Ca^{2+} Signaling During Mammalian Fertilization: Requirements, Players, and Adaptations

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Cold Spring Harbor Perspectives in Biology
www.cshperspectives.org

Changes in the intracellular concentration of calcium ($[Ca^{2+}]_i$) represent a vital signaling mechanism enabling communication among cells and between cells and the environment. The initiation of embryo development depends on a $[Ca^{2+}]_i$ increase(s) in the egg, which is generally induced during fertilization. The $[Ca^{2+}]_i$ increase signals egg activation, which is the first stage in embryo development, and that consist of biochemical and structural changes that transform eggs into zygotes. The spatiotemporal patterns of $[Ca^{2+}]_i$ at fertilization show variability, most likely reflecting adaptations to fertilizing conditions and to the duration of embryonic cell cycles. In mammals, the focus of this review, the fertilization $[Ca^{2+}]_i$ signal displays unique properties in that it is initiated after gamete fusion by release of a sperm-derived factor and by periodic and extended $[Ca^{2+}]_i$ responses. Here, we will discuss the events of egg activation regulated by increases in $[Ca^{2+}]_i$, the possible downstream targets that effect these egg activation events, and the property and identity of molecules both in sperm and eggs that underpin the initiation and persistence of the $[Ca^{2+}]_i$ responses in these species.

An increase in the intracellular concentration of calcium ($[Ca^{2+}]_i$) underlies the initiation, progression and/or completion of a wide variety of cellular processes, including fertilization, muscle contraction, secretion, cell division, and apoptosis (Berridge et al. 2000). To survive and proliferate, cells and organisms must communicate, and changes in $[Ca^{2+}]_i$ allow them to quickly respond to environmental, nutritional, or ligand challenges with responses that regulate cell fate and function. Cells devote significant amounts of their energy reserves to create and maintain ionic gradients between extracellular and intracellular milieus and also within the latter, thereby allowing brief alterations in these gradients to have profound signaling effects. In the case of Ca^{2+}, myriad proteins have acquired the ability to bind Ca^{2+}, which allows them to interpret and transform these elevations into cellular functions. This review will examine the cellular modifications induced by $[Ca^{2+}]_i$ changes during fertilization in mature mammalian oocytes, henceforth referred to as eggs.

Oocytes during maturation ready themselves for fertilization and the initiation of

Editors: Martin D. Bootman, Michael J. Berridge, James W. Putney, and H. Llewelyn Roderick
Additional Perspectives on Calcium Signaling available at www.cshperspectives.org

 doi: 10.1101/cshperspect.a006767
Cite this article as *Cold Spring Harb Perspect Biol* 2011;3:a006767

embryogenesis. During this transition, oocytes undergo changes that include the resumption and progression of meiosis, the development of polyspermy-preventing mechanisms, the reorganization of the cytoskeleton with spindle formation and displacement to the cortex, and the translation, accumulation, and degradation of specific mRNAs and proteins involved in development (Horner and Wolfner 2008b). In most species, and in all mammals, a $[Ca^{2+}]_i$ signal is responsible for breaking the meiosis-imposed developmental pause, causing egg activation, which is the first stage of embryo development (Whitaker 2006; Horner and Wolfner 2008b). The egg activating $[Ca^{2+}]_i$ signal is generally associated with sperm-egg fusion, which occurs at different stages of meiosis depending on the species (Stricker 1999), although in insects, where fertilization is dissociated from activation and where embryos can develop parthenogenetically, the presumed $[Ca^{2+}]_i$ increase is thought to be induced by mechanical stimulation during ovulation/oviductal transport (Page and Orr-Weaver 1997; Horner and Wolfner 2008a).

The $[Ca^{2+}]_i$ responses that underlie egg activation offer a great deal of diversity regarding their spatiotemporal configuration, reflecting both the plasticity of the Ca^{2+} signaling machinery as well as the dissimilar Ca^{2+} requirements for egg activation among species. Generally speaking, species can be categorized either as displaying a single $[Ca^{2+}]_i$ increase, which is the case of sea urchins, starfish, frogs, and fish, or showing multiple $[Ca^{2+}]_i$ changes, also known as oscillations, which is the case of nemertian worms, ascidians, and mammals (Stricker 1999; Miyazaki and Ito 2006). Elucidation of the signaling cascades and identification of the molecules/receptor(s) that initiate the Ca^{2+} signal at fertilization has proven elusive, and this review will not dwell on that literature; readers are referred to excellent recent reviews on the subject (Whitaker 2006; Parrington et al. 2007). Nonetheless, research has found that Src-family kinases (SFKs) and phospholipase Cγ (PLCγ) are involved in the activation of the phosphoinositide pathway and production of inositol 1,4,5-trisphosphate (IP_3) during fertilization in sea urchins, starfish, and frogs, which reflects the contribution of a plasma membrane receptor/signaling complex (Giusti et al. 1999; Sato et al. 2000). Remarkably, a receptor responsible for recruiting and activating SFKs during fertilization remains undiscovered (Mahbub Hasan et al. 2005). Similarly, it has proved difficult to uncover how the sperm initiates oscillations. Research now suggests that this may be accomplished by a novel mechanism whereby the signaling molecule/cargo, known as the sperm factor (SF), is released by the sperm into the ooplasm after fusion of the gametes. Importantly, the SF is not IP_3 or Ca^{2+} but rather it contains a protein moiety (Swann 1990; Wu et al. 1997; Kyozuka et al. 1998; Harada et al. 2007). To date, only the mammalian SF's molecular identity has been resolved, and found to be another member of the PLC family, a novel sperm-specific isoform named PLCζ (Saunders et al. 2002). This review will examine the literature on mammalian PLCζs and will focus as well on the egg molecules that are required to initiate and sustain $[Ca^{2+}]_i$ oscillations in these species.

EGG ACTIVATION

Following the resumption of meiosis during maturation, vertebrate eggs arrest at the metaphase stage of the second meiosis (MII). Sperm entry induces the resumption and completion of meiosis, release of cortical granules (CG), progression into interphase and pronuclear (PN) formation (Fig. 1A); these phenomena, which make possible the transition from egg to embryo, are collectively known as "egg activation" (Schultz and Kopf 1995; Stricker 1999; Ducibella et al. 2002). As stated earlier, an increase in $[Ca^{2+}]_i$ is the universal trigger of egg activation in all species studied to date (Stricker 1999), and in mammals this signal adopts a pattern of brief but periodical increases in $[Ca^{2+}]_i$ that last for several hours after sperm entry (Miyazaki et al. 1986). The spatiotemporal pattern of these $[Ca^{2+}]_i$ responses is decoded by downstream effectors, underpinning the distinct cellular events. We briefly review the events of egg activation that are controlled by

