Chapter 50
Calcium Oscillations, Oocyte Activation, and Phospholipase C zeta

Junaid Kashir, Celine Jones, and Kevin Coward

Abstract In mammals, gamete fusion initiates a succession of oscillations in the intracellular concentration of calcium within the oocyte, prompting a series of events to occur that are collectively known as "oocyte activation". Such events are a fundamental necessity for the initiation of cell division and subsequent embryogenesis. Compelling evidence now indicates that these calcium oscillations are caused by a testis-specific phospholipase C (PLC) termed PLCzeta (PLCζ), released into the oocyte following gamete fusion. A series of recent studies indicate that abnormal expression or aberrant activity of PLCζ is linked to certain types of human male infertility, where oocyte activation ability is impaired or absent altogether. In this chapter, we discuss the critical role of calcium oscillations in the process of oocyte activation, review the role of PLCs in this fundamental biological reaction, describe how PLCζ has been formally linked to male infertility, and postulate the potential roles for PLCζ in terms of clinical diagnosis and therapy.

Keywords Sperm • Oocyte • Calcium • Phospholipase C (PLC) • Phospholipase C zeta (PLCzeta) • Fertilisation • Oocyte activation • Infertility • Assisted reproductive technology (ART) • Assisted oocyte activation (AOA)

Fertilisation and Oocyte Activation

At fertilisation, the fusion of mammalian gametes ensures the successful initiation of development. Mammalian oocytes remain arrested for most of their existence at the dictyate stage of prophase I [74]. These then undergo first meiotic division

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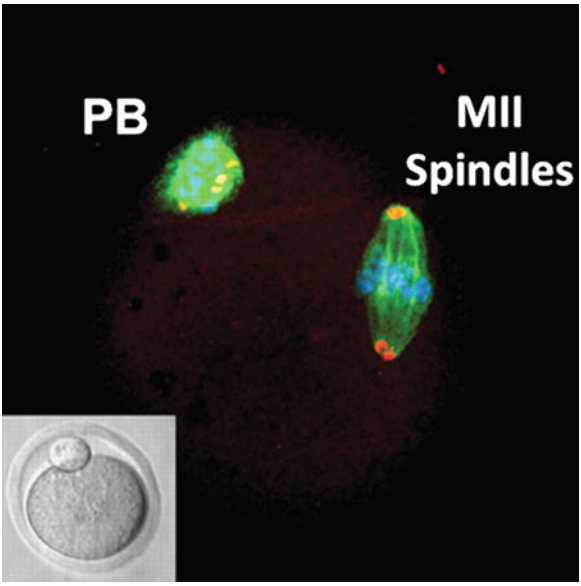

Fig. 50.1 Representative fluorescence micrograph of a normal MII arrested mouse oocyte. Inset shows brightfield image of the same oocyte. *Green*: β-tubulin (polymerized microtubules); *red*/*orange*: pericentrin (microtubule organizing centers); *blue*: chromatin. *PB* polar body (Reproduced from Swain et al. [143] with permission)

within the ovary, immediately after which, and just before ovulation, oocytes become arrested at metaphase of the second meiotic division (MII), following the exclusion of the first polar body [70, 71] (Fig. 50.1). This arrest is maintained by stabilization of the M-Phase promoting factor (MPF), the universal driver for the transition of the cell cycle from the G2- to the M-phase (G2/M transition). MPF is a heterodimer consisting of a regulatory cyclin subunit, cyclin B, and a catalytic subunit, Cdc2 kinase. Active Cdc2 drives entry into M-phase by phosphorylating substrates which lead to nuclear envelope breakdown and spindle formation [54]. Successful sperm/oocyte fusion at fertilisation must alleviate this MII arrest to proceed with embryogenesis.

Alleviation of MII arrest occurs through a series of events, collectively termed "oocyte activation". Characterized by the formation of the second polar body and the male and female pronuclei [105], oocyte activation also involves cortical granule exocytosis, progression of the cell cycle (Fig. 50.2a), and maternal mRNA recruitment, collectively resulting in cell division and embryogenesis (Fig. 50.2b) [79, 105, 123, 144, 145]. The sperm nucleus is remodelled, permitting DNA replication and fusion with the female pronucleus. The sperm nuclear envelope is removed, and sperm-specific protamines or histones are replaced by maternal histone variants [54]. Maternal mRNAs and proteins then undergo several dynamic changes, including regulated degradation, translation, or post-translational modification. The duration and initiation of these events differ between species, beginning at day 1 (2-cell stage) in the mouse. Cytoskeletal rearrangements also occur, presumably to support zygotic growth and development [54].

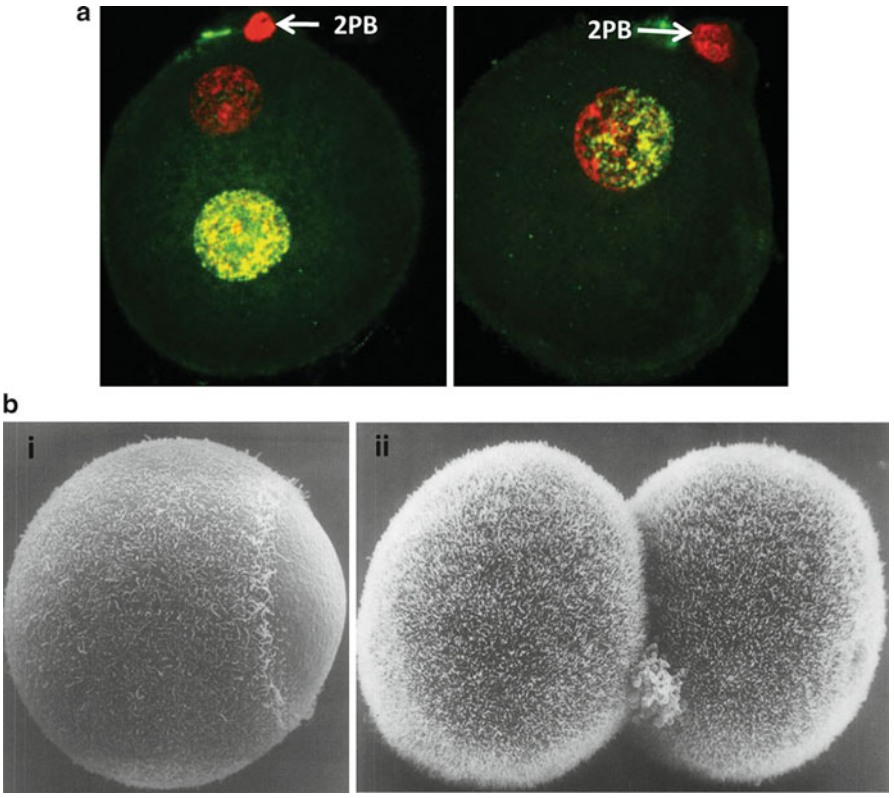

Fig. 50.2 Representative images showing key stages of oocyte activation. (**a**) Fluorescent micrograph of an activated mouse zygote with two pronuclei. *Right panel*: (*yellow*/*green*: larger male pronucleus; *red*: smaller female pronucleus). The nucleus of the second polar body (2PB) is visible above the female pronucleus. *Left panel*: A zygote with a single large pronucleus resulting from male and female pronuclei fusion. The male chromatin (*yellow*/*green*) occupies half of the pronucleus, while the other half is occupied by maternal chromatin (*red*). The nucleus of the 2PB (*red*) is apparent above the pronucleus, slightly to the *right*. (**b**) Scanning electron micrographs of (i) an unfertilised mouse oocyte, and (ii) the early mouse embryo at the 2 cell stage following removal of the zona pellucida (Reproduced from Phillips and Shalgi [121], Liu et al. [96], Krukowska and Tarkowski [83] with permission)

