



Ca²⁺ Signaling and Homeostasis in Mammalian Oocytes and Eggs

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Changes in the intracellular concentration of calcium ([Ca²⁺]_i) represent a vital signaling mechanism enabling communication between and among cells as well as with the environment. Cells have developed a sophisticated set of molecules, “the Ca²⁺ toolkit,” to adapt [Ca²⁺]_i changes to specific cellular functions. Mammalian oocytes and eggs, the subject of this review, are not an exception, and in fact the initiation of embryo development in all species is entirely dependent on distinct [Ca²⁺]_i responses. Here, we review the components of the Ca²⁺ toolkit present in mammalian oocytes and eggs, the regulatory mechanisms that allow these cells to accumulate Ca²⁺ in the endoplasmic reticulum, release it, and maintain basal and stable cytoplasmic concentrations. We also discuss electrophysiological and genetic studies that have uncovered Ca²⁺ influx channels in oocytes and eggs, and we analyze evidence supporting the role of a sperm-specific phospholipase C isoform as the trigger of Ca²⁺ oscillations during mammalian fertilization including its implication in fertility.

An increase in the intracellular concentration of calcium ([Ca²⁺]_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, 2003; Clapham 2007). For Ca²⁺ to perform these duties, cells create and maintain ionic gradients between extracellular and intracellular milieus and within the cellular content, thereby rendering this divalent ion into a signaling messenger. Cells have at their disposal a myriad of proteins with different affinities to bind Ca²⁺, which allows them to interpret and transform these ele-

vations into cellular functions. This review will examine how mammalian oocytes and eggs, namely mouse gametes, which are the most widely studied, have adopted and adapted a variety of molecules involved in establishing cellular Ca²⁺ homeostasis, the “Ca²⁺ toolkit,” to serve their precise physiological functions. These include the processes of maturation, fertilization, and embryo development until the birth of an offspring.

In the egg, the activating Ca²⁺ signal that initiates development has been broadly studied in many mammalian and nonmammalian species (Miyazaki 2006; Whitaker 2006; Horner

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and Wolfner 2008), as their large size and precise cell-cycle stage made them an ideal model to learn about the cellular Ca^{2+} homeostasis machinery when the pharmacological and imaging tools were less sophisticated (Ridgway et al. 1977). Nevertheless, changes in Ca^{2+} homeostasis also occur during maturation and are of pivotal importance for the success of fertilization (Jones et al. 1995; Deguchi et al. 2005; Wakai et al. 2012). These mechanisms have not been well studied, but the recent discoveries in this area will be reviewed here in detail. Similarly, the intracellular Ca^{2+} release channel(s) that underpin most of the Ca^{2+} release during oocyte maturation and fertilization have been broadly investigated and characterized. Conversely, the plasma membrane (PM) channels responsible for the Ca^{2+} influx that is required to support the oscillations and filling of the internal stores in these cells have not been as carefully examined. Recent electrophysiological studies identified the first channels in these cells, and their contribution to Ca^{2+} homeostasis will be covered here. Last, we will discuss evidence supporting the role of phospholipase C ζ (PLC ζ), the sperm-specific PLC isoform (Saunders et al. 2002), as the responsible factor for the majority of Ca^{2+} release at fertilization, and the recent insights gained from genetics and mutational studies.

THE Ca^{2+} TOOLKIT IN MAMMALIAN OOCYTES/EGGS

Changes in the concentrations of Ca^{2+} represent one of the oldest and more widespread modes of cellular communication in life, from bacteria to animals. The relentless pace of evolution that resulted in the appearance of eukaryotes, and subsequently, in the emergence of complex multicellular organisms has caused molecular adaptations and the development of diverse and complex Ca^{2+} -signaling systems (Cai and Clapham 2012). This expanded capacity made possible for Ca^{2+} signaling to operate within different cellular compartments, between cells, and between cells and their extracellular environment (Clapham 2007). To accomplish this, the concentration of free Ca^{2+} must be parsed between

the subcellular compartments and the surrounding environment. In most cell types, the basal cytoplasmic $[\text{Ca}^{2+}]_i$ under resting conditions is 100 nM, which is 10^4 times lower than the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) of ~ 1 mM (Clapham 2007; Bagur and Hajnoczky 2017). Within the cell, the endoplasmic reticulum (ER), which is the main Ca^{2+} reservoir of nonexcitable cells, ($[\text{Ca}^{2+}]_{\text{ER}}$), accumulates Ca^{2+} , reaching luminal Ca^{2+} concentrations >100 μM . $[\text{Ca}^{2+}]_{\text{ER}}$ is maintained via the inositol 1,4,5-trisphosphate receptor (IP $_3$ R), which is the main intracellular Ca^{2+} -release channel of almost all mammalian cell types and is located in the ER (for review, see Berridge et al. 2000; Bootman et al. 2001; Berridge 2009). Other organelles, such as mitochondria and the Golgi apparatus, also sequester Ca^{2+} and are involved in cellular homeostasis and Ca^{2+} signaling (Bagur and Hajnoczky 2017), as is discussed in detail below.

To create these concentration gradients between organelles and the cytoplasm and between the cell and the extracellular medium, cells possess a combination of pumps, channels, exchangers, and buffering mechanisms in the PM and membranes of intracellular organelles known as the Ca^{2+} toolkit, which makes possible the generation of oscillatory $[\text{Ca}^{2+}]_i$ transients with precise spatiotemporal organization (Berridge et al. 2003; Cai and Clapham 2012). These key Ca^{2+} -signaling molecules make Ca^{2+} entry into the cell possible mostly by various types of channels. Ca^{2+} release from intracellular organelles into the cytoplasm is mainly mediated by ion channels, whereas the clearing of the cytosolic Ca^{2+} increases is mainly mediated by adenosine triphosphatase (ATPase) transporters and ion exchangers (Fig. 1; described in further detail below). Further, each cell type presents a particular combination of the Ca^{2+} toolkit components to create a cell type- and stimulus-specific Ca^{2+} signal that suits their physiological requirements (Table 1; Cai and Clapham 2012). The fertilization-associated Ca^{2+} oscillations, which vary among species in duration, amplitude, number of rises, and the stage of meiosis at which they occur are notable examples of this notion (Stricker 1999; Miyazaki 2006). In mam-

