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Actin cytoskeleton modulates calcium signaling during maturation of starfish oocytes

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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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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

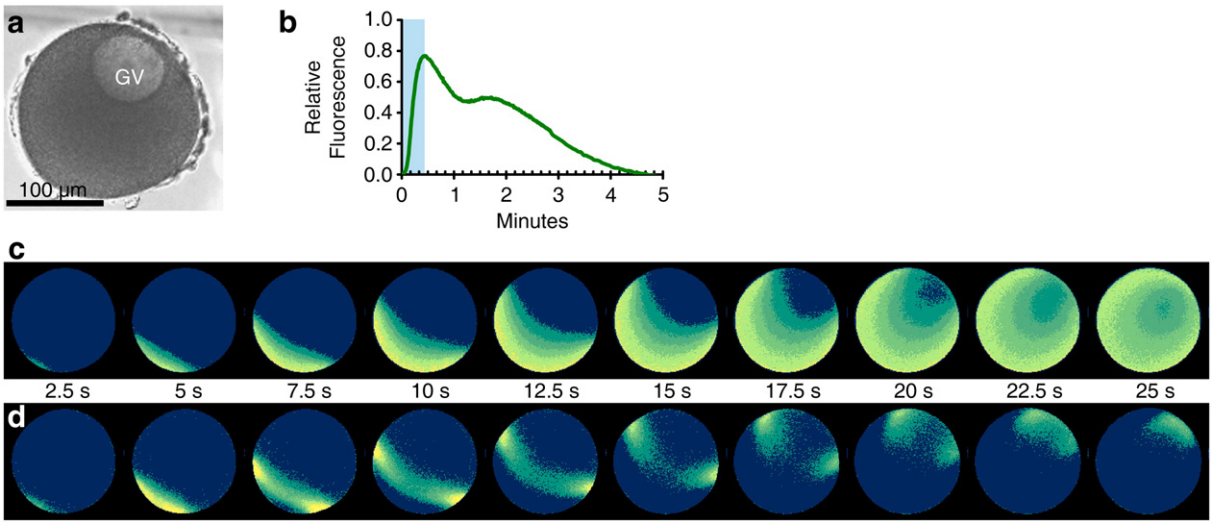

Fig. 1. Mobilization of intracellular Ca^{2+} by 1-MA. (a) A light photomicrograph of an immature *A. pectinifera* oocyte. The germinal vesicle (GV) is located in the animal hemisphere of the oocytes. (b) Quantification of intracellular Ca^{2+} levels induced by 1-MA. Within 1–2 min after the addition of 1-MA, a sharp Ca^{2+} transient was observed. The moment of the first detectable Ca^{2+} spike was taken as $t=0$. The area in blue shade depicts the initial phase of the Ca^{2+} increase, which was further analyzed by a CCD camera in panels c and d. (c) The relative fluorescence pseudo-colored images of the Ca^{2+} indicator. The first Ca^{2+} signal always starts in the vegetal pole. (d) Presentation of the same data following the differential formula (see Materials and methods) to visualize the incremental changes of instantaneous Ca^{2+} release.

existence of a fluorescence gradient from the cortex to the inner cytoplasm. This conclusion was also confirmed by similar analyses with confocal microscopy (Supplementary data 1). Our observation of the cortical propagation of Ca^{2+} signals agrees with the previous findings of others showing that 1-MA can trigger Ca^{2+} release in isolated cortices (Dorée et al., 1978; Meijer and Guerrier 1984). These data suggest that the cortex of the starfish oocyte possesses the capability of storing and releasing Ca^{2+} in response to 1-MA.

Is the 1-MA-induced Ca^{2+} response mediated by an $InsP_3$-dependent pathway?

$InsP_3$ is arguably the most extensively characterized Ca^{2+}-mobilizing second messenger. The 1-MA-induced maturation of starfish oocytes is mediated by the activation of G-protein-linked receptors (Jaffe et al., 1993; Chiba and Hoshi, 1995), which may be linked to the activation of phospholipase C (PLC) that produces $InsP_3$ (reviewed in Berridge, 2007). Thus, we have tested if the inhibitors of $InsP_3$-dependent Ca^{2+} signaling pathways affect the 1-MA-evoked Ca^{2+} response. We treated immature oocytes with the phosphatidylinositol-specific PLC-inhibitor U73122 for 30 min (10 μM final concentration). The results showed that U73122 completely abolished the cortical Ca^{2+} increase induced by 1-MA (Fig. 2a, $n=4$). Likewise, the pre-injection of the oocytes with heparin, the antagonist of $InsP_3$ receptors (pipette concentration 25 to 50 μg/μl), completely inhibited the Ca^{2+} response (Fig. 2b, $n=8$). These results suggest that the $InsP_3$-dependent pathway is likely to mediate the 1-MA-induced Ca^{2+} signaling, assuming that U73122 and heparin respectively block PLC and $InsP_3$ receptors without non-specific side effects.

Considering that $InsP_3$ is soluble and diffusing freely in the cell, the results reported in Fig. 1, showing the Ca^{2+} signals always initiate in the vegetal hemisphere, require that a Ca^{2+} store more sensitive to $InsP_3$ should be present in the vegetal cortex. However, the uncaging of pre-injected $InsP_3$ produced a higher Ca^{2+} response in the *animal* hemisphere (Lim et al., 2003). Although it cannot be excluded that the downstream effects of 1-MA may be polarized in the oocyte to produce disproportionally larger amounts of $InsP_3$ in the vegetal pole, our observation that the 1-MA-induced Ca^{2+} release starts in the vegetal cortex, which is less sensitive to $InsP_3$, raises the possibility that factors other than $InsP_3$ may be involved in the generation of Ca^{2+} signals in response to 1-MA.

