Calcium signaling differentiation during \textit{Xenopus} oocyte maturation

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Abstract

Ca$^{2+}$ is the universal signal for egg activation at fertilization in all sexually reproducing species. The Ca$^{2+}$ signal at fertilization is necessary for egg activation and exhibits specialized spatial and temporal dynamics. Eggs acquire the ability to produce the fertilization-specific Ca$^{2+}$ signal during oocyte maturation. However, the mechanisms regulating Ca$^{2+}$ signaling differentiation during oocyte maturation remain largely unknown.

At fertilization, \textit{Xenopus} eggs produce a cytoplasmic Ca$^{2+}$ transient (Ca$^{2+}_{\text{cyt}}$) that lasts for several minutes, and is required for egg activation. Here, we show that during oocyte maturation Ca$^{2+}$ transport effectors are tightly modulated. The plasma membrane Ca$^{2+}$ ATPase (PMCA) is completely internalized during maturation, and is therefore unable to extrude Ca$^{2+}$ out of the cell. Furthermore, IP$_3$-dependent Ca$^{2+}$ release is required for the sustained Ca$^{2+}_{\text{cyt}}$ rise in eggs, showing that Ca$^{2+}$ that is pumped into the ER leaks back out through IP$_3$ receptors. This apparent futile cycle allows eggs to maintain elevated cytoplasmic Ca$^{2+}$ despite the limited available Ca$^{2+}$ in intracellular stores. Therefore, Ca$^{2+}$ signaling differentiates in a highly orchestrated fashion during \textit{Xenopus} oocyte maturation endowing the egg with the capacity to produce a sustained Ca$^{2+}_{\text{cyt}}$ transient at fertilization, which defines the egg’s competence to activate and initiate embryonic development.

Keywords: Oocyte maturation; \textit{Xenopus}; Ca$^{2+}$ signaling; IP$_3$ receptor; Plasma membrane Ca$^{2+}$ ATPase; Endocytosis

Introduction

At fertilization, vertebrate eggs undergo a major transition from gametogenesis to embryogenesis with dramatic cellular alterations referred to collectively as egg activation. Ca$^{2+}$ is the universal signal for egg activation at fertilization in all sexually reproducing species studied to date, from plants to humans (Stricker, 2000; Antoine et al., 2000). The fertilization-induced Ca$^{2+}$ signal has specific spatial and temporal dynamics, and is essential to activate the egg and initiate embryonic development (Stricker, 2000; Homa et al., 1993). This specialized Ca$^{2+}$ signal takes the form of a single, or multiple Ca$^{2+}$ transients depending on the species (Stricker, 2000). For example, in jellyfish, sea urchin, and \textit{Xenopus}, a single Ca$^{2+}$ wave is observed at fertilization. In contrast, in annelids, ascidians, and mammals, multiple Ca$^{2+}$ transients can be detected (Stricker, 2000). Ca$^{2+}$ is a fitting second messenger to mediate egg activation across phylogeny, because of its ability to induce various Ca$^{2+}$-dependent signaling cascades leading to diverse cellular responses (Bootman et al., 2002). Egg activation provides an elegant example of how Ca$^{2+}$ signals specify complex disparate cellular responses. In \textit{Xenopus}, the Ca$^{2+}$ rise at fertilization mediates the early steps of egg activation, including the block to polyspermy and the transition into the embryonic mitotic cell cycle (Busa and Nuccitelli, 1985; Busa et al., 1985; Nuccitelli et al., 1993; Lora et al., 1993; Morin et al., 1994).

Fertilization of the \textit{Xenopus} egg induces a local Ca$^{2+}$ rise at the site of sperm entry, which gates Ca$^{2+}$-activated Cl$^-$ channels resulting in membrane depolarization and the fast block to polyspermy (Machaca et al., 2001; Kline, 1988). Membrane depolarization blocks sperm entry, because \textit{Xenopus} sperm have a voltage sensitive effector required for fertilization (Jaffe et al., 1983). As the fertilization-induced Ca$^{2+}$ wave sweeps through the egg, it triggers cortical granule fusion (fertilization envelope) and the slow block to poly-
following maturation, eggs become fertilization competent and able to initiate embryonic development. Therefore, fertilization (Yamashita et al., 2000; Masui and Clarke, the frog), enter meiosis and arrest at metaphase II until cell cycle and in response to the proper trigger (progesterone in Ca\(^{2+}\) transport effectors during oocyte maturation. These transient at fertilization. To better understand how Ca\(^{2+}\) signaling differentiation during oocyte maturation remain obscure.

In \textit{Xenopus} at fertilization, Ca\(^{2+}\)_cyt remains elevated for \(\sim 10\) min (Busa and Nuccitelli, 1985). The initial phase of this Ca\(^{2+}\) transient is a sweeping Ca\(^{2+}\) wave that is due to Ca\(^{2+}\) release from intracellular stores through the IP\(_3\) receptor (IP\(_3\)R). Inhibiting IP\(_3\)-dependent Ca\(^{2+}\) release or PIP2 hydrolysis blocks the Ca\(^{2+}\) release wave (Nuccitelli et al., 1993; Larabell and Nuccitelli, 1992; Runft et al., 1999). Furthermore, a wave of IP\(_3\) has been proposed to precede Ca\(^{2+}\) release in eggs at fertilization (Wagner et al., 2004). Ca\(^{2+}\) influx does not contribute to the Ca\(^{2+}\) rise at fertilization (Fontanilla and Nuccitelli, 1998), especially that the primary Ca\(^{2+}\) influx pathway in \textit{Xenopus} oocytes, store-operated Ca\(^{2+}\) entry (SOCE), is inhibited during oocyte maturation due to MPF activation (Machaca and Haun, 2000; Machaca and Haun, 2002). Therefore, fertilization induces IP\(_3\) production, which underlies the wave of Ca\(^{2+}\) release from stores.