The Role of Calcium in Oocyte Activation

Several key experiments have established that the mechanisms underlying mammalian oocyte activation are induced by repeated oscillations in the concentration of intracellular calcium (Ca^{2+}) [79, 123], while in many non-mammalian species such as sea urchins and frogs, there is only a single Ca^{2+} transient (Fig. 50.3) [159]. Experiments involving the application of Ca^{2+}-sensitive dyes to oocytes and eggs

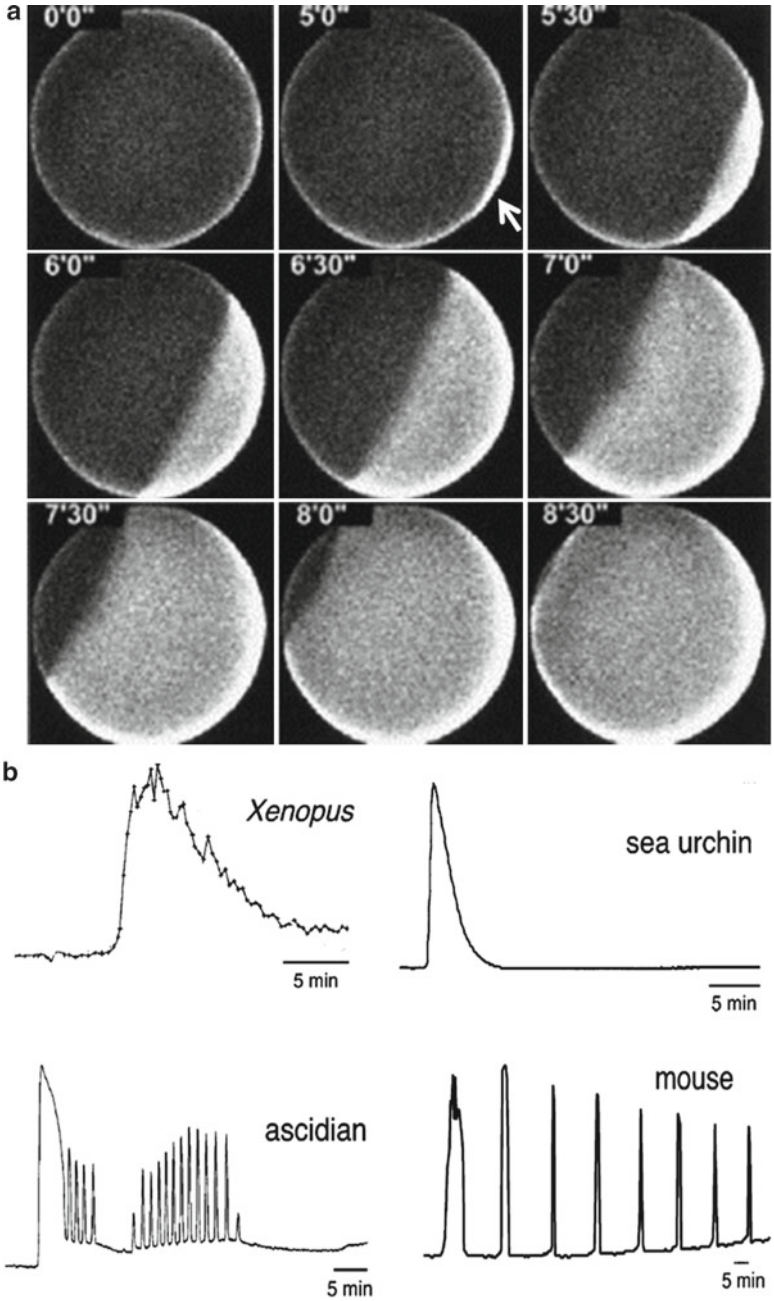

Fig. 50.3 (**a**) Calcium wave as observed using calcium-green-1-dextran in a *Xenopus* egg. Time 0 shows the egg's resting levels of calcium. The sperm-induced calcium wave was initiated by sperm entry (indicated by *white arrow*), and traversed the entire egg. (**b**) Ca^{2+} responses at fertilization in eggs and oocytes of several species (Reproduced from Fontanilla and Nuccitelli [39], Miyazaki [104], Miyazaki and Ito [105] with permission)

from a wide range of species, established the importance of Ca^{2+} transients in oocyte activation and embryogenesis [31], while the microinjection of Ca^{2+} ions alone triggered embryo development up to the blastocyst stage in mice [44, 145]. Repetitive Ca^{2+} oscillations at oocyte activation are thus necessary to alleviate MPF-mediated MII arrest, an insufficiency which may result in altered oocyte to embryo transition [30].

Ca^{2+} oscillations within the oocyte are commonly accepted to occur as a direct result of inositol triphosphate (IP_3)-mediated Ca^{2+} release [117, 119, 133, 146, 148, 159]. Blocking IP_3 receptors (IP_3Rs) within oocytes using specific antibodies [108], down-regulating IP_3Rs using adenophostin [12, 68], and reducing the expression of IP_3Rs by siRNA [164], all lead to the inhibition of Ca^{2+} oscillations. Studies have also reported cytosolic increases in the concentration of IP_3 during fertilization in mammalian oocytes [146], compounding the importance of IP_3 levels and IP3-mediated Ca^{2+} release within this process.

While the type 1 isoform of IP3Rs (IP_3R1) appears predominant in human and mouse oocytes [38, 46, 102, 120], it is thought that cells may express and redistribute ion channels. It is therefore likely that differential localization of such channels may impact upon developmental events [152, 155]. Indeed, the dynamic phosphorylation of IP_3R1 during maturation may increase IP_3R1 sensitivity, while sustained release of IP_3 during oocyte activation may lead to an overall reduction in IP3R1 number, a mechanism possibly responsible for the regulation of Ca^{2+} oscillations during oocyte activation [61, 93, 154].

Mammalian oocytes are released from MII arrest through the degradation of cyclin B1 by proteolysis, mediated by ubiquitin/proteasome activation via Ca^{2+} oscillations at fertilisation [105] (Fig. 50.4). Binding of Ca^{2+} to calmodulin activates calmodulin-dependent kinase II (CaMKII) [97, 105], a repetitive process which reoccurs coincident with each Ca^{2+} peak in fertilised mouse oocytes [100]. Cyclin B1 is then poly-ubiquitinated by the anaphase promoting complex or cyclosome (APC/C), an E3 ubiquitin ligase, resulting in degradation of cyclin B1. This process is prevented in unfertilised oocytes by cytostatic factor (CSF), which assists MPF in maintaining MII arrest [57, 69, 105]. Upon mammalian fertilisation, CaMKII is thought to inhibit CSF components Emi 1, Mad2, or Bub1 [57].