Heparin, the conventional antagonist of $InsP_3$ receptors, not only inhibits 1-MA-triggered Ca^{2+} release, but also dramatically changes the cortical actin cytoskeleton

Microinjection of starfish oocytes with heparin, the widely used antagonist of $InsP_3$ receptors, completely abolished 1-MA-induced Ca^{2+} signaling (Fig. 2b). However, we noticed that pre-injection of 25–50 μg/μl heparin strongly affected the cytoplasmic structure of oocytes. In a few oocytes, heparin injection caused irregular shapes and less transparency of the cytoplasm (Fig. 3b), while the immature oocytes injected with only the Ca^{2+} indicator did not show such changes (Fig. 3a). These morphological changes do not appear to result from the potential toxicity of heparin, as these cells were viable and physiologically functional. The cells still responded to uncaged $InsP_3$ with Ca^{2+} release and may also undergo GVBD, the hallmark of meiotic maturation (not shown). Further analyses of the heparin-microinjected oocytes with the subsequent injection of Alexa Fluor 488-conjugated phalloidin revealed that the cortical actin cytoskeleton was remarkably reorganized (Figs. 3c and d). Assessed with the same sensitivity settings of the confocal microscopy, the heparin-microinjected oocytes exhibited more intense fluorescence labeling in the cortex, reflecting a significant increase of F-actin in comparison to the control. The effect of heparin was so drastic that the phalloidin-labeling of cortical F-actin filaments even reached a saturation point when visualized with the same photomultiplier (PMT) and gain

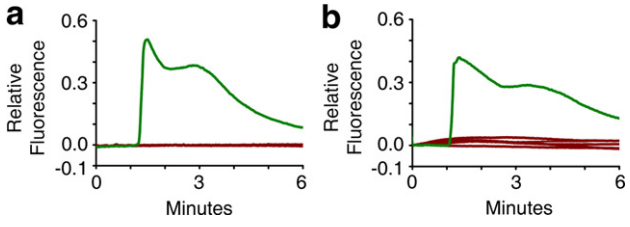

Fig. 2. Inhibitory effects of U73122 and heparin on 1-MA-induced Ca^{2+} signaling. (a) The green curve depicts Ca^{2+} signals in the control oocytes in 1-MA. The inhibitor of PLC (U73122) completely abolishes the 1-MA-induced Ca^{2+} rise ($n=4$, brown curves). (b) The 1-MA-induced Ca^{2+} signal is blocked by heparin, a conventional inhibitor of $InsP_3$ receptors ($n=8$, brown curves). While U73122 was delivered by bath application (10 μM, 30 min), heparin was microinjected into the oocytes (pipette concentration, 50 mg/ml).

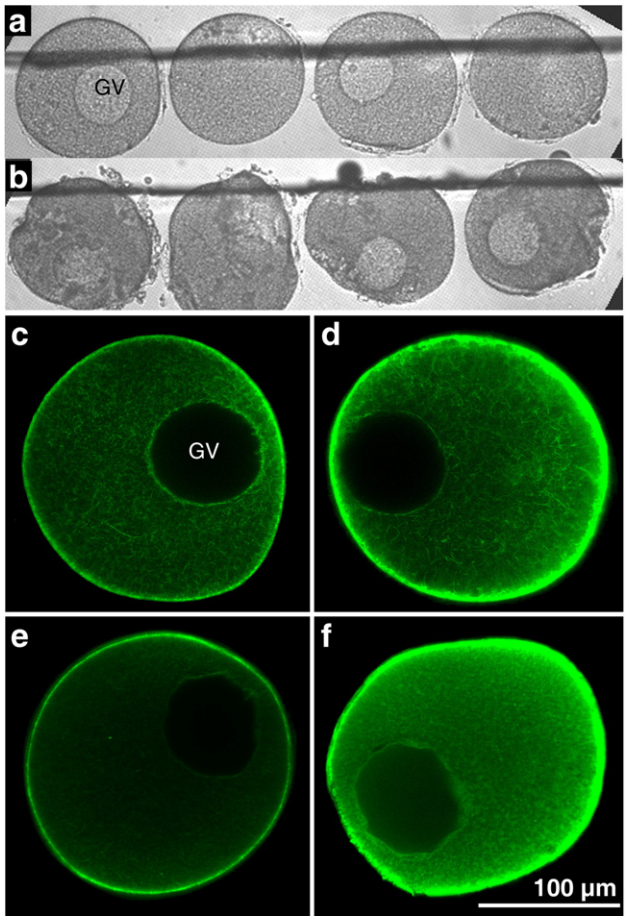

Fig. 3. Additional effects of heparin. Microinjection of heparin induces morphological changes. Light micrographs of the control (a) and the heparin-injected oocytes (b) mounted on cover slips. Heparin-injected oocytes exhibit more opaque cytoplasm and irregular cell surfaces. F-actin was visualized by direct microinjection of live oocytes with Alexa Fluor 488-conjugated phalloidin with (d) or without (c) pre-injection of heparin. Incubation of the heparin-pre-injected oocytes produced a remarkable increase of cortical actin filaments (d). Visualization of F-actin with Alexa Fluor 488-conjugated phalloidin after the fixation of oocytes with (f) or without (e) heparin pre-injection. Although the F-actin staining patterns inside the inner cytoplasm are slightly different in the two staining methods, the remarkable enhancement of the cortical actin staining after heparin pre-injection are the same (d and f). Hence, the increase of cortical actin filaments is not due to the actin-stabilizing effect of phalloidin.

settings that were optimized to show the detailed images of the cytoplasmic actin fibers in control oocytes. This dramatic change of the cortical actin cytoskeleton was not caused by the actin-stabilizing effect of phalloidin itself, which has been reported in several types of cells (Wehland et al., 1977). Heparin increased the recruitment of actin filaments in the cortex to the same extent even when phalloidin was added after the fixation of the oocytes with paraformaldehyde (Figs. 3e and f). Furthermore, the oocytes microinjected with heparin in the absence of phalloidin consistently produced remarkable ultrastructural changes in the cortex. In the cortex of the control oocytes, cortical granules and other vesicles were positioned immediately below the plasma membrane, which was covered with microvilli (Fig. 4a). In contrast, the ultrastructure of the heparin-injected oocytes sectioned at the equivalent plane showed that the cortical vesicles were pushed away from the plasma membrane and moved toward the inner cytoplasm (Fig. 4b, double arrows). Thus, the enhanced staining of the cortical regions by fluorescent phalloidin after heparin injection (Fig. 3d) represents structural changes of the cortical actin cytoskeleton. This drastic rearrangement of the cortical actin cytoskeleton was also reflected in alterations of microvilli, the finger-like extensions of plasma membrane that contain actin filaments. As shown in Fig. 4b (single arrows), heparin treatment led to the retraction of microvilli. Hence, heparin apparently produced two opposite effects in the cortical microdomains: enhanced recruitment of actin filaments in the sub-membraneous region and shortening of microvilli on the cell surface. Because the inhibitory effect of heparin on $InsP_3$ receptors is thus inseparable from the accompanying changes in the actin cytoskeleton, it should be noted that the use of heparin in the living cell as a specific antagonist of $InsP_3$ receptors calls for caution in interpreting such experimental data.

