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Ca^{2+} homeostasis and regulation of ER Ca^{2+} in mammalian oocytes/eggs

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Abstract

The activation of the developmental program in mammalian eggs relies on the initiation at the time of fertilization of repeated rises in the intracellular concentration of free calcium ($[Ca^{2+}]_i$), also known as $[Ca^{2+}]_i$ oscillations. The ability to mount the full complement of oscillations is only achieved at the end of oocyte maturation, at the metaphase stage of meiosis II (MII). Over the last decades research has focused on addressing the mechanisms by which the sperm initiates the oscillations and identification of the channels that mediate intracellular Ca^{2+} release. This review will describe the up-to-date knowledge of other aspects of Ca^{2+} homeostasis in mouse such as the mechanisms that transport Ca^{2+} out of the cytosol into the endoplasmic reticulum (ER), the Ca^{2+} store of the oocyte/egg, into other organelles and also those extrude Ca^{2+}. Evidence pointing to channels in the plasma membrane that mediate Ca^{2+} entry from the extracellular milieu, which is required for the persistence of the oscillations, is also discussed, along with the modifications that these mechanisms undergo during maturation. Lastly, we highlight areas where additional research is needed to obtain a better understating of the molecules and mechanisms that regulate homeostasis in this unique Ca^{2+} signaling system.

Introduction

Before fertilization, mammalian eggs are arrested at the metaphase stage of the second meiosis (MII). Sperm entry triggers a series of increases in the intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$), termed $[Ca^{2+}]_i$ oscillations, which enable exit from the MII and induce egg activation [1]. Importantly, in mammals the Ca^{2+} signal unfolds in a pattern of

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brief but periodical rises that last for several hrs after sperm entry. The spatio-temporal information provided by the pattern of these $[Ca^{2+}]_i$ responses is decoded by downstream effectors that underpin the distinct cellular events of egg activation, which include cortical granule (CG) exocytosis, extrusion of the second polar body (2PB), pronuclear (PN) formation and entry into first mitosis [2]. Although the presence of long-lasting $[Ca^{2+}]_i$ oscillations is a hallmark of mammalian fertilization, the underlying molecular changes that make them possible remain elusive.

Inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from intracellular stores is primarily responsible for the generation of the $[Ca^{2+}]_i$ wave and oscillations at fertilization [3]. Although the general properties of Ca^{2+} release mechanism during oscillations have been described in the context of IP_3 production and regulation of its cognate receptor (IP_3R), the regulation of the $[Ca^{2+}]_i$ responses in these cells may be far more complex. For instance, to continue the long-term $[Ca^{2+}]_i$ oscillations without attenuation, after each $[Ca^{2+}]_i$ rise $[Ca^{2+}]_i$ levels need to be rapidly returned to baseline values, and the Ca^{2+} content of the endoplasmic reticulum (ER), the main Ca^{2+} reservoir in the cell [4], needs to be refilled ($[Ca^{2+}]_{ER}$) in anticipation of the next $[Ca^{2+}]_i$ rise. To bring $[Ca^{2+}]_i$ to baseline levels, cells either reuptake the free cytosolic Ca^{2+} into the ER refilling the store and/or remove it to other organelles or to the extracellular space [5, 6]. Nevertheless, to consistently replenish $[Ca^{2+}]_{ER}$ and maintain oscillations, extracellular Ca^{2+} must enter into the egg/oocyte across the plasma membrane by a variety of channels and mechanisms, one of which is the store operated Ca^{2+} entry (SOCE) mechanism [7]. Despite the pivotal role of these channels/ mechanisms on Ca^{2+} homeostasis and support of the oscillations, few studies have examined the underlying molecules and their function in mammalian oocytes/eggs.