Persistent Ca^{2+} oscillations are thought to be responsible for pronucleus formation via the reduction of mitogen-associated protein kinase (MAPK) activity [30, 105]. The temporal pattern of Ca^{2+} oscillations is largely species-specific in terms of amplitude, duration, and frequency [30, 31, 72, 106]. Remarkably, oocytes appear to "count" each wave of oscillations, with early events such as cortical granule exocytosis requiring fewer oscillations than later events such as the alleviation of MII arrest [98, 140]. Indeed, the frequency and amplitude of Ca^{2+} oscillations have been reported to affect early embryo protein profiles in mice [30], and embryonic development in rabbits, playing a role in compaction, blastocyst formation, and the rate of successful transplantation of 4-cell embryo to host mothers [105, 145].

Spatial and temporal Ca^{2+} and IP_3 gradients assist in the establishment of the dorsal–ventral axis of various developing embryos [118, 158], with similar roles

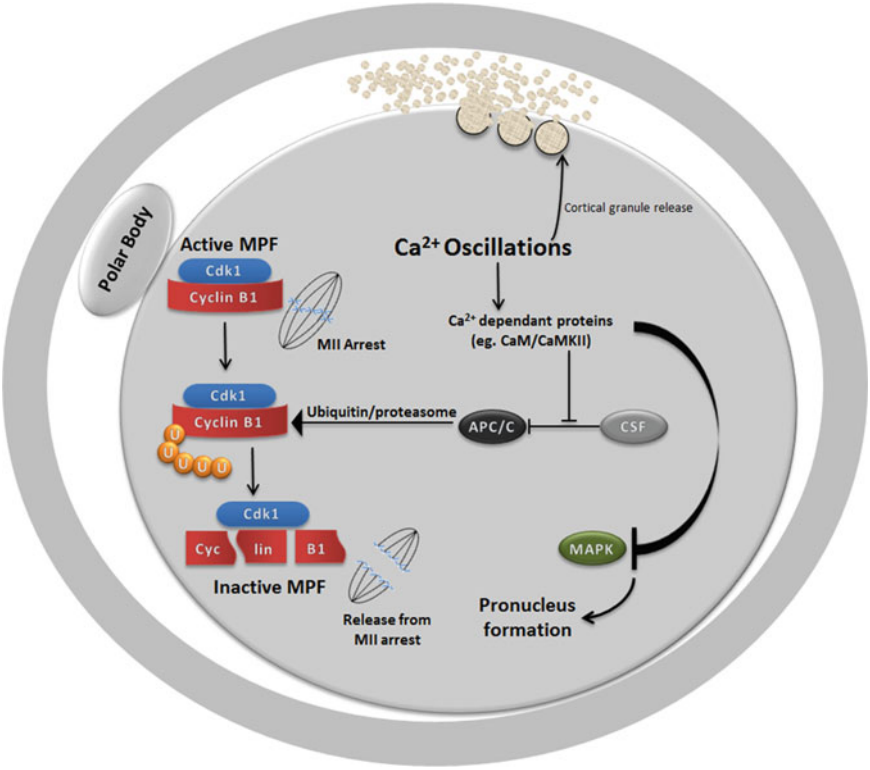

Fig. 50.4 Schematic representation of the signaling mechanism, downstream of Ca^{2+} release at fertilisation, thought to alleviate MII arrest in mammalian oocytes and leading to subsequent embryogenesis

for the velocity and duration of Ca^{2+} waves [65, 160]. Experiments inducing a variety of Ca^{2+} release profiles did not all induce good quality embryos [44, 84, 128, 153]. Ca^{2+} transients at fertilization are also responsible for the activation of Ca^{2+}-sensitive genes or proteins important in subsequent embryo development, such as CAMKII [28, 55]. Indeed, CaMKIIγ (the predominant CamKII isoform in mouse oocytes) is thought to specifically control mouse oocyte activation via cell cycle resumption [3].

Although it is well-established that Ca^{2+} oscillations are of the utmost importance for oocyte activation, the precise mechanism responsible has been the subject of intense debate, particularly in relation to the relative roles played by both gametes during gamete fusion. Three predominant models have been hypothesized: (1) the Ca^{2+} conduit model, (2) the membrane receptor model, and (3) the soluble sperm factor model [119, 133, 148].

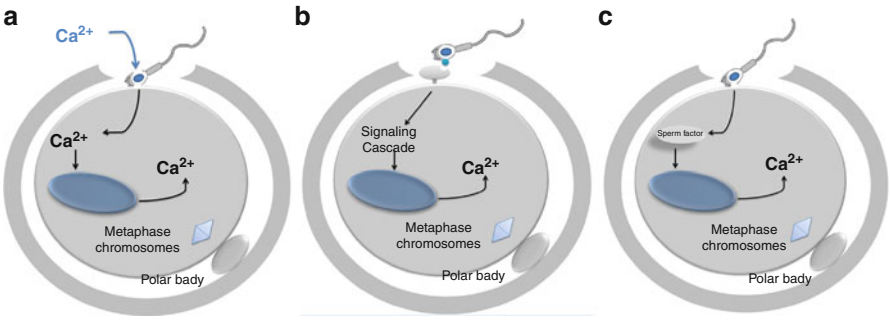

Fig. 50.5 Schematic representations of (**a**) Ca^{2+} conduit, (**b**) membrane receptor, and (**c**) sperm factor models of oocyte activation (Figure adapted from Parrington et al. [119])

The Ca^{2+} Conduit Model

This model hypothesizes the introduction of a bolus of Ca^{2+} directly into the oocyte following gamete fusion, leading to Ca^{2+}-induced Ca^{2+} release [62, 64], and was subsequently modified, based on sea urchin sperm-egg binding, to suggest that channels on the surface of the sperm membrane acted as a conduit following gamete fusion, allowing Ca^{2+} influx into the oocyte (Fig. 50.5a) [23, 24]. While an attractive model initially, particularly for sea urchin egg activation, this model did not take into account mechanisms of activation in other species such as mice or ascidicans. Experimental evidence strongly implicated that IP_3-mediated Ca^{2+} release was an integral aspect of oocyte activation. Furthermore, direct injection of Ca^{2+} into oocytes failed to induce further Ca^{2+} increase [145], whilst other studies failed to detect localised elevations in cytoplasmic concentrations of Ca^{2+} during mouse gamete fusion [72]. Furthermore, sea urchin eggs can be activated by acrosome-reacted sperm in seawater lacking Ca^{2+} [35, 159], rendering it difficult to envisage how Ca^{2+} flux through the sperm could be sustained where Ca^{2+} concentrations are lower or similar to resting concentrations within the egg [72].

