somewhat higher than that observed with cRNA injection and probably reflects the fact that the recombinant protein

may be unstable and a significant proportion of it may not be fully active. Together, the observations with PLCζ cRNA and recombinant protein clearly show that sperm PLCζ is a highly effective mimic of the sperm-induced Ca^{2+} oscillations and that it is more effective in eggs than other isoforms, such as PLCγ.

PLCζ protein has been shown to be present in soluble sperm extracts from mouse, hamsters and pigs [37,40,41]. Antibodies raised against PLCζ have been used to estimate the amount of PLCζ in mouse sperm. Two separate studies, using different antibodies, resulted in estimates of 20–50 fg, and 40–50 fg for the amount of PLCζ in a single sperm [37,41]. These estimates are within the range of the effective concentrations of PLCζ in eggs, suggesting that there is sufficient PLCζ in a single sperm to trigger Ca^{2+} oscillations at fertilization. One of the antibodies to PLCζ has also been used to immunoprecipitate PLCζ protein from sperm extracts. When PLCζ is immunodepleted from extracts they lose their ability to cause Ca^{2+} oscillations in mouse eggs and to trigger Ca^{2+} release in sea urchin egg homogenates [37]. This shows that PLCζ is essential for the activity of the previously described sperm factor that is active in causing Ca^{2+} release in intact eggs and cell-free extracts. Since estimates for the molecular size of the sperm factor activity have been in the 30–80 kDa range, with the PLCζ of ∼70 kDa, it seems unlikely that any other proteins are associated with sperm factor [34,42]. Given that the observed properties of PLCζ are sufficient to explain the sperm factor-induced Ca^{2+} release in eggs, it seems reasonable to conclude that PLCζ protein is synonymous with the previously described soluble sperm factor.

As well as causing Ca^{2+} oscillations, the injection of mouse PLCζ into mouse eggs leads to egg activation and development. Mouse eggs injected with PLCζ cRNA form pronuclei and when they are made diploid (by incubation in cytochalasins) they can develop to the blastocyst stage at similar rates to fertilized eggs [37]. The ability of PLCζ to trigger Ca^{2+} oscillations and development is not species-specific. Injection of the human or monkey PLCζ into mouse eggs also causes Ca^{2+} oscillations and activates development up to at least the blastocyst stage [43]. Injection of the human PLCζ also causes Ca^{2+} oscillations and the activation of preimplantation development in human eggs [44]. Fig. 1c shows that the human PLCζ is also effective at inducing Ca^{2+} oscillations in in vitro matured pig eggs and Fig. 1d shows Ca^{2+} oscillations stimulated by human PLCζ cRNA injection into an aged human egg. These findings of cross-species activity for PLCζ are consistent with previous data showing that the sperm factor activity is present in a range of mammalian sperm and is effective in eggs from different mammalian and non-mammalian species [15,16]. There is now evidence for the presence of a PLCζ gene in 8 different mammalian species. Table 1 shows the predicted molecular mass and isoelectric points of PLCζ from different mammals for which a homologue has been identified so far. It seems reasonable to expect that the different mammalian versions of PLCζ will be able to cause Ca^{2+} oscillations similar to those seen at fertilization in all mammalian species.

The experiments that we discussed above and that lead to the discovery of PLCζ were based upon soluble sperm extracts [31,37]. It was noted above that experiments injecting whole sperm into mouse eggs suggested the existence of an 'insoluble' sperm factor bound to the perinuclear matrix of the sperm [22,23]. The group that first identified this perinuclear sperm factor has carried out a series of fractionation experiments with proteins extracted from the perinuclear matrix of mouse sperm. These fractions were analysed by tandem mass spectrometry. Those fractions that correlated with the ability to activate mouse eggs uniquely contained PLCζ [41]. This data clearly suggests that the soluble and 'insoluble' sperm factors are the same protein, namely PLCζ. It is noteworthy that the approach taken in these studies involved a different starting material and a different assay from the previous studies of the sperm factor, and yet the same protein, PLCζ, was identified as SOAF, as for the sperm factor protein [41]. The data also suggests that PLCζ is localized in the perinuclear matrix of mouse sperm. This may be one reason why mouse sperm proved a difficult source for making active sperm extracts. Hence, PLCζ may be distributed differently in various species. For example, studies on pig sperm have shown that PLCζ is distributed in both the soluble cytosolic fractions of sperm extracts, as well as in the pellet of the extracts that contains the sperm heads [40]. PLCζ may be similar to many signalling proteins and enzymes that can be distributed in both soluble and insoluble fractions of cell extracts.