The Ca\(^{2+}\) release wave in eggs is significantly slower than that in oocytes in response to Ca\(^{2+}\) mobilizing agents (Fontanilla and Nuccitelli, 1998; Machaca, 2004). Furthermore, the mode of wave propagation is saltatory in oocytes, whereas it is continuous in eggs (Callamaras et al., 1998; Machaca, 2004; Nuccitelli et al., 1993; Fontanilla and Nuccitelli, 1998). The speed and mode of propagation of the Ca\(^{2+}\) wave in eggs can be accounted for by the clustering of IP\(_3\)-dependent elementary Ca\(^{2+}\) release events (Ca\(^{2+}\) puffs) during oocyte maturation (Machaca, 2004). This reiterates the dramatic differentiation of the Ca\(^{2+}\) handling machinery during oocyte maturation to produce the specialized sustained Ca\(^{2+}\) transient at fertilization. To better understand how Ca\(^{2+}\) signaling pathways differentiate during oocyte maturation, here we analyze the subcellular distribution and activity of Ca\(^{2+}\) transport effectors during oocyte maturation. These studies show that Ca\(^{2+}\) signals differentiate in two important ways to mediate the sustained Ca\(^{2+}\) rise in eggs. Ca\(^{2+}\) extrusion out of eggs is blocked because the plasma membrane Ca\(^{2+}\)-ATPase is internalized during oocyte maturation. Furthermore, IP\(_3\) receptors in eggs continuously leak Ca\(^{2+}\) out of the ER, which is pumped back by SERCA resulting in Ca\(^{2+}\) recycling between the ER lumen and cytosol. This Ca\(^{2+}\) recycling maintains cytosolic Ca\(^{2+}\) elevated using limited free Ca\(^{2+}\) in intracellular stores. Therefore, during oocyte maturation, the activity and distribution of Ca\(^{2+}\) transport effectors are modulated to produce the sustained, fertilization-specific Ca\(^{2+}\) transient.

**Materials and methods**

**Ca\(^{2+}\) imaging**

\textit{Xenopus} oocytes were obtained, cultured, and matured as previously described (Machaca and Haun, 2002). Oocytes or eggs were injected with Oregon Green BAPTA-1 (OG-1), Fluo-4 (Molecular Probes), Heparin (Sigma), and/or caged-IP\(_3\) (\(\gamma\)-myo-inositol 1,4,5-triphosphate, \(P\_451\) (2-nitrophenyl-\(\beta\)ethyl) ester, tris triethylammonium salt) (Molecular Probes) as indicated. Injections were performed in 110 mM NaCl, 1.3 mM KCl, 20 mM MgSO\(_4\), 0.1 mM EGTA, 5 mM HEPES, pH 7.4 containing 10 mM Chlorobutanol to prevent egg activation (Busa and Nuccitelli, 1985). During imaging, oocytes were incubated in Ca\(^{2+}\)-free Ringer (in mM: 96 NaCl; 2.5 KCl; 4 MgCl\(_2\); 10 HEPES; 0.1 EGTA; pH 7.4). Ca\(^{2+}\) imaging was performed on an Olympus IX70 microscope equipped with a Fluoview 300 confocal box using a 20x PlanoFl objective (NA 0.5). UV pulses to uncage IP\(_3\) were from a 100 W Xenon arc lamp and passed through a filter cube equipped with a 360/40 excitation filter and a 400dp beam splitter (Chroma). OG-1 or Fluo-4 fluorescence emission was passed through a 510 long pass filter before acquisition. UV intensity was modulated using neutral density filters and duration was controlled by a UniBlitz shutter. The shutter and confocal image acquisition were controlled by TTL pulses using pClamp8 software. Ca\(^{2+}\) imaging data were analyzed offline using MetaMorph (Universal Imaging Corp.) and figures were prepared in Adobe Photoshop. Kinetic analyses were performed using Origin software (OriginLab Corp.). Because sperm enter on the animal hemisphere of eggs, all experiments were limited to the animal hemisphere.

**Immunocytochemistry**

Eggs (3 h after germinal vesicle break down ‘GVBD’) and oocytes were fixed in 2% paraformaldehyde for 1 h at 4\(^\circ\)C. After fixation, the cells were embedded in tissue freezing media (Electron Microscopy Science) using Tissue Tek cryomold and frozen at −20\(^\circ\)C overnight. Samples were sliced into 8 \(\mu\)m sections collected on sialenized slides (Electron Microscopy Science). Slides were washed in TBST (20 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween) for 1 h and then blocked in blocking buffer (10 mM Tris–HCl pH 7.5, 1% BSA, 0.3% Triton-X100, 1% Gelatin, 0.02 M Glycine, 150 mM NaCl, and 5% goat serum) for 1 h at room temperature. Slides were then incubated with primary antibodies for 90 min in antibody buffer (20 mM Tris pH 7.4, 150 mM NaCl, and 0.05% goat serum) followed by 1 h incubation with the appropriate Alexa 546 labeled secondary (Molecular Probes). Antibodies used were: a pan-specific anti-PMA antibody that recognizes all 4 PMA isoforms (Affinity BioReagents), an anti-SERCA2 (NOVO CASTRA), an anti-Integrin monoclonal (8C8, Developmental Studies Hybridoma Bank), and an anti-type 1 IP\(_3\)R antibody (gift from S. Joseph; Joseph and Samanta, 1993). For each antigen, the experiment was repeated at least 3 times on cells from different donor frogs and each time at least 5 slices were analyzed. For every experiment, slices were stained with the secondary alone as a control and showed no detectable staining at the confocal settings used to collect the experimental images. For Wheat Germ Agglutinin conjugate (WGA) staining, cells were fixed then incubated for 10 min at 4\(^\circ\)C in 48 \(\mu\)g/ml WGA. Transmission and fluorescence images were collected on a Fluoview confocal (Olympus) coupled to a microscope.
(model IX70; Olympus) at RT, using an UPlanApo 60× oil objective. Figures were compiled using Adobe Photoshop 7.0.