1-MA acts on the cortical Ca^{2+} store that is sensitive to the agents inducing polymerization and depolymerization of actin filaments

The striking reorganization of the cortical region by heparin raises the possibility that the cortical actin cytoskeleton is implicated in 1-MA-induced Ca^{2+} signaling. To test this, we examined the effect of the actin-remodeling drug LAT-A on the onset of Ca^{2+} mobilization. LAT-A induces actin depolymerization in living cells by sequestering monomeric G-actin (Spector et al., 1989). The treatment of oocytes with 0.5 μM LAT-A delayed the Ca^{2+} response to 1-MA (Fig. 5a). In addition, the magnitude of the Ca^{2+} signal was progressively reduced as the incubation time for LAT-A was prolonged. In comparison with the control oocytes displaying the height of the Ca^{2+} peak at 0.56 arbitrary units (green line, $n=16$), the oocytes pre-incubated with LAT-A for 10–15 min exhibited greatly reduced Ca^{2+} peaks, recording 0.27 (brown line, $n=16$) and 0.07 (red line, $n=16$) arbitrary units, respectively. Because the cortical actin layer undergoes remodeling after LAT-A incubation (see below), these results suggest that the 1-MA-induced Ca^{2+} release may be linked to the cortical actin cytoskeleton.

To gain further insights into the role of the cortical actin organization in the regulation of the 1-MA-induced Ca^{2+} signal, we examined the effect of the actin-stabilizing drug, jasplakinolide (JAS).

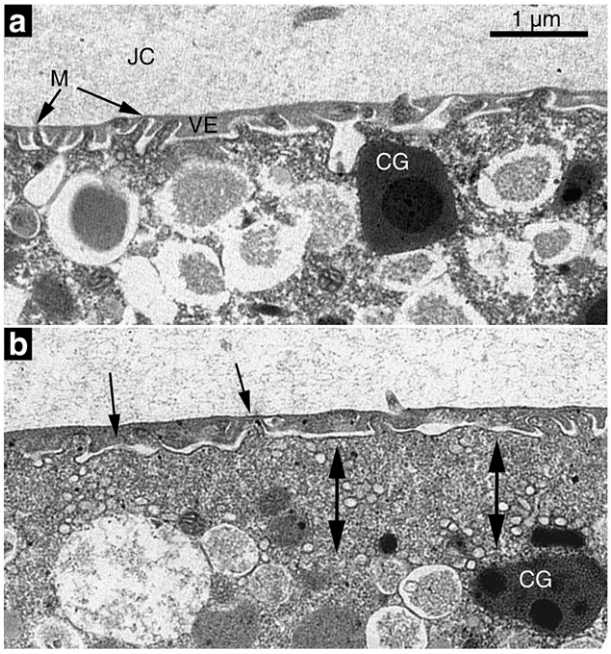

Fig. 4. Ultrastructural analyses of the control (a) and the heparin-injected (b) oocytes by transmission electron microscopy. Within the plane of the section is the area directly under the plasma membrane of the oocyte. The two sections were taken from the equivalent planes, and the scale bar refers to both pictures. In parallel with the changes in the cortical actin cytoskeleton and the blockade of the intracellular Ca^{2+} release, microinjected heparin also caused retraction of the microvilli (single arrows) and dislocated the cortical granules and other vesicles away from the plasma membrane (double arrows in panel b). Abbreviations: JC, jelly coat; CG, cortical granule; VE, vitelline envelope; M, microvilli.

JAS rearranges the actin cytoskeleton by inducing actin polymerization while inhibiting depolymerization of actin filaments *in vivo* (Bubb et al., 2000). We found that pre-treatment of oocytes with JAS inhibited the 1-MA-induced Ca^{2+} release in a dose-dependent manner. As shown in Fig. 5b, the percentage of the oocytes responding to 1-MA was sharply reduced from the 92% of the control cells to 25 and 0% of the oocytes pre-treated with 6 and 12 μM of JAS, respectively. These results indicate that the maintenance of the proper organization of the cortical actin cytoskeleton is crucial for creating physiologically normal 1-MA-induced Ca^{2+} signals.

LAT-A, an actin-depolymerizing agent, produces dual effects on Ca^{2+} release from the $InsP_3$-sensitive stores depending on the subcellular region

As mentioned above, the treatment of oocytes with LAT-A abolishes the 1-MA-induced Ca^{2+} release (Fig. 5a). Even in this condition, however, the basic mechanism to release Ca^{2+} in response to $InsP_3$ is still functional in the inner cytoplasm. In the control oocytes without LAT-A treatment, photo-release of the caged $InsP_3$ first produced Ca^{2+} signals in the cortical regions. The Ca^{2+} wave then spread toward the center of the cytoplasm (Fig. 5c upper panel). Thus, it appears that Ca^{2+} stores are more sensitive to $InsP_3$ at the cortex than in the inner cytoplasm. In contrast, in the oocytes pre-treated with 3 μM LAT-A for 10 min, photo-release of caged $InsP_3$ produced a Ca^{2+} response, but without the characteristic cortical Ca^{2+} release seen in the control cells (Fig. 5c lower panel). Instead, in the inner cytoplasm, LAT-A treatment caused slightly faster Ca^{2+} release than in the controls (Fig. 5d). Hence, LAT-A produced two opposite effects in the cortex and inner cytoplasm with respect to the $InsP_3$-dependent Ca^{2+} release. It remains unknown how LAT-A causes these subtle changes in the kinetics and the spatial patterns of the $InsP_3$-induced Ca^{2+} rise. Because the primary candidate that mediates the effect of uncaged $InsP_3$ is the $InsP_3$ receptor in the membrane of the ER, the changes in the kinetics of the Ca^{2+} rise induced by LAT-A may also be related to the rearrangement of the ER. Although such an event is known to occur in the oocytes of many animal species during meiotic maturation (Stricker, 2006), we did not observe any changes in the DiC18-stained ER following the same treatment with LAT-A (not shown). While the ER of the inner cytoplasm may still undergo subtle remodeling, which may account for the slightly faster release of Ca^{2+}, the efficacy of the Ca^{2+} channels such as the $InsP_3$ receptor might also be modulated by LAT-A through equally subtle microenvironmental changes of the actin cytoskeleton. In summary, these results suggest that the actin-depolymerizing drug, LAT-A, causes different Ca^{2+} responses in the cortex compared to the inner cytoplasm.

