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REVIEW

Molecular triggers of egg activation at fertilization in mammals

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Abstract

In mammals, the sperm activates the development of the egg by triggering a series of oscillations in the cytosolic-free Ca^{2+} concentration (Ca^{2+}_i). The sperm triggers these cytosolic Ca^{2+}_i oscillations after sperm–egg membrane fusion, as well as after intracytoplasmic sperm injection (ICSI). These Ca^{2+}_i oscillations are triggered by a protein located inside the sperm. The identity of the sperm protein has been debated over many years, but all the repeatable data now suggest that it is phospholipase Czeta (PLCζ). The main downstream target of Ca^{2+}_i oscillations is calmodulin-dependent protein kinase II (CAMKII (CAMK2A)), which phosphorylates EMI2 and WEE1B to inactivate the M-phase promoting factor protein kinase activity (MPF) and this ultimately triggers meiotic resumption. A later decline in the activity of mitogen-activated protein kinase (MAPK) then leads to the completion of activation which is marked by the formation of pronuclei and entry into interphase of the first cell cycle. The early cytosolic Ca^{2+} increases also trigger exocytosis via a mechanism that does not involve CAMKII. We discuss some recent developments in our understanding of these triggers for egg activation within the framework of cytosolic Ca^{2+} signaling.

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Introduction

Egg activation refers to the early events that occur at fertilization and that start the development of the embryo. Two of the major events of activation are meiotic resumption and cortical granules exocytosis. In mammalian eggs (metaphase II oocytes), the events of activation are triggered by a transient increase in the cytosolic-free Ca^{2+} concentration (Ca^{2+}_i). In mammals, the Ca^{2+}_i signal consists of a series of repetitive increases that last several hours (Fig. 1). There have been reviews on the roles of other molecules in fertilization including those involved in sperm–egg fusion, or in the changes in the cytoskeleton or the meiotic spindle (Jones 2007, Horner & Wolfner 2008, Clift & Schuh 2013, Okabe 2014). There are also reviews on aspects of Ca^{2+}_i homeostasis during oocyte maturation in preparation for fertilization (Machaca 2007, Wakai & Fissore 2013). Here, we focus on the way the sperm triggers the Ca^{2+}_i signals that activate mammalian eggs. In addition, we shall consider aspects of how Ca^{2+}_i triggers the two most studied events of oocyte activation, namely the completion of meiosis and cortical granule exocytosis. These two events are downstream of the Ca^{2+}_i signal and appear to involve independent pathways.

Ca^{2+}_i oscillations at fertilization

Oscillatory increases in Ca^{2+}_i are both necessary and sufficient for egg activation at fertilization (Kline & Kline 1992, Ozil *et al*. 2005). In the mouse egg, these oscillations start about 1–2 min after sperm fusion (Lawrence *et al*. 1997). The Ca^{2+} release during the first oscillation originates from the sperm-fusion point and the later transients consist of rapid Ca^{2+}_i waves that sweep across the egg (Deguchi *et al*. 2000). These repetitive Ca^{2+}_i transients, or Ca^{2+}_i spikes, often referred as 'Ca^{2+} oscillations', can persist for up to 5 or 6 h, and in mouse eggs, they stop around the time of pronuclei formation (Marangos *et al*. 2003) (Fig. 1). The frequency of sperm-induced Ca^{2+}_i oscillations can vary considerably between species, from one transient every 10–20 min in the mouse zygote, to about one Ca^{2+}_i transient every 30–60 min in bovine or human zygotes (Taylor *et al*. 1993, Fissore *et al*. 1995, Deguchi *et al*. 2000). These are low-frequency Ca^{2+}_i oscillations by comparison to somatic cells.

Ca^{2+}_i oscillations during mammalian fertilization are a result of IP_3-induced Ca^{2+} release. If IP_3 receptors are inhibited, or their expression downregulated before fertilization, no Ca^{2+}_i oscillations are detected (Miyazaki *et al*. 1992). Furthermore, IP_3Rs become physiologically downregulated during Ca^{2+}_i oscillations at fertilization in direct response to increases in IP_3 (Brind *et al*. 2000). The most likely explanation for how the sperm initiate IP_3 production and Ca^{2+}_i oscillations is that sperm introduces a diffusible cytosolic factor into the egg after fusion (Swann 1990). This idea is supported by the finding that the injection of soluble cytosolic extracts from sperm can cause Ca^{2+}_i oscillations and egg