Table 1
Comparative features of different mammalian isoforms of PLCζ

Species	AA	MW	p*I*	Exons	Chromosome	Accession no.
Human	608	70411	9.14	15	12	AF532185
Monkey	641	74551	7.21			AB070108
Monkey	640	74450	7.58			AB070109
Mouse	647	74614	5.44	14	6	AF435950
Rat	645	74315	5.29	13*	4	AY885259
Chicken (*Gallus gallus*)	641	72732	8.44	13*	1	XM416413
Chicken (*Gallus domesticus*)	639	72530	8.53			AY843531
Cow	634	73606	6.60			AY646356
Pig	636	73702	6.13			AB113581
Dog	653	75086	6.04	13*	27	XM543784
Chimpanzee				14		NW124823

The abbreviations used are: AA is the number of amino acids, MW is predicted molecular weight and p*I* is the predicted isoelectric point. The asterisk (*) indicates that the first, non-coding exon is not currently known in these sequences. It is therefore predicted PLCζ from these species will have 14 exons. There are two sequences for cynomolgus monkeys that are both equally active in mouse eggs [43].

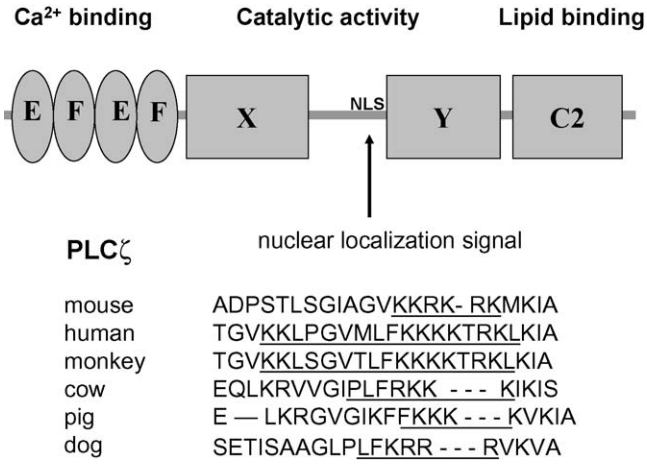

Fig. 2. The predicted domain structure of PLCζ. The putative nuclear localization sequence (NLS) of PLCζ from different species. There is predicted to be 2 pairs of EF hand domains that appear to be involved in Ca^{2+} binding. The catalytic activity is associated with the X and Y domains and the C2 domain is considered to bind to phosphoinositde containing lipids. In mouse there is a nuclear localization sequence that appears to be functional in transporting PLCζ into the pronuclei that form after fertilization [57]. The predicted nuclear localization sequence is shown for the same region of primary sequence for some other mammalian species.

A clear case can now be made that PLCζ is involved in the mechanism of Ca^{2+} oscillations and egg activation following sperm extract injection and ICSI. It is expected that PLCζ will also play the critical role during normal fertilization. To date there is only one piece of direct evidence to show that PLCζ plays a role in normal fertilization. Transgenic mice have been generated that make RNAi that interferes with PLCζ expression in the testis. It was shown that PLCζ levels are about half those of normal mice. During fertilization using sperm from these mice, the Ca^{2+} oscillations terminate prematurely, and litter size is reduced compared to control mice [45]. The premature termination of oscillations might be because these type of sperm contain less PLCζ. What is most intriguing is that no offspring from transgenic males pass the transgene to offspring. Since expression of the transgene is likely to be mosaic in spermatogenic cells, it appears that there is a major defect in sperm that are likely to show the most reduced PLCζ expression [45]. The data, therefore, support the hypothesis that PLCζ plays an important role in activating successful development at fertilization in mammals.