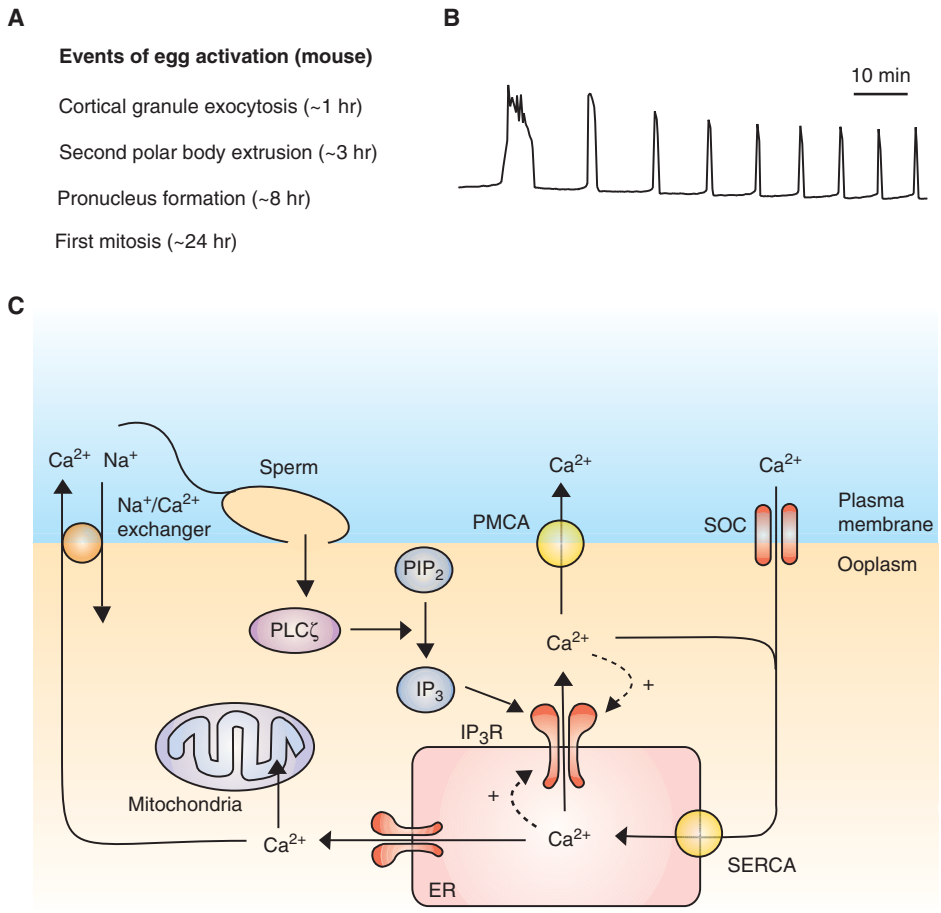

Figure 1. Temporal course of activation events in mouse eggs with a characteristic $[Ca^{2+}]_i$ response and candidate molecules involved in $[Ca^{2+}]_i$ oscillations and Ca^{2+} homeostasis. (*A*) Main cellular events of egg activation and approximate time in hours (hr) required for their completion after sperm entry. (*B*) A typical pattern of $[Ca^{2+}]_i$ oscillations associated with fertilization or with injection of PLCζ cRNA. Note that recordings were terminated prematurely. (*C*) On fusion, the sperm delivers phospholipase C (PLC)ζ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphospahte (IP_3) and dyacyl glycerol (DAG). IP_3 binds its receptor, IP_3R1, causing Ca^{2+} release out of the endoplasmic reticulum (ER). Following Ca^{2+} release, basal $[Ca^{2+}]_i$ levels are regulated by the combined action of the sarcoendoplasmic reticulum Ca^{2+}ATPase (SERCA), plasma membrane Ca^{2+} pump (PMCA), Na/Ca^{2+} exchanger and mitochondria. Store operated Ca^{2+} channels (SOC) are proposed to mediate Ca^{2+} influx required to fill the ER and maintain oscillations. Broken lines suggest feedback action of Ca^{2+} on IP_3R1.

$[Ca^{2+}]_i$ increases and the underlying molecular effectors.

Events of Egg Activation Require $[Ca^{2+}]_i$ Increases

Release from the MII arrest is an early and necessary event of egg activation, as it allows completion of meiosis, establishment of euploidy and progression into interphase with DNA synthesis. The MII block is imposed at the conclusion of maturation prior to ovulation by the action of the cytostatic factor (CSF) (Masui and Markert 1971). The CSF constrains the activity of the anaphase promoting factor (APC), an E3 ubiquitin ligase (Tunquist and Maller 2003), which is responsible for the

ubiquitination and degradation of cyclin B (CycB). CycB and the cyclin-dependent kinase 1 (Cdk1, also known as cdc2) are the molecular components of the maturation promoting factor (MPF) (Swenson et al. 1986; Draetta et al. 1989), and inhibition of CycB degradation by the CSF stabilizes MPF, which results in MII arrest, as MPF activity correlates with metaphase (Masui and Markert 1971). Research has shown that endogenous meiotic inhibitor 2 (Emi2) is an inhibitory component of APC that underlies the MII arrest (Schmidt et al. 2005; Tung et al. 2005; Shoji et al. 2006). In MII oocytes, inhibition of APC also ensures that persistent levels of the separase inhibitor securin prevents sister chromatid separation (Madgwick et al. 2004; Nabti et al. 2008).

The association between intracellular ionic increases and release from meiotic arrest was first proposed at the end of the nineteenth century by Loeb and colleagues who observed that initiation of development in sea urchin eggs was possible simply by varying the concentration and composition of the fertilizing medium (Loeb 1907). Subsequently, it was learned that $[Ca^{2+}]_i$ levels dramatically change after fertilization (Mazia 1935), which focused attention on the role of this ion. Steinhardt and colleagues showed the dominant role of Ca^{2+}, as they were able to promote parthenogenetic development in a variety of species by exposing eggs to Ca^{2+} ionophores (Steinhardt et al. 1974).

How $[Ca^{2+}]_i$ increases induce release from the MII arrest remained unknown for decades. Research showed that $[Ca^{2+}]_i$ increases per se were unable to induce CycB degradation and that instead it required binding to a calmodulin-sensitive enzyme (Lorca et al. 1993), which was later shown to be Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). Nonetheless, how CaMKII relieved the CSF-imposed MII arrest remained unknown. Adding to the confusion was the finding that despite the need for a $[Ca^{2+}]_i$ increase, *Xenopus* egg extracts depleted of Polo-like kinase 1 (Plx1), a kinase known until then more for its role on spindle organization, were unable to overcome the MII arrest (Descombes and Nigg 1998). This quandary was solved when it was discovered that Emi2 is a key component of CSF and that Emi2 phosphorylation by Plx1, which causes its degradation, is required for CycB proteasomal degradation (Schmidt et al. 2005). Subsequent studies found that binding of Plx1 to Emi2 requires a preceding phosphorylation by CaMKII, thereby molecularly linking the need for $[Ca^{2+}]_i$ and Plx1 to exit MII.

Although the aforementioned pathway was elucidated in *Xenopus* eggs and extracts, mouse eggs rely on similar mechanisms to enter and exit the MII arrest. Most prominently, it is well documented that CycB undergoes degradation during fertilization (Nixon et al. 2002) and that each of the sperm-induced $[Ca^{2+}]_i$ increases is accompanied by a parallel increase in CaMKII activity (Markoulaki et al. 2003). The role of CaMKII on mammalian egg activation was convincingly shown by studies in the mouse, in which expression of constitutive active forms of CaMKII into eggs initiated all events of egg activation, except CG exocytosis, and promoted development to the blastocyst stage (Madgwick et al. 2005; Knott et al. 2006). Conversely, depletion of the CaMKIIγ isoform abrogated the ability of these eggs to exit MII in response to $[Ca^{2+}]_i$ stimulation (Backs et al. 2010; Chang et al. 2009), causing infertility. Research also implicated Emi2 in MII arrest in the mouse, as inhibition of Emi2 synthesis prevents cyclin B1 accumulation during maturation (Madgwick et al. 2006), which causes spontaneous activation (Shoji et al. 2006). The role of Plk1 in mouse MII arrest remains unexplored, although our preliminary data show that treatment of eggs with BI2536, a new and selective Plk1 inhibitor, prevents CycB degradation and MII exit in eggs treated with $SrCl_2$ (data not shown). In *Xenopus*, Plx1 phosphorylates xEmi2 within a phosphodegron motif, after which xEmi2 is rapidly targeted for degradation, but this motif, or its canonical replacements, is absent in the mouse homolog (Perry and Verlhac 2008). Although the role of Ca^{2+} and the molecular pathways required for MII exit and embryo development are conserved in vertebrate eggs, it is presently unknown how Plk1 regulates Emi2 function in mammals.