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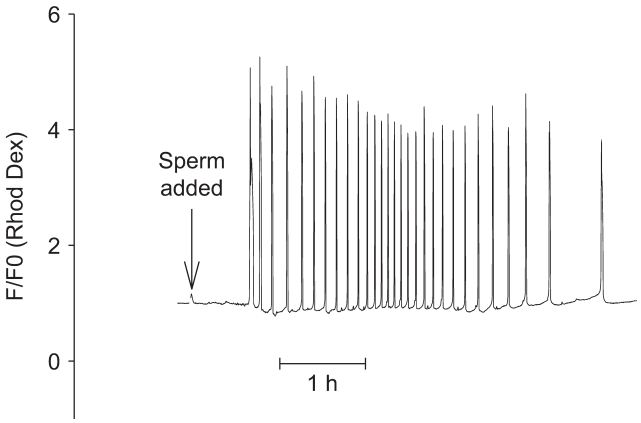

Figure 1 Ca^{2+}_i oscillations at fertilization in a mouse egg. An example is shown of a recording from mouse eggs that were microinjected with the Ca^{2+}-sensitive fluorescent dye Rhod dextran and fluorescence measured every 10 s following the addition of capacitated mouse sperm (at the arrow). Further details in (Saunders *et al*. 2002).

activation (Swann 1990). Sperm extracts from different mammalian species are able to activate eggs from different species showing that the factor causing Ca^{2+} release is not species specific (Swann 1994). However, it is sperm specific since extracts from somatic tissues do not trigger Ca^{2+}_i oscillations (Jones *et al*. 2000). The idea of an egg-activating factor inside the sperm is also supported by the clinical practice of ICSI (intracytoplasmic sperm injection), which triggers Ca^{2+}_i oscillations similar to those at fertilization (Tesarik & Sousa 1994). The active factor in these ICSI studies has been referred to as SOAF (sperm-borne oocyte-activating factor) (Kimura *et al*. 1998). The SOAF is associated with the perinuclear matrix, which lies beneath the plasma membrane around the sperm head (Kimura *et al*. 1998). Proteins extracted from the perinuclear matrix also cause Ca^{2+}_i oscillations in eggs (Perry *et al*. 2000). The SOAF is clearly active in different mammals and is active across species and so, for example, injection of human sperm can cause Ca^{2+}_i oscillations in mouse eggs (Yoon *et al*. 2008). These data confirm that mammalian sperm contain a sperm-specific protein(s) that can cause the physiological pattern of Ca^{2+}_i oscillations in eggs seen at fertilization.

Although each increase in cytosolic Ca^{2+} is a result of intracellular Ca^{2+} release, Ca^{2+} influx across the plasma membrane is important in maintaining Ca^{2+}_i oscillations. Therefore, for example, removal of extracellular Ca^{2+} leads to the cessation of Ca^{2+}_i transients in fertilizing hamster and mouse eggs (Igusa & Miyazaki 1983). We also know that Ca^{2+} influx is stimulated during each Ca^{2+}_i increase since Mn^{2+} influx, used as a surrogate for Ca^{2+}, can be detected following each Ca^{2+}_i rise (McGuinness *et al*. 1996). Ca^{2+} influx in mammalian eggs may involve a number of different channels. In mouse eggs, one of these channels is T-type Ca^{2+} channel CaV 3.2 (Bernhardt *et al*. 2015). This may not be the only channel to bring Ca^{2+} in because CaV 3.2 null female mice are fertile (Bernhardt *et al*. 2015). Another channel in the mouse plasma membrane is the TRPV3 which when stimulated can lead to parthenogenetic egg activation (Carvacho *et al*. 2013, Lee *et al*. 2016). These types of channels are probably the most significant in mouse eggs because store-operated Ca^{2+} influx is downregulated in the mouse oocyte in preparation for fertilization. This is achieved by loss of cortical Stim1 and Orai during oocyte maturation (Lee *et al*. 2013). The reason why the egg has to downregulate the influx seems to be that the release machinery becomes so sensitive to Ca^{2+}-induced Ca^{2+} release that if the influx levels of an immature oocyte are maintained in a mature egg, it causes very high-frequency oscillations that lead to cell death (Lee *et al*. 2013). Although store-operated Ca^{2+} channels does not appear to be the mechanism for Ca^{2+} influx in mouse eggs (Miao *et al*. 2012), it may be significant in some other species. For example, in pig eggs, the experimental downregulation of *STIM1* or *ORAI* leads to premature cessation of Ca^{2+}_i oscillations at fertilization (Wang *et al*. 2015).