The modulation of the Ca^{2+} response by LAT-A, JAS, and U73122 treatment is mirrored by structural reorganization of the cortical actin cytoskeleton

To elucidate the mechanism underlying the actin cytoskeleton-regulated Ca^{2+} release, we next examined the distribution and structure of F-actin filaments in the oocyte cortex. The actin cytoskeleton of starfish oocytes has been usually visualized in fixed cells or isolated cortices (Schroeder and Stricker, 1983; Otto and Schroeder, 1984; Ookata et al., 1992; Heil-Chapdelaine and Otto, 1996). However, the phalloidin-stained images of fixed cells usually fail to reveal finer filamentous actins in the inner cytoplasm. To circumvent this problem, we have developed a microinjection method to visualize actin filaments in living oocytes. An additional advantage of this method is the opportunity to observe the dynamic changes of the actin cytoskeleton in real-time. Within a few minutes, Alexa Fluor 488-conjugated phalloidin clearly showed that the cortex and the inner cytoplasm are organized differently in terms of actin filaments (Fig. 6c). In the post-fixation method, however, the actin filaments were visualized equally well in the cortical area, but not in the inner cytoplasm (Fig. 6a). The improved detection of actin filaments in the inner cytoplasm by the microinjection method might result from better access of the probes to the targets in the living oocytes.

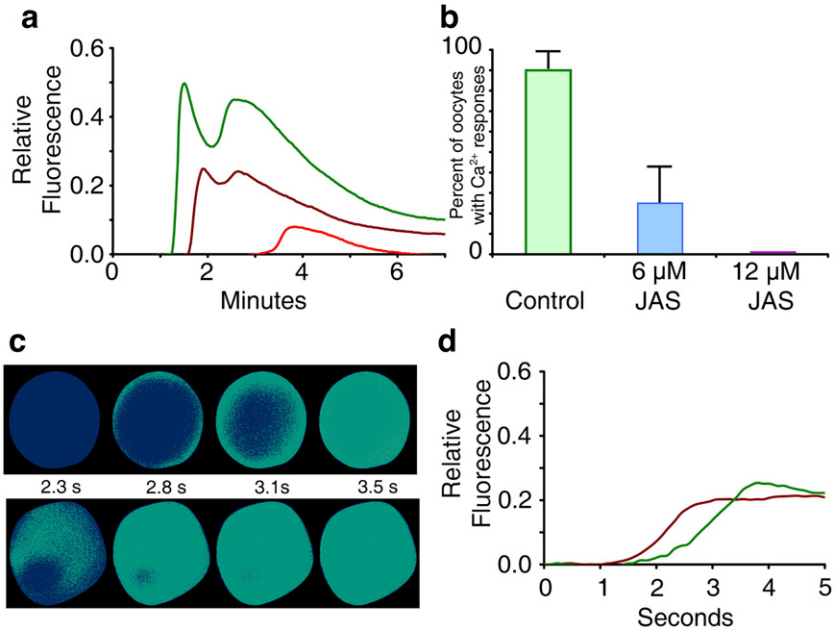

Fig. 5. (a) The actin-depolymerizing agent LAT-A inhibits 1-MA-induced Ca^{2+} signaling in a time-dependent manner. Color codes: green (control), brown (0.5 μM LAT-A for 10 min), and red (0.5 μM LAT-A for 15 min). (b) JAS inhibits the 1-MA-induced Ca^{2+} signaling in a dose-dependent manner. In the absence of JAS, 92% of cells responded to 1-MA with Ca^{2+} release, but the frequency of the cells responding to 1-MA stimulation was reduced to merely 25 and 0% in the presence of 6 or 12 μM JAS, respectively. The histogram and the error bars represent the averaged values and standard deviations of the data pooled from four independent experiments representing four different animals (n=6 in each case). (c and d) The LAT-A treatment of oocytes influences the pattern of Ca^{2+} mobilization by $InsP_3$ uncaging (12 s). The control (c, upper panel) and LAT-A-treated oocytes (c, lower panel) were simultaneously irradiated to liberate caged $InsP_3$. In LAT-A-treated cells, Ca^{2+} is released earlier than in the control cells, but the characteristic Ca^{2+} movement in the cortical area is not evident. The results of the same experiments were presented in a graph (d). Color codes: green (control) and brown (LAT-A).

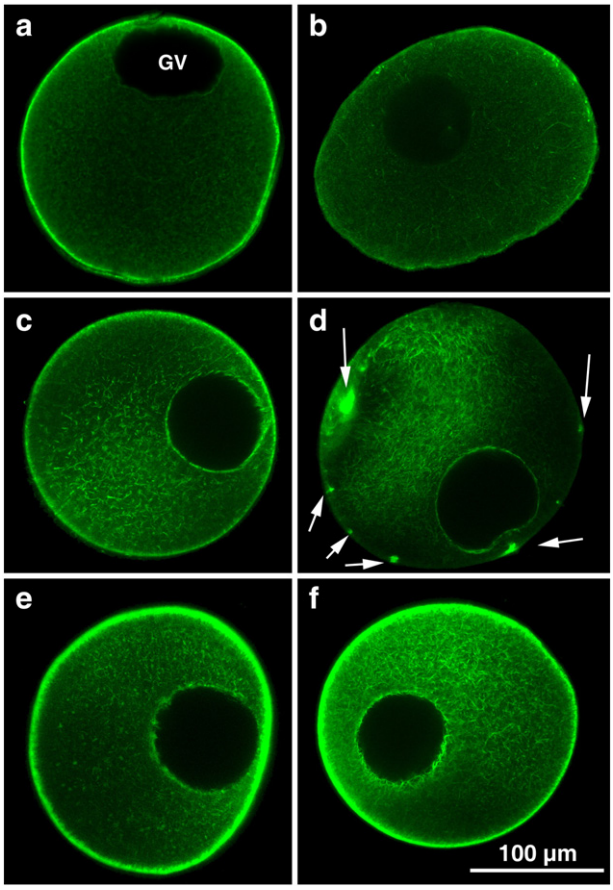

Fig. 6. The cortical actin cytoskeleton is drastically remodeled by LAT-A, JAS, and U73122. Actin filaments were visualized with Alexa Fluor 488-conjugated phalloidin in confocal microscopy in either paraformaldehyde-prefixed (a and b) or live (c-f) starfish oocytes. Compared with the control (a), the oocytes pre-incubated with LAT-A (3 μM, 15 min) prior to cell fixation displayed the disassembly of the cortical actin layer (b). Likewise, the same LAT-A treatment produced comparable results in phalloidin-injected live cells. The distinct cortical actin layer evident in the control cells (c) was disassembled by LAT-A (d). Arrows indicate tentative clusters of phalloidin-stained structures en route to depolymerization. In comparison with the control (c), incubation of live oocytes with JAS (12 μM, 10 min) greatly increased phalloidin-labeled actin filaments in the cortex (e). Incubation of live oocytes with U73122 (10 μM, 30 min) highly enhanced phalloidin-labeling of the actin filaments both in the cortex and in the inner cytoplasm (f). All images were taken from the equivalent confocal plane of the oocytes. GV, germinal vesicle.