**Westerns**

Cells were dounced in lysis buffer (20 mM HEPES-KOH pH 7.5, 1 mM EGTA, 2 mM β-Mercaptoethanol, 10% sucrose, 1 mM PMSF, and 0.1 mM A23187, 5 μM Bestatin, 1.5 μM E–64, 2 μM Leupeptin, and 1 μM Pepstatin A) and centrifuged at 1000 × g for 10 minutes to remove yolk proteins. Samples were separated on 8% polyacrylamide gels, transferred to PVDF membrane, and blotted with the primary antibodies. This was followed by the appropriate HRP conjugated secondary antibody (Jackson Immunoresearch) and detected by ECL-Plus (Amersham Pharmacia Biotech). Westerns were visualized using a STORM™ system (Molecular Dynamics).

**Results**

Figs. 1A–C illustrate differences in Ca²⁺ handling in oocytes and eggs following Ca²⁺ mobilization. In response to a short IP₃ pulse in cells loaded with caged IP₃, oocytes release Ca²⁺ locally in the region of uncaging with minimal spread of the Ca²⁺ release due to diffusion of the uncaged IP₃ (Figs. 1A, B). In contrast, the brief (1 s), localized IP₃ uncaging pulse in the egg produces a self-sustained Ca²⁺ wave that slowly spreads across the entire egg (Figs. 1A, B). Even when Ca²⁺ was released from stores globally, using Ca²⁺ ionophore to eliminate the spatial dimension to the spreading Ca²⁺ wave, Ca²⁺ decayed back to baseline in oocytes but is maintained at high levels in eggs (Fig. 1C). It is possible to consistently image Ca²⁺ transients in eggs for extended times, and because a Ca²⁺ rise activates the egg resulting in a cortical rotation, which precludes Ca²⁺ imaging. These experiments were performed in Ca²⁺-free medium, thus the only source of Ca²⁺ is from intracellular stores. Ca²⁺ decay in oocytes is due to cytoplasmic Ca²⁺ buffering, Ca²⁺ reuptake into the ER, and Ca²⁺ extrusion out of the cell. The persistent Ca²⁺ rise in eggs argues that these mechanisms combined are downregulated. Alternatively, eggs may produce a large Ca²⁺ release transient that overwhelms the Ca²⁺ handling machinery resulting in sustained Ca²⁺ levels. In addition, IP₃ metabolisms and the
regulation of IP3 receptor gating in the egg may also contribute to the sustained Ca2+ rise. Therefore, besides the slow propagation of the Ca2+ wave, other mechanisms are at play in eggs to maintain prolonged high levels of Ca2+_cyt.

**Releasable store Ca2+**

A potential contributing factor to the prolonged Ca2+ rise in eggs is the amount of available Ca2+ in intracellular stores. During maturation, eggs could accumulate Ca2+ in their stores, leading to a sustained Ca2+ rise at fertilization. Although, it is difficult to imagine eggs sequestering enough Ca2+ to sustain an ~10 min Ca2+ rise. Nonetheless, we wanted to directly measure available ER Ca2+ in oocytes and eggs. We devised two strategies to estimate releasable ER Ca2+. First, cells were loaded with a Ca2+ dye (Fluo-4) and heparin to block wave propagation and egg activation. Heparin blocks IP3 receptors, which prevents the Ca2+ release wave. Ca2+ was released from stores using saturating concentrations of Ca2+ ionophore (15 μM A23187) in Ca2+-free Ringer solution. The integral under the rising phase of the Ca2+ signal was taken as an estimate of Ca2+ store content (Fig. 1D). This shows that releasable ER Ca2+ is slightly higher in eggs (1.39 ± 0.1-fold) as compared to oocytes. To support this finding and because Ca2+ mobilization from stores through IP3 receptors has been shown to be the source of Ca2+ signal at fertilization (Nuccitelli et al., 1993; Runft et al., 1999), we wanted to measure IP3-releasable Ca2+.

Measuring IP3-releasable Ca2+ is complicated by the fact that Ca2+ release in eggs produces a sweeping Ca2+ wave and activates mechanisms that are able to maintain Ca2+_cyt high (Figs. 1A–C), which would interfere with direct measurement of IP3 releasable Ca2+. We could not use heparin as for the ionophore experiment (Fig. 1D) because it blocks the IP3 receptor. It was therefore necessary to release Ca2+ without allowing Ca2+-dependent egg activation. This was accomplished by loading cells with caged-IP3 and high concentrations (150 μM) of the high affinity (Kd ~ 170 nM) Ca2+ dye, Oregon Green BAPTA-1. Cells were subjected to a saturating uncaging pulse (15 s) to maximally stimulate IP3-dependent Ca2+ release in the imaging plane. Our rational was that, at such high concentrations of a low Kd Ca2+ dye, released Ca2+ will be bound by the dye and reported as an increase in fluorescence intensity, while preventing free Ca2+ diffusion and thus egg activation. Indeed, local uncaging of IP3 under these conditions prevents the spread of the Ca2+ wave in eggs (not shown). Using this approach, IP3-releasable Ca2+ was 1.4 ± 0.19-fold higher in eggs as compared to oocytes (Fig. 1E). This is in close agreement with the ionophore releasable Ca2+, arguing that releasable ER Ca2+ is ~1.4-fold higher in eggs as compared to oocytes. Furthermore, these data imply that the majority of the available store Ca2+ is mobilized following IP3 production, arguing that egg Ca2+ stores are gated primarily by the IP3 receptor. This shows that a higher level of releasable store Ca2+ contributes to the sustained Ca2+ rise in eggs. However, the rather small 1.4-fold increase is unlikely to fully account for the extended Ca2+ rise at fertilization.