5. Structure and function of PLCζ

It is clear that PLCζ is distinct from other PLCs in its ability to cause Ca^{2+} oscillations in eggs at very low concentrations. Some insight into this unique feature could be gained from an analysis of its structure. The PLCζ enzymes are similar in size for all species so far described, being 70–75 kDa (Table 1). The isoelectric point varies widely, from 5.29 in rat to 9.14 in human, for reasons that are unclear. The domain structure of PLCζ shares many similarities with PLCs of the δ class that have been described in detail previously (Fig. 2) [46,47]. PLCζ has X and Y catalytic domains that are common to all phosphoinositide-specific PLCs. In common with PLCδs there is a set of EF hand domains and a C2 domain (Fig. 2). The one major difference between PLCζ and PLCδ is the absence of a PH domain in all identified versions of PLCζ. This means that PLCζ is the smallest known mammalian PLC and has an overall size and domain structure that is most similar to plant PLCs [47].

By homology with PLCδ, the X–Y catalytic domain of PLCζ is predicted to consist of a barrel-like structure of alternating α-helices and β-sheets [46,47]. The catalytic domain of PLCζ shows significant conservation across species and a close homology with PLCδ1. The active site residues within the catalytic domain of PLCδ1 have been identified from structural studies and have been comprehensively mutated [48]. Alignment of the catalytic domains of PLCζ and rat PLCδ1 reveal that all the active site residues are conserved, or conservatively replaced. We have mutated one of the active site residues (mPLCζ $D^{210}R$; 37) and injection of cRNA encoding this site-directed mutant PLCζ leads to the loss of its ability to generate Ca^{2+} oscillations. This confirms that $InsP_3$ generation by PLCζ is the way it causes Ca^{2+} oscillations and that the main function of its X–Y domain is likely to be similar to that described for PLCδ1—the hydrolysis of $PtdInsP_2$ to produce $InsP_3$.

PLCζ has four EF-hand motifs at the N-terminus that appear to play an important role in the enzymatic activity of PLCζ. The EF-hand motifs are arranged in two pairs of lobes similar to those involved in Ca^{2+} binding in calmodulin and troponin [49]. It is important to examine whether the EF hands are involved in Ca^{2+} binding since PLCζ is unusual in exhibiting a high degree of sensitivity to stimulation by Ca^{2+} [39,49,50]. PLCζ is considerably more sensitive to stimulation by Ca^{2+} than PLCδ1 [39,50], or other somatic isoforms of PLC. This observation could explain why PLCζ is much more effective than other PLCs at causing $InsP_3$ production and Ca^{2+} release in the cytoplasm of eggs. The importance of the EF hands in Ca^{2+} stimulation is supported by our finding that removal of the first pair of EF hands increases the EC_{50} for Ca^{2+} and decreases the Hill coefficient [50]. We also found that the deletion of both pairs of EF hands further raises the Ca^{2+} EC_{50} to 30 μM and also reduces the Hill coefficient to 0.6 [50]. However, the interpretation of these studies is complex since deletion of the EF hand domains may also lead to a loss of in vitro catalytic activity in the EF hand domain-deleted PLCζ [49]. This suggests that the EF hands also play an important structural role in maintaining PLCζ activity. Further complexity is added by the finding that mutating the amino acid residues considered to be essential for Ca^{2+} binding in the first pair of EF hand domains has only a small effect upon Ca^{2+} binding [49]. Nevertheless, it is quite clear that the injection of cRNA to express high levels of protein for either of these truncated forms of PLCζ lacking EF hand domains fails to cause any Ca^{2+} oscillations in mouse eggs [49,50]. These data show that the EF hand domains do play an important role in the way PLCζ generates $InsP_3$ in an egg.