The Membrane Receptor Model

This model proposed that an interaction between a ligand on the sperm surface and a corresponding receptor on the oocyte triggered activation (Fig. 50.5b), and was the dominant hypothesis for many years [36, 63, 119, 134]. Adopting one of the most fundamental features of cell signaling in somatic cells, where such surface mediated interactions are a normal feature, this model proposed that a signaling cascade triggered by sperm/egg interaction, would lead to the activation of a

phospholipase C (PLC) inside the oocyte [36, 63, 134]. At present, the proposed oocyte-sperm receptor is based on indirect evidence, arising from experiments involving the over-expression of G-protein-linked receptors in oocytes and corresponding application of ligands (Santella et al. 2004). Furthermore, sperm-induced Ca^{2+} transients were inhibited by the injection of G-protein antagonists in hamster oocytes, indicating that G-proteins may activate PLCβ in response to sperm-oocyte interaction ([103]; Santella et al. 2004), while injection of the hydrolysis-resistant GTP analogue, GTP-γS, in sea urchin and frog eggs and mammalian oocytes elicited Ca^{2+} release ([66]; Santella et al. 2004). However, while these experiments indeed demonstrated Ca^{2+} release, the transients observed were dissimilar to those observed at fertilisation, particularly in the case of mammalian oocytes. Furthermore, despite many targeted research studies, such a sperm ligand/oocyte receptor remains to be characterized [119]. Critically, the successful nature of intracytoplasmic sperm injection (whereby sperm is directly microinjected into the oocyte cytosol; ICSI) casts significant doubt over such a mechanism as this method bypasses sperm/oocyte membrane interaction completely.

The Soluble Sperm Factor Model

This model proposed that oocyte activation is triggered by a soluble factor released from the sperm into the oocyte during, or immediately following, gamete fusion (Fig. 50.5c). This was supported by the injection of sea urchin sperm extracts into eggs, which induced egg activation [26], followed by similar observations using ascidian sperm [25], suggesting that the activating stimulus was a protein or a Ca^{2+} mobilizing messenger. A series of studies, in which sperm extracts were injected into eggs of a variety of species, including marine worms, and ascidians, resulted in successful Ca^{2+} release and oocyte activation [88, 141, 144]. Sperm extracts from frogs, chickens, and tilapia fish, have also been shown to trigger Ca^{2+} oscillations in mouse oocytes [18, 29], suggesting the existence of a similar sperm-based mechanism throughout a wide spectra of species. However, the precise identity of this "sperm factor" remained a mystery for some time, although considerable research suggested that the IP_3-dependant nature of oocyte activation depended upon a PLC-mediated mechanism [119].

PLCs at Fertilisation and Oocyte Activation

Phosphoinositide metabolism is a vital intracellular signaling system involved in many cellular functions including hormone secretion, neurotransmitter transduction, growth factor signaling, membrane trafficking, cytoskeletal regulation, and have also been linked to fertilisation and embryogenesis ([15, 67, 135]; for reviews see [43, 110]). PLCs are key enzymes within this system, involved in the hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). DAG mediates protein kinase C (PKC) activation, and IP3 triggers Ca^{2+} from

intracellular stores [8, 43, 113]. Ca^{2+} is also a major player in many other aspects of sperm function, including chemotaxis [5, 9, 16, 32, 76, 138]. PLC and IP_3R signaling mechanisms are also involved in sperm thermotaxis, where elevated levels of Ca^{2+} may modify flagellar bending and sperm motion paths [4, 5, 10, 33, 53].

Currently, there are 13 known mammalian PLC isozymes, categorized on the basis of structure and regulatory activation mechanisms: PLC-delta (PLC-δ 1, 3, and 4), PLC beta (PLCβ 1–4), PLC gamma (PLCγ 1 and 2), PLC epsilon (PLCε), PLC zeta (PLCζ), and PLC eta (PLCη 1 and 2) [43, 56, 109, 125, 175]. PLC isozymes may contain catalytic X and Y domains as well as various regulatory domains, including the C2 domain, EF-hand motif, and pleckstrin homology (PH) domain, in various conformations, depending on the isozyme. Some isozymes also consist of subtype-specific domains, thought to contribute to towards specific regulatory mechanisms. These include the Src homology (SH) domain in PLC γ [125] and the Ras-associating and Ras-GTPase exchange factor-like domains in PLC ε [77, 137].

Sperm from PLCδ4 knockout (KO) mice induced activation of fewer oocytes following *in vitro* fertilization (IVF), failing to elicit Ca^{2+} oscillations, indicating an essential role in oocyte activation in sperm for PLCδ4 [41]. Solubilized mouse zona pellucida (a protective glycoprotein layer surrounding the oocyte; ZP) was able to induce the acrosome reaction (an essential step in fertilization involving the digestion of the ZP) in sperm from normal mice, but could not do so in PLC δ4 KO sperm. Elevations of Ca^{2+} levels were thought to have a primary role in this process [11, 27], and sperm from normal mice treated with ZP exhibited continuous elevations in Ca^{2+}, while incubation of ZP with PLCδ4 KO sperm induced only a small increase in Ca^{2+}, suggesting a role for PLCδ4 in the ZP-induced acrosome reaction [42, 43, 110].

Evidence suggests that PLCγ1, modulated by tyrosine phosphorylation, may be activated in mouse spermatozoa [37, 129, 151]. Immunostaining indicated that PLCγ-1 was localised to the sperm head, and that capacitation induced a translocation of this localisation pattern [151]. An increase in PLCγ 1 activity was observed following treatment with ZP, an observation prevented by suppressing ZP-induced acrosomal exocytosis [95, 129]. While there is no direct evidence supporting a role for PLCβ in sperm, both PLCβ1 and β3 have been identified in acrosomal regions of mouse sperm, along with Gαq/11 [41, 157]. Considering that PLCβ is activated by pertussis toxin-insensitive GTP-binding proteins Gq and G11 in somatic cells, the observation that progesterone-stimulated DAG formation was not blocked by pertussis toxin may infer a possible role for PLCβ in acrosomal exocytosis [108, 129]. Indeed, sperm from PLCβ1 KO mice exhibited lower acrosome reaction rates than their normal counterparts [14]. However, more in-depth studies are required to further elucidate the exact roles of these PLC isozymes during fertilisation [129].

Several observations suggest that there may be a role for endogenous oocyte PLCs during oocyte activation [132]. Oocytes contain significant amounts of PLC isoforms, including β, γ, and δ, which may be regulated by the Ca^{2+} oscillations at fertilisation [58]. Reduced levels of oocyte PLCβ1 decreased the amplitude of Ca^{2+} oscillations at oocyte activation, but did not change their duration or frequency.

Over-expression of PLCβ1 in oocytes prior to fertilization did not result in spontaneous Ca^{2+} oscillations, but altered the Ca^{2+} oscillation profile following fertilization, indicating a role for oocyte derived PLCs in sperm-induced oocyte activation in mammals [58]. Furthermore PLCβ1 has been implicated in nuclear translocation following meiotic resumption in mouse oocytes, apparently to perichromatin and interchromatin granules, followed by a subsequent shift to the nucleoplasm [2, 96].