Expression and subcellular localization of Ca2+ transport effectors

Ca2+_cyt dynamics are regulated primarily by channels and transporters that localize to the cell and ER membranes. We used immunofluorescence to determine whether the subcellular distribution of the major Ca2+ transport effectors is modified during oocyte maturation. We localized the IP3 receptor, the only ER Ca2+ release channel known to exist in Xenopus oocytes (Parys and Bezprozvanny, 1995); the plasma membrane Ca2+-ATPase (PMCA), which mediates Ca2+ extrusion out of the cell; and the sarcoendoplasmic reticulum Ca2+-ATPase (SERCA), the pump that sequesters Ca2+ back into the ER. The data show an interesting and significant redistribution of all three, with important functional implications (Fig. 2).

In oocytes, the IP3 receptor has a diffuse reticular distribution consistent with its localization to the ER (Fig. 2A), whereas in eggs, the IP3 receptor redistributes into large clusters throughout the cytoplasm (Fig. 2A), as has been previously reported (Kume et al., 1997; Parys et al., 1994). Consistent with the IP3 receptor physical clustering, we have recently shown that functionally elementary Ca2+ release events (Ca2+ puffs) cluster in eggs (Machaca, 2004). A general remodelling of the ER has been documented in Xenopus and other species during oocyte maturation (Terasaki, 1994; Terasaki et al., 1996; Kline et al., 1999; Terasaki et al., 2001). Importantly, however, the clustering of IP3 receptors in the animal hemisphere of the egg documented here (Fig. 2A) is not due to ER remodelling because although there is dramatic clustering of the ER in the vegetal hemisphere of Xenopus oocytes, such clustering is absent in the animal hemisphere (Terasaki et al., 2001).

Immunostaining shows that in oocytes SERCA localizes to the reticular ER network, whereas in eggs, it localizes to clusters (Fig. 2B), similar to, although smaller than those observed with the IP3 receptor. SERCA immunostaining is technically difficult in eggs, therefore to confirm SERCA distribution, we used bodipy-labeled thapsigargin to stain for SERCA in live oocytes and confirmed the mild clustering observed by immunofluorescence (not shown).

PMCA localizes to the plasma membrane in oocytes, but is completely internalized into an intracellular pool in eggs (Fig. 2C). The plasma membrane was labeled with wheat–germ agglutinin (WGA) in these experiments (Fig. 2C). Close inspection revealed vesicle-like structures below the plasma membrane that stain positive for PMCA (Fig. 2C, inset). Internalization of plasma membrane proteins during Xenopus oocyte maturation is not unique to PMCA, and has been documented for other endogenous and exogenously expressed membrane proteins including β-integrin and the Na–K-ATPase (Muller et al., 1993; Schmalzing et al., 1990; Shcherbatko et al., 2001). Indeed, β-integrin follows a similar redistribution to PMCA during oocyte maturation (Fig. 2D). These data show that during oocyte maturation PMCA is endocytosed, and completely removed from the plasma membrane. Therefore, in eggs, PMCA does not extrude Ca2+ out of the cell, thus greatly enhancing the egg’s ability to maintain high Ca2+_cyt.
Endocytosis is a common mechanism to modulate Na–K-ATPase levels at the plasma membrane. Both PTH and dopamine induce internalization of Na–K-ATPase in kidney epithelial cells (Khundmiri et al., 2004; Chibalin et al., 1998). In this case, Na–K-ATPase is targeted for internalization due to phosphorylation on two Ser residues by PKC and/or ERK (Khundmiri et al., 2004; Chibalin et al., 1998).

To determine whether the expression levels of Ca$^{2+}$ transport effectors are modulated during maturation, we used semi-quantitative Westerns to assess PMCA and IP$_3$ receptor.

Fig. 2. Subcellular localization and expression of Ca$^{2+}$ transport effectors. (A–D) Oocytes and eggs were fixed, sectioned, and stained with specific antibodies as described in the Materials and methods section. Both DIC and immunofluorescence images are shown for each antigen as indicated above the immunofluorescence panel. For PMCA, (C) cells were also stained with wheat germ agglutinin-Oregon green 488 (WGA, green) to stain the plasma membrane. Merged images show the absence of PMCA at the plasma membrane in eggs. The inset in panel C shows an enlarged view of the boxed area with arrows pointing to vesicle-like structures that stain positive for PMCA. Scale bar in all panels is 25 μm. (E) Western blot analysis of IP$_3$ receptor and PMCA levels in oocytes and eggs. The numbers above the gel refer to oocyte or egg equivalents (0.25, 0.5, and 1 cell). One cell equivalent is the volume in which one cell is lysed in and is typically ~25 μg.
levels (Fig. 2E). Densitometry analysis of the Western blots revealed a small increase in IP3 receptor levels (1.25 ± 0.52-fold; n = 3) as previously reported (Kume et al., 1997). PMCA levels also increased in eggs by 1.4 ± 0.43-fold (n = 3) (Fig. 2E). However, the variability between different donor frogs and between different blots was considerable, making the significance of these small changes in protein levels questionable.