Due to the absence of a PH domain, it is unclear how PLCζ is able to target its membrane-bound substrate, $PtdInsP_2$. One candidate is the C2 domain. These are membrane-targeting domains of approximately 120 residues in length, composed of two four-stranded β-sheets, with flexible linkers, or 'loops', connecting

the β-strands [51,52]. C2 domains have been shown to bind to phospholipid containing membranes, e.g. protein kinase Cα and PLCδ1 bind to phosphatidylserine, cytosolic phospholipase $A_2\alpha$ and 5-Lipoxygenase bind to phosphatidylcholine, while synaptotagmin–C2A binds to $PtdInsP_2$ [52]. Lipid binding can be either Ca^{2+}-dependent or -independent, with the Ca^{2+} ions binding to the loop regions at one end of the domain via the side chains of conserved aspartate residues [51]. The effect of Ca^{2+} on the lipid binding of the PLCζ C2 domain is unknown. However, sequence comparisons of PLCδ1 and PLCζ reveals that whilst the Ca^{2+} binding residues of loops 1 and 2 are conserved, the Ca^{2+} binding residues of loop 3 are not present in PLCζ. This removes three potential Ca^{2+} binding sites and suggests that the C2 domain of PLCζ may bind to lipid in a Ca^{2+}-independent manner, similar to the C2 domains in synaptotagmin and PKCα [53,54]. When the C2 domain of PLCζ is deleted there is some loss of enzyme activity but, unlike the EF hand domain deletions, the overall Ca^{2+} sensitivity is only slightly altered, confirming the possibility that this domain does not play a specific role in Ca^{2+} ion binding. However, injection of cRNA encoding PLCζ without the C2 domain does not generate any Ca^{2+} oscillations in mouse eggs, suggesting that the C2 domain retains an essential role in the function of PLCζ in eggs. The isolated C2 domain is able to bind to phosphatidylinositol-3-phosphate and this could play a role in targeting or regulating PLCζ activity [49].

In addition to the EF hand and C2 domains, the other non-catalytic part of PLCζ that could also be involved in regulating its activity is the segment between the X and Y catalytic domains, known as the X–Y linker. This region shows the greatest divergence between species. The length of the X–Y linker is longest in the monkey *Macaca fascicularis*, and shortest for *Homo sapiens*. Compared with that of PLCδ, all PLCζ's exhibit an extended X–Y linker that is rich in charged residues. The significance of these differences is unknown, but the obvious proximity of the X–Y linker to the active site could indicate some involvement in regulating catalytic activity, or $PtdInsP_2$ binding. It is possible to make analogies with PLCγ which has an extended X–Y linker that contains a split PH domain with SH2 and SH3 domains that play an important role in regulating PLCγ activity in cells [47]. However, unlike PLCγ, the PLCζ X–Y linker contain no regions of predicted secondary structure.

There have been reports suggesting the presence of splice variants of PLCζ. A variant of mouse PLCζ, containing a 30 bp insert between bases 159 and 160 in the wild type PLCζ ORF exists in the database (AK006672). However, this insert contains two internal stop codons. If translation were to begin at the next in-frame methionine (residue 111 of the full-length PLCζ), then a truncated 61 kDa protein would be produced. We have described this protein as M111-PLCζ [50], while Kouchi et al. [38,39] have called it s-PLCζ (*short*-PLCζ). This truncated PLCζ is missing one and a half of the EF-hand lobes. Injection of cRNA encoding this M111 (sPLCζ), or sPLCζ recombinant protein does not cause any Ca^{2+} oscillations [39]. This is consistent with the previously discussed data showing that deletion of any of the EF hand domain structure from PLCζ leads to a loss of activity in eggs [49,50]. The exact function of the PLCζ splice variant is, therefore, unclear. Examination of the variant cDNA sequence reveals the 30 bp insert is consistent with the sequence in the flanking intronic region immediately preceding exon 4. Thus, it is likely that the insert is an artifact deriving from incompletely spliced RNA. We have also observed several other incompletely spliced cDNAs, which contain internal stop codons at various points, when cloning PLCζ from human and rat testis cDNA libraries (unpublished observations). Interestingly, these transcripts were not detected when cloning mouse PLCζ from a spermatid library and were not observed in Northern blots of testis mRNA. Since the M111-PLCζ has also not been observed as a 61 kDa protein in sperm extracts, it is possible that it may only exist as a minor transcript.