In starfish eggs, the Ca^{2+} rise associated with activation requires an egg Src family kinase (SFK) that activates PLCγ via a SH2 domain-mediated mechanism involving the endoplasmic reticulum (ER) [130, 150]. It is not yet known if PLCδ, ε, or ζ are present in echinoderm oocytes. However PLCβ may be activated by heterotrimeric G protein-coupled receptors, while PLC γ may be activated by receptor and non-receptor protein tyrosine kinases (PTK), or via translocation to the plasma membrane [130, 166]. However, whether G protein-PLCβ or PTK-PLCγ plays a role during egg activation in other invertebrates, or whether these pathways function synergistically remains controversial [166].

Coward et al. [20] recently identified a novel PLCδ isoform in sea urchin gametes, termed PLCδsu, although its precise role during fertilization and early embryogenesis remains unknown. While a green fluorescent protein tagged PLCδsu PH domain was observed to localise to the plasma membrane, increasing in intensity at fertilization, recombinant PLCδsu failed to elicit Ca^{2+} signals characteristic of fertilization in mouse oocytes and sea urchin eggs, suggesting that PLCδsu may not be directly involved in egg activation, but may play a role in further downstream extracellular signals. Intriguingly, the *in vivo* expression of PLCδsu cRNA did not result in Ca^{2+} transients in either mouse or sea urchin eggs. This observation is consistent with the behaviour of recombinant PLCβ1, PLCγ1, PLCγ2, PLCδ1, PLCδ3 and PLCδ4 protein and cRNA, none of which cause Ca^{2+} release in mouse oocytes. However, the specific PLC isozyme responsible for oocyte activation itself remained a mystery until Saunders et al. [132], using mouse express sequence tag databases, identified a novel, and importantly testis-specific, PLC, termed PLCzeta (PLCζ), a protein ~74 kDa in mice and ~70 kDa in humans, which was proven to play a key role in mammalian oocyte activation.

PLCζ, the Mammalian Oocyte Activation Factor

General consensus agrees that the soluble factor which elicits Ca^{2+} release within oocytes is sperm-specific, as extracts from other tissues do not induce Ca^{2+} increase upon oocyte injection [144, 162]. Data indicated that the mammalian sperm factor was a sperm-specific PLC possessing distinct enzymatic properties compared to other known PLCs [72, 73]. Indeed, this correlated with the hypothesis that oocyte activation involved Ca^{2+} oscillations generated in an IP_3-mediated manner, supporting the notion that the soluble sperm factor was a PLC which mediated the hydrolysis of PIP_2 to IP_3 and DAG [106, 132, 146]. Expression studies in mice concluded that PLCζ mRNA was first detectable in spermatids [132].

More systematic studies of PLCζ mRNA expression during porcine spermatogenesis observed PLCζ mRNA translation in elongating spermatids [168]. Northern blot analyses of testes from post-natal hamsters showed that PLCζ mRNA was present as early as day 17 [171].

A large body of evidence supports the identity of the mammalian oocyte activation factor as PLCζ. The injection of recombinant PLCζ RNA into mouse oocytes resulted in the initiation of Ca^{2+} oscillations and supported embryonic development to the blastocyst stage [82, 132]. Immuno-depletion of PLCζ from sperm extracts suppressed their ability to release Ca^{2+} [132], while sperm fractionation studies correlated the presence of PLCζ in sperm to their ability to induce Ca^{2+} oscillations [40, 86]. Furthermore, RNA interference (RNAi) experiments yielded transgenic mice exhibiting disruption of PLCζ expression in the testis, while sperm from these mice induced Ca^{2+} oscillations that ended prematurely, with a clear reduction in litter size [80]. Data strongly suggests that PLCζ may be a universal feature of vertebrate oocyte activation. For example, sperm extracts and PLCζ cRNA from one species are able to elicit Ca^{2+} release upon microinjection in oocytes from another species [7, 22]. Furthermore, non-mammalian PLCζ homologues have been identified in the chicken [19], medaka fish [60], and quail [107].

Immunofluorescence studies [40, 169, 171] have shown that PLCζ is predominantly localized to post-acrosomal regions of the sperm head in mice, a pattern maintained following the acrosome reaction (Fig. 50.6a) [171]. Intriguingly, however, this pattern of PLCζ immunofluorescence was reported to change during capacitation [171], increasing in intensity and implying modified PLCζ structure or molecular interactions, or that PLCζ becomes more accessible to antibody following capacitation due to physiological changes within the sperm membrane [49, 156, 171]. Intriguingly a second acrosomal population of PLCζ was also identified, suggesting multiple roles for PLCζ besides oocyte activation, such as the acrosome reaction [171]. Immunoblots of porcine sperm extracts revealed a variety of isoforms [40], ranging from the expected full length 74 kDa PLCζ to fragments ranging from 27 to 50 kDa, all of which may exist in a stable complex, retaining enzymatic activity [87].

In non-capacitated human sperm, PLCζ is predominantly localized to equatorial regions of the sperm head (Fig. 50.6b) [47, 170], an ideal location for a sperm factor since the equatorial segment of the sperm remains intact following the acrosome reaction [6, 47, 161], allowing PLCζ to be one of the first sperm proteins to be released into the oocyte following gamete fusion [47]. As in mouse and hamster sperm, a second population of PLCζ was also identified in acrosomal regions of human sperm [47], while capacitation resulted in dynamic changes in localization patterns in post-acrosomal/equatorial regions [47, 171].

Studies suggest that human PLCζ may be much more potent that other isoforms at eliciting Ca^{2+} release [173]. An interesting point to consider is the fundamental requirement of a specific amount of PLCζ for the initiation of Ca^{2+} oscillations within the oocyte. Although, too little or too much PLCζ does indeed cause Ca^{2+} oscillations within the oocyte, it is clear that such oscillations are either not sufficient to activate oocytes, or cause excessive Ca^{2+} signaling, resulting in activation

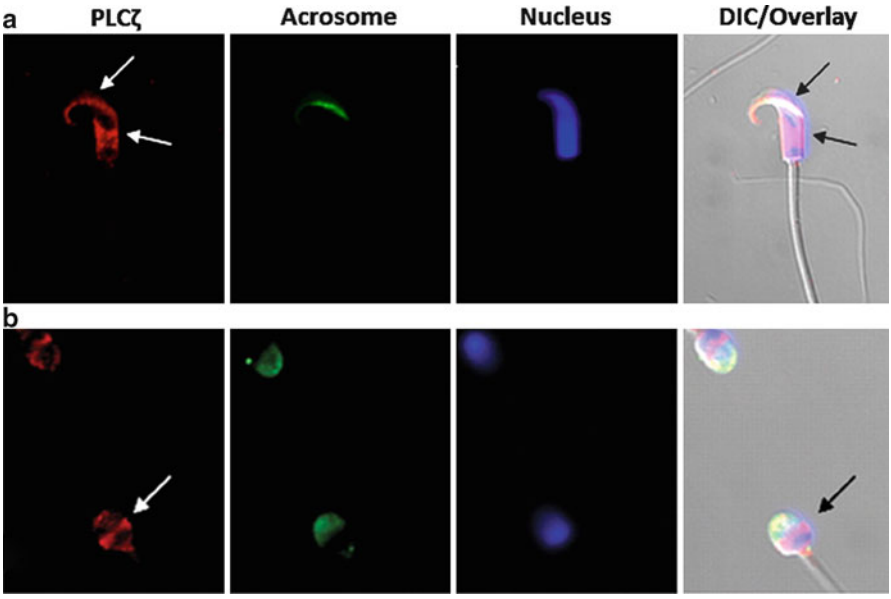

Fig. 50.6 Immunofluorescent localization of PLCζ (*red*) in hamster (**a**) and human (**b**) sperm. Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA)-lectin staining (*green*) identifies the acrosome, and Hoescht-33342 staining (*blue*) identifies the nuclei. *Arrows* indicate the immunolocalization of PLCζ within the acrosome and equatorial segment of hamster and equatorial segment of human sperm (Reproduced from Young et al. [171] and Grasa et al. [47] with permission)