CG exocytosis is another event of egg activation that depends on Ca^{2+} release (Kline and Kline 1992a). CG release underlies, at least in part, the cortical remodeling that occurs after fertilization (Sardet et al. 2002), and modifies the components of zona pellucida to prevent polyspermy, thereby ensuring the formation of a diploid zygote. Although it was believed that the effects of Ca^{2+} on CGs were transduced by activation of protein kinase C (PKC), as PKC agonists promoted CG release and other activation events, later studies using PKC inhibitors failed to prevent fertilization-associated CG release (Ducibella and LeFevre 1997). Importantly, the widespread expression of PKC isoforms in oocytes (Gallicano et al. 1997; Eliyahu et al. 2001; Page Baluch et al. 2004), along with their distinct cellular distribution (Viveiros et al. 2001; Page Baluch et al. 2004), and the implications of their impact on Ca^{2+} influx (Halet 2004), suggest important roles for these enzymes in setting off embryo development. CaMKII was also expected to participate in CG exocytosis, although the aforementioned studies using constitutively active forms of the protein (Knott et al. 2006) or eggs devoid of CaMKII have ruled out this possibility (Backs et al. 2010; Chang et al. 2009). Recent studies have implicated myosin light chain kinase (MLCK), another Ca^{2+}-dependent kinase, as being involved in CG exocytosis in mouse fertilization, as pharmacological inhibitors greatly diminished their release in response to Ca^{2+} stimulation (Matson et al. 2006). The role of MLCK on CG exocytosis is not unexpected, as myosin II, a direct target of MLKC, and actin microfilaments are involved in cortical reorganization in the mouse (Simerly et al. 1998; Deng et al. 2005) and zebrafish eggs (Becker and Hart 1999). Importantly, the molecular regulation of MLCK needs to be determined, as besides its requirement for Ca^{2+}, it is highly sensitive to phosphorylation, and kinases such as ERK and Rho that are active during meiosis might have regulatory roles (Deng et al. 2005). In summary, the molecular effectors for several events of egg activation downstream of Ca^{2+} have been uncovered over the last decade. Although important gaps remain, the requirement for $[Ca^{2+}]_i$ oscillations for initiation of mammalian development is unambiguous.

Single versus Multiple $[Ca^{2+}]_i$ Increases

The early ionophore studies hinted to a pivotal role for Ca^{2+} in the initiation of development, especially with regard to the increases in $[Ca^{2+}]_i$, but not in K^+ or pH, induced all early and late events of egg activation (Steinhardt and Epel 1974). Nevertheless, whether or not such changes happened during normal fertilization and how their inhibition affected development was unknown. Evidence soon accumulated, first using the luminescent protein "aequorin" synthesized by Shimomura and colleagues (Shimomura and Johnson 1970) that explosive $[Ca^{2+}]_i$ increases accompanied fertilization in medaka fish eggs (Ridgway et al. 1977) and in sea urchin eggs (Steinhardt et al. 1977). Unlike the single $[Ca^{2+}]_i$ increases detected in these early recordings, measurements of $[Ca^{2+}]_i$ changes in mammals revealed that their eggs displayed $[Ca^{2+}]_i$ oscillations (Cuthbertson et al. 1981; Miyazaki and Igusa 1981). Although oscillatory $[Ca^{2+}]_i$ responses were subsequently reported in non-mammalian species, mammalian eggs are the only ones whose oscillations extend for over several hours (Stricker 1999). Further, research soon followed demonstrating that abrogation of fertilization-associated $[Ca^{2+}]_i$ increases, which was accomplished with the Ca^{2+} chelator BAPTA, prevented all events of egg activation and prevented the initiation of development (Kline and Kline 1992a). Together, these results confirmed the widespread role of Ca^{2+} as the activation signal for development.

Although the elevation of $[Ca^{2+}]_i$ is ubiquitous in fertilization, the presence of long-lasting oscillations is a hallmark of mammalian fertilization (Fig. 1B). Remarkably, the developmental advantages and underlying molecular changes associated with these oscillations remain unclear. Research by Ducibella et al. underscored the varying sensitivities of egg activation events to $[Ca^{2+}]_i$ increases. For instance, most events of egg activation, such as CG exocytosis, meiotic resumption and recruitment of maternal mRNAs require fewer $[Ca^{2+}]_i$

increases for initiation than for completion, and early events such as CG exocytosis and release from MII arrest require fewer $[Ca^{2+}]_i$ responses for completion than later events, such as PN formation and recruitment of maternal mRNAs (Ducibella et al. 2002). In this context, oscillations make sense, especially to promote CycB degradation and inactivation of MPF, as CycB synthesis is continuous (Nixon et al. 2002; Marangos and Carroll 2004) and a single $[Ca^{2+}]_i$ increase would be unable to promote its complete degradation (Nixon et al, 2002). Nevertheless, if exit of MII arrest is overcome either by a single, overwhelming $[Ca^{2+}]_i$ increase induced by an electrical pulse (Ozil et al. 2005) or by expression of a constitutively active form of CaMKII, development to the blastocyst stage is only mildly impaired (Madgwick et al. 2005; Ozil et al. 2005; Knott et al. 2006). This apparent lack of impact of $[Ca^{2+}]_i$ oscillations on preimplantation development is in contrast to another report showing that both premature termination or excessive Ca^{2+} stimulation negatively impacts preimplantation and postimplantation development, and alters embryonic gene expression (Ozil et al. 2006). Similar research documented that parthenogenotes generated without a $[Ca^{2+}]_i$ increase by exposing eggs to cycloheximide, a protein synthesis inhibitor, showed altered gene expression and poor development to the blastocyst stage (Rogers et al. 2006). Nonetheless, development of these embryos was rescued to the same extent by exposure to a single or multiple $[Ca^{2+}]_i$ increases, casting doubts on the beneficial effects of $[Ca^{2+}]_i$ oscillations on development.

One way that multiple $[Ca^{2+}]_i$ elevations may pose a developmental advantage is by specifically stimulating embryonic gene expression. The recruitment of maternal mRNAs, which mediates new protein synthesis after fertilization, takes place during the period of oscillations and is susceptible to the magnitude of the $[Ca^{2+}]_i$ stimulation; more pulses more protein synthesis (Ducibella et al. 2002). To this end, one of the two transcripts identified after fertilization is cyclin A (Oh et al. 1997; Fuchimoto et al. 2001), which participates in the activation of the embryonic genome (Fuchimoto et al. 2001). Thus, based on research in hippocampal neurons, the suggestion was made that pulsatile activation of CaMKII may underlie the enhanced gene expression observed after repeated $[Ca^{2+}]_i$ pulses (Ducibella et al. 2006). Subsequent research, however, showed that recruitment of mRNAs could occur independently of this kinase (Backs et al. 2010). Furthermore, it might not be under the exclusive control of Ca^{2+}, as in the absence of cell cycle progression, fertilization-initiated oscillations failed to induce recruitment of mRNAs (Backs et al. 2010). Therefore, it might that the total magnitude of the $[Ca^{2+}]_i$ increase, as proposed by Ozil and colleagues (Ducibella et al. 2006; Ozil et al. 2006) rather than the temporal pattern of $[Ca^{2+}]_i$ increases is the determinant factor of egg activation in mammals. Nonetheless, oscillations may be necessary, as besides signaling the stepwise completion of all events of egg activation, it might be the only manner whereby mammalian eggs can attain a Ca^{2+} signal of sufficient magnitude to ensure CycB degradation without undermining other cellular functions.

MOLECULAR PLAYERS RESPONSIBLE FOR $[Ca^{2+}]_i$ OSCILLATIONS DURING FERTILIZATION

The $[Ca^{2+}]_i$ oscillations that underlie egg activation in mammals rely on molecular players widely characterized in other cellular systems in which they mediate $[Ca^{2+}]_i$ responses induced by a variety of agonists such as hormones, growth factors and antigen-presenting mechanisms (Berridge et al. 2000; Clapham 2007). In gametes however, the function and regulation of some of these molecules has been adapted to respond to the unique requirements of fertilization. For instance, oocytes require weeks or months of preparation before being ready for fertilization, because interruptions in the cell cycle are imposed during meiosis to synchronize oocyte and follicular growth before reinitiating meiosis and ovulation. It is believed that during this growth phase, oocytes do not require $[Ca^{2+}]_i$ elevations, and Ca^{2+} release mechanisms are quiescent (Carroll et al. 1994). Importantly, these mechanisms are quickly

reactivated in fully-grown oocytes after receiving an LH surge, which is the endocrinological signal that induces oocyte maturation from the germinal vesicle (GV) stage. During this process, which may last from 12 to 48 hr according to the species, the oocytes' Ca^{2+} release mechanisms undergo reprogramming and optimization so that fertilization can initiate $[Ca^{2+}]_i$ oscillations. The sperm also undergoes a protracted preparation, undergoing changes during transport through the male reproductive tract and more closely as it approaches the site of fertilization in the female tract (Suarez 2008b). Remarkably, some of these changes also involve $[Ca^{2+}]_i$ increases (Suarez 2008a), although they occur while preserving the sperms' Ca^{2+} activating signal.