Changes in the Ca^{2+} content inside stores have been directly measured using a Ca^{2+} probe targeted to the endoplasmic reticulum in mouse eggs (Takahashi *et al*. 2013, Wakai *et al*. 2013). There is a marked decrease in store Ca^{2+} during each cytosolic Ca^{2+}_i increase, and then gradual refilling of Ca^{2+} stores during the intervals between Ca^{2+}_i spikes (Takahashi *et al*. 2013, Wakai *et al*. 2013). It is suggested that refilling of stores sets the timing of each Ca^{2+}_i increase and this is very likely with relatively high-frequency Ca^{2+}_i oscillations (Takahashi *et al*. 2013, Wakai *et al*. 2013). However, whether Ca^{2+} refilling always acts a pacemaker is unclear. For example, with the initial low-frequency oscillations, the level of Ca^{2+} in the store does not correlate with the time of each Ca^{2+} release event (Wakai *et al*. 2013). Furthermore, several cycles of Ca^{2+} release can occur in the presence of the Ca^{2+} pump inhibitor thapsigargin, which inhibits refilling (Wakai *et al*. 2013). The timing of each Ca^{2+} release event may be determined by a number of factors other than Ca^{2+} store loading.

PLCζ is the soluble sperm factor and SOAF

The sperm extracts that trigger Ca^{2+}_i oscillations in eggs contain a high phospholipase C (PLC) activity that is distinctive in being stimulated by Ca^{2+} concentrations equivalent to resting levels in eggs (Jones *et al*. 1998, Rice *et al*. 2000). It was then proposed that the sperm factor is some form of PLC (Jones *et al*. 1998, Swann & Parrington 1999). Such a PLC would have to be distinct from the β, γ, or δ isoforms, which are unable, or much less able, to cause Ca^{2+}_i oscillations in eggs and are present in many somatic tissues (Jones *et al*. 1998).

However, mammalian testes do specifically express a distinct PLC isoform known as PLCζ (zeta) (Saunders *et al*. 2002). Microinjection of PLCζ, as RNA or protein, causes Ca^{2+}_i oscillations in mouse, cow, pig, or human eggs (Saunders *et al*. 2002, Kouchi *et al*. 2004, Rogers *et al*. 2004, Ross *et al*. 2008, Ito & Kashiwazaki 2012, Nomikos *et al*. 2013*b*) (Fig. 2). Subsequently, egg activation and development occurs up to the blastocyst stage (Cox *et al*. 2002, Saunders *et al*. 2002, Rogers *et al*. 2004, Yoneda *et al*. 2006). Crucially, PLCζ is able to cause Ca^{2+}_i oscillations at levels that are comparable to that present in a single sperm (Saunders *et al*. 2002, Ross *et al*. 2008). What is more PLCζ has been found to localize to the equatorial and post-acrosomal regions of the sperm, where the sperm first makes contact with the egg plasma membrane (Fujimoto *et al*. 2004, Heytens *et al*. 2009, Escoffier *et al*. 2016). Finally, sperm extracts depleted of PLCζ using an anti-PLCζ antibody are unable to cause Ca^{2+}_i oscillations when injected into eggs (Saunders *et al*. 2002). These data show that PLCζ is the previously described soluble sperm factor and that it fits the key criteria for triggering the activation of development.

While the identity of the cytosolic 'soluble' sperm factor has been shown to be PLCζ, studies on mouse sperm had always suggested that they contain an insoluble factor (SOAF) that was located within the perinuclear theca (Kimura *et al*. 1998). The active SOAF has been extracted from the perinuclear theca using reducing agents and further purification identified PLCζ as the protein that correlated with the ability to activate mouse eggs (Fujimoto *et al*. 2004). These data clearly suggest that both SOAF and the activity of 'soluble sperm factor' are the same thing: namely PLCζ. The explanation for the original differences in their character may be that mouse sperm contain PLCζ that is tightly bound to the perinuclear matrix, whereas in other species, some of the PLCζ is more soluble. In boar sperm, for example, it has been shown that PLCζ is present in both soluble extracts and the sperm perinuclear matrix (Kurokawa *et al*. 2005). It is notable that the analysis of material extracted from the perinuclear matrix provides biochemical confirmation that PLCζ is localized within the sperm; hence, it is not credible to claim that PLCζ is a membrane protein (Fujimoto *et al*. 2004, Aarabi *et al*. 2012).

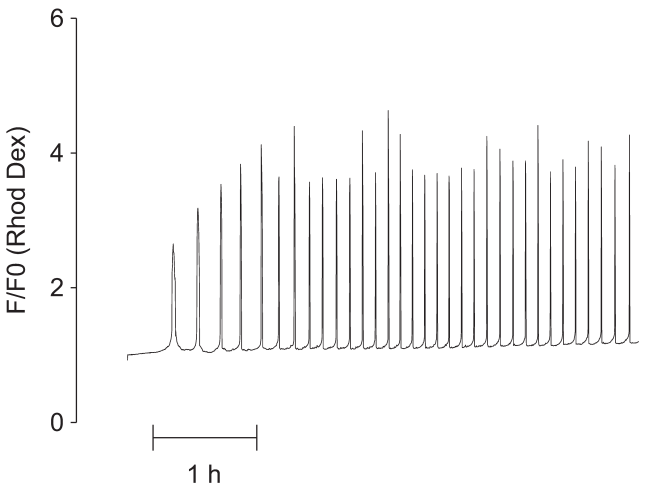

Figure 2 PLC-induced Ca^{2+}_i oscillations in a mouse egg. The conditions are the same as Fig. 1 expect that no sperm were added. The egg had been injected with PLC cRNA (0.02 g/L in the pipette) as described in (Nomikos *et al*. 2005). The trace shows a recording over 5 h.