6. Nuclear localization of PLCζ

Following fertilization by sperm, the cytoplasmic Ca^{2+} oscillations in the egg cease around the time the two pronuclei form [6]. Some Ca^{2+} oscillations are subsequently observed during mitosis in mouse zygotes [6,55]. One proposal suggested to explain why Ca^{2+} oscillations stopped and started in such a cell cycle-dependent manner, was that the sperm factor was localized to pronuclei during interphase [55]. This localization would result in the cessation of Ca^{2+} oscillations, and they would return when pronuclear envelope breakdown occurred during entry into mitosis. This mechanism is consistent with studies showing that there is a Ca^{2+}-releasing activity associated with fertilized, sperm-injected, or sperm extract-injected mouse embryos [56]. The idea of a nuclear-associated sperm factor has now been strengthened by recent experimental observations with PLCζ. When PLCζ is tagged with Venus fluorescent protein or a Myc epitope, the expression of PLCζ can be monitored in eggs and is associated with Ca^{2+} oscillations. When the Ca^{2+} oscillations stop the tagged PLCζ can be seen to be associated with the nascent pronuclei [38,57]. During the first mitosis, the tagged PLCζ returns to the cytoplasm in temporal coincidence with the restart of Ca^{2+} oscillations [57]. Although the Ca^{2+} oscillations have only been recorded to occur during the first mitosis in mouse zygotes, if exogenous PLCζ is introduced into the embryos at later stages of development it continues to undergo nuclear sequestration [58]. This suggests that the nuclear localization is a fundamental feature of mouse PLCζ. Fig. 3 illustrates the idea that PLCζ is released from the sperm and then localized to pronuclei in a manner that is consistent with the termination of Ca^{2+} oscillations.

The nuclear sequestration of PLCζ appears to be the result of a specific signal sequence contained within PLCζ, called a nuclear localisation signal (NLS), essentially consisting of a cluster of basic residues. Bioinformatics analysis identified the presence of a predicted NLS motif in the X–Y linker region in mouse PLCζ, comprising the hexapeptide, KKRKRK. Upon ablation of this putative NLS by introducing an acidic residue in the middle of this basic sequence, the mutated NLS form of mouse PLCζ failed to localize to pronuclei when introduced into eggs [57]. The NLS mutant PLCζ still caused Ca^{2+} oscillations, but the oscillations continued beyond the time of pronuclear formation. This suggests that the pronuclear localization of PLCζ does play a role in terminating Ca^{2+} oscillations at fertiliza-

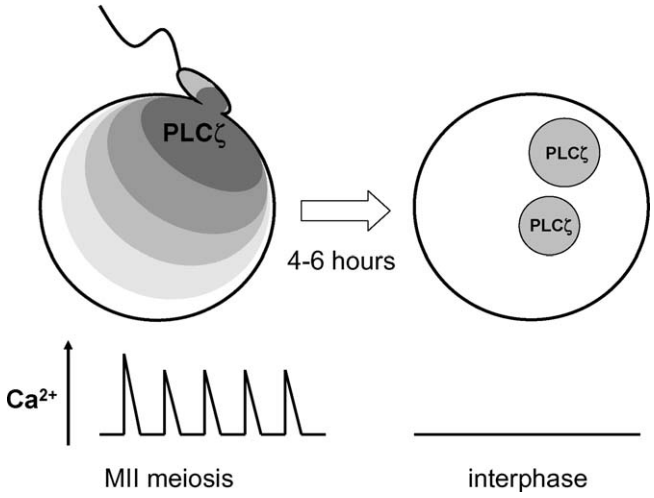

Fig. 3. Schematic diagram of PLCζ localization at fertilization. When the sperm fuses with the eggs the PLCζ is released from the sperm head. PLCζ is released from the sperm in a process that may take at least 15 min in the mouse [72]. It then diffuses into the egg cytoplasm triggering a series of Ca^{2+} oscillations in the egg which is otherwise arrested at metaphase of the second meiotic division. The Ca^{2+} oscillations cease as the egg enters interphase and the zygote then forms two pronuclei into which PLCζ is sequestered.

tion in mouse eggs. Notably, there are changes in the sensitivity to $InsP_3$ in the egg after fertilization and this is likely to also play a role in terminating Ca^{2+} oscillations [59,60]. However, the nuclear-cytoplasmic translocation of PLCζ remains the simplest explanation for why Ca^{2+} oscillations return during the first mitosis in fertilized embryos.