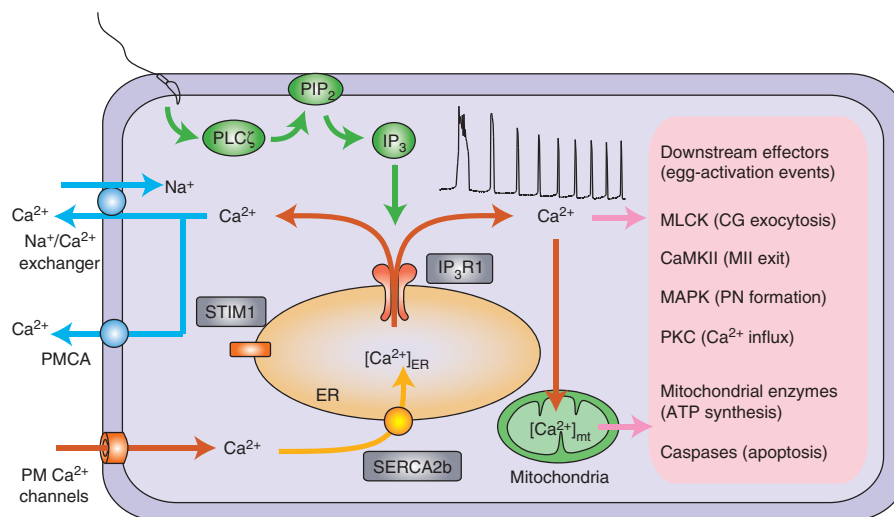


Figure 1. Ca²⁺-signaling toolkit in mammalian oocytes/eggs. At fertilization, the sperm delivers phospholipase C ζ (PLC ζ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) (green). IP₃ binds its receptor, IP₃R1, causing Ca²⁺ release out of the endoplasmic reticulum (ER). In mammals, the Ca²⁺ signal unfolds in brief but periodic rises termed Ca²⁺ oscillations (red). The spatiotemporal information provided by the pattern of these Ca²⁺ responses is decoded by downstream effectors that underpin the distinct cellular events of egg activation (pink), which include cortical granule (CG) exocytosis, exit from metaphase II (MII) arrest, and pronuclear (PN) formation. Part of the cytosolic Ca²⁺ is transduced into the mitochondria where it stimulates mitochondrial enzyme, resulting in adenosine triphosphatase (ATP) synthesis, whereas mitochondrial Ca²⁺ ([Ca²⁺]_{mt}) overload results in a loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspases causing apoptosis. Following Ca²⁺ release, elevated Ca²⁺ levels can be returned to baseline by the actions of plasma membrane (PM) Ca²⁺ ATPases (PMCA) and/or Na⁺/Ca²⁺ exchanger (NCX), which extrudes Ca²⁺ into the external milieu (blue). The sarco-ER Ca²⁺ ATPases (SERCAs) reuptake Ca²⁺ into the ER stores in anticipation of the next Ca²⁺ rise (yellow). Nevertheless, to consistently replenish ER Ca²⁺ ([Ca²⁺]_{ER}) stores and maintain oscillations, extracellular Ca²⁺ must enter into the egg/oocyte across the PM by a variety of channels and mechanisms (Fig. 2). One proposed Ca²⁺ influx mechanism is the store-operated Ca²⁺ entry (SOCE) mechanism. SOCE consists of a two-component system whereby a PM protein, ORAI1, is recruited upon store depletion into clusters with ER-associated, stromal interaction molecule 1 (STIM1), which aggregates toward the PM upon sensing low [Ca²⁺]_{ER} stores. SOCE seems redundant in mouse oocytes and eggs, but it might be more important in other mammalian species. In mice, other channels are the main carriers of Ca²⁺ from the external milieu. MLCK, Myosin light chain kinase; CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; MAPK, mitogen-activated protein kinase; PKC, protein kinase C.

mals, the responses to fertilization are known as Ca²⁺ oscillations, as they unfold in a pattern of brief, periodic rises that last for several hours after sperm entry (Miyazaki 2006). The spatiotemporal complexity of the Ca²⁺ oscillations that unfold in mature, metaphase II–arrested (MII-arrested) oocytes, henceforth referred to as eggs, is in part the consequence that Ca²⁺ from two sources, extracellular media and intracellular stores, must be integrated to produce stereotypical responses. Importantly, the molecules and regulatory mechanisms that underlie the Ca²⁺

oscillations and homeostasis in these cells are largely unknown.

Before ovulation, during maturation, oocytes undergo nuclear and cytoplasmic modifications in preparation for fertilization. Immature oocytes resume meiosis and transition from the germinal vesicle (GV) stage to the MII stage. Remarkably, the precise machinery required to develop the characteristic spatiotemporal pattern of the sperm-induced Ca²⁺ oscillations at the MII stage is acquired during maturation. In fact, in vitro fertilized GV oocytes

Table 1. Components of the Ca²⁺ tool kit in mouse oocytes/eggs

Mechanism	Protein	Expression	Phenotypic changes caused by molecular inhibition	References
Ca ²⁺ release	PLCZ1	Sperm	KO male mice were subfertile and Ca ²⁺ oscillations after fertilization were disturbed; ICSI with KO sperm did not trigger oscillations	Saunders et al. 2002; Hachem et al. 2017; Nozawa et al. 2018
	IP ₃ R1	Oocytes/eggs	Functional blocking antibody prevents Ca ²⁺ oscillations and egg activation	Miyazaki et al. 1992; Parrington et al. 1998; Jellerette et al. 2000
Ca ²⁺ influx	Ca _v 3.2	Oocytes > eggs	KO mice showed normal Ca ²⁺ oscillations but mild decrease of ER Ca ²⁺ store content in eggs	Bernhardt et al. 2015; Carvacho et al. 2018
	TRPV3	Eggs > oocytes	KO mice failed to Sr ²⁺ -induce egg activation	Carvacho et al. 2013
	TRPM7-like	Oocytes and lesser in eggs	Pharmacological inhibition prevents spontaneous Ca ²⁺ oscillations in GV oocytes; cKO eggs showed reduced oscillations after fertilization	Carvacho et al. 2016; Bernhardt et al. 2018
	ORAI1	Oocytes/eggs	KO mice showed normal fertilization-induced oscillations and fertility	Cheon et al. 2013; Bernhardt et al. 2017
	STIM1/2	Oocytes/eggs	KO mice showed normal fertilization-induced oscillations and fertility	Cheon et al. 2013; Bernhardt et al. 2017
Ca ²⁺ pumps	SERCA2b	Oocytes/eggs	Pharmacological inhibition prevents/prematurely terminates Ca ²⁺ oscillations in eggs	Kline and Kline 1992; Lawrence and Cuthbertson 1995; Wakai et al. 2013
	PMCA	Unknown	Pharmacological inhibition enhances Ca ²⁺ oscillations in eggs	Miao et al. 2012; Wakai et al. 2013
Ca ²⁺ buffering	NCX	Unknown	Pharmacological inhibition enhances Ca ²⁺ oscillations in oocytes/eggs	Carroll 2000
	MCU	Unknown	Unknown	
	VDAC	Unknown	Unknown	

PLCZ1, phospholipase C ζ ; KO, knockout; ICSI, intra-cytoplasmic sperm injection; ER, endoplasmic reticulum; IP₃R1, inositol 1,4,5-trisphosphate receptor; TRPV3, transient receptor potential (TRP) vanilloid, member 3; TRPM7, TRP melastatin 7; GV, germinal vesicle; cKO, conditional knockout; STIM1/2, stromal interaction molecule 1/2; SERCA2b, sarco-endoplasmic reticulum Ca²⁺-ATPases; NCX, Na⁺/Ca²⁺ exchanger; PMCA, plasma membrane Ca²⁺-ATPases; MCU, mitochondrial Ca²⁺ uniporter; VDAC, voltage-dependent anion-selective channel.

show fewer [Ca²⁺]_i oscillations and these oscillations show a shorter duration and amplitude than those observed in fertilized MII eggs (Carroll and Swann 1992; Wakai et al. 2012). Despite this knowledge, the mechanisms underlying the enhanced Ca²⁺-releasing ability of MII eggs ver-

sus GV oocytes are not well understood, although multiple parameters (e.g., IP₃R1 post-translational modifications, Ca²⁺ store content, and ER reorganization) are believed to be involved in this process (Ajduk et al. 2008). Spontaneous oscillations cease within a few hours

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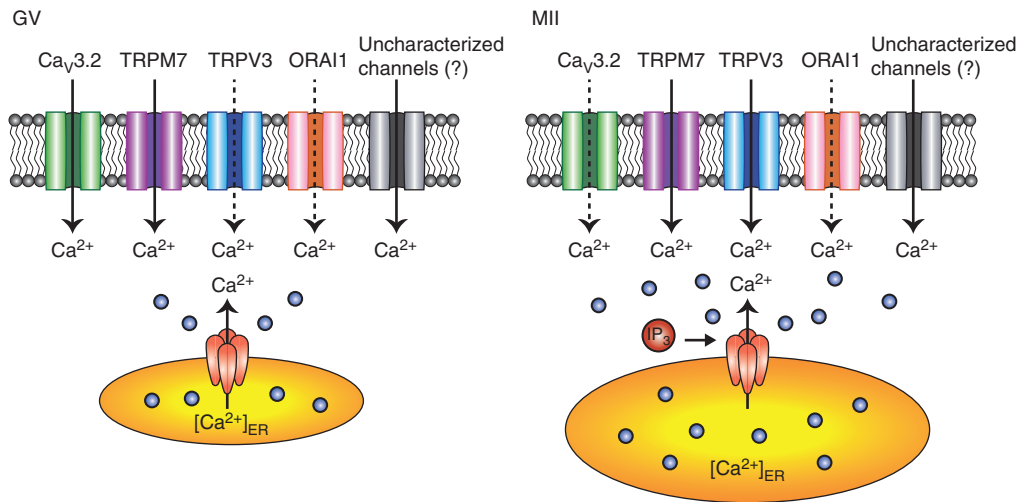