Alternatively, phalloidin itself might have stabilized and thereby increased the amount of F-actin. Although it is difficult to distinguish between these two possibilities, phalloidin-induced actin polymerization is known to take amorphous shapes and require more time (Wehland et al., 1977). It is worth emphasizing that the site of our interest is in the cortical region where the effect of phalloidin on the actin cytoskeleton is minimal or absent. Furthermore, even in the presence of microinjected phalloidin, live oocytes underwent normal maturation in response to 1-MA (not shown) implying that the phalloidin probe does not interfere with meiotic maturation.

To determine if the inhibition of the 1-MA-induced Ca^{2+} release by LAT-A, U73122, and JAS is related to local changes in the cortical actin network, we stained the actin filaments with Alexa Fluor 488-conjugated phalloidin. In the post-fixation method (Figs. 6a and b), LAT-A treatment (3 μM for 15 min) significantly depolymerized cortical actin filaments. Likewise, the same treatment with LAT-A produced similar disassembly of the cortical actin filaments in live oocytes (Figs. 6c and d). Curiously, we have noted that the LAT-A-disassembled cortical actin filaments pass through an intermediate stage where short phalloidin-stained actin filaments display an aggregated form (Fig. 6d, arrows). However, within 30 min the aggregates disappeared completely from the cortex (not shown). In agreement with the dose-dependent inhibition of the 1-MA-induced Ca^{2+} signaling by JAS (Fig. 5b), we have observed that treatment with the same concentration of JAS produced the formation of many more cortical actin filaments (Fig. 6e). Finally, the U73122 treatment, which completely blocked 1-MA-induced Ca^{2+} signals (Fig. 2a), resulted in a remarkable increase of actin filaments in the cortex (Fig. 6f). To our surprise, the effect of the PLC-inhibitor U73122 was almost identical to that of JAS in altering the structure of the cortical actin cytoskeleton (Figs. 6e and f), except that U73122 produced an additional increase of actin filaments in the inner cytoplasm. Taken together with the data indicating the inhibitory effects of these drugs on Ca^{2+} signaling (Figs. 2 and 5), the morphological data demonstrate that the cortical actin

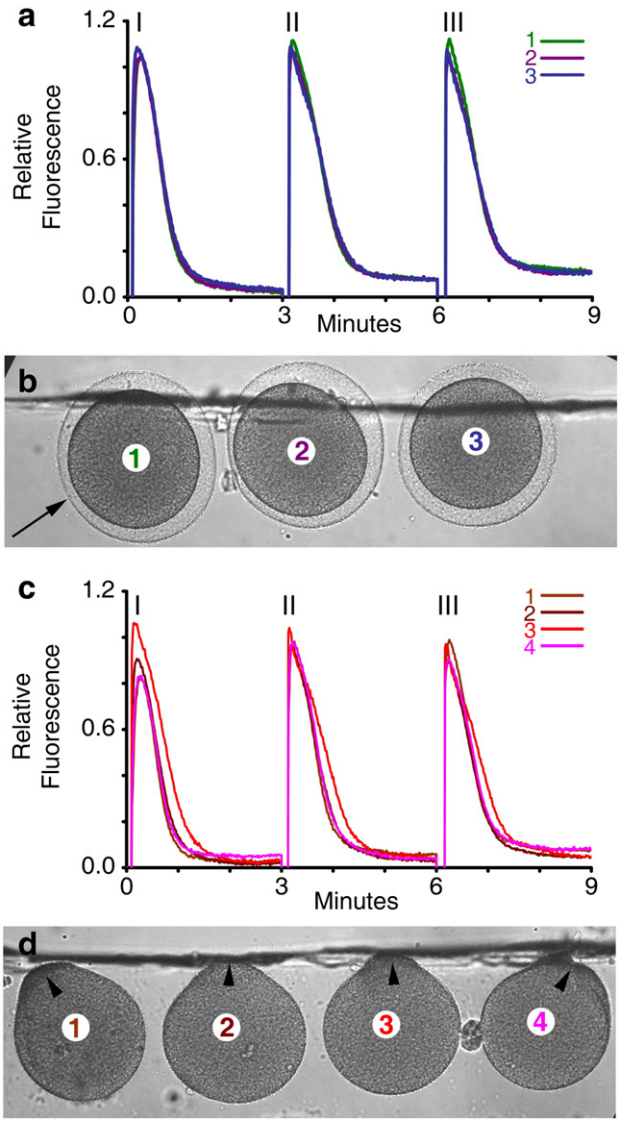

Fig. 7. Treatment of starfish eggs with U73122 does not affect the $InsP_3$-dependent Ca^{2+} release, but blocks exocytosis of cortical granules. Immature oocytes were co-injected with the Ca^{2+} dye and caged $InsP_3$ and then exposed to 1-MA for 1 h. (a) Ca^{2+} was released in response to three repetitive uncagings of $InsP_3$ in the control cells. (b) Elevation of the vitelline layer (arrow) as a result of cortical granule exocytosis at the end of the first uncaging of $InsP_3$. Results in three numbered cells were represented in green, brown, and blue curves. (c and d) Four numbered cells incubated with U73122 (10 μM, 30 min) produced comparable Ca^{2+} release in response to $InsP_3$ uncaging (c), but the elevation of vitelline layer was blocked (d) even in the presence of massive Ca^{2+} release. The changes of cell morphology by U73122 were also evident in one side of the cell surface (arrowheads).

cytoskeleton is implicated in the modulation of 1-MA-induced Ca^{2+} signaling.