A predicted NLS is consistently found in the X–Y linker region of the deduced PLCζ protein sequence from a variety of mammalian species. Fig. 2 schematically illustrates the location of the predicted NLS close to the start of the Y-domain, and gives a sequence alignment of the NLS (underlined) identified in six full-length PLCζ sequences. Although there is some variation in the precise NLS sequence for the PLCζ from different mammals, all the sequences exhibit the reliable features of a cluster of basic residues near the centre of the molecule located immediately preceding the catalytic Y domain (NLS prediction using PSORT II (http://psort.nibb.ac.jp/). This suggests that nuclear localization may be a conserved feature of PLCζ. Nevertheless, to date only the mouse PLCζ NLS has been shown to be functional in eggs.

7. Species variation in sperm PLCζ

There appear to be varying amounts and disparate solubilities of the PLCζ in sperm from different species [40]. This was suggested by early studies which used cytosolic extracts from mechanically-disrupted hamster sperm that were extremely potent in causing Ca^{2+} oscillations in eggs of different species [14,15]. In contrast, mechanical disruption of mouse sperm was unable to yield an active extract, and one of the first reports of a sperm factor activity found that egg activation could not be caused by mouse sperm extracts [61]. More recently, it has been shown that pig sperm contain significantly more PLCζ than mouse sperm [40]. Moreover, whilst some of the PLCζ in pig sperm is soluble and found in the cytosolic extract, a considerable proportion of PLC activity is retained in the sperm head and can only be extracted by high pH treatment [40]. Fractionation of pig sperm also suggests that the 70 kDa, full-length PLCζ does not always correlate with PLC activity and the ability of sperm extracts to cause Ca^{2+} oscillations in eggs [40]. In this species, there is evidence that a proteolytically cleaved version of PLCζ may be the active form.

Not only are there likely to be differences in the amount and solubility of PLCζ from different species but it seems likely that there are substantial differences in the relative potency of the PLCζ from different species. We have found that it takes nearly a hundred times less human PLCζ cRNA than mouse PCLζ cRNA to cause Ca^{2+} oscillations in mouse eggs [43]. We have also found that the mouse PLCζ cRNA is less effective at generating Ca^{2+} oscillations in human eggs ([44], Rogers and Swann, unpublished observations). Whilst the precise expression levels of protein were not measured quantitatively in these experiments, the data suggests that human PLCζ may be unusually effective in causing Ca^{2+} oscillations. When combined with the evidence that sperm from various mammals contain different amounts of PLCζ, it is plausible that the species-dependent variation enables a precise tuning of the effective 'dose' of PLCζ delivered by a sperm. The species-specific sperm PLCζ quantity and quality is thus adjusted to match the size and sensitivity of the recipient egg, that culminates in a series of robust Ca^{2+} oscillations lasting several hours in order to reliably effect egg activation.

The solubility of PLCζ could also be related to the timing of oscillations. For example, at fertilization in hamster eggs, the sperm initiates Ca^{2+} oscillations within about 10 s of sperm-egg fusion, whereas in mouse there appears to be a delay of 1–3 min between sperm-egg fusion and the first Ca^{2+} increase [9,12]. The delay to the first Ca^{2+} increase is related to sperm because if zona-free hamster eggs are inseminated with mouse sperm there are also Ca^{2+} oscillations, but these start several minutes after sperm-egg fusion [62]. The rapid response of the hamster egg is therefore related to the sperm, and may be explained by the highly soluble nature of the 'sperm factor', and by implication PLCζ, in hamster sperm extracts.

8. Future questions

8.1. How is PLCζ localized and regulated in the sperm and egg?

When PLCζ is introduced into the egg by gamete membrane fusion it appears that it is already enzymatically active and able to generate $InsP_3$. One of the reasons that it is more active in eggs compared to other PLC isoforms is probably because of its high sensitivity to Ca^{2+}. In fact, PLCζ is expected to be half-maximally active at resting intracellular Ca^{2+} levels in the eggs [39,49,50]. This raises the question of how PLCζ is contained for weeks within mature sperm, which have a similar resting Ca^{2+} concentration to the egg. It is reasonable to assume that it is somehow maintained in an inert state. This may be connected with the localization of PLCζ in sperm, which appears to be the

perinuclear matrix. It is possible that this matrix holds PLCζ away from its substrate PtdInsP(4,5)P_2, which is expected to be in the plasma membrane of sperm.