Some of the unique capabilities of PLCζ in causing Ca^{2+}_i oscillations in eggs can be explained by its structure. PLCζ consists of X–Y catalytic domains, four EF hand domains, and a C2 domain (Nomikos *et al*. 2005). It also contains an unstructured region between the X and Y domains, referred as the X–Y linker (Nomikos *et al*. 2011). A full review of the relationship between these domains and the activity of PLCζ in eggs can be found elsewhere (Kouchi *et al*. 2005, Nomikos 2015). Here, we briefly highlight some features of PLCζ that are pertinent to function in eggs. The EF hand motifs of PLC account for its extraordinary Ca^{2+} sensitivity and allow PLCζ to generate IP_3 at resting levels in the oocyte cytoplasm (Nomikos *et al*. 2015*b*). Any slight increase in Ca^{2+}_i will also increase IP_3 production, and hence, there is a positive-feedback loop of Ca^{2+}_i and IP_3 increase. This positive-feedback loop accounts for the enhanced 'Ca^{2+}-induced Ca^{2+} release' after fertilization, and it has been shown to be part of the mechanism generating Ca^{2+}_i oscillations in response to PLCζ or fertilization (Swann & Yu 2008) (Fig. 3). Furthermore, oscillations in IP_3 concentration, in synchrony with Ca^{2+}_i oscillations, have been detected in mouse eggs injected with PLCζ (Shirakawa *et al*. 2006). The X–Y linker region of PLCζ is unusual, in that it can account for the ability of PLCζ to bind to PIP_2 (Nomikos *et al*. 2011). For other PLCs, the X–Y linker plays an auto-inhibitory role, but for PLCζ, the X–Y linker is essential for its binding to its substrate. The role of the C2 domain is currently unknown, but it is important because a chimeric protein made of PLCζ, but with the C2 domain of PLCδ1, is unable to cause Ca^{2+}_i oscillations in eggs (Theodoridou *et al*. 2013).

Another unusual feature of PLCζ is that it appears to be only active in eggs. Expression of PLCζ in cell lines fails to cause Ca^{2+}_i oscillations and ectopic expression in somatic tissues has surprisingly little effect (Phillips *et al*. 2011). It is possible that the specific effect in eggs is related to an unusual localization pattern. Numerous studies have failed to find PLCζ in the plasma membrane where most other PLCs localize, and where cells maintain a pool of phosphatidylinositol 4,5-bisphosphate (PIP_2), the substrate for PLCs. However, immunocytochemical studies of PLCζ (at physiological concentrations) have found it localized in multiple vesicles throughout the

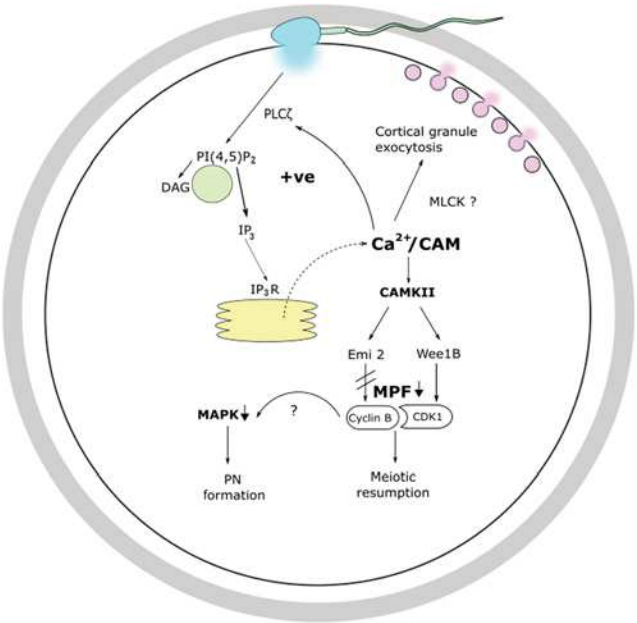

Figure 3 Schematic proposal for the key events triggering egg activation. The sperm fused with the egg and introduces PLCζ which diffuses into the cytosolic space. Vesicular PIP_2 is hydrolyzed to generate IP_3, which releases Ca^{2+}. The Ca^{2+} binds to and activates calmodulin, which stimulates CamKII and possibly other kinases such as MLCK. CamKII phosphorylates EMI2 and WEE1B which ultimately leads to MPF destruction. This in turn causes a much delayed decrease in MAPK activity. PLC activity is further stimulated in response to the increase in Ca^{2+} in the form of a positive-feedback loop (+ve).

egg cytoplasm (Yu *et al*. 2012). There are similar vesicles in mouse eggs that contain PIP_2, and hence, PLCζ may stimulate PIP_2 hydrolysis from an intracellular source (Yu *et al*. 2012). The implication is that IP_3 and DAG are produced from these internal membranes (Yu *et al*. 2012) (Fig. 3).