Two molecules stand out in mammalian fertilization as central to the initiation and maintenance of $[Ca^{2+}]_i$ oscillations; namely, the IP_3R1 receptor in eggs and PLCζ in the sperm. Here we will describe the evidence supporting their role in mammalian fertilization, focusing on regulatory mechanisms and highlighting some of the unanswered questions regarding their regulation. We will also review other molecular mechanisms required to maintain oscillations, especially those affecting Ca^{2+} influx whose function in eggs has not been widely investigated (Fig. 1C).

IP_3R1

IP_3R1 in MII Eggs

The IP_3R is the main intracellular Ca^{2+}-release channel of almost all mammalian cell types and is located in the endoplasmic reticulum (ER), the cells' main Ca^{2+} reservoir (reviewed in Berridge et al. [2000]; Bootman et al. [2001]). The IP_3R is a large protein (>250 kDa) and functions as a tetramer (>1000 kDa). Each monomer consists of more than 2600 amino acids and can be broadly divided into three regions, a cytosolic amino-terminal domain that binds IP_3, a regulatory domain that contains multiple regulatory sites for Ca^{2+}, ATP, and other modulatory molecules/proteins (MacKrill 1999; Patterson et al. 2004) and a carboxy-terminal channel domain that contains six transmembrane domains and a short cytosolic tail. As described by Taylor and Tovey (2010), the activation and opening of the IP_3R1 requires binding by both Ca^{2+} and IP_3, and the regulation of IP_3-induced Ca^{2+} release (IICR) by Ca^{2+} adopts a bell-shape form, as IICR is stimulated at low $[Ca^{2+}]_i$ and inhibited at high $[Ca^{2+}]_i$ (Taylor and Tovey 2010; Iino 1990; Finch et al. 1991). This dual regulation of IP_3R1 by Ca^{2+} and IP_3 makes it especially suited to support long lasting oscillations.

There exists three IP_3R isoforms (reviewed in (Berridge et al. [2000]), and mammalian oocytes and eggs and their surrounding cells express all isoforms (Fissore et al. 1999a; Fissore et al. 1999b; Diaz-Munoz et al. 2008). Importantly, oocytes and eggs overwhelmingly express the type I IP_3R isoform (Kume et al. 1997; Fissore et al. 1999a; Jellerette et al. 2000; Iwasaki et al. 2002). The initial suggestion that IP_3R may play a role during fertilization arose from studies in sea urchin eggs in which an increase in phosphoinositide metabolism accompanied fertilization (Turner et al. 1984), an observation that was soon followed by the demonstration that injection of IP_3 triggered Ca^{2+} release (Clapper and Lee 1985) and cortical granule exocytosis (Turner et al. 1986). Studies followed in hamster oocytes in which injection of IP_3 and guanine nucleotides initiated repeated Ca^{2+} release from intracellular stores (Miyazaki 1988). Purification and identification of the IP_3R protein from the cerebellum occurred in the late 1980s (Maeda et al. 1988; Furuichi et al. 1989), and confirmation of its significance in mammalian fertilization took place soon after when both the initiation of $[Ca^{2+}]_i$ oscillations (Miyazaki et al. 1992) and egg activation (Xu et al. 1994) were prevented by injection of a functional blocking antibody raised against the Carboxy-terminal end of mouse IP_3R1. Subsequent studies confirmed the role of IP_3R1 in fertilization in other species (Parys et al. 1994; Thomas et al. 1998; Yoshida et al. 1998; Runft et al. 1999; Goud et al. 2002; Iwasaki et al. 2002).

Fertilization-associated $[Ca^{2+}]_i$ oscillations in mouse zygotes undergo changes during the transition from the MII stage into interphase, becoming initially less frequent before ceasing altogether at the time of PN formation (Jones

et al. 1995; Kono et al. 1996; Deguchi et al. 2000). During this transition, the IP_3R1 undergo several modifications and it is possible that either singly or collectively these influence the pattern of oscillations. For example, mammalian eggs richly express IP_3R1, as only 20 mouse eggs are required for its detection by Western blotting (Parrington et al. 1998; Jellerette et al. 2000) and within 4 h after sperm entry the IP_3R1 mass is reduced approximately to a half (Parrington et al. 1998; Deguchi et al. 2000; Kurokawa and Fissore 2003). Moreover, recent research shows that IP_3R1 degradation alone can explain the widening of the $[Ca^{2+}]_i$ intervals, although not the termination of oscillations (Lee et al.). Changes in IP_3R1 localization may also affect the pattern of oscillations. In eggs, the IP_3R1 and the ER are organized in clusters near the cortex, a location that might facilitate the initiation of $[Ca^{2+}]_i$ oscillations, as the PLCζ concentration may be higher in this area after sperm-egg fusion. The accumulation of ER clusters in the cortex may also enhance IP_3R1 sensitivity, as $[Ca^{2+}]_i$ oscillations originate from the hemisphere opposite to the MII spindle where ER/IP_3R1 clusters are particularly dense (Kline et al. 1999; Dumollard et al. 2004). Interestingly, in *Xenopus*, IP_3R1s that are more sensitive move to the cortex from the subcortex as oocytes progress to the MII stage (Boulware and Marchant 2005). Importantly, in the mouse, ER (FitzHarris et al. 2003) and possibly IP_3R1 (our unpublished data) cortical clusters disappear ahead of the termination of the oscillations, suggesting that they are not required for the persistence of oscillations. Nevertheless, the precise distribution of IP_3R1 in eggs suggest an important role during fertilization, which may correspond to the need for localized high amplitude $[Ca^{2+}]_i$ increases to facilitate CG release to prevent polyspermy (McAvey et al. 2002).

IP_3R1 function may also be regulated by phosphorylation. Not surprisingly, the first report describing IP_3R1 phosphorylation in eggs suggested an association with cell cycle kinases (Jellerette et al. 2004), which play a prominent role in the MII arrest. IP_3R1 phosphorylation in mouse eggs was first characterized using an antibody that identifies proteins phosphorylated at the MPM-2 epitope, which consists of phosphorylated serines(S)/threonines(T) next to prolines(P) surrounded by hydrophobic amino acids (Westendorf et al. 1994). IP_3R1 becomes phosphorylated at a MPM-2-detectable epitope during maturation (Lee et al. 2006) reaching maximal reactivity at the MII stage (Fig. 2). Following egg activation, it becomes gradually dephosphorylated and phosphorylation is not regained at first mitosis (Lee et al. 2006). The responsible kinases for IP_3R1 MPM-2 phosphorylation remain to be determined, although several M-phase kinases, such as polo-like kinase 1 (Plk1), mitogen-activated protein kinase (MAPK), and Cdk1 can all

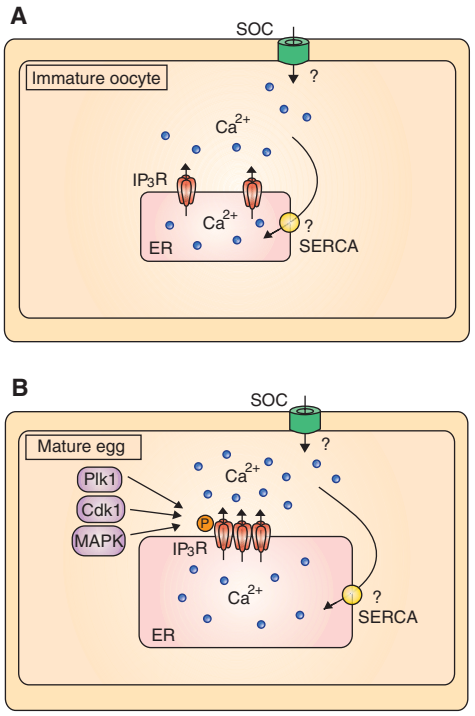

Figure 2. IP_3R1-mediated Ca^{2+} release increases during mouse oocyte maturation. Several factors may contribute to this, including the increased Ca^{2+} content of the stores, IP_3R1 organization into clusters and IP_3R1 phosphorylation. Question marks suggest mechanisms that are suspected to contribute to Ca^{2+} influx and increased Ca^{2+} store content. Phosphorylating kinases are Polo-like kinase-1, Cyclin-dependent kinase-1, and Mitogen-associated protein kinase.