**PMCA activity**

PMCA internalization during oocyte maturation shows that Ca\(^{2+}\) extrusion is eliminated in eggs as compared to oocytes. To functionally confirm this conclusion, we measured PMCA activity using an imaging approach. PMCA extrudes Ca\(^{2+}\) out of the cell, therefore if PMCA is blocked, Ca\(_{cys}\) should decay with slower kinetics. We used Ca\(_{cys}\) decay as an indicator of PMCA activity following ionophore-mediated Ca\(^{2+}\) mobilization in Ca\(^{2+}\)-free media, in the presence or absence of La\(^{3+}\) to block PMCA (Fig. 3). The time required for the Ca\(_{cys}\) signal to reach half-maximal levels was taken as a measure of Ca\(^{2+}\) decay and the data were normalized to control cells (Fig. 3). In oocytes treated with La\(^{3+}\), Ca\(_{cys}\) decays significantly slower than in control oocytes, showing that PMCA-mediated Ca\(^{2+}\) extrusion is an important contributor to Ca\(_{cys}\) decay (Fig. 3). Similar decay rates were obtained when oocytes were injected with heparin to block Ca\(^{2+}\)-release through the IP3 receptor (Fig. 3). It was not possible to perform the same experiment in control eggs, because global Ca\(^{2+}\) release in eggs results in a sustained Ca\(^{2+}\) rise and cortical rotation that precludes prolonged Ca\(^{2+}\) imaging. However, blocking IP3 receptor with heparin prevented Ca\(^{2+}\) wave propagation (Fig. 5), thus allowing measurements of Ca\(_{cys}\) decay. Under these conditions, Ca\(_{cys}\) decayed with similar kinetics whether eggs was treated with La\(^{3+}\) or not (Fig. 3), showing that PMCA-mediated Ca\(^{2+}\) extrusion does not contribute to Ca\(_{cys}\) decay in eggs. Therefore, as expected from the internal localization of PMCA (Fig. 2C), functionally PMCA does not contribute to Ca\(^{2+}\) extrusion in eggs.

Ca\(^{2+}\) extrusion can also be mediated by the Na–Ca-exchanger in variety of cells (Philipson et al., 2002). The presence of the Na–Ca-exchanger (NCX) in *Xenopus* oocytes is controversial (Supplisson et al., 1991; Solis-Garrido et al., 2004). Nonetheless, La\(^{3+}\) has been reported to block NCX (Kimura et al., 1986), and thus if NCX is present in oocytes it is likely blocked by La\(^{3+}\) under our experimental conditions. The fact that La\(^{3+}\) does not alter the decay kinetics in eggs (Fig. 3) argues that if NCX is present in oocytes it does not contribute to Ca\(_{cys}\) decay in eggs.

**Cytosolic Ca\(^{2+}\) clearing capacity**

In addition to Ca\(^{2+}\) efflux out of the cell, Ca\(_{cys}\) transients are dissipated due to Ca\(^{2+}\) sequestration into the ER through the action of SERCA, by uptake into mitochondria and by cytosolic Ca\(^{2+}\) buffers. Together, these mechanisms define the capacity of the cytosol to lower Ca\(^{2+}\) levels. If these pathways are less efficient at clearing Ca\(_{cys}\) in eggs, this may contribute to the sustained Ca\(^{2+}\) transient observed at fertilization. We devised an approach to measure the combined activity of these pathways, which we refer to as the cytosolic Ca\(^{2+}\) clearing capacity. For these experiments, oocytes and eggs were injected with NP-EGTA preloaded with Ca\(^{2+}\) (caged-Ca\(^{2+}\)), which allowed us to generate a localized Ca\(^{2+}\) transient by UV uncaging (Fig. 4A). There was a linear relationship between the duration of UV exposure and the amount of Ca\(^{2+}\) released, showing that NP-EGTA was not limiting (Fig. 4A). In oocytes, uncaging NP-EGTA for different durations (0.5–4 s) produces a transient Ca\(^{2+}\) rise in the uncaging region that gradually returns to baseline (Fig. 4A, Oocyte). In eggs, short uncaging durations (0.5–1.5 s) produced localized Ca\(^{2+}\) decay.
transients similar to those observed in oocytes (Fig. 4A, Egg). However, at longer uncaging durations (≥4 s) in eggs, the localized Ca\(^{2+}\) transient resulted in a global Ca\(^{2+}\) wave that spreads through the entire cell (Fig. 4A, Egg—4 s). This shows that a rise in Ca\(^{2+}\) in eggs is sufficient to induce wave propagation and thus egg activation. However, the localized Ca\(^{2+}\) rise has to be sustained for several seconds before inducing wave propagation. This could be a safety mechanism that prevents stochastic egg activation in the absence of sperm entry. There is evidence in the literature that an IP\(_3\) wave precedes the Ca\(^{2+}\) wave in Xenopus oocytes (Wagner et al., 2004), and a consensus that IP\(_3\) production and functional IP\(_3\) receptors are necessary for wave propagation (Busa et al., 1985; Larabell and Nuccitelli, 1992; Nuccitelli et al., 1993; Snow et al., 1996; Runft et al., 1999). This argues that a rise in Ca\(^{2+}\) activates a PLC-dependent pathway in eggs resulting in
IP3 production and Ca2+ wave propagation. However, incubating eggs with high concentrations of the non-specific PLC inhibitor (U73122, 30 μM) did not consistently block the Ca2+-induced wave propagation in oocytes (data not shown). Furthermore, interfering with PLCβ or PLCγ activity has been reported not to block the Ca2+ wave at fertilization (Runft et al., 1999). Therefore, the identity of the Ca2+ sensor that initiates Ca2+ wave propagation remains a mystery at this point.