Figure 2. Differential expression of plasma membrane (PM) Ca²⁺ channels in mammalian oocytes/eggs. Ca²⁺ influx plays a pivotal role in both spontaneous Ca²⁺ oscillations in germinal vesicle (GV) oocytes (*left*) and fertilization-induced oscillations in MII eggs (*right*). During oocyte maturation, the putative active channel(s) are progressively inactivated, which causes down-regulation of Ca²⁺ influx and termination of spontaneous Ca²⁺ oscillations by the GV breakdown stage, thereby avoiding parthenogenesis at the MII stage. A high [Ca²⁺]_{ER} in MII eggs is important for robust IP₃-induced Ca²⁺ release at the time of fertilization. Note that a variety of Ca²⁺ channels have been functionally detected in the PM of mouse oocytes, including the low-voltage-activated (LVA) T-type calcium channel 3.2 (Ca_v3.2 T-type), transient receptor potential (TRP) family members vanilloid member 3 (TRPV3), and TRP melastatin 7 (TRPM7) as well as ORAI1 channels. These channels are proposed to mediate the majority of the influx, although the PM channels that mediate Ca²⁺ influx during oocyte maturation and fertilization remain to be fully identified, and may be different between these two stages. In mouse GV oocytes, the persistently low [Ca²⁺]_{ER} levels serve as the natural trigger for Ca²⁺ influx, which appears mostly operated through Ca_v3.2 channels. All the other channels are likely also to mediate additional influx, although of lesser magnitude. The only channel whose functional expression clearly increases during maturation in mouse oocytes is TRPV3, as it is nearly absent at the GV stage with its maximal expression being at the MII stage. TRPM7 also seems to change during maturation, as there is higher current density in GV oocytes than in MII eggs. However, fertilization reactivates influx, although how this is accomplished is presently unknown. Thick complete arrows, active influx; broken arrows, reduced expression and/or function.

after resumption of meiosis and the content of the [Ca²⁺]_{ER} steadily increases until the MII stage, suggesting that the molecules responsible for these adjustments in Ca²⁺ homeostasis experience similar dynamic modifications. It is therefore possible that several features of Ca²⁺ signaling in GV oocytes change during maturation so that some of the Ca²⁺ mechanisms/channels active at the GV stage may not be so at the MII stage and vice versa. Moreover, these distinct and changing features of Ca²⁺ homeostasis during maturation are likely to underpin the acquisition of both maturation and developmental competence as well as fulfill stage-specific cellular functions.

THE MECHANISM OF INOSITOL 1,4,5-TRIPHOSPHATE RECEPTOR (IP₃R)-MEDIATED Ca²⁺ RELEASE

The IP₃R is a large protein (>250 kDa) that functions as a tetramer (>1000 kDa). There are three IP₃R isoforms, denoted IP₃R1–3, each with similar, albeit slightly distinctive, properties. Each monomer broadly contains three functional regions, a cytosolic amino-terminal domain that binds IP₃, a regulatory domain with multiple regulatory sites for Ca²⁺, ATP, and other modulatory molecules/proteins, and a carboxy-terminal channel domain that contains six transmembrane domains and a short cyto-



solic tail. The activation and opening of the IP₃R requires binding by both Ca²⁺ and IP₃, and the regulation of IP₃-induced Ca²⁺ release (IICR) by Ca²⁺ adopts a bell-shaped form, as IICR is stimulated at low [Ca²⁺]_i and inhibited at high [Ca²⁺]_i (Iino 1990). This dual regulation of IP₃R by Ca²⁺ and IP₃ makes it especially suited to support long-lasting oscillations.

Mammalian oocytes and eggs and their surrounding cells express all three IP₃R isoforms (Fissore et al. 1999), although oocytes and eggs overwhelmingly express the IP₃R1 isoform (Fig. 1; Parrington et al. 1998; Jellerette et al. 2000). The initial suggestion that IP₃R may play a role during fertilization arose from studies in sea urchin eggs that showed that phosphoinositide metabolism via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) into IP₃ increases after fertilization (Turner et al. 1984), an observation that was soon followed by the demonstration that injection of IP₃ triggered Ca²⁺ release (Clapper and Lee 1985). Similar studies in hamster oocytes (Miyazaki 1988) further defined the realization of the role of IP₃R in mammalian fertilization, which was later firmly established with the demonstration that injection of a functional blocking antibody prevented both the initiation of Ca²⁺ oscillations and egg activation (Miyazaki et al. 1992; Xu et al. 1994). Subsequent studies confirmed the role of IP₃R1 in fertilization in other species (for review, see Parys et al. 1994; Thomas et al. 1998; Yoshida et al. 1998; Runft et al. 1999; Goud et al. 2002; Iwasaki et al. 2002; Wakai et al. 2011).

Several modifications of IP₃R1 may explain some of the idiosyncratic changes of the Ca²⁺ signaling in mammalian oocytes and eggs. The fertilization-associated Ca²⁺ oscillations in mouse zygotes become less frequent as they transition from the MII stage into interphase before ceasing altogether at the time of pronuclear (PN) formation (Kono et al. 1996; Deguchi et al. 2000). During this transition, ~50% of the IP₃R1 mass is lost through ligand-induced degradation (Parrington et al. 1998; Jellerette et al. 2000). Likewise, the organization of IP₃R1s is affected as the ER changes from a predominantly cortical and clustered organization at the MII stage, which is believed to enhance IP₃R1's sen-

sitivity (Ullah et al. 2014), to a more homogeneous distribution that modifies the channel's properties (FitzHarris et al. 2003). Phosphorylation also affects IP₃R1 function in eggs. Cell-cycle kinases play a prominent role in the MII arrest and phosphorylate IP₃R1 (Jellerette et al. 2004). IP₃R1 phosphorylation was first detected in mouse oocytes using an anti-MPM-2 antibody, which recognizes a variety of proteins that are phosphorylated during mitosis, and when in mouse oocytes during maturation, showed maximal reactivity at the MII stage followed by a gradual and persistent decline after egg activation (Lee et al. 2006). The MPM-2 epitope (peptides containing leucine-threonine-proline-leucine-lysine [LTPLK] and phenylalanine-threonine-proline-leucine-glutamine [FTPLQ] domains) can be modified by several mitosis (M-phase) kinases such as polo-like kinase 1 (Plk1), mitogen-activated protein kinase (MAPK), and cyclin-dependent kinase 1 (Cdk1) (Joughin et al. 2009), although it is presently unknown whether one or several of these kinases contribute to the modification of this epitope in IP₃R1 in mammalian eggs. Importantly, subsequent studies using phosphospecific antibodies showed that serine 421 (S421) and threonine 799 (T799), which are Cdk1 consensus sites, are phosphorylated in mouse eggs at the MII stage (Wakai et al. 2012). In somatic cells, for example, phosphorylation of IP₃R1 at these sites, both under in vitro and in vivo conditions, enhanced IP₃ binding and Ca²⁺ (Malathi et al. 2005). Further, in mouse eggs, expression of a heterologous IP₃R1 with phosphomimetic mutations corresponding to three M-phase motifs (which should be phosphorylated by M-phase kinases in MII eggs) resulted in greater Ca²⁺ oscillatory activity and sensitivity to IP₃ compared with expression of wild-type (WT) IP₃R1 (Zhang et al. 2015). Besides M-phase kinases, studies in somatic cells have shown that IP₃R isoforms can be phosphorylated by more wide-ranging kinases (Bezprozvanny 2005). The most commonly implicated kinases include protein kinase A (PKA), protein kinase C (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 laevis* oocytes found that IP₃R1 is uniformly phosphorylated throughout maturation in both PKA consensus motifs (Ser¹⁵⁸⁹ and Ser¹⁷⁵⁵ within the central cytosolic domain of IP₃R1 are the only sites phosphorylated by PKA), whereas IP₃R1 is not phosphorylated on PKC sites (Sun et al. 2009). In mouse oocytes, phosphorylation by PKA, but not by PKC, was shown during maturation, with maximal PKA phosphorylation occurring at the GV stage (Wakai et al. 2012), which is consistent with the high level of cyclic adenosine monophosphate (cAMP) at this stage (Norris et al. 2009). It is therefore possible that down-regulation and reorganization of IP₃R1 along with modifications of its phosphorylation status all contribute to shape the Ca²⁺ oscillations at fertilization in mammals.

