Calcium signaling differentiation during *Xenopus* oocyte maturation

Wassim El-Jouni, Byungwoo Jang, Shirley Haun, Khaled Machaca *

Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, 4301 West Markham St. Slot 505, Little Rock, AR 72205, USA

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, *Xenopus* eggs produce a cytoplasmic Ca^{2+} (Ca^{2+}_{cyt}) rise 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+}_{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 *Xenopus* oocyte maturation endowing the egg with the capacity to produce a sustained Ca^{2+}_{cyt} transient at fertilization, which defines the egg's competence to activate and initiate embryonic development.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Oocyte maturation; *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 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 *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 *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; Lorca et al., 1993; Morin et al., 1994).

Fertilization of the *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 *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-

---

spermy (Wolf, 1974; Grey et al., 1976). On an even slower time scale, the Ca^{2+}-dependent activation of Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) leads to completion of meiosis II with the extrusion of the second polar body and entry into mitosis. CaMKII mediates the meiotic to mitotic transition by inactivating both maturation promoting factor (MPF) and cytostatic factor (CSF) (Lorca et al., 1993; Morin et al., 1994). MPF (cdk1) is the primary kinase that drives the G2/M transition of the cell cycle (Coleman and Dunphy, 1994), and CSF is an activity that maintains the metaphase II arrest (Tunquist and Maller, 2003).

Eggs acquire the potential to produce the specialized Ca^{2+} transient at fertilization following a cellular differentiation pathway referred to as 'oocyte maturation'. Fully grown vertebrate oocytes are arrested at the G2/M transition of the cell cycle and in response to the proper trigger (progesterone in the frog), enter meiosis and arrest at metaphase II until fertilization (Yamashita et al., 2000; Masui and Clarke, 1979). Following maturation, eggs become fertilization competent and able to initiate embryonic development. Therefore, differentiation of Ca^{2+} signaling during oocyte maturation is a fundamental component of preparing the egg for fertilization and embryogenesis. However, the mechanisms regulating Ca^{2+} signaling differentiation during oocyte maturation remain obscure.

In *Xenopus* at fertilization, Ca_{cyt}^{2+} 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_3R). 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 *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-depedent 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

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 (D-*myo*-inositol 1,4,5-triphosphate, $P_{4(5)}$-(1-(2-nitrophenyl)ethyl) ester, tris triethylammonium salt) (Molecular Probes) as indicated. Injections were performed in 110 mM NaCl, 1.3 mM KCl, 20 mM MgSO4, 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 MgCl2; 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 20× UPlanFl 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 400dclp beamsplitter (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°C. After fixation, the cells were embedded in tissue freezing media (Electron Microscopy Science) using Tissue Tek cryomold and frozen at −20°C overnight. Samples were sliced into 8 μm 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-PMCA antibody that recognizes all 4 PMCA isoforms (Affinity BioReagents), an anti-SERCA2 (NOVO CASTRA), an anti-Integrin monoclonal (8C8, Developmental Studies Hybridoma Bank), and an anti-type 1 IP_3R 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°C in 4.8 μg/ml WGA. Transmission and fluorescence images were collected on a Fluoview confocal (Olympus) coupled to a microscope