Prior to ovulation, during oocyte 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. Importantly, the precise spatio-temporal pattern of sperm-associated $[Ca^{2+}]_i$ responses in eggs is established during maturation. In fact, *in vitro* fertilized GV oocytes show fewer $[Ca^{2+}]_i$ oscillations and each $[Ca^{2+}]_i$ rise exhibits lesser duration and amplitude than those observed in fertilized MII eggs [8, 9]. In spite of this knowledge, the mechanisms underlying the enhanced Ca^{2+} releasing ability of matured eggs vs. GV oocytes are not well understood, although multiple parameters (e.g. IP_3R modifications, ER re-organization, increase in $[Ca^{2+}]_{ER}$) are thought to be involved in this process [10]. In addition, given that several of the parameters of Ca^{2+} homeostasis progressively change during maturation, for instance $[Ca^{2+}]_{ER}$ steadily increases during maturation, the molecules responsible for these adjustments in Ca^{2+} homeostasis may experience dynamic modifications during maturation such that some of the mechanisms/channels active at the GV stage may not be so at the MII stage and vice versa.

This review highlights the changes that $[Ca^{2+}]_{ER}$ experiences during maturation and fertilization. The mechanisms that contribute to the regulation of $[Ca^{2+}]_{ER}$ are also discussed, including Ca^{2+} buffering systems and Ca^{2+} influx mechanisms as well as the changes that these mechanisms undergo during oocyte maturation.

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Ca^{2+} homeostasis during fertilization

The initiation of $[Ca^{2+}]_i$ oscillations during mammalian fertilization is thought to be triggered by the release from the sperm of a male specific Phospholipase C (PLC) enzyme, PLCζ, following fusion of the gametes [11, 12]. More details about PLCζ can be found in another chapter of this special issue. Therefore, our focus will be on other components of the Ca^{2+} toolkit that regulate Ca^{2+} homeostasis in oocytes and eggs.

1) Ca^{2+} buffering mechanisms

To bring $[Ca^{2+}]_i$ to baseline levels after a $[Ca^{2+}]_i$ rise, cells either reuptake the free cytosolic Ca^{2+} into the ER by the action of the sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA) and/or remove it to the surrounding environment by the action of plasma membrane Ca^{2+} ATPase (PMCA) and Na^+/Ca^{2+} exchanger [5, 6]. Few studies have addressed the function of these molecules in mammalian oocytes/eggs. 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 [13]. SERCA1a and SERCA1b variants are expressed in skeletal muscle. SERCA2a variant is expressed in cardiac muscle, whereas SERCA2b variant is expressed nearly ubiquitously and is thus considered the housekeeping isoform. SERCA3 is instead expressed in a limited number of non-muscle cells [13]. In *Xenopus* oocytes, expression of the SERCA2 protein was documented using immunofluorescence and it was shown to undergo reorganization similar to that described for IP_3R during oocyte maturation, which is consistent with the fact that both proteins are ER-resident proteins and with the reorganization that the ER undergoes during maturation [14]. The molecular presence and cellular distribution of SERCA2b has not yet been characterized in mammalian oocytes, although transcripts and proteins have been reported [15, 16]. The functional importance of SERCA can be surmised by the alteration of $[Ca^{2+}]_i$ levels caused by exposure to thapsigargin, an specific inhibitor of SERCA [17]. For example, exposure of MII eggs to thapsigargin causes a slow and steady rise in $[Ca^{2+}]_i$ followed by a protracted decline while in fertilized eggs, addition of thapsigargin or other SERCA inhibitors, prematurely terminates $[Ca^{2+}]_i$ oscillations [17, 18]. Nonetheless, the impact that these inhibitors have in $[Ca^{2+}]_{ER}$ has not been investigated, and therefore the regulatory role of $[Ca^{2+}]_{ER}$ on the pattern and/or persistence of oscillations is unknown in this species. Here, we made use D1ER, a FRET-based Ca^{2+} sensor [19], to directly examine $[Ca^{2+}]_{ER}$ levels during oscillations. To initiate $[Ca^{2+}]_i$ oscillations, we injected mouse eggs with mouse PLCζ cRNA, a procedure that we and others have shown to cause long-lasting and fertilization-like oscillations (Fig. 1) [11]. As depicted in the figure, each $[Ca^{2+}]_i$ rise is accompanied by a rapid decline in $[Ca^{2+}]_{ER}$ that is followed by a slow recovery. Compared to the quick return to baseline that cytosolic $[Ca^{2+}]_i$ transients experience, $[Ca^{2+}]_{ER}$ levels return to near basal levels in a very gradual manner, suggesting that other Ca^{2+} buffering systems contribute to cytosolic $[Ca^{2+}]_i$ clearance. Moreover, the upstroke of the next $[Ca^{2+}]_i$ increase occurs before $[Ca^{2+}]_{ER}$ levels are fully recovered, and for approximately the first 3 $[Ca^{2+}]_i$ rises $[Ca^{2+}]_{ER}$ levels progressively decrease, although after that they seemingly reach a plateau, where each $[Ca^{2+}]_i$ increase occurs from the same $[Ca^{2+}]_{ER}$ level.

