Developmental Biology 320 (2008) 426–435

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Actin cytoskeleton modulates calcium signaling during maturation of starfish oocytes

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ARTICLE INFO

Article history:
Received for publication 17 August 2007
Revised 23 May 2008
Accepted 27 May 2008
Available online 6 June 2008

Keywords:
Starfish
Oocyte
1-methyladenine
Actin
Cytoskeleton
$InsP_3$
Heparin
U73122
Cortical granule exocytosis
Meiosis

ABSTRACT

Before successful fertilization can occur, oocytes must undergo meiotic maturation. In starfish, this can be achieved *in vitro* by applying 1-methyladenine (1-MA). The immediate response to 1-MA is the fast Ca^{2+} release in the cell cortex. Here, we show that this Ca^{2+} wave always initiates in the vegetal hemisphere and propagates through the cortex, which is the space immediately under the plasma membrane. We have observed that alteration of the cortical actin cytoskeleton by latrunculin-A and jasplakinolide can potently affect the Ca^{2+} waves triggered by 1-MA. This indicates that the cortical actin cytoskeleton modulates Ca^{2+} release during meiotic maturation. The Ca^{2+} wave was inhibited by the classical antagonists of the $InsP_3$-linked Ca^{2+} signaling pathway, U73122 and heparin. To our surprise, however, these two inhibitors induced remarkable actin hyper-polymerization in the cell cortex, suggesting that their inhibitory effect on Ca^{2+} release may be attributed to the perturbation of the cortical actin cytoskeleton. In post-meiotic eggs, U73122 and jasplakinolide blocked the elevation of the vitelline layer by uncaged $InsP_3$, despite the massive release of Ca^{2+}, implying that exocytosis of the cortical granules requires not only a Ca^{2+} rise, but also regulation of the cortical actin cytoskeleton. Our results suggest that the cortical actin cytoskeleton of starfish oocytes plays critical roles both in generating Ca^{2+} signals and in regulating cortical granule exocytosis.

Introduction

During oogenesis, both the growth and the differentiation of starfish oocytes are arrested at first meiotic prophase (reviewed in Meijer and Guerrier 1984). To become capable of fertilization, oocytes of all animals must overcome the arrest and activate meiotic maturation. The availability of large numbers of synchronized oocytes makes the starfish an excellent experimental model system for studying meiotic maturation. Previous studies have shown that immature starfish oocytes exhibit polarized cell morphology. First, the large nucleus (the germinal vesicle, or GV) is located close to the plasma membrane of the animal hemisphere. The cytoskeletal organization of the animal pole is quite different from that of the other regions in that the F-actin layer is missing in the space between the GV and the plasma membrane. Indeed, it is through this "corridor" that the two polar bodies are extruded during the two reduction divisions which are the visual display of oocyte maturation. Second, the cortical region of the oocyte differs from the inner cytoplasm in that actin filaments are ordered in a cluster just beneath the plasma membrane forming a cortical cytoskeletal layer. In addition, the cortical region is crowded with numerous membrane-bound vesicles filled with stratified contents (Schroeder, 1985). Because of the presence of actin-binding proteins that polymerize or depolymerize actin filaments, the cortical actin cytoskeleton is in a dynamic equilibrium.

The hormone that induces maturation of starfish oocytes is 1-methyladenine (1-MA) (Kanatani et al., 1969). When oocytes are exposed to 1-MA, a series of cytological changes occur. Scanning EM and immunofluorescence microscopy have established that 1-MA immediately stimulates the transient appearance of prominent microvilli on the oocyte surface caused by the rapid assembly and disassembly of the filamentous actin bundles in their inner cores (Schroeder, 1981; Schroeder and Stricker, 1983; Otto and Schroeder, 1984). This is a fast response that occurs within 1 min after the addition of 1-MA. An equally rapid change in response to 1-MA is the quick release of intracellular Ca^{2+} (Moreau et al., 1978; Santella and Kyozuka 1994). Following these early events, 1-MA then induces more extensive reorganization of cytoplasmic actin network and drastic changes in the phosphorylation state of numerous proteins (Labbé et al., 1989; Masui 2001; Prigent and Hunt, 2004). In parallel, intracellular organelles such as the endoplasmic reticulum (ER) undergo structural changes (Jaffe and Terasaki, 1994; Terasaki, 1994). The final event of the maturation process is the breakdown of the nuclear envelope of the GV. Owing to these cytoplasmic changes, the

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doi:10.1016/j.ydbio.2008.05.549

post-meiotic eggs are ready for fertilization. The sperm-induced intracellular Ca^{2+} release can now occur more efficiently, and the Ca^{2+}-dependent exocytosis of cortical granules leads to the elevation of the vitelline layer and its transformation into the fertilization envelope, which aids in preventing polyspermy (Longo et al., 1995; Santella et al., 1999).