In mammalian eggs, there is evidence for some PtdIns(4,5)P_2 in the plasma membrane. The PH domain of PLCδ1 binds very specifically to PtdIns(4,5)P_2 and has been shown to concentrate in the plasma membrane region of mouse eggs [63]. One would therefore expect PLCζ to be targeted to the plasma membrane of eggs. The Ca^{2+} increases after sperm extract injection have been shown to be initiated from regions that are close the egg cortex so it is possible that PLCζ has a cortical localization. However, both $InsP_3$ receptors and the endoplasmic reticulum, which is the relevant Ca^{2+} store, are more concentrated in the egg cortex. So this cortical release may reflect the site of $InsP_3$ action rather than its generation [64]. Interestingly, when the Venus-PLCζ was injected into mouse eggs, it was not detected in the plasma membrane region, but appeared to be dispersed through the egg cytoplasm [38]. Consequently, it remains unclear whether PLCζ is targeted to the plasma membrane. An alternative possibility is that PLCζ is localized in some intracellular organelles. This would be consistent with experiments on sea urchin egg homogenates where it was shown that $InsP_3$ generated by mammalian sperm extracts was due to the hydrolysis of PtdIns(4,5)P_2 associated with yolk vesicles [33]. There are no yolk vesicles of this type in mammalian eggs, but an equivalent organelle is the lysosome.

8.2. Does PLCζ play a role at fertilization in all species?

So far PLCζ has been identified in a number of different mammalian species. Introducing the mouse, human and monkey isoforms of PLCζ all cause Ca^{2+} oscillations in mouse eggs [37,39,41,43]. Hence, it seems likely that the role we propose for PLCζ at fertilization in mouse eggs applies to all mammalian species. However, it is noteworthy that PLCζ has also been found in two species of chickens (Table 1). Introduction of the chicken homologue of PLCζ into mouse eggs has been shown to cause Ca^{2+} oscillations similar to those seen with mouse or human PLCζ [65]. There are indications that a PLCζ-like protein may exist in other vertebrates since sperm extracts from both Xenopus and teleost fish have been shown to cause Ca^{2+} oscillations in mouse eggs [66,67]. This suggests that PLCζ or a similar PLC may play a fertilization role in many vertebrates. How far this extends to other chordates or invertebrates is unclear. There is evidence for the existence of a soluble 'sperm factor' in ascidians, since ascidian sperm extracts have also been shown to cause Ca^{2+} oscillations in ascidian eggs [68]. However no evidence exists to show that ascidian sperm extracts are associated with a PLC activity, or that they can cause Ca^{2+} oscillations when injected into mammalian eggs. So the nature of the ascidian sperm factor and its relationship to PLCζ remains to be established.

8.3. Does PLCζ play a role in human fertility?

PLCζ is present in humans and is suited to play the same role in activating the egg at fertilization that we suggest in the mouse. It is also presumed to play a role in activating human eggs after ICSI. During clinical ICSI, the sperm membrane is disrupted prior to injection of the sperm and this is likely to aid the release of factors such as PLCζ. There are some cases of egg activation failure after ICSI and it is possible that the sperm may sometimes lack the activating factor [69]. In these cases, eggs have been treated with Ca^{2+} ionophores in order to overcome activation failure as a clinical treatment [70]. As well as these rare cases, a relative lack of PLCζ may also lead to problems with fertility that are less obviously connected with what happens during fertilization. For example the 'knockdown' of PLCζ in mice suggests that a reduction of PLCζ and the associated deficiency in Ca^{2+} oscillations could lead to embryo arrest much later during development [45]. This idea is consistent with studies in rabbit eggs where the pattern of Ca^{2+} changes during egg activation have been shown to exert an effect upon the size and morphology of the foetus [71]. If these studies were applicable to humans, then despite the signs of successful egg activation, it is possible that a failure of pregnancy could be related to a deficiency of PLCζ in the sperm that fertilized the egg.

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