The cortical actin cytoskeleton plays an important role in regulating exocytosis of cortical granules

It has been known that the massive exocytosis of egg cortical granules is triggered by Ca^{2+} signals (Vacquier, 1975). In the early studies, the involvement of the cytoskeleton in this process was controversial. Based on the lack of evidence that cytochalasin, colchicine, and other inhibitors of cytoskeletal function influenced the Ca^{2+}-dependent exocytosis in the isolated cortex of sea urchin eggs, it was concluded that participation of actin and tubulin was not likely (Whitaker and Baker, 1983). However, studies on pancreatic acinar cells (Muallem et al., 1995) and neuroendocrine cells (Vitale et al., 1991; Trifarò et al., 1992; Gasman et al., 2004; Malacombe et al., 2006) indicated that remodeling of the cortical actin cytoskeleton is crucial to exocytosis of vesicles. Here, we show that actin polymerization in the cortex has a striking effect on the progress of cortical granule exocytosis in starfish oocytes.

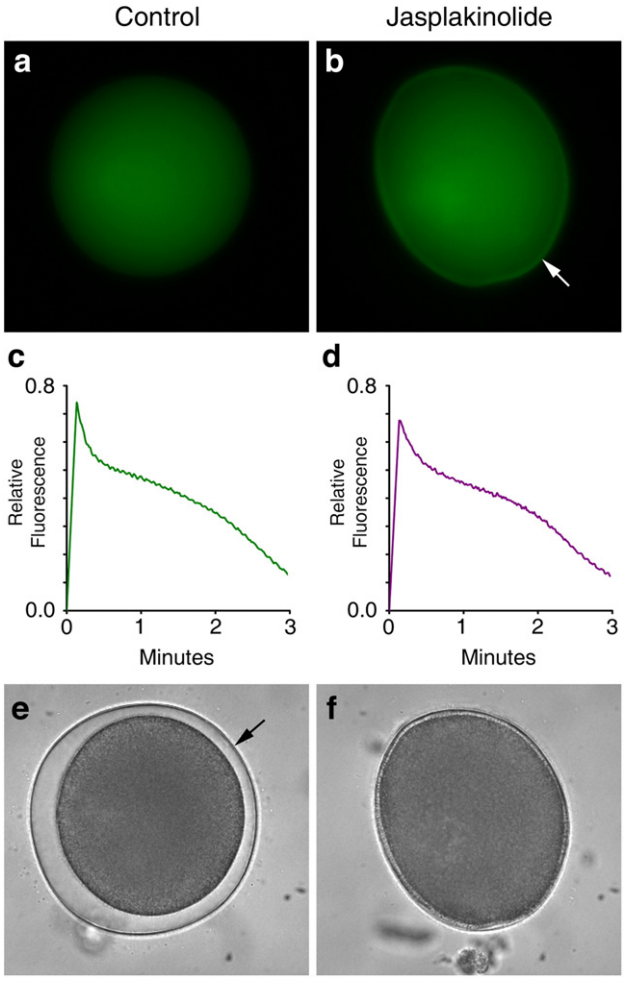

Fig. 8. The actin-polymerizing agent JAS does not affect the $InsP_3$-dependent Ca^{2+} release but blocks exocytosis of cortical granules. Immature oocytes were co-injected with the Ca^{2+} dye and caged $InsP_3$ and then exposed to 1-MA for 1 h. Matured eggs were photoactivated to uncage $InsP_3$ in the presence or absence of JAS (12 μM in the media for 10 min). (a and b) Images of the matured eggs loaded with the fluorescent Ca^{2+} dye before $InsP_3$ uncaging. Note the elevated distribution of Ca^{2+} dyes in the cortex of the JAS-treated egg (an arrow in panel b), presumably reflecting considerable affinity of Ca^{2+} to the enhanced cortical actin filaments, or other structural elements. (c and d) Intracellular Ca^{2+} releases in response to $InsP_3$ uncaging in the control egg (c) and in the JAS-incubated egg (d). (e and f) Even though uncaged $InsP_3$ instantly released Ca^{2+} in both eggs, the JAS-treated cell failed to show the elevation of the fertilization envelope (f) that is evident in the control cell (e, indicated with an arrow).

It has been demonstrated that a massive Ca^{2+} release in response to $InsP_3$ results in elevation of the vitelline layer in starfish oocytes (Lim et al., 2003). As expected, the control eggs responded to three-repeated $InsP_3$ uncagings by showing three sharp rises of intracellular Ca^{2+} (Fig. 7a). Immediately after the first Ca^{2+} transient, the cell responded with the elevation of vitelline layer as a result of cortical granule exocytosis (Fig. 7b, arrow). In the presence of U73122, which increased cortical F-actin (Fig. 6f), the Ca^{2+} transient in response to the first $InsP_3$ uncaging was slightly lower than in the control. However, these cells responded to the following $InsP_3$ uncaging by showing the same magnitude of Ca^{2+} signals as in the control (Fig. 7c). The main difference between the control and the U73122-incubated cells was in the fact that the elevation of the vitelline envelope was totally blocked in the U73122-treated cells (Fig. 7d). Hence, despite the massive release of intracellular Ca^{2+}, U73122 could still block the exocytosis of cortical granules. Supporting the idea that the blockade of cortical granule exocytosis by U73122 is linked to the enhancement of the cortical actin layer, another pharmacological agent inducing cortical actin polymerization, JAS, also produced basically the same results in independent experiments (Fig. 8).

Discussion

The 1-MA-induced maturation of starfish oocytes is first signaled by the immediate increase of intracellular Ca^{2+} (Moreau et al., 1978; Santella and Kyozuka, 1994; Santella et al., 2003). The initial Ca^{2+} transient may trigger a series of Ca^{2+}-dependent events to initiate the changes driving meiosis. In fact, the Ca^{2+} chelators blocking 1-MA-induced Ca^{2+} signals also block the normal maturation process (Santella and Kyozuka, 1994; Santella et al., 2005). Here, we have shown that this quick Ca^{2+} transient always initiates in the vegetal hemisphere and propagates along the cortex to reach the animal pole (Fig. 1d). We have also demonstrated that the 1-MA-induced Ca^{2+} release is restricted to the oocyte's cortex, which is defined as the first 1–5 μm of cytoplasm under the plasma membrane (Vacquier, 1981). The finding that the propagating Ca^{2+} release is mainly in the cortex, and not the inner cytoplasm, suggests the existence of region-specific and structure-based intracellular Ca^{2+} mobilization mechanisms. The 1-MA-induced Ca^{2+} signaling is not caused by Ca^{2+} entry from seawater, because the hormone can evoke Ca^{2+} signals in the absence of extracellular Ca^{2+} (Meijer and Guerrier, 1984). Thus, the downstream effect of 1-MA on Ca^{2+} release must be mediated by an intracellular mechanism. The question is how Ca^{2+} is released in a spatially restricted manner just beneath the oocyte's plasma membrane.