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As mentioned, the lag time between the return of $[Ca^{2+}]_i$ rises to baseline and the recovery of $[Ca^{2+}]_{ER}$ suggests the involvement of other Ca^{2+} buffering mechanisms for clearing $[Ca^{2+}]_i$ out of the cytosol. To this end, the functional activity of Na^+/Ca^{2+} exchanger was demonstrated in mouse eggs [20, 21]. 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 a reverse mode of Na^+-Ca^{2+} exchange. Importantly, in spite of the initial changes in $[Ca^{2+}]_i$ levels, even in the absence of external Na^+, $[Ca^{2+}]_i$ returned to baseline levels, implying that the action of other mechanisms, possibly plasma membrane Ca^{2+} ATPase (PMCA) may be more physiologically relevant [21]. In spite of those initial studies, not until recently was the possible involvement PMCA in mammalian oocytes tested. It was shown that inhibition of PMCA by millimolar concentrations of gadolinium (Gd^{3+}), generating a Ca^{2+} insulation system, as both the influx and efflux of Ca^{2+} are greatly reduced, increased the amplitude and duration of the initial $[Ca^{2+}]_i$ rise and oscillations [22], suggesting the active presence of this mechanism in mouse eggs. Nonetheless, the molecular identification of PMCA was not documented in that study and thus the molecular presence of PMCAs in mammalian oocytes/eggs needs confirmation. In contrast, in *Xenopus* oocytes, both the molecular presence and function of PMCA1 were demonstrated [23].

Besides the aforementioned mechanisms, the mitochondria may also contribute to shape $[Ca^{2+}]_i$ rises during oscillations [24, 25], as these organelles can uptake Ca^{2+} into their matrix, thereby alleviating the overall cytosolic Ca^{2+} load [26]. In support of this function, inhibition of mitochondrial function disrupted $[Ca^{2+}]_i$ oscillations and caused a sustained increase in $[Ca^{2+}]_i$ in mouse eggs [27, 28]. Nonetheless, this does not seem to be its main function in terms of Ca^{2+} homeostasis, as inhibition of mitochondrial depolarization did not immediately terminate sperm-initiated oscillations [28]. Instead, and possibly due to 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 ATP production maintains SERCA activity, which is required to maintaining $[Ca^{2+}]_{ER}$ and to sustain sperm-triggered Ca^{2+} oscillations [17]. Importantly, direct assessment of mitochondrial Ca^{2+} uptake has not been performed because the conventional inhibitors, such as ruthenium red, are somehow ineffective in eggs. Recently, however, an RNAi screen showed that a protein named MICU1 is required for mitochondrial Ca^{2+} uptake [29] and its discovery led to the molecular identification of the mitochondrial Ca^{2+} unipoter [30, 31]. Thus, using knockdown approaches it will be worth investigating the role of these molecules on $[Ca^{2+}]_i$ oscillations in mammalian eggs.