Because both the Ca^{2+} signals and the changes of cell surface actin fibers are induced immediately after the initiation of the maturation process, we were interested in studying the relationship between these two events. We have recently observed that rearrangements of the actin cytoskeleton by use of actin-depolymerizing agents, or actin-binding proteins, can strongly modulate Ca^{2+} signals during the maturation and fertilization processes (Lim et al., 2002, 2003; Nusco et al., 2006). In the present study, we have focused on the first event of the Ca^{2+} release that heralds the onset of meiotic maturation. Previously, we had shown that this Ca^{2+} signal starts at one point of the oocyte surface (Santella et al., 2003). We have now localized the initiation site of the Ca^{2+} release to the oocyte's vegetal hemisphere. Because the oocyte is uniformly exposed to 1-MA, it is remarkable that the Ca^{2+} signal invariably starts from only the vegetal pole. Interestingly, we have noted that the path of the Ca^{2+} wave propagation is mainly through the cortical layer and not through the center of the cytoplasmic mass. This observation, and the consideration of the asymmetric molecular organization of oocytes, led us to ask whether or not the cortical actin cytoskeleton plays an important role in mobilizing intracellular Ca^{2+}.

To address this question, we have investigated the spatiotemporal dynamics of the 1-MA-induced Ca^{2+} signal by simultaneously measuring the Ca^{2+} response in six oocytes exposed to the hormone for the same duration. To study how the Ca^{2+} signaling pattern is influenced by changes of the actin cytoskeleton, we used pharmacological agents that depolymerize or polymerize actin filaments, e.g., latrunculin-A (LAT-A) and jasplakinolide (JAS), respectively. These agents had strong inhibitory effects on the Ca^{2+} signals elicited by 1-MA, or by $InsP_3$-uncaging, indicating that the Ca^{2+} release process could be modulated by the cortical actin cytoskeleton. To our surprise, we observed that even the classical inhibitors of the $InsP_3$-dependent Ca^{2+} release pathway, U73122 and heparin, also induced a remarkable increase of cortical actin filaments, while they inhibited the 1-MA-linked Ca^{2+} response. Hence, the actin cytoskeleton is implicated in this inhibitory process. Finally, we have demonstrated that the exocytosis of cortical granules requires not only a massive release of Ca^{2+}, but also the normal architecture of the cortical cytoskeleton.

Materials and methods

Preparation of oocytes

Starfish (*A. pectinifera*) were captured in Mutzu Bay, Japan, during the breeding season (September) and transported to the *Stazione Zoologica* in Naples, Italy. Animals were maintained in circulating seawater at 16 °C. The gonads were dissected from the central dorsal area near the arms and transferred to cold, filter-sterilized seawater (FSW). Fully-grown immature oocytes were isolated as single cells by sieving through gauze several times in cold FSW. Nearly all the oocytes released were arrested at meiotic prophase I, as judged by the presence of the germinal vesicle. Free oocytes were isolated by repeated rinsing and gentle hand centrifugation in cold FSW. For fertilization experiments, immature oocytes were stimulated with 1 μM 1-MA in FSW for 1 h before being exposed to spermatozoa suspended in FSW.

Microinjection, photoactivation of caged compounds and Ca^{2+} imaging

Microinjection of oocytes was performed with an air-pressure Transjector (Eppendorf). Typically, the amount of injected material was estimated at 1–2% of the oocyte volume. Hence, the final concentration of the injected material inside the oocytes should have been 50 to 100-fold lower than the concentration in the injection pipette. The fluorescent calcium dye, Calcium Green 488, conjugated to 10 kDa dextran was purchased from Molecular Probes (Eugene, Oregon) and used in 5 mg/ml pipette concentration with the injection buffer (10 mM Hepes, pH 7.0, 100 mM potassium aspartate). The same injection buffer was used for delivering heparin (Sigma-Aldrich) and caged $InsP_3$ (Molecular Probes) by microinjection. Caged $InsP_3$ (5 μM pipette concentration) was co-injected with the fluorescent Ca^{2+} indicator into either immature oocytes or matured eggs. To activate the caged $InsP_3$, oocytes were irradiated with 330 nm UV light for 25 s with the use of a computer-controlled shutter system (Lambda 10-2, Sutter Instruments, Co., Novato, CA). Cytosolic Ca^{2+} changes were detected with a cooled CCD camera (MicroMax, Princeton Instruments, Inc., Trenton, NJ) mounted on a Zeiss Axiovert 200 microscope with a Plan-Neofluar 20×/0.50 objective. The quantified Ca^{2+} signal at a given time point was normalized to the baseline fluorescence (F_0) following the formula $F_{rel}=[F-F_0]/F_0$, where F represents the average fluorescence level of the entire oocyte. The equation $F_{inst}=[(F_t-F_{t-1})/F_{t-1}]$ was applied to analyze the incremental changes of the Ca^{2+} rise in order to visualize the site of instantaneous Ca^{2+} release. Fluorescent Ca^{2+} images were analyzed with the MetaMorph Imaging System software (Universal Imaging Corporation, West Chester, PA, USA). The incubation conditions of jasplakinolide (JAS), latrunculin-A (LAT-A), and U73122 are indicated in the figure legends. Unless specified otherwise, the control cells refer to the oocytes from the same batches that were treated with the same vehicle for drug delivery. While JAS, U73122, and LAT-A were dissolved in DMSO, heparin and caged $InsP_3$ were prepared in aqueous solution (injection buffer).