Nonetheless, the fact that short uncaging durations (0.5 s) produced a localized Ca2+ rise in both oocytes and eggs provided us with the ability to compare the cytosolic Ca2+ clearing capacity in these cells. That is, Ca2+ sequestration into the ER, and cytosolic and mitochondrial buffering. Ca2+ was released deep in the cytosol to minimize the contribution of Ca2+ efflux in the cell cortex (Fig. 4). Indeed, performing the same experiments in the presence of La3+ to block PMCA resulted in Ca2+ transients with identical kinetics, indicating that PMCA is not contributing to the Ca2+ decay in the ROI in oocytes. The time course of Ca2+ decay following NP-EGTA uncaging (0.5 s) was recorded. Examples of Ca2+ decay kinetics in an oocyte and an egg are shown in Fig. 4B. The time required for half-maximal decay was taken as a measure of the cytosolic Ca2+ clearing capacity. Interestingly, oocytes are able to clear Ca2+ faster than eggs (T0.37 vs. 10.4 ± 0.98 s, Fig. 4B), showing that the combined activity of SERCA sequestration, and mitochondrial and cytosolic buffers, is less efficient at lowering Ca2+ levels in eggs as compared to oocytes. Therefore, this slower cytosolic Ca2+ clearing capacity in eggs is also likely to contribute to the sustained Ca2+ rise.

Ca2+ recycling

Because of the central role of IP3-dependent Ca2+ release in the propagation of the Ca2+ wave at fertilization, we were interested in determining the role of IP3 receptors in the sustained Ca2+ plateau in eggs. Cells were loaded with NP-EGTA and long UV uncaging (5 s) was used to produce a localized Ca2+ rise (Fig. 5A). In eggs, this results in the propagation of a Ca2+ wave, that is blocked by heparin (Fig. 5A). Analysis of Ca2+ kinetics under these conditions provided important insights into the mechanisms of Ca2+ handling in eggs. Ca2+ dynamics were recorded following NP-EGTA uncaging in three region of interest shown in Fig. 5A: (1) the region of localized NP-EGTA uncaging (‘Uncaging ROI’, magenta and green circles); (2) a region peripheral to the uncaging site (‘Peripheral ROI’, red or cyan rectangles); (3) globally over the entire imaging plane (‘Global’, orange and olive circles). In control eggs (Egg), uncaging NP-EGTA causes a local Ca2+ rise followed by a Ca2+ wave that spreads across the imaging plane (Fig. 5A). In the ‘uncaging ROI’, the Ca2+ rise is due to both NP-EGTA uncaging and Ca2+ release from stores through IP3 receptors (Fig. 5A). In contrast, in the ‘peripheral ROI’, only Ca2+ released from intracellular stores through IP3 receptors contributes to Ca2+ dynamics (Fig. 5A, red). This is because in heparin injected cells, the uncaging pulse does not produce a Ca2+ rise in the ‘peripheral ROI’ (Fig. 5A, cyan).

In the ‘uncaging ROI’, Ca2+ decays significantly more rapidly in heparin injected (green) vs. control (magenta) eggs (Fig. 5A), requiring 54.1 ± 6.6 s to reach half-maximal levels in control eggs vs. 15 ± 1.4 s in heparin injected eggs (Fig. 5C). Even in the ‘peripheral ROI’ (red), in the absence of any contribution from Ca2+ uncaging, the Ca2+ signal still decays significantly slower than in heparin injected eggs, requiring 34.1 ± 3.2 s to reach half-maximal amplitude (Fig. 5C). This result is counter intuitive. In the ‘peripheral ROI’, in control eggs, Ca2+ stores have been depleted by the passing Ca2+ wave, thus producing a large driving force for SERCA to pump Ca2+ back into the ER lumen. In contrast, in heparin injected eggs, Ca2+ stores are full in the ‘uncaging ROI’, and therefore SERCA is faced with a steep Ca2+ gradient against which to pump Ca2+ into the ER lumen. Nonetheless, the Ca2+ signal decays significantly more rapidly in heparin injected vs. control eggs. It is unlikely that additional pathways such as mitochondrial Ca2+ uptake or cytoplasmic Ca2+ buffering capacity are involved when comparing heparin injected and control eggs because they should be similar. These data show that in the absence of Ca2+ release through IP3 receptors in eggs Ca2+ decays significantly more rapidly.

Global Ca2+ dynamics support this conclusion. In eggs, the global Ca2+ signals increase gradually over time due to wave propagation, hold at a plateau phase, and then slowly decline back to baseline (Fig. 5A, orange). In the presence of heparin, the global Ca2+ signal rapidly decays back to baseline (Fig. 5A, olive). Furthermore, when Ca2+ is mobilized from stores with ionomycin, Ca2+ holds at a high plateau in control eggs, whereas in the presence of heparin, Ca2+ decays back to baseline (Fig. 5D). Together, these data show that Ca2+ release through IP3 receptors is required for the sustained Ca2+ rise in eggs. Therefore, during egg activation, IP3 receptors allow continuous Ca2+ leak out of the ER, which explains the slower Ca2+ decay kinetics in control vs. heparin injected eggs.