So far a genetically modified mouse lacking PLCζ or containing an inactive PLCζ mutation has not been reported. One preliminary report suggested that male mice lacking PLCζ fail to make mature sperm (Ito 2010), but this study has yet to be fully presented. Otherwise, it has been shown that knockdown of the levels of PLCζ in sperm, using transgenic mice with RNAi, leads to a reduction in Ca^{2+}_i oscillations at fertilization suggesting that PLCζ must play some role in generating oscillations physiologically (Knott *et al*. 2005). The relevance of PLCζ to fertilization is also suggested from clinical case studies. It was found that there are certain cases of male factor infertility that are associated with reduced levels of PLCζ (Kashir *et al*. 2011). It has also been reported that one male patient who had repeated failed ICSI had *PLCζ* genes with two different mutations on both alleles (Kashir *et al*. 2012*b*). These mutations were in the catalytic domain of PLCζ and lead to a loss of its ability to cause Ca^{2+}_i oscillations in eggs (Kashir *et al*. 2012*b*, Escoffier *et al*. 2015). A loss of functionality for *PLCζ* on both alleles is significant since there is a sharing of gene products during spermatogenesis, which means that a mutation on only one *PLCζ* allele may not lead to a complete loss of *PLCζ* (Kashir *et al*. 2012*b*). A mutation in the *DY19L2* gene, which has previously been associated with the condition globozoospermia, has also been found to correlate with a reduction in the sperm's ability to cause Ca^{2+} oscillations (Escoffier *et al*. 2015). The sperm from patients with this mutation or from *Dy19l2*-knockout mice is either lacking in PLCζ or contains much lower levels than would be physiologically expected (Escoffier *et al*. 2015). A recent study by Arnoult and coworkers has emerged of two brothers who also had failed fertilization after ICSI treatments (Escoffier *et al*. 2016). Whole genome sequencing was carried out on these patients. Only one gene was found to have a homologous mutation that was predicted to be disruptive, and that gene was *PLCζ* (Escoffier *et al*. 2016). The mutation in this case was in the C2 domain, and it lead to a loss of *PLCζ* from the sperm as well as reduced ability to cause Ca^{2+}_i oscillations (Escoffier *et al*. 2016). This case study provides the strongest evidence to date that PLCζ is the critical protein for causing Ca^{2+}_i oscillations and egg activation at fertilization. This clinical evidence also suggests that PLCζ sperm levels could be used for a biomarker of fertility and that the levels of PLCζ within the sperm are vital for successful fertilization and subsequent development. It may be possible that some cases of total fertilization failure that occur following ICSI treatment in the clinic could be a result of reduced levels of PLCζ in the patient's sperm. The clinical applications for PLCζ have been discussed thoroughly in a number of reviews (Ramadan *et al*. 2012, Kashir *et al*. 2012*a*, Nomikos *et al*. 2013*a*, Swann & Lai 2016).

PAWP is not relevant to egg activation

Another protein called post-acrosomal WW domain-binding protein (PAWP) has been proposed as the factor causing Ca^{2+} release and egg activation at fertilization. PAWP is located in the perinuclear theca of the sperm head, previously identified as containing the SOAF (Kimura *et al*. 1998). It has been reported that recombinant PAWP protein can activate eggs from mice, pigs, frogs, and monkeys (Wu *et al*. 2007, Aarabi *et al*. 2014). Significantly, it was also shown that injection of human recombinant protein PAWP or PAWP cRNA into mouse or human eggs elicited Ca^{2+}_i oscillations comparable to those induced by ICSI (Aarabi *et al*. 2014). PAWP protein injection was also reported to induce a Ca^{2+}_i increase in frog eggs (Aarabi *et al*. 2010). PAWP is proposed to work via its binding to yes-associated protein (YAP), which then may activate egg-derived PLCζ by a noncanonical SH3 domain interaction (Wu *et al*. 2007, Aarabi *et al*. 2014). Indeed, injection of a PY-containing peptide into

eggs that competitively binds to YAP abolished ICSI-induced Ca^{2+}_i oscillations and egg activation (Wu *et al*. 2007, Aarabi *et al*. 2014). These data clearly suggest that PAWP could be the physiological sperm factor (or SOAF) causing Ca^{2+}_i oscillations and egg activation.