phosphorylate this epitope (Joughin et al. 2009). Our studies in mouse oocytes using pharmacological inhibitors revealed that Plk1 might be involved in MPM-2 phosphorylation early during maturation (Ito et al. 2008; Vanderheyden et al. 2009) and MAPK during the MI to MII transition, although whether they directly phosphorylate the receptor remains to be shown. Research in DT40 B-cell lymphocytes showed IP_3R1 phosphorylation by MAPK at S^{436}, a residue that lies in the receptor's ligand binding domain within a consensus site for the kinase; using back cross phosphorylation studies, this group also showed IP_3R1 MAPK phosphorylation of mouse MII eggs (Bai et al. 2006). This study did not examine the role of this phosphorylation in eggs, although in microsome preparations MAPK IP_3R1 phosphorylation decreased IP_3 binding and Ca^{2+} release (Bai et al. 2006). In contrast to this, phosphorylation of IP_3R1 in somatic cells by Cdk1, which was observed to occur in several conserved Cdk1 motifs under in vitro and in vivo conditions, enhanced IP_3 binding and Ca^{2+} release (Malathi et al. 2003; Malathi et al. 2005). IP_3R1 phosphorylation within Cdk1 and MAPK consensus sites was reported in *Xenopus* oocytes and independent activation of these kinases increased IP_3R-mediated Ca^{2+} release (Sun et al. 2009). Notably, an earlier study in mouse zygotes had dismissed the role of MPF and MAPK on $[Ca^{2+}]_i$ oscillations, as the continuation was unaltered by the decline in MPF activity, which occurs at the time of second PB extrusion, or after inhibition of the MAPK pathway with U0126 (Marangos et al. 2003). Importantly, the phosphorylation status of IP_3R1 was not examined in that study, and subsequent results found that IP_3R1 MPM-2 phosphorylation outlasts MPF activity (Lee et al. 2006) and that 4 hours U0126 exposure does not eliminate MPM-2 IP_3R1 reactivity (our unpublished observations). A possible interpretation for these results is that IP_3R1 phosphorylation, and the phosphorylation of other M-phase substrates in eggs is safeguarded by suppression of phosphatase(s) activity, a function that has been attributed to the Greatwall kinase in mitotic cells and *Xenopus* egg extracts (Castilho et al. 2009). Therefore, accumulating evidence suggests a regulatory role for phosphorylation on IP_3R1 function during fertilization, although the responsible kinases, phosphorylation sites and their impact on IP_3R1 function remain to be clarified.

IP_3R1 in Maturing Oocytes

The precise spatio-temporal pattern of sperm-associated $[Ca^{2+}]_i$ responses in vertebrate eggs is established during oocyte maturation. For example, in vitro fertilized mouse GV oocytes show fewer $[Ca^{2+}]_i$ oscillations and each $[Ca^{2+}]_i$ increase shows reduced duration and amplitude than those observed in fertilized MII eggs (Jones et al. 1995a; Mehlmann et al. 1996). The molecular events underlying these changes are not understood, although changes in IP_3R1 sensitivity, i.e., the receptor's ability to conduct Ca^{2+} in response to IP_3, are thought to be involved. Importantly, studies to elucidate these mechanisms are needed, but given the recalcitrant nature of some of these changes in MII eggs, it is suggested that they should be performed during maturation.

As discussed above, IP_3R1 phosphorylation during maturation by M-phase kinases is thought to enhance Ca^{2+} release in eggs. MPM-2 reactivity, which is used as a marker of their activity, is first evidenced in IP_3R1 at the time of GV breakdown (GVBD) and persists until the MII stage, a period that closely coincides with the increased function of IP_3R1 during maturation (Mehlmann and Kline 1994). Inhibition of Plk1, a kinase involved in the activation of Cdk1, reduced and delayed MPM-2 IP_3R1 reactivity and decreased $[Ca^{2+}]_i$ release through IP_3R1 at the GVBD stage (Ito et al. 2008a; Vanderheyden et al. 2009). Nonetheless, the persistent presence of BI2556, a nonreversible and specific Plk1 inhibitor, did not eliminate MPM-2 IP_3R1 reactivity, which even experienced a partial recovery, suggesting that other kinases, possibly Cdk1, might phosphorylate IP_3R1 (our unpublished observation). Investigation of the role of Cdk1 on IP_3R1 phosphorylation in mouse oocytes/eggs is hindered by the findings that roscovitine, a specific Cdk1

inhibitor, indirectly inhibits Plk1 activity (our unpublished results) and greatly reduces Ca^{2+} store content ($[Ca^{2+}]_{ER}$) (Deng and Shen 2000), thereby compromising the interpretation of the results. Inhibition of the MAPK pathway, which does not affect the aforementioned kinases and only mildly affects IP_3R1 MPM-2 phosphorylation after the MI stage, greatly reduces $[Ca^{2+}]_i$ responses (Lee et al. 2006). It is therefore possible that IP_3R1 phosphorylation by MAPK is not recognized by the MPM-2 antibody or, alternatively, that the MAPK pathway affects other aspects of Ca^{2+} homeostasis. In this regard, one study found an altered Ca^{2+} content in U1026-treated oocytes (Matson and Ducibella 2007) whereas the other did not (Lee et al. 2006). Therefore, additional mutational studies are needed to clarify the impact of M-phase kinases on IP_3R1-mediated Ca^{2+} release in mammalian oocytes and eggs.

Besides M-phase kinases, numerous studies in somatic cells have shown that IP_3R isoforms can be phosphorylated by various, more wide-ranging kinases that generally increase IP_3R-mediated Ca^{2+} release (Bezprozvanny 2005; Vanderheyden et al. 2009), although phosphorylation by PKB reportedly reduces IP_3R-mediated Ca^{2+} release (Szado et al. 2008). The most commonly implicated kinases include PKA, PKC, and CaMKII, all of which have important physiological functions in oocytes and eggs (Ducibella and Fissore 2008). Extensive phosphopeptide mapping combined with substrate specific antibodies in *Xenopus* oocytes found that IP_3R1 is uniformly phosphorylated throughout maturation in both PKA consensus motifs, whereas PKC sites seemed unperturbed (Sun et al. 2009). Whether or not PKA IP_3R1 phosphorylation has functional consequences in oocytes is unknown, although in somatic cells this phosphorylation has been associated with increased IP_3R1 activity (DeSouza et al. 2002) and reduced $[Ca^{2+}]_{ER}$, which may reportedly underlie the antiapoptotic effects of some members of the Bcl-2 family of proteins (Oakes et al. 2005). Moreover, it has been suggested that these proteins modify the PKA-associated IP_3R1 phosphorylation status (Oakes et al. 2005), although research from other laboratories has not confirmed this mechanism of action of Bcl-2 family protein on IP_3R1 function (Rong et al. 2008; Rong et al. 2009). It is worth noting that $[Ca^{2+}]_{ER}$ is low in GV oocytes in spite of persistent Ca^{2+} influx, as evident by the continuous spontaneous oscillations at this stage (Carroll and Swann 1992). On GVBD, however, $[Ca^{2+}]_{ER}$ undergoes a marked increase (Jones et al. 1995a), which occurs concurrently with the termination of the oscillations that implies suppression/reduction of Ca^{2+} influx. Given that cAMP levels decrease at GVBD (Norris et al. 2009), it is therefore possible that a Ca^{2+} leak mechanism regulated by PKA IP_3R1 phosphorylation may be implicated in Ca^{2+} homeostasis during oocyte maturation. Future studies should examine whether PKA-mediated IP_3R1 phosphorylation changes during mouse oocyte maturation.