2) Ca^{2+} influx mechanisms

Given that only a fraction of Ca^{2+} from each $[Ca^{2+}]_i$ rise is deposited back into the ER Ca^{2+} store by SERCA, extracellular Ca^{2+} must be taken in to replenish $[Ca^{2+}]_{ER}$. Support for the notion that Ca^{2+} influx plays a pivotal role in fertilization is long-standing, as sperm-initiated $[Ca^{2+}]_i$ oscillations cease prematurely in the absence of external Ca^{2+} [22, 32, 33]. Nonetheless, the molecular mechanisms underlining Ca^{2+} influx in mammalian eggs are poorly understood. The cells input extracellular Ca^{2+} across the plasma membrane through a variety of mechanisms, including receptor-operated channels (ROCs), voltage-operated channels (VOCs) [34] and store-operated Ca^{2+} channels (SOCs) [7, 34, 35]. The changes in

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membrane potential associated with $[Ca^{2+}]_i$ responses in fertilized hamster eggs are of very low magnitude to suggest activation of VOCs during this process [36]. Further, several additional findings suggest that the participation of these channels in mammalian fertilization is likely minor, as $[Ca^{2+}]_i$ rises precede changes in membrane potential [33], Ca^{2+} influx continues between $[Ca^{2+}]_i$ rises [37] and, in the mouse, the changes in membrane potential are nearly imperceptible [33]. These findings raise the prospect that Ca^{2+} influx in eggs may be attained, at least in part, by a different mechanism(s). Instead, SOCE, which is associated with $[Ca^{2+}]_{ER}$ levels, may fulfill at least in part this role in mammalian eggs. Towards this end, exposure of MII eggs to thapsigargin evoked a Ca^{2+} rise after addition of extracellular Ca^{2+}, suggesting that SOCE is active in these cells [17]. Subsequent studies implicated SOCE during $[Ca^{2+}]_i$ oscillations, as using the manganese-quenching technique it was found that in mouse eggs the initiation of each $[Ca^{2+}]_i$ rise coincided with divalent cation influx [37, 38]. Nevertheless, additional demonstration of the participation of this mechanism in mammalian fertilization was prevented by lack of knowledge of the molecular components of this mechanism.

Recently, the proteins responsible for SOCE have been identified; stromal interaction molecule 1 (STIM1) is located mostly in the ER where it acts as a Ca^{2+} sensor, as its downregulation was shown to decrease Ca^{2+} influx in response to thapsigargin [39, 40]. 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 [41-43]. The expression and functional analysis using siRNA knockdown of Stim1 and Orai1 were performed using porcine eggs and the downregulation of them prevented persistent $[Ca^{2+}]_i$ oscillations [44, 45]. Although the involvement of Stim1 and Orai1 on $[Ca^{2+}]_i$ oscillations has been demonstrated, their regulation, for instance, the spatio-temporal patterns at each $[Ca^{2+}]_i$ response, and whether or not these molecules overlap during Ca^{2+} influx, remains obscure. In mouse oocytes, studies have also reported the presence of these molecules and their possible involvement in Ca^{2+} homeostasis both during maturation and fertilization. Nevertheless, the cellular organization, re-distribution and expression of these proteins during maturation as well their contribution to fertilization-associated oscillations needs to be clarified [46]. Further, transient receptor potential (TRP) ion channels [47], which are associated with both SOCs and ROCs and are expressed in mammalian eggs [15], may also participate in Ca^{2+} influx during oscillations and their role needs to be further explored.