The above evidence for PAWP at fertilization has, however, now been contradicted by several separate lines of evidence from different research groups. First, as discussed already, the original studies of SOAF identified it as PLCζ (Fujimoto *et al*. 2004). SOAF activity is present in the perinuclear extracts of the mouse sperm and such extracts were shown to contain several different proteins as well as PLCζ, but these extracts did not contain PAWP (Fujimoto *et al*. 2004). Secondly, in marked contrast to PLCζ, the data showing PAWP's ability to cause Ca^{2+}_i increases have not been reproducible (Nomikos *et al*. 2014, 2015*a*). Independent studies injecting mouse eggs with human or mouse PAWP protein or cRNA failed to detect any Ca^{2+}_i transients (Nomikos *et al*. 2014, 2015*a*). The expression of PAWP protein from injected RNA was validated in these experiment with tagged or untagged RNA, and the levels that were similar or greater than those levels reported to be found in sperm (Wu *et al*. 2007). In addition, injecting the same PY-containing peptide (used by Aarabi *et al*.) into eggs did not result in any inhibition of Ca^{2+}_i oscillations induced by IVF or ICSI (Nomikos *et al*. 2015*a*). Thirdly, it has now been shown that PAWP null male mice are fertile and produce sperm that can trigger a normal pattern of Ca^{2+}_i oscillations and embryonic development after ICSI (Satouh *et al*. 2015). Finally, in the above case study of two brothers who have sperm that fail to fertilize eggs in ICSI, and who had a homozygous mutation in PLCζ, it was found that there were no alterations in the sequence or expression of PAWP (Escoffier *et al*. 2016). These data show that PAWP plays no significant role in generating Ca^{2+}_i oscillations during egg activation in mice or humans.

Downstream of Ca^{2+}: meiotic resumption and entry into interphase

The most well-characterized event of mammalian egg activation is the resumption and completion of meiosis which starts with a metaphase-to-anaphase transition, and completes with the formation of two pronuclei (Jones 2007). Meiotic arrest is maintained by high levels of activity of M-phase promoting factor (MPF) that principally consists of cyclin B and a cyclin-dependent kinase (CDK1). The meiotic state also depends on high activity levels of MAPK (Choi *et al*. 1996, Abrieu *et al*. 1997). The key protein linking Ca^{2+}_i oscillations with a decline in MPF activity is calmodulin-dependent protein kinase II (CAMKII (CAMK2A)) (Markoulaki *et al*. 2004). The microinjection of constitutively active CAMKII into mouse eggs triggers meiotic resumption and development up to at least the blastocyst stage (Knott *et al*. 2006). Moreover, eggs from CAMKIIγ knockout mice, or eggs in which CAMKIIγ (CAMK2G) has been knocked down, fail to show any signs of meiotic resumption at fertilization (Backs *et al*. 2010). There are at least two mechanistic pathways linking CAMKII and meiotic resumption. First, active CAMKII phosphorylates EMI2 (Madgwick & Jones 2007), which leads to its phosphorylation by polo kinase, which in turns blocks the ability of EMI2 to inhibit the anaphase-promoting complex (APC). As a consequence, the APC destroys EMI2, as well as cohesion, which holds sister chromatids together, and it destroys cyclin B, which leads to a loss of MPF activity (Hansen *et al*. 2006). The second link between CAMKII and MPF activity involves phosphorylation of the protein kinase WEE1B (Oh *et al*. 2011). WEE1B is a kinase that phosphorylates CDK1 and inhibits MPF activity (Oh *et al*. 2011), and so when WEE1B is phosphorylated, MPF activity falls. In mouse oocytes, WEE1B is essential for inactivation of MPF and cyclin B destruction during oocyte activation (Oh *et al*. 2011). These data suggest a two-pronged action of the Ca^{2+}_i signal on reducing MPF activity (Fig. 3).

While a single Ca^{2+}_i increase at fertilization triggers meiotic resumption, multiple Ca^{2+}_i transients at fertilization are needed to complete the process. A single, physiological sized,Ca^{2+}_i increase can lead to a reduction in MPF activity, but this is only a transient effect (Tatone *et al*. 2002). MPF activity can return after insufficient Ca^{2+}_i increases, and this can lead to a re-establishment of a metaphase arrest: a so-called metaphase III arrest (Kubiak 1989). Using electrical stimulation to mimic the Ca^{2+}_i transients at fertilization, it has been shown that more than eight transients is required to ensure that egg forms pronuclei (Ducibella *et al*. 2002). This is generally consistent with observations on fertilizing mouse eggs, where early termination of Ca^{2+}_i spiking tends to stop 2nd polar body emission and pronuclear formation (Kubiak 1989). It should be noted that Ca^{2+} ionophores only cause a single large Ca^{2+}_i increase in eggs and yet are able to activate development (Winston *et al*. 1991). However, parthenogenetic stimuli that generate a single Ca^{2+}_i transient are not very effective in activating eggs of many species, and particularly poor in activating freshly ovulated eggs (Jones 2007). Ca^{2+} ionophores are generally used in combination with a protein kinase or protein synthesis inhibitor that helps to reduce MPF activity (Jilek *et al*. 2000). Ionophores also cause a much larger and long-lasting Ca^{2+}_i increase than seen physiologically at fertilization.