The major intracellular Ca^{2+} store, the oocyte ER, undergoes extensive restructuring during meiotic maturation, which may partially explain the increased sensitivity of Ca^{2+} release from the $InsP_3$-dependent stores in starfish oocytes (Chiba et al., 1990) and oocytes of other animals (Stricker, 2006). The fact that the fast initial Ca^{2+} transient is generated even before any significant structural changes take place in the ER, while the cortical actin cytoskeleton already undergoes rearrangement in response to 1-MA (Schroeder and Stricker, 1983), provided us with an opportunity to study the role of the actin cytoskeleton in regulating Ca^{2+} release in the cortex.

It is generally accepted that $InsP_3$ receptor is the major channel for Ca^{2+} release from the ER stores. According to the studies in *Xenopus* (Kume et al., 1993) and the hamster (Shiraishi et al., 1995), $InsP_3$ receptors are concentrated in the animal pole of immature oocytes where the GV is located. Furthermore, functional analyses of starfish oocytes with uncaged $InsP_3$ have demonstrated that the Ca^{2+} store at the animal pole is more sensitive to $InsP_3$ (Lim et al., 2003), although $InsP_3$ receptors are evenly distributed throughout the cytoplasm (Iwasaki et al., 2002). Hence, if $InsP_3$ receptors were the major source

of intracellular Ca^{2+}-mobilization, it was expected that the 1-MA-induced Ca^{2+} response would start at the animal hemisphere. Instead, we found that addition of 1-MA always elicited the Ca^{2+} transient starting from the vegetal hemisphere. Thus, our results imply that some other important factors besides $InsP_3$ may play critical roles in regulating intracellular Ca^{2+} release at this early stage of 1-MA induction.

In the course of revaluating the relevance of $InsP_3$ to 1-MA-induced Ca^{2+} mobilization, we made some novel observations. The biggest surprise came from the finding that heparin produced a very pronounced remodeling of the cortical actin network and other conspicuous changes in the ultrastructure of the subplasmalemmal region (Figs. 3 and 4). Hence, the heparin blockade of the 1-MA-induced Ca^{2+} release cannot be solely attributed to pharmacological effects on $InsP_3$ receptors. It is worth noting that the alteration of the actin cytoskeleton might be directly involved in this phenomenon. Further evidence is the observation that the inhibitor of $InsP_3$-dependent Ca^{2+} release pathway, U73122, also resulted in the increase of cortical actin filaments, while it blocked the 1-MA-induced Ca^{2+} release. At this stage of our work, we have no definitive explanation for how U73122 and heparin induce the reorganization of the actin cytoskeleton. However, it is known that a plasma membrane-enriched phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), can affect the status of actin polymerization by modulating the activity of actin-binding proteins (Sechi and Wehland, 2000; Logan and Mandato, 2006). Because the negatively charged head group of PIP_2 physically interacts with the clustered basic amino acids of the actin-binding proteins (Sechi and Wehland, 2000), it is conceivable that the hexagonic carbon ring and the crowded sulfate groups in heparin might mimic PIP_2 and thereby affect actin polymerization. The direction of the net effect on polymerization or depolymerization of the local actin pools may depend on the differential localization of actin-binding proteins. Similarly, inhibition of phospholipase C (PLC) by U73122 is expected to modulate actin filaments by increasing PIP_2, the substrate of the enzyme. Indeed, U73122 treatment increased or redistributed microfilaments in several types of cells (Kimata et al., 2006; Papakonstanti and Stournaras, 2004; Weston et al., 2008). Suggesting a specific role of PLC in Ca^{2+} signaling and reorganization of the actin cytoskeleton, U73343, a structural analog of U73122, affected neither the 1-MA-induced Ca^{2+} signals nor the phalloidin-stained actin cytoskeleton (Supplementary data 3).

Modulation of Ca^{2+} signaling in starfish oocytes by the cortical actin cytoskeleton was also demonstrated in other ways. The treatment of oocytes with JAS, a well-characterized reagent that produces similar hyper-polymerization of cortical actin, also inhibited the 1-MA-induced Ca^{2+} signals in a dose-dependent manner (Fig. 5b). The results of LAT-A experiments further support the idea that the perturbation of actin polymerization or depolymerization can drastically impair 1-MA-triggered Ca^{2+} signaling. The cortical actin network must therefore play an important role in regulating Ca^{2+} release in maturing oocytes.

The molecular mechanism by which the actin cytoskeleton influences the release of intracellular Ca^{2+} is not understood. One hypothesis is that the actin cytoskeleton may modulate the activity of Ca^{2+}-releasing channels, such as $InsP_3$ receptors, by changing their microenvironment (Wang et al., 2002). $InsP_3$ receptors are physically linked to the actin cytoskeleton in several cell types, and this interaction may lead to modulation of Ca^{2+} channel activity (Joseph and Samanta 1993; Fujimoto et al., 1995; Turvey et al., 2005). Actin filaments are thus implicated in the regulation of both Ca^{2+} influx (Patterson et al., 1999; Rosado et al., 2000; Furuyashiki et al., 2002) and the intracellular Ca^{2+} release from the ER stores (Baumann 2001; Wang et al., 2002; Sabala et al., 2002). Taking into account the sensitive Ca^{2+}-induced Ca^{2+} release mechanism of $InsP_3$ receptors (Bosanac et al., 2004), it is conceivable that a subtle impact on the priming of Ca^{2+} release by the actin cytoskeleton may result in a significant effect on the $InsP_3$-evoked intracellular Ca^{2+} signaling. Suggesting that the activities of $InsP_3$ receptors may be modulated by the actin cytoskeleton, disruption of the cortical actin layers with LAT-A also abolished $InsP_3$-evoked Ca^{2+} signals (Fig. 5). However, recombinant fragment of the murine $InsP_3$ receptor did not inhibit the generation of the Ca^{2+} wave in response to 1-MA, while it strongly affected the Ca^{2+} response at fertilization (Iwasaki et al., 2002). Furthermore, although drastic rearrangements of cortical actin filaments with U73122 and JAS completely blocked the 1-MA-induced Ca^{2+} transient, such alteration of the actin cytoskeleton did not impair the $InsP_3$-evoked Ca^{2+} release (Figs. 7 and 8). In summary, these results raise the possibility that $InsP_3$ formation may not be necessary for the initiation of the Ca^{2+} transient triggered by 1-MA. Alternatively, it cannot be ruled out that the actin cytoskeleton may work upstream of $InsP_3$ synthesis and thereby influence Ca^{2+} signaling.