failure [127, 173]. Different species have been shown to possess varying amounts of PLCζ within sperm, with species delivering "fine-tuned" doses of PLCζ, with varying potencies adjusted via evolutionary processes to match the size and sensitivity of the oocyte being activated [148]. The varying solubility of PLCζ in sperm of various species may also contribute to this inference. Hamster sperm initiates Ca^{2+} oscillations within approximately 10 s of gamete fusion, whereas there is a 1–3 min delay before Ca^{2+} oscillations begin in mice [91]. It is therefore possible that PLCζ activity and concentration in mammalian species has naturally been optimised to achieve the precise Ca^{2+} oscillation profile required for normal oocyte activation and embryo development within that species [17]. Recent data also suggests that PLCζ RNA transcripts are present within sperm [89, 122], which may be transcribed during fertilization, sustaining a "long-lived" Ca^{2+} response [89], which may also explain the presence of varying PLCζ isoforms within the sperm. However, the role for PLCζ and other RNA transcripts within sperm remain elusive, and require further examination [74].

Interestingly, while all mammalian isoforms of PLCζ have been testis-specific, Coward et al. [21] identified ovarian and brain forms of PLCζ in puffer-fish. Injection of cRNA corresponding to the ovarian isoform into mouse oocytes did not result in Ca^{2+} oscillations. This is particularly interesting given the debate regarding the

mechanism responsible for oocyte activation [21, 36, 105, 119]. The discovery that PLCζ may be present in oocytes of some species raises the question of how a sperm stimulus may activate the oocyte. While the majority of scientific opinion agrees that PLCζ is the endogenous sperm factor, recent studies have demonstrated possible candidates for sperm factors apart from PLCζ, which are able to induce meiotic progression or elicit Ca^{2+} oscillations in a variety of species. Harada et al. [48] identified a new 45 kDa protein, termed citrate synthase, as the major component responsible for egg activation in the newt *Cynops pyrrhogaster*. Wu et al. [163] also reported another possible candidate for the sperm factor, which resides in the post-acrosomal sheath region of the perinuclear theca, termed post-acrosomal sheath WW domain-binding protein (PAWP), in bovine sperm and other mammalian species. However, the molecular mechanisms underlying the precise function of both PAWP and citrate synthase are currently unknown [1, 163]. It is, of course, possible that oocyte activation involves the collective action of PLCζ and other sperm factors. However, this theory remains to be established [74].

PLCζ Structure and Function

While general consensus now agrees that PLCζ is the physiological agent of mammalian oocyte activation [70, 74, 119, 148, 159], many questions remain regarding its precise mechanism of action. PLCζ is unique from other PLC isoforms in its high sensitivity to Ca^{2+} and Ca^{2+} oscillation induction [81, 124]. In all species in which PLCζ has been characterized, PLCζ ranges from 70 to 75 kDa in molecular weight [148], representing the smallest PLC isoform found to date. PLCζ exhibits typical PLC domain structure [132] with closest homology to PLCδ isoforms [75, 124], particularly PLCδ1 [148]. PLCζ possesses characteristic X and Y catalytic domains which form the active site common to all PLCs [124, 148], a C2 domain and a set of EF hands, similar to PLCδ (Fig. 50.7).

PLCζ is unique from other PLC isoforms in that it lacks a PH domain [124, 132], and it is thus unclear as to how PLCζ targets its membrane-bound substrate PIP_2. Intriguingly, experiments suggest that the PH domain is not integral to the membrane localizing ability of PLCδ4 [92], leaving the possibility open that PLCζ localizes to the plasma membrane through an alternate mechanism. It is possible that indirect interaction binds PLCζ to the membrane, or indeed that PLCζ acts upon cytosolic PIP_2 instead of membrane-bound forms. Regardless, it would seem that the target of PLCζ is membrane-bound PIP_2, since this would increase the local concentration of substrate for the enzyme to act upon, significantly increasing hydrolysis rate [78, 101, 124, 125].

PLCζ has four EF-hand motifs at the N-terminus, which are important for enzymatic activity [148], and are arranged in a similar lobed way to the Ca^{2+} binding domains of calmodulin and troponin [124, 148]. Deletion of the EF hands led to a reduction in Ca^{2+} response upon injection of cRNA into mouse oocytes [114], suggesting that the EF hands play a structural role in maintaining PLCζ activity. However, there is some conflicting data with regards to the importance of EF hands

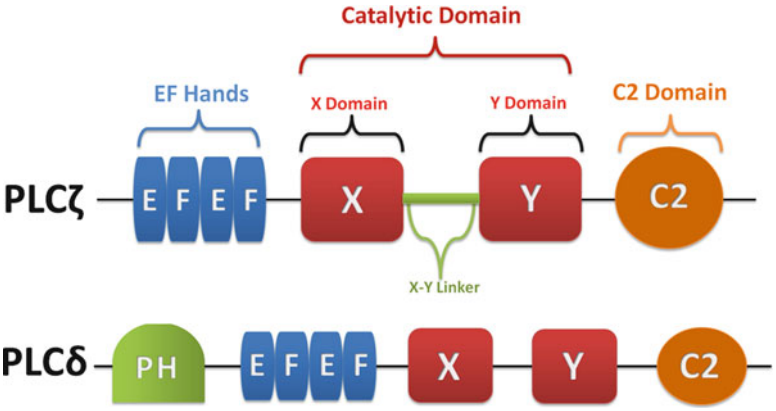

Fig. 50.7 Schematic illustrations of domain structure for PLCζ and it's closest PLC homologue, PLCδ

in the Ca^{2+} oscillation producing ability of PLCζ, where offspring were obtained following round spermatid injection (ROSI) in mouse oocytes, along with the injection of a truncated form of PLCζ [111] reportedly found in the testis where the first three EF hands are lacking [40, 81, 86, 167].

C2 domains are able to bind to phospholipid containing membranes, as is the case with PLCδ1 binding to phosphatidylserine or PLCA2 binding to phosphatidylcholine [126]. Deletion of the PLCζ C2 domain led to some loss of enzymatic activity, and only a slight reduction in Ca^{2+} sensitivity. However, the injection of PLCζ cRNA lacking the C2 domain in mouse oocytes did not induce Ca^{2+} release, implying that the C2 domain is also required for oocyte activation [114, 148].