An important remaining consideration is the site of IP₃ production during fertilization in mammalian oocytes and eggs. Whereas canonical PLCs find most of their substrate, PIP₂, at the PM, this does not appear to be the case with PLCζ1, which is the PLC that accounts for most of the Ca²⁺ release in mouse fertilization (see below for specific discussion of PLCζ function and regulation). PIP₂ can be found in the PM of mouse eggs using specific probes (Halet 2004), although its levels there do not appear to change during fertilization, and in fact it might actually increase (Halet et al. 2002; Halet 2004). Further, depletion of PIP₂ from the PM does not seem to affect Ca²⁺ oscillations (Sanders et al. 2018). Recent research shows that mouse eggs appeared to also store PIP₂ in small cytoplasmic vesicles distributed throughout the ooplasm (Sanders et al. 2018), and a treatment that prevents the utilization of this PIP₂, either by reducing its levels and/or by disrupting its distribution, causes rapid but reversible termination of the Ca²⁺ oscillations (Sanders et al. 2018). Thus, it appears that during mouse fertilization, IP₃ might be generated throughout the ooplasm, which may be a logical choice given the diameter of eggs and the reported speeds of the fertilization-induced Ca²⁺ oscillations in these species. The typical speed of the fertilization Ca²⁺ wave

is >50 μm/sec, crossing the egg in ~1 sec, which cannot be simply explained by the diffusion coefficient of IP₃ in intact cells that is only of <5 μm²/sec (Dickinson et al. 2016). Future studies should identify the nature of the vesicles that contain IP₃ in mouse eggs, determine whether this strategy extends to other species, and uncover how the sperm's PLC finds the correct localization.

THE REGULATION OF [Ca²⁺]_{ER} CONTENT

So far, we have discussed fertilization-induced Ca²⁺ oscillations in the context of IP₃ production and regulation of IP₃R1 function. Nevertheless, the regulation of Ca²⁺ oscillations in eggs is far more complex, as the components of the Ca²⁺ toolkit involved in Ca²⁺ homeostasis impart different spatial and temporal properties to the responses in oocytes and eggs. For example, for Ca²⁺ oscillations to continue uninterrupted and to avoid cellular toxicity following a [Ca²⁺]_i increase, [Ca²⁺]_i levels need to be returned to baseline and stores refilled in anticipation of the next transient. To bring [Ca²⁺]_i to baseline, cells either return free cytosolic Ca²⁺ into the ER by the action of the sarco-ER Ca²⁺ ATPases (SERCAs), and/or extrude it by the action of PM Ca²⁺ ATPases (PMCA) and Na⁺/Ca²⁺ exchangers (NCXs) (Berridge et al. 2003). The mitochondria and Golgi apparatus also contribute to shape [Ca²⁺]_i, as mitochondrial calcium uniporter (MCU) and secretory-pathway Ca²⁺-transport ATPases (SPCAs) uptake Ca²⁺ into organelles. Three different SERCA genes (*ATP2A1–3*) encode three main isoforms (*SERCA1–3*), each of which undergoes tissue-specific splicing, further increasing the diversity of these pumps. *SERCA1a* and *SERCA1b* variants are expressed in skeletal muscle (Brini and Carafoli 2009). The *SERCA2a* variant is expressed in cardiac muscle, whereas the *SERCA2b* variant is expressed nearly ubiquitously and is thus considered the housekeeping isoform. *SERCA3* is instead expressed in a limited number of nonmuscle cells. The expression of SERCA proteins in mammalian eggs was surmised by earlier reports using pharmacological inhibition of SERCA, as the exposure of eggs to

thapsigargin (a specific SERCA inhibitor) prevents continuation of Ca^{2+} oscillations (Kline and Kline 1992; Lawrence and Cuthbertson 1995). A follow-up study revealed the presence of the SERCA2b protein (Wakai et al. 2013), and found that it was expressed throughout oocyte maturation and attained a conspicuous cortical cluster organization in mature eggs (Wakai et al. 2013). The colocalization of SERCA2b with the $\text{IP}_3\text{R1}$ and ER cortical clusters may facilitate the initiation of robust Ca^{2+} oscillations at the time of fertilization.

Whether the $[\text{Ca}^{2+}]_{\text{ER}}$ stores undergo changes in content simultaneously with the fertilization-initiated Ca^{2+} oscillations remain unknown. Recent studies, however, using fluorescence resonance energy transfer (FRET)-based Ca^{2+} indicators have provided evidence that indeed this is the case (Takahashi et al. 2013; Wakai and Fissore 2013). It was shown that $[\text{Ca}^{2+}]_{\text{ER}}$ displays oscillatory responses such that with each Ca^{2+} oscillation, the $[\text{Ca}^{2+}]_{\text{ER}}$ undergoes a simultaneous but opposite change in its content. Further, each Ca^{2+} oscillation is accompanied by a rapid decline in $[\text{Ca}^{2+}]_{\text{ER}}$ levels, which suggests direct contribution of the $[\text{Ca}^{2+}]_{\text{ER}}$ content to the cytosolic $[\text{Ca}^{2+}]_{\text{i}}$ transient. Nevertheless, unlike the quick return to baseline that cytosolic $[\text{Ca}^{2+}]_{\text{i}}$ transients experience, $[\text{Ca}^{2+}]_{\text{ER}}$ level's return to basal levels is protracted, suggesting that other Ca^{2+} buffering systems contribute to cytosolic $[\text{Ca}^{2+}]_{\text{i}}$ clearance. It is worth noting that for approximately the first three Ca^{2+} oscillations after sperm entry, the upstroke of the next Ca^{2+} oscillation occurs before $[\text{Ca}^{2+}]_{\text{ER}}$ levels are fully recovered, showing that the $[\text{Ca}^{2+}]_{\text{ER}}$ levels progressively decrease during this time. Thereafter, $[\text{Ca}^{2+}]_{\text{ER}}$ levels seemingly reach a steady state in which each Ca^{2+} oscillation occurs from the same $[\text{Ca}^{2+}]_{\text{ER}}$ level, which represents a partly refilled $[\text{Ca}^{2+}]_{\text{ER}}$, suggesting that $[\text{Ca}^{2+}]_{\text{ER}}$ levels set the pace of the oscillations during the mid- to late-stage oscillations.

The filling status of $[\text{Ca}^{2+}]_{\text{ER}}$ depends on Ca^{2+} influx, Ca^{2+} efflux, and sequestering mechanisms. Given our previous demonstration that only a fraction of Ca^{2+} from each Ca^{2+} oscillation is recycled into the $[\text{Ca}^{2+}]_{\text{ER}}$ by SERCA, $[\text{Ca}^{2+}]_{\text{o}}$ must be taken in to replenish $[\text{Ca}^{2+}]_{\text{ER}}$. Evidence

for the need of Ca^{2+} influx to support Ca^{2+} oscillations during fertilization is long-standing, as sperm-initiated $[\text{Ca}^{2+}]_{\text{i}}$ and $[\text{Ca}^{2+}]_{\text{ER}}$ oscillations cease prematurely in the absence of $[\text{Ca}^{2+}]_{\text{o}}$ (Kline and Kline 1994). Nevertheless, the identity of the channels responsible for Ca^{2+} influx in mouse and other mammalian eggs remains to be determined (see below for up-to-date detailed description of Ca^{2+} influx in mechanisms in these eggs). In terms of extrusion mechanisms, elevated $[\text{Ca}^{2+}]_{\text{i}}$ can also be returned to baseline by the actions of PMCA and/or of the NCX, which release Ca^{2+} into the external milieu. There is functional evidence for both mechanisms in rodent oocytes and eggs. The functional activity of the NCX was evidenced by elimination of Na^+ from the external media (i.e., to block the NCX from operating in “forward mode” and extruding Ca^{2+}), which caused Ca^{2+} oscillations or accelerated existing ones, suggesting that a reverse mode of operation of the NCX (i.e., Ca^{2+} influx and Na^+ efflux) can be induced in these cells (Carroll 2000; Machaty et al. 2002; Cui et al. 2003). Importantly, even in the absence of external Na^+ , $[\text{Ca}^{2+}]_{\text{i}}$ returned to baseline levels (Carroll 2000), implying that the action of other mechanisms, especially PMCA, may be physiologically more relevant. Despite these initial findings, the participation of PMCA in Ca^{2+} homeostasis in mammalian oocytes had not been tested. Demonstration of its functional presence was recently accomplished using high concentrations of gadolinium (Gd^{3+}), which besides inhibiting Ca^{2+} channels, blocks PMCA activity, effectively preventing Ca^{2+} influx and efflux. This “ Ca^{2+} insulation” method resulted in increased amplitude and duration of the sperm-initiated Ca^{2+} oscillations, suggesting the functional presence of PMCA(s) in mouse eggs (Miao et al. 2012). Nonetheless, the molecular identification of PMCA in mammalian oocytes/eggs has yet to be shown.