In contrast to eggs, in oocytes, both local and global Ca2+ decay with similar kinetics in the presence or absence of heparin showing that IP3-dependent Ca2+ release does not contribute to Ca2+ signal dynamics in oocytes (Figs. 5B, C).

The requirement for functional IP3 receptors for the sustained Ca2+ signal in eggs argues that Ca2+ ions that are pumped into the ER lumen by SERCA will be free to diffuse back into the cytosol through IP3 receptors. This apparently futile cycle has the advantage of maintaining Ca2+ levels high for extended time periods, a requirement for egg activation, with the limited amount of available Ca2+ in intracellular stores. We refer to this phenomenon as ‘Ca2+ recycling’ to illustrate the fact that in eggs Ca2+ is able to continuously recycle between the cytosol and ER lumen to produce the prolonged Ca2+ transient.

Discussion

Based on our analysis of the subcellular localization, expression levels and activity of the major Ca2+ transport pathways in oocytes and eggs, we propose a model of Ca2+ signaling differentiation during oocyte maturation (Fig. 6). This differentiation of Ca2+ signaling pathways endows the egg with
the capacity to produce the sustained Ca\(^{2+}\) rise at fertilization, which is essential for egg activation and the egg-to-embryo transition (Nuccitelli et al., 1993; Larabell and Nuccitelli, 1992; Runft et al., 1999; Kline, 1988). In oocytes, Ca\(^{2+}\) transients are produced following Ca\(^{2+}\) release from intracellular stores through IP\(_3\) receptors (Fig. 6), since the type 1 IP\(_3\) receptor is the only Ca\(^{2+}\) release channel known to be expressed in *Xenopus* oocytes (Parys and Bezprozvanny, 1995). If sufficient store depletion is achieved during the Ca\(^{2+}\) release phase, it will lead to Ca\(^{2+}\) influx at the cell membrane through the store-operated Ca\(^{2+}\) entry (SOCE) pathway (Hartzell, 1996; Yao et al., 1999; Machaca and Haun, 2000).

Fig. 5. Ca\(^{2+}\) recycling. (A) Oocytes and eggs were injected with NP-EGTA (0.9 mM) and Fluo-4 (10 µM) with (Hep) or without heparin (150 µg/ml) as indicated. NP-EGTA was uncaged for 5 s in a localized region (uncaging ROI) in the center of the imaging field (magenta and green). Ca\(^{2+}\)-dependent fluorescence intensity was monitored in three regions of interest (ROI): (1) In the uncaging ROI (magenta and green circles); (2) In a peripheral ROI (red and cyan rectangles); and (3) Globally over the entire imaging field (orange and olive circles). Color matched traces show Ca\(^{2+}\) decay kinetics in control and heparin injected eggs in the three ROIs. (B) An example of local Ca\(^{2+}\) decay kinetics in a control and heparin injected oocyte treated in as described in panel A for eggs. (C) Summary of decay kinetics in oocytes and eggs. The time required for the Ca\(^{2+}\) signal to reach half-maximal levels following NP-EGTA uncaging was taken as a measure of the speed of Ca\(^{2+}\) decay. Data are color matched to panels A and B. Local decay kinetics (top panel) refer to the uncaging ROI for both oocytes (Ooc) and eggs, except for Egg-Per, which refers to Ca\(^{2+}\) decay in the peripheral ROI in control eggs (red rectangle in panel A). Asterisks indicate significantly different datasets (\(P \leq 8 \times 10^{-5}, n = 9 – 14\)). Same labeling applies to global decay kinetics (bottom panel) measured from the entire imaging plane. Asterisks indicate significantly different data sets (\(P \leq 0.012, n = 9 – 14\)). (D) Ca\(^{2+}\) decay in eggs following ionomycin-mediated Ca\(^{2+}\) mobilization. Ca\(^{2+}\) was released from stores with the Ca\(^{2+}\)-ionophore ionomycin (10 µM) in control and heparin injected cells (200 µg/ml) and Ca\(^{2+}\) decay monitored over time. The traces show the average decay time course from 8 cells each for oocytes and eggs. The mean ± SE is plotted.
is through the SERCA pump (Fig. 6), most likely the SERCA2 isoform (Fig. 2) (Kobrinsky and Kirchberger, 2001).