A non-catalytic PLCζ domain possibly involved in the regulation of activity is a segment between the X and Y catalytic domains, referred to as the X-Y linker [115, 148]. This apparently unstructured cluster of residues shows greatest divergence between species, and is longest in *Macaca fascicularis*, but shortest in humans [132, 148]. It remains unclear what these differences mean in terms of biological importance, but the proximity of the X-Y linker to the active site implies potential involvement in regulating catalytic activity, or by sequestering PIP_2 in the locality of PLCζ in an electrostatic manner [115, 148]. However, unlike PLCγ, the PLCζ X-Y linker does not contain any regions of predicted secondary structure, casting doubt as to how the X-Y linker would play a role in anchoring PLCζ to PIP_2 [148]. There is also evidence to suggest that PLCζ remains functional following proteolytic cleavage at the X-Y linker, which can form complexes that retain activity [87]. Indeed, proteolytic cleavage may be required for PLCζ to be able to bind to the membrane and act upon PIP_2 [132]. Intriguingly, studies show that the X-Y linker in some, if not all, PLC isoforms play an auto-inhibitory role, the deletion of which results in elevated activity [52].

Following fertilization, Ca^{2+} oscillations in the oocyte cease after pronuclei formation [99], following which some oscillations are subsequently observed in

mouse zygotes during mitosis [13, 99]. One possible explanation of this cell-cycle dependant termination and resumption, was that PLCζ was localized to the pronuclei during interphase [13, 148], resulting in the cessation of Ca^{2+} oscillations which resume following pronuclear envelope breakdown during the oocytes entry into mitosis [148]. When pronuclear formation was inhibited, Ca^{2+} oscillations persisted for much longer than normal [60, 99]. cRNA coding for mouse PLCζ tagged with either a Venus fluorescent protein or a Myc epitope, revealed that PLCζ was associated with the nascent pronuclei [85, 90, 135, 167]. Tagged PLCζ was also observed to return to the cytoplasm during first mitosis, coinciding with the resumption of Ca^{2+} oscillations [90].

This "nuclear sequestration" activity appears to be the result of a specific signal sequence within the X-Y Linker region of PLCζ, termed the nuclear localisation signal (NLS) [85, 90, 148]. Mutational analysis of the NLS resulted in termination of PLCζ nuclear sequestration, but still induced Ca^{2+} oscillations which continued beyond pronuclear formation, suggesting that PLCζ pronuclear sequestration plays a role in regulating Ca^{2+} oscillations at fertilization [90, 148]. While a NLS has been predicted in a variety of mammalian PLCζs, located in a similar location within the PLCζ structure [148], only the mouse PLCζ NLS has been shown to be functional in oocytes [148]; further study is required for other species. Alternate explanations for the termination of Ca^{2+} oscillations have also been reported. For example, changes in the cellular distribution of IP_3Rs [46] may also be involved. Indeed, IP_3 is known to be down-regulated following fertilization [12, 68]. Alternatively, a negative feedback mechanism involving the production of DAG and subsequently protein kinase C (PKC) might affect PLCζ activity [172].

PLCζ is known to re-accumulate in blastomere nuclei [85, 135]. It is therefore possible that PLCζ may promote further cell division during embryogenesis in a Ca^{2+} dependant manner. As PLCζ targets nuclei specifically during interphase [135], there may be a role for PLCζ at this stage of the cell cycle. Indeed, PLCβ1 translocates to nuclei during G2 to M transition in immature mouse oocytes, contributing towards the breakdown of the germinal vesicle via DAG and PKC [2, 45, 142]. PLCδ1 also possesses a NLS [165], which in a manner similar to PLCζ, localises to nuclear structures at the G_1/S phase boundary of the cell cycle [139, 142]. Suppressing PLCδ1 results in increased levels of cyclin E, thus inhibiting cellular proliferation [139]. When considered with reports that concentrations of nuclear PIP_2 are significantly increased at the G_1/S boundary, and at least doubled at G_0, one can speculate that PLCδ1 may regulate certain nuclear functions [142, 174], a possibility that should be investigated for PLCζ.

The Role of PLCζ in Human Infertility

Considering the pivotal role of PLCζ in mammalian fertilisation, defective forms of PLCζ may contribute to cases of male infertility where oocyte activation is deficient (OAD) [74]. Infertility represents a major problem to a growing proportion of the global population. Consequently, worldwide investment in assisted reproductive

technology (ART) has significantly increased over recent years. ART now accounts for ~7% of all births in some developed countries [112], and over eight million ART babies have been born worldwide thus far [59]. A key technique in ART is intracytoplasmic sperm injection (ICSI), whereby a sperm is microinjected directly into the oocyte cytosol. Predominantly used to treat male factor infertility following the failure of conventional *in vitro* fertilisation (IVF), ICSI remains a highly successful technique that, on average, results in normal fertilization in 70% of cases [112]. However, a noted phenomenon associated with male infertility is the failure of some oocytes to activate, even following ICSI (for review, see [74]). Recent studies have successfully linked cases of oocyte activation failure to aberrant expression, localization and structure of PLCζ in sperm from patients diagnosed with oocyte activation deficiency, particularly in cases of globozoospermia (round headed, acrosome-less sperm) [50, 170].

Sperm from some infertile men who consistently fail IVF and ICSI are either unable to elicit Ca^{2+} oscillations upon injection into mouse oocytes, or produce oscillations uncharacteristic of fertilisation, showing reduced frequency and amplitude (Fig. 50.8a). Furthermore, immunofluorescence and immunoblots have revealed that sperm from ICSI failed infertile patients showed abnormal PLCζ expression (Fig. 50.8b) [50, 170]. Interestingly, and of crucial importance to future therapeutic options, the activating ability of ICSI failed human sperm can be rescued upon co-injection with mouse PLCζ mRNA (Fig. 50.8c) [170]. Interestingly, sperm from wobbler mice (a strain of mice which suffer from motor neuron disease caused by a naturally occurring mutation, in whom males are infertile) elicit low rates of fertilisation using ICSI, and exhibit an abnormal PLCζ localisation profile. Intriguingly, sperm from these mice produce healthy pups following ICSI and artificial oocyte activation (AOA) [51]. Taylor et al. [149] further showed that successful pregnancy and birth could be achieved using globozoospermic sperm lacking PLCζ by utilising ICSI and AOA [149] (for review on AOA see [74, 112]).

Importantly, Heytens et al. [50] identified a substitution mutation in a non-globozoospermic infertile male within the Y domain of PLCζ at position 398, resulting in a histidine to proline substitution (H398P) (Fig. 50.9a). Predictive modelling suggested that this residue change may disrupt the active site loop (Fig. 50.9b), and the ability of PLCζ to interact with PIP_2 [116]. Multiple sequence alignments confirmed that the histidine at this position is highly conserved across all mammalian [22, 132, 168, 171], chicken [19], and medaka isoforms of PLCζ [60], as well as all PLCδ isoforms [34, 132], indicating that this residue may play a critical role within the active protein [50]. Furthermore, the injection of H398P PLCζ cRNA into mouse oocytes resulted in highly abnormal Ca^{2+} release which was insufficient for oocyte activation (Fig. 50.9c) [50].