THE ROLES OF MITOCHONDRIAL Ca^{2+} ($[\text{Ca}^{2+}]_{\text{mt}}$) UPTAKE

Besides the ER, mitochondria also contribute to the shaping of Ca^{2+} oscillations during oscillations (Duchen 2000; Rizzuto et al. 2000), as they

take up Ca^{2+} into the matrix, thereby alleviating the overall cytosolic Ca^{2+} load (Rizzuto et al. 2000). Despite early evidence to the contrary (Liu et al. 2001), a role as a Ca^{2+} reservoir does not seem to be the main function of these organelles in eggs, as inhibition of Ca^{2+} intake by the mitochondria does not immediately terminate the sperm-initiated oscillations (Dumollard et al. 2004). Importantly, besides contributing to Ca^{2+} buffering, propagation of Ca^{2+} signals from the ER into the mitochondria promote several events that sustain ATP levels in cells (Hajnoczky et al. 1995). Given the proximity between the ER and $\text{IP}_3\text{R1s}$, the Ca^{2+} -driven ATP output may be the mitochondria's most critical contribution to Ca^{2+} homeostasis in mammalian eggs. Simultaneous measurement of $[\text{Ca}^{2+}]_i$ and mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{mt}}$) showed that Ca^{2+} oscillations are accompanied by parallel increases in $[\text{Ca}^{2+}]_{\text{mt}}$, which stimulates ATP output (Dumollard et al. 2008). To this end, inhibition of mitochondrial function with oligomycin reduced $[\text{Ca}^{2+}]_{\text{ER}}$ levels by impairing its refilling (Wakai et al. 2013), suggesting that Ca^{2+} -driven ATP output is required to sustain SERCA activity and the sperm-triggered Ca^{2+} oscillations.

The contributions of $[\text{Ca}^{2+}]_{\text{mt}}$ to Ca^{2+} homeostasis have been known for decades, but the molecular identities of the channels and transporters responsible for the influx of Ca^{2+} into the mitochondria have not been identified until very recently. Cytosolic Ca^{2+} reaches the mitochondrial matrix by permeating the outer mitochondrial membrane through the voltage-dependent anion-selective channel (VDAC) (Blachly-Dyson et al. 1993) and the inner mitochondrial membrane (IMM) via the Ca^{2+} selective MCU (Baughman et al. 2011; De Stefani et al. 2011). The close association between the mitochondria and ER at contact sites (MERCs; also known as mitochondria-associated ER membranes [MAMs]) creates localized sites of high Ca^{2+} concentration that favor its influx into the mitochondrial matrix. Despite the pivotal role that Ca^{2+} oscillations play in mitochondrial ATP production and cellular homeostasis in mammalian oocytes/eggs, no studies have examined the function and regulation of the un-

derlying molecules that mediate its influx into this organelle in oocytes and eggs. Future studies should identify their regulation and function using genetic approaches, because thus far conventional and nonspecific inhibitors of $[\text{Ca}^{2+}]_{\text{mt}}$ intake such as ruthenium red appear to be ineffective in eggs (Dumollard et al. 2008).

Ca^{2+} INFLUX THROUGH THE PUTATIVE PLASMA MEMBRANE (PM) Ca^{2+} CHANNELS

The relationship between Ca^{2+} influx and spontaneous oscillations was first noted in the legend to Figure 2. Nevertheless, the molecules that mediate Ca^{2+} influx, and their regulation, remain poorly characterized. During maturation, the $[\text{Ca}^{2+}]_{\text{ER}}$ stores in mouse oocytes must be filled in preparation for fertilization (Jones et al. 1995; Wakai et al. 2012), then to sustain Ca^{2+} oscillations after fertilization, the $[\text{Ca}^{2+}]_{\text{ER}}$ stores need to be refilled between oscillations in anticipation of the next Ca^{2+} rise. These processes share dependency on sequestration and extrusion mechanisms as well as on the influx of external Ca^{2+} , the latter of which is accomplished by Ca^{2+} channels and exchangers. For instance, store-operated Ca^{2+} entry (SOCE) is one of these proposed mechanisms that is activated by the depletion of $[\text{Ca}^{2+}]_{\text{ER}}$ (Putney 2011). Eggs and somatic cells alike use several mechanisms to accomplish Ca^{2+} influx including receptor-operated channels (ROCs), voltage-gated channels (VGCs), as well as other PM channels such as ORAI 1–3, which is the channel component of SOCE (Bernhardt et al. 2017; Putney 2017), and the transient receptor potential (TRP) family of channels, which includes six subfamilies and nearly 30 human members that are expressed in multiple cell types and tissues (Wortzman-Show et al. 2007).

VGCs are classified according to the voltage needed to activate them: high-voltage activated (HVA) and low-voltage activated (LVA). The mouse egg predominantly expresses an LVA VGC known as $\text{Ca}_v3.2$ (T-type) (Peres 1986; Kang et al. 2007; Bernhardt et al. 2015). Evidence suggests that the functional expression of these channels changes during maturation, as mouse GV oocytes display larger $\text{Ca}_v3.2$ -

mediated currents than MII eggs (Bernhardt et al. 2015; Carvacho et al. 2018), although the nature of these changes and mode of regulation remains to be well established. It is apparent, however, that mice null for the $Ca_v3.2$ gene, *Cacna1h*, only show mild subfertility and sperm-induced oscillations are largely unaffected (Bernhardt et al. 2015). Thus, the function of $Ca_v3.2$, at least in mouse oocytes and eggs, is believed to support Ca^{2+} influx during the GV stage and to prepare oocytes for fertilization, as after it, the current magnitude and functional properties vary (Yamashita 1982; Kang et al. 2007; Bernhardt et al. 2015). Besides, mouse oocytes/eggs are largely nonexcitable cells, and in contrast to invertebrate species, experience only a small change in membrane potential during fertilization (Igusa et al. 1983; Jaffe and Cross 1984). Thus, it remains to be determined why these VGCs are functionally expressed in mammalian oocytes and eggs. It is possible that their function as “window currents”—operating in a window of voltage in which activation and inactivation overlap, and that allows a sustained but low flux of Ca^{2+} into the cell (Rossier 2016)—is their most important contribution, especially in steady-state situations such as GV-stage oocytes of growing follicles (Bernhardt et al. 2015).

There is growing evidence for TRP channel expression in mouse oocytes and eggs and of their importance in the regulation of Ca^{2+} homeostasis in these cells. The first TRP channel to be reported in eggs was TRP vanilloid, member 3 (TRPV3) (Carvacho et al. 2013), although its expression is predominant in skin cells. TRP channels are modulated by a myriad of stimuli including chemical, mechanical, and temperature. Besides Ca^{2+} , TRPV3 permeates other divalent cations such as strontium (Sr^{2+}), which is essential for triggering parthenogenetic embryonic development (Carvacho et al. 2013). Remarkably, TRPV3 is differentially expressed during maturation, as it is nearly absent at the beginning of maturation with its maximal expression being at the MII stage, although it is not required for normal fertility either, as null females are fertile (Chen et al. 2003; Carvacho et al. 2013). The role of TRPV3 needs reexamination as well, as single knockout (KO) *Trpv3*^{-/-}

eggs do not show changes in oscillation frequency postfertilization, whereas elimination of both TRPV3 and $Ca_v3.2$ channels has devastating effects on the eggs' Ca^{2+} store content and oscillations postfertilization (A Mehregan, G Ardestani, I Carvacho, et al., unpubl.).

The reason for the differential expression of $Ca_v3.2$ and TRPV3 channels in mouse oocytes and eggs also requires further investigation, but their simultaneous deletion as noted greatly impacts Ca^{2+} homeostasis in oocytes and eggs, and compromises the ability to initiate regularly spaced, frequent Ca^{2+} transients after fertilization. Females of this double KO group displayed subfertility, but were fertile nonetheless, suggesting that these two channels could potentially have redundant, compensatory functions that sustain normal oscillations in the loss of a single channel (A Mehregan, G Ardestani, I Carvacho, et al., unpubl.). It is also plausible that there are undetermined endogenous modulators of TRPV3 and $Ca_v3.2$ that are present in oocytes and eggs, but how their activity is regulated requires further investigation.