The differential redistribution of ER/IP_3R1 may also enhance IP_3R1 function during oocyte maturation. Before the initiation of maturation, the ER in mouse oocytes shows a homogeneous distribution with slight accumulation around the GV, although by the MII stage, the ER displays a fine tubular network appearance with dense accumulation in the cortex (Mehlmann et al. 1995), which is thought to facilitate the initiation of sperm-induced $[Ca^{2+}]_i$ oscillations (Kline et al. 1999). The dramatic reorganization ensues at about the time of GVBD and is underpinned by distinct components of the cytoskeleton (FitzHarris et al. 2007), as the migration of the ER toward the condensing chromosomes is dependent on microtubules, whereas its dispersal from the MI spindle to the egg's cortex relies on actin microfilaments (FitzHarris et al. 2003). The IP_3R1 are also organized in cortical clusters at the MII stage (Mehlmann et al. 1996; Fissore et al. 1999a; Ito et al. 2008a), although it remains to be established whether the same cytoskeletal mechanisms that control ER organization control IP_3R1 distribution. Curiously, in spite of the large remodeling that the oocyte's ER undertakes, it is unknown whether this reorganization affects IP_3R1's sensitivity and the ability to initiate and support oscillations in mammalian eggs. Thus, preventing ER and possibly IP_3R1 cortical cluster organization with appropriate cytoskeleton inhibitors would

help elucidate the influence of their reorganization on IP_3R1 function in oocytes.

Lastly, changes in other cytoplasmic parameters are likely to contribute to increase IP_3R1 sensitivity in oocytes. For example, the increase in $[Ca^{2+}]_{ER}$ during maturation (Kline and Kline 1992a; Jones et al. 1995a) may not only increase the amount of available Ca^{2+} for release, but may also increase the receptor's sensitivity (Missiaen et al. 1991). The increase in $[Ca^{2+}]_{ER}$ is likely the result of careful regulation of the pathways that control Ca^{2+} influx and efflux, which in *Xenopus* oocytes are known to be actively regulated during maturation (El-Jouni et al. 2005; Yu et al. 2009). Remarkably, the molecular identity and significance of these mechanisms in mammalian oocytes remain largely unknown and will be discussed below.

Ca^{2+} Homeostasis in Oocytes and Eggs

$[Ca^{2+}]_i$ Clearing Mechanisms

$[Ca^{2+}]_i$ oscillations in mammals continue for long periods that can exceed 20 h (Fissore et al. 1992; Sun et al. 1992). For $[Ca^{2+}]_i$ responses to continue without attenuation following a $[Ca^{2+}]_i$ increase, $[Ca^{2+}]_i$ levels need to be returned to baseline and stores refilled in anticipation of the next $[Ca^{2+}]_i$ response. To bring $[Ca^{2+}]_i$ to baseline, cells either return free cytosolic Ca^{2+} into the ER by the action of the sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs), and/or extrude it by the action of plasma membrane (PM) Ca^{2+} ATPases (PMCAs) and Na^+/Ca^{2+} exchangers (Berridge et al. 2000; Bootman et al. 2001). Few studies have addressed the function of these molecules in mammalian oocytes/eggs, although the presence of SERCA2b can be surmised by the alteration of $[Ca^{2+}]_i$ levels caused by exposure to thapsigargin, an inhibitor of SERCA (Kline and Kline 1992b; Lawrence and Cuthbertson 1995; Machaty et al. 2002). Exposure of MII eggs to thapsigargin causes a slow and steady increase in $[Ca^{2+}]_i$ followed by a protracted decline, whereas in fertilized eggs it prevents the continuation of oscillations (Kline and Kline 1992b). Importantly, the molecular presence and cellular distribution of SERCA2b has not yet been examined in mammalian oocytes, although transcripts have been found in GV and MII stage oocytes (Su et al. 2007). In *Xenopus* oocytes, expression of the SERCA2 protein was documented by immunofluorescence and it was shown to undergo reorganization similar to that described for IP_3R (El-Jouni et al. 2005). Given that the levels of $[Ca^{2+}]_{ER}$ change dramatically during maturation, it is possible that SERCA activity may be actively regulated during this process. In somatic cells, SERCA activity can be regulated by different mechanisms, including binding to regulatory proteins such phospholamban, sarcolipin, and by several posttranslational modifications (reviewed in Brini and Carafoli [2009]). An earlier report in *Xenopus* oocytes showed that SERCA2b activity could be regulated by association with the ER chaperone protein, calnexin, which inhibited the pump's activity; phosphorylation of calnexin relieved both the association with SERCA and its inhibition (Roderick et al. 2000). Although additional studies are needed to understand the conservation of this mechanism, it is worth noting that addition of roscovitine prematurely, albeit reversibly, terminates $[Ca^{2+}]_i$ oscillations during fertilization (Deng and Shen 2000). Although the inhibitor's target was not elucidated, $[Ca^{2+}]_{ER}$ levels were severely depleted, suggesting an effect either on SERCA activity or on the Ca^{2+} influx mechanism(s). Future studies should explore the pathways involved in regulation of SERCA activity in mammalian eggs, as its function in sustaining long-term $[Ca^{2+}]_i$ oscillations after fertilization.

Besides the ER sequestration of Ca^{2+}, cytosolic $[Ca^{2+}]_i$ can also be returned to baseline by the action of PMCA and the Na^+/Ca^{2+} exchanger, which release Ca^{2+} into the external media. The functional activity of Na/Ca^{2+} exchanger was shown in mouse eggs by two different reports (Pepperell et al. 1999; Carroll 2000). It was shown that elimination of Na^+ from the external media caused $[Ca^{2+}]_i$ responses, or accelerated existing ones, and these responses were ascribed to reverse mode Na^+-Ca^{2+} exchange. In spite of the initial changes, even in the absence of external Na^+, $[Ca^{2+}]_i$ levels returned to baseline levels, implying that the

action of PMCA may be more physiologically relevant (Carroll 2000). The molecular presence of PMCA has not been documented in mammalian oocytes/eggs, although in *Xenopus* oocytes PMCA1 seems to contribute to the shaping of $[Ca^{2+}]_i$ responses (El-Jouni et al. 2005; El-Jouni et al. 2008). For instance, in GV oocytes the presence of 1 mM La^{3+}, which is known to inhibit PMCA function, delay the half-time decay of a $[Ca^{2+}]_i$ increase induced by ionononmycin, but the same treatment was without effect in MII eggs, suggesting that the pump's activity is down regulated during maturation (El-Jouni et al. 2005). Research by these investigators also showed that most of the PMCA in the plasma membrane becomes internalized during maturation explaining, at least in part, the lower $[Ca^{2+}]_i$ clearing capacity of eggs versus oocytes (El-Jouni et al. 2005). Nevertheless, it remains to be shown how PMCA plays a role in mammalian fertilization and what are the putative regulatory mechanisms, as complete internalization seems unlikely in this system, given that $[Ca^{2+}]_i$ increases occur uninterruptedly for hours in these eggs.

The mitochondria may also contribute to regulate baseline $[Ca^{2+}]_i$ in the presence of oscillations (Duchen 2000; Rizzuto et al. 2000), as they can sequester Ca^{2+} into the matrix thereby decreasing the overall cytosolic Ca^{2+} load (Rizzuto et al. 1998). Despite early evidence to the contrary (Liu et al. 2001), this does not seem to be the main function in eggs, as inhibition of Ca^{2+} mitochondrial uptake does not immediately terminate sperm-initiated oscillations (Dumollard et al. 2004). Instead, and possibly because of its vicinity to the IP_3R1/ER, the Ca^{2+}-driven ATP output may be the mitochondria's most critical contribution to Ca^{2+} homeostasis in MII eggs, as it maintains SERCA activity, which is required to sustain sperm-triggered Ca^{2+} oscillations (Dumollard et al. 2004).