Although the biological significance of $[Ca^{2+}]_i$ oscillations in mammalian eggs seems to be ambiguous in terms of embryo development, it might be that episodic $[Ca^{2+}]_i$ responses are important for the stepwise completion of the events of egg activation [10, 48]. Generally, different events of egg activation show differential susceptibility to $[Ca^{2+}]_i$ increases, and their initiation and completion also have differential Ca^{2+} requirements. For instance, early events such as CG exocytosis and 2PB extrusion require fewer $[Ca^{2+}]_i$ responses than later events such as PN formation and maternal recruitment of mRNAs [49]. While these results associated the completion of egg activation events to the number of $[Ca^{2+}]_i$ rises, Miao and colleagues recently demonstrated using the Ca^{2+} insulation system with millimolar levels of extracellular Gd^{3+} that even if $[Ca^{2+}]_i$ oscillations occur, eggs fails to extrude 2PB. Given that Ca^{2+} influx is obliterated under those conditions, the conclusion was drawn that, besides replenishing $[Ca^{2+}]_{ER}$, Ca^{2+} influx is required for completion of certain events of egg

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activation [22]. Up to now, the role of $[Ca^{2+}]_i$ rises on egg activation events have been considered in the context of activation of the Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII). It is well established that each sperm-induced $[Ca^{2+}]_i$ rise is accompanied by a parallel increase in Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) activity [50]. Further, the role of CaMKII on egg activation in mammals was documented first by a series of studies where expression of constitutive active forms of CaMKII initiated all events of egg activation, except CG exocytosis, and promoted development to the blastocyst stage [51, 52], and second by genetic studies whereby depletion of CaMKIIγ isoform abrogated the ability of these eggs to exit MII in response to $[Ca^{2+}]_i$ stimulation [53, 54] and caused infertility [54]. Thus, the authors speculated that Ca^{2+} influx activates critical signaling pathways upstream of CaMKIIγ that are required for 2PB emission [22]. Since the detection of Ca^{2+} by target molecules can be highly selective due to space, time and concentration constrains, as it should be when the signal involved regulates the completion of specific cellular events, the regulation of 2PB extrusion by Ca^{2+} influx could be viewed in the context of local vs. global Ca^{2+} sensing [55]. Regardless, further studies are needed to address the signals linking $[Ca^{2+}]_i$ increases and each egg activation event. Further, while the suggested role of Ca^{2+} influx on 2PB extrusion is an important observation, the effects of high Gd^{3+} on 2PB extrusion should also be examined after use of treatments that induce egg activation and 2PB extrusion without increasing $[Ca^{2+}]_i$ and presumably without inducing Ca^{2+} influx, such as the case after addition of the CDK1 And MAPK inhibitors [56].

Ca^{2+} homeostasis during oocyte maturation

As described above, mouse eggs acquire fertilization-competence during oocyte maturation. Multiple factors are involved in the increased Ca^{2+} releasing ability of MII eggs, including IP_3R1 phosphorylation and increased $[Ca^{2+}]_{ER}$ content [18], although more in-depth studies are needed to determine the contribution of each of these changes to the overall increase in Ca^{2+} responsiveness. Following the theme of this review, here we continue the discussion of factors that regulate the increase in $[Ca^{2+}]_{ER}$. The increase in $[Ca^{2+}]_{ER}$ during maturation is a well-documented phenomenon [8, 9, 18] and here we show that addition of ionomycin induces $[Ca^{2+}]_i$ responses of increasing magnitude as maturation progresses (Fig. 2). How the increase in $[Ca^{2+}]_{ER}$ contributes to $[Ca^{2+}]_i$ oscillations in MII eggs was investigated by using cyclopiazonic acid (CPA), a reversible SERCA inhibitor [18]. CPA-treated GV oocytes advanced to the MII stage without delay or gross abnormalities, although without increasing their $[Ca^{2+}]_{ER}$. In this condition, CPA-matured eggs injected with PLCζ cRNA showed a shortened first $[Ca^{2+}]_i$ rise even though CPA was washed away before initiating the oscillations. These results suggest that the increase in $[Ca^{2+}]_{ER}$ directly impact $[Ca^{2+}]_i$ oscillations, especially the robust first $[Ca^{2+}]_i$ rise.