Another TRP channel member, melastatin 7 (TRPM7), was shown to be required for early embryonic development, as its global disruption resulted in embryonic lethality before day 7 (Jin et al. 2008). TRPM7 is a bifunctional protein known as a chanzyme with both ion channel and serine/threonine kinase activities, which can be modulated by Mg^{2+} , pH, and PIP_2 (Wu et al. 2010; Fleig and Chubanov 2014). A recent report has shown the functional expression of TRPM7-like channels in mouse oocytes and eggs (Carvacho et al. 2016), and like $Ca_v3.2$ and TRPV3, the functional expression of TRPM7 seems to change during maturation, as there is higher current density in GV oocytes than in MII eggs. Further, its functional expression is reactivated after fertilization, as noted by increased current in two-cell zygotes, which is consistent with its reported requirement during early embryogenesis and the survival of pluripotent stem cells alike; it is, however, not required after embryonic day 14 or for the survival of many differentiated cells (Jin et al. 2008).

The modulation of TRPM7 permeability and function by intracellular and extracellular

Mg²⁺ confirmed its role in the regulation of physiological Mg²⁺ homeostasis. Disruption of such a function can lead to developmental problems such as impaired gastrulation in *Xenopus*, hypomagnesemia, and birth defects such as spina bifida in humans (Schmitz et al. 2003; Ryazanova et al. 2010; Liu et al. 2011). In mammalian oocytes and eggs, TRPM7 may also be mediating Mg²⁺ homeostasis, as it has recently been shown that fertilization-induced embryo development in several species is increased in media with lower concentrations of Mg²⁺ (Herrick et al. 2015), and that sperm-initiated Ca²⁺ oscillations were increased when measurements were performed in the presence of low levels of extracellular Mg²⁺ (Ozil et al. 2017).

Interestingly, the Mg²⁺-sensitive properties of TRPM7 are what differentiated it from Ca²⁺ release-activated current ([CRAC], the ion current that underlies SOCE). Initial studies in *Xenopus* oocytes revealed the molecular mechanism of SOCE in these cells, which manifests around the GV breakdown (GVBD) phase by the uncoupling of Ca²⁺ store depletion and influx (Machaca and Haun 2002). SOCE consists of a two-component system whereby a PM protein, ORAI1 (the Ca²⁺ influx channel), is recruited upon [Ca²⁺]_{ER} store depletion into clusters with ER-associated protein, STIM1, which aggregates toward the PM upon sensing low [Ca²⁺]_{ER} (Cheon et al. 2013; Putney et al. 2017). Evidence for SOCE in mammalian oocytes was first observed after the application of thapsigargin in Ca²⁺-free media (to empty the [Ca²⁺]_{ER} store and thereby activate SOCE), causing a large influx of Ca²⁺ after adding Ca²⁺ back to the media (Kline and Kline 1992; Machaty et al. 2002). Coexpression of human STIM1 and ORAI1 induces a persistent increase in basal [Ca²⁺]_i only in GV oocytes, but enhances Ca²⁺ influx at all stages of maturation (Cheon et al. 2013). Nevertheless, the magnitude of the Ca²⁺ influx decreases as maturation progresses, suggesting a dispensable role for this mechanism at the fertilization stage and/or that it is reactivated by fertilization. Recent studies appear to support the first possibility, as oocyte-specific conditional knockout (cKO) mice for *Stim1* and *Stim2*, or *Stim1/2* double cKO mice as well as *Orai1*-null

mice showed normal fertilization-induced oscillations and fertility (Bernhardt et al. 2017). The role of SOCE may be more prominent in porcine oocytes, as knockdown of STIM1 and ORAI1 significantly disturbed sperm-induced Ca²⁺ oscillations in these species (Lee et al. 2012; Zhang et al. 2018). Whether these results extend to other large mammalian species is not known, as it is also unknown how inactivation of SOCE affects fertility in these species.

Alternatively, TRPM7 could be mediating a SOCE-like Ca²⁺ influx in mammalian oocytes. It was shown that TRPM7 can be a mediator of SOCE-like Ca²⁺ influx in somatic cells, such as in macrophages, which use TRPM7-mediated Ca²⁺ influx to support downstream-signaling pathways independent of STIM and ORAI proteins (Schappe et al. 2018). A similar pattern may be occurring in oocytes and eggs, as oocytes and eggs of cKO *Trpm7* mice displayed greatly reduced Ca²⁺ influx induced by emptying the internal stores (Bernhardt et al. 2018). Nevertheless, these eggs were able to mount persistent Ca²⁺ oscillations, and the females were only mildly subfertile (Bernhardt et al. 2018). A stronger Ca²⁺ oscillation phenotype was observed following deletion of *Trpm7* and *Cacna1h* channels, as the sperm-induced oscillations were of shorter duration and displayed an abnormal pattern (Bernhardt et al. 2018), suggesting that the oscillations of fertilization are supported by multiple channels.

Aside from these channels, there are other players mediating Ca²⁺ influx, although to a lesser extent, in mouse oocytes and eggs. Ca²⁺-activated chloride channels (CACCs) are activated in response to sperm-induced Ca²⁺ release, which causes the channel to conduct Cl⁻ out of the cell, thereby inducing membrane depolarization to effectively block polyspermy (Cross and Elinson 1980; Jaffe et al. 1983; Wozniak et al. 2018). Although this is the predominant mechanism of polyspermy prevention at fertilization in *Xenopus* eggs (Wozniak et al. 2018), a CACC was also observed in two-cell-stage mouse embryos, in which exposure to a selective CACC inhibitor, niflumic acid (NFA), blocked anion-driven outward currents and impaired development (Li et al. 2007, 2009).

Ca^{2+} -activated potassium ($\text{K}_{(\text{Ca})}$) channels have been reported in hamster eggs and human eggs. Fertilization and activation in hamster eggs is driven by large, hyperpolarizing currents that have been implicated with the activity of $\text{K}_{(\text{Ca})}$ channels (Miyazaki and Igusa 1981, 1982), and were abolished by addition of the Ca^{2+} chelator, egtazic acid (EGTA) (Georgiou et al. 1983; Igusa and Miyazaki 1983). Similar to the hamster, in unfertilized human eggs, the bell-shaped current after application of Ca^{2+} ionophore, A23187, was blocked by a selective inhibitor of big conductance (BK) $\text{K}_{(\text{Ca})}$ channels, iberiotoxin (Homa and Swann 1994). However, the mechanism in mouse eggs remains different from the hamster and human, whereby a voltage-gated K^+ channel (K_V) that is not mediated by cytosolic Ca^{2+} and whose activity is dependent on the cell-cycle stage of the oocyte, was reported (Day et al. 1993). The contributions of these channels to fertilization and early development are not well characterized.

In conclusion, the identification of ion channels in mammalian oocytes remains a technical challenge. There are few commercially available antibodies and there is a lack of specific pharmacological agents. Thus, we must look to the generation of KO models to study the effect of functional loss of a channel. Although current reports indicate that these KO models can elicit a change in the behavior of expression of the remaining channels, in which eggs may consequently develop a compensatory mechanism to maintain such a crucial process as Ca^{2+} influx. Thus, only the simultaneous development of electrophysiological methods, genetic models, and pharmacological reagents will allow for the initial discovery and subsequently, the manipulation of all ion channels in mammalian oocytes and eggs.

DISTINCT REGULATION OF Ca^{2+} HOMEOSTASIS DURING OOCYTE MATURATION

A peculiar aspect of the Ca^{2+} stores of GV-stage oocytes is that they fail to accumulate Ca^{2+} , despite the presence of spontaneous Ca^{2+} oscillations and persistent Ca^{2+} influx (Carroll and

Swann 1992; Carroll et al. 1994). In contrast to the sperm-induced Ca^{2+} oscillations in MII eggs, the spontaneous, repetitive Ca^{2+} rises in GV oocytes are of small amplitude and occur every 1–3 min. These Ca^{2+} oscillations appear to be stimulus-independent and are constitutive, although the mechanisms that underpin them and their functions remain poorly investigated. It is noteworthy that these spontaneous Ca^{2+} oscillations persist for a few hours and cease around the time of GVBD, which is when oocytes simultaneously undergo the most drastic increase in $[\text{Ca}^{2+}]_{\text{ER}}$ store content and experience a sharp down-regulation in Ca^{2+} influx (Cheon et al. 2013; Wakai and Fissore 2013). The temporal coincidence of these phenomena implicates $[\text{Ca}^{2+}]_{\text{ER}}$ -associated mechanisms in the occurrence of spontaneous Ca^{2+} oscillations. A later study uncovered a unique regulatory mechanism of $[\text{Ca}^{2+}]_{\text{ER}}$ in GV oocytes, which is reversed in MII eggs; namely, in GV oocytes, $[\text{Ca}^{2+}]_{\text{ER}}$ levels are maintained persistently low by a constitutive Ca^{2+} “leak” through $\text{IP}_3\text{R1s}$ that is manifested in the form of spontaneous Ca^{2+} oscillations (Wakai and Fissore 2019). These Ca^{2+} oscillations are actuated by nearly constitutive Ca^{2+} influx and its down-regulation and inactivation of the $[\text{Ca}^{2+}]_{\text{ER}}$ leak, which together terminate the oscillations, and appear to be linked to the progressive increase in the content of the $[\text{Ca}^{2+}]_{\text{ER}}$ that occurs during oocyte maturation.