Yoon et al. [170] and Heytens et al. [50] provided the first key link between defective PLCζ and human male infertility. However, further analytical studies are needed to explore the precise functional effects of the H398P mutation identified by Heytens et al. [50]. Studies investigating larger cohorts of patients, from a range of

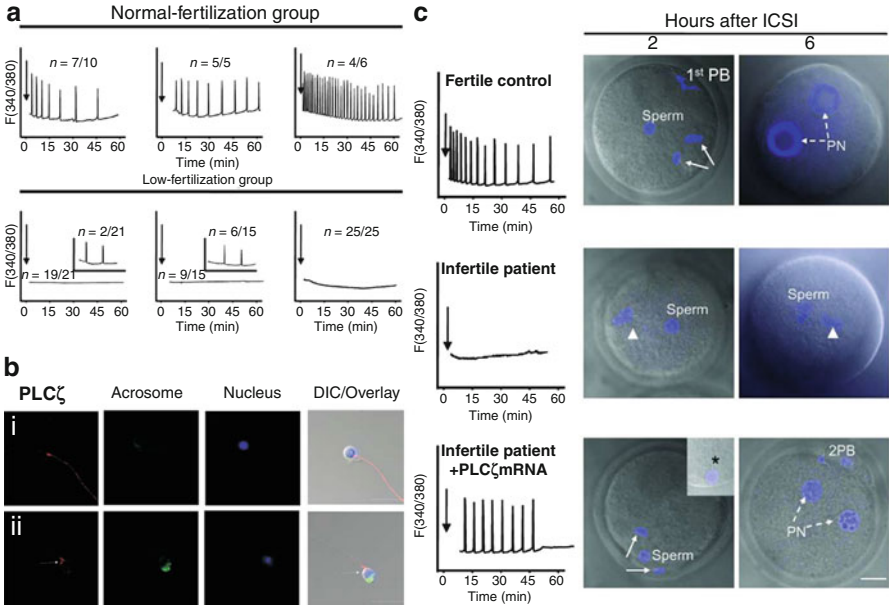

Fig. 50.8 (**a**) Ca^{2+} oscillation profiles following the injection of sperm from normal fertile humans (*top panel*) and infertile patients who had previously failed or exhibited low fertilisation rates following ICSI (*bottom panel*). *Arrows* denote time of sperm injection, and *n* indicates the number of mouse oocytes exhibiting the corresponding Ca^{2+} oscillation profile. (**b**) Reduced PLCζ immunostaining (*red*) in sperm from infertile ICSI failed patients exhibiting abnormal morphology (in this case globozoospermia; acrosome-less, round-headed sperm) (i), and normal morphology (ii). Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA)-lectin staining (*green*) identifies the acrosome, and Hoescht-33342 staining (*blue*) identifies the nuclei. *Arrow* indicates reduced PLCζ immunolocalisation of within the equatorial segment. Scale bars indicate 5 μm. (**c**) (*Top panel*) Injection of a sperm from a fertile control was able to elicit Ca^{2+} oscillations and activate mouse oocytes. (*Middle panel*) Sperm from an infertile patient with a history of ICSI failure was unable to elicit Ca^{2+} oscillations, and was unable to activate mouse oocytes following injection, but was able to do so following the co-injection of PLCζ mRNA (*Bottom panel*). *Arrows* denote time of sperm injection. *1st PB* first polar body, *2PB* second polar body. TO-PRO-3 staining (*blue*) stains chromatin. *Asterisk* in inset points to the persistence of the human sperm tail in mouse oocytes. Scale bar indicates 10 μm (Reproduced from Yoon et al. [170] and Heytens et al. [50] with permission)

biogeographical regions, targeting specific male factor conditions, and utilizing high-throughput genetic screening techniques may be invaluable in discovering the prevalence of such mutations in human infertility.

Further analyses should also systematically map out specific clinical conditions in which aberrant forms or reduced expression of PLCζ may play a role, and thus provide the foundations for the development of a therapeutic version of PLCζ to aid such patients as an alternative to current AOA protocols which remain the source of

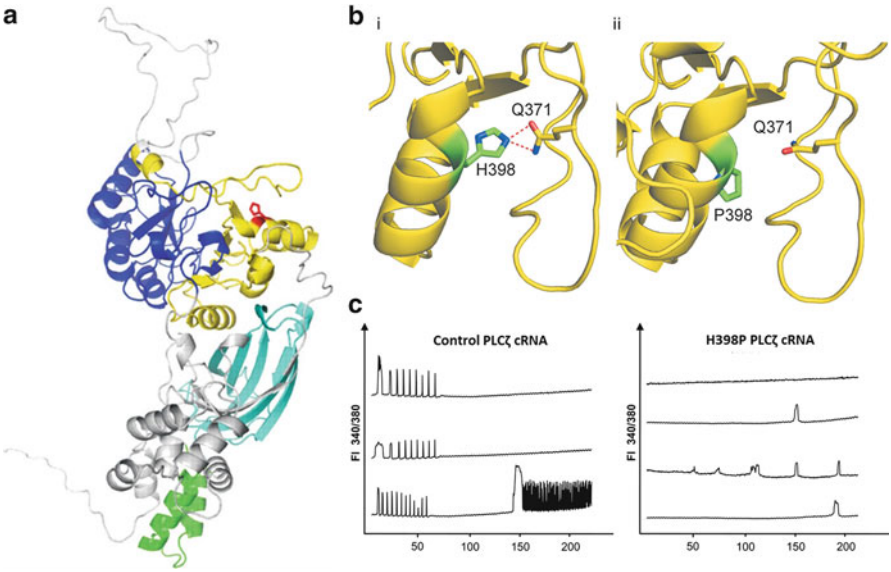

Fig. 50.9 Histidine>Proline point mutation (H398P) identified by Heytens et al. [50] in an infertile male patient diagnosed with oocyte activation deficiency. (**a**) Model of human PLCζ functional domains (*green* – EF hands, *blue* – X catalytic domain, *yellow* – Y catalytic domain and *cyan* – C2 domain). Histidine398 (H398) is shown in *red*. (**b**) Close-up of H398 showing side-chain-side-chain hydrogen bonds (bi), alongside a close-up of P398 in mutant PLCζ showing no side-chain-side-chain hydrogen bonds (bii). (**c**) Microinjection of wild type and mutant PLCζ into mouse oocytes and resulting calcium release patterns (Reproduced from Heytens et al. [50] with permission)

some concern [74]. While Yoon et al. [170] provided the first real evidence the PLCζ as a novel therapeutic, Rogers et al. [127] provided additional supportive evidence by activating aged human oocytes incapable of being activated by any other means.

Currently, we know little of the precise mechanisms of PLCζ action, the regulatory pathways involved, and the relative roles of the various localisation patterns reported in the literature. Systematic analysis of these issues represents a critical challenge in the translation of PLCζ into a routine therapeutic agent for the clinic. In addition, immunofluorescence and immunoblot analysis of PLCζ expression and localization in human sperm, combined with assays of PLCζ enzymatic activity, may have potential use as diagnostic tools with which to identify and treat men with oocyte activation deficiency. While much remains unknown with regards to PLCζ and its mode of activity within oocytes, and in particular it's potential role in embryogenesis, the utilization of PLCζ as a clinical therapeutic holds much promise for the future treatment of human infertility.

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