Many studies of egg activation concern the reinitiation of meiosis. The end of meiosis is marked by the formation of pronuclei and the trigger sequence for this event is less well understood. It is known that the completion of meiosis and entry into interphase depends upon a fall in the activities of MAPK (principally ERK1 and ERK 2) (Moos *et al*. 1996). The activity of ERK1/2 kinase is kept high by phosphorylation by another kinase MEK, which

in turn is kept active through phosphorylation by MOS, which is specifically expressed in oocytes (Dupre *et al*. 2011). A fall in MAPK (ERK1/2) activity is essential for entry into interphase since preventing its decline using phosphatase inhibitors, or by injecting constitutively activate MEK, prevents pronuclear formation (Moos *et al*. 1996). By contrast, the MEK inhibitor U0126 induces pronuclear formation (Phillips *et al*. 2002). The trigger for the fall in MAPK activity is the decline in MPF since drugs that inhibit CDK1, such as roscovitine, initiate a decrease in MAPK activity with a delay that mimics fertilization (Gonzalez-Garcia *et al*. 2014). Hence, there is a sequence of triggers in which a decline in MPF leads to a decline in ERK1/2 (MAPK) activity that leads to pronuclear formation (Fig. 3).

The sequence of MPF and MAPK inactivation at fertilization is seen in many vertebrate eggs (Haccard *et al*. 1995, Bogliolo *et al*. 2000, McDougall *et al*. 2011). Nevertheless, the story in mammals is unusual, in that there is a long delay, of several hours, between the fall in MPF activity and the fall in MAPK activity. The use of a luciferase probe of ERK1/2 kinase activity shows that decline in MAPK activity in mouse zygotes is initiated about 2 h after the start of Ca^{2+}_i oscillations, which is about 1.5 h after the decline in cyclin B levels (Gonzalez-Garcia *et al*. 2014). Once started, the decline in MAPK then precedes gradually over the next few hours continuing well after pronuclear formation, which may explain why some reports show a MAPK decline after pronuclear formation (Gonzalez-Garcia *et al*. 2014). Exposing egg to a series of electrical pulses has also shown that MPF can be fully inactivated for about 2 h before a fall in MAP kinase activity is detected (Tatemoto & Muto 2001). Consequently, there is a substantial delay between the fall in activity of MPF and MAPK (Gonzalez-Garcia *et al*. 2014).This delay is not explained by a slow decline in MOS because MOS levels do not decline significantly in the first few hours after fertilization (Gonzalez-Garcia *et al*. 2014). Also, MOS overexpression in mouse eggs does not affect the timing of the fall in ERK1/2 kinase activity (Gonzalez-Garcia *et al*. 2014). The delayed fall in ERK1/2 activity could be driven by an increase in a protein phosphatase activity (Gonzalez-Garcia *et al*. 2014). However, the molecular link between the fall in MPF activity and the stimulation of such phosphatases is unknown.

Downstream of Ca^{2+}: cortical granule exocytosis

Another key event of egg activation in mammals is cortical granule exocytosis. The kinetics of exocytosis at fertilization has been accurately measured by membrane capacitance in hamster eggs (Igusa & Miyazaki 1986, Kline & Stewart-Savage 1994). Most of the change in capacitance occurs with the first Ca^{2+}_i increase at fertilization (Kline & Stewart-Savage 1994). Exposing eggs to electrical pulses to generate Ca^{2+}_i increases also suggests that the first four Ca^{2+}_i spikes trigger the majority of cortical granule to be released. These data are consistent with the role of exocytosis in releasing enzymes that modify the zona pellucida to prevent further sperm entry (Horvath *et al*. 1993). The signals for meiotic resumption are different from those involved in cortical granule exocytosis because injection of constitutively active CamKII triggers meiotic resumption and pronuclear formation, but not exocytosis (Knott *et al*. 2006, Gardner *et al*. 2007, Backs *et al*. 2010). Moreover, fertilization of oocytes from *CAMKII*$^{-/-}$ mice, or from WEE1B knockdown oocytes, does not lead to meiotic resumption, and yet in both cases, cortical granule exocytosis occurs (Ducibella & LeFevre 1997, Backs *et al*. 2010, Oh *et al*. 2011). Hence, meiotic resumption and cortical granule exocytosis appear to be separate downstream events that diverge early on in the Ca^{2+}_i signaling pathway (Fig. 3).