Ca^{2+} Influx Mechanisms

Given that a fraction of Ca^{2+} from each $[Ca^{2+}]_i$ increase is secreted out of the egg by the action of PMCA or the Na^+/Ca^{2+} exchanger, external Ca^{2+} must be taken in to maintain $[Ca^{2+}]_{ER}$. Ca^{2+} influx plays a pivotal role in fertilization, as sperm-initiated $[Ca^{2+}]_i$ oscillations cease prematurely in the absence of external Ca^{2+} (Igusa and Miyazaki 1983; Igusa et al. 1983; Winston et al. 1995). Nevertheless, the molecules that mediate Ca^{2+} influx and their regulation remain poorly characterized. Oocytes/eggs and somatic cells use several Ca^{2+} influx mechanisms, including receptor-operated channels (ROCs) and voltage-operated Ca^{2+} channels (VOCs) (Berridge et al. 2000; Smyth et al. 2006), the last of which is active in mammalian oocytes and eggs (Tosti and Boni 2004). Notably, although changes in membrane potential accompany fertilization in mammals, several findings suggest that they might not be causally linked to the replenishment of the stores, as $[Ca^{2+}]_i$ increases precede changes in membrane potential (Igusa et al. 1983), Ca^{2+} influx continues between $[Ca^{2+}]_i$ increases (McGuinness et al. 1996) and, in the mouse, the changes in membrane potential are almost imperceptible (Igusa et al. 1983). These findings raise the prospect that Ca^{2+} influx in oocytes may be attained, at least in part, by a different mechanism(s). Store-operated Ca^{2+} entry (SOCE), which is associated with $[Ca^{2+}]_{ER}$ levels (Putney 1986), may fulfill this role in oocytes/eggs. The presence of store operated Ca^{2+} channels (SOC) to mediate SOCE and their electrophysiological properties were surmised in mast cells and in T-cells more than 10 years (Lewis and Cahalan 1989; Zweifach and Lewis 1993), although their molecular identity remained elusive until recently. Using a small RNA interference (RNAi) screen, two groups found that STIM1 was required for SOCE, as its reduction decreased Ca^{2+} influx in response to thapsigargin (Liou et al. 2005; Roos et al. 2005). Given that STIM1 lacks an obvious channel, the search was on to find the required channel partner protein, one of which was quickly identified as Orai1 (Feske et al. 2006; Vig et al. 2006). Besides the recent demonstration of molecular coupling at the cellular level between STIM1 and Orai1 (Park et al. 2009), spontaneous mutations of these proteins in humans have been linked to related immune response-related diseases, conclusively implicating their function in the same

Ca^{2+} influx pathway (Feske et al. 2005; Picard et al. 2009). For general information about Stim and Orai family of proteins, see Lewis 2011.

Evidence for SOCE in mammalian eggs was first observed after the application of thapsigargin, which caused a large Ca^{2+} influx after adding Ca^{2+} back to the media, (Kline and Kline 1992b; Machaty et al. 2002). Subsequent studies implicated SOCE in fertilization, as using the manganese-quenching technique it was found that in mouse eggs the initiation of each $[Ca^{2+}]_i$ increase coincided with divalent cation influx (McGuinness et al. 1996). Although SOCE was also described in human eggs (Martin-Romero et al. 2008), the understanding of the molecular underpinning of this influx in mammalian oocytes remains poor.

Transient receptor potential (TRP) ion channels (Venkatachalam and Montell 2007), which show widespread cellular distribution and display numerous regulatory mechanisms, were considered as possible mediators of Ca^{2+} influx in eggs. Expression at the transcript level was noted in porcine and mouse oocytes for several of the TRP family members (Machaty et al. 2002; Su et al. 2007), although evidence for their involvement in fertilization has yet to materialize. In contrast, two recent manuscripts examined the expression and function of STIM1 in oocytes. In porcine oocytes, STIM1 expression was detected at the mRNA level, and over-expression or knock down of STIM1 enhanced/reduced, respectively, thapsigargin-promoted Ca^{2+} influx. Expression of YFP-tagged STIM1 suggested ER localization and "puncta" reorganization in these oocytes, although more conclusive studies are needed (Koh et al. 2009). In mouse eggs, STIM1 was detected by western blotting, although the apparent molecular weight (Gomez-Fernandez et al. 2009) seems lower compared to published data in mouse somatic cells (Manji et al. 2000). Further, the detection of endogenous STIM1 by immunofluorescence revealed large patches (Gomez-Fernandez et al. 2009), which seem disproportionate to the reportedly low abundance of this protein in most cell types (Park et al. 2009). Lastly, whereas transcripts of Orai1 and two have been detected in mouse oocytes and eggs (Su et al. 2007), their protein expression has not been confirmed, and therefore their involvement in Ca^{2+} influx during mammalian fertilization remains to be shown.

A better understanding of SOCE's molecular effectors and regulatory mechanisms already exists in *Xenopus* oocytes and eggs (Machaca and Haun 2002; Yu et al. 2009). Initial research showed inactivation of SOCE, which is manifested by the uncoupling of Ca^{2+} store depletion and Ca^{2+} influx, around the time of GVBD (Machaca and Haun 2002). Although the inactivating mechanism was not known, it was determined to be associated with the activities of the M-phase kinases that regulate GVBD (Machaca and Haun, 2002). A follow up study found that during GVBD Orai1 is internalized from the plasma membrane, and STIM1's ability to form clusters and puncta is obliterated, which together disable SOCE (Yu et al. 2009). Earlier, an uncoupling between Ca^{2+} influx and Ca^{2+} store content was reported in somatic cells during mitosis (Preston et al. 1991). Those findings were recently extended, and SOCE inactivation during mitosis was associated with Stim1 phosphorylation by Cdk1, which prevents its rearrangement and precludes coupling and activation of Orai1 (Smyth et al. 2009). STIM1 phosphorylation by Cdk1 was also noted in the foregoing *Xenopus* study, although it was deemed to have minor impact on STIM1 function (Yu et al. 2009). It is noteworthy that a conserved Cdk1 phosphorylation site present in mammalian STIM1 is absent from *Xenopus* STIM1. Importantly, unlike the previous examples, SOCE is operational during the MII stage of mammalian fertilization. Therefore, future studies should examine the regulatory mechanism(s) that control SOCE during maturation and fertilization in mammals.

PLCζ

There has been much debate and speculation as to the mechanism(s) that triggers $[Ca^{2+}]_i$ oscillations during mammalian fertilization. Several

excellent recent reviews have addressed this topic in depth (Swann et al. 2006; Parrington et al. 2007; Horner and Wolfner 2008b) and therefore only the most salient and outstanding aspects of PLCζ will be discussed here. As noted earlier, research in a variety of species including mammals showed that fertilization-associated $[Ca^{2+}]_i$ responses require the same agonists and signaling cascades that cause Ca^{2+} release in somatic cells (Miyazaki et al. 1993; Miyazaki and Ito 2006). Nevertheless, although stimulation of these pathways induced $[Ca^{2+}]_i$ responses, they failed to reproduce the pattern of $[Ca^{2+}]_i$ oscillations associated with mammalian fertilization, leaving open the possibility that a different mechanism may underpin oscillations in these species (Swann et al. 1989). Observations first in sea urchin eggs and then in ascidian eggs noted that injection of sperm extracts caused PM currents similar to that observed in fertilization (Dale et al. 1985; Dale 1988). Subsequently, studies in mammals showed that injections of sperm extracts initiated fertilization-like oscillations and egg activation (Stice and Robl 1990; Swann 1990). Based on these results and in light of the protracted nature of the $[Ca^{2+}]_i$ oscillations, which can vastly exceed the interaction time of gametes at the PM, a hypothesis was proposed whereby a SF acts as the trigger of oscillations after fusion of the gametes (Swann and Lai 1997). Although this hypothesis was received with skepticism, support for it grew steadily, as injection of sperm extracts initiated oscillations in several mammalian and nonmammalian species (Stricker 1997; Wu et al. 1997). Furthermore, physiological support for this concept was provided both when intracytoplasmic sperm injection into eggs (ICSI) resulted in the birth of young (Palermo et al. 1992), and the subsequent demonstration that ICSI initiated fertilization-like oscillations in several mammalian species (Tesarik and Testart 1994; Nakano et al. 1997; Kurokawa and Fissore 2003; Malcuit et al. 2006). Together, these studies consolidated the concept of the SF as the initiator of oscillations in mammalian eggs, although identification of the active principle would have to wait for another decade.