Little is known about how oocytes accumulate Ca^{2+} in the stores during maturation, although our preliminary results indicate that external Ca^{2+} is the source of the increased $[Ca^{2+}]_{ER}$ during maturation (unpublished observations). Further, how Ca^{2+} loading occurs under basal $[Ca^{2+}]_i$ levels is also unclear as well as the mechanisms/channels that might mediate this influx. Nevertheless, it is noteworthy that GV oocytes display spontaneous $[Ca^{2+}]_i$ oscillations [57]. Importantly, these oscillations cease around the GV breakdown

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(GVBD) stage, which is when the most drastic increase in $[Ca^{2+}]_{ER}$ is observed [8]. The temporal coincidence of these observations implies the low levels of $[Ca^{2+}]_{ER}$ at the GV stage, possibly caused by a constitutive Ca^{2+} leak out of the ER, could promote Ca^{2+} influx. While additional studies are needed to clarify how spontaneous oscillations are terminated and the mechanism(s) that keeps low levels of $[Ca^{2+}]_{ER}$ in GV mouse oocytes, given that Ca^{2+} influx is required for the GV-stage $[Ca^{2+}]_i$ oscillations and the known regulation of Ca^{2+} influx by Ca^{2+} store content [58], it stands to reason that Ca^{2+} influx might be regulated during oocyte mouse maturation. In this regard, in *Xenopus* oocytes Ca^{2+} homeostasis and molecules associated with it undergo profound modifications during maturation and more precisely at the GVBD stage. For example, the function of the Ca^{2+} influx, SOCE, and efflux, PMCA, pathways is significantly downregulated at the GVBD stage [14, 59]. One of the key mechanisms underlying these downregulation is the dynamic reorganization of the plasma membrane during maturation, as channels and pumps are specifically internalized [14, 60], depriving these oocytes from quick shuttling of Ca^{2+} to and from the external milieu; seemingly, the membrane trafficking mechanism appear, at least in part, to be conserved between *Xenopus* and mammalian oocytes [61, 62]. Nonetheless, the contribution of plasma membrane reorganization as a regulatory element of Ca^{2+} homeostasis in mouse oocytes needs additional investigation. Further, the similarities between mouse and *Xenopus* oocytes must end at some point during maturation, as mouse eggs at the MII stage undergo oscillations after fertilization and, as noted before, these oscillations rely on Ca^{2+} influx. Therefore, important questions remain unresolved regarding the regulation of Ca^{2+} influx in mouse oocytes and eggs. For example, is the same mechanism that mediates Ca^{2+} influx at the GV stage, but that is inactivated at GVBD, capable of mediating influx in MII eggs following fertilization? Are there other Ca^{2+} channels that undergo differential regulation during maturation and fertilization? Is it possible that some Ca^{2+} channels are synthesized/incorporated into the plasma membrane after the GV stage? How conserved are the Ca^{2+} influx mechanisms during maturation and fertilization among mammals, considering that other elements of the Ca^{2+} oscillation toolkit such as IP_3R1 sensitivity and PlCζ specific activity are widely different among these species. Therefore, studies to answer these questions should occupy researchers interested in this field for some time. Besides providing a deeper understanding of oocyte physiology, identification of these mechanisms is likely to have clinical applications both to improve developmental competence of *in vitro* matured oocytes as well as in the design of novel egg activation procedures.