Results of our studies on $InsP_3$ raise the intriguing question; what is the second messenger involved in the 1-MA-induced Ca^{2+} release? The intracellular Ca^{2+} release is generally mediated by three major second messengers: inositol 1,4,5-trisphosphate ($InsP_3$), cyclic-ADP-ribose (cADPr), and nicotinic acid adenine dinucleotide phosphate (NAADP) (Lee 1997 and 2005; Carafoli et al., 2001; Galione and Petersen, 2005; Santella, 2005; Yamasaki et al., 2005; Guse, 2005; Berridge 2006; Mikoshiba, 2007). Although cADPr and NAADP often play distinct roles in concert with $InsP_3$ toward the creation of the characteristic Ca^{2+} waves in response to external cues (Albrieux et al., 1998; Santella et al., 2004), the oocytes used in our studies did not respond to uncaged cADPr (Nusco et al., 2002, 2006). This observation implies that ryanodine receptors (RyRs) might not contribute to the 1-MA-induced Ca^{2+} signaling in *A. pectinifera*. This conclusion was further substantiated by the observation that the specific inhibitor of cADPr, 8-NH_2-cADPr, did not block the 1-MA-induced Ca^{2+} signals (Supplementary data 2).

The finding that 1-MA can induce a Ca^{2+} response without external Ca^{2+} (Meijer and Guerrier 1984), while NAADP mainly mediates Ca^{2+} influx from outside (Santella et al., 2000; Lim et al., 2001; Moccia et al., 2003), suggests that NAADP is less likely to make significant contributions to the 1-MA-induced Ca^{2+} release. This idea was further supported by the use of a recently characterized inhibitor of NAADP receptor, named Ned-14 (a generous gift from Dr. G. Churchill, University of Oxford, UK). While incubation of starfish oocytes with 100 μM Ned-14 for 10 min completely abolished NAADP-evoked (pipette concentration, 1 mM) Ca^{2+} signals, the same treatment did not block the 1-MA-induced Ca^{2+} signaling (not shown). Taken together, these observations suggest that cADPr and NAADP are not likely to play major roles in the 1-MA-induced Ca^{2+} release, unless 1-MA sparks a huge amount of cADPr or NAADP that cannot be surmounted by the inhibitors.

An alternative possibility to explain how the actin cytoskeleton influences the intracellular Ca^{2+} signaling is that actin filaments themselves might play direct roles in storing and releasing Ca^{2+} by a still unknown mechanism. Since Ca^{2+} ions bind with high affinity to double-helical actin filaments, F-actin may serve as a stable intracellular reservoir for Ca^{2+} storage especially in microvilli and the cortical areas beneath the plasma membrane (Carlier et al., 1986; Lange, 1999; Lange and Gartzke, 2006). Disruption of actin filaments can release Ca^{2+} ions *in vitro*, and therefore 'treadmilling' of actin filaments was suggested as a mechanism to release Ca^{2+} (Lange, 1999). Interestingly, the 1-MA-induced Ca^{2+} mobilization coincides exactly with the timing of the dynamic changes of the microvillar actin filaments (Schroeder and Stricker, 1983). Supporting the idea that microvilli may play a role in Ca^{2+} signaling, the total elimination of the 1-MA-induced Ca^{2+} signaling by heparin (Fig. 2b) was also accompanied by the retraction of microvilli (Fig. 4b, single arrows). Disruption of actin filaments by LAT-A spontaneously produced Ca^{2+} spikes in starfish oocytes, and these Ca^{2+} signals lasted for hours as long as the dynamic structure of microvilli was maintained (Lim et al.,

2002). Hence, it is conceivable that the dynamic changes of actin filaments could liberate Ca^{2+} in the oocyte cortex. This alternative view on the direct role of actin filaments in intracellular Ca^{2+} signaling requires further investigation.

In living cells, the dynamic reorganization of the actin cytoskeleton is mediated by actin-binding proteins (Chun and Santella, 2007). In agreement with the idea that the actin network plays a modulatory role in intracellular Ca^{2+} signaling, the actin-binding protein cofilin was found to potentiate the Ca^{2+} responses during meiotic maturation and fertilization. Interestingly, cofilin also showed dual effects on the sperm-induced Ca^{2+} signaling pattern. While the cortical Ca^{2+} release was abolished, Ca^{2+} mobilization in the inner cytoplasm was instead enhanced by cofilin (Nusco et al., 2006). How cofilin brings about the opposite effects in the two different subcellular regions remains unknown. Because the actin pools in the cortex and the inner cytoplasm are subtly different with respect to F-actin structures and polymerization dynamics (Heil-Chapdelaine and Otto, 1996), whether or not such differences in the actin cytoskeleton may contribute to the dual effects of cofilin would be an interesting subject for further investigation.

The results of our study indicate that the actin cytoskeleton is important in modulating the Ca^{2+} release from the $InsP_3$-sensitive stores, both in the cortex and in the inner cytoplasm. Hence, one of the physiological roles of the rearrangement of the actin cytoskeleton during meiotic maturation may be to sensitize the Ca^{2+} release mechanism in preparation for fertilization (Lim et al., 2001; Santella et al., 2004; Moccia et al., 2006). Likewise, the actin cytoskeletal changes during meiotic maturation may also be important for the formation of the fertilization envelope. Our data showing that the robust exocytosis of cortical granules by massive Ca^{2+} release can be blocked by the enhancement of the cortical actin network (hyper-structuralization of the cortex) suggests that the timely regulation of the cortical actin cytoskeleton is crucial not only in generating Ca^{2+} signals, but also in mediating their cytological outcomes (Figs. 7 and 8). Similar prevention of cortical granule exocytosis by JAS-induced F-actin rearrangement was also observed in mammalian eggs (Terada et al., 2000). Whether the dense subplasmalemmal actin network simply serves as a physical barrier or plays other functional roles for exocytosis would require further investigations.

Acknowledgments

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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.2008.05.549.

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