Numerous studies of exocytosis in mammalian eggs have implicated protein kinase C in triggering exocytosis. Increasing PKC activity, for example, using phorbol esters or synthetic DAGs, can stimulate exocytosis in mammalian eggs (Eliyahu & Shalgi 2002). Ca^{2+} ionophores can also stimulate exocytosis in a manner that is blocked by PKC inhibitors (Ducibella & LeFevre 1997). However, the relevance of these results is unclear since PKC inhibitors do not block exocytosis at fertilization in the mouse (Ducibella & LeFevre 1997). Also, both phorbol esters and Ca^{2+} ionophores are nonphysiological in the way they stimulate eggs. For example, a probe made of GFP linked to a DAG-sensing C1 domains shows a distinctive increase in the plasma membrane of mouse eggs in response to phorbol esters, or Ca^{2+} ionophores (Swann & Yu 2008). However, the same probes show no translocation in the plasma membrane at fertilization, or after injection of physiological amounts of PLCζ (Yu *et al*. 2008). Interestingly, these data suggest that DAG is not produced in significant amounts in the plasma membrane at fertilization in mouse eggs (Halet 2004, Yu *et al*. 2012). This idea is consistent with our suggestion that the PI turnover, and hence DAG production, at fertilization is principally occurring on cytoplasmic vesicles. There may be no DAG produced to stimulate PKC in the plasma membrane at fertilization. The detectable PKC stimulation in the plasma membrane of eggs may be mainly due to a Ca^{2+}_i increase (Halet 2004, Yu *et al*. 2008). One idea is that the PKC and calmodulin pathways converge by the translocation of myristoylated alanine-rich C kinase substrate (MARCKS). Exocytosis is likely to be a multiple stage process that requires the reorganization of the dense actin cytoskeleton in the cortex and the translocation of vesicles to the plasma membrane (Ducibella & Matson 2007). MARCKS has a role in reorganizing actin filaments in the cortex following its translocation either as a result of phosphorylation by PKC or by binding to calmodulin and has been shown to be associated with exocytosis in other cell types as well

as cortical granule exocytosis in eggs (Eliyahu *et al*. 2006, Tsaadon *et al*. 2008). Translocation of the vesicles could involve the Ca^{2+} calmodulin-dependent enzyme myosin light-chain kinase (MLCK). MLCK targets myosin II in neuroendocrine cells and is responsible for translocating vesicles to the synaptic membrane (Ducibella & Matson 2007). Inhibitors of MLCK such as ML7 inhibit cortical granule exocytosis at fertilization in mouse eggs (Matson *et al*. 2006).

Recent studies have shown that cortical granules in mammalian oocytes contain Zn^{2+} (Kim *et al*. 2011, Que *et al*. 2015). At fertilization, or after oocyte activation with Ca^{2+} ionophores, exocytosis triggers release of this Zn^{2+} into the extracellular space, and this release can be detected using fluorescence dyes (Kong *et al*. 2015). It has been proposed that the loss of Zn^{2+} from the egg at fertilization facilitates the process of meiotic resumption (Kim *et al*. 2011). This is plausible since EMI2 is a Zn^{2+}-dependent enzyme and Zn^{2+} chelators, such as TPEN, can trigger meiotic resumption and embryo development (Kim *et al*. 2011). However, for Zn^{2+} released from cortical granules to affect EMI2, there would have to be a decrease in cytosolic Zn^{2+} levels, and it has yet to be shown that cytosolic-free Zn^{2+} levels change in eggs at fertilization. The idea that Zn^{2+} loss via exocytosis plays a role in meiotic resumption is inconsistent with previous studies showing that exocytosis and meiotic resumption are independent events downstream of the Ca^{2+}_i signal.

Conclusions and perspectives

Some of the key molecules in egg activation in mammals are known. CAMKII appears to be cemented in as the hub for all the Ca^{2+}-dependent events triggering meiotic resumption. All the indications are that PLCζ is the molecule that initiates the Ca^{2+}_i signals that stimulate CAMKII. Questions remain with regards to PLCζ's localization and targeting in eggs. We also eagerly await the confirmation phenotype of sperm from a mouse either lacking PLCζ or else containing an inactive PLCζ protein. Other important questions that remain concern the nature of Ca^{2+} influx channels and their regulation during the oscillation cycle, as well as the factors determining the long delay between Ca^{2+}_i spikes at fertilization. It will also be interesting to determine the signaling pathway for exocytosis, since it does not appear to involve CAMKII. Finally, we suggest that it is important to establish the link between the fall in MPF and the decline in MAPK activity since it represents the last in a sequence of triggers of egg activation in mammals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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