The Ca^{2+} oscillations of mouse GV oocytes may be important for cellular homeostasis. The mitochondria are a common downstream target of Ca^{2+} increases and $[\text{Ca}^{2+}]_{\text{mt}}$ homeostasis is important for the regulation of mitochondrial enzymes associated with the production of oxidizable substrates. Basal uptake of Ca^{2+} by the mitochondria in resting cells is important to maintain the reduced form of nicotinamide adenine dinucleotide (NADH) production to support oxidative phosphorylation (Cardenas et al. 2010). As noted above, after fertilization, the function of the mitochondria is indispensable for Ca^{2+} oscillations because it supplies the ATP necessary for the Ca^{2+} pumps required to maintain $[\text{Ca}^{2+}]_{\text{ER}}$ levels provably through SERCA activity. Remarkably, the spontaneous

Ca^{2+} oscillations in GV oocytes and their counterparts in the mitochondria have a seemingly similar function in these cells. During the spontaneous Ca^{2+} oscillations in GV oocytes part of the cytosolic Ca^{2+} signal is transduced into the mitochondria where it stimulates mitochondrial metabolism, thereby increasing the levels of ATP (Wakai and Fissore 2019). Future studies should ascertain whether and how enhanced ATP content affects oocyte maturation and developmental competence.

The $[\text{Ca}^{2+}]_{\text{ER}}$ store content increases markedly during maturation (Jones et al. 1995; Wakai et al. 2012). This increase in $[\text{Ca}^{2+}]_{\text{ER}}$ contributes to Ca^{2+} oscillations in MII eggs, as established using cyclopiazonic acid (CPA), a reversible SERCA inhibitor. CPA-treated GV oocytes advanced to the MII stage without delay or gross abnormalities, although without increase in $[\text{Ca}^{2+}]_{\text{ER}}$ (Wakai et al. 2012). In this condition, CPA-matured eggs injected with PLC ζ complementary RNA (cRNA) showed a shortened first Ca^{2+} oscillation even though CPA was washed away before initiating the oscillations. These results suggest that the increase in $[\text{Ca}^{2+}]_{\text{ER}}$ directly impacts fertilization-initiated Ca^{2+} oscillations, especially the robust first Ca^{2+} oscillation (Wakai et al. 2012). How the shape, amplitude, and number of Ca^{2+} oscillations postfertilization impacts embryo development remains to be firmly established.

$[\text{Ca}^{2+}]_i$ Oscillations Postfertilization: The Role and Function of PLC ζ

How the sperm initiates the signal responsible for egg activation has interested researchers for >100 years. There have been comprehensive reviews on this topic in the last few years (Machaty 2016; Swann and Lai 2016; Parrington et al. 2019) and here we will only highlight the most salient points, recent advances, and unanswered questions. Suffice it to say that the impossibility to replicate the pattern of fertilization-induced Ca^{2+} oscillations in mammals following experimentation with the growing list of proposed hypotheses, resulted in the re-emergence in the late 1980s of the sperm factor (SF) idea originally articulated by Jacques Loeb in the early 1900s

(Loeb 1913). This theory proposed that the sperm contains a soluble factor that, upon gamete fusion, enters the egg triggering the initiation of development. Subsequent studies showed that the trigger for development is a $[\text{Ca}^{2+}]_i$ increase, which largely originates from the intracellular Ca^{2+} stores (for review, see Stricker 1999). The first evidence supporting this notion in mammals was provided by Karl Swann, who injected SF extracts into eggs, which induced Ca^{2+} oscillations similar to those of fertilization (Swann 1990), and later studies showed that the extracts induced all the events of egg activation (Stice and Robl 1990). The physiological significance of this concept was strengthened when it was realized that egg activation could be induced by injection of a sperm into the ooplasm without interaction of the gametes' PMs (Uehara and Yanagimachi 1976; Yanagida et al. 1991). The intra-cytoplasmic sperm injection (ICSI) method, as it became known, was later perfected for application in humans and represents an essential tool to treat human male infertility (Palermo et al. 1992). Studies showed that ICSI induces Ca^{2+} oscillations similar to those initiated by in vitro fertilization (IVF), albeit with slight differences in the initiation of oscillations and in the propagation of the first rise (Tesarik et al. 1994; Nakano et al. 1997). Collectively, these results advanced the idea that a soluble SF carried in by the sperm caused egg activation. Despite this evidence, the molecular identity of the SF remained unclear for many years, including several misidentifications (Parrington et al. 1996; Sette et al. 1997; Wolosker et al. 1998; Wolny et al. 1999).

Logically, because PLCs are directly responsible for IP_3 production, they were always potential SF candidates. It was a study of the Ca^{2+} -releasing properties of mammalian sperm extracts using the cell-free sea urchin egg homogenate as the readout that first suggested that the SF might be a PLC (Jones et al. 1998). This was followed by a demonstration that the ability of mammalian SF fractions to trigger $[\text{Ca}^{2+}]_i$ oscillations after chromatographic fractionation coincided with a PLC activity (Parrington et al. 1999). Multiple PLC isoforms are expressed in sperm (Mehlmann et al. 1998; Fu-

kami et al. 2001; Parrington et al. 2002), although more in-depth in vitro PLC assays revealed that the sperm extracts' PLC activity was highly sensitive to Ca^{2+} (Rice et al. 2000). A subsequent genomic search for testis/sperm-specific PLCs led to the discovery of a novel sperm-specific PLC, PLC ζ , which was first reported in the mouse (Saunders et al. 2002) and then in other mammals (Cox et al. 2002). PLC ζ was shown to be ~ 100 -fold more sensitive to Ca^{2+} compared with PLC δ and displays a half-maximal PLC activity at a $[\text{Ca}^{2+}]_i$ concentration around the reported resting levels in mammalian eggs (Kouchi et al. 2004, 2005; Nomikos et al. 2005). Other distinct features of PLC ζ were that it showed sperm-specific expression, injection of its messenger RNA (mRNA) triggered fertilization-like Ca^{2+} oscillations in a variety of mammalian eggs (Cox et al. 2002), and it was predominantly localized to the equatorial/post-acrosomal region of mammalian sperm heads (Grasa et al. 2008; Yoon et al. 2008). Electron microscopy (EM) studies later found it to be in close association with the inner acrosomal membrane (Escoffier et al. 2015).

PLC ζ has a basic domain structure consisting of four EF hands, an X and Y catalytic domain, and a C2 domain, but lacks the amino-terminal pleckstrin homology domain, which is present in all other PLCs to target membrane-bound substrate PIP_2 (Saunders et al. 2002). EF hands are a Ca^{2+} -binding motif that allows proteins to sense and respond to changes in Ca^{2+} levels. The X and Y domains are the conserved catalytic domains of PLC ζ (Nomikos et al. 2011), but the discrete linker region that separates the X and Y domains in PLC ζ contains a distinct cluster of basic amino acid residues not found in other PLCs, and a predicted nuclear localization signal (Kuroda et al. 2006; Ito et al. 2008). The C2 domain, which is believed to play an important role in the Ca^{2+} -dependent subcellular membrane targeting in a variety of lipid-metabolizing enzymes (Clark et al. 1991), appears to play a similar role in PLC ζ , as in vitro studies showed that the C2 domain of PLC ζ binds to membrane phospholipids such as phosphatidylinositol 3-phosphate (PI_3P) and phosphatidylinositol 5-phosphate (PI_5P) (Kouchi

et al. 2005; Nomikos et al. 2017). Disturbing the C2 domain may affect the function of PLC ζ , which may explain the infertility caused in humans by the naturally occurring Ile489Phe mutation (Escoffier et al. 2016).