During oocyte maturation, the Ca\textsuperscript{2+} handling machinery differentiates dramatically to endow the egg with the capacity to produce the sustained Ca\textsuperscript{2+} transient at fertilization (Fig. 6). Fertilization or Ca\textsuperscript{2+} mobilizing agents produce a sustained Ca\textsuperscript{2+} rise in eggs, which occurs in the absence of Ca\textsuperscript{2+} influx through SOCE, because this pathway is inactivated in eggs due to MPF-dependent inhibition of the coupling mechanism between store depletion and SOCE activation (Fig. 6) (Machaca and Haun, 2000; Machaca and Haun, 2002). The Ca\textsuperscript{2+} rise in eggs differs from that in oocytes in two important ways. First, the time to peak is slower due to the slow propagation of the Ca\textsuperscript{2+} release wave. Second, Ca\textsuperscript{2+} holds at a high plateau to fully induce egg activation (Fig. 6). In eggs, a localized Ca\textsuperscript{2+} rise induces IP\textsubscript{3} production through an undefined signaling cascade (Nuccitelli et al., 1993; Larabell and Nuccitelli, 1992; Runft et al., 1999; Wagner et al., 2004). This induces Ca\textsuperscript{2+} release from stores and Ca\textsuperscript{2+} wave propagation (Fig. 4). Physically, IP\textsubscript{3} receptors cluster in eggs (Fig. 2A) (Kume et al., 1997; Parys et al., 1994), and this clustering is observed functionally in eggs as elementary Ca\textsuperscript{2+} release events with a larger spatial spread (Machaca, 2004). Furthermore, the sensitivity of the IP\textsubscript{3} receptor to IP\textsubscript{3} is greatly enhanced in eggs (Machaca, 2004; Terasaki et al., 2001). The self-sustained Ca\textsuperscript{2+}-induced IP\textsubscript{3} production in eggs coupled to the increased sensitivity and clustering of IP\textsubscript{3} receptors combine to produce a robust Ca\textsuperscript{2+} release signal. Our data show that IP\textsubscript{3} levels remain at high enough levels to gate the channel. This is likely to be the case. Resting IP\textsubscript{3} in Xenopus eggs is <50 nM and goes up to 400–600 nM at fertilization (Wagner et al., 2004; Stith et al., 1993), as an IP\textsubscript{3} wave precedes the Ca\textsuperscript{2+} wave at fertilization (Wagner et al., 2004). High IP\textsubscript{3} is maintained for ~5 min, the time required for the Ca\textsuperscript{2+} wave to sweep across the entire egg, and begins to fall after that time (Stith et al., 1993). Furthermore, IP\textsubscript{3} in Xenopus oocytes has a half-life of >5 min (Sims and Allbritton, 1998). If one assumes a similar IP\textsubscript{3} half-life in eggs, then IP\textsubscript{3} levels are likely to stay at >200 nM for 10–15 min, allowing gating of IP\textsubscript{3} receptors.

In addition, Ca\textsuperscript{2+} itself is an important modulator of IP\textsubscript{3} receptor function. Ca\textsuperscript{2+} is a required co-agonist to gate the IP\textsubscript{3} receptor, yet high cytosolic Ca\textsuperscript{2+} levels inhibit IP\textsubscript{3} receptor gating resulting in a bell-shaped Ca\textsuperscript{2+} dependence of IP\textsubscript{3} receptor gating (Finch et al., 1991; Bezprozvanny et al., 1991; Mak et al., 1998). Our data show that IP\textsubscript{3} receptors as a population are open in eggs despite high cytosolic Ca\textsuperscript{2+} levels, arguing that Ca\textsuperscript{2+}-dependent inhibition of the IP\textsubscript{3} receptors is somehow down-regulated during oocyte maturation. It is not possible to directly test this prediction by recording IP\textsubscript{3} receptor biophysical properties in eggs, because the nucleus breaks down during oocyte maturation thus preventing patch clamping studies. The Xenopus oocyte IP\textsubscript{3} receptor biophysical properties have been studied primarily by patch clamping on the large oocyte nucleus (germinal vesicle) (Mak and Foskett, 1997; Mak et al., 1998). Therefore, understanding the regulation of IP\textsubscript{3} receptor gating and permeation in eggs will require indirect approaches.

Nonetheless, the studies presented here show that in eggs Ca\textsubscript{Cyt} that is taken up into stores by SERCA leaks back into the cytosol through open IP\textsubscript{3} receptors, which maintains Ca\textsubscript{Cyt} levels high for several minutes using the limited free Ca\textsuperscript{2+} that is available in Ca\textsuperscript{2+} stores (Fig. 6).
Another important factor in the sustained Ca\(^{2+}\) rise in eggs is PMCA internalization during oocyte maturation. PMCA is removed from the cell membrane (Fig. 2C), and functionally PMCA does not modulate Ca\(^{2+}\) transients in eggs (Fig. 3). Therefore, the primary Ca\(^{2+}\) extrusion pathway in oocytes is inactivated during maturation, which contributes to maintaining cytosolic Ca\(^{2+}\) levels high for several minutes (Fig. 6).

In summary, Ca\(^{2+}\) signaling pathways differentiate in a highly orchestrated fashion during Xenopus oocyte maturation endowing the egg with the capacity to maintain Ca\(^{2+}\)cyt levels high for several minutes to induce egg activation and initiate development. Xenopus eggs are laid in the wild in pond water, which varies significantly in its ionic content. Therefore, the egg apparently relies exclusively on its intracellular Ca\(^{2+}\) handling machinery to produce the fertilization-specific sustained Ca\(^{2+}\) rise. The two most significant adaptations of the Ca\(^{2+}\) signaling machinery in eggs are the gating of IP\(_3\) receptors and PMCA internalization (Fig. 6).

IP\(_3\) receptor gating coupled to its increased sensitivity and clustering in eggs potentiates IP\(_3\)-dependent Ca\(^{2+}\) release from stores with two important consequences: it underlies the slow propagation of the Ca\(^{2+}\) wave (Machaca, 2004), and it allows continuous Ca\(^{2+}\) leak out of the ER (Fig. 6). This results in Ca\(^{2+}\) recycling between the ER lumen and cytosol, which sustains high Ca\(^{2+}\)cyt levels. Furthermore, PMCA internalization eliminates Ca\(^{2+}\) extrusion out of the egg, thus preserving released Ca\(^{2+}\) into the cytosol and contributing to the sustained Ca\(^{2+}\) rise. This differentiation of Ca\(^{2+}\) signaling pathways during oocyte maturation represents an ingenious solution to the difficult problem of maintaining Ca\(^{2+}\)cyt levels high for several minutes that the large Xenopus egg is faced with at fertilization. Together, these studies provide important insights into how oocytes regulate their Ca\(^{2+}\) signaling machinery in preparation for fertilization and embryogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.10.034.

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