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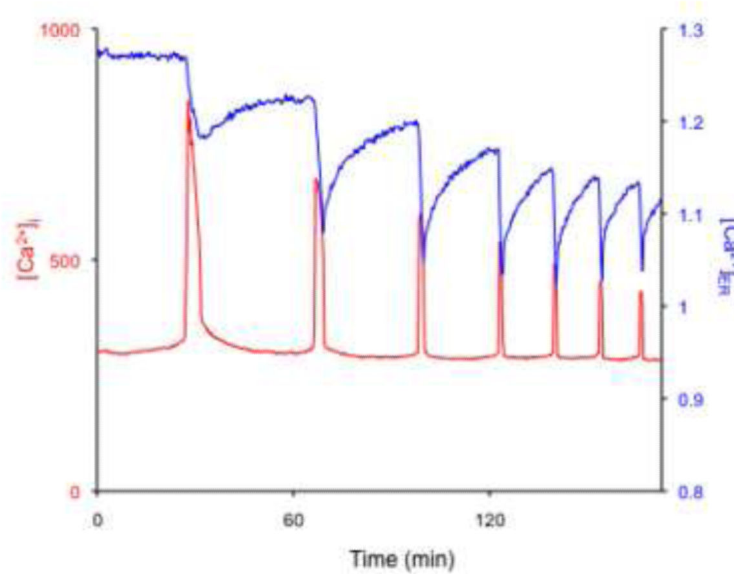

Fig. 1.

$[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_i$ undergo simultaneous but opposite changes in concentration during oscillations in mouse MII eggs. Ca^{2+} responses were induced by injection of 0.05 μg/μl mouse PLCζ cRNA into MII eggs. In vitro transcribed D1ER RNA was injected into eggs 5 hr before the initiation of $[Ca^{2+}]_i$ measurements. The emission ratio of D1ER (YFP/CFP) was used to estimate relative changes in $[Ca^{2+}]_{ER}$ (right axis, blue trace). $[Ca^{2+}]_i$ (left axis, red trace) was recorded using Rhod-2. Rhod-2 is generally used to measure mitochondrial Ca^{2+}, although given that it fails to target into mitochondria in mouse eggs, it is possible to use it as a reported of $[Ca^{2+}]_i$ (Dumollard, R. et al.).

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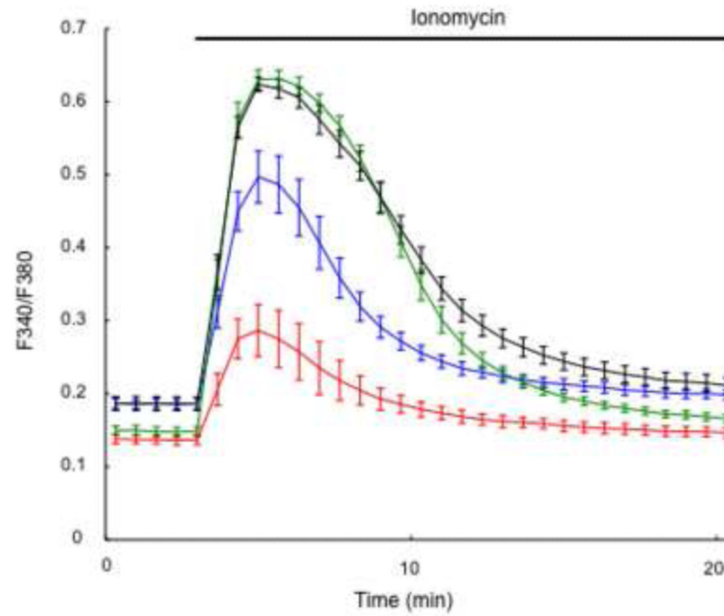

Fig. 2.

$[Ca^{2+}]_{ER}$ content increases during mouse oocyte maturation. $[Ca^{2+}]_{ER}$ was estimated from the $[Ca^{2+}]_i$ responses induced by the addition of 2 μM ionomycin under Ca^{2+} free conditions. The mean fluorescent peak (Fura-2) was compared at 0 (red), 4 (blue), 8 (green) and 12 (black) hr of *in vitro* maturation, which corresponded with GV, GVBD, MI and MII stages of meiotic progression (n=5).