A question that has remained unanswered for some time is how PLC ζ changes the intracellular concentration of IP_3 ($[\text{IP}_3]_i$) during the Ca^{2+} oscillations. Do basal $[\text{IP}_3]_i$ levels oscillate with each $[\text{Ca}^{2+}]_i$ rise, or are the Ca^{2+} oscillations driven by persistently elevated levels of IP_3 ? A recent study appears to have resolved this question using a FRET construct capable of detecting the low magnitude changes in $[\text{IP}_3]_i$ that occur after mammalian fertilization (Matsu-Ura et al. 2019). It is shown that following fertilization, a persistent increase in the $[\text{IP}_3]_i$ is observed, which remains high throughout the oscillations. Remarkably, with each $[\text{Ca}^{2+}]_i$ rise, a small increase in $[\text{IP}_3]_i$ is induced, reflecting the high Ca^{2+} sensitivity of PLC ζ . Nevertheless, the fact that the peak in $[\text{IP}_3]_i$ occurs slightly after the initiation of the $[\text{Ca}^{2+}]_i$ rise, suggests the periodic $[\text{Ca}^{2+}]_i$ rises during the fertilization-induced oscillations are primarily governed by changes in the sensitivity of $\text{IP}_3\text{R1}$ (Iino 1990), which has the major role in triggering the rising phase $[\text{Ca}^{2+}]_i$ rise (Matsu-Ura et al. 2019), and the refilling of the stores by influx from the extracellular media (Wakai et al. 2013).

PLC ζ and Fertility

The advent of ICSI represented a major advance in the treatment of severe male factor infertility cases. However, ICSI is not successful in all couples, and in cases of repeated ICSI failure $\sim 80\%$ of the eggs fail to exit the MII stage, suggesting a defect in egg activation (Flaherty et al. 1995). Yoon et al. (2008) first showed that sperm from certain patients that had failed ICSI were also unable to trigger Ca^{2+} oscillations and egg activation upon injection into mouse eggs. Analysis of PLC ζ expression in these sperm revealed absence of PLC ζ protein, although analysis of the *Plcz1* gene failed to identify the molecular defects. Heytens et al. (2009) and subsequently Kashir et al. (2012) provided the first link between human male infertility and

PLC ζ mutations with debilitating functional effects on the PLC ζ protein. Most recently, Escoffier et al. (2016) used whole exomic sequencing to trace back the infertility of two brothers with complete fertilization failure after ICSI despite normal sperm morphology to a homozygous mutation in the *Plcz1* gene. The mutation, Ile489Phe (I489F), which is in the C2 domain of PLC ζ , significantly reduced its expression, and using I489F PLC ζ mRNA, it was shown that the construct had a reduced ability to generate Ca $^{2+}$ oscillations and displayed abnormal distribution in oocytes and eggs (Escoffier et al. 2016). In addition, analysis of I489F PLC ζ by Nomikos et al. (2017) showed diminished binding to PI $_3$ P- and PI $_5$ P-containing liposomes, confirming the role of the C2 domain in binding to such lipids. It is also consistent with modeling predictions in which the I489F mutation creates an unusually large hydrophobic area in the C2 domain that might compromise the targeting and/or binding of PLC ζ to specific substrate(s) (Escoffier et al. 2016). These human studies, besides identifying the molecular basis of certain types of male infertility, revealed important clues about the structure–function relationships of the PLC ζ protein. Thus far, PLC ζ remains the only protein whose presence is required for the initiation of Ca $^{2+}$ oscillations during human fertilization.

To show the role of PLC ζ in the fertility of other mammals, it was necessary to prevent its expression by genetic approaches. The initial studies only accomplished partial down-regulation of PLC ζ expression (Knott et al. 2005) and/or caused off-target effects that resulted in the arrest of spermatogenesis (Ito and Ikawa 2010), which prevented gaining insight into the mechanism and/or role of PLC ζ in initiating Ca $^{2+}$ oscillations and fertility. A full PLC ζ gene KO was recently accomplished using clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) genome-editing technology (Hachem et al. 2017). These animals displayed normal spermatogenesis but lacked expression of WT *Plcz1* in the testis and full-length PLC ζ protein in the sperm. When used in fertilization studies, both by ICSI or IVF methods, *Plcz1* KO sperm failed

to trigger Ca $^{2+}$ oscillations, which provided the first definitive evidence that PLC ζ is the physiological trigger of the Ca $^{2+}$ oscillations in mammals. Remarkably, when the outcome of fertilization was assessed soon after sperm entry, increased rates of polyspermy were observed both after IVF or in vivo mating. These results suggest that the lack of Ca $^{2+}$ oscillations noted above compromised the ability of the fertilized eggs to engage the Ca $^{2+}$ -sensitive mechanisms responsible for blocking polyspermy (Avella et al. 2013). Notably, a few of these zygotes generated using KO sperm and/or KO males developed to the blastocyst stage, and after naturally mating, male PLC ζ KO mice were subfertile, generating a few pups per litter. This constituted the first demonstration that in vivo fertilization without the normal physiological trigger of egg activation can result in offspring, at least in mice.

The study of Hachem et al. (2017) relied heavily on the use of cryopreserved sperm, which could have further compromised the ability of the KO sperm to initiate a [Ca $^{2+}$] $_i$ response, even an atypical one. This might have been the case, as in a recent study using a similar CRISPR/Cas9 genome-editing technology to generate *Plcz1* KO mice, the KO sperm initiated a [Ca $^{2+}$] $_i$ response, albeit highly abnormal (Nozawa et al. 2018) with oscillations starting after a delay of ~1 h after sperm entry and consisting of only a few Ca $^{2+}$ oscillations. Remarkably, fresh *Plcz1* KO sperm also failed to induce Ca $^{2+}$ oscillations following fertilization by ICSI, and *Plcz1* KO males were subfertile with embryos displaying high rates of polyspermy, just as noted by the previous study (Nozawa et al. 2018). Together, these studies show the importance of PLC ζ in the initiation of Ca $^{2+}$ oscillations and normal embryo development in mammals. Whereas there appears to be residual Ca $^{2+}$ -releasing activity in mouse sperm that does not express functional PLC ζ , it seems highly unlikely this activity is associated with PAWP (gene name *Wbp2nl*), PAWP has been proposed for some time to represent an alternative egg-activating mechanism in mammals capable of initiating Ca $^{2+}$ oscillations (Aarabi et al. 2014). Nevertheless, this does not seem to be the case, as a

Wbp2nl KO mouse showed no obvious defects in spermatogenesis and ability to initiate Ca^{2+} oscillations (Satouh et al. 2015).

CONCLUSIONS

The study of the mechanisms that underlie Ca^{2+} homeostasis in oocytes and eggs during maturation and fertilization, respectively, in mammals has resulted in important contributions to the Ca^{2+} -signaling field in general and for the field of gamete biology by providing important insights into the molecules and parameters required to initiate normal embryo development. For example, the indispensable role of $\text{IP}_3\text{R1}$ -mediated Ca^{2+} release in regulating cellular functions was first unequivocally shown in mouse fertilization (Miyazaki et al. 1992). Also, the initiation of Ca^{2+} oscillations by the sperm's cargo $\text{PLC}\zeta$ (Sanders et al. 2018) represents a unique, intimate, and highly controlled method of activation of embryo development hardly seen in other animal models. The precise source of PIP_2 as a substrate for $\text{PLC}\zeta$ in mammalian oocytes remains to be elucidated, and may also constitute a novel adaptation of Ca^{2+} homeostasis and wave propagation. Last, significant progress has been made regarding identification of the channels that make Ca^{2+} influx possible in mouse oocytes and eggs. Nevertheless, we are still unaware of the full repertoire of Ca^{2+} channels in mammalian oocytes/eggs, knowing even less about their regulation, and we remain largely ignorant of what channel(s) are present in human eggs. Furthermore, the fact that these channels' function may be differentially regulated during maturation and fertilization has the potential to uncover specific and novel mechanisms of Ca^{2+} influx regulation. Identification of all the components of the Ca^{2+} toolkit in these cells as well as elucidation of their regulatory mechanism(s) will deepen our understanding of maturation and fertilization, information that could then be used for more precise diagnosis of infertility, to improve the development of embryos generated by assisted reproductive technology procedures, and to develop novel and nonhormonal methods of contraception.

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