Identification of PLCζ

The search for the SF's active component(s) was the subject of intense interest and it was not without some false starts. A turning point came when studies using sea urchin egg extracts and in vitro PLC assays revealed that cytosolic preparations from mammalian sperm possessed high PLC activity, which was nearly twice as high as the activity present in other tissues (Parrington et al. 1999; Jones et al. 2000; Rice et al. 2000). In addition, it was discovered that the sperm's PLC activity displayed high sensitivity to Ca^{2+}, meaning that it shows near maximal activity in the presence of basal $[Ca^{2+}]$ concentrations (Rice et al. 2000), which in most cells are of ~100 nM (Clapham 2007). This feature made the putative SF a credible candidate to trigger oscillations, because to attain high specific activity most PLCs require $[Ca^{2+}]$ concentrations in excess of 1 μM (Rebecchi and Pentyala 2000; Nomikos et al. 2005), concentrations that are not compatible with MII arrest. It was therefore not surprising that injection of recombinant proteins representing most of the known isoforms expressed in sperm (Choi et al. 2001; Fukami et al. 2001) failed to initiate oscillations in mouse eggs (Parrington et al. 2002), or if they did, they did so at nonphysiological concentrations (Mehlmann et al. 2001). Hence, it became evident that if a PLC were to be the SF, it had to be a novel PLC. Toward this end, the novel sperm-specific PLCζ (Saunders et al. 2002) was identified in a PLC homology screen of mouse testis expressed sequence tags. Data in the latter study and in follow up reports provided strong evidence to support the concept that PLCζ is the pivotal, and possibly exclusive, initiator of $[Ca^{2+}]_i$ oscillations in mammals. Specifically, injection of recombinant PLCζ (Fujimoto et al. 2004; Kouchi et al. 2004) or PLCζ cRNA evoked sperm-like oscillations in mouse (Saunders et al. 2002), rat (Ito et al. 2008b), human (Rogers et al. 2004), bovine (Malcuit et al. 2005; Ross et al. 2008), porcine (Yoneda et al. 2006), and equine (Bedford-Guaus et al. 2008) eggs. In vitro PLC assays, using recombinant PLCζ confirmed the enzyme's high sensitivity to Ca^{2+},

which render it nearly fully active at basal $[Ca^{2+}]_i$ concentrations (Kouchi et al. 2004). Immunolocalization studies localized PLCζ to the postacrosomal region of mouse sperm (Fujimoto et al. 2004) and to the equatorial area of bull and human sperm (Yoon and Fissore 2007; Grasa et al. 2008; Yoon et al. 2008), regions that first come in contact with the ooplasm following gamete fusion, respectively (Sutovsky et al. 2003).

Recent evidence linking PLCζ expression and fertility further strengthened the role of PLCζ as the initiator of $[Ca^{2+}]_i$ oscillations in mammals. One study examined the ability of sperm from patients with repeated ICSI failure to initiate $[Ca^{2+}]_i$ oscillations in mouse eggs. The sperm from a few of these patients were incapable of initiating $[Ca^{2+}]_i$ responses, and examination of PLCζ expression by immunofluorescence and by Western blotting found reduced/absent levels of the enzyme in these sperm (Yoon et al. 2008). The results suggest that the inability of these sperm to activate eggs might be the main cause of their infertility. Consistent with this notion, studies have shown that the infertility of patients with globozoospermia, an affliction where even after ICSI most patients remain sterile, can be overcome by ICSI followed by Ca^{2+} ionophore-aided egg activation (Taylor et al. 2010; Heindryckx et al. 2005). A second study in patients with ICSI failure found that, in addition to reduced expression of PLCζ, a point mutation was identified that compromises PLCζ's ability to initiate $[Ca^{2+}]_i$ oscillations (Heytens et al. 2009). Collectively, the evidence supporting PLCζ as the mammalian SF is compelling. Nevertheless, questions remain regarding its expression during spermatogenesis and storage in sperm, its mechanism of release into the ooplasm, and mechanism(s) of activation once in the egg.

Despite evidence that PLCζ serves as the principal trigger of oscillations in mammals, research has unearthed species-specific differences that might prove useful in elucidating how PLCζ is regulated during fertilization. For example, although mouse PLCζ, which is the most studied, accumulates into the nucleus following PN formation (Saunders et al. 2002; Yoda et al. 2004), none of the other PLCζ isoforms tested display this localization despite sharing a nuclear localization signal (Cooney et al.; Ito et al. 2008b). There seems also to be significant differences in specific activity. For instance, based on the concentrations of cRNAs required to initiate oscillations, human PLCζ seems ~40-fold more active than mouse PLCζ, which itself is significantly more active than the rat enzyme (Cox et al. 2002; Rogers et al. 2004; Ito et al. 2008b). Although the role of these species-specific variations has not been explored carefully, it is tempting to speculate that they are the result of adaptations to promote the optimal activation signal. To this end, it is revealing that the species with the weakest PLCζ, the rat, has the easiest oocytes to activate (Zernicka-Goetz 1991; Ito et al. 2007). Future studies should examine whether an inverse association exists between expression levels/activity of PLCζ in sperm and IP_3R1 sensitivity/strength of the CSF-arresting machinery in eggs. Similarly, future studies should elucidate the molecular changes that underlie the differences in PLCζ activity among species. For example, despite missing the pleckstrin homology (PH) domain, PLCζ shows the modular organization characteristic of other PLCs, which consists of 4 EF hand Ca^{2+}-binding domains, X and Y catalytic domains, and the Ca^{2+}-dependent phospholipid-binding C2 domain (Rebecchi and Pentyala 2000). The EF-hand domains, and especially the EF3-hand domain, have been suggested to confer the high Ca^{2+} sensitivity of PLCζ through in vitro studies (Kouchi et al. 2005; Nomikos et al. 2005); whether sequence differences in this or other EF-hand domains underlie PLCζ species-specific differences should be examined.

CONCLUSIONS

The study of the Ca^{2+} mechanisms that underlie fertilization in mammals has resulted in important contributions to the Ca^{2+} signaling field in general and to the field of fertilization in particular. For example, the indispensable role of IP_3R1-mediated Ca^{2+} release in regulating cellular functions was unequivocally shown in mouse

fertilization (Miyazaki et al. 1992). Likewise, the discovery of the SF's active component, PLCζ (Saunders et al. 2002), not only provided evidence for a novel way of activating Ca^{2+} signaling in a host cell, but also added a new member with unique properties to the all important family of PLC enzymes. Importantly, and despite progress in the role of these two molecules in fertilization, we are still unaware of their fine regulatory mechanisms. For example, IP_3R1 function is greatly optimized during oocyte maturation, but the precise underlying molecular mechanisms responsible for these changes remain undetermined. Similarly, how the seemingly constitutive activity of PLCζ is provisionally restrained in the sperm and how its expression is regulated during spermatogenesis are questions that need addressing. Lastly, although $[Ca^{2+}]_i$ oscillations trigger mammalian development, we remain uninformed of the regulation of SERCA, which recycles Ca^{2+} into the ER, and of the molecules that underpin Ca^{2+} influx, which sustain the oscillations. Identification and elucidation of these regulatory mechanisms in oocytes will deepen our understating of fertilization, information that could be then used in the clinic for the diagnosis of infertility, and to enhance developmental competence of embryos generated by a variety of Assisted Reproductive Technology procedures.

ACKNOWLEDGMENTS

This work was supported by grants from the USDA and the NIH/N.I.C.H.D-HD051872 to R.A.F. The authors wish to thank all members of the Fissore lab for their generous contributions and suggestions. We apologize to those whose work was not cited because of space limitations.

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