F-actin staining, laser-scanning confocal microscopy and transmission electron microscopy

F-actin was visualized by two methods, either with or without cell fixation. To visualize F-actin in living oocytes, the microinjection pipette was loaded with 50 μM Alexa Fluor 488-conjugated phalloidin in DMSO. Oocytes maintained in 16 °C FSW were microinjected with the phalloidin probe and visualized with confocal microscopy after 10 min incubation. To stain polymerized actin in fixed oocytes, we followed a method described previously (Strickland et al., 2004). Briefly, cells were incubated in FSW containing 3% paraformaldehyde for 30 min at room temperature. Oocytes were then resuspended in wash buffer (50 mM HEPES, pH 7.0, 50 mM PIPES, 600 mM mannitol, 3 mM $MgCl_2$ FSW) containing 0.1% Triton X-100. After rinsing a few times in wash buffer, oocytes were incubated with 3–10 U/ml of Alexa Fluor 488-conjugated phalloidin in PBT (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.1% Triton X-100, final pH 7.2) for 30 min. All steps were performed at room temperature. After staining, oocytes were transferred to an experimental chamber and were observed with an Olympus Fluoview 200 laser-scanning microscope with a 60× (1.20 NA) objective. Transmitted light and fluorescent confocal images were acquired from the equivalent cytoplasmic planes containing the GV. Images of F-actin stained with Alexa Fluor 488-conjugated phalloidin were recorded through a BP 510540 emission filter. For transmission electron microscopy, oocytes were first fixed in 1% glutaraldehyde in FSW (pH 8.0) for 1 h at room temperature and then rinsed extensively in FSW before being treated 1 h with FSW containing 1% osmium tetroxide. Specimens were dehydrated in increasing concentrations of alcohol and embedded in EPON 812. Sections were stained with 2% uranyl acetate and 0.2% lead citrate and examined with a LEO 912 AB energy filter transmission electron microscope.

Results

The 1-MA-induced Ca^{2+} signal initiates in the vegetal hemisphere and propagates through the cortical region

We have investigated the origin and the spatiotemporal characteristics of the Ca^{2+} increase induced by 1-MA. Immature oocytes isolated from *A. pectinifera* contain a large GV in the animal hemisphere (Fig. 1a). Approximately 2 min after the addition of 1-MA, the oocytes respond with a sharp rise of intracellular Ca^{2+} (Fig. 1b). The analyses of the relative fluorescence of the global Ca^{2+} signals indicated that the propagating Ca^{2+} wave reached its peak within 30 s ($[F-F0]/F0=0.7$, $n=70$). Further analyses of the spatiotemporal distribution of intracellular Ca^{2+} showed that the Ca^{2+} wave always originated from the vegetal hemisphere and propagated toward the animal pole in the form of a crescent (Fig. 1c). At the later stage, the Ca^{2+} signals appear in the GV (Santella and Kyozuka 1994; Santella et al., 1998; Supplementary data 1). The relative fluorescence images of the Ca^{2+} signals presented in this manner leave the impression that the slightly concave Ca^{2+} front was propagating into the central cytoplasm. However, when we applied the equation $F_{inst}=[(F_t-F_{t-1})/F_{t-1}]$ to analyze the incremental changes of the regional Ca^{2+} rise, and visualized the site of instantaneous Ca^{2+} release, we observed that the sites of the maximal changes of Ca^{2+} were always restricted to the cortex of the oocyte. As shown in Fig. 1d, the focal sites of the most active Ca^{2+} release did not move centripetally, but progressively moved to the opposite pole along the cortex. There was only a residual Ca^{2+} change occurring in the inner cytoplasm, suggesting the